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Amino-acids Peptides and Proteins

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Amino-acids, Peptides, and Proteins

Volume 7

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Senior Reporter

R. C. Sheppard, *MRC Laboratory of Molecular Biology, Cambridge*

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Organic formulae composed by Wright's Symbolset method

Preface

This seventh Report reviews papers relevant to the chemistry of amino-acids, peptides and proteins published in the main journals during 1974. In the two preceding volumes, Chapter 5 on 'Chemical Structure and Biological Activity' dealt almost exclusively with peptide hormones and related compounds. This year it covers enzymes for the first time. The biennial review of metal derivatives appears this year in Chapter 6. The other main areas surveyed are the same as those in Volume 6.

Once again it is a pleasure to thank most warmly the many contributors to this volume.

R. C. SHEPPARD

Contents

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 and 5 in this series.

Other abbreviations which have been used are:

Adoc	adamantyloxycarbonyl
Aoc	t-amyloxycarbonyl
Asu	α-aminosuberic acid
Asx	aspartic acid or asparagine (not yet determined)
ATP	adenosine 5′-triphosphate
Bpoc	2-(4-biphenylyl)-isopropoxycarbonyl
BSA	bovine serum albumin
c.d.	circular dichroism
Cha	cyclohexylamine
CIMS	chemical ionization mass spectrometry
Cm	carboxymethyl
Cmc	*S*-carboxymethylcysteine
Dce	2,2-diethoxycarbonyl
Dcha	dicyclohexylamine
DMF	*NN*-dimethylformamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
Dnp	2,4-dinitrophenyl
Dns	1-dimethylaminonaphthalene-5-sulphonyl (dansyl)
Dopa	3,4-dihydroxyphenylalanine
DP	degree of polymerization
DTNB	5,5′-dithiobis-(2-nitrobenzoic acid)
Ec	ethylcarbamoyl
edta	ethylenediamine tetra-acetate
e.p.r.	electron paramagnetic resonance
e.s.r.	electron spin resonance
Gal	galactose
GC–MS	gas chromatograph–mass spectrometer combination
g.l.c.	gas-liquid chromatography
Glc	glucose
Glp	pyrrolid-2-one-5-carboxylic acid
Glx	glutamic acid or glutamine (not yet determined)
GTP	guanosine 5′-triphosphate

i.r.	infrared
Man	mannose
NAD	nicotinamide-adenine dinucleotide (NAD^+ oxidized; NADH, reduced)
n.m.r.	nuclear magnetic resonance
OPfp	pentafluorophenoxy
ONSu	succinimido-oxy
OPcp	pentachlorophenoxy
ONp	*p*-nitrophenoxy
ONp(*o*)	*o*-nitrophenoxy
OPic	4-picolyloxy
o.r.d.	optical rotatory dispersion
OTcp	2,4,5-trichlorophenoxy
Pipoc	piperidino-oxycarbonyl
Pth-Gly	the phenylthiohydantoin derived from glycine, *etc.*
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
Ser(P)	*O*-phosphorylserine
t.l.c.	thin-layer chromatography
u.v.	ultraviolet
Ztf	1-benzyloxycarbonylamino-2,2,2-trifluoroethyl

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Amino-acids

BY G. C. BARRETT

1 Introduction

No substantial new emphasis on some aspect of amino-acid science has arisen in the recent literature, and the present Chapter, reviewing the literature of 1974, is subdivided as in previous Volumes of this series. As before, the coverage is intended to be thorough, but excludes most of the biological literature dealing with biosynthetic, metabolic, physiological, and microbiological aspects.

Textbooks and Reviews.—The laboratory synthesis [1-3] and large-scale production [2, 3] of amino-acids, and their technological applications,[1, 2] have been surveyed. Other more specific reviews are cited in the appropriate sections.

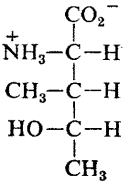
2 Naturally Occurring Amino-acids

Occurrence of Known Amino-acids.—Increasing attention is being given to the identification of organic compounds in geological samples, and the analysis of ancient cyanite schists from the Kola peninsula [4] (six free amino-acids and seven in a bound form) uses routine techniques; more information can be inferred from the degree of racemization of amino-acids present in fossils (see p. 21).

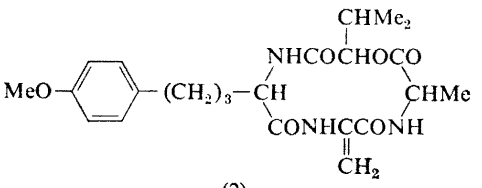
Aspects of the distribution of non-protein amino-acids in plants have been reviewed.[5] Among the more notable reports of the appearance of known amino-acids in plant sources are the presence of *cis*-4-hydroxy-L-proline in three genera (four species) of *Santalaceae*,[6] suggesting a useful taxonomic index for the species; also, the isolation from *Crotalaria juncea* seeds of δ-hydroxy-norleucine (5-hydroxy-2-aminohexanoic acid),[7] previously noted to be a constituent of the ilamycins. Partly racemized (*R*)-2-aminobut-3-enoic acid ('D-vinylglycine') isolated from *Rhodophyllus nidorosus* [8] is shown to exist in the plant in optically impure form. Tissues of *Medicago sativa* contain several amino-acid betaines, including stachydrine and homostachydrine (*NN*-dimethylproline and *NN*-dimethylpipecolic acid betaines, respectively),[9] and a careful study has established links between betaine content and growth rate.

[1] V. M. Belikov, *Vestnik Akad. Nauk S.S.S.R.*, 1973, 33.
[2] 'Synthetic Production and Utilization of Amino-acids', ed. T. Kaneko, Y. Izumi, I. Chibata, and T. Itoh, Kodansha, Tokyo, and Wiley, New York, 1974.
[3] E. N. Safonova and V. M. Belikov, *Uspekhi Khim.*, 1974, **43**, 1575.
[4] I. Z. Sergienko, M. I. Bobyleva, S. A. Sidorenko, and I. A. Egorov, *Doklady Akad. Nauk S.S.S.R.*, 1974, **215**, 474.
[5] L. Fowden, *Ann. Proc. Phytochem. Soc.*, 1972, **9**, 323.
[6] R. Kuttan, K. S. V. Pattabhiraman, and A. N. Radhakrishnan, *Phytochemistry*, 1974, **13**, 453.
[7] R. Pant and H. M. Fales, *Phytochemistry*, 1974, **13**, 1626.
[8] G. Dardenne, J. Casimir, M. Marlier, and P. O. Larsen, *Phytochemistry*, 1974, **13**, 1897.
[9] J. K. Sethi and D. P. Carew, *Phytochemistry*, 1974, **13**, 321.

Chirality at side-chain asymmetric centres may differ from species to species. The demonstration [10] that enniatin A is a mixture of diastereoisomers containing both *N*-methyl-L-isoleucine and *N*-methyl-L-alloisoleucine residues is incorrectly claimed (see refs. 12, 31, 32) to be the first report of the co-occurrence of both epimers of an L-amino-acid with two chiral centres in the same group of natural products. γ-L-Glutamyl-*S*-(*trans*-prop-1-enyl)-L-cysteine sulphoxide isolated from *Santalum album* leaves has the opposite configuration at sulphur from that in the same dipeptide isolated from onion.[11] The γ-hydroxyisoleucine residue in γ-amanatin is shown by *X*-ray analysis of its lactone hydrobromide [12] to be (2*S*,3*R*,4*S*)-2-amino-3-methyl-4-hydroxyvaleric acid (1), from which there follows a re-formulation of the absolute configuration of γδ-dihydroxyisoleucine present in the α- and β-amanatins to (2*S*, 3*R*, 4*R*)-2-amino-3-methyl-4,5-dihydroxyvaleric acid on the basis of chemical correlation.[12] Revision of the



(1)



(2)

configurational assignments to the γ-hydroxyisoleucine diastereoisomers found recently (in unequal amounts) in plants, for the first time (see Volume 6, p. 2), may now be necessary. Alternariolide (2), a host-specific toxin produced by *Alternaria mali* (responsible for apple blotch), contains two non-protein amino-acids;[13] structure (2) is assigned [13] to the toxin on the basis of spectroscopic data, but no evidence for absolute configuration was obtained.

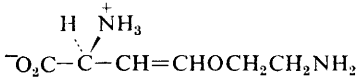
N-Methyl amino-acids of various types are represented for this Section by *N*-methyl-L-methionine-*S*-sulphoxide which is found, together with the corresponding primary amino-acid, in the red alga *Grateloupia turuturu*;[14] the proposal [15] that promine and retine, from calf liver and thymus, are N^{ε}-trimethyllysine and N^{G}-dimethyl-arginine respectively is not borne out by the physical and chemical properties of the compounds.[16, 17]

Where appropriate, mention is made in this Chapter of β- and γ-amino-acids, although most amino-acids mentioned in the literature are of the α-series. γ-Amino-L-α-hydroxybutyric acid has been established as a component of 4′-deoxybutirosins.[18]

[10] T. K. Audhya and D. W. Russell, *J.C.S. Perkin I*, 1974, 743.
[11] R. Kuttan, N. G. Nair, A. N. Radhakrishnan, T. F. Spande, H. J. Yeh, and B. Witkop, *Biochemistry*, 1974, **13**, 4394.
[12] A. Gieren, P. Narayanan, W. Hoppe, M. Hasan, K. Michl, T. Wieland, H. O. Smith, G. Jung, and E. Breitmayer, *Annalen*, 1974, 1561.
[13] T. Okuno, Y. Ishita, K. Sawai, and T. Matsumoto, *Chem. Letters*, 1974, 635.
[14] K. Miyazawa and K. Ito, *Nippon Suisan Gakkaishi*, 1974, **40**, 655.
[15] E. Tyihak and A. Patthy, *Acta Agron. Acad. Sci. Hung.*, 1973, **22**, 445.
[16] C. Marmasse, *Acta Agron. Acad. Sci. Hung.*, 1974, **23**, 216.
[17] T. Nakajima, *Acta Agron. Acad. Sci. Hung.*, 1974, **23**, 236.
[18] M. Konishi, K. Numata, K. Shimoda, H. Tsukiura, and H. Kawaguchi, *J. Antibiotics*, 1974, **27**, 471.

Microbial synthesis of amino-acids continues to provide an expanding literature, and only representative papers can be cited here. L-Amino-acids produced through biosynthesis include isoleucine[19] and *cyclo*-isoleucylisoleucine,[20] threonine,[21] *O*-alkyl-homoserines,[22] arginine and citrulline,[23] indole-substituted tryptophans,[24] phenylalanine,[25] histidine,[26] dopa[27] and *N*-Z, *N*-Boc, and *N*-formyl derivatives of dopa,[28] and azetidine-2-carboxylic acid.[29]

New Natural Free Amino-acids.—Further details have been provided[30] of the acetylenic amino-acids present in *Tricholomopsis rutilans*. Both *threo* and *erythro* diastereoisomers of L-2-amino-3-hydroxyhex-4-ynoic acid are present, adding a further example to those reported in the past two years[10, 12, 31, 32] of the occurrence of epimeric amino-acids in the same species. A number of other unsaturated amino-acids have been isolated from plant sources, and from bacterial and fungal cultures, and reported during the year under review. (2*S*,3*S*)-3-Hydroxy-4-methylene-glutamic acid is present in seeds of *Gleditsia caspica* [the known amino-acids (2*S*,4*R*)-4-methyl-glutamic acid and its (2*S*,3*S*,-4*R*)-3-hydroxy analogue are also present],[33] and L-2-amino-4-chloropent-4-enoic acid (from *Amanita pseudoporphyria*)[34] and L-2-amino-4-(2-aminoethoxy)-*trans*-but-3-enoic acid (3) (from an unidentified *Streptomycete*)[35] are further



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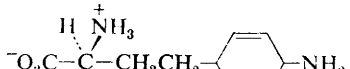


(4)

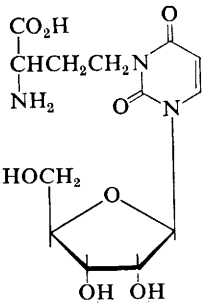
acyclic examples, with an unusual alicyclic derivative, L-2-amino-4-(4′-amino-2′,5′-cyclohexadienyl)butyric acid (4), being a new amino-acid antibiotic.[36] The stereochemistry of the cyclohexadienyl moiety in (4) is not yet established.

[19] H. Matsushima, K. Murata, and Y. Mase, *Hakko Kogaku Zasshi*, 1974, **52**, 20.
[20] Y. Yamada, S. Sawada, and H. Okada, *Hakko Kogaku Zasshi*, 1974, **52**, 143.
[21] T. Hirakawa and K. Watanabe, *Agric. and Biol. Chem.* (*Japan*), 1974, **38**, 77.
[22] N. Ogasawara, T. Sato, M. Kato, and K. Sakaguchi, *Agric. and Biol. Chem.* (*Japan*), 1974, **38**, 515.
[23] K. Kubota, T. Onoda, H. Kamijo, F. Yoshinaga, and S. Okumura, *J. Gen. Appl. Microbiol.*, 1973, **19**, 339.
[24] M. Wilcox, *Analyt. Biochem.*, 1974, **59**, 436.
[25] H. Hagino and K. Nakayama, *Agric. and Biol. Chem.* (*Japan*), 1974, **38**, 157.
[26] K. Araki, F. Kato, Y. Arai, and K. Nakayama, *Agric. and Biol. Chem.* (*Japan*), 1974, **38**, 189.
[27] H. Yoshida, Y. Tanaka, and K. Nakayama, *Agric. and Biol. Chem.* (*Japan*), 1974, **38**, 455, 633.
[28] J. Rosazza, P. Foss, M. Lemberger, and C. J. Sih, *J. Pharm. Sci.*, 1974, **63**, 544.
[29] E. Leete, G. E. Davis, C. R. Hutchinson, K. W. Woo, and M. R. Chedekel, *Phytochemistry*, 1974, **13**, 427.
[30] Y. Niimura and S. Hatanaka, *Phytochemistry*, 1974, **13**, 175.
[31] G. A. Dardenne, J. Casimir, E. A. Bell, and J. R. Nulu, *Phytochemistry*, 1972, **11**, 787.
[32] G. A. Dardenne, E. A. Bell, J. R. Nulu, and C. Cone, *Phytochemistry*, 1972, **11**, 791.
[33] G. A. Dardenne, J. Casimir, and H. Sorensen, *Phytochemistry*, 1974, **13**, 2195.
[34] S. Hatanaka, S. Kaneko, Y. Niimura, F. Kinoshita, and G. Soma, *Tetrahedron Letters*, 1974, 3931.
[35] D. L. Pruess, J. P. Scannell, M. Kellett, H. A. Ax, J. Janecek, T. H. Williams, A. Stempel, and J. Berger, *J. Antibiotics*, 1974, **27**, 229.
[36] Y. Okami, T. Kitihara, M. Hamada, H. Naganawa, S. Kondo, K. Maeda, T. Takeuchi, and H. Umezawa, *J. Antibiotics*, 1974, **27**, 656.

α-Amino-γ-(isoxazolin-5-on-2-yl)butyric acid has been isolated from *Lathyrus odoratus*,[37] together with β-(isoxazolin-5-on-2-yl)alanine and β-(2-β-D-glucopyranosyl-isoxazolin-5-on-4-yl)alanine which were previously found in *Pisum sativum* seedlings. 4-(4-Hydroxy-3-methyl-Δ^2-butenyl)tryptophan has been isolated from cultures of *Claviceps purpurea*, the structural assignment resting on mass spectrometric study of its *N*-trifluoroacetyl methyl ester so that no configurational assignment could be made.[38] A further new heterocyclic amino-acid, of particular interest, is 3-(3-amino-3-carboxypropyl)uridine (5), a novel modified nucleoside from *E. coli* tRNA representing the site of reaction with phenoxyacetic acid.[39, 40]

N-(3-Aminopropyl)-4-aminobutyric acid, $NH_2(CH_2)_3NH(CH_2)_3CO_2H$, appears in rabbit urine as a metabolite of bleomycin A_5.[41]

New Amino-acids from Hydrolysates.—Peptide antibiotics continue to provide novel amino-acids, often closely related in structure to the protein amino-acids. Hydrolysates of longicatenamycin contain 5-chloro-D-tryptophan,[42] and antibiotic SF-1293 contains an L-2-amino-4-(methylphosphino)butyric acid residue (6).[43] The structure of SF-1293, the tripeptide (6)-L-Ala-L-Ala,[43a] has an extra-



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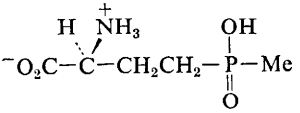

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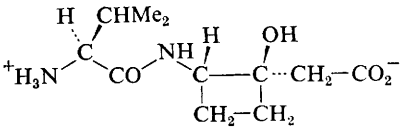

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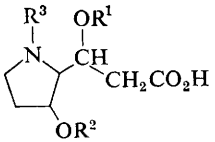


(8) R^1 = (+)-(*S*)-2-methylbutyryl-L-phenylalanine
R^2 = Ac, EtCO, Pr^{i}CO, Pr^{n}CO, Me_2CHCH_2CO
R^3 = L-valyl

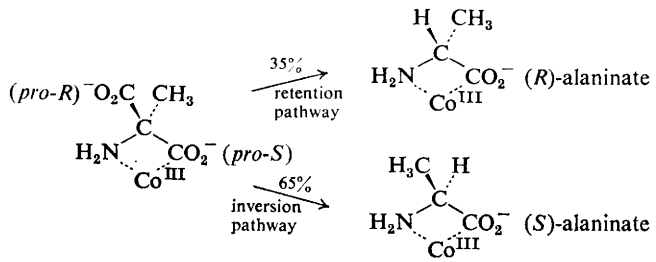
[37] F. Lambien and R. Van Parijs, *Biochem. Biophys. Res. Comm.*, 1974, **61**, 155.
[38] J. A. Anderson and M. S. Saini, *Tetrahedron Letters*, 1974, 2107.
[39] Z. Ohashi, M. Maeda, J. A. McCloskey, and S. Nishimura, *Biochemistry*, 1974, **13**, 2620.
[40] S. Friedman, H. J. Li, K. Nakanishi, and G. Van Lear, *Biochemistry*, 1974, **13**, 2932.
[41] S. Hori, T. Sawa, T. Yoshioka, T. Takita, T. Takeuchi, and H. Umezawa, *J. Antibiotics*, 1974, **27**, 489.
[42] T. Shiba, Y. Mukunoki, and H. Akiyama, *Tetrahedron Letters*, 1974, 3085.
[43] (*a*) Y. Ogawa, T. Tsuruoka, S. Inoue, and T. Niida, *Meiji Seika Kenkyu Nempo*, 1973, No. 13, 42; (*b*) Y. Ogawa, H. Yoshida, S. Inoue, and T. Niida, *ibid.*, p. 49; (*c*) N. Ezaka, S. Amano, K. Fukushima, S. Inoue, and T. Niida, *ibid.*, p. 60 (*Chem. Abs.*, 1974, **81**, 37 806, 37 788, and 37 805).

ordinary similarity with an L-glutamine antimetabolite, (X)-L-Ala-L-Ala[where (X) = L-(N^5-phosphono)methionine-*S*-sulphoximine residue], mentioned in last year's review (Volume 6, p. 7). Antibiotic LL-AV 290 contains 3-chloro-4-hydroxyphenylglycine and *p*-hydroxyphenylsarcosine residues.[44]

New β-amino-acids and higher homologues have been reported. γ-Hydroxy-β-lysine is a new basic amino-acid from hydrolysates of tuberactinomycins A and N;[45] a metabolite from an unclassified *Streptomycete* is a dipeptide (7) containing a 2-aminocyclobutane-1-acetic acid moiety.[46] The novel amino-acid detoxinine (8; $R^1 = R^2 = R^3 = H$) is a constituent of a group of depsipeptide antibiotics, the detoxins.[47]

3 Chemical Synthesis and Resolution of Amino-acids

Asymmetric Synthesis.—Decarboxylation of α-amino-α-methylmalonic acid after binding to $\Lambda(-)_{436}$-α-[(2*S*,9*S*)-2,9-diamino-4,7-diazadecanecobalt(III) dichloride] cation leads to the corresponding (*R*,*S*)-alanine complex in which the (*S*)-enantiomer is present in 30% excess.[48] This is the first example of the absolute chiral recognition of a prochiral centre by a small molecule – the process is otherwise well illustrated in enzymic reactions. The crystal structure of $\Lambda(-)_{436}$-β_2-[(2*S*,9*S*)-2,9-diamino-4,7-diazadecanecobalt(III) α-amino-α-methylmalonate] perchlorate monohydrate[49] shows that a Λ-β-*R*-conformation is adopted, with the pro-*S*-carboxy-group of the malonate moiety co-ordinated to cobalt, rather than the pro-*R*-carboxy-group, and the considerable asymmetric induction caused by the dissymmetric cobalt centre in favour of inversion accompanying decarboxylation (Scheme 1) is due to a less obstructed pathway



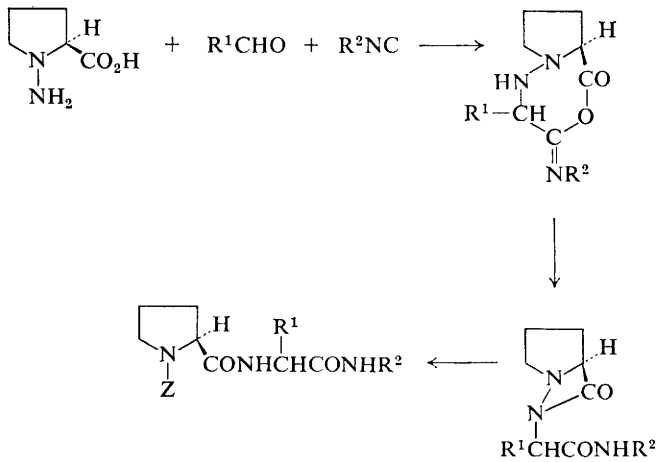
Scheme 1

for the incoming proton in this direction.[49] A late stage in the classical malonic ester synthesis of α-amino-acids is represented in these decarboxylation studies, and the opportunity has now been created for developing a new asymmetric synthesis based on otherwise well-established reactions.

[44] J. J. Hlavka, P. Bitha, J. H. Boothe, and G. Morton, *Tetrahedron Letters*, 1974, 175.
[45] T. Wakamiya, T. Teshima, I. Kubota, T. Shiba, and T. Kaneko, *Bull. Chem. Soc. Japan*, 1974, **49**, 2292.
[46] D. L. Pruess, J. P. Scannell, J. F. Blount, H. A. Ax, M. Kellett, T. H. Williams, and A. Stempel, *J. Antibiotics*, 1974, **27**, 754.
[47] N. Otake, K. Furihata, K. Kakinuma, and H. Yonehara, *J. Antibiotics*, 1974, **27**, 484.
[48] R. C. Job and T. C. Bruice, *J. Amer. Chem. Soc.*, 1974, **96**, 809.
[49] J. P. Glusker, H. L. Carrell, R. Job, and T. C. Bruice, *J. Amer. Chem. Soc.*, 1974, **96**, 5741.

Treatment of a Schiff base derived from (−)-(*S*)-1-(4-pyridyl)ethylamine and an α-keto-ester with base in Bu^{t}OH solution gives the rearranged (*S*)-α-amino-acid ester Schiff base (see Scheme 6, p. 23). This stereospecific (suprafacial) proton transfer depends on the presence of bulky substituents to sustain the geometry of the starting material through the transition state. In Bu^{t}OD, the α-deuteriated (*S*)-α-amino-acid is formed. Efficient asymmetric hydrogenation of α-acetamidocinnamic acids is catalysed by chiral phosphine–rhodium complexes;[50] in a partial asymmetric synthesis, chiral isocyanides are converted into their lithium aldimine homologues, *e.g.* PhCMe(Et)N=CRLi, followed by carboxylation or ethoxycarbonylation.[51]

A novel procedure favouring the formation of D-amino-acids[52] based on *N*-amino-L-proline and an isocyanide is displayed in Scheme 2.

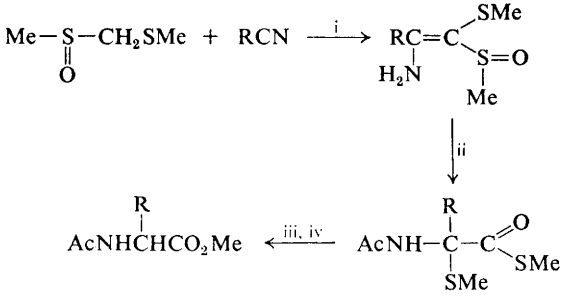


Scheme 2

General Methods of Synthesis.—Further examples of the Ugi reaction have been provided, illustrating a synthesis of L-prolyl-D-amino-acids[52] and a synthesis of 1,4-dihydrophenylalanine,[53] for which a conventional Strecker synthesis was inappropriate.[53] A review has appeared[54] of the uses of α-metallated isocyanides in organic synthesis, including the synthesis of β-functional α-amino-acids (see Volume 6, p. 9). An outstanding new synthesis of α-amino-acids from nitriles (Scheme 3)[55] involves a rearrangement step whose characteristics are not yet fully understood.

Full details of the use of malonic acid half-esters in a modified Curtius reaction (diphenylphosphoryl azide) for amino-acid synthesis are available,[56] supplement-

[50] W. S. Knowles, M. J. Sabacky, and B. D. Vineyard, in 'Homogeneous Catalysis—II', Advances in Chemistry Series No. 132, American Chemical Society, 1974, p. 274.
[51] N. Hirowatari and M. H. Walborsky, *J. Org. Chem.*, 1974, **39**, 604.
[52] K. Achiwa and S. Yamada, *Tetrahedron Letters*, 1974, 1799.
[53] D. Scholz and U. Schmidt, *Chem. Ber.*, 1974, **107**, 2295.
[54] D. Hoppe, *Angew. Chem. Internat. Edn.*, 1974, **13**, 789.
[55] K. Ogura and G. Tsuchihashi, *J. Amer. Chem. Soc.*, 1974, **96**, 1960.
[56] K. Ninomiya, T. Shioiri, and S. Yamada, *Chem. and Pharm. Bull. (Japan)*, 1974, **22**, 1398.



Reagents: i, NaH; ii, Ac_2O; iii, MeOH; iv, desulphurization

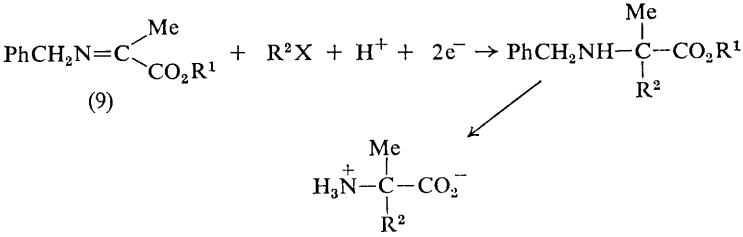
Scheme 3

ing the preliminary communication mentioned in Volume 6 (p. 11). The use of the α-acylamino-malonic ester route is exemplified in many papers, as usual, for the synthesis of specific α-amino-acids,[34, 43, 57–59] and the hydantoin synthesis [60–64] and azlactone synthesis,[65–68] Strecker synthesis,[69, 70] and the α-halogeno-acid amination procedure,[64, 70–72] have been employed.

Schiff bases are already counted among the more valuable starting materials for amino-acid synthesis, and further such applications have been devised. Electroreductive coupling with an alkyl halide using constant potential electrolysis can give 38—86% yields of α-methyl-α-amino-acids from a pyruvate ester Schiff base (9; see Scheme 4).[73] β-Amino-acid amides $R^1NHCHR^2CH_2CONR^3_2$ and corresponding esters may be prepared from Schiff bases through the Reformatzky reaction.[74]

General methods for the synthesis of β-carboxy-α-aminosulphonic acids [75] and αβ-unsaturated α-amino-acids [76] have been reported; *N*-trimethylsilylmethylglycinamide, $Me_3SiCH_2NHCH_2CONH_2$, has been synthesized from $Me_3SiCH_2NH_2$ and $ClCH_2CONH_2$ in a method suitable for general application.[72]

[57] K. Matsumoto, T. Miyahara, M. Suzuki, and M. Miyoshi, *Agric. and Biol. Chem. (Japan)*, 1974, **38**, 1097.
[58] H. Maehr and M. Leach, *J. Org. Chem.*, 1974, **39**, 1166.
[59] L. Pichat and J. P. Beaucourt, *J. Labelled Compounds*, 1974, **10**, 103.
[60] A. Arendt, A. Kolodziejczyk, T. Sokolowska, and M. Mrozowski, *Roczniki Chem.*, 1974, **48**, 883.
[61] J. J. Ellington and I. L. Honigberg, *J. Org. Chem.*, 1974, **39**, 104.
[62] M. M. Abdel-Monem, N. E. Newton, and C. E. Weeks, *J. Medicin. Chem.*, 1974, **17**, 447.
[63] M. M. Ames and N. Castagnoli, *J. Labelled Compounds*, 1974, **10**, 195.
[64] J. Mizon and C. Mizon, *J. Labelled Compounds*, 1974, **10**, 229.
[65] R. T. Coutts and J. L. Malicky, *Canad. J. Chem.*, 1974, **52**, 390.
[66] M. L. Anhoury, P. Crooy, R. De Neys, and J. Eliaers, *Bull. Soc. chim. belges*, 1974, **83**, 117.
[67] T. S. T. Wang and J. A. Vida, *J. Medicin. Chem.*, 1974, **17**, 1120.
[68] G. W. Kirby and M. J. Varley, *J.C.S. Chem. Comm.*, 1974, 833.
[69] D. J. Aberhardt and L. J. Lin, *J.C.S. Perkin I*, 1974, 2320.
[70] P. Friis, P. Helboe, and P. O. Larsen, *Acta Chem. Scand. (B)*, 1974, **28**, 317.
[71] C. Eguchi and A. Kakuta, *Bull. Chem. Soc. Japan*, 1974, **47**, 1704.
[72] W. Fink, *Helv. Chim. Acta*, 1974, **57**, 1042.
[73] T. Iwasaki and K. Harada, *J.C.S. Chem. Comm.*, 1974, 338.
[74] F. Dardoize and M. Gaudemar, *Bull. Soc. chim. France*, 1974, 939.
[75] A. Le Berre, A. Etienne, and J. Coquelin, *Bull. Soc. chim. France*, 1974, 221.
[76] D. H. Rich, J. Tam, P. Mathiaparanam, J. A. Grant, and C. Mabuni, *J.C.S. Chem. Comm.*, 1974, 897.



Scheme 4

Prebiotic Synthesis; Model Reactions.—Electrical discharge studies with CH_4–CO_2–NH_3 [77] and CH_4–NH_3–H_2O [78] mixtures continue to demonstrate the formation of amino-acid mixtures, and the synthesis of amino-acids and high molecular weight proteins under radiofrequency cold plasma conditions has been reported.[79] Polymeric material obtained from aqueous methylammonium bicarbonate after n,γ-irradiation gave glycine, alanine, and lysine on hydrolysis;[80] trimethylammonium bicarbonate gave in addition γ-aminobutyric acid and valine, and n-pentylammonium bicarbonate gave norleucine, γ-aminobutyric acid, alanine, and 6-aminohexanoic acid, on similar treatment. A related study, but with some preparative value, has shown that aliphatic carboxylic acids subjected to contact glow discharge electrolysis in concentrated aqueous ammonia give a wide variety of amino-acids in yields up to 13%.[81] Propionic acid, for example, under these conditions (75 mA at 15 °C for 3 h) gives 6.9% alanine, 5.3% β-alanine, and 1% glycine.

Exposure to sunlight of solutions of formaldehyde, ammonium molybdate, ammonium phosphate, and mineral salts gives appreciable amounts of amino-acids after 80 h,[82] with some dependence of relative proportions of the different amino-acids upon the concentrations of formaldehyde and ammonium molybdate.

Hydrogen cyanide oligomers have been shown earlier to be a source of amino-acids on hydrolysis, even though the oligomers themselves do not appear to be closely related to polypeptides. Fractionation of the oligomers into acidic, neutral, and basic components, followed by hydrolysis and analysis by g.l.c. and mass spectrometry, shows [83] that a wider range of protein amino-acids is available from this source than previously supposed. Glutamic acid is obtained by hydrolysis of the neutral oligomers, but not from the acidic and basic fractions which give glycine, aspartic acid, and *meso*- and DL-diaminosuccinic acids, with smaller amounts of alanine, isoleucine, and α-aminoisobutyric acid.[83] In comparison with the somewhat disappointing earlier evidence that only the more esoteric amino-acids could be generated by hydrolysis of hydrogen cyanide oligomers,

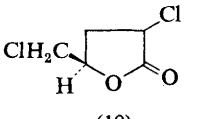
[77] E. F. Simonov, V. B. Lukyanov, and E. R. Roshal, *Vestnik Muskov Univ., Khim.*, 1974, **15**, 365.
[78] D. Stefanescu, *Stud. Cercet. Biochim.*, 1974, **12**, 205.
[79] C. I. Simionescu, F. Denes, and M. Dragnea, *Compt. rend.* 1974, **278**, *C*, 29; C. I. Simionescu, F. Denes, D. Onac, and G. Bloos, *Biopolymers*, 1974, **13**, 943.
[80] L. N. Zhigunova, G. V. Manuilova, and E. P. Petryaev, *Vestsi Akad. Navuk B.S.S.R., Ser. Fiz. Energ. Navuk*, 1974, 33 (*Chem. Abs.*, 1974, **81**, 152 607).
[81] K. Harada and T. Iwasaki, *Nature*, 1974, **250**, 426.
[82] K. Bahadur, M. L. Verma, and Y. P. Singh, *Z. allg. Mikrobiol.*, 1974, **14**, 87.
[83] J. P. Ferris, J. D. Wos, D. W. Nooner, and J. Oro, *J. Mol. Evol.*, 1974, **3**, 225.

these results will enliven the arguments of those who advocate the origin of life within the chemistry of hydrogen cyanide.

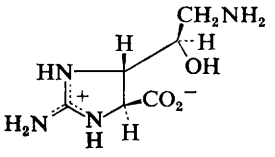
Protein and Other Naturally Occurring Amino-acids.—New syntheses described in the preceding sections have employed some of the well-known protein amino-acids as synthetic objectives. This section reports specific syntheses which are interesting in their own right, and also capable of being developed into routes to close analogues of natural products.

A synthesis of lysine from butadiene[84] involves conversion with nitrogen pentoxide into 1-nitrobuta-1,3-diene followed by addition to ethyl nitroacetate or diethyl 2-nitromalonate, and hydrogenation and acid hydrolysis.

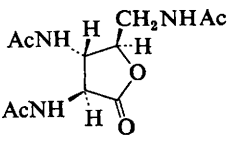
A simple synthesis of L-proline from L-pyroglutamic acid[85] (2-oxopyrrolidine-5*S*-carboxylic acid) employs the method used by the same author in a cucurbitine synthesis described in Volume 6 (p. 15), in which the amide grouping is converted into an imidate ester with triethyloxonium fluoroborate [—CO—NH— → —C(OEt)=N—], which on reduction ($NaBH_4$) gives the secondary amine —CH_2—NH—. D-Glutamic acid gives a mixture of L-hydroxyproline and D-allohydroxyproline through a route involving the butyrolactone (10);[71] amination of (10) followed by hydrolysis of the resulting amide gives a mixture of the diastereoisomeric hydroxyprolines from which an enhanced yield of L-hydroxyproline may be obtained[86] by equilibration of the cyclic dipeptide of the D-allo-isomer, followed by acid hydrolysis.



(10)



(11)



(12)

Specific examples of syntheses of less-common naturally occurring amino-acids, using well-established methods, are αα′-diaminopimelic acid,[60] 3-(3-amino-3-carboxypropyl)uridine,[39] and 4-methylphosphino-L-butyrine.[43]

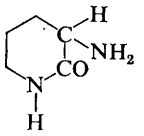
Elegant syntheses[87, 88] of roseonine [(11) *alias* streptolidine or geamine] starting from D-ribose have been reported, involving the lactone (12) as intermediate.

Mention has already been made (p. 3) of microbiological syntheses of natural amino-acids and close relatives, and the possibilities are intriguing when the continuous production implicit in the use of *E. coli* cells immobilized in polyacrylamide gel is taken into account; the feasibility of this has been demonstrated[89] for the synthesis of L-aspartic acid from ammonium fumarate.

[84] T. I. Samoilovich, A. S. Polyanskaya, and V. V. Perekalin, *Doklady Akad. Nauk S.S.S.R.*, 1974, **217**, 1335.
[85] H. G. Monteiro, *Synthesis*, 1974, 137.
[86] C. Eguchi and A. Kakuta, *Bull. Chem. Soc. Japan*, 1974, **47**, 2277.
[87] T. Goto and T. Ohgi, *Tetrahedron Letters*, 1974, 1413.
[88] S. Kusumoto, S. Tsuji, and T. Shiba, *Tetrahedron Letters*, 1974, 1417.
[89] T. Tosa, T. Sato, T. Mori, and I. Chibata, *Appl. Microbiol.*, 1974, **27**, 886.

α-Alkyl and αα-Dialkyl Amino-acids.—A novel synthesis of a chloroalkyl amino-acid in which L-methionine methyl ester is converted in 28% yield into L-2-amino-4,4,4-trichlorobutanoic acid, $Cl_3CCH_2CH(NH_2)CO_2H$, by chlorine in chloroform, followed by hydrolysis,[90] should be more generally applicable where amino-acid side-chains carry functional groups capable of activating an adjacent saturated carbon centre towards halogenation.

Syntheses of α-methylproline[61] and α-methylornithine[61, 62] by the hydantoin route have been reported. An alternative synthesis[62] of α-methylornithine from the parent amino-acid involves treatment of the derived amide (13) with phenyllithium followed by methyl iodide; less direct methods are usually employed in the synthesis of α-alkyl-α-amino-acids.[91–93]



(13)

Amino-acids with Unsaturated Functional Groups in Side-chains.—α-Cyanoglycine is a reactive amino-acid obtained in the past by enzymic deacylation of acetamidocyanoacetic acid; an alternative method[94] involves careful hydrolysis of its *N*-carboxyanhydride prepared from ethyl aminocyanoacetate. Syntheses of DL-vinylglycine (2-aminobut-3-enoic acid)[8, 70] and its enzymic resolution[70] have been reported; 2*S*-amino-4-chloropent-4-enoic acid[34] and diastereoisomers of 2-amino-3-hydroxyhex-4-ynoic acid[30] have been synthesized by standard methods.

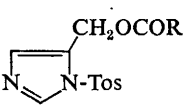
β-Bromo-αβ-unsaturated-α-amino-acids, $RCBr{=}C(NH_2)CO_2H$, are accessible (in low yield) from α-hydroxyaminoalkanoic esters by reaction with bromoacetyl bromide, or in quantitative yield from *N*-acyl-αβ-unsaturated amino-acids by reaction with *N*-bromosuccinimide.[95] *N*-Bromoamide intermediates are involved in these processes.

Amino-acids with Hydroxyalkyl Side-chains.—Syntheses of β-hydroxy-α-amino-acids by standard methods are illustrated in the 1974 literature for 2-amino-3-hydroxyhex-4-ynoic acid[30] and β-hydroxy-methionine and -homomethionine.[96] Nucleophilic addition of the corresponding aldehydes to cupric glycinate in alkaline solution[96] is one of several minor variations of a standard procedure.

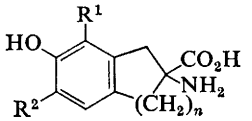
γ-Hydroxy-β-lysine, a component of tuberactinomycins A and N, has been synthesized[45] by the Arndt–Eistert route from β-hydroxyornithine. A biogenetically modelled synthesis has been described for (2*S*,3*S*,4*R*)-4-amino-3-hydroxy-2-methyl-n-valeric acid.[97]

[90] Y. Urabe, T. Okawara, K. Okumura, M. Miyoshi, and K. Matsumoto, *Synthesis*, 1974, 440.
[91] J. G. Cannon, J. P. O'Donnell, J. P. Rosazza, and C. R. Hoppin, *J. Medicin. Chem.*, 1974, **17**, 565.
[92] N. Zenker, V. H. Morgenroth, and J. Wright, *J. Medicin. Chem.*, 1974, **17**, 1223.
[93] M. Suzuki, T. Miyahara, R. Yoshioka, M. Miyoshi, and K. Matsumoto, *Agric. and Biol. Chem. (Japan)*, 1974, **38**, 1709.
[94] C. B. Warren, R. D. Minard, and C. N. Matthews, *J. Org. Chem.*, 1974, **39**, 3375.
[95] C. Shin, K. Nanjo, and J. Yoshimura, *Tetrahedron Letters*, 1974, 521.
[96] T. T. Otani and M. R. Briley, *J. Pharm. Sci.*, 1974, **63**, 1253.
[97] T. Yoshioka, T. Hara, T. Takita, and H. Umezawa, *J. Antibiotics*, 1974, **27**, 356.

Aromatic and Heteroaromatic Amino-acids.—Important new approaches to the synthesis of β-aryl- and β-heteroaryl-alanines, $ArCH_2CH(NH_2)CO_2H$,[57] and α-methyl analogues[93] have been described. In an improved synthesis of DL-histidine[57] an N-protected acyloxymethyl-imidazole (14) is condensed with diethyl acetamidomalonate; and the same intermediate is used in a synthesis of α-methylhistidine by condensation with ethyl isocyanopropionate.[93] α-Methyl-tryptophan and α-methyl-dopa syntheses have also been recorded,[93] employing



(14)



(15) n = 1 or 2; R^1 = H, R^2 = OH or R^1 = OH, R^2 = H

isocyanopropionate esters and gramine methiodide and O-protected 3,4-dihydroxybenzyl bromides, respectively. Cyclic analogues (15) of α-methyl-dopa have been synthesized[91] and employed in studies of the mode of action of the amino-acid.

Synthetic analogues of L-dopa and their biological evaluation have been reviewed.[98]

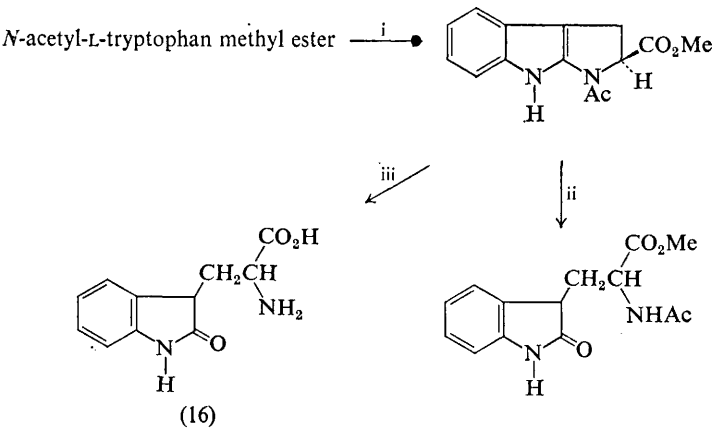
A new synthesis of 2′-mercapto-DL-histidine[99] and syntheses of DL-α-(2-thiazolyl)-glycines[100] illustrate continuing minor improvements in synthetic methods.

Most of the new analogues of aromatic and heteroaromatic amino-acids reported this year, as in previous years, have been prepared by substitution and other elaboration reactions of the protein amino-acids. Ring substitution of phenylalanine is readily brought about through straightforward procedures, but mixtures of products are often troublesome to separate. Pure *p*-chlorophenylalanine is best obtained[101] through a roundabout route; nitration gives a mixture of 55% *p*-nitro-, 25% *m*-nitro-, and 20% *o*-nitro-phenylalanines from which the *p*-isomer is conveniently separated, reduced, and the product obtained through application of the Sandmeyer reaction. *N*-Benzyloxycarbonyl-L-tyrosine and -DL-α-methyltyrosine have been converted into their 3-(hydroxymethyl) derivatives through reaction with formaldehyde;[102] a quite different approach to DL-3-(hydroxymethyl)tyrosine[67] uses 3-hydroxymethyl-4-hydroxybenzaldehyde as starting material for a conventional azlactone synthesis. Thyroxine analogues annelated between positions 3 and 4 of the 'outer' ring have been prepared.[103]

Tryptophan analogues of particular interest have been described by Witkop[104a] and by Wieland.[104b] 2-Hydroxy-L-tryptophan (16) is now more realistically

[98] A. Brossi, W. Pool, H. Sheppard, J. J. Burns, A. Kaiser, R. Bigler, G. Bartholini, and A. Pletscher, *Adv. Neurol.*, 1974, **5**, 291.
[99] J. Fernandez-Bolanos, D. Martinez Ruiz, and M. Menendez Gallego, *An. Quim.*, 1974, **70**, 94.
[100] M. Hatanaka and T. Ishimaru, *Bull. Chem. Soc. Japan*, 1973, **46**, 3600.
[101] R. A. Houghten and H. Rapoport, *J. Medicin. Chem.*, 1974, **17**, 556.
[102] M. Atkinson, D. Hartley, L. H. C. Lunts, and A. C. Ritchie, *J. Medicin. Chem.*, 1974, **17**, 248.
[103] M. T. Cox, W. G. Bowness, and J. J. Holohan, *J. Medicin. Chem.*, 1974, **17**, 1125.
[104] (*a*) M. Ohno, T. F. Spande, and B. Witkop, *J. Org. Chem.*, 1974, **39**, 2635; (*b*) T. Wieland, M. P. Jordan de Urries, H. Indest, H. Faulstich, A. Gieren, M. Sturm, and W. Hoppe, *Annalen*, 1974, 1570.

available through reactions shown in Scheme 5,[104a] and the 2-ethylsulphinyl-L-tryptophan residue present in a toxic phalloidin has the (*R*)-configuration at sulphur, as shown through synthesis from tryptophan by treatment with ethanesulphenyl chloride, oxidation using hydrogen peroxide, and separation of the



(16)

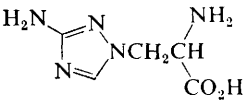
Reagents: i, Bu^t OCl; ii, 20% aq. AcOH, room temperature; iii, 6N-HCl, 110 °C

Scheme 5

diastereoisomeric sulphoxides; that which was identical with the natural amino-acid (comparison of c.d. curves) was shown by *X*-ray analysis to be the (*R*)-sulphoxide.[104b]

α-Methylphenylalanine has been used as starting material for the synthesis of DL-3-(5-benzimidazolyl)-2-methylalanine;[92] *N*-acetyl-L-aspartic-α-thioamide β-methyl ester, on condensation with diethyl bromoacetal, gives L(+)-β-(2-thiazolyl)-β-alanine, identical with a component of bottromycin.[105]

Further studies of the use of enzyme preparations from *Pisum sativum* (and similar extracts from water melon and *Leucaena leucoephala*) for the conversion of *O*-acetyl serine and a five-membered nitrogen heterocycle into a (1-heteroaryl)-alanine have been reported,[106] 3-amino-1,2,4-triazole giving (17).



(17)

N-Substituted Amino-acids.—This section deals with *N*-methyl and *N*-hydroxy-amino-acids synthesized for their importance as natural products; N-protected amino-acids as intermediates in peptide synthesis are excluded.

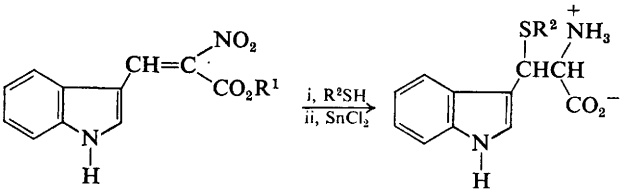
[105] Y. Seto, K. Torii, K. Bori, K. Inabata, S. Kuwata, and H. Watanabe, *Bull. Chem. Soc. Japan*, 1974, **47**, 151.

[106] I. Murakoshi, F. Kato, and J. Haginiwa, *Chem. and Pharm. Bull. (Japan)*, 1974, **22**, 480.

Condensation of L-ornithine with *S*-methyl-iso-*N*-methylthiourea gives N^7-methyl-L-arginine.[107]

The announcement last year (Volume 6, p. 5) that N^5-hydroxy-L-arginine from natural sources possesses antimicrobial properties has been followed by descriptions of methods for its synthesis.[58, 108] Methyl 2-acetamido-5-iodovalerate can be converted into the 5-(α-phenylnitrone) which, by selective hydrolysis, gives N^2-acetyl-N^5-hydroxyornithinamide; reaction with *S*-methyl isothiourea gives N^5-hydroxy-DL-arginine.[58] The relatively limited range of alternative methods available for the synthesis of hydroxylamines has been well tried in recent years, and further examples are provided this year for the synthesis of N^4-hydroxy-L-lysine (a component of mycobactins),[109] and O-protected α-hydroxylamino-acids.[110]

Amino-acids containing Sulphur.—An entry into series of β-mercapto analogues of some of the protein amino-acids is provided by the sequence (18) → (19),[111] illustrating the synthesis of tryptophan analogues.



(18) (19) R^2 = Ac, $PhCH_2$, or H

S-(Pyrimidin-2-yl)-L-cysteine may be synthesized by reaction of *N*-benzyloxycarbonyl-*O*-toluene-*p*-sulphonyl-L-serine *p*-nitrobenzyl ester with the sodium salt of 2-mercaptopyrimidine, though a partly racemized protected product is obtained;[112] 2-chloro-L-alanine gives racemic product with the mercaptopyrimidine but enzymic combination of these reactants gives optically pure material.[112]

A List of α-Amino-acids which have been Synthesized for the First Time

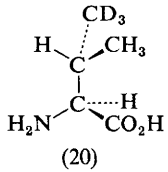
Compound [a]	*Ref.*
L-*threo*-2-Amino-3-hydroxyhex-4-ynoic acid	30
L-*erythro*-2-Amino-3-hydroxyhex-4-ynoic acid	30
(2*S*,3*S*,4*S*)-3-Hydroxy-4-methylglutamic acid	33
L-2-Amino-4-chloropent-4-enoic acid	34
3-(3-Amino-3-carboxypropyl)uridine	39
L-2-Amino-4-(methylphosphino)butyric acid	43
3-(2,5-Dimethoxyphenyl)alanine	65
3-(2,5-Dimethoxy-4-methylphenyl)alanine	65
3-(4-Bromo-2,5-dimethoxyphenyl)alanine	65

[107] J. L. Corbin and M. Reporter, *Analyt. Biochem.*, 1974, **57**, 310.
[108] K. Widmer and W. Keller-Schierlein, *Helv. Chim. Acta*, 1974, **57**, 657.
[109] Y. Isowa and M. Ohmori, *Bull. Chem. Soc. Japan*, 1974, **47**, 2672.
[110] T. Polonski and A. Chimiak, *Tetrahedron Letters*, 1974, 2453; T. Kolasa and A. Chimiak, *Tetrahedron*, 1974, **30**, 3591.
[111] L. K. Vinograd, O. D. Shalygina, N. P. Kostyuchenko, and N. N. Suvorov, *Khim. geterotsikl. Soedinenii*, 1974, 1236.
[112] A. Holy, I. Votruba, and K. Jost, *Coll. Czech. Chem. Comm.*, 1974, **39**, 634.

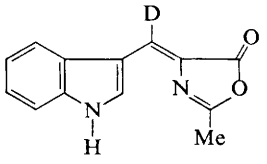
2,6-Dibromo-L-dopa	66
5,6-Dibromo-L-dopa	66
2,3,5-Tribromo-L-dopa	66
3,5,3′-Trimethyl-L-thyronine	114
3,5-Dimethyl-3′-iodo-L-thyronine	114
3,5′-Dimethyl-3′-isopropyl-L-thyronine	114
S-Adenosyl-L-homocysteine sulphoxide [b]	113*a*
S-Adenosyl-L-homocysteine sulphone	113*a*
S-4-Chloro[(β-D-ribofuranosyl)imidazo(4,5-*c*)pyrid-5′-yl]-L-homocysteine	113*b*
S-4-Amino[(β-D-ribofuranosyl)imidazo(4,5-*c*)pyrid-5′-yl]-L-homocysteine	113*b*
S-4-Methylamino[(β-D-ribofuranosyl)imidazo(4,5-*c*)pyrid-5′-yl]-L-homocysteine	113*b*
S-4-Dimethylamino[(β-D-ribofuranosyl)imidazo(4,5-*c*)pyrid-5′-yl]-L-homocysteine	113*b*
4-(1,4-Benzodioxan-6-yloxy)-3,5-di-iodo-L-phenylalanine	103

[a] Other new amino-acids, and labelled analogues of known amino-acids, mentioned elsewhere in this Chapter, are not repeated in this Table. [b] Both (*R*) and (*S*) sulphoxides synthesized and separated.

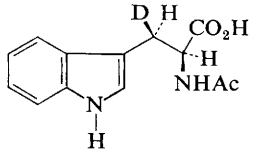
Labelled Amino-acids.—Full details have been published [69] of a route to labelled valines, described last year for the synthesis of (2*RS*,3*S*)-[4,4,4-$^2H_3$]-valine (Volume 6, p. 21) and now extended to the (2*RS*,3*S*)- and (2*RS*,3*R*)-[4-^{3}H]- and (2*RS*,3*S*)-[4-^{13}C]-analogues. A synthesis of (2*S*,3*S*)-[4,4,4-$^2H_3$]-valine (20) has been described [115] using the route established [116] for the [4-^{13}C]-analogue (Volume



(20)



(22)



(21)

6, p. 21) but with CD_3I used in place of $^{13}CH_3I$. (3*R*) and (3*S*) forms of [3-^{2}H]-tryptophan [*e.g.* (21), the 2*S*,3*R*-isomer] have been synthesized [68] by hydrogenation of the *Z*-arylidene-oxazolone (22), followed by hydrolysis. The hydrogenation step proceeds with greater than 95% *cis*-stereospecificity. The corresponding [3-^{3}H]tryptophans have been prepared by the same route.[68]

Deuteriation (with D_2–Pd or $NaBD_4$) of 2-bromo-, 4-bromo-, 2,5-dibromo-, and 3,4-dibromo-phenylalanines gives the corresponding ring-labelled amino-acids.[117]

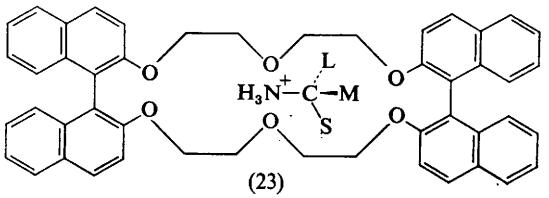
[113] (*a*) R. T. Borchardt and Y. S. Wu, *J. Medicin. Chem.*, 1974, **17**, 862; (*b*) R. T. Borchardt, J. A. Huber, and Y. S. Wu, *ibid.*, p. 868.
[114] E. C. Jorgensen, W. T. Murray, and P. Block, *J. Medicin. Chem.*, 1974, **17**, 434.
[115] H. Kleunder, F.-C. Huang, A. Fritzberg, H. Schnoes, C. J. Sih, P. Fawcett, and E. P. Abraham, *J. Amer. Chem. Soc.*, 1974, **96**, 4054.
[116] H. Kleunder, C. H. Bradley, C. J. Sih, P. Fawcett, and E. P. Abraham, *J. Amer. Chem. Soc.*, 1973, **95**, 6149.
[117] H. Faulstich and H. Trischmann, *Analyt. Biochem.*, 1974, **62**, 615.

α-Methyldopa carrying a β-^{13}C label has been synthesized[63] from 3,4-dibenzyloxyphenyl-lithium by reaction with $^{13}CO_2$, and successive conversion into the aldehyde, 1-(3,4-dibenzyloxyphenyl)-2-nitropropene, and then the benzyl methyl ketone, from which the DL-amino-acid was prepared *via* the hydantoin route. 2-(Methylthio)[1-^{14}C]acetic acid has been converted into [3-^{14}C]methionine, *via* the correspondingly labelled 2-(methylthio)ethyl chloride and acetamidomalonate.[59] L-[3-^{14}C]Serine, on condensation with substituted indoles mediated by *E. coli* tryptophan synthetase, gives side-chain ^{14}C-labelled tryptophan analogues.[24]

The distribution of the ^{14}C label in lysine may be elucidated through permanganate oxidation to γ-aminopentanoic acid, γ-aminobutyric acid, β-alanine, and glycine, and assessing the ^{14}C content of these and the ninhydrin decarboxylation product of glycine formed in this way.[118] The ^{14}C content of methionine labelled in the methyl group may be determined through assay of $^{14}CH_3SCN$ formed by its treatment with CNBr.[119]

^{13}N-Labelled glutamic acid and glutamine have been prepared from $^{13}NH_3$ by enzymic synthesis.[120] DL-Lysine labelled either at N^{α} or N^{ϵ} with ^{15}N has been synthesized from potassium [^{15}N]phthalimide by reaction with $EtO_2CCHBr(CH_2)_4NHCOPh$ or with 5-(4-bromobutyl)hydantoin, respectively.[64]

Resolution of Amino-acids.—A larger number of papers than usual has appeared this year, partly due to progress in the design of chiral complexing agents capable of differentiation between enantiomers. Hexafluorophosphate salts of α-amino-acid esters have been shown to be resolvable by selective complexation with 3,3′-bis(hydroxymethyl)-2,2′-dihydroxy-1,1′-binaphthyl,[121] and pyridyl-bridged analogues.[122] The total optical resolution of an α-amino-acid ester by liquid–liquid chromatography based on the selective complexation principle has been further illustrated[123] using aqueous $NaPF_6$ or $LiPF_6$ on Celite as stationary phase, with a chloroform solution of the (*R*,*R*)-macrocycle (23) as mobile phase.



(23)

The mode of complexation of a chiral amine is shown in (23), and methyl *p*-hydroxyphenylglycinate is efficiently resolved by the technique;[123] the molecular architecture of the complexing agent has specific limitations if it is to recognize

[118] I. J. Christensen, P. O. Larsen, and B. L. Moller, *Analyt. Biochem.*, 1974, **60**, 531.
[119] B. R. Clark, H. Ashe, R. M. Halpern, and R. A. Smith, *Analyt. Biochem.*, 1974, **61**, 243.
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[121] R. C. Helgeson, J. M. Timko, P. Moreau, S. C. Peacock, J. M. Mayer, and D. J. Cram, *J. Amer. Chem. Soc.*, 1974, **96**, 6762.
[122] M. Newcomb, G. W. Gokel, and D. J. Cram, *J. Amer. Chem. Soc.*, 1974, **96**, 6810.
[123] L. R. Sousa, D. H. Hoffmann, L. Kaplan, and D. J. Cram, *J. Amer. Chem. Soc.*, 1974, **96**, 7100.

one enantiomer preferentially, and the broader basis of complexation by chiral crown ether complexes has been reviewed.[124] To use the authors' words, 'a molecular basis has been provided by these studies for building an amino ester resolving machine'.[121] The principle is likely to be illustrated often in natural products, since there are indications that relatively simple structural requirements must be met; thus, *cyclo*-(L-Pro-Gly)$_n$ (n = 3 or 4) forms complexes with D- and L-amino-acid ester salts,[125] involving the carbonyl groups of the cyclic peptide and the protonated amino-group of the salt, and ^{13}C n.m.r. resonances for several carbon atoms of the complexed D-enantiomer are shifted relative to those of the L-enantiomer. This is taken as evidence for enantiomeric differentiation.[125]

Arising from a study of the transport of amino-acids through organic liquid membranes (toluene separating two aqueous phases),[126] the suggestion is made that chirospecific transport could be exploited in a novel resolution technique employing a chiral organic membrane.

A series of papers has appeared dealing with a more conventional ligand-exchange principle for the resolution of amino-acids.[127] Chloromethylated polystyrene treated with an L- or D-amino-acid ester, and hydrolysis of the product, provides a chiral phase which, after co-ordination to copper(II) ions, is suitable for column chromatographic resolution of amino-acids. Powdered paper is advocated for the column chromatographic resolution of DL-tryptophan-α-^{14}C,[128] and paper impregnated with alginic acid and silica gel provides an ion-exchange medium for the resolution of amino-acids.[129]

Leaving discussion of gas–liquid chromatographic resolution to Section 6 of this Chapter, standard techniques are illustrated in the use of *N*-carvomenthoxyacetyl derivatives for resolution by fractional crystallization,[130] differentiation by α-chymotrypsin between D- and L-*N*-acyl phenylalanine esters,[131] and resolution of diaminopimelic acid as the bis-benzyloxycarbonyl derivative by treatment with aniline in the presence of papain, to give the crystalline L,L-monoanilide.[132]

4 Physical and Stereochemical Studies of Amino-acids

Crystal Structures of Amino-acids.—(See also Chapter 2, Part II.) Precision neutron diffraction studies of α-amino-acids continue to be reported, with definitive hydrogen locations and conformational features of L-valine hydrochloride,[133] hippuric acid,[134] and L-cystine dihydrochloride.[135]

[124] D. J. Cram and J. M. Cram, *Science*, 1974, **183**, 803.
[125] C. M. Deber and E. R. Blout, *J. Amer. Chem. Soc.*, 1974, **96**, 7566.
[126] J. P. Behr and J.-M. Lehn, *J. Amer. Chem. Soc.*, 1974, **96**, 6108.
[127] V. A. Davankov, S. V. Rogozhin, and A. V. Semechkin, *J. Chromatog.*, 1974, **91**, 493; I. Peslekas, S. V. Rogozhin, and V. A. Davankov, *Izvest. Akad. Nauk S.S.S.R., Ser. khim.*, 1974, 174, 1872; *Zhur. obshchei Khim.*, 1974, **44**, 468.
[128] L. V. Handes, R. Kido, and M. Schmaeler, *Prep. Biochem.*, 1974, **4**, 47.
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[130] K. Witkiewicz, F. Rulko, and Z. Chabudzinski, *Roczniki Chem.*, 1974, **48**, 651.
[131] M. S. Matta, J. A. Kelley, A. J. Tietz, and M. F. Rohde, *J. Org. Chem.*, 1974, **39**, 2291.
[132] A. Arendt, A. Kolodziejczyk, T. Sokolowska, and E. Szufler, *Roczniki Chem.*, 1974, **48**, 635.
[133] T. F. Koetzle, L. Golic, M. S. Lehmann, J. J. Verbist, and W. C. Hamilton, *J. Chem. Phys.*, 1974, **60**, 4690.
[134] M. Currie and A. L. MacDonald, *J.C.S. Perkin II*, 1974, 784.
[135] S. C. Gupta, A. Sequeira, and R. Chidambaram, *Acta Cryst.*, 1974, **B30**, 562.

X-Ray crystal analysis of other naturally occurring amino-acids reported during the year deal with DL-serine and anhydrous L-serine,[136] calcium L-glutamate trihydrate,[137] pyroglutamic acid,[138] 3,5,3′-tri-iodo-L-thyronine,[139] L-thyronine ethyl ester hydrochloride monohydrate,[140] and D-β-tyrosine hydrobromide and hydrochloride.[141] L-Cysteine is bound to methylmercury(II) *via* a deprotonated thiol group, whereas L-methionine is co-ordinated *via* nitrogen, in their respective 1 : 1 complexes.[142]

Proof of structure for (2*S*,3*S*,4*R*)-4-amino-3-hydroxy-2-methyl-n-valeric acid[143] (a component of bleomycins), and (2*S*,3*R*,4*S*)-2-amino-3-methyl-4-hydroxy-n-valeric acid[12] (a component of γ-amanatin) has been supplied by *X*-ray analysis, for the latter in the form of its lactone hydrobromide. The crystal structure of the potassium salt of *N*-(purin-6-ylcarbamyl)-L-threonine, isolated in 1969 from the total tRNA of yeast, has been determined[144] for its relevance to the conformations of anticodon loops of tRNA, and the base pairing and base stacking interactions of modified nucleosides.

Less-common amino-acids and their derivatives studied include: bis copper(II) D-penicillamine disulphide nonahydrate,[145] L-mimosine sulphate hydrate,[146] meso-3,3′-dithiobisvaline dihydrate,[147] meso-lanthionine dihydrochloride,[148] L-thioproline,[149] *N*-acetyl-L-norvaline,[150] N^{α}-acetyl-L-glutamine,[151] and *N*-benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester.[152]

3,4-Dehydro-DL-proline readily dimerizes;[153] *X*-ray crystal analysis shows that dimerization leads to only D,D- and L,L-stereoisomers.

N.M.R. Spectroscopy.—^{1}H N.m.r. studies of amino-acids and their derivatives continue to provide information on fundamental structural features, such as protonation equilibria for L-cysteine as a function of pH,[154] and ligand sites in mercury(II) complexes of cysteine, cysteine methyl ester, and *S*-methyl-cysteine.[155] Conformational studies of a familiar type, employing both ^{1}H and ^{13}C n.m.r., deal with *N*-formyl alanine amide and *N*-methylamide,[156] *N*-acetyl-*N*-methyl

[136] T. J. Kistenmacher, G. A. Rand, and R. E. Marsh, *Acta Cryst.*, 1974, **B30**, 2573.
[137] H. Einspahr and C. E. Bugg, *Acta Cryst.*, 1974, **B30**, 1037.
[138] V. Pattabhi and K. Venkatesan, *J.C.S. Perkin II*, 1974, 1085.
[139] V. Cody, *J. Amer. Chem. Soc.*, 1974, **96**, 6720.
[140] A. Camerman and N. Camerman, *Canad. J. Chem.*, 1974, **52**, 3042.
[141] A. N. Chekhlov, Y. T. Struchkov, and A. I. Kitaigorodskii, *Kristallografiya*, 1974, **19**, 981.
[142] Y. S. Wong, N. J. Taylor, P. C. Chieh, and A. J. Carty, *J.C.S. Chem. Comm.*, 1974, 625.
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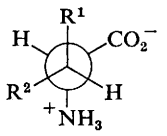
[144] R. Parthasarathy, J. M. Ohrt, and G. B. Chheda, *J. Amer. Chem. Soc.*, 1974, **96**, 8087.
[145] J. A. Thich, D. Mastropaolo, J. Potenza, and H. J. Schugar, *J. Amer. Chem. Soc.*, 1974, **96**, 726.
[146] A. Mostad, E. Rosenqvist, and C. Romming, *Acta Chem. Scand.* (*B*), 1974, **28**, 249.
[147] L. G. Warner, T. Ottersen, and K. Seff, *Acta Cryst.*, 1974, **B30**, 1077.
[148] R. E. Rosenfield and R. Parthasarathy, *J. Amer. Chem. Soc.*, 1974, **96**, 1925.
[149] K. K. Chacko, *Cryst. Struct. Comm.*, 1974, **3**, 561.
[150] G. Lovas, A. Kalman, and G. Argay, *Acta Cryst.*, 1974, **B30**, 2882.
[151] M. R. Narasimhamurthy, K. Venkatesan, and F. Winkler, *Cryst. Struct. Comm.*, 1974, **3**, 743.
[152] V. M. Coiro, F. Mazza, and G. Mignucci, *Acta Cryst.*, 1974, **B30**, 2607.
[153] I. L. Karle, H. C. J. Ottenheym, and B. Witkop, *J. Amer. Chem. Soc.*, 1974, **96**, 539.
[154] D. B. Walters and D. E. Leyden, *Analyt. Chim. Acta*, 1974, **72**, 275.
[155] G. A. Neville and T. Drakenberg, *Canad. J. Chem.*, 1974, **52**, 616.
[156] V. N. Solkan and V. F. Bystrov, *Izvest. Akad. Nauk S.S.S.R., Ser. khim.*, 1974, 1308.

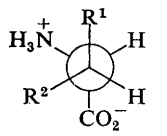
alanine methyl ester,[157] and Boc-glycine.[158] A study of the pH dependence of vicinal coupling constants for histidine and its N^{im}-benzyl, N^{α}-acyl, and *O*-methyl derivatives indicates a *gauche* conformation for histidine in basic solution[159] due to electrostatic interaction between the carboxy and imidazole groups, but in acidic or isoelectric solutions 'the ions show equally populated conformations' (*sic*). ^{1}H N.m.r. data for proline[160, 161] and for hydroxy-L-proline and the *allo*-diastereoisomer[162] have been interpreted in terms of conformational mobility of the pyrrolidine ring[160, 161] and in terms of the torsion angle of the plane of the carboxy-group (around the C_{α}–C^{O} axis),[162] respectively. A study of amino-acids in the solid state has been published.[163]

An extensive literature is accumulating on ^{13}C n.m.r. characteristics of the common amino-acids. Data for eight amino-acids are available,[164] and exploration of some of the factors determining chemical shifts has yielded ^{13}C n.m.r. titration curves of individual carbon atoms of representative amino-acids, with interpretation of the observed shifts.[165] ^{13}C Spin lattice relaxation times T_1 of several amino-acids as a function of pD and of concentration,[166] including data for glycine and lysine,[167] reveal a strong dependence of carboxy carbon T_1 on these parameters, accounted for[166] by intermolecular association.

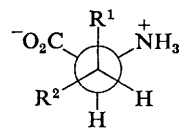
^{13}C–C–C–^{1}H Coupling constants for the carboxy carbon and β-hydrogen atoms in amino-acids give information on side-chain conformation which is not available from ^{1}H–^{1}H coupling constants alone.[168] Data for 1M solutions of amino-acids (^{13}C in natural abundance) are interpreted[168] to show that aspartic acid exists at pH 11 in conformations (24), (25), and (26) ($R^1 = CO_2H$, R^2 = Me)



(24)



(25)



(26)

in proportions 15 : 62 : 23, and that for valine at pH 5.7, the respective proportions ($R^1 = R^2$ = Me) are 17 : *ca.* 60 : *ca.* 20. ^{13}C–^{13}C Coupling constants for ^{13}C-enriched amino-acids[169] (including 85% ^{13}C-enriched alanine, valine, leucine, and isoleucine[170]) have been reported, as has their dependence on pH,[170] though

[157] M. Goodman, F. Chen, and C. Y. Lee, *J. Amer. Chem. Soc.*, 1974, **96**, 1479.
[158] M. Branik and H. Kessler, *Tetrahedron*, 1974, **30**, 781.
[159] R. C. Weinkam and E. C. Jorgensen, *J. Amer. Chem. Soc.*, 1974, **96**, 6084.
[160] M. Ellenberger and L. Pogliani, *Biochem. Biophys. Res. Comm.*, 1974, **58**, 613.
[161] M. Ellenberger, L. Pogliani, K. Haeuser, and J. Valat, *Chem. Phys. Letters*, 1974, **27**, 419.
[162] L. Pogliani and M. Ellenberger, *J. Amer. Chem. Soc.*, 1974, **96**, 1621.
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[164] W. Voelter, S. Fuchs, R. H. Seuffer, and K. Zech, *Monatsh.*, 1974, **105**, 1110.
[165] A. R. Quirt, J. R. Lyerla, I. R. Peat, J. S. Cohen, W. F. Reynolds, and M. H. Freedman, *J. Amer. Chem. Soc.*, 1974, **96**, 570.
[166] I. M. Armitage, H. Huber, H. Pearson, and J. D. Roberts, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 2096.
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[168] J. Feeney, P. E. Hansen, and G. C. K. Roberts, *J.C.S. Chem. Comm.*, 1974, 465.
[169] J. A. Sogn, L. C. Craig, and W. A. Gibbons, *J. Amer. Chem. Soc.*, 1974, **96**, 4694.
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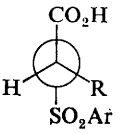
their interpretation in terms of electronic structure and conformation cannot yet be attempted.[169]

Magnetic resonance studies of nitrogen nuclei are still in their pioneering phase, and recent papers deal with ^{14}N n.m.r. of amino-acids, peptides, and derivatives in 0.2M-aqueous solution,[171] ^{14}N,^{2}H pure nuclear quadrupole resonance of deuteriated amino-acids at 77 K,[172] and natural-abundance ^{15}N n.m.r. of eight amino-acids, including L-arginine,[173] for which the pH-dependence of ^{15}N chemical shifts may be related to various ionized species.

O.R.D. and C.D. Spectra.—Studies of the chiroptical properties of amino-acids are being entered into with more scope for interpretation of spectra in terms of conformational isomerism; excepting a study of transitions in the far-u.v., all the more superficial collecting of data has already been carried out for amino-acids. Recent studies of aromatic amino-acids (D-phenylglycine,[174] α-methyl-L-tyrosine,[175] and *p*-hydroxy-D-phenylglycine [176]) include a thorough analysis of the conformations in solution of *p*-hydroxyphenylglycine and its amide; judged by o.r.d. and c.d. data, torsion angles ψ near $-5°$ and χ near 75° are assumed by these compounds.[176]

A good deal of data collected for L-cystine can be interpreted in terms of the chirality of the disulphide chromophore, and the near-u.v. c.d. of solutions of this amino-acid has been analysed in terms of conformer populations.[177] Effects of temperature and salt concentration on the c.d.[178, 179] and o.r.d.[179] of *N*-acetyl-L-alanine *N'*-methylamide [178, 179] and other alanine derivatives [178] have been studied. The *N*-acetyl *N'*-methylamide in 1,2-dichloroethane adopts an intramolecularly hydrogen-bonded conformation at lower temperatures and an increasing amount of a non-hydrogen-bonded form appears as the temperature of the solution is raised,[179] ΔH^0 for the transition between the two forms being 2570 ± 5 cal mol^{-1} and $\Delta S^0 = 6.56 \pm 0.1$ e.u.[179]

Dansyl-L-amino-acids show a positive Cotton effect centred near 260 nm in MeOH, in most cases studied.[180] The assumption [180] that conformation (27) is adopted by dansyl-L-amino-acids showing a positive Cotton effect, but that an alternative staggered conformation (28) accounts for a negative Cotton effect, appears to need more justification than has been provided.



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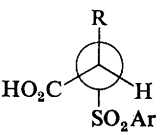

(28)

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[172] M. J. Hunt and A. L. Mackay, *J. Magn. Resonance*, 1974, **15**, 402.
[173] T. Suzuki, T. Yamaguchi, and M. Imanari, *Tetrahedron Letters*, 1974, 1809.
[174] S. Takagi, H. Nomori, and M. Hatano, *Chem. Letters*, 1974, 611.
[175] W. J. Goux, D. B. Cooke, R. E. Rodriguez, and T. M. Hooker, *Biopolymers*, 1974, **13**, 2315.
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[178] W. L. Mattice, *Biopolymers*, 1974, **13**, 169.
[179] G. M. Crippen and J. T. Yang, *J. Phys. Chem.*, 1974, **78**, 1127.
[180] T. Polonski, A. Chimiak, and M. Kochman, *Tetrahedron*, 1974, **30**, 641.

Mass Spectrometry.—The two categories into which mass spectrometric studies of amino-acids might be divided, one covering interpretation of fragmentation modes, the other dealing with analytical applications, are both represented this year. The mass spectra of dimethyl glutamate and its deuteriated derivatives [181] indicate sequential loss of MeOCO and MeOH giving a prominent *m/e* 116 peak, and base peak *m/e* 84, respectively, the latter being formed by hydrogen transfer from C-3 with the formation of a protonated pyrrol-5(4*H*)-one. The mass spectrum of arginine obtained using fast heating rates at the probe shows an $M + 1$ peak (*m/e* 175), while slower heating rates give spectra with highest-mass fragments shown to be *m/e* 158 and 157 (loss of NH_3 or H_2O, respectively, from parent ion);[182] an abundant *m/e* 115 peak may be useful in interpretation of the mass spectra of underivatized arginine peptides. High-pressure chemical ionization mass spectrometric studies continue [183] with studies of association occurring between valine and proline with their protonated ions under CIMS conditions; it is suggested [183] that such studies will contribute knowledge of the energetics of ions of biologically important molecules in their biological environments.

Analytical studies by g.c.–m.s. of volatile derivatives of amino-acids deal with β-aminoisobutyric acid in urine at 1 ng levels,[184] and 12 amino-acids in biological samples [185] (quadrupole m.s. of *N*-trifluoroacetyl n-butyl esters); also of *N*-methylamino-acids present in hydrolysates of actomyosin from heart-cell cultures.[186] Trimethylsilyl derivatives have been employed in similar studies, for the analysis of thyroxine at 100 pg levels,[187] and common amino-acids at 10—100 femtomole levels.[188]

Other Physical and Theoretical Studies.—Non-routine physical studies (and their possible value in a biological context) include surface tension of solutions in 0.1M-NaCl (comparison of hydrophobicity of side-chains),[189] dielectric increments of homologous series of αω-amino-acids (adoption of extended conformations),[190] depolarized Rayleigh scattering (ψ for glycine is $170 \pm 10°$),[191] polarized i.r. of N-deuteriated L-alanine,[192] and studies of the temperature dependence of u.v. spectra of tyrosine and tryptophan [193] and of *N*-acetyltryptophan ethyl ester and tyrosine and phenylalanine analogues (in comparison with temperature effects on protein spectra).[194]

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[184] W. E. Pereira, R. E. Summons, W. E. Reynolds, T. C. Rindfleisch, and A. M. Duffield, *Clin. Chim. Acta*, 1973, **49**, 401.
[185] R. E. Summons, W. E. Pereira, W. E. Reynolds, T. C. Rindfleisch, and A. M. Duffield, *Analyt. Chem.*, 1974, **46**, 582.
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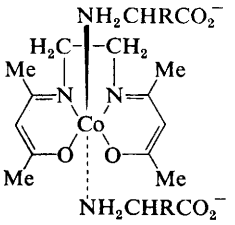
[187] R. Hoffenberg, A. M. Lawson, D. B. Ramsden, and P. J. Raw, in ref. 186, p. 303.
[188] F. P. Abramson, M. W. McCaman, and R. E. McCaman, *Analyt. Biochem.*, 1974, **57**, 482.
[189] H. B. Bull and K. Breese, *Arch. Biochem. Biophys.*, 1974, **161**, 665.
[190] J. T. Edward, P. G. Farrell, and J. L. Job, *J. Amer. Chem. Soc.*, 1974, **96**, 902.
[191] C. Destrade and C. Clement, *Compt. rend.*, 1974, **278**, *C*, 733.
[192] R. F. Adamowicz and M. L. Sage, *Spectrochim. Acta*, 1974, **30A**, 1007.
[193] A. P. Demchenko and V. L. Zima, *Ukrain. Biokhim. Zhur.*, 1974, **46**, 345.
[194] A. Cooper, *F.E.B.S. Letters*, 1974, **48**, 101.

MO calculations of conformations adopted by γ-aminobutyric acid [195, 196] and alanine [196] present different conclusions from those published earlier; notably,[195] γ-aminobutyric acid adopts a highly folded structure.

Determination of Absolute Configuration and Optical Purity of Amino-acids.—A modification of Horeau's procedure has been described,[197] so that it may be applied to amines and amino-acids. 1-(2-Phenylbutyroyl)imidazole is advocated as reagent for the new procedure, and its validity was established using esters of alanine, leucine, and tryptophan.[197]

The optical purity of samples of amino-acids available only in 50–100 mg quantities can be determined by exploiting the greatly enhanced rotation resulting from the equilibration of the sample with $K_2[Co(acac_2en)(Gly)_2]$ to give an equilibrium concentration of the complex (29) after *ca.* 10 h at pH 10.[198] Comparison of observed specific rotations with those for standards prepared from



(29)

optically pure samples under identical conditions gives a measure of the optical purity of the test sample. An enzymic method for the assessment of the optical purity of radioactively labelled L-amino-acids employs t.l.c. estimation of the α-keto-acid formed by D-amino-acid oxidase.[199]

5 Chemical Studies of Amino-acids

Racemization.—An extensive review has appeared dealing with amino-acid racemases.[200] The mechanism of the racemization of L-cystine (the only protein amino-acid to suffer racemization during the hydrolysis of proteins with 6N hydrochloric acid) is considered to involve the formation of an acid enol stabilized by the inductive effect of the partially or fully charged β-heteroatom;[201] some support for this hypothesis is given by the fact that *S*-(2,4-dinitrophenyl)-L-cysteine and 2,3-diaminopropionic acid both racemize at rates similar to that of L-cystine, in acid solutions.[201]

Continuation of studies described last year (Volume 6, p. 28) on the use of aspartic acid racemization as an index for the age of fossil bones leads to an estimate of ⩽50 000 years for the age of several Californian Paleo-Indian

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[196] S. Parthasarathy, M. M. Dhingra, and G. Govil, *Indian J. Chem.*, 1974, **12**, 805.
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[198] Y. Fujii and H. Yoneda, *Chem. Letters*, 1974, 43; Y. Fujii, *Bull. Chem. Soc. Japan*, 1974, **47**, 2856.
[199] M. J. Hardy, *Analyt. Biochem.*, 1974, **57**, 529.
[200] K. Soda, *Seikagaku*, 1974, **46**, 203.
[201] S. J. Jacobson, C. G. Wilson, and H. Rapoport, *J. Org. Chem.*, 1974, **39**, 1075.

skeletons.[202] The technique is useful for dating bones which are either too old or too small for radiocarbon dating.[203] Misgivings about the possible influence of the molecular environment of the amino-acid on the timekeeping of this novel chemical clock are largely overcome by the way in which the method is applied;[203] a bone which can be accurately dated by the radiocarbon technique is used to estimate the first-order racemization rate constant for the aspartic acid it contains, for a particular site, so that other bones at the same site can be dated on the basis of their D- and L-aspartic acid content.[203]

General Reactions.—Mutarotation of solutions of L-amino-acids in aqueous potassium carbonate is ascribed to carbamate formation.[204] The general trend towards more positive rotations with time may be the basis of a method of assigning absolute configuration to acid-sensitive amino-acids (*i.e.* amino-acids which would not survive the conditions required for the application of the Clough–Lutz–Jirgensons rule).[204]

General reactions which will enhance the value of amino-acids as intermediates in organic synthesis include conversion of amino-acid esters into α-alkoxycarbonyl diazoalkanes under mild conditions with *p*-nitro- or 2,4-dinitrobenzenediazonium tetrafluoroborate,[205] synthesis of α-fluoro-acids by diazotization of amino-acids in 70% HF in pyridine,[206] reduction with diborane to 2-aminoalkanols (reductive dehalogenation of ring-chloro- or ring-bromo-phenylalanines is avoided),[207] and dianion formation from *NN*-dimethylglycine followed by nucleophilic addition to benzophenone.[208]

Five mechanistic schemes can be considered for the Dakin–West synthesis of α-acetamidoalkyl methyl ketones from amino-acids with acetic anhydride and pyridine, and rate studies support [209] the mechanism [210] involving a 4-(pyrid-4-yl)oxazolone intermediate.

Interaction of amino-acids with *p*-benzoquinones provides a basis for colorimetric quantitation, previously assumed to involve charge-transfer complex formation but now shown [211] to lead to 2,5-disubstituted quinones (30).

Stereospecific transamination and isotopic exchange of the α-proton of an amino-acid (Scheme 6),[212] involving suprafacial proton transfer, depends on the ability of the bulky pyridyl and t-butyl groups to maintain conformation through the transition state.

Nitrosation studies of imines under mildly acidic conditions have been reported for proline,[213, 214] hydroxyproline,[214] sarcosine,[214] and *N*-acyl tryptophans,[215] a

[202] J. L. Bada, R. A. Schroeder, and G. F. Carter, *Science*, 1974, **184**, 791.
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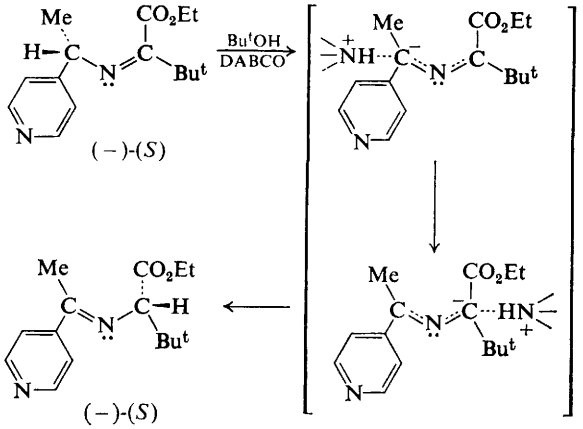
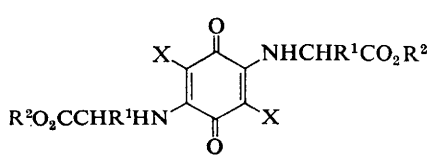
[215] R. Bonnett and R. Holleyhead, *J.C.S. Perkin I*, 1974, 962; see also K. L. Agarwal, G. W. Kenner, and R. C. Sheppard, *J. Chem. Soc.*, (*C*), 1969, 954.



Scheme 6

prime concern being the assessment of maximum amounts of *N*-nitroso-compounds which could be formed in the stomach or in stored food (less than 0.9 p.p.b. in canned meat [213]).

Reactions of amino-acids with carbohydrates include a preparation of 3-phenylfuran from phenylalanine and glucose,[216] an unusual Ugi reaction involving arabinose, glycine, and cyclohexyl isocyanide to give (31) and open-chain



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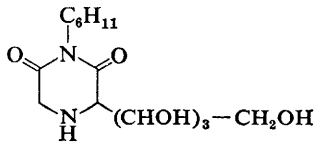

(31)

analogues,[217] and formation of a free radical derived from L-scorbamic acid through reaction of an amino-acid with dehydro-L-ascorbic acid.[218] Pyrolysis of alkyl amino-acids at 500 °C gives decarboxylation and condensation products including amines and pyridines.[219] Valine treated similarly, in the presence of tricaproin, gives caproamide, *N*-isobutylcaproamide, and capronitrile.[220]

Papers dealing with the synthesis of amino-acid derivatives for purposes other than peptide synthesis include vinyl ester synthesis by ester interchange,[221] *N*-methylation of Z- and Boc-amino-acids with MeI–Ag_2O–DMF,[222] synthesis of

[216] K. Misselhorn and H. Bruckner, *Chem., Mikrobiol., Technol. Lebensmitteln*, 1974, **3**, 25.
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[221] K. Geckeler and E. Bayer, *Chem. Ber.*, 1974, **107**, 1271.
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N-TFA amino-acid trimethylsilyl esters by successive treatment with Me_3SiCl and TFA-anhydride,[223] and *O*- and *N*-diethylborylation of amino-acids with Et_3B.[224] Comparison has been made[225] of the various reagents which may be used for the synthesis of trimethylsilyl derivatives of amino-acids. Most of the common *N*- and side-chain-protecting groups may be stripped from amino-acids with boron tribromide[226] or with trifluoromethanesulphonic acid,[227] without additional complications with methionine, tryptophan, or tyrosine.

Specific Reactions.—Syntheses of natural products starting from glutamic acid have been recorded for both enantiomers of disparlure (*cis*-7,8-epoxy-2-methyloctadecane),[228] for D-ribose,[229] and proline[85] and hydroxyproline.[71] A synthesis of trichotomine, the blue pigment from *Clerodendron trichotomum*, starts with L-tryptophan;[230] particularly interesting microbiological syntheses, the conversion of amino-acids into higher alcohols by auxotrophic mutants of *Saccharomyces cerevisiae*,[231] and the biosynthesis of linalool from leucine in *Cinnamomum camphora* var. *linalooliferium* (the first demonstration of isoprenoid biosynthesis in a higher plant),[232] have been reported.

Decarboxylation of tryptophan is achieved in 45% yield by heating the chelates obtained with cupric or zinc acetates;[233] decarboxylation kinetics have been established for *N*-salicylidene-DL-valine,[234] and $Cu(dpa)^{2+}$-catalysed hydrolysis of glycine methyl esters [dpa = bis-(2-pyridylmethyl)amine] has been studied.[235]

Aziridines react with methionine in dilute aqueous solutions at pH 7.4 to give sulphonium salts,[236] indicating a likely site for attack by biological alkylating agents. Under defined conditions, L-methionine-*S*- (or -*R*-) sulphoximine is converted into the sulphoxide with retention of configuration at sulphur, without affecting the α-amino-group.[237] Phosgene reacts with methionine at nitrogen, to give carbonylbis(L-methionine), which in the form of its bis(*p*-nitrophenyl ester) is advocated[238] as a cross-linking agent for insulin, removable by CNBr.

Specific Reactions of Amino-acids Related to Biochemical Processes.—Interactions between amino-acids and nucleotides which can be surmised to have occurred in the prebiotic milieu have been discussed.[239]

Oxidative transformations of aromatic amino-acids have received careful quantitative study, including the cyclization of dopa to dopachrome (32) *en*

[223] H. R. Kricheldorf and M. Fehrle, *Synthesis*, 1974, 420.
[224] R. Koster and E. Rothgery, *Annalen*, 1974, 112.
[225] S. V. Rogozhin, Y. A. Davidovich, S. M. Andreev, N. V. Mironova, and A. I. Yurtanov, *Izvest. Akad. Nauk S.S.S.R., Ser. khim.*, 1974, 1868.
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[233] T. Kametani, T. Suzuki, K. Takahashi, and K. Fukumoto, *Synthesis*, 1974, 131.
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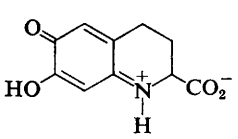
[235] R. Nakon, P. R. Rechani, and R. J. Angelici, *J. Amer. Chem. Soc.*, 1974, **96**, 2117.
[236] P. A. Capps and A. R. Jones, *J.C.S. Chem. Comm.*, 1974, 320.
[237] R. A. Stephani and A. Meister, *Tetrahedron Letters*, 1974, 2307.
[238] W. D. Busse and F. H. Carpenter, *J. Amer. Chem. Soc.*, 1974, **96**, 5947.
[239] C. Saxinger and C. Ponnamperuma, *Origins Life*, 1974, **5**, 189.

route to melanin,[240] autoxidation of tryptophan at 100 °C (maximum rate at pH 5.3 through the acid pH range),[241] and photosensitized oxygenation of tryptophan methyl ester to give (33).[242] Evidence for the formation of a 1 : 1



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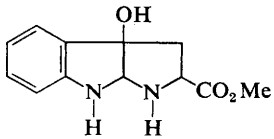

(33)

molecular complex between ATP and tryptophan, or *N*-acetyltryptophanamide, has been derived [243] from difference u.v. spectrometry.

Binding of Ca^{2+} ions by amino-acids in aqueous solutions has been studied.[244]

Effects of Electromagnetic Radiation on Amino-acids.—Reversible effects dealing with concentration dependence of fluorescence quenching of tyrosine,[245] and luminescence [246] and phosphorescence [247] studies of tryptophan, the latter in relation to the phosphorescence of a Trp residue at different protein locations, have been reported. Photoelectron quantum yields of representative amino-acids in the 180—240 nm wavelength range exhibit wavelength dependences similar to those of corresponding poly(amino-acids).[248]

Pulse radiolysis of selenomethionine,[249] and a study of radicals formed by γ-irradiation of solid threonine [250] and by photolysis of amino-acids in solution in the presence of ferricyanide ions or tris-(1,10-phenanthroline)iron,[251] are continuations of earlier studies of a conventional type, while an extraordinary implication has emerged from positron annihilation studies of amino-acids.[252] Since triplet intensities of positron annihilation time spectra of a D-amino-acid differ from those of its enantiomer, subsequent β-decay can lead to a minute initial asymmetry of otherwise racemic amino-acids, from which the natural state of affairs favouring one enantiomer could have developed.[252]

6 Analytical Methods

General.—An extensive survey of modern assay procedures, including several protein amino-acids, is available.[253] Microdetermination of amino-acids using

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[241] M. Stewart and C. H. Nicholls, *Austral. J. Chem.*, 1974, **27**, 205.
[242] M. Nakagawa, T. Kaneko, K. Yoshikawa, and T. Hino, *J. Amer. Chem. Soc.*, 1974, **96**, 624.
[243] F. Morita, *Biochim. Biophys. Acta*, 1974, **343**, 674.
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[245] J. Chrysochoos, *Spectroscopy Letters*, 1974, **7**, 235.
[246] J. Moan, *J. Chem. Phys.*, 1974, **60**, 3859; D. Muller, M. Ewald, and G. Durocher, *Canad. J. Chem.*, 1974, **52**, 407.
[247] J. U. von Schutz, J. Zuclich, and A. H. Maki, *J. Amer. Chem. Soc.*, 1974, **96**, 714.
[248] R. J. Dam, C. A. Burke, and O. H. Griffith, *Biophys. J.*, 1974, **14**, 467.
[249] M. Tamba, S. Bonora, and R. Badiello, *Z. Naturforsch.*, 1974, **29b**, 571.
[250] D. M. Close and R. S. Anderson, *J. Chem. Phys.*, 1974, **60**, 2828.
[251] A. L. Poznyak, S. I. Arzhankov, and G. A. Shagisultanova, *Biofizika*, 1974, **19**, 233.
[252] S. A. Garay, L. Keszthelyi, I. Demeter, and P. Hrasko, *Nature*, 1974, **250**, 332.
[253] 'Methods of Enzymatic Analysis', second English edition, ed. H. U. Bergmeyer, Vol. 4, Academic Press, New York, 1974.

dansyl chloride,[254] and analysis of amino-acids in micro-quantities in nerve tissue and individual neurons,[255] are subjects of recent reviews.

Gas–Liquid Chromatography.—The derivatives of amino-acids which are suitable for g.l.c. analysis have been reviewed,[256] with particular emphasis on silyl derivatives.[256a] There is the customary expression of preference for one or another of the perfluoroacyl N-substituents and alkyl ester groups as a means of converting amino-acids in mixtures into volatile derivatives for g.l.c. analysis, and *N*-trifluoroacetyl n-butyl esters,[257–261] *N*-heptafluorobutyryl n-propyl[262, 263] and isobutyl[264] esters, and *N*-acetyl n-propyl esters[265, 266] are represented in this year's literature. Reaction of amino-acids with 1,3-dichloro-1,1,3,3-tetrafluoropropanone gives 2,2-bis(chlorodifluoromethyl)oxazolidinones which are advocated[267] as volatile derivatives for g.l.c. analysis. The identification of histidine by standard methods, *e.g.* as the N^{α},N^{im}-bis(trifluoroacetyl) derivative n-butyl ester, involves a derivative with a tendency to decompose during handling, and the more stable N^{im}-ethoxycarbonyl-N^{α}-trifluoroacetyl n-butyl ester gives more reliable results.[261]

Sample preparation techniques during these derivative-forming procedures are crucial if the identification of nanomole quantities of amino-acids is to be achieved, and the experience of some of the leading innovators in the field has been summarized.[268] An improved procedure for the preparation of *N*-trifluoroacetyl amino-acid n-butyl esters of nanomole amounts of amino-acids has been described.[257]

Methylthiohydantoins obtained by Edman degradation may be identified by g.l.c. of their trimethylsilyl derivatives,[269] and all protein amino-acids give satisfactory derivatives for g.l.c., except arginine, hydroxyproline, and hydroxylysine. If a flame photometric sulphur detector is used with the gas chromatograph, in place of the flame ionization detector, a number of extraneous peaks accompanying the silylated methylthiohydantoins may be avoided.[270]

Two main approaches to the estimation of the enantiomeric composition of partly racemic amino-acids have been further illustrated this year. *N*-Trifluoroacetyl cyclohexyl esters of L,L-dipeptides may be used as stationary phases for separation of enantiomeric amino-acids in the form of a volatile derivative, and isoleucyl-isoleucine, norvalyl-norvaline, and butyryl-butyrine are superior as

[254] V. Neuhoff, *Mol. Biol., Biochem. Biophys.*, 1973, **14**, 85.
[255] N. N. Osborne, *Progr. Neurobiol.*, 1973, **1**, Part IV, 299.
[256] (*a*) V. Marek, *Chem. Listy*, 1974, **68**, 250; (*b*) A. Darbre, *Biochem. Soc. Trans.*, 1974, **2**, 70.
[257] P. Cancalon and J. D. Klingman, *J. Chromatog. Sci.*, 1974, **12**, 349.
[258] B. Thom and J. W. Parsons, *J. Chromatog.*, 1974, **90**, 370.
[259] J. P. Ussary, *Food Prod. Develop.*, 1973, **7**, 84, 86, 88.
[260] M. Sakamoto, K. I. Kajiyama, and H. Tonami, *J. Chromatog.*, 1974, **94**, 189.
[261] I. M. Moodie, *J. Chromatog.*, 1974, **99**, 495.
[262] M. A. Kirkman, *J. Chromatog.*, 1974, **97**, 175.
[263] C. W. Moss and M. A. Lambert, *Analyt. Biochem.*, 1974, **59**, 259.
[264] S. L. Mackenzie and D. Tenaschuk, *J. Chromatog.*, 1974, **97**, 19.
[265] R. F. Adams, *J. Chromatog.*, 1974, **95**, 189.
[266] B. W. Hanny and C. D. Elmore, *J. Agric. Food Chem.*, 1974, **22**, 476.
[267] P. Husek, *J. Chromatog.*, 1974, **91**, 475, 483.
[268] F. E. Kaiser, C. W. Gehrke, R. W. Zumwalt, and K. C. Kuo, *J. Chromatog.*, 1974, **94**, 113.
[269] W. M. Lamkin, J. W. Weatherford, N. S. Jones, T. Pan, and D. N. Ward, *Analyt. Biochem.*, 1974, **58**, 422.
[270] W. M. Lamkin, N. S. Jones, T. Pan, and D. N. Ward, *Analyt. Biochem.*, 1974, **58**, 549.

bases for the stationary phase in comparison with dipeptide derivatives reported earlier (Volume 6, p. 34).[271] The alternative approach, using an achiral stationary phase, involves conversion of the sample into a mixture of diastereoisomeric amides or esters, for example with (+)-isoketopinyl, (−)-dihydroteresantalinyl, or (−)-teresantalinyl chlorides,[272] or with perfluoroacyl-L-prolyl chlorides;[273, 274] extensions of these studies[274, 275] have employed *N*-trifluoroacetyl-L-prolyl,[274] -L-hydroxyprolyl,[275] and -L-thiazolidin-4-carbonyl[275] n-butyl esters of the amino-acid.

Ion-exchange and Partition Column Chromatography.—Most of the papers eligible for inclusion in this Section describe improvements to amino-acid analyser technique, including aspects of sample preparation and colorimetric estimation. Older generation analysers can be up-dated to accommodate wider ranges of amino-acids,[276] though with correct use of buffers a single-column analysis of 44 compounds can be performed in 5½ h.[277]

The operating conditions of the amino-acid analyser for threonine and serine,[278] lysine,[279, 280] ornithine, $\alpha\gamma$-diaminobutyric acid, and $\alpha\beta$-diaminopropionic acid,[280] and several less-common dibasic amino-acids[281] have been discussed, as have the effects of glucosamine on routine amino-acid assay.[282] Components of a mixture of acidic and neutral amino-acids emerge from a column of SE-Sephadex C-25 in order of their pK_a values.[283]

6-Acetimidyl-lysine residues in proteins subjected to routine hydrolysis prior to amino-acid analysis are only slowly hydrolysed to lysine (6M-hydrochloric acid at 110 °C), and partial hydrolysis to estimate the protein content of this residue has been investigated.[284] Normal conditions of hydrolysis may be used for synthetic peptides containing β-(uracil-1-yl)alanine, β-(thymin-1-yl)alanine, and β-(cytosin-1-yl)alanine, but β-(adenin-9-yl)alanine residues are converted into glycine;[285] however, treatment with 60% perchloric acid at 130 °C during 15 min was satisfactory for peptides containing these residues.[285]

The importance of pre-treatment of materials is illustrated[286] for Dowex-50 cation-exchange resins, which when used straight from the bottle contribute a contaminant equivalent in ninhydrin colour intensity to 27 μmol glycine.

Examples of more recent techniques are separation of phenylthiohydantoins by high-pressure liquid chromatography,[287] and separations of amino-acids as

[271] P. Y. Howard and W. Parr, *Chromatographia*, 1974, **7**, 283.
[272] T. Nambara, J. Goto, K. Taguchi, and T. Iwata, *J. Chromatog.*, 1974, **100**, 180.
[273] H. Iwase and A. Murai, *Chem. and Pharm. Bull. (Japan)*, 1974, **22**, 8, 1455.
[274] H. Iwase, *Chem. and Pharm. Bull. (Japan)*, 1974, **22**, 2075.
[275] H. Iwase, *Chem. and Pharm. Bull. (Japan)*, 1974, **22**, 1663.
[276] H. E. Eick, P. H. Ward, and B. Kassell, *Analyt. Biochem.*, 1974, **59**, 482.
[277] P. L. Y. Lee, *Biochem. Med.*, 1974, **10**, 107.
[278] J. Oulevey and R. Heitefuss, *J. Chromatog.*, 1974, **94**, 283.
[279] C. Dennison, *J. Chromatog.*, 1974, **89**, 84.
[280] R. W. Longton, V. J. Berzinskas, A. Y. Balekjian, and S. B. Needleman, *Analyt. Biochem.*, 1974, **57**, 343.
[281] M. M. Herbst, B. G. Baltimore, G. Bozler, and H. A. Barker, *Analyt. Biochem.*, 1974, **58**, 322.
[282] J. F. Bouhours, *J. Chromatog.*, 1974, **94**, 307.
[283] D. P. Thornhill, *J. Chromatog.*, 1974, **90**, 354.
[284] B. V. Plapp and J. C. Kim, *Analyt. Biochem.*, 1974, **62**, 291.
[285] M. T. Doel, A. S. Jones, and R. T. Walker, *Tetrahedron*, 1974, **30**, 2755.
[286] R. D. Greenland, W. Roth, T. Gieske, and I. A. Michaelson, *Analyt. Biochem.*, 1974, **62**, 305.
[287] A. Haag and K. Langer, *Chromatographia*, 1974, **7**, 659.

perchlorate salts in organic solvents on porous silica by the ion-pair partition principle.[288]

Thin-layer Chromatography.—Techniques for minimizing sources of error in the quantitative analysis of amino-acids by t.l.c. have been reviewed,[289] and a review of non-destructive detection methods in t.l.c.[290] has appeared.

T.l.c. analysis of amino-acids in blood [291] and in protein hydrolysates [292] has been described, including an example of non-destructive location of separated amino-acids.[292] Conditions for the separation of hydroxyproline from allo-hydroxyproline have been established.[293] Further illustration of the sensitivity of the fluorescamine method for the detection of primary amines and amino-acids on t.l.c. plates is provided by separations at the 10 ng [294] and <100 pmol [295] levels. A complex multi-step reaction mechanism is involved in the fluorescamine procedure.[296]

Two-dimensional t.l.c. separation of 24 amino-acid phenylthiohydantoins [297] employs routine detection methods, but the use of the calcein fluorophore procedure permits the detection of phenylthiohydantoins at less than nanomole amounts.[298] Dansyl derivatives [266, 299] and related 5-dibutylaminonaphthalene-1-sulphonyl derivatives [300] of amino-acids still offer increased sensitivity even with the improvements contributed by the fluorescamine procedure to the analysis of un-derivatized amino-acids; picomole amounts can be detected,[300] and the analysis of the amino-acids present in *ca.* 1 mg of brain tissue is feasible using dansyl-^{14}C derivatives.[299]

Colorimetric Procedures for the Analysis of Amino-acids.—Such drawbacks as may still be associated with the major procedures for amino-acid colorimetry and fluorimetry are either readily overcome or are of a minor nature. The ninhydrin reaction is not quantitative with cysteine and lysine, and possible reasons for the non-ideal stoicheiometry have been discussed.[301] The isatin procedure is superior to the ninhydrin method for proline in amino-acid mixtures.[302] Further examples are provided of the conversion of imines into fluorescamine-sensitive primary amines;[303] N^{α}-methylamino-acids give methylamine with *N*-chlorosuccinimide, by which *N*-methylalanine may be detected at 100 picomole levels.[303]

[288] B. A. Persson and B. L. Karger, *J. Chromatog. Sci.*, 1974, **12**, 521.
[289] H. K. Berry, in 'Quantitative Thin Layer Chromatography', ed. J. C. Touchstone, Wiley, New York, 1973, p. 113.
[290] G. C. Barrett, *Adv. Chromatog.*, 1974, **11**, 145.
[291] R. Giguere, D. Shapcott, and B. Lemieux, *J. Chromatog.*, 1974, **95**, 122; M. Lato, S. Rufini, M. Ghebregzabher, G. Ciuffini, and T. Mezzetti, *Clin. Chim. Acta*, 1974, **53**, 273.
[292] M. D. Lutsik, I. I. Litvin, V. A. Monastyrskii, and Y. I. Aleksevich, *Khim. prirod. Soedineniya*, 1974, **10**, 197.
[293] F. Drawert and H. Barton, *Z. physiol. Chem.*, 1974, **355**, 902.
[294] J. Sherma and J. C. Touchstone, *Analyt. Letters*, 1974, **7**, 279.
[295] K. Imai, P. Bohlen, S. Stein, and S. Udenfriend, *Arch. Biochem. Biophys.*, 1974, **161**, 161.
[296] S. Stein, P. Bohlen, and S. Udenfriend, *Arch. Biochem. Biophys.*, 1974, **163**, 400.
[297] K. D. Kulbe and H. Konschewski, *Analyt. Biochem.*, 1974, **59**, 564.
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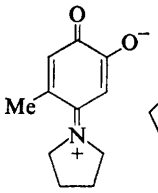
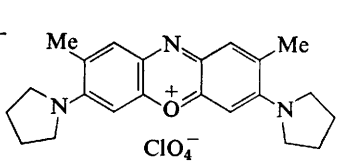
[299] B. E. Leonard, V. Neuhoff, and S. R. Tonge, *Z. Naturforsch.*, 1974, **29c**, 184.
[300] N. Seiler and B. Knoedgen, *J. Chromatog.*, 1974, **97**, 286.
[301] M. Friedman and L. D. Williams, *Bio-org. Chem.*, 1974, **3**, 267.
[302] R. J. Elliott and D. L. Gardner, *Biochem. Soc. Trans.*, 1974, **2**, 741.
[303] A. M. Felix and G. Ferkelsen, *Analyt. Biochem.*, 1974, **60**, 78.

The use of *o*-phthalaldehyde is advocated for the detection of histidine and related primary amines at 4–5 ng ml^{-1} levels by fluorimetry,[304] and the enhancement of the fluorescence of dansyl amino-acids by the addition of *cyclo*-heptaamylose permits their detection at 50 picomol ml^{-1} levels.[305] Fluorophores formed from dopa and from dopamine by successive treatment with formaldehyde and glyoxylic acid show markedly different excitation and emission spectra, so that these amines should be identifiable in tissue.[306]

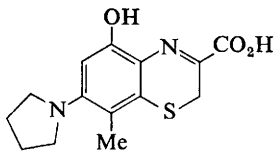
Oxidative deamination of amino-acids by the model aminochrome (34) gives the greenish-blue phenoxazine dye (35);[307] although proline and hydroxyproline



(34)



(35)



(36)

do not react, and cysteine reacts atypically in giving (36). The reaction is suggested to have some diagnostic value. The related *p*-benzoquinone reaction (λ_{max} 490 nm for amino-acids, λ_{max} 525 nm for prolines) is highly sensitive;[308] incidentally, the interpretation of this process as charge-transfer complex formation [308] needs revision in view of the isolation of 2,5-disubstituted quinones (30) in preparative scale studies.[211]

Other Analytical Methods.—A paper surveying rapid automated microbioassay of amino-acids [309] provides an entry to the extensive literature covering the technique. Further illustration of a method based on the addition of a known quantity of labelled amino-acid to an 'unknown' mixture, followed by addition to specific tRNA, has been provided,[310] with an indication of the lower limit in the nanomole range, and an accuracy ±15%. Low voltage electrophoresis on thin layers of microcrystalline cellulose offers an alternative means of separating amino-acids in biological samples.[311]

Determination of Specific Amino-acids.—Relatively well-established methods are applied to the assay of cysteine,[312] ergothioneine,[313] and hydroxyproline.[314, 315] The nitroprusside–methionine colour reaction provides the basis for an automated analysis of the amino-acids in biological samples,[316] and a sensitive pro-

[304] R. Hakanson, A. L. Ronnberg, and K. Sjolund, *Analyt. Biochem.*, 1974, **59**, 98.
[305] T. Kinoshita, F. Iinuma, and A. Tsuji, *Chem. and Pharm. Bull. (Japan)*, 1974, **22**, 2413.
[306] O. Lindvall, A. Bjorklund, and L. A. Svensson, *Histochemistry*, 1974, **39**, 197.
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[311] R. S. Ersser and S. Krywawych, *Med. Lab. Technol.*, 1974, **31**, 235.
[312] K. K. Verma and S. Bose, *Analyt. Chim. Acta*, 1974, **70**, 227.
[313] J. Carlsson, M. P. J. Kierstan, and K. Brocklehurst, *Biochem. J.*, 1974, **139**, 237.
[314] A. Walter and G. A. Schoenenberger, *Arzneim.-Forsch.*, 1974, **24**, 841.
[315] B. F. Gibbs, K. Itiaba, and J. C. Crawhall, *Clin. Chim. Acta*, 1974, **54**, 395.
[316] C. W. Gehrke and T. E. Neuner, *J. Assoc. Off. Analyt. Chem.*, 1974, **57**, 682.

cedure for the analysis of *N*-methylalanine uses nitroprusside and acetaldehyde.[317]

The cystine-binding protein isolated from *E. coli* has been exploited in an assay for this amino-acid, using the uptake of added [^{14}C]cystine as the basis for quantitation.[318] Other biologically oriented assays include immobilized amino-acid decarboxylases as electrodes in a simple cell, with electrode response in the presence of the appropriate amino-acid being proportional to concentration with a precision in the order of 2%;[319] immobilized tryptophanase or tryptophanase–lactate dehydrogenase has been used in an L-tryptophan assay.[320] Fluorimetric methods are particularly suitable for tryptophan[321–323] and 5-hydroxytryptophan,[324] and for tyrosine.[325, 326] Representative papers[327–331] describe thyroxine assay procedures, involving the ^{125}I-labelled amino-acid,[327] a Sephadex binding method,[328, 329] and the Thyopac-4 kit for routine assay.[330, 331]

[317] C. M. L. Lin and C. Wagner, *Analyt. Biochem.*, 1974, **60**, 278.
[318] R. G. Oshima, R. C. Willis, C. E. Furlong, and J. A. Schneider, *J. Biol. Chem.*, 1974, **249**, 6033.
[319] A. M. Berjonneau, D. Thomas, and G. Bronn, *Pathol.-Biol.*, 1974, **22**, 497.
[320] S. Ikeda and S. Fukui, *F.E.B.S. Letters*, 1974, **41**, 216.
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[322] M. K. Gaitonde, *Biochem. J.*, 1974, **139**, 625.
[323] G. G. Guilbault and P. M. Froehlich, *Clin. Chem.*, 1974, **20**, 812.
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[325] J. A. Ambrose, *Clin. Chem.*, 1974, **20**, 505.
[326] A. Grenier and C. Laberge, *Clin. Chim. Acta*, 1974, **57**, 71.
[327] F. Wilson, S. Rankel, E. G. Linke, and J. B. Henry, *Amer. J. Clin. Pathol.*, 1974, **62**, 383.
[328] C. H. G. Irvine, *J. Clin. Endocrinol. Metab.*, 1974, **38**, 655.
[329] S. Hamada, T. Kosaka, T. Mori, Y. Takeda, K. Ikekubo, K. Torizuka, and T. Uete, *Kaku Igaku*, 1974, **11**, 15 (*Chem. Abs.*, 1974, **81**, 101 517).
[330] S. Hamada, K. Torizuka, and T. Uete, *J. Clin. Pathol.*, 1974, **27**, 377; T. K. Bell, D. A. Boyle. D. A. D. Montgomery, and S. J. Todd, *J. Clin. Pathol.*, 1974, **27**, 372.
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2
Structural Investigations of Peptides and Proteins

BY J. BRIDGEN, I. D. WALKER, J. E. WALKER, H. MUIRHEAD, AND R. H. PAIN

PART I: Primary Structure and Chemical Modification *by J. Bridgen, I. D. Walker, and J. E. Walker*

1 Introduction

In this article, the seventh of the series, we have for the first time attempted a complete compilation of every amino-acid sequence published during 1974 as well as including large sections on protein characterization and chemical modification. In doing so, several points have become apparent. One is the increasing trend towards publication in duplicate, or even triplicate, of the same sequence from the same laboratory. Occasionally, virtually identical papers are found in different journals. Perhaps this practice should only be used in those few cases where the amino-acid sequence is of such importance that a rapid preliminary publication is necessary. We have also noted that, with certain admirable exceptions, different workers often produce remarkably different sequences for the same protein from the same source. Obviously, the determination of the primary structure of a protein is a complex task and errors are bound to occur, particularly in amide assignments. However, it does appear that in some cases the pressure for rapid publication seems to be taking precedence over the need for absolute accuracy. Finally, the way in which the completed sequences are published, particularly the amount of experimental detail included, varies not only between journals but between different papers in the same journal. While one would not like to see a uniformity of style or presentation, it would be useful to have better defined criteria for the publication of amino-acid sequences so that the author may submit his paper, the referee may edit it, and the reader may read it with a uniform degree of confidence.

2 Protein Isolation and Characterization

Before the molecular features which determine the function of a protein can be investigated, its purification is necessary. This section attempts to summarize the methods used and the results obtained from proteins purified during the past year. Table 2 (p. 48) lists the molecular weights and subunit compositions of proteins and indicates whether amino-acid compositions are available. Affinity chromatography is increasingly being used to purify proteins whose isolation would otherwise be laborious and Table 1 (p. 44) summarizes the salient features of affinity chromatography separations which have been reported in the past year. The data presented in the Tables are quite comprehensive but the text is, by necessity, selective. Polyacrylamide gel electrophoresis is almost universally

used to determine the purity of a protein sample as well as for investigations of its subunit structure, and a short section is devoted to recent reports on its uses. New techniques for the separation of proteins and advances in affinity chromatography are discussed in another section, and finally examples are given of proteins whose subunit structures give an indication of their biological functions.

Polyacrylamide Gel Electrophoresis – Molecular Weight Determination.—Several variations have appeared for the determination of subunit molecular weights on polyacrylamide gels. Fluorescamine can be used to prelabel proteins which can then be detected by their fluorescence after SDS-gel electrophoresis.[1] Reaction with fluorescamine does not affect the migration of proteins on SDS-gels and their molecular weights may be calculated from mobilities in the usual way.[2] Fluorescamine-labelled proteins however, have also been shown to migrate solely as a function of their molecular weight even on gels containing no detergent.[3] A method has appeared [4] for peptide mapping of very small amounts of protein obtained from SDS-gels. It involves prelabelling with fluorescamine, elution of the protein band after SDS-gel electrophoresis, enzymic digestion, and separation of peptides by t.l.c. on cellulose plates. Fluorescamine was used to detect the peptides which could be subsequently eluted and sequenced. Frequently only small amounts of protein are available and methods which permit purification and characterization at the nanomole level should prove useful.

Artefacts occurring during SDS-gel electrophoresis have been documented in previous Reports, and in the past year TEMED, a catalyst in polymerization, has been shown to impair resolution on SDS-gels;[5] removal of TEMED by pre-electrophoresis improved resolution. Samples of SDS from various sources were found to produce different banding patterns with a mixture of defined proteins [6] indicating the importance of using pure SDS. The presence of SDS in gel and electrode buffers is claimed to be unnecessary for electrophoresis of SDS–protein complexes whose mobility in such gels is apparently related to molecular weight.[7] An alternative method [8] of determining molecular weights of SDS–protein complexes involves gel-filtration on agarose columns in the presence of SDS, of S-carboxymethylated proteins, dansylated to facilitate their detection. This method, it is claimed, can be applied to as little as 2 μg of protein, an amount barely detectable on an SDS-gel. Alternative methods of molecular weight determination are necessary owing to the anomalous behaviour of some proteins during gel electrophoresis or gel filtration. As a recent example,[9] the apparent molecular weight of *Malbranchea pulchella* thermomycolase was found to be 11 000—17 000 when determined by gel filtration but 32 000—33 000 by either SDS-gel electrophoresis or ultracentrifugation.

Isolation of Proteins.—Hydrophobic chromatography of protein mixtures has been mentioned in previous Reports but its use in protein purification is not yet

[1] W. L. Ragland, J. L. Pace, and D. L. Kemper, *Analyt. Biochem.*, 1974, **59**, 24.
[2] P. R. Eng and C. O. Parkes, *Analyt. Biochem.*, 1974, **59**, 323.
[3] J. L. Pace, D. L. Kemper, and W. L. Ragland, *Biochem. Biophys. Res. Comm.*, 1974, **57**, 482.
[4] J. Vandekerckhove and M. Van Montagu, *European J. Biochem.*, 1974, **44**, 279.
[5] J. H. Allison, H. C. Agrawal, and B. W. Moore, *Analyt. Biochem.*, 1974, **58**, 592.
[6] J. B. Swaney, G. F. Van de Woude, and H. L. Bachrach, *Analyt. Biochem.*, 1974, **58**, 337.
[7] J. T. Stoklosa and H. W. Latz, *Biochem. Biophys. Res. Comm.*, 1974, **58**, 74.
[8] M. J. Gleason and A. B. Rawitch, *Biochem. Biophys. Res. Comm.*, 1974, **57**, 993.
[9] G. Voordouw, G. M. Gaucher, and R. S. Roche, *Biochem. Biophys. Res., Comm.*, 1974, **57**, 8.

widespread. The purification of wheat germ aspartate transcarbamoylase has been carried out by hydrophobic chromatography on Sepharose matrices to which C_{10} spacer-arms were attached.[10] The spacer of one matrix carried a single carboxy-group and this bound the enzyme which could then be eluted with buffer containing 0.2% n-butanol. The other matrix contained three carboxy-groups and the enzyme failed to bind, presumably owing to electrostatic repulsion between the matrix and the enzyme surface. The adjustment of both the spacer-arm length and the number of charged substituents it carries should enable further refinement in the fractionation of proteins by hydrophobic chromatography.

Two reports have appeared in which proteins with reactive thiol groups have been purified by virtue of their covalent binding to mercury derivatives linked to Sepharose. Thus, sea-urchin *S*-adenosyl-L-methionine decarboxylase could be bound to mercurial-Sepharose and then eluted by an excess of dithiothreitol.[11] The two subunits of lombricine kinase were separated by a variation of this procedure;[12] this enzyme is a dimer of two subunits very similar in size and amino-acid composition but only one of which contains a single, essential, thiol group. This was treated with FDNB and after the remaining cysteine residues had been blocked by carboxymethylation, the *S*-DNP-cysteine was thiolysed to produce cysteine. Application of the mixture to a mercurial-Sepharose column resulted in the binding of only one chain (that containing the reactive cysteine), while the other chain which contained no free cysteine residues emerged unretarded. The bound chain was eluted by buffer containing Hg^{2+} ions. Subsequently, the peptide containing the reactive cysteine was specifically isolated after tryptic digestion of the bound chain and application of the digest to mercurial-Sepharose on which it alone was retained.

Affinity Chromatography.—Affinity chromatography depends upon the specific interaction of protein molecules with ligands immobilized on inert matrices. It is important to eliminate interactions between proteins and affinity resin other than the specific binding between the protein of interest and its complementary ligand. One important factor contributing to incomplete specificity is the hydrophobic interaction which may occur between many proteins and the alkyl spacer-arm often used for linking the ligand to the matrix. As an alternative to alkyl spacer-arms, hydrophilic chains containing amide bonds and hydroxy-groups have been used.[13] Lactate dehydrogenase (LDH), glyceraldehyde-3-phosphate dehydrogenase (GPD), and alcohol dehydrogenase (ADH) were submitted to affinity chromatography on resins containing hydrophilic spacers with specific ligands attached. Both LDH and GPD bound to these affinity resins and could be eluted specifically with the appropriate ligand even in the absence of the rather high salt concentrations normally used to prevent hydrophobic interactions with the alkyl spacer-arms. However, ADH did not bind to NAD^+ attached to Sepharose by a hydrophilic spacer indicating that the binding of ADH to the hydrophobic analogue of this resin was not specific and was likely to be an example of hydrophobic chromatography (described earlier). The

[10] R. J. Yon, *Biochem. J.*, 1974, **137**, 127.
[11] C. Manen and D. H. Russel, *Biochemistry*, 1974, **13**, 4729.
[12] E. Der Terrossian, L. Pradel, R. Kassab, and G. Desvages, *European J. Biochem.*, 1974, **45**, 243.
[13] P. O'Carra, S. Barry, and T. Griffin, *F.E.B.S. Letters*, 1974, **43**, 169.

value of hydrophilic spacer-arms may be in their use in highly substituted affinity resins for large-scale enzyme preparations where non-specific hydrophobic interactions are a serious problem. The effect of varying the length of the spacer-arm on the specific binding of enzyme to attached ligand has also been investigated,[14] and alkyl chains of six or seven methylene groups were found to be optimal.

The specific retention of many NAD^+-requiring dehydrogenases to affinity columns of N^6-(6-aminohexyl)-AMP-Sepharose is well known. In a series of

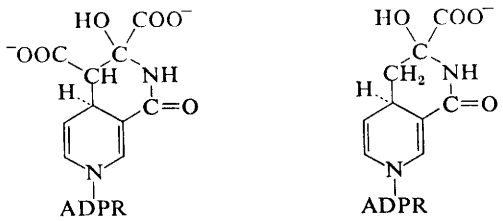

Figure 1 *Structure of reduced adducts. Left, reduced NAD–oxaloacetate adduct; right, reduced NAD–pyruvate adduct*

elegant investigations Kaplan *et al.*[15] have found a means of specifically eluting different dehydrogenases from a mixture bound to AMP-Sepharose. The method depends upon the ability of certain reduced adducts of NAD to form dead-end complexes with only one of the dehydrogenases, thereby detaching it specifically from the resin. Thus the reduced NAD–oxaloacetate adduct (Figure 1) eluted malate dehydrogenase (MDH) but not LDH or ADH. LDH was eluted with reduced NAD–pyruvate adduct (Figure 1). The power of the method was convincingly established by the purification of dogfish LDH from a crude extract by a single affinity chromatography step, in which reduced NAD–pyruvate adduct was used to elute LDH bound to AMP-Sepharose. The apparently pure enzyme was recovered in a yield of 90%. The prospect of refining the specificity of affinity chromatography by designing ligands not only to attach enzymes to resins but also to subsequently elute them in a manner exploiting a further facet of the enzyme's biospecificity is exciting. An approach, similar in principle, has been used to purify certain plant lectins.[16] The affinity adsorbent used was formalinized erythrocytes which, depending upon their source, bind plant lectins of differing sugar specificities. A monospecific lectin may be obtained in high purity from such erythrocytes by elution with its conjugate sugar. Concanavalin A, lima bean agglutinin, wheat germ agglutinin, and a previously uncharacterized lectin from *Ulex europeus* were purified by this method. Use of erythrocytes already possessing a range of different lectin-binding sites obviates the need to synthesize different affinity resins each specific for only one lectin.

A novel and potentially useful approach for purification of specific amino-acyl-tRNA synthetases has been described by Joyce and Knowles.[17] It relies

[14] M. C. Hipwell, M. J. Harvey, and P. D. G. Dean, *F.E.B.S. Letters*, 1974, **42**, 355.
[15] N. O. Kaplan, J. Everse, J. E. Dixon, F. E. Stolzenbach, C. Lee, C. T. Lee, S. S. Taylor, and K. Mosbach, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 3450.
[16] R. W. Reitherman, S. D. Rosen, and S. H. Barondes, *Nature*, 1974, **248**, 599.
[17] C. M. Joyce and J. R. Knowles, *Biochem. Biophys. Res. Comm.*, 1974, **60**, 1278.

upon the binding of a specific synthetase to its conjugate tRNA covalently linked to Sepharose. The main problem was to differentiate chemically the valyl-tRNA of interest from other tRNA's in a crude mixture. This was accomplished (see Figure 2) by charging the tRNA mixture with a single amino-acid, valine, using a crude synthetase preparation. The 2′,3′-diol linkages at the 3′ termini of the tRNA

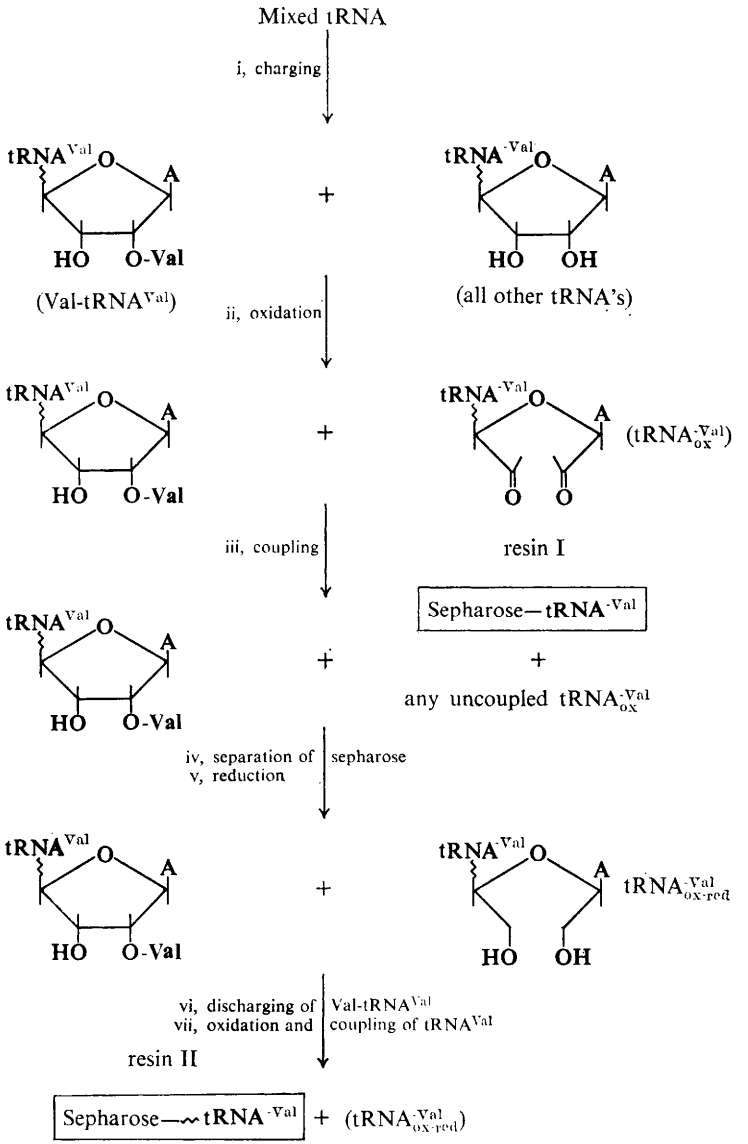

Figure 2 *Preparation of affinity and anti-affinity columns*
(Reproduced by permission from *Biochem. Biophys. Res. Comm.*, 1974, **60**, 1280)

molecules were oxidized with periodate and subsequently coupled to Sepharose (resin I), a treatment from which the aminoacylated valyl-tRNA molecules alone were protected. The valyl-tRNA, free of contaminants, was then deacylated, oxidized with periodate, and coupled to Sepharose as before (resin II). Valyl-tRNA synthetase in a crude mixture emerged unretarded from resin I but was bound to resin II and could be subsequently eluted free from other synthetases and was electrophoretically fairly pure.

Isozymes of LDH have been separated from one another by affinity chromatography on oxamate-Sepharose.[18] Oxamate is an analogue of pyruvate, a substrate for both the M4 and H4 forms of LDH. However, H4 may be specifically retained in the presence of NAD^+ which apparently allows formation of an abortive complex between the H4 form and the oxamate moiety. The stability of this complex is sufficient for binding to the affinity resin. Removal of NAD^+ from the buffer causes elution of the H4 isozyme. The separation of two non-identical subunits of pigeon liver fatty acid synthetase, hitherto inseparable, has been accomplished by affinity chromatography of the dissociated mixture on Sepharose to which ε-aminocaproylpantetheine, a substrate analogue, had been attached.[19] One subunit, the reductase, emerged from the affinity column unbound and almost devoid of transacetylase activity which was subsequently eluted free of reductase.

The synthesis of affinity adsorbents suitable for purification of NADPH specific dehydrogenases [20, 21] and the development of new methods of covalently coupling sugar moieties to matrices for affinity chromatography of amylases [22] and lectins [23] respectively have been reported. Use of pH gradients [24] and temperature gradients [25] for elution of enzymes bound to affinity columns has been investigated.

Immunoadsorbent chromatography relies on the ability of specific antibody molecules covalently bound to a matrix to bind antigens. This approach has been used in the purification of a shortened nonsense mutant of *E. coli* aspartokinase L-homoserine dehydrogenase (mol. wt. 84 000) possessing only aspartokinase activity which binds to antibodies specific for the wild-type enzyme, immobilized on a Sepharose column.[26] The mutant protein (mol. wt. 48 000) was obtained essentially pure after its elution from the column with 8M urea. Immunoadsorbent chromatography has also been used in an attempt to purify interferon from human fibroblasts.[27] Sheep antibodies, partially directed against human interferon, were raised by injection of a crude leucocyte interferon preparation and these were then attached to Sepharose. Crude fibroblast interferon was applied to the column and unbound impurities eluted, leaving only antigens

[18] P. O'Carra, S. Barry, and E. Corcoran, *F.E.B.S. Letters*, 1974, **43**, 163.
[19] F. A. Lornitzo, A. A. Qureshi, and J. W. Porter, *J. Biol. Chem.*, 1974, **249**, 1654.
[20] P. Brodelius, P. Larsson, and K. Mosbach, *European J. Biochem.*, 1974, **47**, 81.
[21] C. R. Lowe and K. Mosbach, *European J. Biochem.*, 1974, **49**, 511.
[22] P. Vretblad, *F.E.B.S. Letters*, 1974, **47**, 86.
[23] R. Bloch and M. M. Burger, *F.E.B.S. Letters*, 1974, **44**, 286.
[24] C. R. Lowe, M. J. Harvey, and P. D. G. Dean, *European J. Biochem.*, 1974, **41**, 347.
[25] M. J. Harvey, C. R. Lowe, and P. D. G. Dean, *European J. Biochem.*, 1974, **41**, 353.
[26] N. Guiso and P. Truffa-Bachi, *European J. Biochem.*, 1974, **42**, 401.
[27] C. B. Anfinsen, S. Bose, L. Corley, and D. Gurari-Rotman, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 3139.

(interferon in particular) common to both cell types bound. Elution of bound antigens with citrate buffer resulted in the selective enrichment of interferon. The purification of rat α1-foetoprotein [28] and of spinach leaf phosphenolpyruvate carboxylase [29] by variations of the immunoadsorption technique have also been described.

Affinity chromatography seems ideally suited for the purification of proteins that recognize specific RNA or DNA sequences and the purification of an apparently homogeneous poly-A binding protein from HeLa cell cytoplasm by poly-A-Sepharose chromatography has been reported.[30] However, in another report,[31] it was shown that many of the proteins which bound to poly-A-Sepharose also bound under the same conditions to poly-U-Sepharose and to CM-cellulose emphasizing the importance of eliminating non-specific binding effects.

3 Subunit Structure and Biological Function

The topics of this section have been selected to illustrate the importance of determining subunit structures of proteins and topographical relationships between their constituent chains.

Enzymes.—The determination of the subunit structure of a protein often involves the determination of its molecular weight in the native state, and the molecular weight of its subunits after dissociation.

Lysine decarboxylase from *E. coli* is composed of subunits of molecular weight 80 000,[32] and its native molecular weight (780 000) indicated an oligomer of 10 subunits. Examination by electron microscopy has confirmed the molecular weight evidence and further indicated that the subunits of the native enzyme are arranged as two regular pentagons lying face to face.

Dimethyl adipimidate, a lysine-specific bifunctional reagent capable of covalently cross-linking chains close to one another, was shown to produce predominantly monomers, dimers, and trimers of glutamate dehydrogenase [33] by analysis of cross-linked products by SDS-gel electrophoresis indicating that the chains of hexameric glutamate dehydrogenase exist as two equivalent trimers.

The subunit molecular weight of phosphofructokinase (PFK) from different vertebrate tissues remains in doubt. That of rabbit muscle (see previous Reports) is believed to be 80 000—90 000 and the molecular weight of its counterpart from rat liver has been determined [34] as 82 000. The subunit molecular weight of chicken liver PFK was previously determined as 60 000. Sheep heart PFK subunits have apparent molecular weights of 80 000—90 000 when examined by either SDS-gel electrophoresis or ultracentrifugation [35] but after maleylation and S-carboxymethylation, procedures not believed to break peptide bonds, the apparent molecular weight was reduced to 39 000—40 000 as determined by

[28] N. Cittanova, A. M. Grigorova, C. Benassayag, E. Nunez, and M. F. Jayle, *F.E.B.S. Letters*, 1974, **41**, 21.
[29] H. M. Miziorko, T. Nowak, and A. S. Mildvan, *Arch. Biochem. Biophys.*, 1974, **163**, 378.
[30] J. M. Blanchard, C. Brissac, and P. Jeanteur, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 1882.
[31] A. Shweiger and G. Mazur, *F.E.B.S. Letters*, 1974, **46**, 255.
[32] D. L. Sabo, E. A. Boeker, B. Byers, H. Waron, and E. H. Fischer, *Biochemistry*, 1974, **13**, 662.
[33] F. Hucho and M. Janda, *Biochem. Biophys. Res. Comm.*, 1974, **57**, 1081.
[34] I. H. Brand and H. Söling, *J. Biol. Chem.*, 1974, **249**, 7824.
[35] S. O. Brennan, P. F. Davis, and G. G. Midwinter, *European J. Biochem.*, 1974, **42**, 489.

ultracentrifugation. The problem is intriguing since rabbit muscle PFK produces only half the number of tryptic peptides expected on the basis of a molecular weight of 80 000 and amino-acid composition, a finding which has recently been confirmed for the sheep heart enzyme.[35]

Two recent reports [36, 37] have indicated that tryptic peptide mapping of selected aminoacyl tRNA synthetases also gives a much smaller number of peptides than expected. Koch *et al.*[37] have found that the methionyl, valyl, and leucyl tRNA synthetases contain many sequences present at least twice in each chain, and suggested that each of these three enzymes consists of two tandemly linked repeated sequences.

The subunit structures of RNA polymerases have been extensively studied recently. The RNA polymerase specified by phage T7 is a single chain of molecular weight 110 000 [38] and that of yeast mitochondria consists of a single type of subunit, molecular weight 67 000. However, the structures of RNA polymerases are generally more complex than this, commonly consisting of at least four different subunits. An RNA polymerase from *E. coli* containing a new type of subunit, δ' (mol. wt. 56 000), replacing δ (mol. wt. 95 000) has been isolated;[39] it will be interesting to know if the presence of δ' alters the transcriptional specificity of the enzyme, in which the remaining subunits are identical with those of the normal *E. coli* RNA polymerase. Infection of *B. subtilis* with the phage SP82 was also shown to result in the replacement of δ by a new subunit, χ,[40] of molecular weight 16 000, whereas *B. subtilis* infected with phage PBS2 was shown to contain an RNA polymerase with four chains in equimolar amounts all of which differed from their counterparts in uninfected cells.[41] The subunit structures of many RNA polymerases from eukaryotic cells seem to be more complex than those of their bacterial counterparts. Rat liver contains at least three RNA polymerases and one of these has been reported to contain eight subunits, six of which differ in molecular weight.[42] All three polymerases have some subunits in common whilst others are unique. One problem in determining the subunit structure of eukaryotic RNA polymerases is that the detection of component chains of low molecular weight on SDS-gels is much less sensitive and reliable than for the larger chains. Consequently, amounts of minor impurities, undetectable on gels of native RNA polymerase, may appear on SDS-gels of the same sample as bands of comparable intensity to those of low molecular weight subunits. Two groups have examined the subunits of the α-amanitin-resistant RNA polymerase from *Physarum polycephalum*: one group [43] report five chains of molecular weights 200 000, 135 000, 45 000, 24 000, and 17 000, respectively, whereas the others [44] feel that significance can be attached only to the two subunits of highest molecular weight (205 000 and 125 000 in their hands) and that the

[36] R. M. Waterson and W. H. Konisberg, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 377.
[37] G. L. E. Koch, Y. Boulanger, and B. S. Hartley, *Nature*, 1974, **249**, 316.
[38] E. G. Niles, S. W. Conlon, and W. C. Summers, *Biochemistry*, 1974, **13**, 3904.
[39] R. Fukuda, Y. Iwakura, and A. Ishihama, *J. Mol. Biol.*, 1974, **83**, 353.
[40] G. B. Spiegelman and H. R. Whiteley, *J. Biol. Chem.*, 1974, **249**, 1476.
[41] S. Clark, R. Losick, and J. Pero, *Nature*, 1974, **252**, 21.
[42] C. Kedinger, F. Gissinger, and P. Chambon, *European J. Biochem.*, 1974, **44**, 421.
[43] S. Z. Gornicki, S. B. Vuturo, T. V. West, and R. F. Weaver, *J. Biol. Chem.*, 1974, **249**, 1792.
[44] H. B. Burgess and R. R. Burgess, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 1174.

bands representing chains of lower molecular weight could be ascribed to impurities.

Yeast phosphoglucose isomerase has recently been purified and shown to consist of three isozymes all of molecular weight 120 000.[45] Each isozyme is a dimer of two identical chains[46] and peptide mapping of the three different subunit types revealed that all three contained many sequences in common but that some unique peptides were also present in each type of subunit. No hybrid forms containing two different types of subunit were detected despite the many sequence similarities between the subunits found by peptide mapping. The absence of hybrids of chains whose respective genes were presumably derived from a common ancestor may be due to sequence alterations during evolution which precluded hybrid formation, and in this regard it would be of interest to know if hybrid formation were possible by *in vitro* reconstitution of different subunits. The ability of subunits from different enzymes to form hybrids can be taken to indicate evolutionary relatedness, and the isolation of an enzyme from *Pseudomonas* containing both 4-aminobutanol dehydrogenase and semialdehyde dehydrogenase activities[47] and composed of subunits characteristic of both purified enzymes suggests a common ancestor for both.

Rabbit brain aldolase is a tetramer composed of two different types of subunits (A and C) which exist *in vivo* as a five-membered set of isozymes each differing in the ratio of A to C subunits. The C4 form has been purified[48] and, although its sequence is clearly homologous to the A subunits, the C subunit is shorter by *ca.* 40 residues and also differs in its amino-acid composition.

Ribosomal Proteins.—Ribosomes are made up of two subunits each containing RNA molecules and a large number of different proteins. An examination of the proteins of cytoplasmic ribosomes from *Xenopus laevis* has revealed the presence of 37 proteins in the larger subunit and 34 proteins in the smaller subunit.[49] The corresponding subunits from *Xenopus* mitochondria contain 44 and 40 different proteins respectively and few proteins, if any, from one type of ribosomal subunit were commonly present in subunits of different types. Similar conclusions were reached for the ribosomal proteins of plant cytoplasm and chloroplasts.[50] Quantitation of the amount of individual proteins relative to the total protein content in the large and small ribosomal subunits of *Dictyostelium discoideum*[51] revealed that two or more molecules of some protein chains were present in the same subunit whereas other chains were present only in some of the subunits. A protein claimed to be present only in mouse kidney ribosomes has been identified,[52] but a careful search for tissue-specific ribosomal proteins in rat skeletal muscle and liver was unsuccessful.[53]

[45] T. D. Kempe, Y. Nakagawa, and E. A. Noltmann, *J. Biol. Chem.*, 1974, **249**, 4617.
[46] T. D. Kempe, D. M. Gee, G. M. Hathaway, and E. A. Noltmann, *J. Biol. Chem.*, 1974, **249**, 4625.
[47] M. S. Rosemblatt, D. M. Callewaert, and T. T. Tchen, *Biochemistry*, 1974, **13**, 4176.
[48] Y. Lee and B. L. Horecker, *Arch. Biochem. Biophys.*, 1974, **162**, 401.
[49] D. E. Leister and I. B. Dawid, *J. Biol. Chem.*, 1974, **249**, 5108.
[50] C. Gualerzi, H. G. Janda, H. Passow, and G. Stoffler, *J. Biol. Chem.*, 1974, **249**, 3347.
[51] F. Kanda, H. Ochiai, and M. Iwabuchi, *European J. Biochem.*, 1974, **44**, 469.
[52] M. S. Kaulenas and B. R. Unsworth, *Biochem. Biophys. Res. Comm.*, 1974, **61**, 135.
[53] C. C. Sherton and I. G. Wool, *J. Biol. Chem.*, 1974, **249**, 2258.

Use of chemical cross-linking reagents to establish proximity relationships between ribosomal proteins was described in the 1973 Report, and a number of groups have managed to confirm and extend the topography map.[54-56] The protein, S16, has been shown to be at the junction of the large and small subunits in *E. coli* ribosomes.[57] Radioactively labelled 50S subunits were recombined with unlabelled 30S subunits and the resultant 70S complex cross-linked with formaldehyde or glutaraldehyde, a treatment which prevented the normal dissociation into 50S and 30S subunits. Ribosomal proteins were prepared from these cross-linked 70S particles and antiserum to each of the 30S proteins was tested for its ability to precipitate radioactively labelled 50S proteins. Only antiserum to S-16 caused precipitation, suggesting that this protein alone in the 30S particle lies at the junction of the two ribosomal subunits.

Membrane Proteins.—A sensitive method for the separation of membrane proteins from human erythrocytes has been reported recently.[58] It involves isoelectric focusing in polyacrylamide gels containing 8M urea of edta-soluble erythrocyte ghost proteins, followed by SDS-gel electrophoresis at right angles to the first dimension. Many more proteins were identified by this procedure than by SDS-gel electrophoresis alone and the method may be useful for the comparison of membrane proteins from different cell types or from normal cells and mutants in which specific membrane proteins are altered or absent.

A recent report has suggested that spectrin, a prominent constituent of human erythrocyte membranes, exists as a single chain [59] despite its very high molecular weight (240 000) rather than being composed of subunits as previously suggested. Another group, however, have demonstrated the presence in spectrin preparations of multiple *N*-terminal amino-acids [60] which they believe represent the existence either of multiple spectrin chains all of approximate molecular weight 200 000 or of smaller subunits. The former alternative was favoured by the appearance on the two-dimensional gel system described above of multiple protein species all with molecular weights characteristic of spectrin chains but differing in isoelectric point.[58]

Two groups have isolated the *E. coli* membrane ATPase responsible for transhydrogenation.[61, 62] Both groups identified the α, β, γ, and ε subunits by SDS-gel electrophoresis. An additional subunit, δ, was also found to be present in the preparation of only one group.[62] The unique property of the enzyme containing the δ subunit was its ability to restore transhydrogenase activity to deficient membranes, thereby implicating δ in the recognition of membrane binding sites.

Two reports describing the proteins of the photosynthetic reaction centres of *Rhodopseudomonas spheroides* have appeared.[63, 64] Reaction centres, prepared

[54] C. Clegg and D. Hayes, *European J. Biochem.*, 1974, **42**, 21.
[55] A. Sommer and R. R. Traut, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 3946.
[56] U. Bode, L. C. Lutter, and G. Stöffler, *F.E.B.S. Letters*, 1974, **45**, 232.
[57] T. Sun, R. R. Traut, and L. Kahan, *J. Mol. Biol.*, 1974, **87**, 509.
[58] S. Bhakdi, H. Knufermann, and D. F. H. Wallach, *Biochim. Biophys. Acta*, 1974, **345**, 448.
[59] F. W. Hulla, *Biochim. Biophys. Acta*, 1974, **345**, 430.
[60] G. M. Fuller, J. M. Boughter, and M. Morazzani, *Biochemistry*, 1974, **13**, 3036.
[61] N. Nelson, B. I. Kanner, and D. L. Gutnick, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 2720.
[62] M. Futai, P. C. Sterweis, and L. A. Heppel, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 2725.
[63] M. Y. Okamura, L. A. Steiner, and G. Feher, *Biochemistry*, 1974, **13**, 1394.
[64] L. A. Steiner, M. Y. Okamura, A. D. Lopes, E. Moskowitz, and G. Feher, *Biochemistry*, 1974, **13**, 1403.

from chromatophores, can accept a photon of light promoting the excitation of electrons which can then reduce electron acceptors. They are believed to be the sites in photosynthetic membranes at which the primary event in photosynthesis takes place. Three chains in equal molar amounts were found to be the sole protein constituents of *R. spheroides* reaction centres. The reaction centres of *Rhodospirillum rubrum*, another photosynthetic bacterium, also contained three chains with molecular weights similar to those of *R. spheroides*. Further dissociation of reaction centres produced a particle containing only two of the three chains which was only slightly less photochemically active than the intact particle. The apparent simplicity of these bacterial reaction centres recommends them as a suitable system for studying the role of individual protein chains in photosynthesis.

The topography of protein subunits of membranes has been studied by chemically cross-linking adjacent chains with bifunctional reagents followed by analysis of the products by SDS-gel electrophoresis. The bifunctional imido-ester, dimethyl malonimidate, has been used to cross-link two glycoproteins GP-1 and GP-2 in human erythrocyte membranes.[65] The composition of the cross-linked product was confirmed by ammonolysis of the presumptive dimer obtained from SDS-gels, a treatment which breaks the covalent cross-links; analysis of the resultant product on SDS-gels revealed the two expected bands corresponding to glycoproteins GP-1 and GP-2. Membrane proteins from human erythrocytes have also been cross-linked by catalysing interchain disulphide bond formation between chains in close proximity with the *o*-phenanthroline cupric ion complex, and also with the imido-ester dimethyl dithiobispropionimidate,[66] producing oligomers whose interchain disulphide bonds were susceptible to reductive cleavage. The products of cross-linking were subjected to two-dimensional SDS-gel electrophoresis, as oxidized cross-linked chains in the first dimension and after reduction in the second. This analysis indicated an unexpectedly high number of homopolymers although some hereropolymers were also detected. The authors suggest that many proteins occur in erythrocyte membranes as oligomers of identical subunits.

Semiliki Forest Virus (SFV) consists of a nucleocapsid surrounded by a membrane whose lipid composition closely resembles that of the host cell from which it is derived. The nucleocapsid contains the viral (RNA) genome and one lysine-rich protein of molecular weight 33 000. The viral membrane has been shown to contain three glycoproteins in equimolar amounts, two of molecular weight *ca.* 50 000 and one of 10 000.[69] A hydrophobic portion of each of the two larger chains, presumably that situated within the lipid bilayer of the membrane, was found to be resistant to thermolytic cleavage whereas the remainder of each chain, those parts comprising the spikes on the external surface of the membrane, was digested.[70] Formyl-*S*-methionyl sulphone methyl phosphate, a lysine-specific reagent impenetrable to lipid membranes, was shown by peptide mapping to label different portions of the two larger chains when treated separately with

[65] T. H. Ji and I. Ji, *J. Mol. Biol.*, 1974, **86**, 129.
[66] K. Wang and F. M. Richards, *J. Biol. Chem.*, 1974, **249**, 8005.
[69] H. Garoff, K. Simons, and O. Renkonen, *Virology*, 1974, **61**, 493.
[70] G. Uttermann and K. Simons, *J. Mol. Biol.*, 1974, **85**, 569.

intact virus and with membrane fragments exposing both inside and outside surfaces to the reagent. This would indicate that both proteins span the viral membrane.[71] Dimethyl suberimidate, treated with intact virus, was shown to cross-link both large chains to the nucleocapsid protein, establishing the close proximity of at least part of the two membrane proteins to the nucleocapsid. On the basis of these results, Garoff and Simons [71] present a model of SFV structure and assembly which accounts for the exclusion of host cell proteins from the viral membrane.

Histocompatibility antigens are proteins present on the surface of mammalian cells which determine whether donor tissue of defined allogenic specificity will be accepted or rejected by a recipient after transplantation. An earlier report (see last year's Report, p. 155) that one of the subunits of purified histocompatibility antigens was identical with β_2 microglobulin, a urinary protein of molecular weight 11 000—12 000, homologous in sequence to the *C*-terminal region of immunoglobulin chains, has been confirmed by three groups.[72–77] Collectively, the data indicate that β_2 microglobulin is a subunit of both human and mouse histocompatibility antigens regardless of whether it is solubilized from membranes by papain digestion or with detergents. The other chain, the alloantigenic subunit in which the allogenic specificity must reside, has been shown by immunological methods [73] to be closely associated in human lymphocyte membranes with the β_2 microglobulin subunit, arguing against a non-specific association of the two chains occurring during purification.

Miscellaneous.—*Colicin E3 Immunity Protein.* Colicin E3 is an extracellular antibacterial protein produced in *E. coli* cells carrying a specific plasmid, the Col E3 factor. Cells capable of synthesizing colicin E3 are immune to its effect, which is to damage irreversibly and lethally the ribosomes of sensitive cells. E3 immunity protein, which has previously been shown to inhibit the action of colicin E3 *in vitro* but not *in vivo*, has recently been purified [78, 79] and found to be a single chain of molecular weight 10 000. Furthermore, purified preparations of colicin E3 have been shown to consist of two subunits, one of high molecular weight and another, indistinguishable in molecular weight from E3 immunity protein. These subunits are present in a 1 : 1 molar ratio.[80] The smaller chain was purified from colicin E3 and shown to be immunologically identical with authentic E3 immunity protein and identical also in its ability to protect ribosomes from inactivation by colicin E3. Jakes and Zinder [80] have proposed that the large chain of the colicin E3 complex, but not the immunity protein to which it is bound, can enter bacterial cells thus accounting for the ineffectiveness of extracellular immunity protein in the protection of sensitive cells. Cells carrying the Col E3 plasmid, however, are immune to colicin E3 owing to an intracellular

[71] H. Garoff and K. Simons, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 3988.
[72] P. A. Peterson, L. Rask, and J. B. Lindblom, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 35.
[73] L. Östberg, J. B. Lindblom, and P. A. Peterson, *Nature*, 1974, **249**, 263.
[74] L. Rask, J. B. Lindblom, and P. A. Peterson, *Nature*, 1974, **249**, 833.
[75] P. Cresswell, R. J. Robb, M. J. Turner, and J. L. Strominger, *J. Biol. Chem.*, 1974, **249**, 2828.
[76] P. Cresswell, T. Springer, J. L. Strominger, M. J. Turner, H. M. Grey, and R. T. Kubo, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 2123.
[77] J. Silver and L. Hood, *Nature*, 1974, **249**, 764.
[78] J. Sidikaro and M. Nomura, *J. Biol. Chem.*, 1974, **249**, 445.
[79] K. Jakes, N. D. Zinder, and T. Boon, *J. Biol. Chem.*, 1974, **249**, 438.
[80] K. Jakes and N. D. Zinder, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 3380.

excess of immunity protein capable of forming an inactive complex with free intracellular colicin E3.

Chromosomal Proteins. A method for the rapid and efficient fractionation of chick erythrocyte histones involving only two chromatography steps has been developed [81] and should be applicable to histones from other sources. Examination of the histones of *Drosophila melanogaster* [82] embryos and of *Ceratitis capitata* [83] indicated that the five major classes of histones were present in both organisms. Amino-acid compositions of *Drosophila* histones indicated that histone F2A1 was most similar and F1 least similar to their counterparts in different species. The non-histone chromosomal proteins from rat hepatoma and normal liver have been examined [84] by SDS-gel electrophoresis and more proteins than occur in either adult or foetal liver were found in hepatoma. Their significance, if any, to gene expression was not clear.

Muscle Proteins. Parvalbumin, hitherto isolated only from fish and amphibian muscles, has been purified from the skeletal muscles of turtle, chicken, rabbit, and mouse:[85] its apparent ubiquity suggests that it may have an unsuspected role to play in the contractile process.

Examination by two-dimensional gel electrophoresis of canine C, myosin light chains from myocardial muscle, has revealed the presence of four different components,[86] all of the same molecular weight. Myosin light chains G1 and G2 from chick smooth muscle have been shown by gel electrophoresis and peptide mapping to be indistinguishable from their counterparts in fibroblasts.[87] However, the myosin heavy chains from these two tissues were shown to differ by examination on SDS-gels of their respective peptides obtained by mild digestion with proteolytic enzymes. Phosphorylase kinases from red and white muscle tissues have both been shown to consist of three types of subunit, α(136 000), β(118 000), and γ(42 000).[88] The β and γ subunits were found to be common to the enzymes of both tissues but different types of the α subunit were found in red and white muscles. Any detailed mechanism proposed for the contractile process in different tissues must take into account the tissue specificity of many of the protein components of the contractile apparatus.

Interferon. Interferon is a protein that confers resistance against viral infection upon cells in which it is synthesized. It is a very potent antiviral agent and the very small quantities present in cells have prevented its purification. Notwithstanding, a report has recently appeared in which the size of the mouse L cell interferon subunit has been estimated.[89] The previous demonstration that interferon could be renatured after its dissociation by SDS allowed the separation of

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Table 1 *Affinity chromatography of proteins*

Protein	*Ligand*	*Matrix*	*Elutant*	*Ref.*
Acetylcholine receptor protein (*Electrophorus*)	flaxedil analogue	Sepharose	flaxedil	91
Acetylcholine receptor protein I (*Electrophorus*)	quaternary ammonium salt	Sepharose	quaternary ammonium salt	92
Acetylcholine receptor protein (*Torpedo*)	*Naja naja* neurotoxin	Sepharose	carbamyl choline chloride	93
ADP-glucose pyrophosphorylase	P^1-(6-phospho-1-hexyl)-P^2-(6-amino-1-hexyl)-pyrophosphate	Sepharose	AMP	94
D-Alanine carboxypeptidase	6-amino-penicillanic acid	Sepharose	hydroxylamine	95
Aeromonas aminopeptidase	*N*-(3-amino-5-methyl-2-oxohexyl)-succinnamic acid	Cellulose	low pH	96
Anti-glycoside antibodies	globomide or hematoside	Glass beads	NaI	97
Antithrombin III	heparin	Sepharose	NaCl	98
Asparaginase	L-aspartic acid	Sepharose	NaCl or L-aspartic acid	99
Aspartase	L-aspartic acid	Sepharose	NaCl or L-aspartic acid	99
Aspartate β-decarboxylase	L-aspartic acid	Sepharose	NaCl	99
Carbonic anhydrase	Sulfamylon	Sepharose	perchlorate	100
Cyclic AMP receptor protein	3′,5′-cyclic AMP	Agarose	urea	101
Cytochrome b2	oxamate	Sepharose	phosphate	102
T7 DNA binding protein	DNA	Cellulose	NaCl	103
DNAase	DNA	Sepharose	NaCl	104
Dopamine-β-hydroxylase	concanavalin A	Sepharose	α-methyl-D-mannoside	105
E. coli exonuclease I	DNA	Cellulose	NaCl	106
Venom 3′-exonuclease	concanavalin A	Sepharose	α-methyl-D-mannoside	107
Formiminotransferase	tetrahydrofolate	Sepharose	formiminoglutamate	108
α-L-fucosidase	fucosamine	Agarose	fucose	109
Galactosyl transferase	α-lactalbumin	Sepharose	UDP-galactose	110
Glutamine synthetase	glutamate	Sepharose	glutamate	111
Glycerol-3-phosphate dehydrogenase	6-phosphogluconate	Agarose	NaCl	112
Glycerol-3-phosphate dehydrogenase(s)	trinitrobenzene	Sepharose	NADH	113
Glycoproteins (rat brain)	concanavalin A	Sepharose	α-methyl-glucoside	114
GTP cyclohydrolase	GTP	Sepharose	GTP	115

Galactose binding lectins:				
A. precatorius	none	Sepharose	α-D-galactose	116
B. simplicifolia	melibiose aldonic acid	Biogel	α-D-galactose	117
R. communis		Sepharose	α-D-galactose	116
Hemagglutinins:				
M. amurensis	glycopeptides	Sepharose		118
C. sessilifolius	tri-*N*-acetylchitotriose	Starch	pH 2.0, NaCl	119
Eel serum	α-fucose	Starch	pH 3.0, NaCl	119
L. polyphemus	mucin	Sepharose	citrate	120

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Table 1 (*cont.*)

Protein	*Ligand*	*Matrix*	*Elutant*	*Ref.*
Hexosaminidases	β-D-glucopyranosylamine	Sepharose	pH 8.0	121
Hydroxysteroid dehydrogenase	blue dextran	Sepharose	$NADP^+$	122
Interferon (human)	crude anti-interferon antibodies	Sepharose	citrate	27
Interferon (human)	albumin	Sepharose	ethylene glycol	123
Kallikrein	*C*-methyl arginine	Sepharose	NaCl	124
Lactate dehydrogenase	AMP	Sepharose	reduced pyruvate–NAD adduct	15
Lipoprotein lipase	concanavalin A	Sepharose	α-methyl-D-mannoside	125
	heparin	Sepharose	NaCl	125
Luciferase	FMN	Sepharose	FMN	127
Lysyl oxidase	collagen	Sepharose	NaCl, urea	126
Membrane receptors (Human erythrocyte):				
R. communis agglutinin (RCA)	RCA	Sepharose	lactose	128
Wheat germ agglutinin (WGA)	WGA	Sepharose	*N*-acetylglucosamine	128
Concanavalin A	concanavalin A	Sepharose	α-methyl-mannoside	129
L. culinaris PHA	*L. culinaris* PHA	Sepharose	α-methyl-mannoside	129
N-Acetylgalactosamine transferase	none	Agarose	UDP	130
N-Acetyl-β-D-hexosaminidase A	*N*-acetyl-β-D-thioglucosamine	Agarose	borate	131
Neurophysin	vasopressin	Sepharose	urea	132
Phospholipase C	lipoprotein	Agarose	NaCl	133
Poly-A binding protein	poly-A	Sepharose	NaCl	30
Prolactin (amnionic fluid)	anti (pituitary) prolactin antibodies	Sepharose	NaCl	134
Prolactin receptor protein	human growth hormone	Agarose	$MgCl_2$	135
Pteroyl-α-oligoglutamyl endopeptidase	α-oligoglutamyl peptides	Sepharose	NaCl	136
S-Adenosyl methionine decarboxylase	methylglyoxal bis(guanylhydrazone)	Sepharose	methylglyoxal bis(guanyl-hydrazone)	137
Thymidilate synthetase	thymidilate analogue	Sepharose	phosphate	138
Tryptophan synthase	indolpropionate	Sepharose	imidazole	139
Wheat germ agglutinin	chitin	None	HCl	140

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Table 2 *Isolation and subunit structure of proteins*

Protein	*Source*	*Molecular weight*	*Subunit structure*	*Amino-acid analysis*	*Ref.*
Acetyl-CoA: *S*-(β-aminoethyl)-L-cysteine acetyltransferase	*Aerobacter aerogenes*	100 000			141
Acetoacetyl-CoA thiolase	rat liver	170 000	4 × 44 000	–	142
Acetyl-CoA carboxylase	*E. coli*	130 000	2 × 33 000 2 × 35 000	–	143
	chicken liver	470 000—510 000	2 × 117 000 1 × 129 000 1 × 139 000	–	144
Acetylcholinesterase	*Electrophorus electricus*	320 000	4 × 80 000	–	145
Acetylcholine receptor	*Electrophorus electricus*	—	43 000 48 000	+	91
	Electrophorus electricus	—	41 500	–	92
	Torpedo nobiliana	—	70 000 43 500 38 500 35 500 33 500		93
Acyl carrier protein	pigeon liver	9000	1 × 9000	+	146
Adenylate cyclase	*Brevibacterium liquefaciens*	92 400	2 × 46 000	+	147
Adenylosuccinate synthetase	rabbit muscle	54 000	1 × 54 000	–	148
Agglutinin	soya bean	120 000	4 × 30 000	+	149
	Homanus americanus	—	55 000		150
D-Alanine carboxypeptidase	*Bacillus stearothermophilus*	—	46 500		95
Aldolase	*Streptococcus faecalis*	56 000	2 × 28 000	–	151
	Pediococcus cereoisae	118 000	4 × 29 500	–	151
	Lactobacillus casei	176 000	6 × 29 500	–	151
Alkaline phosphatase	*Bacillus subtilis*	110 000	2 × 55 000	+	152
	rat liver	154 000	2 × 75 000	–	153
	calf intestine	140 000	2 × 69 000	+	154
	pig kidney	150 00	4 × 39 000	+	155
	human placenta	—	70 000		156
L-Amino-acid decarboxylase	*Micrococcus pericitreus*	101 000	—	–	157

D-Amino-acid aminotransferase	*Bacillus sphaericus*	58 000	—	−	158
4-Aminobutanal dehydrogenase	*Pseudomonas*	228 000	3 × 75 000	−	159
L-3-Aminobutyryl-CoA deaminase	*Clostridium SB4*	64 000	—	−	160
δ-Aminolaevulinic acid dehydratase	bovine liver	289 000	8 × 349 000	−	161
Aminopeptidase	human liver	235 000	2 × 118 000	+	162
3-Aminopropanal dehydrogenase	*Pseudomonas*	226 000	3 × 74 000		163
Aminotransferase	*Salmonella typhimurium*	183 000	6 × 31 000	+	164
AMP-dependent protein kinase: AMP-binding protein	calf ovary	135 000	—	−	
AMP-independent protein kinase		375 000	26 000 64 000 94 000	−	165

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Table 2 (*cont.*)

Protein	*Source*	*Molecular weight*	*Subunit structure*	*Amino-acid analysis*	*Ref.*
α-Amylase	human saliva	54 000 57 000	— —	–	166
Angiotensin-converting enzyme	rat lung	27 000	—	–	167
	rabbit lung	136 000	1 × 136 000	–	168
Antithrombin III–heparin cofactor	dog plasma	77 000	1 × 77 000	–	98
Arginase	*Saccharomyces cerevisiae*	114 000	3 × 39 000	–	169
Ascorbate oxidase	*Cucurbita pepomedullosa*	140 000	2 × 28 000 2 × 38 000	–	170
Aspartate transcarbamylase	*Phaseolus aureus*	128 000	—	–	171
	Streptococcus faecalis	125 000	4 × 32 500		172
ATPase	*E. coli*	(see text)			61, 62
	Electrophorus electricus	— —	93 000 47 000	–	173
Azodye-protein conjugate	rat liver	80 000	2 × 44 000		174
Bromelain	pineapple stem	28 500	1 × 28 500	–	175
Calcium-activated photoproteins					
Mnemiopsin	*Ctenophores mnemiopsis* sp.	—	24 000 27 000	–	176
Berovin	*Beroë ovata*	—	25 000		176
Calcium-binding protein	chick	28 000	1 × 28 000	+	177
	pig parathyroid gland	14 500	—	+	178
	pig intestine	9000	—	+	179
Carbamyl phosphate synthetase (glutamine dependent)	*E. coli*	— —	130 000 42 000	+ +	180
Carbonic anhydrase	dog erythrocytes	28 000	1 × 28 000	+	181
Carboxylesterase	pig liver	180 000	3 × 58 000	–	183
	ox liver	180 000	3 × 59 000	–	183
	human liver	186 000	3 × 61 500	–	184
3-Carboxyl-*cis*,*cis*-muconate cyclase	*Aspergillus niger*	200 000	8 × 24 000	–	185, 186
Carboxypeptidase	yeast	62 000	1 × 62 000	+	187
Carboxypeptidase inhibitor	yeast	25 000	—	–	188
	potatoes	3100	1 × 3100	+	189

DD-Carboxypeptidase-transpeptidase	*Streptomyces R39*	53 300	1 × 53 000	+	190
Carotenoprotein	*Rhodospirillum rubrum*	—	11 000	+	182
Cell surface glycoprotein	chick fibroblast	220 000	—		191
Chymotrypsin	human	23 000	—	+	192
Citrate lyase	*Klebsiella aerogenes*	560 000	55 800 33 800 11 900	−	193
Citrate synthetase	*Azobacter vinelandii*	260 000	4 × 69 000	−	194
	Acinetobacter anitratum	260 000	4 × 69 000	−	194

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Table 2 (*cont.*)

Protein	*Source*	*Molecular weight*	*Subunit structure*	*Amino-acid analysis*	*Ref.*
Chorismate mutase	*Streptomyces aureofaciens*	63 000	4 × 14 500	–	195
Chorismate synthetase	*Neurospora crassa*	110 000	2 × 55 000	–	196
Colicin E3 immunity protein	*E. coli*	10 000	—	+	78, 79
Collagen (α1 chain)	bovine cardiac muscle	—	94 000	+	198
Collagen (α1 and α2 chains)	rabbit lung	—	100 000	+	197
Crotoxin components:	*Crotalis terrificus* serum				
Phospholipase A		15 800	1 × 15 800	+	
Crotaponin		8900	1 × 4274	+	199
			1 × 3658	+	
			1 × 1558	+	
CTP-D-glucose pyrophosphorylase	*Pasteurella pseudo-tuberculosis*	120 000	1 × 120 000	+	200
Cyclic 3′,5′ nucleotide phosphodiesterase activator protein	bovine brain	15 000	1 × 15 000	+	201
Cytochrome *b*	*Neurospora crassa* mitochondria	—	2 × 32 000	–	202
Cytochrome *b*2	yeast	230 000	4 × 58 000	+	102
Cytochrome *b*559	spinach chloroplast	—	5600	+	203, 204
Cytochrome P450	rabbit liver	—	49 000	–	205
	rat liver	—	79 000	–	206
Cytochrome *b*561	bovine adrenal gland	—	4380	+	207
3-Deoxyarabinohepulsonate 7-phosphate synthetase-chorismate mutase	*Bacillus subtilis*	160 000	4 × 38 500	+	208
Deoxyribose-5-phosphate aldolase	*Salmonella typhimuriuum*	57 000	2 × 28 500	–	209
Deoxythymidine kinase	*Bacillus stearothermophilus*	52 000	2 × 28 500	–	210
Dihydroneopterin triphosphate pyrophosphohydrolase	*E. coli*	17 000	—	–	211
Dihydropicolinic acid reductase	*E. coli*	110 000	—	–	212
DNAase:	Vaccinia virus (single-strand specific)	105 000	2 × 50 000	–	213
	Aspergillus sajae	15 600	—	–	214
	human	38 000	—	–	215

DNA-binding protein	T7 infected *E. coli*	—	31 000	–	103
DNA polymerase	*Tetrahymena pyriformis*	80 000 93 000	— 1 × 93 000	–	216
	Acinetobacter calcoaceticus	130 000	1 × 130 000	–	217
	chick embryo (nuclear)	50 000	2 × 27 000	–	218
DNA-unwinding protein	*E. coli*	90 000	4 × 22 000	–	219
EGF-binding protein	mouse submaxillary gland	29 300	—	+	220
Elastase	human leukaemia granulocytes	—	36 000	+	221
Electron-transferring protein	*Peptostreptoccus elsendii*	75 000	1 × 33 000 1 × 41 000	+	222

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Table 2 (*cont.*)

Protein	*Source*	*Molecular weight*	*Subunit structure*	*Amino-acid analysis*	*Ref.*
Elongation factor I	mouse ascites cells	—	47 000	–	223
Endo-β-(1 → 3) glucanase	*Schizosaccharomyces*	—	97 000	–	224
Endopeptidase (neutral)	rabbit kidney	93 000	1 × 93 000	+	225
Envelope protein	*E. coli*	—	36 500	+	226
Esterase	*Bacillus stearothermophilus*	47 000	1 × 47 000	–	227
Exonuclease I	rat liver microsomes	140 000	2 × 70 000	–	228
	E. coli	140 000	2 × 70 000	–	106
Ferritin	rat intestinal mucosa	—	19 000	+	229
Ficin:					
S	*Ficus carica*	—	26 000	–	230
I	fig latex	—	26 000	+	231
II	fig latex	—	26 000	+	231
Follicle-stimulating hormone	ovine pituitary glands	33 000	1 × 15 500 1 × 18 500	+	232
	equine pituitary glands	—	2 × 16 000	+	233
Formaldehyde dehydrogenase	human liver	81 400	2 × 39 500	–	234
Fructose 1,6-diphosphatase	chicken liver	142 000	4 × 38 000	+	235
Galactose binding lectin	*Bandeiraea simplicifolia*	114 000	4 × 31 000	+	117
	Abrus precatorius	134 000	2 × 33 000 1 × 35 000 1 × 36 000	–	116
	Ricinus communis	120 000	2 × 31 000 2 × 34 000	–	116
D-Galactose dehydrogenase	*Pseudomonas fluorescens*	64 000 42 000	2 × 32 000 —	+	236
Galactosyl transferase	bovine milk	55 000	—	–	110
GMI-ganglioside β-galactosidase A	human liver	72 000	1 × 72 000	–	237
Glucose-6-phosphate dehydrogenase	mouse liver	121 000	2 × 62 000	+	238
	Leuconostoc mesenteroides	103 700	2 × 55 000	+	239
	rat mammary gland	120 000	2 × 64 000	–	240
	bovine adrenal gland	285 000	4 × 64 600	–	241
Glucokinase	rat liver	57 000	1 × 57 000	–	242

β-Glucuronidase	rabbit liver	300 000	4 × 75 000	−	243
Glutamate dehydrogenase (NAD requiring)	*Neurospora crassa*	480 000	4 × 116 000	+	244
Glutamine amidotransferase	*Aerobacter aerogenes*	227 000	1 × 51 500 1 × 175 000	−	246
Glutamine synthetase	*Bacillus stearothermophilus*	—	12 × 51 000	−	111
	Bacillus stearothermophilus	630 000	12 × 54 000		245
Glutathione peroxidase	ovine erythrocyte	88 000	4 × 22 000	−	247
	rat liver	78 500	4 × 19 000	+	248
Glutathione reductase	sea-urchin egg	102 000	2 × 52 300	−	249

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Table 2 (*cont.*)

Protein	*Source*	*Molecular weight*	*Subunit structure*	*Amino-acid analysis*	*Ref.*
Glutathione *S*-transferase	rat liver	45 000	2 × 25 000	+	250
Glycogen synthetase (glucose-6-phosphate dependent)	yeast	300 000	4 × 77 000	–	251
Glycoprotein	human erythrocyte	—	23 000	+	252
GTPase inhibitor	*E. coli*	23 000	1 × 23 000	–	253
Guanine deaminase	rabbit liver	55 000	1 × 55 000	–	254
Haemagglutinin:					
I	*Phaseolus coccineus* L.	120 000	—	+	256
II	*Phaseolus coccineus* L.	120 000	4 × 34 000	–	256
anti-H	eel serum	140 000	—	–	119
anti-H	*Cystisus sessilifolius*	110 000	—	–	119
	Maackia amurensis	130 000	4 × 33 000	–	118
Haemocyanin	*Cherax destructor*	—	75 000	–	255
Hemerythrin	*Phascolosoma agassizii*	32 200	3 × 13 000	–	257
Hexokinase	wheat germ	51 000	1 × 51 000	–	258
Hexon protein (type 2)	adenovirus	363 000	3 × 120 000	–	259
High-density lipoprotein	human serum	—	9500 13 000	–	260
Histidine decarboxylase	*Micrococcus* sp.n.	110 000	3 × 7000 3 × 29 000	–	261
Hyaluronidase	bovine testis	61 000	1 × 61 000	–	262
3-Hydroxybenzoate 4-hydroxylase	*Pseudomonas testosteroni*	145 000	—	–	263
17β-Hydroxysteroid dehydrogenase		35 500	—	–	264
Δ^5-3β-Hydroxysteroid dehydrogenase	sheep adrenal gland	—	40 000	–	265
Hypoxanthine-guanine phosphoribosyl transferase	chinese hamster brain	78 000	3 × 25 000		266
	human erythrocyte	—	26 000		267
Inhibitor of chitin synthetase activation	*Saccharomyces cerevisiae*	8500	1 × 8500	+	268
Initiation factor:					
1F-2a	*Caulobacter crescentus*	—	84 000	–	269
1F-3	*E. coli*	—	22 600	–	270
1F-3	*Caulobacter crescentus*	—	25 000	–	271

Interferon	mouse L-cell	—	38 000 22 000	−	89
Isocitrate dehydrogenase (NAD specific)	pig heart	40 000	1 × 40 000	+	272
Isocitrate lyase	*Turbatrix aceti*	480 000	4 × 123 000	−	273
Kallikrein	rat urine	35 300	1 × 35 300	−	274
	rat urine	33 100	1 × 33 100	−	124
	human urine	43 600	—	+	275
α-Ketoglutarate semialdehyde dehydrogenase	*Pseudomonas putida*	120 000	2 × 60 000	−	276

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Table 2 (*cont.*)

Protein	*Source*	*Molecular weight*	*Subunit structure*	*Amino-acid analysis*	*Ref.*
Ketopantoyl lactone reductase	yeast	27 000	—	–	277
Kynurenine formanudase	chick liver	59 000	2 × 30 000	–	278
		22 000	1 × 22 000	+	279
Lactamase(s)	*Bacillus cereus*	28 000	1 × 28 000	+	279
Lactate oxygenase	*Mycobacterium phlei*	350 000	6 × 57 000	+	280
Lectin	*Abrus precatorius*	60 100	2 × 32 000	+	
		68 000	1 × 28 000 1 × 33 000 2 × 7000	+	281
	Pisum sativum	49 000	2 × 17 000	–	282
	Ricinus communis	118 000	1 × 29 500 1 × 34 000	–	
			1 × 65 000		283
		65 000	1 × 29 500 1 × 34 000	–	
	Ulex europeus	160 000	2 × 43 000 2 × 45 000	–	16
Legumin	*Vicia faba*	—	20 100 20 900 23 800 37 000	+	284
Ligandine	pig liver	55 000	2 × 25 000	+	285
Lipase	rat liver lysosomes	—	58 000	–	286
Lipoprotein lipase	rat plasma	—	37 000	+	287
	pig adipose tissue	62 000	1 × 62 000	–	125
Low-density lipoprotein	human plasma	—	33 000	+	288
Luteinizing hormone	horse pituitary gland	34 000	1 × 12 500 1 × 23 000	+	289
Lysine decarboxylase	*E. coli*	—	5 × 80 000	–	32
Lysine mono-oxygenase	*Pseudomonas fluorescens*	246 000	4 × 61 000	+	290
Lysine-rich protein	*Mytilus edulus* sperm	—	5000	+	291
Maltose binding protein	*E. coli*	44 000	1 × 44 000	–	292
Mandelate racemase	*Pseudomonas putida*	—	4 × 69 500		293

Membrane proteins	Semiliki forest virus	—	2 × 50 000 1 × 10 000	+	69
	E. coli	—	33 400		294
Metallothionein	horse liver and kidney	6000	1 × 6000	+	295
	human liver	—	6600	+	296
Methionine synthetase activating protein	*E. coli*	—	27 000 19 400	−	297
Methylenetetrahydrofolate dehydrogenase	*Clostridium formicoaceticum*	60 000	2 × 32 000	+	298
Methylmalonyl-CoA mutase	*Pseudomonas shermanii*	124 000	1 × 61 000 1 × 66 000	−	299
Mitogen(s)	*Phytolacca americana*	—	19 000 21 000 22 000 25 000 31 000	+ + + + +	300

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Table 2 (*cont.*)

Protein	*Source*	*Molecular weight*	*Subunit structure*	*Amino-acid analysis*	*Ref.*
cis,cis-Muconate cycloisomerase	*Pseudomonas putida*	252 000	6 × 42 000	–	301
Muconolactone isomerase	*Acinetobacter calcoaceticus*	100 000	8 × 12 000		302
	Pseudomonas putida	94 000	8 × 11 500	+	302
N-Acetylgalactosaminyltransferase	human plasma	85 000	1 × 85 000	+	303
N-Acetylglucosaminidase	*Streptomyces griseus*	28 000	1 × 28 000	+	304
	human plasma	105 000	1 × 105 000	+	305
N-Acetylhexosaminidases (A and B)	human placenta	—	4 × 18 000	+	306, 307
	human placenta	120 000	4 × 33 000	–	308
NADPH-cytochrome reductase	pig kidney	71 000	—	–	309
NADPH-sulphite reductase	*E. coli*	700 000	12 × 60 000	+	310
Neuraminidase	*Streptococcus* K6646	110 000	—	–	311
Neurotoxin	*Clostridium tetani*	150 000	1 × 10 700 1 × 53 000	–	312
Nitrate reductase	*E. coli*	720 000	4 × 58 000 4 × 142 000	–	313
	E. coli	320 000	—	–	314
	Neurospora crassa (nit-3 mutant)	160 000	—	–	315
Nitrite reductase	*Achromobacter fisheri*	80 000	2 × 39 000	–	316
Nitrogenase Mo-Fe protein	soya bean nodule bacteroids	200 000	4 × 50 000	+	317
Nucleoside diphosphokinase	bovine heart	108 000	6 × 18 000	–	318
Nucleoside triphosphate phosphohydrolase I	vaccinia virus	68 000	1 × 68 000	–	319
L-Ornithine carbamoyltransferase	*Saccharomyces cereoisiae*	125 000	3 × 37 000	–	169
Ornithine cyclase	*Clostridium sporogenes*	81 000	2 × 41 500	+	320
Ovarian growth factor	bovine pituitary gland	13 400	1 × 13 460	+	321
2-Oxoglutarate dehydrogenase	pig heart	216 000 15 600 16 100	2 × 113 000 — —	+ + +	322
Pepsin inhibitor(s)	*Ascaris lumbricoides*	17 500 31 700	— —	+ +	323
Pesticin	*Yersinia pestis*	65 000	1 × 65 000	+	324
Phosphatidylserine decarboxylase	*E. coli*	—	35 000	+	325

1-Phosphofructokinase	*Clostridium pasteurianum*	63 000	1 × 40 000	−	326
6-Phosphofructokinase	rat liver	365 000	4 × 82 000	−	34
	sheep heart	—	40 000	−	35
Phosphoglucomutase	*Bacillus subtilis*	130 000	2 × 60 000	−	327, 328
6-Phosphogluconate dehydrogenase	*Bacillus stearothermophilus*	101 000	2 × 51 000	−	329
	human erythrocyte	104 000	2 × 52 000	+	330, 331
Phosphoglucose isomerase	yeast	120 000	2 × 60 000	+	45, 46
		142 000	2 × 68 000	+	

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Table 2 (*cont.*)

Protein	*Source*	*Molecular weight*	*Subunit structure*	*Amino-acid analysis*	*Ref.*
Phospho-β-glucosidases (A and B)	*E. coli*	132 000	2 × 65 000	+	332
Phospholipase A1	*Myobacterium phlei*	—	45 000	−	333
Phospholipase C	*Clostridium perfingens*	—	43 000	−	133
Phosphoribosyl pyrophosphate synthetase	rat liver		40 500	−	334
Phosphorylase a	*Callinectes danae* muscle	176 000	1 × 176 000	−	335
Phosphorylase kinase	rabbit muscle	—	42 000 118 000 136 000		88
Phytohemagglutinin	lentil	52 000	2 × 8000 2 × 18 000	+	336
Phytohemagglutinin receptor protein	human erythrocyte	—	100 000		129
Poly-A binding protein	HeLa cells	158 000	4 × 38 000	−	30
Prekeratin	cow nose	375 000	2 × 60 000 1 × 72 000	−	337
Proelastin	*Protopterus aethiopicus*	—	25 000	−	338
Prolactin receptor protein	rabbit mammary gland	220 000	—	−	135
Prolidase	pig intestine	108 000	2 × 53 000	+	339
Protease	*Staphylococcus aureus*	29 000	1 × 29 000	+	340
		—	24 000	+	341
	Escherichia freundii	51 000	1 × 51 000	+	342
	Tremeresurus gramineus	29 500	—	+	343
	Tritirachium album	18 500	—	−	344
	Physarum polycephalum	35 000	1 × 35 000	−	345
Protease inhibitor	yeast	61 000	4 × 23 000	−	346
	yeast	10 000	1 × 10 000	−	347
Protein kinase	*Neurospora crassa*	60 000	—	−	348
Proteolipid P-7	rat brain	—	—	+	349
Prothrombin	chicken plasma	73 000	1 × 73 000	+	350
Protyrosinase	*Rana pipiens*	200 000	4 × 54 100	−	351
Pteroyl-α-oligoglutamyl endopeptidase	chick intestine	80 000	—	−	136
Pyruvate carboxylase	*Azotobacter vinelandii*	296 000	—	−	353

	pig liver	—	47 000		354
Pyruvate kinase (fructose 1,6-diphosphate activated)	*E. coli*	240 000	4 × 60 000	+	352
Quinolinic acid phosphoribosyltransferase	*Ricinus communis*	72 000	2 × 35 000	−	355
Reaction centre proteins	*Rhodopseudomonas spheroides*	—	21 000 24 000 28 000	+	63, 64
Relaxin	pig ovary	65 000	—	+	356
Reverse transcriptase	avian myeloblastosis virus	—	60 000 100 000	−	357
Ribonuclease	*Penicillium janthinellum*	30 000	—		358

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Table 2 (*cont.*)

Protein	*Source*	*Molecular weight*	*Subunit structure*	*Amino-acid analysis*	*Ref.*
	rat liver	125 000	1 × 43 000 1 × 85 000	–	359
	bovine brain	39 000	1 × 39 000	–	360
Ribonucleotide reductase	*Euglena gracilis*	440 000	4 × 100 000	–	361
Ribose binding protein	*E. coli*	29 000	1 × 29 500	–	362
D-Ribulose 1,5-diphosphate carboxylase	*Rhodospirillum rubrum*	114 000	2 × 56 000	+	363, 364
RNA polymerase	T7 infected *E. coli*	110 000	1 × 100 000	+	38
	Bacillus stearothermophilus	—	40 000 95 000 140 000 148 000	–	365
	Thermus aquaticus	—	45 000 99 000 160 000 170 000	–	365
	Agrobacterium tumifaciens	—	41 000 98 000 150 000 160 000	–	366
	PBS 2 infected *Bacillus subtilis*	262 000	1 × 48 000 1 × 58 000 1 × 76 000 1 × 80 000	–	40
	yeast mitochondria	—	67 000	–	367
	yeast	—	29 000 41 000 135 000 190 000	–	368
	Zea mays chloroplast	—	180 000 140 000	–	369
	Physarum polycephalum	—	125 000 205 000	–	44

	Physarum polycephalum	—	17 000 24 000 45 000 135 000 200 000	−	43
	mouse myeloma	500 000	1 × 16 500 1 × 27 000 1 × 50 500 1 × 60 500 1 × 117 000 1 × 195 000	−	370
	rat liver	—	16 500 25 000 44 000 51 000 126 000 197 000	−	42
Salicylate 1-mono-oxygenase	*Pseudomonas putida*	—	454 000	−	371
Sexual inducer	*Volvox carteri*	32 000	—	+	90
S-factor	*E. coli*	26 000	2 × 11 500	−	372
S-formylglutathione hydrolase	human liver	55 500	2 × 30 000	−	373
Spectrin	human erythrocyte	—	240 000	+	59
Stearyl-CoA desaturase	rat liver	—	53 000	−	374

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Table 2 (*cont.*)

Protein	*Source*	*Molecular weight*	*Subunit structure*	*Amino-acid analysis*	*Ref.*
Steroid β-D-glucosidase	rabbit liver	58 000	1 × 58 000	–	375
Stringent factor	*E. coli*	—	77 000	–	376
Sucrase	*Bacillus subtilis*	—	55 000	–	377
Superoxide dismutase	*Photobacterium lecognathi*	33 700	1 × 15 500 1 × 17 000	–	378
Thermomycolase	*Malbranchea pulchella*	33 000	1 × 33 000	–	9
Thioredoxin	calf liver	—	12 000	+	380
L-Threonine deaminase	*Clostridium tetanomorphum*	184 000	4 × 46 000	+	381
Thymidilate synthetase	T2-infected *E. coli*	64 400	2 × 31 500	+	379
Thyroxine-binding protein	dog liver and kidney	70 000	—	–	382
Trimethylamine dehydrogenase	bacterium 4B6	161 000	—	–	383
tRNA synthetases:					
Histidyl	*E. coli*	85 000	2 × 42 500	+	384
	Salmonella typhimurium	78 000	2 × 46 000 2 × 39 000	–	385
Phenylalanyl	*E. coli*	267 000	2 × 94 000	–	386
Tryptophan	yeast	110 000	2 × 50 000	–	387
Tyrosyl	*Bacillus stearothermophilus*	95 000	2 × 45 000	+	388
Valyl	*Bacillus stearothermophilus*	110 000	1 × 110 000	–	389
Trypsin	human	21 000	—	+	192
Trypsin inhibitor	guinea-pig serum	—	78 000	+	390
	human pancreas	6200	1 × 6200	+	391
Tryptophan synthase	yeast	143 000	4 × 37 000	–	139
	Neurospora crassa	—	75 000	–	392
	human placenta	125 000	2 × 58 000	–	393
Tubulin	*Naegleria gruberi*	—	55 000	+	394
	bovine thyroid gland	114 000	2 × 55 000	+	395
T11	T4	70 000	3 × 24 000	+	396
Uricase	pig liver	125 000	4 × 32 000	–	397
Uridine phosphohydrolase	rat liver	102 500	—	–	398
Vitamin B12-binding protein	human amnionic fluid	59 300	1 × 59 300	–	399
	human milk and saliva	63 000	1 × 63 000	+	400

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interferon subunits by SDS-gel electrophoresis and their detection by measurement of activity in gel slices after renaturation. Two interferon subunits of molecular weights 38 000 and 22 000 respectively were detected by this technique, which is independent of the purity of the sample.

Sexual Inducer. Male cultures of *Volvox carteri* can produce a substance which induces the appearance of female colonies in asexual cultures. The sexual inducer substance has been purified [90] and shown to be a glycoprotein of minimum molecular weight 32 000.

4 Chemical Modification

Methods—*Thiol Groups.* For the quantitative estimation of thiol groups a new reagent, thio-Michler's ketone (1) has been proposed.[401] An intensely coloured

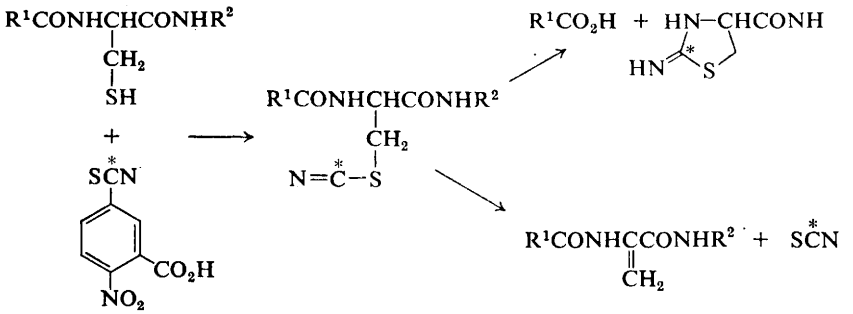

Figure 3 *Cleavage and β-elimination in a cysteine residue modified by NTCB* (Reproduced by permission from *Biochemistry*, 1974, **13**, 1)

product with a high extinction coefficient is formed. In addition, two fluorescent thiol-specific reagents have been described. One is a maleimide derivative, *N-p*-(2-benzimidazoyl)phenyl maleimide (2);[402] the other is *S*-mercuric *N*-dansylcysteine.[403] These reagents can be used both for the quantitative estimation of thiol groups and also for the introduction of fluorescent probes into proteins. The mechanism of *S*-cyanylation of cysteine residues and their alkaline cleavage has been studied further.[404] Increasing the pH above 8.0 accelerated the cleavage reaction but also caused the β-elimination of thiocyanate to a degree dependent upon the structural properties of the β-thiocyanoalanine peptides (Figure 3). Also the extent of modification of small thiol peptides by 2-nitro-5-thiocyanobenzoic acid was found to be decreased at high concentrations of reagents and hence it is recommended that the concentration of thiol groups should not exceed 0.5 mmol l^{-1} during modification.

Two new reagents for the oxidation of intracellular thiol groups have been described.[405] The reagents are diazene dicarboxylic acid bis(*N'*-methylpiperazide)

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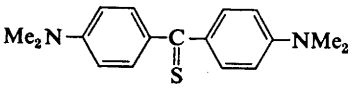
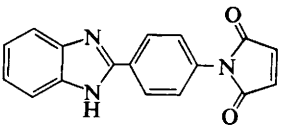
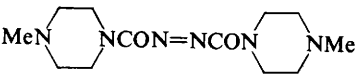


(1)



(2)



(3)

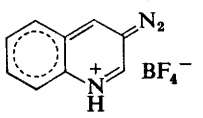
[DIP, (3)], the *N'*-methyl iodide salt of DIP, and the bis(*N'*-methyl iodide) salt of DIP; these have been shown to oxidize rapidly intracellular glutathione.

Disulphide Bridges. The simultaneous reduction and mercuration of disulphide bonds by univalent mercury has been shown to be a one-step process which excludes the reoxidation of free thiol groups.[406] It may be performed under mild conditions and hence may be a useful method for the preparation of heavy-atom derivatives of proteins.

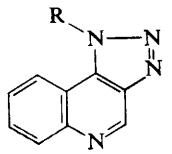
Amino-groups. The introduction of heavy atoms at specific amino-groups in tobacco mosaic virus and the preparation of an isomorphous heavy-atom derivative have been achieved by modification with 4-sulphophenylisothiocyanate and subsequent reaction with methylmercury nitrate.[407] In the isolated coat protein of strain U2 the *N*-terminal proline residue and lysine-53 reacted, whereas in the intact virus only the *N*-terminal proline residue reacted.

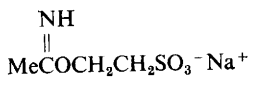
A cyclohepta-amylose dansyl chloride complex, a soluble form of dansyl chloride prepared by the precipitation of dansyl chloride on cyclohepta-amylose, has been introduced for the fluorescent labelling of proteins.[408] Its advantage is that dansylation may be efficiently effected in aqueous media, under conditions where dansyl chloride itself would be insoluble and hence would not react readily with proteins.

Lysyl residues in trypsin, lysozyme, and insulin have been specifically modified at pH 8.0 and 4 °C with the reagent 3-diazoquinoline tetrafluoroborate (4).[409]

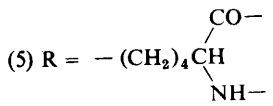


(4)





(6)



(5) R = $-(CH_2)_4CH$ (CO– / NH–)

[406] M. M. David, R. Sperling, and I. Z. Steinberg, *Biochim. Biophys. Acta*, 1974, **359**, 101.
[407] U. Gallwitz, L. King, and R. N. Perham, *J. Mol. Biol.*, 1974, **87**, 257.
[408] T. Kinoshita, F. Iinuma, and A. Tsuji, *Analyt. Biochem.*, 1974, **61**, 632.
[409] M. Kanazawa and S. Ishii, *Biochim. Biophys. Acta*, 1974, **342**, 155.

The lysyl residues are converted into the 1-substituted 1,2,3-triazolo-[4,5-*c*]-quinoline (5).

A novel imido-ester, isoethionyl acetimidate (6), has been introduced for the modification of amino-groups of membranes.[410] Its main advantage over ethyl acetimidate is that it does not penetrate the erythrocyte membrane.

Phosphoserine. The instability of phosphoserine under acidic conditions makes its estimation in phosphoproteins difficult. It has recently been shown, however, that such residues may be modified with methylamine to produce the stable product β-*N*-methyldiaminopropionic acid (*via* dehydroalanine) (Figure 4) which may then be estimated by amino-acid analysis.[411]

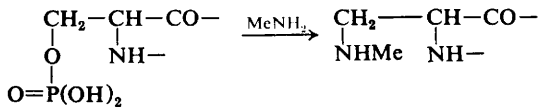


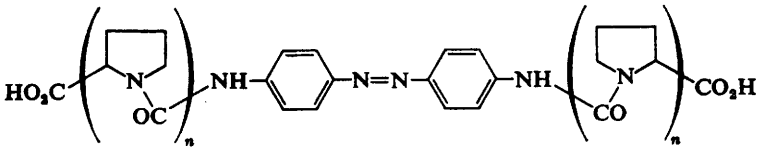
Figure 4 *Conversion of phosphoserine residues into* β-N-*methyldiaminopropionic acid* (Reproduced by permission from *Biokhimiya*, 1974, **39**, 235)

Iodination. Bovine lactoperoxidase attached to Sepharose-4B has been shown to be a versatile reagent which catalyses the iodination of proteins under denaturing conditions in the presence of either urea or SDS.[412] Lactoperoxidase coupled to polyacrylamide has also been shown to catalyse iodination of proteins efficiently.[413] These insolubilized enzymes have the advantage over the soluble form of the enzyme that they and contaminating proteins often present in enzyme preparations can be readily removed after iodination.

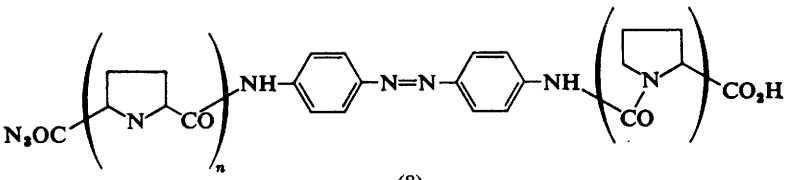
Cross-linking Reagents. Two new cleavable types of cross-linking reagent have been described. One type of reagent contains an azo-dye moiety which can be cleaved by treatment with mild dithionite.[414] The key intermediate (7) may be converted into a reagent (8) suitable for the cross-linking of amino-groups and also into (9), which can be used for the cross-linking of thiol groups. A trifunctional reagent (10) is also described. The second series of reagents (11)—(13) contains the tartaryl moiety which can be cleaved quantitatively with periodate.[415] The utility of this latter series of reagents has been demonstrated by reaction with *E. coli* ribosomes. After cross-linking of the ribosomal proteins, cross-linked pairs were isolated and their components identified by gel electrophoresis after cleavage with periodate.

Another frequently used method has been employed for the identification of the components of cross-linked species after cross-linking of *E. coli* ribosomes with di-imido-esters. It involves the use of antibodies to the individual protein components of the ribosomal particle.[416]

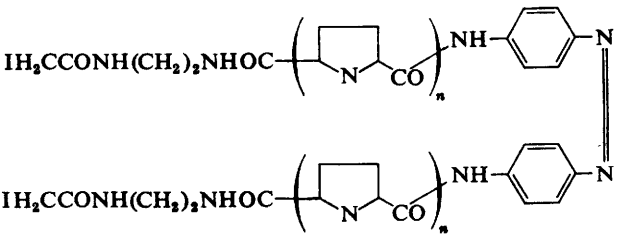
[410] N. M. Whitely and H. C. Berg, *J. Mol. Biol.*, 1974, **87**, 541.
[411] V. Yu Kolesnikova, V. A. Sklyankina, L. A. Baratova, T. I. Nazarova, and S. M. Araeva, *Biokhimiya*, 1974, **39**, 235.
[412] G. S. David and R. A. Reisfeld, *Biochemistry*, 1974, **13**, 1014.
[413] J. I. Thorell and I. Larsson, *Immunochemistry*, 1974, **11**, 203.
[414] K. Wetz, H. Fasold, and Ch. Meyer, *Analyt. Biochem.*, 1974, **58**, 347.
[415] L. C. Lutter, F. Ortanderl, and H. Fasold, *F.E.B.S. Letters*, 1974, **48**, 288.
[416] L. C. Lutter, U. Bode, and C. G. Kurland, *Molec. gen. Genet*, 1974, **129**, 167.



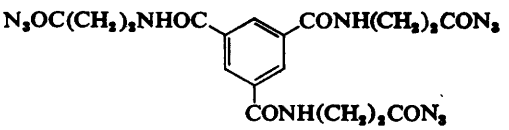
(7) $n = 4 - 13$



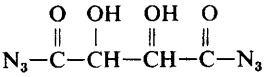
(8)



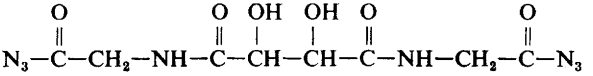
(9)



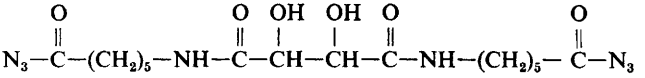
(10)



(11)



(12)



(13)

Applications – General Summary.—The uses of chemical techniques for the modification of proteins are extremely numerous. The application of such techniques to the study of the architecture of membranes and ribosomal particles is discussed in the following section.

The many other uses of the chemical modification of proteins are summarized in Table 3 (p. 80).

The technique of affinity labelling is discussed separately in a further section.

Topographical Studies.—The dispositions of proteins within larger structures can be examined by chemical modification techniques. Bifunctional cross-linking agents, for example, can be used to determine which pairs of molecules are neighbours within the structures. By the use of reagents of different lengths estimates of their distances apart may be obtained.

Also, differential reactivity to chemical reagents may be used to determine the degree of exposure of chemical groups in the proteins, which in turn gives an indication of the degree of exposure of the protein itself. The interpretation of both kinds of experiment, however, depends upon the degree to which the reagent penetrates the structure.

Membrane Proteins. Several reagents which are unable to penetrate the membranes of intact cells but which form stable adducts with proteins are available. These include iodide generated in the presence of lactoperoxidase, diazotized sulphanilic acid, formylmethionyl sulphone methyl phosphate, and the novel imido-ester, isoethionyl acetimidate (6) described above.

An additional advantage of lactoperoxidase catalysed iodination is that it is a mild procedure which often does not impair membrane function. For instance, it has been found that iodinated HeLa cells grow at the same rate as untreated cells.[417] The iodinated surface proteins could be removed by treatment with Pronase and their molecular weights were estimated as 170 000, 145 000, 130 000, 93 000, and 53 000.[418]

Lactoperoxidase catalysed iodination of the erythrocyte membrane showed that one protein component of molecular weight 100 000 (component 111) was labelled from both inside (resealed ghosts) and outside of the cell suggesting that it spans the membrane. The major glycoprotein component was labelled from outside only and spectrin, a glycoprotein, from inside only.[419] Independently the major protein component and a sialoglycoprotein component were shown to extend through the erythrocyte membrane.[420]

Lactoperoxidase iodination has also been used in a comparison of the surface proteins of circulating human platelets and lymphocytes.[421] It appears that a set of surface proteins is common to both cell types as judged by the criteria of polyacrylamide gel electrophoresis and peptide mapping.

Imido-esters also do not appear seriously to impair membrane function. For example, erythrocyte membranes saturated with isoethionyl acetimidate (a non-penetrating reagent) functioned relatively as normal as judged by the

[417] C. M. Tsai, C. C. Huang, and E. S. Canellakis, *Biochim. Biophys. Acta*, 1974, **332**, 47.
[418] C. C. Huang, C. M. Tsai, and E. S. Canellakis, *Biochim. Biophys. Acta*, 1974, **332**, 59.
[419] B. C. Shin and K. L. Carraway, *Biochim. Biophys. Acta*, 1974, **345**, 141.
[420] D. H. Boxer, R. E. Jenkins, and M. J. A. Tanner, *Biochem. J.*, 1974, **137**, 531.
[421] M. J. A. Tanner, D. H. Boxer, J. Cumming, and J. Verrier Jones, *Biochem. J.*, 1974, **141**, 909.

criteria of acetylcholinesterase activity, rate of potassium loss, and facilitated diffusion of glucose.[410] Cross-linkage of this kind of membrane with dimethyl adipimidate (a penetrating reagent) did not affect potassium ion selectivity.[422]

Double labelling experiments with [^{14}C]isoethionyl acetimidate and [^{3}H]ethyl acetimidate have been performed on the erythrocyte membrane.[410] The former, which does not penetrate the membrane, was first used to saturate the amino-groups on the outer surface of the cell and then the remaining sites were saturated with ethyl acetimidate which does penetrate the membrane. Quantitative experiments of this type suggest that 10 times as many reactive groups in protein are to be found on the inner surface than on the outer surface of the erythrocyte membrane. Further it appears that as many as three major polypeptide components and a glycoprotein component may span the membrane. The polypeptides have molecular weights of 170 000, 93 000, and 78 000 and the glycoprotein migrates in a 5% gel with the same mobility as a protein of molecular weight 93 000. Many other polypeptides are exposed only on the inner surface of the membrane.

Two glycoproteins have been successfully cross-linked in erythrocyte membrane ghosts with dimethyladipimidate indicating their proximity.[423–425] The cleavable cross-linking reagent, dithiobispropionimidate, has been used in more extensive nearest-neighbour analysis of erythrocyte membrane proteins.[426] The cross-linking pattern observed was consistent with a picture of trans-membrane and cytoplasmic surface components anchored to a submembrane network of spectrin fibres.

Evidence has been presented for lysine-derived cross-links in the proteins of human erythrocyte membranes.[427] After reduction of the membranes with sodium borohydroxide and hydrolysis, ε-hydroxynorleucine, which is the reduction product of α-aminoadipic-δ-semialdehyde, and ε-chlorononorleucine, which may be formed from ε-hydroxynorleucine during hydrolysis in 6M-HCl, were detected.

An example of the use of the non-penetrating reagent, formyl-methionyl sulphone methyl phosphate, is provided by a study of the spike glycoproteins in the Semliki Forest virus.[428] On the basis of these experiments and of cross-linking studies with dimethylsuberimidate, it is suggested that the glycoproteins extend through the viral membrane and are in close contact with the nucleocapsid.

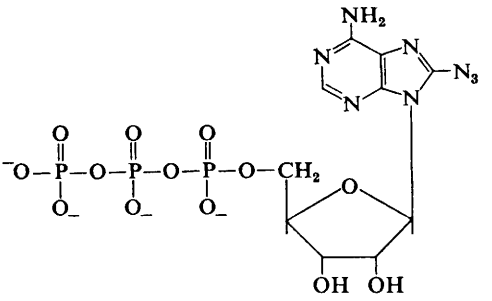
Diazobenzene sulphonate, another non-penetrating membrane probe, has been used to study the localization of components in the inner membrane isolated from rat-liver mitochondria.[429] The membrane appeared to be asymmetric since two polypeptides with molecular weights of 66 000 and 26 000 were labelled at the outer surface of the membrane and five polypeptides were labelled on its inner surface.

[422] N. I. Krinsky, E. N. Bymun, and L. Packer, *Arch. Biochem. Biophys.*, 1974, **160**, 350.
[423] T. H. Ji, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 93.
[424] T. H. Ji and I. Ji, *J. Mol. Biol.*, 1974, **86**, 129.
[425] T. H. Ji, *J. Biol. Chem.*, 1974, **249**, 7841.
[426] K. Wang and F. M. Richards, *J. Biol. Chem.*, 1974, **249**, 8005.
[427] R. G. Langdon, *Biochim. Biophys. Acta*, 1974, **342**, 229.
[428] H. Garoff and K. Simons, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 3988.
[429] H. M. Tinberg, R. L. Melnick, J. Maguire, and L. Packer, *Biochim. Biophys. Acta*, 1974, **345**, 118.

Chemical labelling techniques such as lactoperoxidase catalysed iodination may be exploited to study changes in the topography of membranes in different physiological states. Hence, the degree of exposure of plasma membrane proteins of baby hamster kidney cells was shown to vary with the growth state,[430] and a protein of molecular weight 78 000 was preferentially exposed on the surface of differentiated as compared with undifferentiated murine neuroblastoma cells.[431] Also, a heavily iodinated component (mol. wt. 250 000) was detected in untransformed but not in transformed fibroblasts.[432] Erythrocytes are known to exist in two distinct states, oxygenated and deoxygenated. Recently, it has been shown that membrane proteins (and also glycolipids) react much more readily with dansyl chloride in the deoxygenated than in the oxygenated state.[433]

Membrane components have also been photochemically labelled by reagents which are able to penetrate into the lipid bilayer of the membrane. The active species may then be generated *in situ* by irradiation. When this approach was used to label liposomes and sarcoplasmic reticulum membranes (with 1-azidonaphthalene and 1-azido-4-iodobenzene), 50% of the label was attached to protein.[434] Similarly, radioactive 2-azido-4-nitrophenol, an uncoupler of oxidative phosphorylation, was used to label mitochondrial components presumably concerned with the uncoupling process; 40% of the incorporated radioactivity was found to be associated with protein.[435]

When an enzyme activity can be attributed to a membrane protein the technique of affinity labelling can be used. Hence, the cation-stimulated ATPases of human red-cell membranes have been investigated with the photoaffinity ATP analogue, δ-azido adenosine triphosphate (14).[436] In the absence of light, this



(14)

substance is a substrate for both the Mg-ATPase and the ouabain-sensitive Na,K-ATPase. Photolysis causes inactivation of both ATPases and covalent incorporation of labels into three protein components of the membrane.

Ribosomal Proteins. Techniques similar to those described for the investigation of membrane topography have been used for the study of the architecture of

[430] A. M. Mastro, C. T. Beer, and G. C. Mueller, *Biochim. Biophys. Acta*, 1974, **352**, 38.
[431] R. Truding, M. L. Shelanski, M. P. Daniels, and P. Morell, *J. Biol. Chem.*, 1974, **249**, 3973.
[432] N. M. Hogg, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 489.
[433] O. Tukenaka, T. Sakai, T. Yora, and Y. Inada, *Biochem. Biophys. Res. Comm.*, 1974, **59**, 742.
[434] A. Klip and C. Gitler, *Biochem. Biophys. Res. Comm.*, 1974, **60**, 1155.
[435] W. G. Hanstein and Y. Hatefi, *J. Biol. Chem.*, 1974, **249**, 1356.
[436] B. E. Haley and J. F. Hoffman, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 3367.

ribosomes. Thus, many cross-linked pairs have been identified in *E. coli* 30S ribosomal sub-particles and a lesser number in 50S sub-particles after cross-linking with the bifunctional cross-linking reagent dimethyl suberimidate.[437] This allows a comparison to be made between the 30S ribosomal protein 'neighbourhoods' identified by this technique with the assembly map of reconstitution *in vitro* (Figure 5). (There is as yet no corresponding map for the 50S

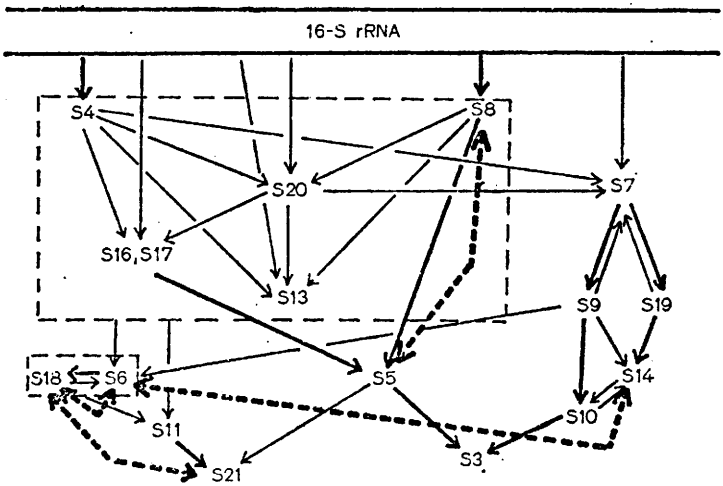


Figure 5 *Comparison of 30S ribosomal protein neighbourhoods identified by treatment with cross-linking agents with the assembly map of reconstitution* in vitro. *Solid arrows indicate interaction in reconstitution* in vitro, *dashed arrows join proteins shown to be neighbours by the use of cross-linking agents. S1, S2, S12, and S15 have not yet been placed*
(Reproduced by permission from *European J. Biochem.*, 1974, **42**, 21)

particle since a total reassembly of the *E. coli* 50S particle has only recently been achieved.[438]) By use of a novel cleavable cross-linking agent containing the tartryl moiety (9) the previously established 'neighbourhoods' S5–S8, S7–S9, and S13–S19 have been confirmed and in addition the protein 'neighbourhoods' S6–S18, S2–S8, S2–S5, S3–S10, S2–S3, and S4–S5 have been identified.[415] Cross-linking experiments with dimethyl suberimidate have also helped to elucidate the mechanism of action of initiation factor-3 (IF-3) in protein synthesis.[439] As illustrated in Figure 6, IF-3 binds to 70S ribosomes and is in contact with both subunits (A-II). This causes a structural loosening (A-III) which exposes the mRNA binding site. The mRNA then binds to the 70S particle and IF-3 is released (A-IV). When the subunits are covalently cross-linked (B-1′) IF-3 can no longer cause the structural loosening required for exposure of the mRNA binding site.

[437] C. Clegg and D. Hayes, *European J. Biochem.*, 1974, **42**, 21.
[438] K. N. Nierhaus and F. Dohme, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 4713.
[439] D. A. Hawley, M. J. Miller, L. I. Slobin, and A. J. Wahba, *Biochem. Biophys. Res. Comm.*, 1974, **61**, 329.

Differential reactivity to chemical reagents has also been used as a probe of *E. coli* 70S ribosomal topography. The complexity of the interpretation of these experiments has been well illustrated by Benkov and Delias who have summarized data relating to the chemical modification of proteins of 30S and 50S ribosomal subparticles using a variety of reagents.[440] These include kethoxal, iodine, fluorescein thiocyanate, glutaradehyde, *N*-ethylmaleimide, and trypsin. The agreement between the various results is not very great.

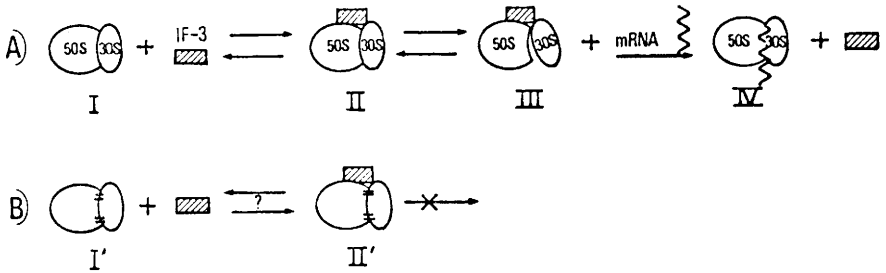


Figure 6 *Model for the mechanism of action of IF-3 on 70S ribosomes*
(Reproduced by permission from *Biochem. Biophys. Res. Comm.*, 1974, **61**, 329)

More readily interpretable are results obtained by iodination with lactoperoxidase. Litman and Cantor have shown by the use of this technique that the proteins L2, L26, L28, and L18 are labelled more in isolated 50S subparticles than in intact 70S ribosomes, suggesting that these proteins are at least partially protected by the 30S subunit.[441] Similarly the same group of workers has shown that proteins S9 and S18 are also near the subunit interface or are involved in interface related functions.[442]

The difficulties mentioned above notwithstanding, several groups have investigated the degree of exposure of the thiol groups of ribosomal proteins with alkylating agents. The total numbers of thiol groups in *E. coli* ribosomal subunits have been determined to be 16—17 (30S) and 26—27 (50S) and the proteins reacting in the native subunits with *N*-ethylmaleimide and iodoacetamide have been identified as S1, S2, S12, S13, S18, S21, L2, L5, L6, L10, L11, L15, L17, L20, L26 + L28, and L27.[443] In an independent study of aminoethylation of isolated *E. coli* 30S subunits, proteins S4, S8, S11, and S14 were found to have reacted in addition to the 30S proteins listed above.[444] Moreover, reconstitution experiments demonstrated that aminoethylation of S4, S13, S17, and S18 did not affect their functional activities in poly(U)-dependent poly(Phe) synthesis. In contrast, aminoethylation of S2, S11, S12, S14, and S21 forms a kinetic or thermodynamic barrier to assembly of active 30S particles *in vitro*. The reactions of thiol groups with *N*-ethylmaleimide and DTNB have been to study the available thiol groups of mammalian ribosomes in different functional states.[445] Changes in

[440] K. Benkov and N. Delihas, *Biochem. Biophys. Res. Comm.*, 1974, **60**, 901.
[441] D. J. Litman and C. R. Cantor, *Biochemistry*, 1974, **13**, 512.
[442] D. J. Litman, C. C. Lee, and C. R. Cantor, *F.E.B.S. Letters*, 1974, **47**, 268.
[443] A. Bakardjiera and R. R. Crichton, *Biochem. J.*, 1974, **143**, 599.
[444] L. Kahan, W. A. Held, and M. Nomura, *J. Mol. Biol.*, 1974, **88**, 797.
[445] P. M. Steinert, B. S. Baliga, and H. N. Munro, *J. Mol. Biol.*, 1974, **88**, 895.

availability have been suggested to reflect conformational changes associated with an opening up of ribosomal structure as it progresses from having the peptidyl-tRNA at the A-position to the D-position, and then binds EF2 and GTP followed by restoration of the more compact form when the incoming aminoacyl-tRNA is bound. A nitroxide derivative of *N*-ethylmaleimide has been used to spin-label 30S subunits and intact *E. coli* ribosomes before and after the addition of streptomycin.[446] These experiments indicate that an effect of the antibiotic is to loosen up the structure of ribosomes.

Reductive alkylation of amino-groups of proteins with aldehydes in the presence of $NaBH_4$ is another technique that has been used as a topographical probe of ribosomal structure. The advantages of the method are that rapid modification proceeds under mild conditions (0 °C, pH 9.0), that the reaction is specific for protein amino-groups (amino-groups of RNA are not modified) and that ε-N-alkylated lysine residues are resistant to trypsin thus facilitating the detection of peptides containing such residues by conventional peptide-mapping methods. The pattern of alkylation of intact *E. coli* ribosomes was independent of the size of the side-chain of the aldehyde employed, although differences in the degree of labelling of different proteins were observed.[447] Every protein in the 30S and 50S subunits was modified indicating that every protein of the ribosome has at least one lysyl residue exposed at or near the surface of the ribonucleoprotein complex. Reductive methylation of proteins L7 and L12 in isolation and in intact 50S subparticles indicated that the lysyl residues were more reactive when the proteins were integrated into the ribosome.[448] The methylated proteins remain active in the GTPase reaction dependent on elongation factor *G*. This technique and, independently, reaction with trinitrobenzenesulphonic acid have been employed also for the finding that lysyl residues in protein S4 are involved in its binding to 16S RNA.[449, 450]

In addition to the techniques of cross-linking and differential labelling with chemical agents, a third powerful chemical modification technique, namely that of affinity labelling, has been employed for the elucidation of ribosomal architecture. The affinity label, usually a derivative of some component of the protein-synthesizing machinery which interacts with the ribosome, may be introduced chemically or photochemically. For example, the donor site of the *E. coli* ribosome was chemically labelled with an analogue of Met-$tRNA_f^{Met}$, *p*-nitrophenylcarbamoyl-methionyl-$tRNA_f^{Met}$. In the presence of initiation factors and phage R-17 mRNA, protein L27 was predominantly labelled, thereby indicating its presence at the donor site.[451, 452] Similarly the peptidyl-site (P-site) was investigated with the peptidyl-tRNA analogue, bromoacetyl-[^{3}H]Phe-$tRNA^{Phe}$.[453, 454] This affinity label reacted covalently with only proteins L2 and

[446] L. Brakier-Gingras, L. Provost, and H. Dugas, *Biochem. Biophys. Res. Comm.*, 1974, **60**, 1238.
[447] G. Moore and R. R. Crichton, *Biochem. J.*, 1974, **143**, 607.
[448] R. Amons and W. Möller, *European J. Biochem.*, 1974, **44**, 97.
[449] R. Amons, W. Möller, E. Schiltz, and J. Reinbolt, *F.E.B.S. Letters*, **41**, 135.
[450] L. Daya-Grosjean, J. Reinbolt, O. Pongs, and R. A. Garrett, *F.E.B.S. Letters*, 1974, **44**, 253.
[451] A. P. Czernilofsky, G. Stöffler, and E. Küchler, *Z. physiol. Chem.*, 1974, **355**, 89.
[452] R. Hauptmann, A. P. Czernilofsky, H. O. Voorma, G. Stöffler, and E. Kuechler, *Biochem. Biophys. Res. Comm.*, 1974, **56**, 331.
[453] M. Pellegrini, H. Oen, D. Eilat, and C. R. Cantor, *J. Mol. Biol.*, 1974, **88**, 809.
[454] D. Eilat, M. Pellegrini, H. Oen, N. de Groot, Y. Lapidot, and C. R. Cantor, *Nature*, 1974, **250**, 514.

L26–L27. These two proteins, and also L14, L15, and L16, were found to be labelled by *p*-nitrophenylcarbamoyl-Phe-tRNA^{Phe} and hence these proteins are either at or near rRNA binding sites within the 50S ribosomal subunit.[455] Estimates of the relative proximities of the 50S ribosomal proteins L2, L16, L24, L26–27, and L32–33 to the 3′-terminus of tRNA bound to the ribosomal P-site have been obtained by affinity labelling of ribosomes with a series of peptidyl-tRNA analogues, bromoacetyl-(Gly)$_{0-16}$-Phe-tRNA^{Phe}.[456] The results suggest

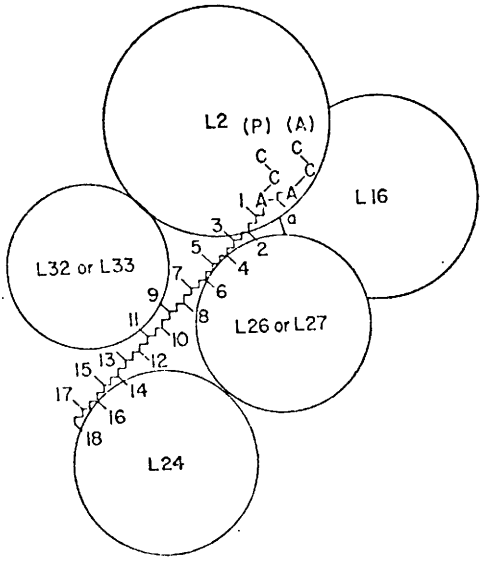


Figure 7 *A model for the arrangement of 50S proteins along the ribosome-bound peptide chain of peptidyl-tRNA. The ribosomal proteins are shown as spheres with a volume proportional to molecular weight. The peptide chain is shown as fully extended.* (P) *and* (A) *the 3′-terminus of the tRNA bound to the 50S ribosomal* P *and* A *binding sites* (Reproduced by permission from *J. Mol. Biol.*, 1974, **88**, 838)

that L2 is closest to the 3′-end of the tRNA followed by L26–L27, L32–L33, and lastly L–24 and that the growing peptide chain points away from the acceptor-site (Figure 7). The photoaffinity label 4-azido-2-nitrophenoxy-4′-phenacetyl-Phe-tRNA^{Phe} (15) reacted with proteins L11 and L18.[457] Since the peptidyl moiety could be released by puromycin prior to photolysis, this indicates that these proteins also form part of the peptidyl transferase centre or are adjacent to it. α-Halogenocarbonyl affinity reagents had previously failed to label these proteins presumably because of lack of accessible reactive nucleophilic groups.

Affinity Labelling.—*Immunoglobulins.* Several studies have been directed towards defining which parts of the polypeptide chains of antibodies are involved in the antigen binding site. The binding sites of rabbit antibodies with affinity

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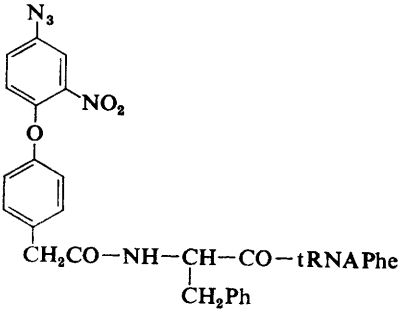
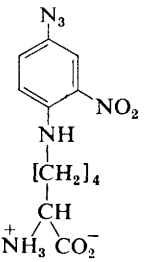


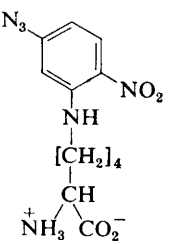
(15)

for the hapten, 4-azido-2-nitrophenyl-L-lysine, have been specifically labelled by photolysis of the hapten–antibody complex.[458] The antibody was specifically labelled in the binding site and the ratio of labelling of heavy to light chains was 3.3—5.0. Two regions of the heavy chain residues 29—34 and 95—114, together contained *ca.* 80% of the label. These two regions include two of the hypervariable regions of rabbit heavy chain. A unique lysyl residue within the second hypervariable region of the γ-chain of guinea-pig anti-*p*-azobenzene-arsonate antibodies was stoicheiometrically labelled by *N*-[1-^{14}C]bromoacetyl-mono(*p*-azobenzene-arsonic acid)-L-tyrosine.[459] The labelled residue was identified as lysine-59 of the γ_2 chain. In the case of protein 460, a mouse IgA myeloma protein with DNP binding activity, the heavy-chain labelling pattern obtained by photoaffinity labelling with the reagent 2,4-dinitrophenylazide has recently been examined.[460] Reaction occurred with residues 33 and 88, both tyrosyl residues to be found in the heavy-chain variable region.

An attempt has been made to determine whether photogenerated reagents can be used to 'map' different parts of the antigen binding site of antibodies by labelling with reagents of slightly different structures structurally related to the hapten.[461] Hence, specific antibodies against the 4-azidonitrophenyl determinant were photochemically labelled with the homologous hapten, ε-(4-azido-2-nitrophenyl)-L-lysine (16) and by the close structural isomer, ε-(5-azido-2-nitrophenyl)-L-lysine (17). The labelling pattern of the heavy and light chains



(16)



(17)

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Table 3 *Chemical modification of peptides and proteins*

Protein	*Source*	*Reagent*	*Residue modified*	*Comments*	*Ref.*
Acetylcholine receptor	electric eel	thiol reagents	Cys	still binds α-bungarotoxin	462
Acetylcholinesterase	electric eel	tetranitromethane	Tyr	inactivation accelerated by quaternary oximes	463
N-Acetylglucosamine kinase	human gastric mucosa	thiol reagents	Cys	inactivated	465
N-Acetylneuraminate lyase	*Cl. perfringens*	chloropyruvate	—	active site label	464
Actin	—	diethylpyrocarbonate	His	His-40 essential for polymerization	466
c-AMP receptor	*E. coli*	*N*-(iodoacetylaminoethyl)-1-naphthylamine-5-sulphonate	—	no effect on c-AMP binding	467
ATPase	mitochondria	spin label probes	—	no loss of activity	468
ATPase	myosin	thiol reagents	—	convergence of activation by actin and SH_1 modification	469
Albumin	bovine serum	photo-oxidation	Tyr	Tyr-130	470
Albumin	bovine serum	whitening agents, photochemical	—		471
Alcohol dehydrogenase	horse liver	formaldehyde, $NaBH_4$	Lys	two lysines per subunit protected by NADH	472
Alcohol dehydrogenase	horse liver	various	—	activation by imido-esters	473
Alcohol dehydrogenase	horse liver	$Pt(CN)_4^{2-}$, $Au(CN)_2^-$	Arg	inhibition	474
Alcohol dehydrogenase S-S isozyme	horse liver	iodoacetate	Cys	inhibition	475
Alcohol dehydrogenase	yeast	thiol reagents	Cys	pH dependent	476
Alcohol dehydrogenase	yeast	iodoacetate	Cys	inactivation	477
Alcohol dehydrogenase	human/horse liver, yeast	butane-2,3-dione, phenyl glyoxal	Arg	NADH protects	478
Aldehyde oxidase	rabbit liver	cyanide	—	active site persulphide	479
Aldolase	rabbit muscle	bromoacetamide	Cys	alkylation induced by freezing	480
Aldolase	rabbit muscle	acetic anhydride	Lys	active centre peptide sequenced	481
Aldolase	rabbit muscle	citraconic anhydride	—		482

Aldolase	rabbit muscle	citraconic anhydride	—		483
Aldolase	—	formaldehyde, cyanide, $NaBH_4$	Lys	inactivation	484
Aldolase	*Salmonella typhimurium*	acetaldehyde, $NaBH_4$	Lys	peptide isolated	485
Aldolase	*Lactobacillus casei*	dihydroxyacetone-Ⓟ, $NaBH_4$	Lys	a Class I and a Class II enzyme identified	486
Alkaline phosphatase	*E. coli*	butane-2,3-dione	Arg	transferase and hydrolase activities lost together	487
Alkaline phosphatase	—	pulse radiolysis	—	active after extensive radiolytic damage	488

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Table 3 (*cont.*)

Protein	*Source*	*Reagent*	*Residue modified*	*Comments*	*Ref.*
Alloantibodies	mouse	fluorescent labels	—	fully active	489
Amine oxidase	bovine plasma	benzylamine, $NaBH_4$	Lys	ε-*N*-benzyl lysine, found after acid hydrolysis	490
L-3-Aminobutyrylcoenzyme A deaminase	*Clostridium* sp.	thiol reagents	Cys	inhibition	491
Aminotransferase	*Salmonella typhimurium*	thiol reagents	Cys	no inhibition	492
α-Amylase	*B. subtilis*	acetic anhydride	—	partial inhibition	493
Antigen E	ragweed pollen	glycinamide; carbodiimide	—	effect on allergenicity depends on extent of reaction	494
Aspartate aminotransferase	pig heart	*N*-dansyl cysteine	Cys	polar environment for Cys-45 and Cys-82	495
Aspartate aminotransferase	pig heart	pyridoxal-5′-sulphate	Lys	sulphate formed	496
Aspartate transcarbamylase	*E. coli*	(*a*) Pyridoxal-5′-phosphate	Lys	(*a*) co-operative interaction in hybrids	497
		(*b*) 3,4,5,6-tetrahydrophthalic anhydride	—	(*b*) chromatographic handle	497
Carbonic anhydrase	human	bromoacetazolamide	His	His-64 modified in solution and in crystals	498
Carboxypeptidase A	bovine	reduction, alkylation	Cys	esterase, peptidase activities retained	499
Carboxypeptidase C_N	*Citrus natsudaidai* exocarp	phenylglyoxal	Arg	inactivation	500
Cathepsin B1	bovine spleen	mercuric chloride	Cys	binds papain inhibitor	501
Cholera toxin	*Vibrio cholera*	cyclohexanedione; phenanthraquinone; butanedione	Arg	binds less to ganglioside GM1	502
Cholesterol hydroxylase	mitochondria	*p*-hydroxymercuribenzoate	Cys	26-hydroxylation inhibited	503
Choline acetyl transferase	squid ganglia	photo-oxidation	His	inactivation	504
α-Chymotrypsin	bovine	4-benzylidene-2-phenyl-Δ^2-oxazolin-5-one	—	α-benzamidocinnamoyl enzyme formed	505
Chymotrypsinogen	bovine	H_2O_2	Met	Met-192 oxidized; increased reactivity	506

Chymotrypsinogen	bovine	1-fluoro-2,4-dinitrobenzene	His	His-40 and His-57 activities compared	507
Citrate synthase	*Acinetobacter iwoffi*	photo-oxidation	His	desensitized to NADH inhibition	508
Clotting factor XIIIa	human plasma	thiol reagents	Cys	inhibition	509
Collagen	carp swim bladder, rat-tail	cyanide, ammonia	aldehyde cross-links	cross-links stabilized	510
Collagen	human tendon	$NaBT_4$	cross-links	reaction decreases with age	511
Concanavalin A	—	maleic anhydride	—	tetramer dissociates to dimer; binding site activity retained	512
γ-Cystathionase	rat liver	thiol reagents	—		513

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Table 3 (*cont.*)

Protein	*Source*	*Reagent*	*Residue modified*	*Comments*	*Ref.*
Cytochrome P-450$_{cam}$	—	mercurials, thiols	Cys	interconversion with P-420	514
Daunorubicin reductase	rat liver	*p*-chloromercuribenzoate	Cys	inhibition	515
3-Deoxy-D-arabino-heptulosonate-7-phosphate synthetase	cauliflower	thiol reagents	Cys	inhibition	516
3-Deoxy-D-arabino-heptulosonate-7-phosphate synthetase: chorismate mutase	*B. subtilis*	*p*-hydroxymercuribenzoate	Cys	DAHP synthetase but not chorismate mutase inhibited	517
Deoxycytidylate amino-hydrolase	donkey spleen	*p*-chloromercuribenzoate	Cys	inhibition	518
Deoxyribonuclease A	bovine pancreas	(*a*) *O*-methylisourea	Lys	active, can be inactivated by methanesulphonyl chloride	519
		(*b*) carbodi-imide	—	inhibition	509
DNA polymerase	mouse myeloma	*p*-hydroxymercuribenzoate and DTT	—	stimulation	520
Deoxyribonucleohistone	calf thymus	5,5′-dithiobisnitro-benzoate (DTNB)	Cys		521
Dihydrofolate reductase	*E. coli*	ethoxyformate	His	one histidine of five protected by NADPH	522
Dihydrofolate reductase	*Lactobacillus casei*	*N*-bromosuccinimide	Trp	$NADP^+$ but not folate protects	523
Dihydrofolate reductase	*Streptococcus faecium*	iodoacetate	Met	Met-28 and Met-50 protected by aminopterin	524
Dynein	cilia from *Tetrahymena pyriformis*	*N*-ethylmaleimide (NEM)	Cys	thiol of dynein required for binding to axenomes	525
Elastase	pig pancreas	maleic and acetic anhydrides	—	*N*-terminal valine reacts at high pH	526
Elongation factor G	*E. coli*	*p*-chloromercuribenzoate	Cys	translocation inhibited	527
Elongation factors Tu and G	*E. coli*	$NaBH_4$, oxidized GTP	—	no Schiff base	528

Elongation factor Tu	*E. coli*	*N*-tosyl-phenylalanine-chloroketone	—	inhibition in bacterial and heterologous bacterial avian system, but not in homologous avian or mammalian system	529
Elongation factor Tu	*E. coli*	spin-labelled NEM derivatives	Cys	specific label in the presence of GDP	530
Elongation factors Tu and Ts	*E. coli*	thiol reagents	Cys		531
Ferricytochrome *c*	horse heart	*O*-methylisourea	Lys	Lys-79 is probably ligand	532
Fibrin stabilizing factor	human platelets/ plasma	iodoacetate	Cys	Ca^{2+} required for reaction with active thiol	533, 534
Ficin	*Papaya* latex	*N*-alkyl maleimides	Cys	inactivation, proflavin binds	535
Formaldehyde dehydrogenase	human liver	mercuric chloride; iodoacetate	Cys	protection by NAD and NADH	536

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Table 3 (*cont.*)

Protein	*Source*	*Reagent*	*Residue modified*	*Comments*	*Ref.*
S-Formylglutathione hydrolase	human liver	various	—	thiol reagents and dansyl chloride inhibition	537
Fructose-1,6-diphosphatase	rabbit muscle	pyridoxal-5′-phosphate	Lys	protection by substrate	538
Fructose-1,6-diphosphatase	rabbit muscle	DTNB	Cys		539
Fructose-1,6-diphosphatase	—	thiol reagents	Cys	no longer enhances inhibition of phospho-fructokinase by ATP	540
D-Galactose dehydrogenase	*Pseudomonas saccharophila*	DTNB	Cys	NAD protects	542
α-D-Galactosyl-binding lectin	*Bandeiraea simplicifolia*	DTNB	Cys	no hemagglutination activity	541
Glutamate dehydrogenase	*Neurospora crassa*	various	—	inhibition by pyridoxal-phosphate and thiol reagents; labelled peptides sequenced	544
Glutamate dehydrogenase	bovine liver	2-hydroxy-5-nitrobromobenzene	Trp		545
Glyceraldehyde-3-phosphate dehydrogenase	pig muscle	dimedone; olefins	sulphenic acid derivative of Cys	acyl phosphatase activity inhibited	546
Glyceraldehyde-3-phosphate dehydrogenase	pig muscle	*p*-hydroxymercuribenzoate	Cys		547
Glyceraldehyde-3-phosphate dehydrogenase	rabbit muscle	7-chloro-4-nitrobenzo-2-oxa-1,3-diazole	Cys	fluorescence probe for coenzyme induced changes	548
Glyceraldehyde-3-phosphate dehydrogenase	rabbit muscle	trinitrobenzenesulphonic acid (TNBS)	Lys	two essential reactive lysine residues/tetramer	549
Glyceraldehyde-3-phosphate dehydrogenase	ox muscle/liver	various	—		550
Glycogen synthetase D	rat liver	oxidized glutathione	Cys	inactivation, dissociation from glycogen; protection by glucose-6-Ⓟ	551

Glycogen synthetase D	rat liver	—TNBS; pyridoxal-5′-phosphate, $NaBH_4$	—	inactivation; protection by glucose-6-Ⓟ	552
Gonadotropin	human	tetranitromethane	Tyr		553
Growth hormone	bovine	hydrogen peroxide	Met	activity unchanged	554
Guanine deaminase	rabbit liver	thiol reagents	Cys	inhibition	555
Hemoglobin	cat	*p*-hydroxymercuribenzoate	Cys	exists in three forms	556
Hemoglobin	chicken	thiol reagents	Cys	exposed thiol localized reactivity affected by oxygenation	557
Hemoglobin, deoxy-sickle	human	pyridoxal derivatives	—	inhibits gelatin; increases solubility of deoxy-sickle Hb	558
—					
Hexosediphosphatase	spinach chloroplasts	thiol reagents	Cys	activation	559

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Table 3 (*cont.*)

Protein	*Source*	*Reagent*	*Residue modified*	*Comments*	*Ref.*
Hexokinase	rat brain	DTNB	Cys	inactivation	560
Histidine ammonia lyase	*Pseudomonas testosteroni*	iodoacetate	Cys	peptide sequenced	561
Histidine ammonia lyase	*Pseudomonas* sp.	L-cysteine	Cys	inactivation; protection by L-histidine	562
Histidyl-tRNA synthetase	*E. coli*	thiol reagents	Cys		563
Histidyl-tRNA synthetase	*Salmonella typhimurium*	thiol reagents	Cys		564
Histone F2A1	calf thymus	cyanogen bromide	Met	recognition of deacylating enzyme	565
d11-Immunoglobulin-G	rabbit	chloramine T; iodoacetate	Met	d11 antigenicity lost	566
Immunoglobulin	human	citraconic anhydride	—	proteins expand; immune reactivity lost	567
Immunoglobulin	human	NEM	Cys	0.8% of every half cysteine residue in reduced form	568
Insulin	bovine	iodine monochloride; enzymic iodination	—	comparison of methods	569
Insulin	bovine	enzymic iodination	Tyr	76% of label at Tyr-A14 fully active	570
Insulin	bovine	ethylthiotrifluoroacetate	—	60—89% active	571
Isocitrate dehydrogenase	bovine heart	thiol reagents	Cys	protection by manganous isocitrate	572
Isocitrate dehydrogenase	bovine heart	amidination, arylation, acetylation, carbamylation	—	protection by manganous isocitrate	573
Isocitrate dehydrogenase	bovine heart	pyridoxal-5′-phosphate	Lys	amino-group at catalytic centre	574
Isocitrate dehydrogenase	*Azobacter vinelandii*	iodoacetate	Met	Met-31 reacts	575
Isoleucyl-tRNA synthetase	*E. coli*	NEM	Cys	peptide sequenced	576
Lactate dehydrogenase	bovine heart	*N*-alkylmaleimides	Cys	inactivation	577
Lipase	bovine pancreas	various	—	thiol, amino at binding site	578

Luteinizing hormone	porcine	carbodi-imide	—	dissociation in presence of ammonium chloride; cross-linking in its absence	579
Luteinizing hormone	sheep	guanidination, carbamylation, reductive methylation	Lys	no activity	580
Luteinizing hormone	sheep	formaldehyde, $NaBT_4$	Lys	activity unchanged	581
Luteinizing hormone	—	iodoacetate	Cys	location of disulphides in α-subunit	582
Luteinizing hormone	sheep	maleic anhydride; cyanate	—	acylated LH inactive	583
Lysozyme	hen	glycine-methyl ester; histidine-methyl ester	—	Asp-119 and *C*-terminal Leu modified; no role in antigenicity	584, 585

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Table 3 (*cont.*)

Protein	*Source*	*Reagent*	*Residue modified*	*Comments*	*Ref.*
Lysozyme	hen	*N*-bromosuccinimide	Trp	affects binding of anomers of *N*-acetylglucosamine	586
Lysozyme	hen	peroxyacetylnitrate	Cys	slight inactivation	587
L-Malate binding protein	*B. subtilis* membrane	NEM	Cys	protection by L-malate	588
Malate dehydrogenase	—	4,4-bis(dimethylamino) phenylcarbinol	Cys	NAD^+ protects; peptide isolated	589
Malate dehydrogenase	pig heart mitochondria	butanedione	Arg	NADH protects	590
Malate dehydrogenase	pig heart supernatant	diethylpyrocarbonate	His	inhibition	591
Malate dehydrogenase	pig heart	tetrachloroplatinate(II)	Met	inhibition reversed by DL-methionine	592
Membrane proteins	*Myco. phlei*	thiol reagents	Cys	proline transport and substrate oxidation dissociated	593
Membrane proteins	*E. coli* vesicles	carbonylcyamide *m*-chloro-phenylhydrazone	—	acts as thiol reagent	594
Membrane proteins	rat adipocytes	enzymic iodination	—	20 proteins labelled	595
Membrane proteins	mouse fibroblasts	enzymic iodination; TNBS	—	fewer proteins labelled by TNBS	596
Membrane proteins	chloroplasts	diazoniumbenzenesulphonic acid	—	O_2 evolution inhibited in light, but not in dark	597, 598
Membrane proteins	*Acholeplasma laidlawii*	biotinyl-*N*-hydroxysuccinimide ester	—	visualized in E.M. by ferritin-linked avidin	599
Membrane proteins	human erythrocytes	various	—	thiol, amino, and imidazole required for sugar transport	600
Membrane proteins	human erythrocytes	1-fluoro-2,4-dinitrobenzene	—	glucose carriers inhibited	601
Membrane proteins	human erythrocytes	succinic anhydride	—	permeability affected	602

Membrane proteins	rat liver mitochondria	*p*-diazobenzenesulphonate; thiol reagents	—	phosphate transport inhibited	603, 604
Membrane proteins	rat liver mitochondria	2,4-dinitro-5-(bromo-acetoxyethoxyl)phenol	—	alkylates mitochondrial protein	605
Membrane proteins	*Sacch. carls-bergiensis* mitochondria	pyridoxal-5′-phosphate, $NaBT_4$	Lys		606
Myosin	—	NEM in presence of ADP	Cys	myosin not activated by actin	607
Nitrate reductase	*Neurospora crassa*	*p*-hydroxymercuribenzoate	Cys	inhibition of pyridine nucleotide-linked activities	608, 609
Nuclease	*Staphylococcus*	Woodward's reagent K	—	ketoketenimine is reactive species	610

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Table 3 (*cont.*)

Protein	*Source*	*Reagent*	*Residue modified*	*Comments*	*Ref.*
Ovotransferrin	—	tetranitromethane	Tyr	some tyrosine residues protected by iron	611
Olfactory transducers	silk moth	thiol reagents, maleic anhydride	—	olfactory response inhibited	612
Papain	*Papaya* latex	$(CNS)_2^-$ and Br_2^- radicals	—	inactivation	613
Papain	*Papaya* latex	*N*-alkylmaleimides	Cys	inactivated, binds proflavin	535
Papain	*Papaya* latex	peroxyacetyl nitrate	Cys	inactivation reversed by thiol	587
Papain	*Papaya* latex	2-bromoacetamide-4-nitro-phenol	Cys	Cys-25 reacts; reporter group	614
Papain	*Papaya* latex	mercuric chloride	Cys	binding of peptides not prevented	615
Papain	*Papaya* latex	NEM	Cys		616
Papain	*Papaya* latex	glycine ethyl ester, water-soluble carbodi-imide	—	single carboxyl modified	617
Papain	*Papaya* latex	aryl-α-haloalkyl ketoximes	—	Cys-25 reacts	618
Pepsinogen	pig	pyridoxal-5′-phosphate, $NaBH_4$	Lys	α-NH_2, Leu-1, and Lys-258 react; can be activated	619
Phenylalanyl-tRNA	*E. coli*	diethylpyrocarbonate; photo-oxidation	His	protection by Phe, ATP, Mg^{2+}	620
Phosphoglycerate kinase	yeast	Woodward's reagent K	—	single carboxyl reacts	621
6-Phosphogluconate dehydrogenase	*B. stearo-thermophilus*	thiol reagents	Cys	inactivation	622
Phosphorylase b	rabbit muscle	fluorescent and spin-labelled probes	Cys	flash activation of glycogen particles followed	623
Phosphorylase	*Micrococcus luteus*	NEM	Cys		624
Poly-histidine	—	enzymic iodination	His	iodination at C-4	625
Prealbumin	—	2-hydroxy-5-nitrobenzyl bromide	Trp	binds retinol; retinol-binding protein	626
Protease	*Mucor miehei*	tetranitromethane	Tyr		627

Protease	*Rhodotorula glutinis*	*N*-diazoacetyl-*N'*-2,4-dinitrophenylethylenediamine	—	peptide containing modified carboxyl sequenced	628
Protein A	*Staph. aureus*	KI_3	His, Tyr	reactivity toward Fc part of IgG decreased	629
Protocatechuate 3,4-dioxygenase	*Achinetobacter calcoaceticus*	cross-linking reagents	—		630
Pyruvate kinase	yeast	DTNB	Cys	allosteric effector increased rate of reaction	631
Pyruvate kinase	yeast	diethylpyrocarbonate	His	inactivation decreased by ATP, ADP, PEP	632, 633
Retinol-binding protein	—	2-hydroxyl-5-nitrobenzyl bromide	Trp	did not bind to pre-albumin	626
Ribonuclease A	bovine pancreas	cyanogen bromide	*S*-Me-Cys	cleavage	634

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Table 3 (*cont.*)

Protein	*Source*	*Reagent*	*Residue modified*	*Comments*	*Ref.*
RNA polymerase	*E. coli*	2-hydroxy-5-nitrobenzyl bromide	Trp, Cys	inhibition	635
RNA polymerase	*E. coli*	tetrathionate	Cys		636
Ribosomal proteins	*Pinus lambertiana*	dansyl chloride	—		637
Ribosomal proteins	*E. coli*	thiol reagents	Cys	'non-enzymic translocation'	638
Sucrose	*B. subtilis*	*p*-hydroxymercuribenzoate	Cys	inhibition	639
Thermolysin	*B. thermoproteolyticus*	ethoxyformic anhydride	His	possibly His-231	640
Thermolysin	—	tetranitromethane	Tyr		641
Thymidylate synthetase	—	thiol reagents	Cys	inactivation	642
Thyroglobulin	—	iodination by I_3 or enzymically	Tyr		643
Toxin B	*Naja naja*	tetranitromethane	Tyr	activity unaffected	644
Toxin	*Naja naja siamensis*	alanine-*N*-carboxyanhydride	—	antigenic activity decreased	645
Toxin	*Naja nivea*	reduction, alkylation	disulphide	disulphide 26—30 cleaved preferentially	646
Trypsin	pronase	acetic anhydride	—	active derivatives	647
Trypsin inhibitor	bovine pancreas	guanidination, malelyation, anthraniloylation	Lys	association with trypsin but not chymotrypsin affected	648, 649
Trypsin inhibitor	bovine pancreas	cyanogen bromide	Met	bond between Met-52 and Arg-53 cleaved; reforms spontaneously	650
Trypsin inhibitor	bovine pancreas	cross-linking reagent	—		651
Trypsin inhibitor	cow colostrum	$NaBH_4$	disulphide	selective reduction of disulphide 17—41	652
Trypsin inhibitor	sweet potato	various	—		653
Tubulin	porcine brain	thiol reagents	Cys	thiols implicated in microtubule assembly	654
Tyrosine aminotransferase	—	antineoplastic agents	—	carbamylation	655
Wool	sheep	reduction, alkylation	disulphide		656
Wool	sheep	whitening agents	—	photochemical reaction	471

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Table 4 *Affinity labelling of proteins*

Protein	*Source*	*Reagent*	*Comments*	*Ref.*
Acetylcholinesterase	electric eel, human erythrocytes	trimethyloxonium ion	protection by tetramethylammonium ion	657
F-Actin	rabbit muscle	*S*-dinitrophenyl-6-mercaptopurine riboside triphosphate	ATP-analogue	658, 659
Albumin	bovine serum	4-azidobenzoylpentagastrin and others	photoaffinity labels; competition by oleic acid; label located	660
Alcohol dehydrogenase	horse liver, yeast	3-chloroacetylpyridine adenine nucleotide	NAD^+-analogue	661
Aldolase	rabbit muscle	*N*-bromoacetylethanolamine	penultimate residue (His) modified	662
Aminoacyl-tRNA synthetases	—	(*a*) ATP-γ-*p*-azidoanilide (*b*) tRNA containing $N_3C_6H_4$.$NHCOCH_2$ attached to a 4-thiouridine residue	photoaffinity reagents	663
Aminopeptidase	*Aeromonas* sp.	leucine methyl ketone derivatives	reversible	664
Ancrod	*Agkistrodon rhodostroma* venom	(*a*) di-isopropylphosphofluoridate (DIFP) (*b*) α-*N*-nitrobenzyloxycarbonyl-L-arginine chloromethyl ketone	(*a*) attached to Ser residue (*b*) attached to His residue	665
Asparaginase	*E. coli*	2-amino-4-oxo-5-chlorovaleric acid	no inhibition	666
Aspartate aminotransferase	pig heart cytoplasm	bromopyruvate	syn-catalytic modification	667
Carboxypeptidase A	bovine pancreas	*N*-bromoacetyl-*N*-methyl-L-phenylalanine	acetylation of Tyr-240 has no effect	668
Carboxypeptidase B	porcine	bromoacetyl-*p*-aminobenzylsuccinic acid	methionine modified	669
α-Chymotrypsin	bovine pancreas	phenylalaninol and tryptophanol derivatives	suggestion	670
α-Chymotrypsin	bovine pancreas	*p*-nitrophenyl ester of *p*-azido[^{14}C]cinnamate	photo-label; localized in C-chain in aromatic binding locus	671
α-Chymotrypsin	bovine pancreas	phenacyl and naphthacyl derivatives	photo-label	672
α-Chymotrypsin	bovine pancreas	(*a*) *p*-nitrophenylalanine, (*b*) *p*-azido-, *m*-azido-, and *o*-azido-phenylalanine	(*a*) good photo-label	673

Chymotrypsinogen	bovine pancreas	DIFP	zymogen has catalytic site	674
Deoxyribonuclease A	bovine pancreas	methanesulphonyl chloride	single serine modified	519
Esterase	pig liver	[^{14}C]-bis-4-nitrophenylphosphate		675
Factor VII	bovine plasma	DIFP		676
17β-Hydroxysteroid dehydrogenase	human placenta	3-chloroacetylpyridine adenine nucleotide	NAD^+ analogue	661
β-Glucosidase A_3	*Aspergillus wentii*	[^{3}H]conduritol B-epoxide	esterifies Asp; sequence of peptide determined	677
Glutamate synthase	*Aerobacter aerogenes*	L-2-amino-4-oxo-5-chloropentanoic acid chloroketone	glutamine-dependent activity inhibited	678
Kallikrein	porcine pancreas	(*a*) DIFP (*b*) chloro-3-tosylamido-7-amino-2-heptanone (TLCK)	(*a*) inhibitory (*b*) non-inhibitory	679
Lipase	*Corynebacterium acnes*	DIFP	inhibitory	680

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Table 4 (*cont.*)

Protein	*Source*	*Reagent*	*Comments*	*Ref.*
Lysozyme	hen	2′,3′-epoxypropyl-β-glycoside of di-(*N*-acetyl-D-glucosamine)	Asp-52 reacted	681
Mandelate vacemase	*Pseudomonas putida*	DL-α-phenylglycidate	mandelic acid analogue	682
Myosin	rabbit muscle	cobalt(III)-ATP complex	displaced by thiol reagents	683
Phenylalanine-tRNA ligase	*E. coli*	*N*-chlorambucilyl-[^{14}C]phenyl-alanine tRNA		684
Phospholipase A2	porcine pancreas	[^{14}C]-*p*-bromophenacyl bromide	His-53 reacted	685
Plasminogen	human	TLCK	pre-activation peptide not degraded	686
Plasminogen activator	SV40 transformed hamster cells	DIFP	inhibited	687, 688
Proteases	—	peptides with *C*-terminal lysine chloromethyl ketone	enzyme specific reagents within trypsin group	689
Protease	L5178Y murine leukaemic cells	DIFP	inhibited	690
Protease	insect moulting fluid	DIFP	inhibited	691
Protease	*Plasmodium berghei*	*p*-methyl sulphonyl fluoride (PMSF)	inhibited	692
Protease	*Plasmodium falciparum*; *P. knowlesi*	PMSF	not inhibited	693
Protease	human serum low density lipoprotein	PMSF	inhibited	694
Proteases	gut of *Locusta migratoria*	PMSF	inhibited	695
Proteases	heterokaryons of HeLa cells and erythrocytes	TLCK	erythrocyte nuclear enlargement inhibited	696
Proteases	bovine pancreas	spin-labelled sulphonyl fluorides	active sites of chymotrypsin and trypsin compared	697, 698
Protease B	*Streptomyces griseus*	peptide chloromethyl ketones	His-33 reacted	699
Protein synthesis	SV40 transformed cells	TPCK, TLCK	non-selective inhibitors of protein synthesis	700

Rennin	mouse submaxillary gland	diazoacetyl-DL-norleucine (DAN) in presence of Cu^{2+}	carboxyl reacted	701
Rennin	bovine	DAN + Cu^{2+}; 1,2-epoxy-3-(*p*-nitrophenoxy)propane	two different Asp residues identified; sequences	702
RNA polymerase	*E. coli*	5-formyl uridine-5′-triphosphate	forms Schiff base with lysine	703
RNA polymerase	*E. coli*	fluorescent nucleotide	attached to β-subunit	704
Thrombin	human	nitrophenyl-*p*-amidophenylmethane sulphonate		705

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was dependent upon the reagent used. This was a consequence of the presence of subpopulations in the preparation of antibodies, and molecules that can be labelled by one reagent cannot be labelled by the other. In other words, different subpopulations from the original specific antibody preparation are attacked independently by the two isomeric nitrene reagents. This demonstrates that the discrimination of the labelling is very high since generation of nitrene at centres only 0.3 nm apart leads to a mutually exclusive labelling pattern and stresses the effectiveness of photolabelling both for the mapping of sites in homogeneous systems and for probing the heterogeneity in systems that are not.

Miscellaneous. Other uses of affinity labelling are summarized in Table 4 (p. 96).

5 Protein Sequence Methods

Cleavage Methods.—*Enzymic Methods.* The specificity of Aspergillopeptidase B has been examined [707] using the two chains of insulin as substrates. At pH 10.2 and 0 °C, the enzyme cleaved predominantly at a Glu–Asn bond in the A chain and at a Leu–Tyr bond in the B chain. This contrasts with its chymotrypsin-like specificity at 37 °C. Whether the enzyme will be generally useful or, indeed, whether other proteases display such limited specificity at low temperatures is unknown.

The enzymes ficin and bromelain belong to the same family of thiol plant proteases as papain and have the same broad specificity. In a kinetic study,[708] using mainly synthetic substrates, it has been shown that major cleavage sites occur on the *C*-terminal side of glycyl, alanyl, and leucyl residues with minor sites after valine, phenylalanine, and tyrosine. Although interesting, these data are unlikely to lead to more widespread use of the enzymes in protein chemistry.

Since the description by Houmard and Drapeau of a protease which could specifically cleave peptide bonds after glutamic or aspartic acid residues, several protein sequence papers have appeared in which the enzyme has been used. A new report [709] deals with the isolation of a very similar enzyme from a mutant of *S. aureus* 8325N which produces only one extracellular protease. This of course greatly simplifies the purification procedure. The enzyme appears to cleave preferentially at the *C*-terminal side of glutamic acid bonds, although some aspartic acid bonds are split slowly.

A new pyrrolidonecarboxylate peptidase, the enzyme which removes pyroglutamic acid (Glp) from the *N*-terminus of peptides, has been described.[710] The protein was isolated from *Klebsiella cloacae* and was found to cleave Glp–Pro bonds as well as to remove the Glp blocking group from intact immunoglobulins. It also appeared to be much more stable than previously isolated pyrrolidone decarboxylases.

Many carboxypeptidases have been described which release all *C*-terminal amino-acids including proline. These have been variously termed: carboxypeptidase C, carboxypeptidase Y, phaseolain *etc.*, and all appear to have similar specificity. The specificity of a new enzyme, penicillocarboxypeptidase-Sl, has

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[708] A. A. Kortt, J. A. Hinds, and B. Zerner, *Biochemistry*, 1974, **13**, 2029.
[709] A.-C. Rydén, L. Rydén, and L. Philipson, *European J. Biochem.*, 1974, **44**, 105.
[710] J. Kwiatkowska, B. Torain, and G. G. Glenner, *J. Biol. Chem.*, 1974, **249**, 7729.

been investigated [711] and again found to be broadly similar to the others in its class.

An interesting enzyme, acyl-lysine deacylase, has been used [712] to remove quantitatively acetyl groups from the ε-NH_2 side-chain of a lysine residue in trypsin inhibitor. If the deacylation is reliably quantitative the method could be a very useful one for the removal under mild conditions of ε-amino blocking groups.

Chemical Methods. No new useful chemical methods for the cleavage of protein chains were reported in 1974. The usefulness of the CNBr reaction for cleavage at *S*-methylcysteine residues has been confirmed and a mechanism established for the reaction.[713]

An investigation has also been made of the reaction between CNBr and peptides with *N*-terminal acetylmethionine.[714] It was found that *ca.* 10% of the methionyl bond remained uncleaved due to the formation of *O*-acetyl-homoserine. This is reminiscent of the situation encountered with Met-Ser or Met-Thr sequences where the intermediate iminolactone is attacked by the neighbouring hydroxy-group to yield homoseryl-*O*-seryl (or threonyl) peptides. Neutralization results in an O → N migration, the production of an internal homoserine residue, and no bond cleavage.

Detection Methods.—*Fluorescamine.* The use of this reagent for the detection both of proteins on polyacrylamide-SDS gels and of peptides on t.l.c. plates has been described.[715] Sensitivity on the gel was found to be comparable with that obtained by staining with Coomassie Blue but the method has the advantage that the fluorescent protein may be eluted and used for subsequent structural analysis. Similar conclusions were drawn after comparing peptide fingerprints stained with fluorescamine and ninhydrin.

A revised spray procedure for fluorescamine on t.l.c. plates has been claimed [716] to give greater sensitivity and longer fluorescence stability. The plates are presprayed with a 10% solution of triethylamine in methylene chloride, dried, sprayed with fluorescamine, dried, and then resprayed with the triethylamine solution. The limit of sensitivity of the reagent, *ca.* 0.5 nmol, is unchanged but the fluorescence is claimed to be stable for at least 24 h. The effect of parameters such as pH, solvent, concentration, and temperature on the reaction has been investigated.[717, 718] Finally, it has been shown,[719] not surprisingly, that peptides with *N*-terminal pyrrolidonecarboxylic acid or proline do not react with the reagent. Also, large fragments with *N*-terminal aspartic acid, asparagine, or glutamic acid and the peptide, Trp-Arg, failed to produce significant fluorescence. The lower level of fluorescence produced by acidic peptides appears to be a

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[715] J. Vandekerckhove and M. Van Montagu, *European J. Biochem.*, 1974, **44**, 279.
[716] A. M. Felix and M. H. Jimenez, *J. Chromatog.*, 1974, **89**, 361.
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[718] S. Stein, P. Böhlen, and S. Udenfriend, *Arch. Biochem. Biophys.*, 1974, **163**, 400.
[719] N. Nakai, C. Y. Lai, and B. L. Horecker, *Analyt. Biochem.*, 1974, **58**, 563.

fairly general phenomenon and care should be taken to ensure detection of these peptides when fluorescamine is used.

End-group Analysis.—Most new methods of *N*-terminal analysis aim either at quantitating the dansyl reaction or at replacing dansyl chloride with a new fluorescent reagent. In general, the replacements have little or nothing advantageous to offer and the attempts at quantitation are at best tedious and at worst unreliable. This year, [^{14}C]dansyl chloride in SDS solution has been used [720] in an attempt to quantitate not only the *N*-terminal amino-acid but also the number of lysine and tyrosine residues in the chain. The results are somewhat variable. Another method is to photograph the t.l.c. plates under u.v. light and scan the films with a densitometer. Accuracy is claimed [721] to be ± 4% at the 10—100 pmol level, a figure which may prove difficult to achieve in practice. The dimethylamino-group on the naphthalene ring of the dansyl reagent has been replaced by a dibutylamino-group which is claimed [722] to give higher fluorescence quantum yields thereby increasing sensitivity.

One new non-fluorescence method [723] involves acylation of the amino-group with pivalyl or benzoyl chloride, cleavage with MeOH–HCl, and esterification of carboxy- and phenolic hydroxy-groups with diazomethane. The derivatives may be identified by g.l.c. with a claimed yield of 70—80%.

Amino-acid Analysis.—*Hydrolysis.* Two new hydrolysis techniques have been proposed. The first,[724] which takes only 15 min, uses 1 ml of propionic acid mixed with 1 ml of 12N-HCl. Hydrolysis is carried out at 160 °C. It should be noted that this method has only been tried on micromolar quantities of synthetic peptides. The second method [725] uses 1 ml of 3N-mercaptoethanesulphonic acid at 110 °C for 22 h. All amino-acids, including tryptophan, are recovered and yields are said to be better than with toluenesulphonic acid or HCl + thioglycollic acid. The results are not compared with those obtained by using the current reagent of choice for tryptophan analysis, methanesulphonic acid.

Ion-exchange Chromatography. Several methods have been described for quantitation of unusual or derivatized amino-acids. The reaction product of lysine with the imido-ester, ethyl acetimidate, has been found [726] to elute after ammonia on the short column of an amino-acid analyser. A correction for partial hydrolysis to lysine is made by extrapolation of the yields of ε-acetimidyl-lysine from various hydrolysis times to zero. The amino-acids ornithine, lysine, αγ-diaminobutyric acid, and αβ-diaminopropionic acid, along with several other basic amino-acids, have been separated on a column of Beckman PA 35 resin.[727] Three buffers are used together with a temperature gradient. A similar short-column system has been used for resolving various diaminohexanoic and diaminopentanoic acid derivatives.[728]

[720] L. Casola, G. Di Matteo, G. Di Prisco, and F. Cervone, *Analyt. Biochem.*, 1974, **57**, 38.
[721] A. O. Chilingarov and P. A. Kometiany, *Vopr. Med. Khim.*, 1974, **20**, 31.
[722] N. Seiler and B. Knödgen, *J. Chromatog.*, 1974, **97**, 286.
[723] J. C. Cavadore, G. Nota, G. Prota, and A. Previero, *Analyt. Biochem.*, 1974, **60**, 608.
[724] F. Westall and H. Hesser, *Analyt. Biochem.*, 1974, **61**, 610.
[725] B. Penke, R. Ferenczi, and K. Kovács, *Analyt. Biochem.*, 1974, **60**, 45.
[726] B. V. Plapp and J. C. Kim, *Analyt. Biochem.*, 1974, **62**, 291.
[727] R. W. Longton, V. J. Berzinskas, A. Y. Balekjian, and S. B. Needleman, *Analyt. Biochem.*, 1974, **57**, 343.
[728] M. M. Herbst, B. G. Baltimore, G. Bozler, and H. A. Barker, *Analyt. Biochem.*, 1974, **58**, 322.

A fluorescamine system has been described [729] for measuring methylated basic amino-acids but problems with large variations in fluorescence yield may detract from its use.

Gas–Liquid Chromatography. The potential advantages of g.l.c. for the quantitation of amino-acids are speed, sensitivity, and economy. The disadvantages include the need for prior modification to form volatile derivatives, incomplete resolution of all amino-acids, poor quantitation of some of the less stable derivatives, and the necessity to eliminate volatile contaminants. A method for removing the sample contaminants on cation-exchange resin has been described and the procedure tested by g.l.c. analysis of hydrolysates of ribonuclease, fish meal, corn, and blood plasma.[730] The hydrolysis conditions, 6N-HCl for 4 h at 145 °C gave results comparable with those obtained after hydrolysis at 110 °C for 18—24 h. However, it is noteworthy that in a method where sensitivity is claimed as a major advantage, 20—25 mg of each protein was used as starting material.

A comparison of the sensitivity and reliability of two analytical methods, g.l.c. and cation exchange chromatography, has been made [731] using various sorghum samples. The results were broadly similar but the g.l.c. figures for histidine were variable, possibly as a result of the instability of the n-heptafluorobutyryl n-propyl ester derivative being used. Figures for methionine and lysine were also somewhat less accurate. The same derivatives have been used [732] for the quantitative g.l.c. determination of hydroxyproline and hydroxylysine on a column of 15% Dexsil 300GC.

A separation system for amino-acid n-heptafluorobutyryl isobutyl esters on a 3% SE-30 column has also been described [733] but few quantitative data have been presented. Again, difficulties were encountered in the derivatization of histidine. Various *S*-substituted cysteines have been separated [734] using *N*-trifluoroacetyl n-butyl ester derivatives on columns of OV-17 and Dexsil 300GC.

For detection of the amino-acid derivatives, a nitrogen-sensitive thermionic detector has the theoretical advantages over a conventional flame ionization detector of increased sensitivity and selectivity for particular compounds. A recent study [735] has shown that the increase in sensitivity may vary from a factor of 22 for glycine to 3.5 for valine and phenylalanine. However, a serious disadvantage was the variability introduced by slight movements of the electrode; a vertical displacement of 0.25 mm was found to cause a 10-fold change in the detector response.

Integration of Results. The increased speed of amino-acid analysers has meant that up to 400 peaks may be produced by a single instrument in one day. The manual calculation of all these peak areas is clearly obsolete and on-line computer systems are becoming a necessary adjunct to this type of equipment.

[729] A. M. Felix and G. Terkelsen, *Analyt. Biochem.*, 1974, **60**, 78.
[730] F. E. Kaiser, C. W. Gehrke, R. W. Zumwalt, and K. C. Kuo, *J. Chromatog.*, 1974, **94**, 113.
[731] M. A. Kirkman, *J. Chromatog.*, 1974, **97**, 175.
[732] C. W. Moss and M. A. Lambert, *Analyt. Biochem.*, 1974, **59**, 259.
[733] S. L. Mackenzie and D. Tenaschuk, *J. Chromatog.*, 1974, **97**, 19.
[734] M. Sakamoto, K.-I. Kajiyama, and H. Tonami, *J. Chromatog.*, 1974, **94**, 189.
[735] M. Butler and A. Darbre, *J. Chromatog.*, 1974, **101**, 51.

One system which has recently been described [736] records the chromatograms on paper tape and then uses an off-line computer programmed in FORTRAN to perform the calculations. Unfortunately, there are no details of the programme.

An alternative method [737] is to record the data on a magnetic tape cassette which, provided the computer has the necessary peripherals, has economic as well as convenience advantages. Again, no detailed programme is given in this paper although there is general information.

A system for further analysis of integrated peak areas has been described.[738] Although avoiding the most difficult part of the integration process, this system may be of value to people with those commercial integrators which produce only limited data. The complete programme, written in BASIC, is given.

Automatic Sequencers.—Sodium 2-bromoethanesulphonate has been suggested as a means of modifying cysteine residues prior to automatic sequence analysis.[739] The increased charge helps to minimize the loss of peptide material from the spinning cup during the organic solvent washes. However, cysteine is one of the rarest amino-acids and the number of charges introduced may be very small. In this respect the sulphonylated naphthaleneisothiocyanate derivatives introduced by Braunitzer *et al.* are clearly superior. For derivatization of cysteine residues, performic acid oxidation to cysteic acid may be preferable since all of the charged derivatives (cysteic acid, histidine, and arginine) may be identified after HI hydrolysis. However, this procedure does have the disadvantage of loss of tryptophan.

Other sequencer developments have been concerned with reducing the amount of protein needed for the degradation. The problem is that to form a stable film in the cup requires a minimum of 2 mg of protein. One obvious approach is to use a carrier molecule to stabilize the film and various polymers have been suggested for this purpose. An ideal carrier should be completely inert to the sequencing reagents, should not interfere with the degradation, and should be charged throughout the sequencing cycle to avoid extractive losses during the solvent wash steps. It should also have the correct physical properties required to form an even, stable film. One suggestion [740] has been to use a succinylated polyornithine derivative but this carrier will be charged only after the acid cleavage steps and solubility proplems may also be encountered. The copolymer, $H(Norleu\text{-}Arg)_{27}NH_2$, has also been used as a carrier [741] and quite good results were obtained on a 6 nmol sample of myoglobin. It is interesting that a linear rather than exponential decrease in yield was observed for this degradation. These workers point out that this carrier is not ideal since arginine released from the polymer would confuse the identification of the real amino-acid in the sequence. Also, to avoid solvent losses, a longer polymer would be more suitable.

A novel but perhaps rather esoteric approach to high-sensitivity sequencing has been described [742] for proteins which can be radioactively labelled *in vivo*.

[736] B. D. Young, *J. Chromatog.*, 1974, **92**, 113.
[737] J. M. Owen, A. D. Dale, A. Youngson, and P. T. Grant, *J. Chromatog.*, 1974, **96**, 235.
[738] K. D. Hapner and K. R. Hamilton, *J. Chromatog.*, 1974, **93**, 99.
[739] V. Niketic, J. Thomsen, and K. Kristiansen, *European J. Biochem.*, 1974, **46**, 547.
[740] J. Silver and L. E. Hood, *Analyt. Biochem.*, 1974, **60**, 285.
[741] H. D. Niall, J. W. Jacobs, J. Van Rietschoten, and G. W. Tregear, *F.E.B.S. Letters*, 1974, **41**, 62.
[742] D. J. McKean, E. H. Peters, J. I. Waldby, and O. Smithies, *Biochemistry*, 1974, **13**, 3048.

Briefly, the procedure is to sequence the labelled protein automatically in the presence of any unlabelled carrier protein. The released thiohydantoins are then hydrolysed back to the parent amino-acid, 20 nmol of amino-acid mixture is added, and each fraction is then subjected to amino-acid analysis. The analyser is modified so that the effluent is directed into a fraction collector which advances one tube at the end of each peak. At the end of the analysis the tubes are counted for radioactivity and the counts are matched to an amino-acid by reference to the chart recorder. There are problems: the hydrolysis system does not allow one to distinguish between serine, alanine, or carboxymethylcysteine, and amides, methionine, and tryptophan are all destroyed. These problems could be avoided in part by using an additional hydrolysis system but this of course would require further sample material. Other snags are that only proteins capable of being labelled *in vivo* can be used and that the hardware costs around £60 000—70 000.

Further results obtained by automatic sequencing at high sensitivity may be found in the section on hormone sequence. The sophistication and complexity of these methods should be compared with the relative simplicity of high-sensitivity solid-phase sequencing methods.

Thiohydantoin Analysis.—*Gas–Liquid Chromatography.* A method has been described for the separation of the trimethylsilyl derivatives of methylthiohydantoin (MTH) amino-acids.[743] A column (165 cm × 4 m) of 2% OV-17 on Gas Chrom Q was used and the complete run took 45 min. MTH-arginine was not detected and the derivatives of asparagine and phenylalanine were not resolved. However, this pair of amino-acids could be separated on a column of 1.7% OV-25 coated onto Supekoport. The same workers have experimented with a sulphur detector on this system to try to gain selectivity in the detection.[744] However, there were considerable deviations from linearity over a low concentration range. Derivatives of Asn, Gln, Pro, Gly, His, and Lys gave abnormally low responses below 2.5 nmol whereas MTH-methionine deviated from linearity at concentrations greater than 2 nmol.

Thin-layer Chromatography. In order to scale down the amount of derivative required for t.l.c. identification one may either miniaturize the separation system or increase the sensitivity of the detection system. The former approach has been used[745] to obtain complete resolution of all the PTH-amino-acids on 5 × 5 cm polyamide layers. Sensitivity is claimed to be 0.05—0.2 nmol. If one prefers 20 × 20 cm plates and improved resolution, a report has appeared[746] that exposure of these plates to iodine vapour before u.v. visualization increases the detection sensitivity 10-fold. In a later paper[747] these authors report that they have found an even better detection method, whereby silica plates containing a fluorescent indicator are used for the separation and these are then sprayed with a palladium-calcein reagent. The resulting fluorescent spots are visible, with one

[743] W. M. Lamkin, J. W. Weatherford, N. S. Jones, T. Pan, and D. N. Ward, *Analyt. Biochem.*, 1974, **58**, 422.
[744] W. M. Lamkin, N. S. Jones, T. Pan, and D. N. Ward, *Analyt. Biochem.*, 1974, **58**, 549.
[745] K. D. Kulbe, *Analyt. Biochem.*, 1974, **59**, 564.
[746] A. S. Inglis, P. W. Nicholls, and L. G. Sparrow, *J. Chromatog.*, 1974, **90**, 362.
[747] A. S. Inglis and P. W. Nicholls, *J. Chromatog.*, 1974, **97**, 289.

exception, at a level of 0.1 nmol. The exception, PTH-proline, is only just visible at a concentration of 0.5 nmol. Apparently it is important to use plates with a fluorescent indicator as this markedly enhances the calcein fluorescence.

A two-dimensional separation of MTH-amino-acids has been published.[748] Solvent 1 is toluene-n-heptane-acetic acid (6 : 3 : 2) and solvent 2 is 35% acetic acid. The leucine/isoleucine and glutamic acid/glutamine pairs are not resolved.

Mass Spectrometry.—Several papers have appeared which seek to develop the theory and methods used for the application of mass spectrometry to peptide sequencing. These provide an interesting contrast with the relatively small number of publications which have actually used the technique for deriving peptide sequences.

Six fragment ions which are diagnostic of particular peptide sequences have been reported.[749] These 'non-sequence' ions are produced by two consecutive N—C cleavages with hydrogen rearrangement at aspartic acid, asparagine, phenylalanine, histidine, tyrosine, or tryptophan. Recognition of these ions can clearly be helpful in determining unknown peptide sequences.

A new series of volatile derivatives prepared by reduction of *N*-perfluoroalkyl peptide methyl esters with lithium aluminium deuteride and subsequent *O*-trimethylsilylation has been introduced.[750] Several synthetic peptides have been treated in this way and it is claimed that the high volatility of the derivative gives an increase in sensitivity so that as little as one nanomole of peptide may be detected. No problems were encountered with arginine or histidine derivatives.

It should be remembered that mass spectrometry cannot normally differentiate between leucine and isoleucine in a peptide sequence. A new method[751] shows how these two amino-acids may be distinguished by unimolecular metastable ion and collisional activation spectra yielded by the immonium ion ($\overset{+}{Y}NH{=}CHC_4H_9$), which is always found when one of these amino-acids is present.

The proton transfer spectra of certain underivatized peptides have been studied in a tandem mass spectrometer system. Ammonium ion was used as the ionizing molecule and a new rapid heating method which involves dispersal of the sample on a Teflon probe gave considerable enhancement of volatility. It was found[752, 753] that underivatized pentapeptides could be sequenced using quantities as low as two nanomoles.

One of the few applications of mass spectrometry as a major method for determining the amino-acid sequence of a protein may be found in a study of the structure of the enzyme, ribitol dehydrogenase.[754] The approach used was to study simple peptide mixtures by low-resolution mass spectrometry, and the authors' success at deriving more than 200 residues of amino-acid sequence indicates the power and utility of the method in practical, rather than theoretical, terms. A partial sequence of dihydrofolate reductase has also been obtained in this way (see sequence section, p. 111).

[748] P. Rabin and A. Darbre, *J. Chromatog.*, 1974, **90**, 226.
[749] A. Dell and H. R. Morris, *Biochem. Biophys. Res. Comm.*, 1974, **61**, 1125.
[750] H. Nau, *Biochem. Biophys. Res. Comm.*, 1974, **59**, 1088.
[751] K. Levsen, H.-K. Wipf, and F. W. McLafferty, *Org. Mass Spectrometry*, 1974, **8**, 117.
[752] R. J. Beuhler, E. Flanigan, L. J. Greene, and L. Friedman, *J. Amer. Chem. Soc.*, 1974, **96**, 3990.
[753] R. J. Beuhler, E. Flanigan, L. J. Greene, and L. Friedman, *Biochemistry*, 1974, **13**, 5060.
[754] H. R. Morris, D. H. Williams, G. G. Midwinter, and B. S. Hartley, *Biochem. J.*, 1974, **141**, 701.

Finally, proton magnetic resonance spectroscopy has been applied to peptide sequencing.[755] Peptides of up to six residues could be analysed on a 100 MHz instrument but the quantities required are too large for the method to be of any value. For instance, a minimum of one micromole of a tripeptide is necessary.

6 Primary Structures

This section includes information on proteins whose partial or complete sequence has been published during 1974. The bulk of this information has been summarized in Table 5 (p. 130) for the complete, or virtually complete, sequences and in Table 6 (p. 138) for the partial sequences. These tables also include notes as to which results are discussed further in the text.

Structural Proteins.—*Collagen.* Now that the entire sequence of the α1 chain of collagen is complete for calf or rat skin, attention is being focused on comparative studies and on the sequence of the α2 chain. Although the *N*-terminus of this chain is blocked in calf skin, limited pepsin digestion removes the *N*-terminal dipeptide, Glp-Phe, allowing the automatic determination of the next 17 residues.[756] Further studies have been performed on peptide CB3-5 from the same source and on the corresponding fragment from rat skin.[757] This work, along with comparative sequences obtained from peptide CB2 of the α2 chains of calf, human, rabbit, and pig skin collagen,[758] shows that there is considerable conservation of sequence between species but considerable variation between the α1 and α2 chains within one species. Preliminary information has also appeared on sea-anemone collagen,[759] which is apparently composed of three identical α-chains, and on the α(111) chain of human collagen.[760] Two new cross-links, 5-keto-5′-hydroxy-lysinonorleucine and 5-keto-lysinonorleucine, have been isolated[761] from bovine bone. Further topics relating to collagen biochemistry will be found under the sections on complement and glycoproteins.

Elastin. Sequence studies on elastin are complicated by the protein's high degree of cross-linking and its insolubility. Some progress has been made on a soluble precursor, tropoelastin, which is not cross-linked, but now two groups have reported methods for the isolation and characterization of cross-linked peptides from mature elastin. The first approach[762] is simply to isolate a peptide containing a desmosine cross-link and to sequence both chains simultaneously in a sequencer. The results are complicated by the large number of alanine residues present and by the fact that the cross-link is attached through four loci, resulting in no PTH identification at the first three attachment positions. However, careful quantitation and, perhaps more important, correlation with the corresponding peptides of tropoelastin has allowed the structures of two peptides to be deduced.

[755] J. S. Bradbury, M. W. Crompton, and B. Warren, *Analyt. Biochem.*, 1974, **62**, 310.
[756] P. P. Fietzek, D. Breitkreutz, and K. Kühn, *Biochim. Biophys. Acta*, 1974, **365**, 305.
[757] P. P. Fietzek and K. Kühn, *Z. physiol. Chem.*, 1974, **355**, 647.
[758] P. P. Fietzek, H. Furthmayr, and K. Kühn, *European J. Biochem.*, 1974, **47**, 257.
[759] H. Nowack and A. Nordwig, *European J. Biochem.*, 1974, **45**, 333.
[760] E. Chung, E. M. Keele, and E. J. Miller, *Biochemistry*, 1974, **13**, 3459.
[761] G. L. Mechanic, *Biochem. Biophys. Res. Comm.*, 1974, **56**, 923.
[762] J. A. Foster, L. Rubin, H. M. Kagan, C. Franzblau, E. Bruenger, and L. B. Sandberg, *J. Biol. Chem.*, 1974, **249**, 6191.

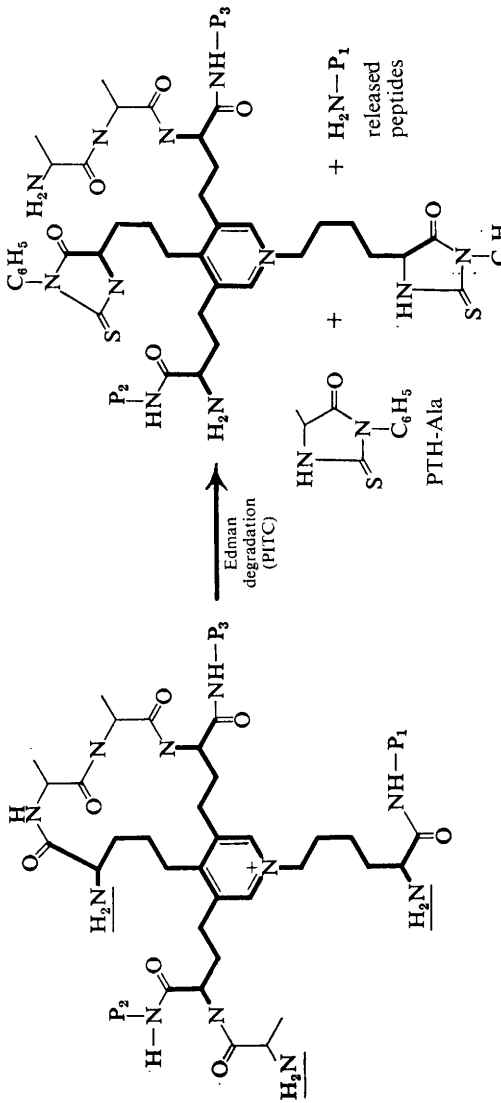


Figure 8 *The use of Edman degradation for the release of* COOH-*terminal peptides from the elastolytic desmosine-containing peptides. The figure indicates one of several possible structures present and shows the release of single-chain peptides from the* COOH-*terminals of the desmosine cross-link* (*dark lines*)
(Reproduced by permission from *J. Biol. Chem.*, 1974, **249**, 5204)

The second approach [763] requires the isolation of cross-linked peptides from an elastase digest, their purification on Cellex P and Sephadex LH-20, and the removal of *N*-terminal lysine residues involved in cross-linking the two peptide chains by one cycle of Edman degradation (Figure 8). Following normal sequencing procedures ten sequences from bovine elastin and five from porcine elastin have been deduced. The combined results indicate that the sequence Lys-Ala-Ala-Lys is cross-linked by desmosine or isodesmosine to Lys-Ala-Ala-Ala-Lys (Figure 8).

Muscle Proteins.—*Myosin.* Apart from a reinvestigation of the sequence around two reactive thiol groups in the head region of myosin,[764] no further structural information has appeared for the main (heavy) chains of this molecule. However, complete sequences have now appeared for the A1 and A2 (alkali) light chains [765] and several significant conclusions may be drawn from these data.

Rabbit skeletal muscle myosin contains two classes of light chains attached to the myosin 'head'. The 'DTNB' light chain may be removed with no loss of the ATPase activity. However, removal of the two 'alkali' light chains (A1 and A2) causes total loss of activity although their function *in vivo* is not clear. Sequence analysis of the A1 and A2 chains shows that there are an additional 41 residues at the *N*-terminal end of A1 followed by eight residues, five of which differ from their counterparts in the first eight residues of the shorter A2 chain. Apart from this, the sequences are identical for the remaining 141 amino-acids. Since there are sequence differences in the first few residues one chain cannot simply be a proteolytic fragment of the other and the proteins are clearly the products of separate genes. The significance of the additional 41 residues of A2 is not clear but the sequence is most unusual. A combination of quantitative automatic sequencing and mass spectrometry [766] has produced the structure:

X-Pro-Lys-Lys-Asn-Val-Lys-Lys-Pro-Ala-Ala-Ala-Ala-Ala-Pro-Ala-Pro-Lys-Ala-

Pro-Ala-Pro-Ala-Pro-Ala-Pro-Ala-Pro-Ala-Pro-Ala-Pro-Lys-...

for residues 1—30.

The stoicheiometry of the light chains is not equimolar and varies between muscle types. However, cross-reinervation of, for instance, fast-twitch and slow-twitch myosins produces a corresponding transformation in light-chain stoicheiometry indicating that the myosin phenotype is controlled *via* the central nervous system and supporting the theory that the 'alkali' light chains are involved in the myosin ATPase activity.

Comparison of the A2 sequence with that from the calcium-binding component of troponin C, and with a calcium-binding protein from carp muscle, shows extensive homologies.[767, 768] In particular, the core of hydrophobic residues which forms the calcium-binding site in the carp protein is also present in the A2

[763] G. E. Gerber and R. A. Anwar, *J. Biol. Chem.*, 1974, **249**, 5200.
[764] T. Yamashita, Y. Soma, S. Kobayashi, and T. Sekine, *J. Biochem.*, 1974, **75**, 447.
[765] G. Frank and A. G. Weeds, *European J. Biochem.*, 1974, **44**, 317.
[766] J. Bridgen and H. R. Morris, *European J. Biochem.*, 1974, **44**, 333.
[767] J. H. Collins, *Biochem. Biophys. Res. Comm.*, 1974, **58**, 301.
[768] A. G. Weeds and A. D. McLachlan, *Nature*, 1974, **252**, 646.

chain. However, the light chains do not appear to bind calcium, and it is possible that during evolution this ability has been lost and an alternative system of calcium regulation, the troponin–tropomyosin system (see below) has evolved. It will be interesting to compare these sequences with that from the 'EDTA' light chain of scallop myosin which does bind calcium.

Troponin. Troponin is a complex of three proteins responsible for conferring calcium-sensitivity to the actomyosin contractile system. One of the proteins, TN-C, binds calcium and is of known sequence. Another, TN-T, binds the troponin to tropomyosin which, in turn, binds to the myofilament protein, actin. The third component, TN-1, is responsible for inhibiting the Mg^{2+}-activated ATPase of actomyosin in the absence of calcium. CNBr fragments of TN-1 have now been purified as an adjunct to sequence work [769] and two reports [770, 771] have appeared on the nature of phosphorylation sites in this protein. One major site was found and the sequence around the phosphorylated residue,

Ⓟ
|
Ala-Ile-Thr-Ala-Arg

was found to be similar to that around the serine phosphate of phosphorylase a,

Ⓟ
|
Gln-Ile-Ser-Val-Arg

A minor site, however, showed no such similarity. The significance of the phosphorylation is not clear.

Enzymes.—*Anthranilate Synthetase.* This enzyme is composed of two non-identical subunits. One subunit catalyses the conversion of chorismate into anthranilate in the presence of glutamine, which is provided by the glutamine amidotransferase activity of the second subunit. In all bacteria, subunit I has a molecular weight of 60 000—65 000 whereas the size of the second subunit is variable. For example, in *E. coli* or *S. typhimurium* the molecular weight of subunit I is also 60 000—65 000 and this subunit has the additional function of catalysing the second step of tryptophan biosynthesis, phosphoribosyl transfer to the anthranilate. In certain other bacteria, *e.g. Serratia marcescens*, *B. subtilis*, the latter activity is absent and the second subunit now has a molecular weight of only 14 000—21 000 and there is a separate enzyme of molecular weight 45 000 for phosphoribosyl transfer. Two very similar papers [772, 773] have now appeared on the *N*-terminal sequences of the type II subunit from both classes of bacteria. All the sequences are clearly homologous, suggesting that the gene encoding the 65 000 dalton subunit resulted during evolution from the fusion of the amidotransferase and the phosphoribosyl transferase genes.

Triose Phosphate Isomerase. An interesting comparison of sequencing techniques can be made from three papers dealing with the primary structure of this enzyme.

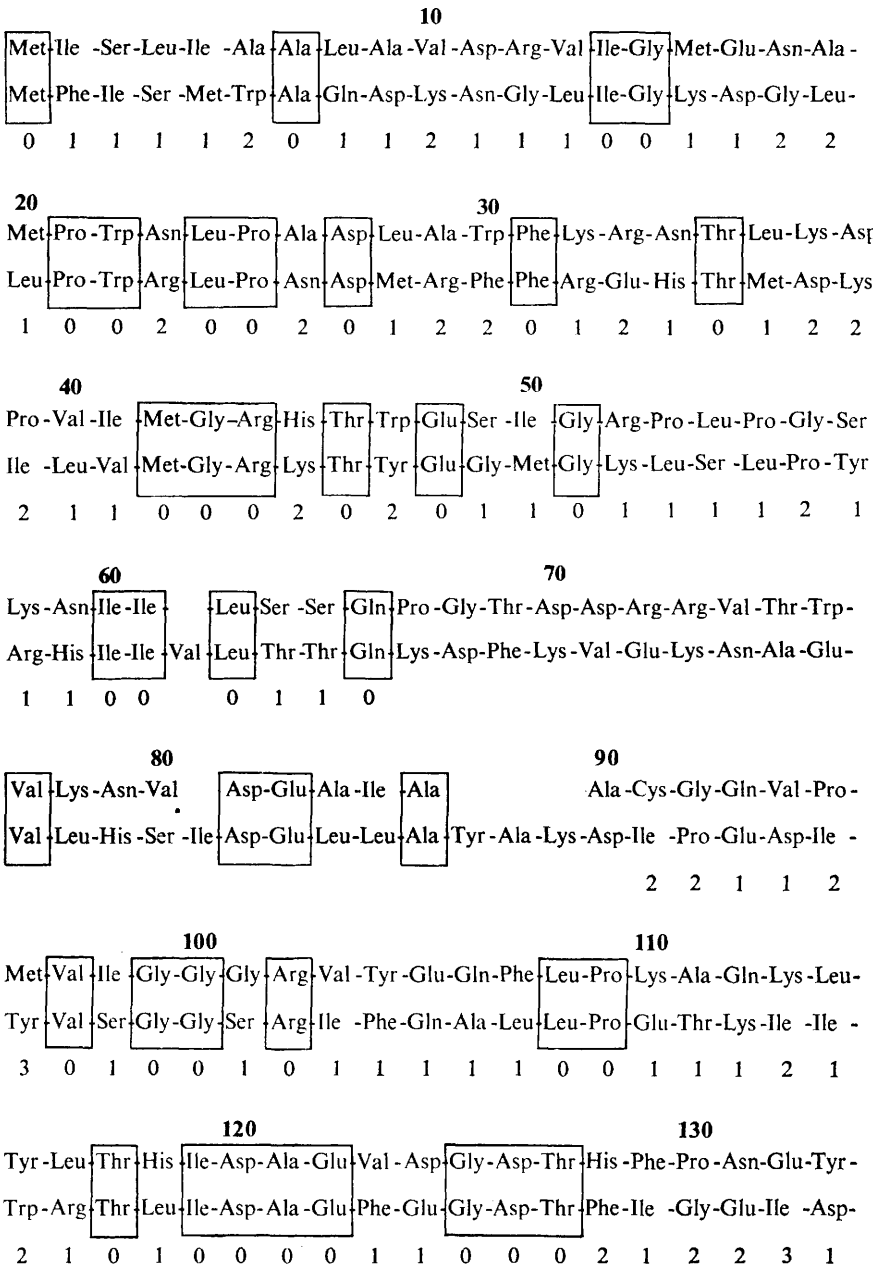
[769] J. M. Wilkinson, *F.E.B.S. Letters*, 1974, **41**, 166.
[770] T. S. Huang, D. B. Bylund, J. T. Stull, and E. G. Krebs, *F.E.B.S. Letters*, 1974, **42**, 249.
[771] A. J. G. Moir, J. M. Wilkinson, and S. V. Perry, *F.E.B.S. Letters*, 1974, **42**, 253.
[772] S.-L. Li, J. Hanlon, and C. Yanofsky, *Biochemistry*, 1974, **13**, 1736.
[773] S.-L. Li, J. Hanlon, and C. Yanofsky, *Nature*, 1974, **248**, 48.

In two cases the sequence is derived from rabbit [774] and chicken [775] muscle using conventional methods and in the other case the corresponding sequence from coelacanth muscle [776] is described but derived mainly by automated methods. By the use of conventional methods *ca.* 80 peptides were isolated and characterized simply to establish the sequence of the tryptic fragments. In contrast only 20 fragments were required to complete the sequence of the coelacanth protein and 75% of the structure was established by automated analysis of only four fragments. In this way the structure was determined using much less material and in a far shorter time than by conventional techniques.

Dihydrofolate Reductase. A major reason for research into the structure of this enzyme is an attempt to define the mode of action of drugs such as methotrexate, trimethoprim, and pyrimethamine with which it interacts specifically. Two complete sequences have been reported for methotrexate-resistant mutants of *E. coli* B [777] and *Streptococcus faecium* var. Durans,[778] and there is a partial sequence of the enzyme from *Lactobacillus casei* MTX/R.[779] In the case of the *E. coli* enzyme, an attempt has been made to demonstrate sequence homologies with other nucleotide-binding proteins but the comparisons made are not convincing. For instance it is well known that the cofactor NAD binds to the *N*-terminal region of glyceraldehyde-3-phosphate dehydrogenase but the region compared here is from the *C*-terminal catalytic domain. Also, to construct a coenzyme binding site of the type found in several dehydrogenases would require almost the whole of the dihydrofolate reductase molecule. Comparison of the two complete dihydrofolate reductase sequences (Figure 9) shows that they are remarkably dissimilar, with only 42 identical residues over the entire chain and unless the tertiary structures are more conserved, designing drugs to interact at specific sites will not be easy. With the *Lactobacillus* enzyme,[779] emphasis is placed on sequencing methods rather than on results. An elastase digest was found to yield small soluble fragments which were very amenable to mass spectrometric sequencing methods, particularly mixture analysis. A total of 105 residues has been sequenced but not overlapped.

Glutamate Dehydrogenase. Following the determination of the structure of this hexameric protein from bovine and chicken liver, a sequence has now appeared for the 452 residue NADP-specific protein from *Neurospora crassa.*[780] The analysis was complicated by partial cleavages after tryptophan residues with CNBr and the structure presented is only tentative. However, comparison with the bovine sequence shows that of 82 identical residues, 62 occur in the *N*-terminal half and only 20 in the *C*-terminal half. This correlates well with the position of the catalytically reactive lysine at residue 113, suggesting that it is the *C*-terminal

[774] P. H. Corran and S. G. Waley, *Biochem. J.*, 1974, **139**, 1.
[775] A. J. Furth, J. D. Milman, J. D. Priddle, and R. E. Offord, *Biochem. J.*, 1974, **139**, 11.
[776] E. Kolb, J. I. Harris, and J. Bridgen, *Biochem. J.*, 1974, **137**, 185.
[777] C. D. Bennett, *Nature*, 1974, **248**, 67.
[778] J. M. Gleisner, D. L. Peterson, and K. L. Blakley, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 3001.
[779] H. R. Morris, K. E. Batley, N. G. L. Harding, R. A. Bjur, J. G. Dann, and R. W. King, *Biochem. J.*, 1974, **137**, 409.
[780] J. C. Wootton, G. K. Chambers, A. A. Holder, A. J. Baron, J. G. Taylor, J. R. S. Fincham, K. M. Blumenthal, K. Moon, and E. L. Smith, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 4361.


10
Met Ile -Ser-Leu-Ile -Ala Ala Leu-Ala -Val -Asp-Arg-Val Ile-Gly Met-Glu-Asn-Ala -
Met Phe-Ile -Ser -Met-Trp Ala Gln-Asp-Lys -Asn-Gly-Leu Ile-Gly Lys -Asp-Gly-Leu-
0 1 1 1 1 2 0 1 1 2 1 1 1 0 0 1 1 2 2
20
30
Met Pro -Trp Asn Leu-Pro Ala Asp Leu-Ala -Trp Phe Lys - Arg-Asn Thr Leu-Lys -Asp-
Leu Pro -Trp Arg Leu-Pro Asn Asp Met-Arg-Phe Phe Arg-Glu- His Thr Met-Asp-Lys -
1 0 0 2 0 0 2 0 1 2 2 0 1 2 1 0 1 2 2
40
50
Pro -Val -Ile Met-Gly–Arg His Thr Trp Glu Ser -Ile Gly Arg-Pro -Leu-Pro -Gly -Ser -
Ile -Leu-Val Met-Gly - Arg Lys Thr Tyr Glu Gly -Met Gly Lys -Leu-Ser -Leu-Pro -Tyr -
2 1 1 0 0 0 2 0 2 0 1 1 0 1 1 1 1 2 1
60
70
Lys -Asn Ile -Ile Leu Ser -Ser Gln Pro -Gly -Thr -Asp-Asp-Arg-Arg-Val -Thr -Trp -
Arg-His Ile -Ile Val Leu Thr -Thr Gln Lys -Asp-Phe-Lys -Val -Glu-Lys -Asn-Ala -Glu-
1 1 0 0 0 1 1 0
80
90
Val Lys -Asn-Val Asp-Glu Ala -Ile Ala Ala -Cys -Gly -Gln -Val -Pro -
Val Leu-His -Ser -Ile Asp-Glu Leu-Leu Ala Tyr - Ala -Lys -Asp-Ile -Pro -Glu -Asp-Ile -
2 2 1 1 2
100
110
Met Val Ile Gly -Gly Gly Arg Val -Tyr -Glu -Gln -Phe Leu-Pro Lys -Ala -Gln -Lys -Leu-
Tyr Val Ser Gly -Gly Ser Arg Ile -Phe -Gln -Ala -Leu Leu-Pro Glu-Thr -Lys -Ile -Ile -
3 0 1 0 0 1 0 1 1 1 1 1 0 0 1 1 1 2 1
120
130
Tyr -Leu Thr His Ile -Asp -Ala -Glu Val - Asp Gly - Asp - Thr His -Phe -Pro -Asn -Glu -Tyr -
Trp -Arg Thr Leu Ile -Asp -Ala -Glu Phe -Glu Gly - Asp - Thr Phe -Ile -Gly -Glu -Ile -Asp -
2 1 0 1 0 0 0 0 1 1 0 0 0 2 1 2 2 3 1

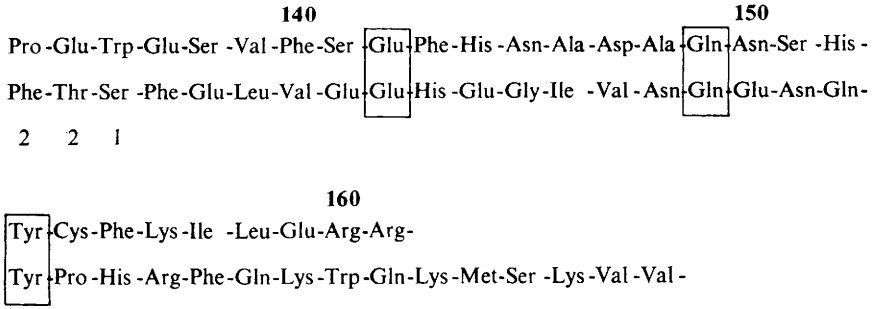


Figure 9 *Comparison of the amino-acid sequences of dihydrofolate reductase from* E. coli *(upper sequence) and* S. faecium *(lower sequence). Numbers below residues indicate the minimum number of base changes needed to change the amino-acid in one sequence to the residue in the other sequence*

part of the molecule which binds the coenzyme and may therefore be less conserved. An analysis of the sequence, based on rules for predicting protein secondary structure,[781] shows that there is indeed a coenzyme binding domain analogous to that found in other dehydrogenases between residues 219 and 344 in the *Neurospora* sequence. However, these rules also predict a second binding site in the *N*-terminal region between residues 1 and 115 although it appears that only one molecule of NADPH is bound per subunit.

In contrast, the NAD-specific glutamate dehydrogenase from *N. crassa* has been found[782] to be a tetrameric enzyme of subunit molecular weight 116 000. Peptides isolated after selective modification of one cysteine and five lysine residues showed no homology with the sequence of the NADP-specific enzyme and it appears that these proteins are not closely related nor is the NAD-specific dehydrogenase at all similar to its invertebrate counterparts.

Superoxide Dismutase. Interest in this protein stems from interest in structure–function relationships in metalloenzymes, its role as a component of the aerobic defence mechanism against oxygen toxicity, its ability to act as a tool for assessing the participation of superoxide radicals in the mechanism of chemical and biochemical reactions, and as a means of investigating evolutionary relationships between eukaryotes, mitochondria, and prokaryotes.

In determining the structure of the 151 residue Cu-Zn bovine enzyme,[783, 784] use was made of tryptic, maleylated tryptic, CNBr, peptic, plasmin, hydroxylamine, chymotryptic, subtilisin, and dilute acid hydrolysis peptides. Amongst other points it was found that addition of 0.5M-urea to the elution buffers reduced absorptive losses of peptide on ion-exchange columns and that NH_2OH also cleaved an Asn-Ala bond as well as Asn-Gly linkages. Plasmin was found to be a useful enzyme for preparing large fragments. No homology could be found with carbonic anhydrase B which also binds zinc and there is also no homology with mitochondrial or bacterial dismutases. A 3 Å resolution *X*-ray

[781] J. C. Wootton, *Nature*, 1974, **252**, 542.
[782] F. M. Veronese, Y. Degani, J. F. Nye, and E. L. Smith, *J. Biol. Chem.*, 1974, **249**, 7936.
[783] H. J. Evans, H. M. Steinman, and R. L. Hill, *J. Biol. Chem.*, 1974, **249**, 7315.
[784] H. M. Steinman, V. R. Naik, J. L. Abernethy, and R. L. Hill, *J. Biol. Chem.*, 1974, **249**, 7326.

diffraction study shows that the copper atom is liganded to four histidine residues at positions 44, 46, 61, and 118. Finally, it has been shown [785] that the single S—S bond links cysteine residues 55 and 144.

Blood Clotting Proteins. The process of blood clotting is one of the more complex physiological phenonema, particularly from the point of view of protein chemistry. A simplified 'flow-chart' of the clotting process is shown in Figure 10

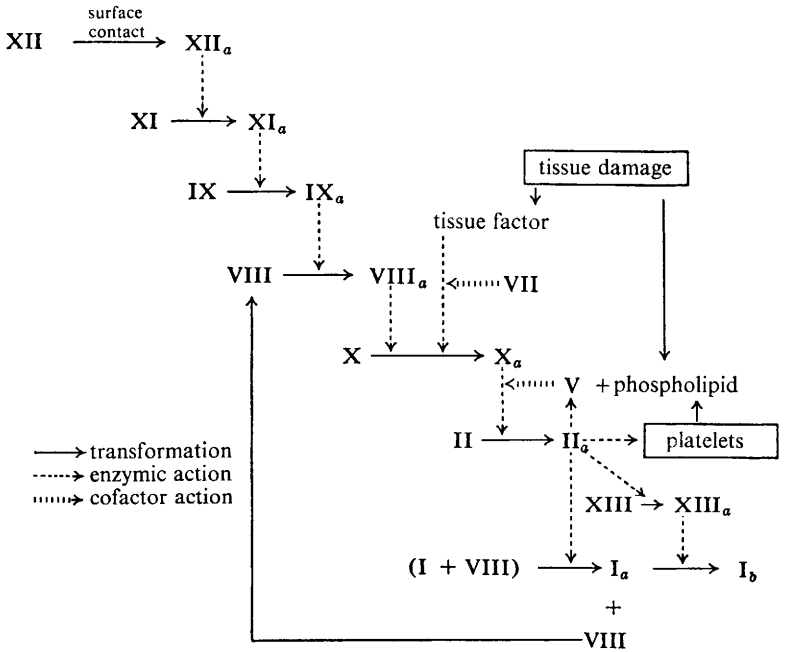


Figure 10 *A scheme of the interaction of clotting factors. The subscript a indicates the activated form or product.* II_a = *thrombin*; I_a = *fibrin*; I_b = *stabilized fibrin*
(Reproduced by permission from *Adv. Enzymol.*, 1968, **30**, 270)

from which it can be seen that clotting consists of a cascade of sequential enzymic cleavages and activations. Clotting Factors VII, IX, X, and prothrombin are dependent on vitamin K for their synthesis. Amino-terminal sequences of three of these,[786] Factor IX, the light chain of Factor X, and prothrombin, show considerable homology, suggesting a common ancestral gene. Since these molecules have been shown to be homologous in their *C*-terminal regions and since they differ considerably in size, they must have evolved by removal or addition of polypeptide segments from the centre of the chain. Furthermore, the activation products of these three factors, Factors IXa, Xa, and thrombin, have also been shown [787] to have homologous *N*-terminal sequences as well as being homologous around a reactive serine residue.

[785] J. L. Abernethy, H. M. Steinman, and R. L. Hill, *J. Biol. Chem.*, 1974, **249**, 7339.
[786] K. Fujikawa, M. H. Coan, D. L. Enfield, K. Titani, L. H. Ericsson, and E. W. Davie, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 427.
[787] D. L. Enfield, L. H. Ericsson, K. Fujikawa, K. Titani, K. A. Walsh, and H. Neurath, *F.E.B.S. Letters*, 1974, **47**, 132.

The mechanism of activation of each of these factors has been studied in detail. It appears [788] that Factor IX is of molecular weight 55 400 with *N*-terminal sequence: Tyr-Asn-Ser-Gly. This is cleaved by XIa into IXa light and heavy chains. The heavy chain is then cleaved further into a 27 300 molecular weight fragment whose *N*-terminal sequence is homologous with those of factor Xa and thrombin (see above). A similar multi-step activation process has also been described for Factor X.[789, 790]

The released thrombin acts on fibrinogen to produce fibrin and fibrinopeptides (see below) and also on Factor XIII, releasing a small peptide and producing a transamidase-type enzyme which cross-links the fibrin. The sequence of the released peptides from human and bovine plasma and platelets has been determined.[791, 792] These studies show that thrombin cleaves an Arg-Gly bond in the *N*-terminal region of the a chain of Factor XIII in both the plasma and platelet systems.

The three peptides released by thrombin from canine fibrinogen have been isolated and sequenced.[793] Two of the peptides differed only in the phosphorylation of a serine residue.

Several groups have discussed the nature of the vitamin K-induced modification of prothrombin. In the absence of this vitamin a prothrombin is formed which differs from the normal form in that it does not bind to $BaSO_4$, binds less calcium and is slightly less negatively charged. During activation of normal prothrombin a 20 000—25 000 molecular weight fragment is cleaved from the *N*-terminus of the molecule which possesses all of these features. This fragment has now been isolated [794, 795] from human prothrombin and from dicoumarol (a vitamin K analogue) induced prothrombin, and it has been shown that the calcium-binding fragments are identical in composition but differ in charge. ^1H N.m.r. and mass spectra [796] of a tetrapeptide comprising residues 6—9 of normal prothrombin established the sequence of this peptide to be: Leu-γ-Glu-γ-Glu-Val, thus explaining the increased negative charge of this region. A second report [797] describes finding a similar peptide with sequence: γ-Glu-Ser. Finally, the complete structure of residues 1—42 of bovine prothrombin has shown the presence of no less than 10γ-glutamic acid residues,[798] and it appears, therefore, that the function of vitamin K in blood clotting is to cause the post-translational modification of glutamic acid residues in Factors X, IX, VII, and prothrombin causing them to become strongly calcium-binding and activated in the normal Ca^{2+}-phospholipid systems.

[788] K. Fujikawa, M. E. Legaz, H. Kato, and E. W. Davie, *Biochemistry*, 1974, **13**, 4508.
[789] J. Jesty, A. K. Spencer, and Y. Nemerson, *J. Biol. Chem.*, 1974, **249**, 5614.
[790] K. Fujikawa, M. H. Coan, M. E. Legaz, and E. W. Davie, *Biochemistry*, 1974, **13**, 5290.
[791] T. Takagi and R. F. Doolittle, *Biochemistry*, 1974, **13**, 750.
[792] S. Nakamura, S. Iwanaga, and T. Suzuki, *Biochem. Biophys. Res. Comm.*, 1974, **58**, 250.
[793] A. J. Osbahr and R. W. Colman, *Biochim. Biophys. Acta*, 1974, **336**, 273.
[794] J. Stenflo, *J. Biol. Chem.*, 1974, **249**, 5527.
[795] T. Skotland, T. Holm, B. Østerud, R. Flengsrud, and H. Prydz, *Biochem. J.*, 1974, **143**, 29.
[796] J. Stenflo, P. Fernlund, W. Egan, and P. Roepstorff, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 2730.
[797] G. L. Nelsestuen, T. H. Zytkovicz, and J. B. Howard, *J. Biol. Chem.*, 1974, **249**, 6347.
[798] S. Magnusson, L. Sottrup-Jensen, T. E. Petersen, H. R. Morris, and A. Dell, *F.E.B.S. Letters*, 1974, **44**, 189.

The reverse process of clot dissolution is mediated by the enzyme plasmin. This is formed by proteolysis of its precursor, plasminogen, by the action of urokinase or streptokinase. Amino-terminal sequencing has been used to show [799] that the urokinase activation is a two-step process involving at least two cleavages at the *N*-terminal end of the precursor. A similar study [800, 800a] has been made on the mechanism of streptokinase activation as well as on the proteolytic modification of the activator itself.

Pronase. The commercial product, Pronase, is a mixture of various endo- and exo-peptidases. The sequences of two of the endopeptidases, Proteases A [801] and B,[802] have now been determined and a comparison made with the pancreatic proteases. Both enzymes contain the active-site Asp-Ser-Gly sequence which has been found in other 'serine' proteases (Figure 11) and a comparison of the complete sequences has allowed predictions of the tertiary structure of the microbial proteins. Protease A is predicted to have a very similar structure to the pancreatic proteases around the two S—S bridges and active site residues but to differ in three surface regions. Two of these surface differences have also been found previously in the microbial enzyme, *Myxobacter* α-lytic protease. Protease B has 61% identity with protease A, and the same structural differences that were found between the A protein and the pancreatic enzymes can also be predicted for this protease. It would appear therefore that the tertiary structures of the A and B enzymes are very similar. The extra stability of the B enzyme (it is active in concentrated urea and guanidine solutions) must therefore arise from detailed rather than gross structural differences.

Phospholipase A. This enzyme catalyses the hydrolysis of fatty acid ester bonds at the 2-carbon position of 3-*sn*-phosphoglycerides. The structure of the enzyme from pig pancreas is known and reports have now appeared on sequences from venom of the bee, *Apis mellifica*,[803] and from the snakes, *Agkistrodon halys blomhoffii* [804] and *Bitis gabonica*.[805] In the case of the bee enzyme, use was made of a protease from the basidiomycete *Armillaria mellea* which specifically cleaves peptide bonds adjacent to lysine α-amino groups. No cleavage occurred at Lys-Pro bonds but Pro-Lys and X-aminoethylcysteine bonds were broken. There was no cleavage at lysine-14, presumably as a consequence of the carbohydrate bound to asparagine-13. The four disulphide bridges of this protein have also been assigned.[806] The homology between the enzyme from bee venom appears to be lower than that between the snake venom phospholipases and the porcine pancreatic enzyme. However, in comparing any of the sequences many insertions and deletions have to be introduced to obtain acceptable homologies and it

[799] P. J. Walther, H. M. Steinman, R. L. Hill, and P. A. McKee, *J. Biol. Chem.*, 1974, **249**, 1173.
[800] D. K. McClintock, M. E. Englert, C. Dziobkowski, E. H. Snedeker, and P. H. Bell, *Biochemistry*, 1974, **13**, 5334.
[800a] W. J. Brockway and F. J. Castellino, *Biochemistry*, 1974, **13**, 2063.
[801] P. Johnson and L. B. Smillie, *F.E.B.S. Letters*, 1974, **47**, 1.
[802] L. Jurášek, M. R. Carpenter, L. B. Smillie, A. Gertler, S. Levy, and L. H. Ericsson, *Biochem. Biophys. Res. Comm.*, 1974, **61**, 1095.
[803] R. A. Shipolini, G. L. Callewaert, R. C. Cottrell, and C. A. Vernon, *European J. Biochem.*, 1974, **48**, 465.
[804] Y. Samesjima, S. Iwanaga, and T. Suzuki, *F.E.B.S. Letters*, 1974, **47**, 348.
[805] D. P. Botes and C. C. Viljoen, *J. Biol. Chem.*, 1974, **249**, 3827.
[806] R. A. Shipolini, S. Doonan, and C. A. Vernon, *European J. Biochem.*, 1974, **48**, 477.

appears that the phospholipase A structure can tolerate considerable primary structure variation but still maintain biological activity.

Protein Synthesis Factors.—*Elongation Factor* 2. This factor catalyses the translocation of newly lengthened peptidyl tRNA from the acceptor site to the

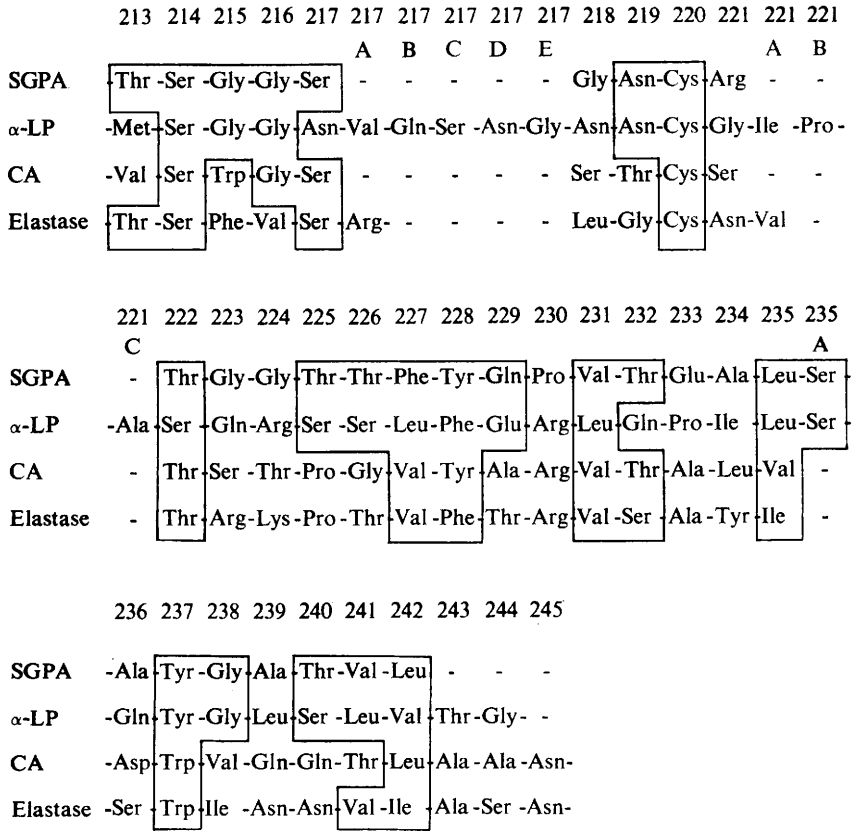
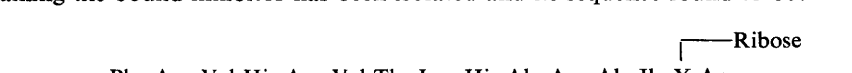


Figure 11 *The amino-acid sequence of* SGPA *aligned with other Asp-Ser-Gly proteases. The numbering system is that of bovine chymotrypsinogen A, and chemically similar residues are enclosed in solid lines. The proteases compared are Myxobacter α-lytic protease* (α-LP), *bovine chymotrypsinogen A* (CA), *and porcine elastase*
(Reproduced by permission from *F.E.B.S. Letters*, 1974, **47**, 4)

donor site on the ribosome. This reaction may be inhibited by ADP ribosylation of the protein using the ADP ribose moiety of NAD^+. A tryptic peptide containing the bound inhibitor has been isolated and its sequence found to be:[807]



[807] E. A. Robinson, O. Henriksen, and E. S. Maxwell, *J. Biol. Chem.*, 1974, **249**, 5088.

The nature of X is unknown. The amino-acid elutes between phenylalanine and histidine on the amino-acid analyser and is also found in the unmodified protein. It is speculated that it represents a new amino-acid but its significance to protein synthesis *in vivo* remains to be investigated.

tRNA Synthetases. Aminoacyl tRNA synthetases occupy a central position in maintaining the fidelity of the genetic code and it would be reasonable to expect that they had evolved from a common precursor and were all closely related in structure. However, a diversity of structures of varying subunit molecular weight and composition have been found. It is now apparent that this diversity may be explained, at least in part, by a theory of partial or total gene duplication and fusion. In support of this theory, evidence has recently been presented for internal sequence repetition in the tRNA synthetases from *E. coli* (Leu),[808] *E. coli* (Met),[809] and *B. stearothermophilus* (Val, Leu, and Met).[810] In some of the latter cases the two parts of the sequence appear very similar and possibly reflect the enormous selective pressure to maintain the exact structure, and hence the exact specificities, of these enzymes. It also appears that there is a basic sequence unit of molecular weight about 50 000 and it is by addition to this structure that diversity is produced. Whether this hypothesis is correct must await the determination of complete sequences of these enzymes.

Histones.—*Histone I* (*histone KAP*, *f*1). The amino-acid sequence of the hydrophobic region of this protein, comprising residues 72—107, has been determined.[811] This completes residues 1—107 of the structure.

Histone IIb (*histone ALK*, *f*2*a*2). The structure of this protein from calf thymus has been determined,[812] principally by analysis of thermolytic peptides. The sequence is identical with that described in 1972 for the same protein from the same source but from a different laboratory.

Histone III (*histone ARE*, *f*3). By sequential application of chemical cleavage methods using CNBr, *N*-bromosuccinimide, and dilute HCl, it has been possible to generate a complete set of peptide fragments from chicken erythrocyte histone III.[813] The cleavage methods were selected so that no more than three peptides were generated at any step. A knowledge of the terminal sequences and/or end-groups then eliminates the need to produce overlapping fragments. The peptides were sequenced automatically[814] using 3-(*NN*-dimethylamino)propyne as buffer. This revised structure is now identical with that from calf thymus except that Cys-96 in the latter protein becomes a serine.

An identical approach has been used by the same group to elucidate the structure of histone III from shark erythrocytes.[815] The sequence is identical with that from chicken which suggests that a study of these proteins from a purely evolutionary standpoint will yield little useful information. However, the method is

[808] R. M. Waterson and W. H. Konigsberg, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 376.
[809] C. J. Bruton, R. Jakes, and G. L. E. Koch, *F.E.B.S. Letters*, 1974, **45**, 26.
[810] G. L. E. Koch, Y. Boulanger, and B. S. Hartley, *Nature*, 1974, **249**, 316.
[811] G. M. T. Jones, S. C. Rall, and R. D. Cole, *J. Biol. Chem.*, 1974, **249**, 2548.
[812] P. Sautière, D. Tyrou, B. Laine, J. Mizon, P. Ruffin, and G. Biserte, *European J. Biochem.*, 1974, **41**, 563.
[813] W. F. Brandt and C. Von Holt, *European J. Biochem.*, 1974, **46**, 407.
[814] W. F. Brandt and C. Von Holt, *European J. Biochem.*, 1974, **46**, 419.
[815] W. F. Brandt, W. N. Strickland, and C. Von Holt, *F.E.B.S. Letters*, 1974, **40**, 349.

clearly a good one, particularly for small proteins where there is a very homologous sequence to assist in selecting cleavage methods.

Histone IV (*histone GRK*, *f2a1*). This is the most conserved of all histones. Amino-acid analysis has shown only one difference between the sea-urchin and calf thymus proteins and this substitution has now been shown to be: $Thr_{73} \rightarrow Cys$.[816] Out of 102 residues, 52 have been sequenced.

Lipoproteins and Membrane Proteins.—*Lipoproteins.* Human plasma low-density lipoproteins are responsible for the transport of endogenous triglyceride in blood. The major protein constituents have been designated apo LP-Ser, apo LP-Glu, apo LP-Ala-1, and apo LP-Ala-2, the last two differing only in their sialic acid contents. High-density plasma lipoproteins contain only two major constituents, Gln-1 and Gln-2, the latter being a 77-residue protein of known sequence. The 245-residue structure of Gln-1 has now been determined[817] and this shows several structural features which may account for its high helical content and lipid-binding properties. There is a high concentration of helical-forming residues (Glu, Ala, Leu), and model-building shows[818] that these helices are amphipathic with a polar and an apolar face. Furthermore, acidic residues invariably occur in a narrow strip along the centre of the polar face whereas the basic amino-acids are located on the lateral edges, an arrangement which should permit close orientation of the polar head of the phospholipid with the ion pairs of the protein.

The low-density lipoproteins contain about 90% lipid and 10% protein. The sequence of one of these proteins, the 57-residue LP-Ser, has been determined[819] and confirms a previous report. A second protein, LP-Ala, has also been sequenced.[820, 821] Of the 79 residues, the first 41 were placed by automated Edman degradation and the remainder was completed by analysis of tryptic peptides. Carbohydrate was attached to a threonine residue in the sequence: Val-Arg-Pro-Thr-Ser-Ala- . . .. Interestingly, neither this sequence, nor that of LP-Gln 2, contains long stretches of hydrophobic residues which could interact with the lipid. This is in contrast to integral membrane proteins such as glycophorin where there is a defined amphipathic structure with an internal hydrophobic region thought to be important for protein–lipid interaction.

Myelin. Myelin from the central nervous system has a rather simple protein composition consisting of 30% of a basic protein and 70% proteolipid. In rats, two forms of the basic protein are found which differ in molecular weight by *ca.* 4000 daltons, and it has now been shown[822] that this is due to an internal

[816] M. Strickland, W. N. Strickland, W. F. Brandt, and C. Von Holt, *F.E.B.S. Letters*, 1974, **40**, 346.
[817] H. N. Baker, T. Delahunty, A. M. Gotto, and R. L. Jackson, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 3631.
[818] J. P. Segrest, R. L. Jackson, J. D. Morrisett, and A. M. Gotto, *F.E.B.S. Letters*, 1974, **38**, 247.
[819] R. L. Jackson, J. T. Sparrow, H. N. Baker, J. D. Morrisett, O. D. Taunton, and A. M. Gotto, *J. Biol. Chem.*, 1974, **249**, 5308.
[820] R. S. Shulman, P. N. Herbert, D. S. Fredrickson, K. Wehrly, and H. B. Brewer, *J. Biol. Chem.*, 1974, **249**, 4969.
[821] H. B. Brewer, R. S. Shulman, P. N. Herbert, R. Ronan, and K. Wehrly, *J. Biol. Chem.*, 1974, **249**, 4975.
[822] P. R. Dunkley and P. R. Carnegie, *Biochem. J.*, 1974, **141**, 243.

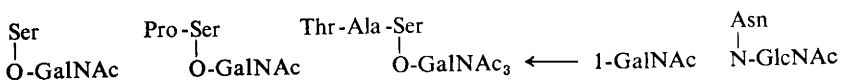
deletion of 40 residues in the smaller protein. The deletion appears to be between residues 120 and 160 of the 173-residue protein and possible mechanisms for its formation include unequal cross-over during synapsis or misrepair of DNA coding for the large protein after enzymic or mechanical breakage.

The sites of phosphorylation of the corresponding protein from bovine spinal cord have been determined.[823] Using an endogenous kinase, a serine residue (Ser*) in the sequence: Arg-Gly-Ser*-Gly-Lys, was predominantly labelled, whereas with an exogenous kinase from rabbit muscle, a serine in the sequence: Gly-Leu-Ser-Leu-Ser*-Arg, was phosphorylated. Finally, identical *N*-terminal sequences have been presented for one of the myelin lipoproteins, protein P7, from human [824] and rat [825] brain.

Cytochromes b. Cytochrome b_5 consists of a hydrophilic segment of molecular weight 11 000 daltons which contains the catalytic site and a hydrophobic segment (mol. wt. 6000 daltons) which binds the protein to endoplasmic membranes. The structures of several b_5 catalytic segments are known and preliminary data have now been presented for the membranous portion of the molecule.[826] Not surprisingly, a preponderance of hydrophobic amino-acids is found. Cytochrome b_5 appears to be homologous with cytochrome b_2 core,[827] a proteolytic haem-binding fragment of the enzyme, responsible for the oxidation of lactate to pyruvate. Not only are there sequence similarities but the b_2 core primary structure may be accommodated into the tertiary structure of the corresponding region of the microsomal b_5 protein. This would suggest a common ancestor.

Glycoproteins.—*Blood-group-specific Glycoproteins.* The major problem in determining the structures of these molecules, and of all glycoproteins carrying large amounts of carbohydrate, is the lack of a suitable method for removal of the sugar chains. The range of purified glycosidases is insufficient. For instance, no enzyme has yet been reported which will cleave the *O*-glycosidic linkage between *N*-acetylgalactosamine and a hydroxyamino-acid side-chain. Chemical methods invariably lead to either hydrolysis or rearrangement of the peptide chain.

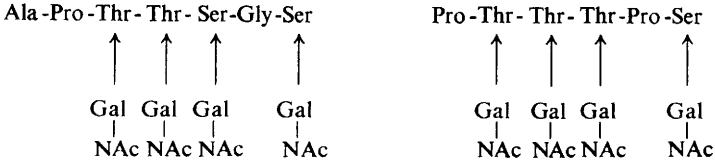
One approach has been to use a combination of the Smith degradation and enzymic hydrolysis by a mixed glycosidase preparation from *Clostridium.* Application to blood-group proteins from pig stomach lining has allowed the identification of the following peptides:[828]



GalNAc = *N*-acetylgalactosamine; GlcNAc = *N*-acetylgalactosamine

[823] P. R. Carnegie, P. R. Dunkley, B. E. Kemp, and A. W. Murray, *Nature*, 1974, **249**, 147.
[824] J. L. Nussbaum, J. F. Rouayrenc, J. Jollès, P. Jollès, and P. Mandel, *F.E.B.S. Letters*, 1974, **45**, 295.
[825] J. L. Nussbaum, J. F. Rouayrenc, P. Mandel, J. Jollès, and P. Jollès, *Biochem. Biophys. Res. Comm.*, 1974, **57**, 1240.
[826] J. Ozols, *Biochemistry*, 1974, **13**, 426.
[827] B. Guiard, O. Groudinsky, and F. Lederer, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 2539.
[828] N. K. Kochetkov, V. A. Derevitskaya, L. M. Likhosherstov, and S. A. Medvedev, *Biochem. Biophys. Res. Comm.*, 1974, **56**, 311.

A second approach is to digest the glycoprotein with pronase, incubate for 24 h in 0.25M-H_2SO_4 in glacial acetic acid at 60 °C, and then further hydrolyse in 0.2M-HCl for 24 h at 110 °C. Peptides of up to seven residues have been isolated from human blood-group-A specific protein using this method,[829] and the sequences are broadly similar to those determined from the hog protein. Certain of the sequences such as:



are incompatible with the proposed recognition sequence for glycosylating enzymes, namely, Thr-X-Pro.

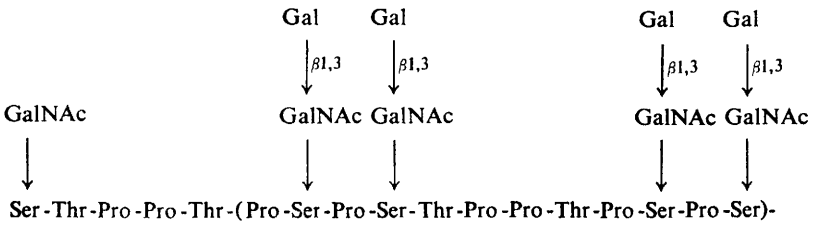
Fetuin. Exhaustive pronase digestion has also been used to prepare glycopeptides from 8 g of fetuin,[830] an α-globulin isolated from bovine serum. The peptides were fractionated by partition in 80% ethanol followed by conventional gel-filtration and ion-exchange procedures. The carbohydrate units were found to fall into two distinct classes. The more abundant Type A, representing at least 80% of the total hexose of fetuin, consisted of mannose (3—4 mol mol^{-1}), galactose (4—6 mol), *N*-acetylglucosamine (5—7 mol), and *N*-acetylneuraminic acid attached to the protein *via* an asparagine side-chain. These glycopeptides were insoluble in 80% ethanol. Type B glycopeptides contained galactose (2—5 mol), *N*-acetylgalactosamine (2—3 mol), and *N*-acetylneuraminic acid (0—3 mol) attached *O*-glycosidically to serine in a proline-rich region of the peptide chain.

Collagen. In all collagens so far investigated the carbohydrate units are linked *O*-glycosidically to hydroxylysine. Pronase digestion of bovine tracheal cartilage collagen [831] has yielded six glycopeptides of which five have the general structure Gly-X-Hyl-Gly-Y-Arg, generally found in collagen glycopeptides. However, in the sixth peptide, the arginine residue is replaced by hydroxyproline, hence the precise nature of the recognition site for the galactosyl-transferase remains to be determined.

Immunoglobulins. Human myeloma IgA contains both *N*- and *O*-glycosidically linked carbohydrate units, and the structures of both types have recently been published. Exhaustive digestion of 2—9 g of protein with pronase was followed by fractionation of the peptides by conventional column methods monitored by the phenol–sulphuric acid reaction. Carbohydrate residues were released by periodate oxidation and treatment with various glycosidases, and the released sugars were then identified by a combination of g.l.c. and mass spectrometry. The *N*-glycosidically linked carbohydrate was found to be attached to an

[829] S. D. Goodwin and W. M. Watkins, *European J. Biochem.*, 1974, **47**, 371.
[830] R. Begbie, *Biochim. Biophys. Acta*, 1974, **371**, 549.
[831] M. Isemura, T. Ikenaka, T. Mega, and Y. Matsushima, *Biochem. Biophys. Res. Comm.*, 1974, **57**, 751.

asparagine residue [832] whereas the *O*-glycosidically linked sugars were attached to serine residues [833, 834] in the sequence:



This correlates with the structure of the 'hinge' peptide previously isolated from IgA H-chains and it also preserves the symmetry of the repeating octapeptide sepuence.

Immunoglobulins.—By far the largest amount of sequence information published each year is on some aspect of immunoglobulin structure. Much of this work is concerned with delineation of sub-groups within species, determination of hypervariable regions, the positioning of genetic markers, *etc.*, and it is this class of work which is providing evidence on the mode of immunoglobulin evolution as well as on the generation of antibody diversity. Because of space limitations the bulk of this work is listed in Tables 5 and 6 below. Elucidation of the mechanism of antibody function has been aided by crystal structures for various antibody fragments, and the corresponding sequences from a human myeloma light(L)-chain [835] (2.8 Å map), human mcgλ L-chain [836] (3.5 Å map), and the heavy(H)-chain variable region of a phosphorylcholine binding myeloma protein, McPC 603 [837] (4.5 Å map) have now been published.

Heterogeneous Immunoglobulins. On the basis of antigenic properties and chemical structure, there are two classes of Ig L-chains, κ and λ. Each class appears to be coded by a small number of constant region genes (C-genes) plus a pool of variable region genes (V-genes) and it is the number of V-genes which is the current topic of controversy in immunology. The L-chains appear to overlap in function so that in species such as the mouse there are almost exclusively κ-chains whereas in the horse, λ-chains predominate. Since most of the λ-chains are acetylated at the *N*-terminus, automated Edman degradation of the Ig mixture from pooled horse serum has allowed [838] the major and minor residues present at each position of the κ-chain to be identified. This type of analysis requires very careful quantitation and in this case there was a further complication of *ca.* 20% of unblocked λ-chains. The proportion of κ-chains was found to be 7—8% of the total and a major sequence was assembled for the first 28 residues. This showed a number of unique residues when compared with human κ-chains

[832] J. P. J. Despont and C. A. Abel, *J. Immunol.*, 1974, **112**, 1623.
[833] J. Baenziger and S. Kornfeld, *J. Biol. Chem.*, 1974, **249**, 7260.
[834] J. Baenziger and S. Kornfeld, *J. Biol. Chem.*, 1974, **249**, 7270.
[835] B. L. Chen and R. J. Poljak, *Biochemistry*, 1974, **13**, 1295.
[836] J. W. Fett and H. F. Deutsch, *Biochemistry*, 1974. **13**, 4102.
[837] S. Rudikoff and M. Potter, *Biochemistry*, 1974, **13**, 4033.
[838] D. Gibson, *Biochemistry*, 1974, **13**, 2776.

and several positions in the sequence were clearly heterogeneous, suggesting that most of the horse κ-chains derive from a single ancestral sequence. Similar experiments have also been performed on avian H-chains.[839] This type of analysis can also yield clues to the nature of generation of antibody diversity and the number of genes encoding λ and κ L-chains. Assuming that levels of κ- and λ-chains reflect the relative number of germ-line V-genes for each type, and that, for example, these were only 20 L-chain V-region genes, then the minimum level for the minor type would be 5%, *i.e.* one gene. If the number of germ-line genes is indeed small, there should be a minimum level for the κ/λ ratio.

Homogeneous Immunoglobulins. Comparison of the V-region sequences of H- and L-chains from the same or related species has been used to estimate the time of divergence of the L- and H-chain genes. Analysis of the V-region from the H-chain of a rabbit antibody to type III pneumococcal polysaccharide[840,841] has shown that the homology between V-regions of H- and L-chains is no greater than that between this V_H region and other L-chain V-regions. *N*-terminal analysis of a related antibody from the nurse shark[842] leads to the similar conclusion that the V_H region is closer to mammalian V_H regions than to shark V_L sequences. This suggests that the L- and H-genes diverged before elasmobranchs diverged from mammals, that is at least 400 million years ago.

The origin of diversity in mouse myeloma H-chains has been investigated by *N*-terminal sequence analysis of 13 of these proteins.[843] Correlation with 15 other known H-chain sequences shows that the proteins fall into four major sets (these authors prefer the term 'set' to 'subgroup') and that the major set has at least five subsets. This indicates at least eight genes coding for the V-region of mouse H-chains. Furthermore, there is a distinct correlation between these sets and the hapten-binding activity of the individual chains (Figure 12). The complete primary structure of one of these H-chain V-regions, that of protein 315 which possesses anti-dnp binding activity, has been determined.[844] An identity of 33% was found with the L-chain V-region but since very few sequences of H- and L-chain V-regions are available the significance of this figure for ligand binding remains unclear. The 'switch' peptide, the constant region sequence immediately following the V-region, was found to be Val-Ser-Ser, which is identical with switch peptides previously found in human H-chains. However, a recent survey of four human IgM H-chains[845] has also revealed the switch sequence: Ile-Ser-Ser. This common dipeptide sequence, representing only six nucleotides, seems an insufficient recognition signal for the union of V- and C-genes.

The structures of the carbohydrate units of human IgA have been determined and are discussed in the glycoprotein section (p. 121).

[839] R. L. Wasserman, J. M. Kehoe, and J. P. Capra, *J. Immunol.*, 1974, **113**, 954.
[840] J.-C. Jaton and J. Haimovich, *Biochem. J.*, 1974, **139**, 281.
[841] J.-C. Jaton, *Biochem. J.*, 1974, **143**, 723.
[842] C. Sledge, L. W. Clem, and L. Hood, *J. Immunol.*, 1974, **112**, 941.
[843] P. Barstad, V. Farnsworth, M. Weigert, M. Cohn, and L. Hood, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 4096.
[844] S. H. Francis, R. G. Q. Leslie, L. Hood, and H. N. Eisen, *Proc. Nat. Acad. Sci. U.S.A.* 1974, **71**, 1123.
[845] G. Florent, D. Lehman, and F. W. Putnam, *Biochemistry*, 1974, **12**, 2482.

Mutant Immunoglobulins. Heavy-chain disease is a condition associated with the production of low molecular weight H-chains. The nature of the defect has now been characterized for a human IgA disease protein [846] and it appears that, following a short variable region, there is an internal deletion comprising the

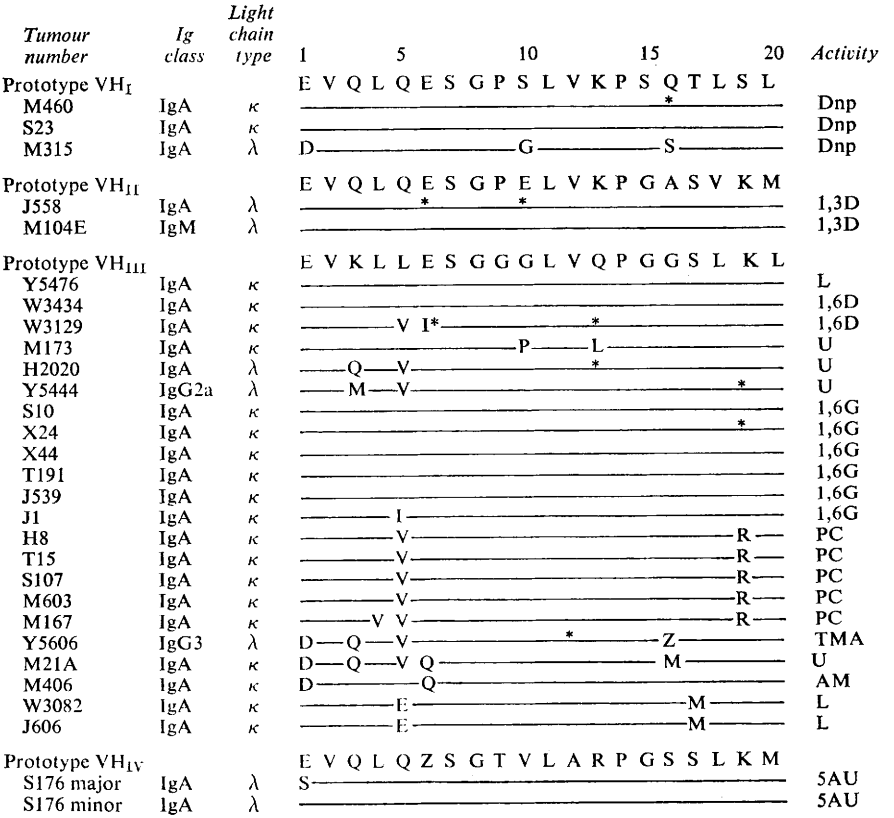


Figure 12 N-*terminal sequences of BALB/c heavy chains, using the one-letter code.* 1,3D *indicates* α-1 → 3 *dextran, and* 1,6D α-1 → 6 *dextran.* U *indicates unknown specificity,* L *is levan,* AM *is* N-*acetyl*-D-*mannosamine,* 1,6G *is* β-1 → 6 *galactan,* PC *is phosphorylcholine,* TMA *is trimethylamine, and* 5AU *is* 5-*acetyluracil. Asterisks mark regions of uncertainty about amide or residue assignment*

whole of the C_H1 domain. The remainder of the chain, immediately preceding the hinge peptide, is normal. There is also some *N*-terminal heterogeneity presumably arising from post-translational proteolysis. However, preliminary characterization of an abnormal mouse IgA chain has shown that, in this case,

[846] C. Wolfenstein-Todel, E. Mihaesco, and B. Frangione, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 974.

most of the deletion of *ca.* 100 residues occurs at the *C*-terminus of the protein.[847] In addition, there are as yet undefined differences in the hinge region.

Preliminary details have also been published on the inter-chain disulphide bonds of a μ H-chain disease protein.[848]

Complement Fixation. Whereas the variable region of the immunoglobulin is responsible for antigen binding, the constant region of the heavy chain carries out the binding of complement. The sequence of the major portion of a murine IgG C_H2 domain, which had previously been shown to be capable of fixing significant quantities of complement, has now been determined.[849] Unexpectedly, only 60% identity was found on comparison with the corresponding region from other mammalian IgG H-chains, a figure which is considerably lower than inter-species homologies for certain parts of the H-chain *variable* region. This would suggest that complement binding depends on a conformational feature of this region rather than on recognition of a specific sequence.

Complement.—The subcomponent, Cl_q, of the first component of complement has previously been shown to contain hydroxyproline and hydroxylysine, and to be susceptible to digestion with the enzyme collagenase. A recent report[850] shows that a region of the A-chain of Cl_q contains a 78-residue collagen-like repeating sequence of Gly-X-Y, where X is often proline and Y is often hydroxyproline. Further studies[851] have indicated that this type of sequence may also be present in the Cl_q B- and C-chains.

Hormones.—*Parathyroid and Proparathyroid Hormones.* Human parathyroid hormone contains 84 amino-acids and, following the publication of the sequence in 1972, synthetic studies were reported the following year. Unfortunately 1974 saw a new version[852] of part of the sequence which differs in three positions in the first 37 residues. The latest results were obtained by quantitative automatic degradation of 140 nmol of hormone using [^{35}S]PITC as coupling reagent. Histidine residues were identified by radioactive labelling with ^{125}I and detected by the release of radioactivity during the degradation. The discrepancies between the two reports involve three charge differences in nine residues so that synthetic analogues are likely to differ in immunoreactivity and possibly also in biological activity. Since there is widespread interest in the synthesis of this hormone for physiological and clinical studies it is important that these differences are rapidly resolved. A possible indication of which structure is correct may be found in the sequence of the porcine hormone[853] which is identical with the latest human sequence except for one substitution at position 16 (Asn → Ser).

The same group has also reported on the sequence of the precursor, pro-parathyroid hormone, from bovine and human species. In the case of the bovine

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prohormone[854] a hexapeptide, Lys-Ser-Val-Lys-Lys-Arg, was found to be attached to the *N*-terminus of the active molecule but since the molecular weight indicates about 20 extra amino-acids to be present; there may also be an additional sequence at the *C*-terminal end of the molecule. Alternatively, proteolysis during the isolation procedure may have resulted in the loss of part of the sequence.

Because the human precursor hormone was available in very limited quantities it was necessary to adopt a labelling technique *in vivo* based on the knowledge of the corresponding bovine sequence. Thus, human parathyroid adenomas were incubated in a medium containing [^{3}H]lysine and [^{14}C]arginine or [^{3}H]valine and [^{14}C]serine.[855] After degradation in the sequencer in the presence of myoglobin carrier, the radioactive fractions were identified by t.l.c. and a sequence identical with bovine proparathyroid hormone was derived. The utility of this *in vivo* labelling technique will be limited in mammalian systems by experimental difficulties such as isotope dilutions in whole animals or in tissue culture and by economic necessities where there is no homologous sequence and a large number of labelled amino-acids have to be used. In the present case only four types of residue were present and by the use of two radioactive labels only two incorporation experiments were required.

Prolactin. An alternative radioactive sequencing procedure has been used for determining the first 25 residues of human amniotic fluid prolactin.[856] In this case an initial coupling was performed with 0.5% [^{35}S]PITC followed by a second coupling with 5% unlabelled PITC. Hence, most of the radioactivity is incorporated into the protein, Using 1 mg of hormone its sequence was found to be identical with that of pituitary prolactin. The structure of bovine pituitary prolactin has also appeared.

Intestinal Peptides. The enzyme, kallikrein, has been used to cleave specifically an Arg-Lys bond in porcine vasoactive intestinal octacosapeptide. The complete structure[857] shows similarities with glucagon and secretin (Figure 13) and this may reflect the secretin-like action of this peptide on the exocrine pancreas as well as its glucagon-like action on blood sugar concentration. Homologies with other intestinal peptides are less convincing and may be assessed by reference to Figure 13.

Haemoglobin.—The characterization of innumerable haemoglobin mutants (see Table 5) and comparative sequence studies on the proteins isolated from diverse sources may not be of particular interest to workers outside the field. However, every year several papers appear on haemoglobin which are of considerable general interest from either a genetic or an evolutionary standpoint.

It is known that production of haemoglobin β-chains is preceded by the biosynthesis of γ-chains in foetal haemoglobin which has the structure $\alpha_2\gamma_2$. It has

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Polypeptide	*Amino-acid sequence*
Substance P	R P K P Q Q F F G L M ■
Glucagon	H S Q G T F T S D Y S K Y L D S R R A Q D F V Q W L M N T
Octacosapeptide	H S D A V F T D N Y T R L R K Q M A V K K Y L N S I L N ■
Secretin	H S D G T F T S E L S R L R D S A R L Q R L L Q G L V ■
Cholecystokinin-pancreozymin[a]	K A P S G R V S M I K N L Q S L D P S H R I S D R D Y M G W M D F ■
Motilin	F V P I F T Y G E L Q R M E E K E R N K G Q
Gastric inhibitory polypeptide	Y A E G T F I S D Y S I A M D K I R Q Q D F V N W L L A Q Q K G K K S D W K H N I T Q

[a] Tyrosine residue esterified with sulphuric acid

Figure 13 *Amino-acid sequences of substance P, glucagon, the vasoactive intestinal octacosapeptide, secretin, cholecystokinin-pancreozymin, motilin, and the gastric inhibitory polypeptide. The sequences are aligned in parallel starting with the* N-*terminal residues, except for substance P where the* C-*terminal residue coincides with that of secretin and cholecystokinin-pancreozymin where it coincides with that of the octacospeptide. Residues that are situated in the same position in two or more of the peptides are printed in bold face type. Substance P is of equine, all the other peptides of porcine origin*
(Reproduced by permission from *European J. Biochem.*, 1974, **42**, 588)

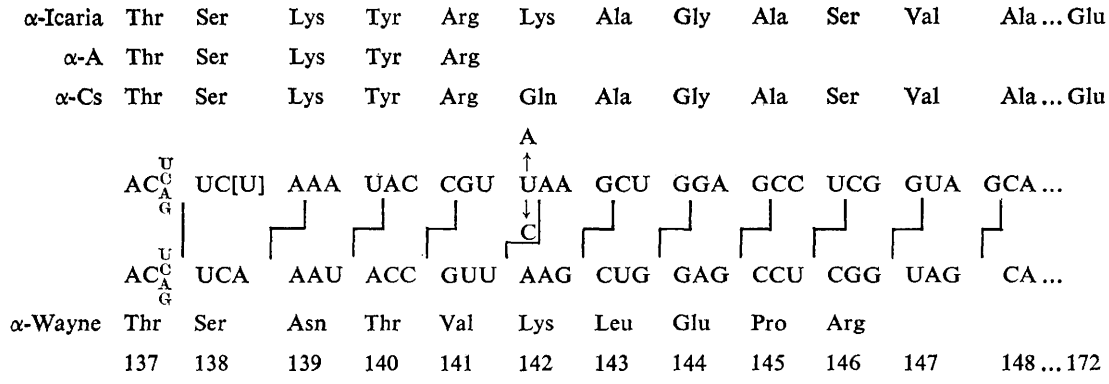

Figure 14 *Partial amino-acid and nucleotide sequences for α-chains of haemoglobins A, CS, Icaria, and Wayne. The base sequences for codons* 138 *and* 139 *have been chosen arbitrarily. Deletion of any one of the four bases before codon* 140 *produces the nucleotide sequence which defines the amino-acid sequence at the C-terminal end of the α-chain of Hb Wayne. In this diagram the third base of the codon for Ser-*138 *has been deleted; the base sequence of the α mRNA is thus uniquely defined from codon* 140 *to the second base of codon* 148
(Reproduced by permission from *Nature*, 1974, **251**, 246)

now been shown [858, 859] that the synthesis of α-chains is preceded during embryonic development by that of ξ-chains, and at some point during embryogenesis there is a gene switch from ξ- to α-chain synthesis. Since there is more sequence similarity between ξ-chains of different species than between ξ- and α-chains of the same species, there must have been an early divergence of their encoding genes, thus constituting a further example of globin gene duplication during evolution. It is possible that in α-thalassaemia, where no α-chains are produced,

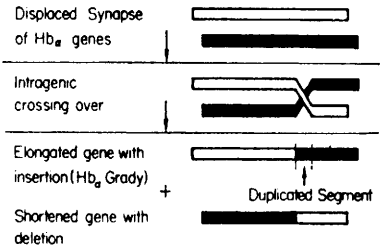

Figure 15 *Possible origin of the Hb_α Grady gene carrying a duplicated segment, and of a Hb_α gene with a deletion*
(Reproduced by permission from *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 3270)

there is a failure to switch from ξ- to α-chain production. Thus, tetramers of the type $\xi_2\gamma_2$ are produced which exhibit no co-operative oxygen-binding.

A chain-termination mutant, Hb Icaria, which also causes α-thalassaemia, has been characterized.[860] In this case the normal α-chain terminating codon (UAA) at position 42 has mutated into a glutamine codon (CAA) allowing read-through for an additional 30 residues. Comparison with other terminator and frameshift mutants allows a unique globin mRNA sequence of 26 nucleotides to be deduced (Figure 14).

Another haemoglobin, Hb Grady, has also been found to have an extended α-chain.[861] However, in this case the mutation was found to be a tandem intragenic duplication resulting in two copies of the sequence Glu-Phe-Thr, between residues 116 and 118. Assuming that there is one Hb_α locus on each chromosome it appears that the *N*-terminal segment (residues 11—118) of the elongated Hb_α gene originates from the Hb_α locus of one chromosome and the *C*-terminal segment (residues 116—141) from the Hb_α locus of the other (Figure 15). If the chromosome carries two repeated loci in tandem, the elongation could arise by mismatched intragenic cross-over between the unequally paired Hb_α loci of such a repetition.

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Table 5 *Amino-acid sequence completed in* 1974

Protein	*Source*	*No. of residues*	*Notes*	*Ref.*
α_1-Acid glycoprotein	human plasma	181	S–S bridges, Cys 5-147, Cys 72-164	862
Adenylate kinase	pig	194	tertiary structure	865
Adrenocorticotrophic hormone	sheep	—	revision, Asp_{25} → Asn	863
Adrenocorticotrophic hormone	dogfish	39		864
Aldolase	rabbit muscle	364	remarkable differences between these structures	866
Aldolase	rabbit muscle			867
Amyloid, A.S.	human liver	76	non-Ig type sequence	868
Apolipoprotein, Ala	human plasma	79	see text	820, 821
Apolipoprotein, Gln-1	human plasma	245	see text	817
Apolipoprotein, Ser	human plasma	57	see text	819
Apovitellenin I	emu egg yolk	84	lipoprotein	869
Arginine kinase	lobster muscle		tertiary structure	870
L-Asparaginase	*E. coli*	321		871
Aspartate aminotransferase	pig heart	412	confirmation	872
Carbonic anhydrase CI, C11	bovine erythrocytes		isozymes, Arg_{56} → Gln	873
Carbonic anhydrase CI	bovine erythrocytes	261		874
Carbonic anhydrase B	human erythrocytes	260		874, 875
Carbonic anhydrase C	human erythrocytes	261		874
Carbonic anhydrase C	human erythrocytes	259		876
Carbonic anhydrase C	ovine erythrocytes	259		877
β-Casein, E variant	bovine	—	Glu_{36} → Lys	878
κA-Casein	sheep	171		879
Chymotryptic inhibitor I	potato	84—85	apparent internal repetition, four subunits	880, 881
Colipase II	pig pancreas	84	three S–S bridges placed	882, 883
α-Crystallin	bovine	175	αB_2 chain	884
Cytochrome *c*	box-elder	112		885
Cytochrome *c*	elder	111		885
Cytochrome *c*	nasturtium	111		885
Cytochrome	ostrich			886
Cytochrome *c*	parsnip	111		885
Cytochrome *c*	potato	111		887

Cytochrome c_3	*Desulphovibrio vulgaris*	107		888
Cytochrome c_{551}	*Pseudomonas aeruginosa*	82	nine strains	889
Cytochrome c_{551}	*Pseudomonas fluorescens*	82	six strains	889
Cytochrome c_{552}	*Euglena gracilis*	87		890
Iso-1-cytochrome *c*	yeast		missense mutants	891
Deoxyribonuclease *D*			identical with C + 2 carbohydrate units	892

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Table 5 (*cont.*)

Protein	*Source*	*No. of residues*	*Notes*	*Ref.*
Dihydrofolate reductase	*E. coli* B	156	methotrexate-resistant strain, see text	777
Dihydrofolate reductase	*Streptococcus faecium*	163	methotrexate-resistant strain, see text	778
Gene 5 protein	phage fd	88	DNA-binding protein	893
Gene 5 protein	phage M13	87	identical to fd protein	894
Ferredoxin	*Chlorobium limicola*	60	no Met, Phe, Trp, Lys, His, Arg	895
Ferredoxin	*Clostridium* M-E	55	lacks aromatic residue at position 30	896
Flavodoxin	*Clostridium* MP	138	1.9 Å map	897
Flavodoxin	*P. elsdenii*		revision, Leu → Gly	898
Glutamate dehydrogenase	*Neurospora crassa*	452	NADP-specific enzyme, see text	780
	echidna		Hb 1-A	899
Haemoglobin	kidney bean	145	leghaemoglobin	900
Haemoglobin	opossum	141	α-chain	901
Haemoglobin	rabbit	—	revision; β_{112}, Ile → Val	902
Haemoglobin	viper	141	α-chain	903
Haemoglobin mutants				
Hb Abruzzo	human	—	β_{143}, His → Arg, increased O_2 affinity	904
Hb Ankara	human	—	β_{10}, Ala → Asp, neutral	905
Hb F Auckland	human	—	$G\gamma_7$, Asp → Asn	906
Hb J Birmingham	human	—	α_{120}, Ala → Glu, neutral	907
Hb J Buda	human	—	α_6, Lys → Asn	908
Hb F Fort Royal	human	—	$\alpha_2{}^G\gamma_2$, Glu_{125} → Ala	909
Hb Grady	human	144	three-residue insertion in α-chain, see text	861
Hb J Habana	human	—	α_{71}, Ala → Glu, neutral	910
Hb Icaria	human	172	α_{142} → Lys, elongated α-chain, see text	860
Hb Lyon	human	—	β_{17} Lys and β_{18} Val deleted	911
Hb F Malaysia	human	—	$\alpha_2\gamma_2$, Gly-Cys; 136, Gly	912
Hb J Meerut	human	—	α_{120}, Ala-Glu	913
Hb A_2 Melbourne	human	—	$\alpha_2\delta_2$, Glu_{43} → Lys, neutral	914
Hb Moscva	human	—	β_{24}, Gly → Asp, unstable	915
Hb A_2NYU	human	—	$\alpha_2\delta_2$, Asn_{12} → Lys	916
Hb Ottawa	human	—	α_{15}, Gly → Arg, neutral	917
Hb G-Pest	human	—	α_{72}, Asp → Asn	908

Hb J Rovigo	human	—	α_{53}, Ala → Asp, unstable	918
Hb St. Claude Or	human	—	α_{127}, Lys → Thr, neutral	919
Hb San Diego	human	—	β_{109}, Val → Met, causes erythrocytosis	920
Hb Siam	human	—	α_{15}, Gly → Arg	921
Hb J Sicilia	human	—	β_{65}, Lys → Asn, neutral	922
High potential Fe–S protein	*Thiocapsa pfennigii*	81		923

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Table 5 (*cont.*)

Protein	*Source*	*No. of residues*	*Notes*	*Ref.*
Histone $f2a_2(IIb_1)$	calf thymus	129	homologies with histone f_{2a}(IV), see text	812
Histone f3	chicken erythrocytes	135	one substitution relative to calf thymus, $Ser_{96} \rightarrow Cys$, see text	814
Histone f3	shark erythrocytes	135	identical with chicken, see text	815
Ig light chain	human	211	λ-type, sub-group IV, protein Bau	924
Ig light chain	human	213	λ-type, new sub-group, protein Del	925
Ig light chain	human	220	κ-type, sub-group IV, protein Len	926
Ig light chain	human		2.8 Å map, protein IgG New, see text	835
Ig light chain	rabbit	210	Vκ1 sub-group allotype b4	927
Ig light chain	human	216	3.5 Å map, λ-type, see text	836
Insulin	hagfish	21-A 31-B	A and B chains sequenced	928
Insulin C	guinea-pig	29		929
Keratin	silver gull feather	98		930
Malformin A	*Aspergillus niger*	5	cyclic pentapeptide	931
Melanocyte-stimulating hormone	dogfish	16 = β	α and β forms	932
Myelin basic protein	rat brain		see text	822
Myoglobin	badger	153		933
Myoglobin	california sealion			934
Myoglobin	chicken			935
Myoglobin	horse		revision, $Asp_{122} \rightarrow Asn$	936
Myoglobin	human		skeletal and heart muscle forms identical	937
Myoglobin	sperm whale		revision, $Asp_{122} \rightarrow Asn$	936
Myoglobin	tree-shrew			938
Myosin, light chain A1	rabbit muscle	190	see text	765
Myosin, light chain A2	rabbit muscle	149	*C*-terminal 141 residues identical with A1, see text	765, 766
Neocarzinostatin	*Streptomyces carzinostaticus*	109		939
Neurophysin II	bovine	97	seven S–S bridges, internal duplication	940
Parathyroid hormone	pig	84	see text	853
Pepsin	hog	326	revised version	941
Phospholipase A_2-II	*Agkistrodon halys blomhoffii*	127	see text	804

Phospholipase A_2	bee venom	128	four S–S bridges placed, see text	803
Phospholipase A	gaboon adder	118	six S–S bridges, see text	805
Plastocyanin	broad bean	99		942
Plastocyanin	*Chlorella fusca*	98	sequence around Cys similar to azurin	943
Plastocyanin	elder	99		944
Plastocyanin	french bean	99		945
Plastocyanin	potato leaves	99		946
Plastocyanin	vegetable marrow	99		947
Prolactin	bovine	199		948
Protease A	*Streptomyces griseus*	182	pronase component, see text	801
Protease B	*Streptomyces griseus*	242	pronase component, see text	802

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Table 5 (*cont.*)

Protein	*Source*	*No. of residues*	*Notes*	*Ref.*
Protease inhibitor	*Streptomyces albogriseolus*	113	inhibits microbial alkaline proteases	949
Putidaredoxin	*Pseudomonas putida*	106	Fe–S protein	950
Red pigment-concentrating hormone	shrimp	8	confirmation	951
Ribitol dehydrogenase	*Klebsiella aerogenes*	249	mainly by mass spectrometry	
Ribonuclease	giraffe	124		952
Ribonuclease	goat	124		953
Ribonuclease	horse	125	carbohydrate sites identified, residue 39 deleted	954
Ribonuclease	sheep	124	identical with goat	953
Ribosomal protein S8	*E. coli*	109	16S RNA binding protein	955
Superoxide dismutase	bovine erythrocytes	151	S–S bridge between Cys 55-144, see text	784
Tentoxin	*Alternaria tenuis* Nees	4	cyclic tetrapeptide alternative structure	956
Toxin components (arranged in alphabetical order of species)				
Toxin II	*Androctonus australis* Hector	—	S–S bridges (scorpion)	957
Venom peptide	*Askistrodon halys blomhoffi*	3		958
Neurotoxin variants	*Centruroides sculpturatus* Ewing	66	four S–S bridges (scorpion)	959
Toxin TA2	*Dendroaspis angusticeps*	60	four S–S bridges, non-toxic (green mamba)	960
Venom protein	*Dendroaspis viridis*	60	four S–S bridges, low toxicity (green mamba)	961
Neurotoxic protein 1, 2	*Dendroaspis viridis*	72	five S–S bridges	962
Neurotoxic protein 1, 2	*Dendroaspis viridis*	60		962
Neurotoxin	*Laticauda semifasciata* III	66	five S–S bridges (sea snake)	963
Venom protein $V^{II}1$	*Naja melanoleuca*	60	cytotoxin (forest cobra)	964
Venom proteins $V^{II}2$ and 3	*Naja melanoleuca*	61	four S–S bridges, cytotoxins	965
Neurotoxin	*Naja melanoleuca*	71		966
Cytotoxins $V^{II}1$, 2, and 3	*Naja mossambica mossambica*	60		967
Cytotoxin $V^{II}4$	*Naja mossambica mossambica*	60	four S–S bridges	968
Cytotoxin	*Naja naja oxiana*	60	(middle Asian cobra)	969
Neurotoxin α	*Naja naja oxiana*	61	four S–S bridges	970
Neurotoxin 1	*Naja naja oxiana*	73	five S–S bridges	971
Neurotoxin	*Naja naja phillippinensis*	61		972
Proteinase inhibitor II	*Vipera ruselli*	60	three S–S bridges, 50% homologous with trypsin inhibitor	973, 974

Triose phosphate isomerase	coelacanth	247	see text	776
Vasoactive octacosapeptide	porcine intestine	28	see text	857

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Table 6 *Partial amino-acid sequences published in* 1974

Protein	*Source*	*No. of residues*	*Notes*	*Ref.*
A-1 protein	chicken	9	FSWGGEGHK, encephalitogenic-inducing peptide	975
Adenovirus II hexon	adenovirus	4	Ac-ATPS, *N*-terminal peptide	976
Adenovirus II hexon	adenovirus	97	six SH sequences, sub-units probably identical	977
Albumin	bovine serum	96	residues 87—183	978
Albumin	dog serum	24	residues 1—24, lacks Cu-binding His-3	979
Albumin	rat serum	13	precursor protein with additional five residues at *N*-terminus	980
D-Amino-acid oxidase	—	21	*N*-terminal	981
α-Amylase	*B. subtilis*	12	VNGTLMQYFEWY	982
Amyloid fibrils	guinea-pig	20	*N*-terminal, non-Ig protein	983
Anthranilate synthetase	*E. coli*	55	*N*-terminal, see text	772, 773
Anthranilate synthetase	*Salmonella typhimurium*	55	*N*-terminal, see text	772, 773
Anthranilate synthetase	*Serratia marcescens*	55	*N*-terminal, see text P |	772, 773
Aspartate aminotransferase	chicken heart	4	S-K-N-F, pyridoxal phosphate peptide	985
Aspartate aminotransferase	pig heart mitochondria	303 (70%)	homologous with heart enzyme	986
Assembly protein	phage MS2	54		984
Blood group protein	pig stomach lining	7	glycopeptides, see text	828
Blood group protein	human	62	glycopeptides, see text	829
Carbonic anhydrase	dog erythrocytes	6	Ac-SSSSNW, *N*-terminal peptide	987
Carboxypeptidase B	bovine	85	six SH peptides	988
para-κA-casein	sheep	108	*N*-terminal	989
Collagen	bovine tracheal cartilage	43	hydroxylysyl and glycopeptides, see text	831
Collagen, α1(11) chain	bovine cartilage	75	hydroxylysyl sequences	990
Collagen	calf skin	19	Glp-FDAKGGGPGPMGLMGPAG, *N*-terminal, see text	756
Collagen, α2-CB2	calf skin	30	see text	758
Collagen, α2-CB3.5	calf skin	36	see text	757

Collagen, α2-CB2	human, rabbit skin	22	see text	758
Collagen, α1(111) chain	human skin	—	CNBr fragments purified, see text	760
Collagen, α1 chain	pig skin	20		991
Collagen, α2 chain	pig skin	16	Pca-YDGKGVAGPGPMGLM	991
Collagen, α2-CB2	pig skin	26	see text	758
Collagen, α1-CB3	rat skin	149	five differences relative to calf skin	992
Collagen, α2-CB3	rat skin	48	see text	757
Collagen	sea anemone	—	CNBr fragments, see text	759
Collagen cross-links	bovine	'4'	lysinonorleucine derivatives, see text	761
Creatine kinase	chicken muscle	13	SH peptide from normal and dystrophic muscle	993
α-Crystallin, αB_2 chain	bovine	68	*N*-terminal	994
Cytochrome b_2	yeast	95	homologous to cytochrome b_5, see text	827
Cytochrome b_5	bovine, equine, porcine liver	28	*N*- and *C*-terminal sequences, see text	826
Cytochrome *c*	*Ceratitis capitata*	41	*N*-terminal, identical with *Drosophila*	995

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Table 6 (*cont.*)

Protein	*Source*	*No. of residues*	*Notes*	*Ref.*
Cytochrome *c*	*Humicola lanuginosa*	6	corrections	996
Cytochrome *c*	*Neurospora crassa*	43	corrections	996
Cytochrome c_2	*Rhodopseudomonas palustris*	8	KQCMTCHR, haem peptide	997
Cytochrome c_{552}	*Chromatium*	7	flavin peptides; YTCY, TCY	998
Dihydrofolate reductase	*Lactobacillus caseii*	105	elastase peptides, see text	779
DNA polymerase I	*E. coli*	15	MVZIPZGB *N*-terminal and two fragments	999
Elastin	bovine	43+	desmosine cross-linked peptides, see text	763
Elastin	porcine		desmosine cross-linked peptides, see text	762
Elongation factor 2	rat liver	15	^{14}C-ADP labelled peptide, see text	807
Envelope protein	*E. coli*	8	*N*-terminal	1000
UDP-Gal.4-epimerase	*E. coli*	28	*N*-terminal, correlates with mRNA sequence	1001
Fd coat protein	phage Fd	7	YAWAMVV, correction	1002
Ferredoxin	*Aphanothece sacrum* Okada	58	*N* + *C*-terminal	1003
Ferredoxin	*E. coli*	7	PKIVILY, *N*-terminal	1004
Fetuin	foetal bovine serum	27	glycopeptides, see text	830
Fibrinogen	dog	35	see text	793
Flagellin	*Salmonella paratyphi* BSL167	22	five residues differ from strain BSL877	1005
Flavodoxin	*Azotobacter vinelandii*	46	*N*-terminal, includes FMNP binding site	1006
Flavodoxin	*Rhodospirillum rubrum*	38	*N*-terminal, includes FMNP binding site	1006
α_2-Gliadin	wheat	25	*N*-terminal	1007
β-Glucanase	*Fungi imperfecti*	4	DAGS, *C*-terminal	1008
β-Glucosidase	*Aspergillus wentii*	16	'active centre' peptide	1009
Glutamate dehydrogenase	*Neurospora crassa*	47	NAD-specific enzyme, ^{14}C-NEM peptides, see text	782
Growth hormone	bovine		corrections	1010
Haemoglobin	beaver			1011
Haemoglobin	bullfrog	105	β-chain	1012
Haemoglobin	chicken	α-43 β-42	component P1	1013
Haemoglobin	coelacanth	15	VLWAADERAIITIVY	1014

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Haemoglobin	human		embryonic ξ-chain	
Haemoglobin	*Macaca nemestrina*		β-chain	1015
Haemoglobin	mouse		embryonic ξ-chain, see text	859
Haemoglobin	rabbit		embryonic ξ-chain, see text	859
Haemoglobin	rat	17	three SH peptides	1016
Haemoglobin	viper	58	residues 35—92, α-chain	1017
Haptoglobin	human	171	β-chain	1018
Histidine ammonia-lyase	*P. testoteroni*	17	'active-centre' peptide	1019
Histidinol dehydrogenase	*Salmonella*	59	frameshift mutants	1020
Histone $F2a_1(IV)$	sea-urchin	52	Cys peptide, $Thr_{73} \rightarrow Cys$, see text	816
Histone 1 (lysine-rich)	rabbit thymus	46	residues 72—107, see text	811

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Table 6 (*cont.*)

Protein	*Source*	*No. of residues*	*Notes*	*Ref.*
Immunoglobulins				
Cryoglobulin	human		κ- and λ-type, sub-group $V\kappa_1$ and $V\lambda_{III}$	1021
L-Chain	horse	28	κ-chains, see text	838
L-Chain	human myeloma Car		κ_{1b} sub-group V-region + part of C	1022
L-Chain	human, Bence-Jones Cro	6	genetic markers, κ-chain	1023
L-Chain	human, Bence-Jones Scw		κ-type	1024
L-Chain	human, Bence-Jones Vor	216	λ-type; V-region and part of C	1025
L-Chain	various animals	5	RFSGS pentapeptide, restricted sequence	1026
L-Chain	human IgM (17 proteins)		κ-chains	1027
L-Chain	rabbit	139	*N*-terminal	1028, 1029
L-Chain	rabbit	23	four SH peptides, λ-chains	1030
H-Chain	avian (5 species)	24	*N*-terminal, VH_{III} sub-group, see text	839
H-Chain	guinea-pig	93	IgG_2C_H2 domain	1031
H-Chain	guinea-pig	85	IgG_2C_H3 domain	1032
H-Chain	human (5)		V-regions, VH_{III} sub-group IgA + IgG	1033
H-Chain	human myeloma	5	IgA, glycopeptides, see text	833, 834
H-Chain	human myeloma	38	IgA, glycopeptides, see text	832
H-Chain	human	155	IgE, $C_\varepsilon 3$ domain, ε-chain	1034
H-Chain	human		IgM, Fcμ fragment	1035
H-Chain	human	121	IgM, V-region	1036
H-Chain	human		IgM switch points, see text	845
H-Chain	rabbit	48	*N*-terminal, anti-pneumococcal antibody, see text	840
H-Chain	mouse myeloma 315	156	IgA, V-region, see text	844
H-Chain	mouse myeloma (13 chains)	20	*N*-terminal, see text	843
H-Chain	mouse	62	IgG_{2a} complement fixing peptide, see text	849
H-Chain	mouse	212	IgG_{2a} Fc fragment	1037
H-Chain	myeloma McPC 603	120	V-region, 4.5 Å map, see text	837
H-Chain	rabbit	47	V-region, anti-pneumococcal a.b., see text	841
H-Chain	human, Def		IgA internal deletions, see text	846
H-Chain	mouse myeloma MOPC 47A		IgA, *C*-terminal deletion, see text	847
H-Chain		64	S–S bridged peptides, see text	848

Bridge peptide	human myeloma		IgA_2	1038
L and H chains	human myeloma	30-L, 5-H	IgA_1, IgG_2, λ-type L-chains	1039
L and H chains	human myeloma	26-L, 50-H	IgG_1, κ-type, $V\kappa_1$ and VH_{III} sub-groups	1040
L and H chains	shark	28-L, 28-H	see text	842
Hapten-binding regions	guinea-pig	83-H	IgG_2, three different antibodies	1041
Hapten-binding peptide	mouse myeloma	7	IgA_2, VLSG(E,A)CRP	1042
J-chain	human	13	*C*-terminal CNBr fragment	1043
Inorganic pyrophosphatase	yeast	50	*N*-terminal	1044
Isocitrate dehydrogenase	*Azetobacter vinelandii*	30	'active-site' peptide	1045

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Table 6 (*cont.*)

Protein	*Source*	*No. of residues*	*Notes*	*Ref.*
Lactoferrin		84	SH and *C*-terminal peptides	1046
Lactotransferrin	human	—	CNBr fragments	1047
Lactotransferrin	human	27	glycopeptides	1048
Lipoamide dehydrogenase	pig heart	23	'active-centre' peptide	1049
Lipoamide dehydrogenase	pig heart	15	SH peptides	1050
Luteinizing hormone	human	19	*N*-terminal, β-subunit	1051
Lysozyme	phage T4	1	cold-sensitive phenotype, $Tyr_{88} \rightarrow$ His	1052
Malate dehydrogenase	pig	9	VSVPIHGG(V, A, G)K, 'active-centre' peptide	1053
Motilin	pig	1	correction, $Glu_{14} \rightarrow$ Gln	1054
Myelin P7	rat brain	20	*N*-terminal, P7 apoprotein, see text	825
Myelin P7	human	20	*N*-terminal, identical with rat brain, see text	824
Myelin			phosphorylation sites in basic protein, see text	823
Myosin	rabbit muscle	3	CNG, see text	764
Neurophysin I	human	54	*N*-terminal, 89% identity with bovine	1055
Nucleoside diphosphate kinase	pea seed	13	AIBIGRBVIHGSB, phosphorylated peptide	1056
Ovotransferrin	chicken egg white		CNBr fragments	1057
Parathyroid hormone	human	37	*N*-terminal, see text	852
Proparathyroid hormone	bovine	6	KSVKKR, *N*-terminal, see text	854
Proparathyroid hormone	human	6	KSVKKR, see text	855
Pepsin	hog	55		1058
Pepsinogen	bovine	45		1059
C-Phycocyanin	*Synechococcus* sp. (*Anacystis nidulans*)	α-14 β-19	*N*-terminal, α- and β-subunits	1060
Plasminogen	human	20	activation system, see text	799
Polyhedral protein	polyhedrosis virus (*B. mori*)	77	tryptic peptides	1061
Polyhedral protein	polyhedrosis virus (*B. bombycis*)	130	chymotryptic peptides	1062

Prealbumin	human plasma	127		1063
Proelastase	African lungfish	A-27, B-44	*N*-terminal	1064
Prolactin	human amniotic fluid	25	*N*-terminal, see text	856
			P |	
Pyruvate kinase	pig liver	6	LRRASL, phosphopeptide	1065
Rennin	bovine	10	DTGTSL, DTGSS, 'active-centre' peptides	1066
RNA polymerase	T4-infected *E. coli*	3	TVR, peptide labelled by ADP ribose	1067
RNA synthesis stimulating peptide	red kidney beans	50	tryptic peptides	1068
Ribonuclease	whale pancreas	108		1069
Ribonuclease U_1	—	101	60% different from RNase T_1	1070

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Table 5 (*cont.*)

Protein	*Source*	*No. of residues*	*Notes*	*Ref.*
Ribosomal protein L7, L12	*B. stearothermophilus*	15	*N*-terminal	1071
Ribosomal protein	*B. stearothermophilus*			1072
Ribosomal protein	*E. coli*	15	*N*-terminal	1072
Ribosomal protein L20	*Halobacterium cutirubrum*	45		1073
Secretory component	human	16	KSPIFGPEEVDSVEGG, *N*-terminal	1074
SV40 capsid	simian virus	7	APTKRKG, *N*-terminal	1075
Streptokinase	human	12	native and 'altered' forms, see text	800, 800*a*
Superoxide dismutase	*B. stearothermophilus*	60	*N*-terminal	1076
Testis basic protein	rat testis	23	54-residue protein, no E, Q, C, I, W, F	1077
Thrombin	human	36	A-chain, see text	787, 1078
Prothrombin	human	>42	contains γ-Glu, see text	798, 786
Factor IX	bovine	35	20 *N*-terminal, 15 around active site serine	786
Factor X	bovine	35	20 *N*-terminal, 15 around active site serine	786
Factor XIII	human and bovine	37	see text	791, 792
Factor IXa	bovine	15	*N*-terminal (H-chain), see text	787
Factor Xa	bovine	15	*N*-terminal (H-chain), see text	787
Transferrin	baboon, bovine	—	CNBr fragments	1079
Transferrin	human serum	57	two CNBr fragments	1080, 1081
Triose phosphate isomerase	chicken muscle	15	VVLAYEPVWAIGTGK, 'active-site' peptide	1082
Triose phosphate isomerase	chicken muscle	~250	tryptic peptides, see text	775
Triose phosphate isomerase	rabbit muscle	~250	tryptic peptides, see text	774
tRNA synthetase-Ile	*E. coli*	15	IESMVADRPNWCISR	1083
tRNA synthetase-Leu, Met, Val	*B. stearothermophilus*		see text	810
tRNA synthetase-Leu, Ser	*E. coli*	—	see text	808
tRNA synthetase-Met	*E. coli*		see text	809
Trypsinogen	elephant seal	8	FPTDDDDK, *N*-terminal	1084
Toxin	cholera	20	*N*-terminal, two subunit types	1085
Toxin	*Naja nigricollis mossambica* Peters	37—46	three toxins, *N*- and *C*-terminal	1086
Troponin	rabbit muscle	50	phosphorylation sites, see text	770, 771
Troponin	rabbit muscle	—	CNBr fragments, see text	769
Type C viral antigens	mouse	24	*N*-terminal, five strains	1087

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

1 Introduction

This has been a year of consolidation. Relatively few completely new protein structures have been reported but several structures have been improved and refined and the emphasis has been on the comparison of different structures and a description of their active sites.

On the theoretical side very accurate refinements of protein structure have been carried out showing that it is better to use phases calculated from approximate atomic positions rather than experimental phases obtained by the multiple isomorphous replacement technique when attempting to refine the structure. More work has been done on the extension of the resolution of a protein structure when only the native intensities are available for the higher resolution data. The use of non-crystallographic symmetry in the molecular replacement method for the determination of protein structure has been further developed.

Work on antibodies has continued and the binding of small haptens to antibody fragments has been studied in detail. Comparisons of the structures of dehydrogenases and kinases have highlighted the great similarities between the coenzyme binding portions of these structures. It seems reasonable to assume that a relatively large number of enzymes could have developed from a small number of basic, fairly non-specific enzymes, probably by a process of gene duplication and subsequent divergence which could have led to the great diversity of function which is found today. On this basis it should be possible to group enzymes into a number of families, each derived from a common ancestor and having elements of tertiary structure in common, since a three-dimensional stable structure might be expected to change more slowly than an amino-acid sequence. The existence of a common nucleotide binding structure found in the dehydrogenases and kinases would suggest the evolution of these structures from a common nucleotide binding protein. It should be possible to establish evolutionary relationships by making sequence comparisons in the light of tertiary structural information. The alternative explanation is that the observed similarities could be due to a combination of convergent evolution and a limitation on the number of ways of forming certain super-secondary structures.

Considerable progress is being made in the determination of the structures of complex proteins and viruses, including the structures of some of the filamentous bacterial viruses.

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

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

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

2 Data Collection and Protein-structure Analysis

Krieger *et al.*[1] have discussed ways of correcting for the variation of background as a function of the different setting angles involved in collecting data on a diffractometer. Interest in the collection and processing of photographic data continues to increase, and Ford[2] has shown that a method of intensity determination using a profile-fitting algorithm applied to precession photographs reduces the likely random error in intensity measurements. Milch and Minor[3] have discussed the indexing of single-crystal *X*-ray rotation photographs using rotation photographs from crystals of lysozyme as an example.

Holmes and Barrington Leigh[4] have considered the problem of data collection from non-crystalline fibres. They have shown that, provided the layer line-width is small, a correction factor can be derived which, when applied to the intensity function in reciprocal space for a non-crystalline fibre, yields the intensity which would be obtained from a perfectly oriented fibre. The general problem can be treated by a numerical deconvolution procedure. Stubbs[5] has calculated such a correction factor and applied it to data obtained from tobacco mosaic virus gels.

Matthews[6] has suggested a new method for the determination of molecular weight from protein crystals. He reviews current methods, emphasizes the importance of allowing for bound water, and suggests a method based on determining the protein content of a known mass of wet crystals. The density of the crystals must be measured as well. Assuming that a crystal consists of protein, free solvent, and bound water, then if m_m is the mass of protein per unit mass of wet crystal, D_c is the density of the crystal, and V is the volume of the unit cell then

$$\text{mol. wt.} = \frac{NVm_m D_c}{n}$$

More workers are applying direct methods to the analysis of isomorphous heavy-atom derivatives. Such techniques are particularly useful when partial substitution occurs at multiple sites and direct interpretation of the difference Patterson synthesis is difficult. Navia and Sigler[7] have applied the tangent formula in three dimensions to the E_Δ's, the normalized moduli of the differences between the scaled amplitudes of the derivative and parent structure factors. A full three-dimensional set of starting phases was computed from trial heavy-atom constellations derived from symbolic addition analysis of the centrosymmetric axial projections. They define E_Δ by

$$E_\Delta^2 = (\Delta F)^2 \Big/ \left(\varepsilon^2 \sum_{j=1}^{N} f_j^2\right)$$

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

Compound	*Space group*	*a*/Å	*b*/Å	*c*/Å	α	β	γ	*Z*	*R*	*Ref.*
N-Ac-L-Tyr-methylamide [a]	$P4_1$	8.588		17.221				4	0.041	*b*
Calcium L-Glu-trihydrate [c]	$P3_121$	8.863		20.863				6	0.021	*d*
N-Ac-Pro-lactyl-methylamide [a]	$P2_12_12_1$	22.58	8.45	6.49				4	0.082	*e*
N-acetyl-DL-Ala-*N*-methylamide [f]	$I4_1/a$	13.81		16.50				16	0.072	*g*
N-acetyl-L-Ala-*N*-methylamide [f]	$P2_12_12_1$	13.87	6.98	16.29				8	0.12	*g*
Tryptamine picrate [h]	$P2_1/c$	15.93	6.91	21.84		133.88		4	0.056	*i*
DL-Tryptamine picrate methanol [h]	$P\bar{1}$	11.73	11.55	7.97	100.34	81.31	97.98	2	0.088	*i*
N-(t-Butyloxy carbonyl)-L-Pro	$P2_12_12_1$	12.11	8.15	12.10				4	0.05	*j*
N-Ac-L-norvaline	$P2_12_12_1$	5.81	9.96	16.18				4	0.113	*k*
DL-His	$P2_1/c$	8.98	8.09	9.42		97.65		4	0.13	*l*
Carboxobenzoxy-L-Leu-*p*-nitrophenyl ester	$P2_12_12_1$	8.49	9.40	25.21				4	0.105	*m*
N-Ac-L-Trp-methyl ester	$P2_12_12_1$	14.12	11.23	8.53				4	0.041	*n*
DL-Ser	$P2_1/a$	10.74	9.15	4.83		106.42		4	0.032	*o*
Anhydrous L-Ser	$P2_12_12_1$	8.60	9.35	5.62				4	0.059	*o*
Ac-Gly-*N*-methylamide	$C2/c$	8.35	4.93	17.64		94.47		4	0.128	*p*
N-Ac-L-Pro-D-lactyl-methylamide [a]	$P2_12_12_1$	12.45	10.00	10.33				4	0.066	*q*
N-Ac-DL-Phe-*N*-methylamide [f]	$P2_1/n$	19.45	4.98	12.29		91.92		4	0.06	*r*
L-Cystine dihydrochloride [s]	$C2$	18.60	5.24	7.23		103.74		2	0.034	*t*
L-Cystine dihydrochloride [s]	$C2$	18.58	5.24	7.23		103.74		2	0.055	*u*
L-Cystine	$P4_1$	6.71		21.73				4	0.097	*v*
Gly-Tyr dihydrate [a]	$P2_12_12_1$	12.08	22.41	4.85				4	0.037	*b*
Ala-Phe [a]	$P2_1$	9.78	9.15	8.92		105.2		2	0.043	*w*
Gly-Phe [a]	$P2_12_12_1$	8.66	26.90	6.04				4	0.046	*w*
N-t-Butyloxycarbonyl-*S*-benzyl-Cys-Gly-methyl ester [f]	$P2_1$	16.97	5.02	12.14		96.99		2	0.064	*x*
Gly-Gly sulphate monohydrate	$P2_1/c$	9.72	8.48	13.47		105.22		4		*y*
DL-Ala-LD-Met [z]	$P2_1/c$	13.32	5.33	16.05		106.87		4	0.068	*aa*
t-Amyloxycarbonyl-Pro-Pro-Pro [z,bb]	$P2_12_12_1$	14.32	9.92	15.92				4	0.06	*cc*
Pro-Pro-Pro [dd]	$P2_12_12_1$	15.95	19.09	9.23				8	0.13	*ee*
Pro-Pro-Hyp [dd]	$P2_12_12_1$	16.28	9.45	9.23				4	0.06	*ee*

Ilamycin B_1 [dd]	$P2_1$	14.32	19.69	11.88	93.97	2	0.08	*ff*
Na-antamanide(II) (analogue) [dd]	$P2_12_12_1$	12.88	38.86	13 96		4	0.12	*gg*
Cytosine-Gly-Gly-Cu^{II} [hh]	$P2_1/c$	10.64	8.08	17.79	124.29	4	0.09	*ii*
Guanidine, HCl and Gly-Gly complex [hh]	$P2_12_12_1$	14.60	15.03	4.78		4	0.054	*jj*
Glucopyranosyl-NAc-Asn [kk]	$P2_1$	4.94	24.26	7.77	97.7	2	0.054	*ll*
Glc-Asn [kk]	$P2_1$	4.94	16.68	8.08	96.1	2	0.072	*ll*

(*a*) Gives information on preferred orientations and conformations of amino-acid side-chains. (*b*) M. Cotrait and J.-P. Bideau, *Acta Cryst.*, 1974, **B30**, 1024. (*c*) Model for chelation of calcium by proteins. (*d*) H. Einspahr and C. E. Bugg, *Acta Cryst.*, 1974, **B30**, 1037. (*e*) C. Lecomte, A. Aubry, J. Protas, G. Boussard, and M. Marraud, *Acta Cryst.*, 1974, **B30**, 1992. (*f*) Hydrogen bonds as in β-pleated sheet structure. (*g*) Y. Harada and Y. Iitaka, *Acta Cryst.*, 1974, **B30**, 1452. (*h*) Study of interaction of indoles with aromatic acceptors. (*i*) G. L. Gartland, G. R. Freeman, and C. E. Bugg, *Acta Cryst.*, 1974, **B30**, 1841. (*j*) E. Benedetti, M. R. Ciajolo, and A. Maisto, *Acta Cryst.*, 1974, **B30**, 1783. (*k*) G. Lovas, A. Kálmán, and G. Argay, *Acta Cryst.*, 1974, **B30**, 2882. (*l*) P. Edington and M. M. Harding, *Acta Cryst.*, 1974, **B30**, 204. (*m*) V. M. Coiro, F. Mazza, and G. Mignucci, *Acta Cryst.*, 1974, **B30**, 2607. (*n*) M. Cotrait and Y. Barrans, *Acta Cryst.*, 1974, **B30**, 510. (*o*) T. J. Kistenmacher, G. A. Rand, and R. E. Marsh, *Acta Cryst.*, 1974, **B30**, 2573. (*p*) F. Iwasaki, *Acta Cryst.*, 1974, **B30**, 2503. (*q*) C. Lecomte, A. Aubry, J. Protas, G. Boussard, and M. Marraud, *Acta Cryst.*, 1974, **B30**, 2343. (*r*) Y. Harada and Y. Iitaka, *Acta Cryst.*, 1974, **B30**, 726. (*s*) Neutron diffraction study. (*t*) D. D. Jones, I. Bernal, M. N. Frey, and T. F. Koetzle, *Acta Cryst.*, 1974, **B30**, 1220. (*u*) S. C. Gupta, A. Sequeira, and R. Chidambaram, *Acta Cryst.*, 1974, **B30**, 562. (*v*) M. O. Chaney and L. K. Steinrauf, *Acta Cryst.*, 1974, **B30**, 711. (*w*) M. Cotrait and Y. Barrans, *Acta Cryst.*, 1974, **B30**, 1018. (*x*) S. Kashino, T. Ashida, and M. Kakudo, *Acta Cryst.*, 1974, **B30**, 2074. (*y*) F. H. Cano and S. Martínez-Carrera, *Acta Cryst.*, 1974, **B30**, 2729. (*z*) Peptide groups are significantly non-planar. (*aa*) R. E. Steinkamp and L. H. Jensen, *Acta Cryst.*, 1974, **B30**, 1541. (*bb*) Collagen-like structure. (*cc*) G. Kartha, T. Ashida, and M. Kakudo, *Acta Cryst.*, 1974, **B30**, 1861. (*dd*) Structure contains *cis*-peptide bonds. (*ee*) G. Kartha, G. Ambady, and P. V. Shankar, *Nature*, 1974, **247**, 204. (*ff*) Y. Iitaka, H. Nakamura, K. Takada, and T. Takita, *Acta Cryst.*, 1974, **B30**, 2817. (*gg*) I. L. Karle, *Biochemistry*, 1974, **13**, 2155. (*hh*) Model for protein–nucleic acid complexes. (*ii*) K. Saito, R. Terashima, T. Sakaki, and K. Tomita, *Biochim. Biophys. Res. Comm.*, 1974, **61**, 83. (*jj*) F. A. Cotton, T. La Cour, E. E. Hazen, and M. Legg, *Biochim. Biophys. Acta*, 1974, **359**, 7. (*kk*) Model for protein–carbohydrate complexes. (*ll*) L. T. J. Delbaere, *Biochem. J.*, 1974, **143**, 197.

Table 2 *Preliminary X-ray data on proteins*

Protein	*Space group*	*a*/Å	*b*/Å	*c*/Å	*β*	*Mol. wt. of asymmetric unit*	*Ref.*
L-3-Hydroxyacyl coenzyme A	$P3_121$ or $P3_112$	139.7		90.9		67 000 (dimer)	*a*
Dehydrogenase from pig heart	$P2_1$	141.5	84.9	89.2	90.1	201 000 (3 dimers)	*a*
	$P2_1$	141.3	81.4	90.7	90.1	201 000 (3 dimers)	*a*
Wheat germ agglutinin	$C2$	51.31	73.35	91.45	97.75	35 000 (dimer with heterologous subunit contacts)	*b*, *c*
	$P2_1$	73.8	51.2	90.8	90.0 (γ)	70 000 (2 dimers)	*c*
Spinach superoxide dismutase	$C2$	166.2	46.1	85.6	99.3	64 000 (2 molecules)	*d*
Hemerythrin	$P4$	96.9		100.4		108 000 (1 molecule)	*e*
Favin, a lectin from fava bean	$P2_12_12_1$	90.0	89.3	67.4		53 000 (1 molecule)	*f*
Lac repressor protein	$P222_1$	140	91	117		150 000 (1 molecule)	*g*
Horse spleen apoferritin	$P42_12$	147		154.4		— ($\frac{1}{4}$ molecule)	*h*
L-Arabinose-binding protein – *E. coli*	$P2_12_12_1$	55.3	71.4	77.5		38 000 (1 molecule)	*i*
Yeast inorganic pyrophosphatase	$P2_1$	70.4	95.4	52.2	99.8	68 000 (dimer)	*j*
D-Xylose isomerase	$I222$	93.9	99.4	102.7		40 000 ($\frac{1}{4}$ molecule)	*k*
	$I222$	94.5	98.9	87.0			*k*
Cytochrome b_{562}	$P1$	33.71	50.48	32.73	86.55 (α = 102.8, β = 106.7)	26 000 (2 molecules)	*l*
Thrombin	$P4_22_12$	87		101		38 000 (1 molecule)	*m*
		or 88		98			*m*
Myohemerythrin from sipunculid worm	$P2_12_12_1$	41.6	80.0	37.8		13 900 (1 molecule)	*n*
Abrin – seed extract of *Abrus precatorius*	$P2_1$	113	72	71	103	128 000 (2 molecules)	*o*
Insulin – proinsulin complex	—	257	125	83		12 hexamers/unit cell	*p*
Hagfish insulin	$P4_12_12$	38.51		85.37		6000 (monomer)	*q*
Cytochrome c^1 *Rhodopseudomonas palustris*	$P2_12_12_1$	39.5	77.6	107.8		28 000 (dimer)	*r*
Aspartokinase I – homoserine dehydrogenase I	$I422$ or $I4_122$	180.5		493		350 000 (tetramer)	*s*
B. subtilis levansucrase	$P2_12_12_1$	68	125	54		56 000 (1 molecule)	*t*
Concanavalin B	$P6_1$	81		101		30 000 (1 molecule)	*u*

Serum albumin	$C2$	126.5	39.2	135.2	93.3	65 000 (1 molecule)	*v*
	$P4_12_12$	84.0		276		65 000	
	$P2_12_12_1$	155	83	122		130 000 (dimer)	
	$P6_1$	96.6		144.5		65 000	
Bacteriochlorophyll – protein from green photosynthetic bacterium *Chlorobium limicola*	$P6_3$	112.4		98.4		50 000 ($\frac{1}{3}$ molecule)	*w*
	$P3_1$	83.2		165.8		50 000	
Human immunoglobulin G	$P3_121$	135.6		82.1		75 000 ($\frac{1}{2}$ molecule)	*x*
Fragment of Bence-Jones protein (C regions of λ-type light chains)	$P2_12_12$	54.64	52.22	42.62		12 500 ($\frac{1}{2}$ molecule)	*y*

(*a*) M. Weininger, B. E. Noyes, R. A. Bradshaw, and L. J. Banaszak, *J. Mol. Biol.*, 1974, **90**, 409. (*b*) C. S. Wright, *J. Mol. Biol.*, 1974, **87**, 835. (*c*) C. S. Wright, C. Keith, R. Langridge, Y. Nagata, and M. M. Burger, *J. Mol. Biol.*, 1974, **87**, 843. (*d*) Y. Morita and K. Asada, *J. Mol. Biol.*, 1974, **86**, 685. (*e*) A. C. T. North and G. J. Stubbs, *J. Mol. Biol.*, 1974, **88**, 125. (*f*) J. L. Wang, J. W. Becker, G. N. Reeke, and G. M. Edelman, *J. Mol. Biol.*, 1974, **88**, 259. (*g*) T. A. Steitz, T. J. Richmond, D. Wise, and D. Engelman, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 593. (*h*) T. G. Hoy, P. M. Harrison, and R. J. Hoare, *J. Mol. Biol.*, 1974, **86**, 301. (*i*) F. A. Quiocho, G. N. Phillips, R. G. Parsons, and R. W. Hogg, *J. Mol. Biol.*, 1974, **86**, 491. (*j*) G. Bunick, G. P. McKenna, R. Colton, and D. Voet, *J. Biol. Chem.*, 1974, **249**, 4647. (*k*) H. M. Berman, B. H. Rubin, H. L. Carrell, and J. P. Glusker, *J. Biol. Chem.*, 1974, **249**, 3983. (*l*) E. W. Czerwinski and F. S. Mathews, *J. Mol. Biol.*, 1974, **86**, 49. (*m*) D. Tsernoglou, D. A. Walz, L. E. McCoy, and W. H. Seegers, *J. Biol. Chem.*, 1974, **249**, 999. (*n*) W. A. Hendrickson and G. L. Klippenstein, *J. Mol. Biol.*, 1974, **87**, 147. (*o*) C. H. Wei and J. R. Einstein, *J. Biol. Chem.*, 1974, **249**, 2985. (*p*) B. W. Low, W. W. Fullerton, and L. S. Rosen, *Nature*, 1974, **248**, 339. (*q*) J. D. Peterson, C. L. Coulter, D. F. Steiner, S. O. Emdin, and S. Falkmer, *Nature*, 1974, **251**, 239. (*r*) F. R. Salemme, *Arch. Biochem. Biophys.*, 1974, **163**, 423. (*s*) J. Janin, *F.E.B.S. Letters*, 1974, **45**, 318. (*t*) J. Berthou, A. Laurent, E. Lebrun, and R. Van Rapenbusch, *J. Mol. Biol.*, 1974, **82**, 111. (*u*) A. McPherson, J. Geller, and A. Rich, *Biochem. Biophys. Res. Comm.*, 1974, **57**, 494. (*v*) R. J. McClure and B. M. Craven, *J. Mol. Biol.*, 1974, **83**, 551. (*w*) R. E. Fenna, B. W. Matthews, J. M. Olson, and E. K. Shaw, *J. Mol. Biol.*, 1974, **84**, 231. (*x*) W. Palm and P. M. Colman, *J. Mol. Biol.*, 1974, **82**, 587. (*y*) B.-C. Wang and M. Sax, *J. Mol. Biol.*, 1974, **87**, 505.

Table 3 *Low-resolution structures*

Protein	*Resolution*/Å	*Comments*	*Ref.*
Hagfish insulin	6.0	dimer with crystallographic two-fold axis; no hexameric aggregation	*a*
Leghaemoglobin of *Lupinus luteus* L.	5.0	monomer of mol. wt. 16 000. Shows myoglobin type structure	*b*
Bovine carboxypeptidase B	5.5	rotation and translation search methods show that carboxypeptidases A and B have similar structures	*c*
Bovine Cu^{2+}, Zn^{2+} superoxide dismutase	5.5	extensive β-structure	*d*
Phosphorylase b	6.0	structure in presence of IMP	*e*
Yeast hexokinase	7.0	structure of heterologous dimer plus binding of sugar substrates and inhibitors	*f*
Porcine phosphoglucose isomerase	6.0	structure and binding of inhibitor	*g*
Cat muscle pyruvate kinase	6.0	structure and substrate binding	*h*
E. coli aspartate transcarbamylase	5.5	binding of CTP	*i*

(*a*) J. F. Cutfield, S. M. Cutfield, E. J. Dodson, G. G. Dodson, and M. N. Sabesan, *J. Mol. Biol.*, 1974, **87**, 23. (*b*) B. K. Vainshtein, E. H. Harutyunyan, I. P. Kuranova, V. V. Borisov, N. I. Sosfenov, A. G. Pavlovsky, A. I. Grebenko, and N. V. Konareva, *Doklady Akad. Nauk U.S.S.R.*, 1974, **216**, 690. (*c*) M. F. Schmid, J. R. Herriott, and E. E. Lattman, *J. Mol. Biol.*, 1974, **84**, 97. (*d*) K. A. Thomas, B. H. Rubin, C. J. Bier, J. S. Richardson, and D. C. Richardson, *J. Biol. Chem.*, 1974, **249**, 5677. (*e*) L. N. Johnson, N. B. Madsen, J. Mosley, and K. S. Wilson, *J. Mol. Biol.*, 1974, **90**, 703. (*f*) W. F. Anderson, R. J. Fletterick, and T. A. Steitz, *J. Mol. Biol.*, 1974, **86**, 261. (*g*) H. Muirhead and P. J. Shaw, *J. Mol. Biol.*, 1974, **89**, 195. (*h*) H. Muirhead and D. K. Stammers, *Biochem. Soc. Trans.*, 1974, **2**, 49. (*i*) B. F. P. Edwards, D. R. Evans, S. G. Warren, H. L. Monaco, S. M. Landfear, G. Eisele, J. L. Crawford, D. C. Wiley, and W. N. Lipscomb, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 4437.

Table 4 *Low angle X-ray scattering*

Structure	*Mol. wt.*	R_G/Å	V/Å^3	*Solvent* (g/g)	*Comments*	*Ref.*
E. coli pyruvate dehydrogenase core complex	3.78×10^6	156.5	1.07×10^7	1.03	diameter 433 Å	*a*
T2 Bacteriophage	375×10^6	400	4.9×10^8	0.55		*b*
S_D Bacteriophage	160×10^6	300	2.1×10^8	0.5		*b*
DD2 Bacteriophage	95×10^6	255	1.2×10^8	0.5		*b*
Porcine plasma high density lipoprotein		55			lipid core of radius 42 Å polar annulus 12 Å thick	*c*
Human plasma lipoproteins HDL_3	2.1×10^5	57			lipid core plus outer polar shell	*d, e*
HDL_2	3.7×10^5				11 Å thick	
Bacteriophage MS2 viral RNA	1.09×10^6	176			elliptic cylinder, max diameter 620 Å	*f*
Yeast lysine-tRNA ligase	1.14×10^5	37.5	2.95×10^5		oblate ellipsoid	*g*
Native hog thyroglobulin	6.7×10^5	64	1.5×10^6	0.63	prolate ellipsoid, axial ratio 2 : 1	*h*
Anti-poly-D-alanyl antibodies + haptens		60			volume contraction when haptens bind	*i*
Helix pomatia haemocyanin ($\frac{1}{2}$ molecule)	4.3×10^6	140	8.4×10^6	0.45	hollow cylinder composed of spherical subunits	*j*
Sepia officinalis haemocyanin	4.0×10^6	140	8.0×10^6	0.44		

(*a*) H. Durchschlag, *J. Appl. Cryst.*, 1974, **7**, 167. (*b*) L. A. Feigin, A. T. Dembo, and A. K. Boyorintseva, *J. Appl. Cryst.*, 1974, **7**, 1614. (*c*) D. Atkinson and J, P. Green, *J. Appl. Cryst.*, 1974, **7**, 179. (*d*) L. Mateu, A. Tardieu, C. Sardet, V. Luzzatti, and A. M. Scanu, *J. Appl. Cryst.*, 1974, **7**, 180. (*e*) K. Müller, P. Laggner, O. Kratky, G. Kostner, A. Holasek, and O. Glatter, *F.E.B.S. Letters*, 1974, **40**, 213. (*f*) P. Zipper, W. Folkhard, and J. Clauwaert, *J. Appl. Cryst.*, 1974, **7**, 168. (*g*) R. Österberg, L. Rymo, and U. Lagerkvist, *J. Appl. Cryst.*, 1974, **7**, 168. (*h*) H. Brumberger, J. L. Lipton, G. Dorfman, and E. Nakana, *Biochem. J.*, 1974, **143**, 495. (*i*) I. Pilz, O. Kratky, A. Licht, and M. Sela, *J. Appl. Cryst.*, 1974, **7**, 187. (*j*) I. Pilz, Y. Engelborghs, R. Witters, and R. Lontie, *European J. Biochem.*, 1974, **42**, 195.

where the f_j are the scattering factors of N heavy atoms and ε is a symmetry factor. The method was applied successfully to derivatives of concanavalin A and formylmethionine tRNA using 3 Å resolution projection data and 5 Å resolution three-dimensional data.

Argos and Rossmann[8] have used non-crystallographic symmetry to determine heavy-atom positions in isomorphous derivatives. If there are a number of chemically identical molecular subunits in the crystallographic asymmetric unit, then heavy atoms attached specifically to the subunits of the native molecule should show the same symmetry. Patterson vectors between non-crystallographically equivalent heavy atoms can be generated for arbitrary trial positions and compared with actual Patterson syntheses. A search of all positions within the molecular subunit can be used to establish the sites of the larger heavy-atom substitutions. This is an extension of other vector search methods and was used successfully for crystals of glyceraldehyde-3-phosphate dehydrogenase from lobster, which have four molecular subunits in the crystallographic asymmetric unit. A precise knowledge of the direction and location of non-crystallographic symmetry axes is necessary when using this information in the isomorphous replacement method as described above or for the molecular replacement technique. Lentz and Strandberg[9] have interpreted the 10 Å rotation function of the satellite tobacco necrosis virus assuming the known icosahedral symmetry. The rotation function was fitted numerically to an icosahedral axis set. The root mean square angular deviation of the observed peak maxima from the calculated model axis set was 0.67° with a maximum of 1.4°. There was no significant deviation from icosahedral symmetry at 10 Å resolution. The best resolution was obtained by using only a thin shell of the highest resolution data available and a radius of integration no larger than the estimated diameter of the virus particle subunit. The use of such a shell emphasizes the inter-subunit vectors and minimizes lattice packing effects. Similarly a rotation function for apoferritin at 9 Å resolution shows that it has 24 subunits arranged with 432 (octahedral) symmetry.[10]

Bricogne[11] has developed the use of non-crystallographic symmetry in phase determination. Instead of working in reciprocal space he has derived linear equations in direct space which express the relation between the electron densities of crystals built from the same molecules but with different lattices or with several identical subunits in their crystallographic asymmetric units. These equations are shown to be equivalent to the most general molecular replacement equations in reciprocal space. He discusses the solution of these phase equations by the method of successive projections and shows that this algorithm, which is best implemented in direct space by averaging operations, is convergent for over-determined problems and is equivalent to a least-squares solution to the phase equations.

Another problem of great interest to protein crystallographers is that of the feasibility of extending the resolution of a structure determination when only

[8] P. Argos and M. G. Rossmann, *Acta Cryst.*, 1974, **A30**, 672.
[9] P. J. Lentz and B. Strandberg, *Acta Cryst.*, 1974, **A30**, 552.
[10] T. G. Hoy, P. M. Harrison, and R. J. Hoare, *J. Mol. Biol.*, 1974, **84**, 515.
[11] G. Bricogne, *Acta Cryst.*, 1974, **A30**, 395.

the native protein intensities are available for the higher resolution data. Sayre [12] has applied a technique for the direct least-squares refinement of phases to the problem of phasing a 1.5 Å data set of observed structure factor magnitudes for *Clostridium pasteurianum* rubredoxin, a small protein of molecular weight 6100, given a set of phases to 2.5 Å from isomorphous heavy-atom derivatives. The resulting map, which is obtained without chemical assumptions and with a considerable saving of effort, confirmed that obtained by the method of refinement of an approximate atomic model. It seems probable that the method is limited to the 15 000—30 000 molecular weight range, and attempts to extend the resolution from 3 Å phases were not interpretable in terms of protein structure. This method is expensive in the amount of computing time which it uses. According to a short note [13] D. M. Collins has extended the resolution of the electron-density map of rubredoxin using a method which works directly with the electron-density map and assumes that there are no areas of negative electron density. He has devised a function which shapes the map so that the Fourier series converges to the ultimate value more rapidly. This is said to be much faster and cheaper than Sayre's method.

Once a high-resolution electron-density map has been obtained the problem is one of interpretation and the refinement of atomic co-ordinates. Greer [14] has attempted an automated interpretation of an electron-density map. His method is to reduce a high-resolution electron-density map to a set of connected thin lines which follow the density. Side-chains are removed so that only main chain, disulphide bridges, and very strong hydrogen bonds remain. Procedures have been devised for separating a molecule from neighbouring molecules and listing provisional α-carbon positions. This method has been used on the 2 Å map of ribonuclease S and appears to be a useful way of isolating a single molecule and predicting α-carbon positions with reasonable accuracy. Diamond [15] has published the real space refinement of hen egg-white lysozyme. This refinement technique gives optimal co-ordinates for well-resolved features which were located to *ca.* 0.1 Å. The folding of the polypeptide chain is adjusted with standard bond lengths and bond angles to obtain the optimum fit to the electron-density map. This work is based on the 2 Å multiple isomorphous replacement map, and Diamond points out that phases calculated from atomic co-ordinates would have been better than multiple isomorphous replacement phases as shown by Huber's work on the trypsin inhibitor. Levitt has applied his energy refinement programs to the resulting co-ordinates and obtained a standard crystallographic *R*-factor of 0.35.[16]

3 Antibody Structure and Antigen Binding

In 1973 the three-dimensional structures of various fragments of antibody molecules were published. In 1974 the emphasis has been on improving the resolution of these structures and on studying the binding of antigens. Antibodies have a common basic structure in which the antigen-binding function is

[12] D. Sayre, *Acta Cryst.*, 1974, **A30**, 180.
[13] T. H. Maugh, *Science*, 1974, **186**, 913.
[14] J. Greer, *J. Mol. Biol.*, 1974, **82**, 279.
[15] R. Diamond, *J. Mol. Biol.*, 1974, **82**, 371.
[16] M. Levitt, *J. Mol. Biol.*, 1974, **82**, 393.

carried out by the variable domain whose hypervariable regions are involved in the binding process. This variable domain is composed of the amino-terminal half of the light chain and the amino-terminal quarter of the heavy chain. Repetitive amino-acid sequences along the heavy and light chains were suggestive of a number of very similar tertiary structural domains along the polypeptide chains, and this was confirmed by the structures.

Poljak and his collaborators [17] have published the structure of the Fab fragment of a human myeloma immunoglobulin (IgG New) at 2.0 Å resolution. Each of the structural subunits or domains corresponding to the variable and to the constant homology regions of the light and heavy chains contain two irregular β-sheets which are roughly parallel to each other and surround a tightly packed interior of hydrophobic side-chains. The regions of hypervariable sequence in the light and heavy chains occur in close spatial proximity at one end of the molecule. The binding of a γ-hydroxy-derivative of vitamin K_1 has been studied at a resolution of 3.5 Å.[18] This hapten binds in a shallow groove between the light and heavy chains in close proximity to the polypeptide segments containing the hypervariable regions. At least 12 residues are in close contact with it and there is a high density of aromatic side-chains lining the crevice (Figure 1). The hypervariable loop of the variable region of the heavy chain does not conform to the approximate two-fold axis of symmetry relating the two variable domains and it is likely that variations in the length of this loop could change the width and depth of the active site pocket. At this resolution no conformational changes can be seen although such changes would be needed for complement binding or for B-cell activation reactions. It is possible that the interactions needed to trigger such conformational changes are not present when relatively small haptens are bound. Such conformational changes could produce changes in the relative orientations of the different domains.[17] Segal *et al.*[19] have studied the binding of phosphorylcholine to Fab McPC 603 and obtained similar results, although the shape of the binding pocket is somewhat different. They argue that the hypervariability of the sequence in the region of the pocket could be sufficient structural explanation for antibody diversity.

The structure of a λ-type Bence-Jones dimer which consists of two identical light chains resembles an Fab fragment, in which one light chain plays the structural role of the heavy chain. It is interesting to note that in such circumstances two polypeptide chains with the same primary sequence can take up different conformations. The two constant domains are held together by close interactions between opposing surfaces while there is a solvent channel between the two variable domains which consists of a conical cavity terminating in a pocket. This Bence-Jones protein will bind various dinitrophenyl derivatives and these hapten-like molecules bind at two sites in the main cavity and at a third site within the pocket.[20] Most of the side-chains lining the cavity are constituents of the

[17] R. J. Poljak, L. M. Amzel, B. L. Chen, R. P. Phizackerley, and F. Saul, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 3440.

[18] L. M. Amzel, R. J. Poljak, F. Saul, J. M. Varga, and F. F. Richards, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 1427.

[19] D. M. Segal, E. A. Padlan, G. H. Cohen, S. Rudikoff, M. Potter, and D. R. Davies, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 4298.

[20] A. B. Edmundson, K. R. Ely, R. L. Girling, E. E. Abola, M. Schiffer, F. A. Westholm, M. D. Fausch, and H. F. Deutsch, *Biochemistry*, 1974, **13**, 3816.

three hypervariable regions and some of these have been identified as being at or near the binding sites of anti-dinitrophenyl antibodies. Residues suitable for both hydrogen bonding and hydrophobic interactions are present but the binding is primarily dependent on hydrophobic interactions. Small conformational changes were observed around one site only. However, large entering groups

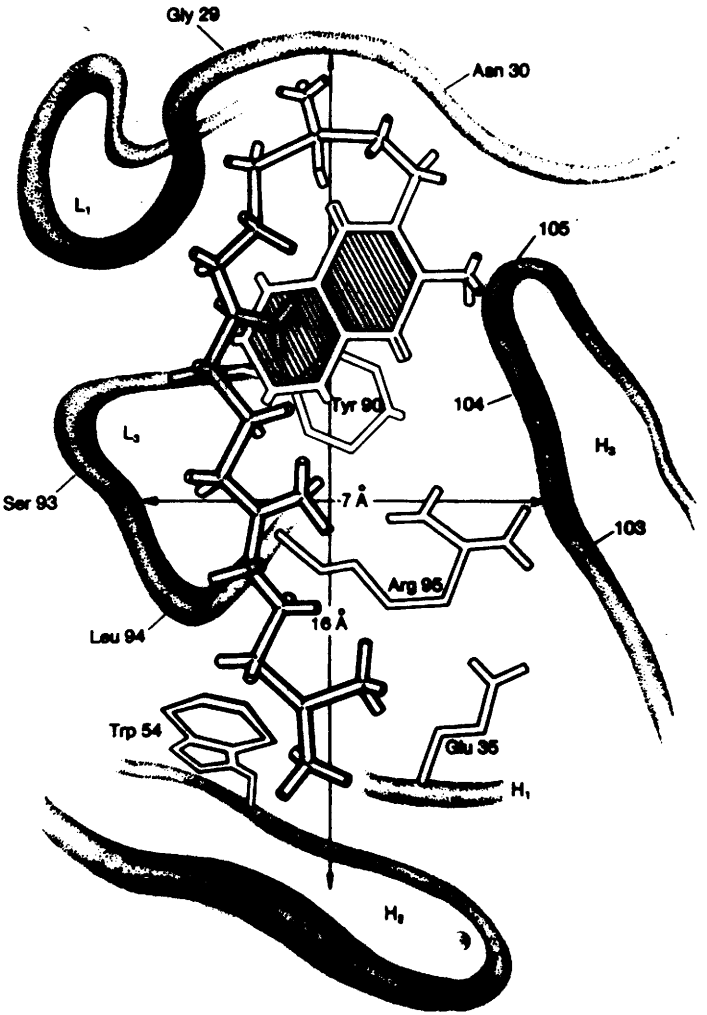

Figure 1 *Schematic drawing of vitamin* K_1OH *bound to the combining region of* IgG New. L_1 *and* L_3 *indicate the approximate location of the first and third hypervariable regions of the light chain.* H_1, H_2, *and* H_3 *designate hypervariable regions of the heavy chain. Light chain residues* Tyr-90 *and* Arg-95 *are at the bottom of the shallow groove or crevice between the heavy and light chains.* Trp-54 (*an invariable residue in human heavy-chain sequences*) *and* Glu-35 (*tentative assignment, not based on actual sequence data*) *are in close contact with the end of the phytyl chain of vitamin* K_1OH
(Reproduced by permission from *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 1427)

with the proper affinity can disrupt the crystal structure and must be breaking the weak interactions along the solvent channel while binding at the sites which are more deeply buried in the molecule.

The molecular structure of a dimer composed of the variable portions of a χ-type Bence-Jones protein has shown that the folding of the chains and the spatial relationship of the two monomers are the same as in the λ-type Bence-Jones protein dimer and the Fab fragment.[21, 22] More recently, crystals of the fragments containing the constant regions of both light and heavy chains have been studied, and these constant regions appear to have the same tertiary structure,[23, 24] thus confirming the domain concept for the structure of the immunoglobulins in which the four repeating homology regions of the heavy chains and the two of the light chains have very similar tertiary structures.

Palm and Colman have obtained crystals of an intact IgG molecule which should be suitable for high-resolution work.[25]

4 Dehydrogenases and Kinases

The polypeptide chain of many globular proteins is folded into two or more structural domains, which are spatially separated from one another and frequently are based on different architectural principles. Among known tertiary structures several recurring folds of the polypeptide chain have been recognized. While structural and functional similarities within such domains may be evidence for gene duplication this should be confirmed by the comparison of amino-acid sequences. However, when the three-dimensional structure is known it should be taken into account when making sequence alignments. Rossman and Liljas [26] have suggested a general method for the recognition and comparison of such structural domains based on the distances between equivalent residues within a domain.

Three-dimensional homologies have been studied by comparing the structures of the coenzyme binding domains and the bound coenzyme molecules in liver alcohol dehydrogenase, lactate dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase.[27] The domains were superposed pairwise and distances between corresponding α-carbons as well as between equivalent atoms of the coenzyme molecules were compared. Striking structural similarities were found in the polypeptide folds of those residues that compose six strands of parallel β-pleated sheet as well as in those that form the loops and helices in the central regions of the domains (Figure 2). Most important is the fact that the sequence of the elements of secondary structure in the coenzyme binding structure is the same in all dehydrogenases which have been investigated so far. The structures of the coenzyme molecules and their mode of binding to this coenzyme binding domain

[21] O. Epp, P. Colman, H. Felhhammer, W. Bode, M. Schiffer, and R. Huber, *European J. Biochem.*, 1974, **45**, 513.
[22] R. Huber and W. Steigemann, *F.E.B.S. Letters*, 1974, **48**, 235.
[23] P. M. Colman, O. Epp, H. Fehlhammer, W. Bode, M. Schiffer, E. E. Lattman, and T. A. Jones, *F.E.B.S. Letters*, 1974, **44**, 194.
[24] B.-C. Wang and M. Sax, *J. Mol. Biol.*, 1974, **87**, 505.
[25] W. Palm and P. M. Colman, *J. Mol. Biol.*, 1974, **82**, 587.
[26] M. G. Rossmann and A. Liljas, *J. Mol. Biol.*, 1974, **85**, 177.
[27] I. Ohlsson, B. Nordström, and C.-I. Brändén, *J. Mol. Biol.*, 1974, **89**, 339.

were also very similar. These three-dimensional homologies were used to align and compare the amino-acid sequences showing that there were only four invariant residues of which three were glycines. This apparent lack of sequence homology may be related to the time involved since divergence. Ohlsson *et al.*[27] suggest that sophisticated enzymes may have evolved by the fusion of primordial

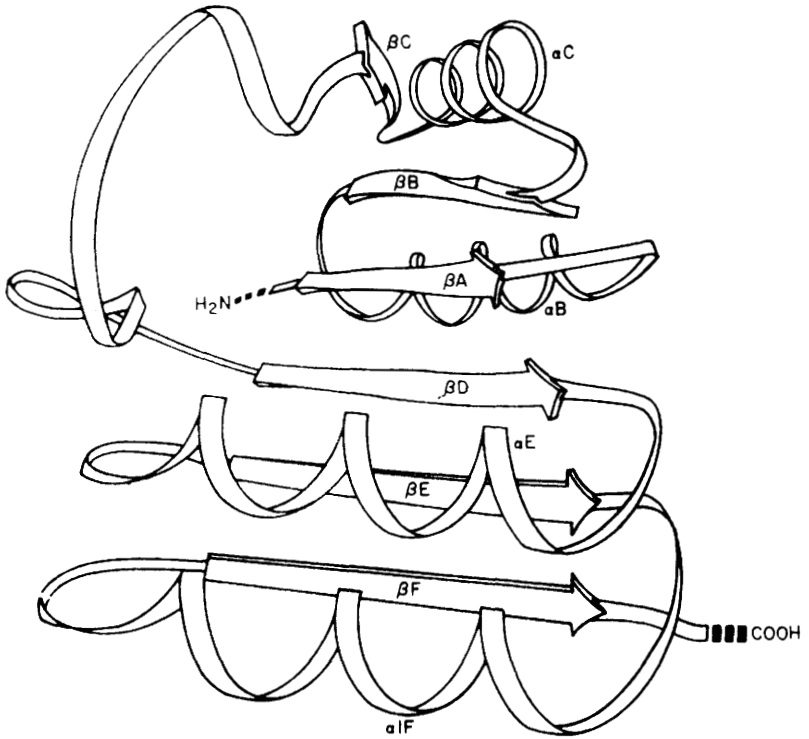

Figure 2 *Schematic representation of the structure of the coenzyme binding domain in dehydrogenases.* βA *to* βF *represent the six parallel strands of pleated sheet and* αB, αC, αE, *and* α1F *represent the four helices*
(Reproduced by permission from *J. Mol. Biol.*, 1974, **89**, 339)

genes coding for small, monofunctional units and that subunits are divided into domains, each of which is associated with a particular function. Rossmann *et al.*[28] have done more extensive comparisons and have demonstrated the structural relationship between the nucleotide binding structures in several proteins including dehydrogenases, kinases, and flavodoxins. They suggest that this common nucleotide binding structure was present during precellular evolution.

Lobster D-Glyceraldehyde-3-phosphate Dehydrogenase.—This structure has been determined at a resolution of 3 Å.[29] Multiple isomorphous replacement phases

[28] M. G. Rossmann, D. Moras, and K. W. Olsen, *Nature*, 1974, **250**, 194.
[29] M. Buehner, G. C. Ford, D. Moras, K. W. Olsen, and M. G. Rossmann, *J. Mol. Biol.*, 1974, **82**, 563.

were used at low resolution and single isomorphous replacement phases at high resolution. The asymmetric unit is the tetramer, which consists of four identical polypeptide chains, and the electron-density map was averaged over the four subunits. This use of non-crystallographic symmetry to improve the electron-density map is equivalent to the molecular replacement method. It may introduce some small errors if there are real conformational differences between the subunits. It was possible to trace the entire polypeptide chain in the averaged map. As stated above,[27] the nucleotide binding domain was similar to those found in other dehydrogenases. However, the association of the subunits is different; *e.g.*, in lactate dehydrogenase the four nucleotide binding sites are far apart, whereas in glyceraldehyde-3-phosphate dehydrogenase they are close together and form two pairs of associated sites. The co-operativity and the half-of-the-sites-reaction properties of glyceraldehyde-3-phosphate dehydrogenase are probably associated with this characteristic. A more accurate map[30] enabled Rossmann and his colleagues to describe a mechanism for the enzyme based on the following observations. The conformation of the coenzyme NAD^+ is the same as in lactate dehydrogenase apart from a rotation of 180° about the glycosidic bond which exposes the B side of the nicotinamide ring to the substrate. The second domain contains His-176 situated near the essential cysteine, Cys-149, and presumably acting as a base during catalysis. Lys-83 binds to pyrophosphate in the active site of an adjacent subunit so that the catalytic centre contains residues from two different subunits. Thus the NAD binding sites are directly linked in pairs forming a functional dimer. The position of two sulphate anion sites suggests the position of binding of the substrate phosphate and the inorganic phosphate to be subsequently incorporated into the product. Support for the interpretation of the map comes from the fact that the amino-acids in the active centre region and those involved in subunit contacts are more highly conserved than other residues.

Horse-liver Alcohol Dehydrogenase.—The resolution of the structure of liver alcohol dehydrogenase has been extended to 2.4 Å.[31] This enzyme is a dimer of molecular weight 80 000 and the two subunits are identical, each containing two firmly bound zinc atoms. One of these zincs is necessary for the catalytic activity and the other for the structural stability of the enzyme. At this resolution it was possible to position all the side-chains in the known amino-acid sequence and, using the information obtained by studying complexes of the enzymes with a coenzyme analogue ADP-ribose and various inhibitors, to derive a plausible mechanism of action. The subunit consists of two domains, the nucleotide binding domain and the catalytic domain which contains the two zinc atoms and a complicated network of β-structures. The catalytic zinc is at the bottom of a deep pocket between the two domains. It is bound to three protein ligands, Cys-46, Cys-174, and His-67, and to a water molecule. This bound water molecule makes an internal hydrogen bond to Ser-48 which in turn is hydrogen bonded to His-51. This network of hydrogen bonds could provide a framework

[30] M. Buehner, G. C. Ford, D. Moras, K. W. Olsen, and M. G. Rossmann, *J. Mol. Biol.*, 1974, **90**, 25.

[31] H. Eklund, B. Nordström, E. Zeppezauer, G. Söderlund, I. Ohlsson, T. Boiwe, and C.-I. Brändén, *F.E.B.S. Letters*, 1974, **44**, 200.

for the proton release which takes place during the reaction. By model building, assuming the position of the coenzyme moiety from the ADP-ribose position, a plausible mechanism for alcohol oxidation is that of electrophilic catalysis mediated by the active-site zinc atom. The binding of the coenzyme, NAD^+, perturbs the value of the pK_a of the zinc-bound water molecule with a concomitant proton release. Alcohol then binds to the zinc as the alcoholate ion, displacing the hydroxide ion. The zinc atom polarizes the alcoholate so that direct hydrogen transfer and subsequent rearrangement to aldehyde can occur. The co-ordination of the second zinc to four cysteine residues is reminiscent of the structural arrangement around the iron atom in rubredoxin and around the iron–sulphur clusters in the bacterial ferredoxins. In a later paper [32] the binding of salicylate is described and it is suggested that salicylate should bind to all enzymes with a similar coenzyme-binding domain; a fluorometric method could be used to screen for salicylate binding to such enzymes.

Phosphoglycerate Kinase and Adenylate Kinase.—The structure of horse muscle phosphoglycerate kinase has been solved at a resolution of 3 Å and that of yeast phosphoglycerate kinase at 3.5 Å.[33, 34] The two structures are similar. Phosphoglycerate kinase catalyses the following reaction in the glycolytic pathway

$$\text{1,3-diphosphoglycerate} + \text{ADP} \underset{}{\overset{Mg^{2+}}{\rightleftharpoons}} \text{3-phosphoglycerate} + \text{ATP}$$

It is a monomeric enzyme which contains about 355 amino-acids although its amino-acid sequence is not known. The monomer has a markedly dinuclear or two-lobed structure consisting of two domains which are structurally independent. Each domain has a central β-sheet core with α-helices aligned parallel with the strands of the sheets. The Mg–ADP and Mg–ATP binding site has been located from low-resolution difference maps. The adenine ring fits into a slot at the carboxy-end of the six-stranded β-pleated sheet in one lobe. The metal is linked to both α- and β-phosphates and to an enzyme side-chain. The γ-phosphate of ATP is near to a side-chain from the second domain.[33] This suggests that one domain carries the ADP, ATP binding site and the other the phosphoglycerate binding site and the catalytic site. The ADP, ATP binding domain bears a strong resemblance to the nucleotide binding structure found in the dehydrogenases. All the main secondary structure elements are defined but since the sequence is not known the connectivity is less reliable. However, it is possible to interpret the structure such that the connectivity is the same as in the dehydrogenases although the initial interpretation was different. The orientation of ATP in the binding site is slightly different from that of NAD in the dehydrogenases.

This suggests the possibility of an evolutionary relationship in the nucleotide binding units of the kinases and the dehydrogenases. Further evidence is supplied by the structure of adenylate kinase at a resolution of 3 Å. Again the domains which contained the AMP and ATP binding sites had a similar structure.[35, 35a]

[32] R. Einarsson, H. Eklund, E. Zeppezauer, T. Boiwe, and C.-I. Brändén, *European J. Biochem.*, 1974, **49**, 41.
[33] C. C. F. Blake and P. R. Evans, *J. Mol. Biol.*, 1974, **84**, 585.
[34] T. N. Bryant, H. C. Watson, and P. L. Wendell, *Nature*, 1974, **247**, 14.
[35] G. E. Schulz, M. Elzinga, F. Marx, and R. H. Schirmer, *Nature*, 1974, **250**, 120.
[35a] G. E. Schulz and R. H. Schirmer, *Nature*, 1974, **250**, 142.

Phosphoglycerate mutase, where no nucleotide binding is required, is another glycolytic enzyme and its structure has been determined at 3.5 Å resolution. This structure also contains a central β-pleated sheet flanked with α-helices which could be related to the nucleotide binding fold, although the similarity is not so marked as for the two kinase structures.[36]

Yeast Hexokinase B.—The B isoenzyme of yeast hexokinase has been crystallized in three crystal forms: I, II, and III. In forms I and II the subunits associate to form dimers. The quaternary structures in the two forms are different although they both show heterologous interactions between the two subunits. Unlike BI the BII crystal form binds both nucleotide and sugar substrates and there are interactions between the different ligand sites.[37] The overall tertiary structure at low resolution is the same as in BI. The quaternary structure of the dimer is completely different and the two subunits are related by a rotation of 160° and a translation of 13 Å. A deep cleft divides each subunit into two domains of roughly equal size. There is considerable secondary structure but the structure is not homologous in detail to the other kinases. Sugar substrates and inhibitors bind deeply in the cleft which separates the two lobes and produce substantial conformational changes in the protein structure. Substrate binding to the two subunits is not identical. This could be explained by the different environments of the binding sites. Recently the structure of the BIII crystal form, in which hexokinase does not appear to associate into dimers, has been determined at 2.7 Å resolution.[38] At this resolution it can be seen that the monomer contains three structural domains rather than two, of which one is predominantly α-helical and the other two contain β-pleated sheet flanked by helices. Both substrates, glucose and AMP, bind and produce significant alterations in the protein structure. Glucose binds in the deep cleft as in the BII crystal of the dimeric enzyme. AMP binds at the end of one of the β-pleated sheets at a site which is different from the major inter-subunit site observed in the crystalline dimer. From the current interpretation of the electron-density map neither of the two nucleotide binding regions has the same structure as that observed for the nucleotide-binding region of the dehydrogenases and other kinases, although some similarities exist. The inter-subunit site is completely different from the dehydrogenase fold, while the other site is only superficially similar. An unsuccessful attempt was made to reinterpret the map in a manner consistent with the dehydrogenase fold. Fletterick *et al.*[38] have suggested that the observed similarities could be due to a combination of convergent evolution and a limitation on the number of ways of forming certain super-secondary structures.

5 Proteolytic Enzymes

Chymotrypsin and Trypsin.—Changes in the structure of α-chymotrypsin over the pH range 1.0—10.0 have been studied at a resolution of 2.8 Å.[39] Structural transitions occur at pH values close to the known pK values of ionizable groups. One such change, around pH 8.0, is produced by deprotonation of the N-terminal

[36] J. W. Campbell, H. C. Watson, and G. I. Hodgson, *Nature*, 1974, **250**, 301.
[37] W. F. Anderson, R. J. Fletterick, and T. A. Steitz, *J. Mol. Biol.*, 1974, **86**, 261.
[38] R. J. Fletterick, D. J. Bates, and T. A. Steitz, *Proc. Nat. Acad. Sci. U.S.A.*, 1975, **72**, 38.
[39] A. Mavridis, A. Tulinsky, and M. N. Liebman, *Biochemistry*, 1974, **13**, 3661.

amino-group of Ile-16. This leads to the disruption of an internal ion pair which is followed by a severe reorganization within the active site region. In the transition range asymmetric changes can occur within the dimer interface due to slight pK differences between equivalent groups in different environments in the two halves of the dimer. The high pH conformer, which contains completely deprotonated Ile-16, is inactive since the conformational changes affect the enzyme–substrate dissociation constant.

The structure of an inhibited form of trypsin, di-isopropylphosphoryl trypsin, at a resolution of 2.7 Å and in the pH range (7—8) at which the enzyme is active has been published.[40] The inhibited enzyme was used instead of the active form in order to reduce the possibility of autolysis. The molecule shows an extensive homology to α-chymotrypsin and elastase. However, the conformation at the active site is slightly different in that the structure of di-isopropylphosphoryl trypsin resembles that of the tetrahedral intermediate in the reaction mechanism, the phosphorus atom of the inhibitor occupying the position of the carbonyl carbon of the peptide bond to be cleaved in the postulated tetrahedral intermediate. The binding of the competitive inhibitor benzamidine was used to locate the specific side-chain binding pocket of trypsin, which normally binds arginine or lysine during proteolysis.[41] There are conformational differences between di-isopropylphosphoryl and benzamidine trypsins, and the binding of benzamidine in the pocket appears to provide a good model for real substrate binding. If it is assumed that the side-chains of the catalytic site in the native enzyme are arranged as in chymotrypsin, then there is a transition to an acyl-like position on binding benzamidine. It seems likely that the structure of benzamidine trypsin resembles an activated conformation, although the enzyme is inhibited, since the presence of benzamidine inhibits productive substrate binding by steric hindrance. However, the small alkyl amines or guanidines, which activate trypsin, would induce similar small changes in the catalytic site of trypsin and these changes would be responsible for activating the enzyme and affecting k_{cat}. Specific side-chains of real substrates should produce similar rearrangements.

Silver ion is a potent inhibitor of the serine proteases. Difference Fourier syntheses have shown that the primary binding site is between the carboxy-group of Asp-102 and the δ nitrogen of His-57, so that the silver ion interferes primarily with the acylation rate constant and does not significantly affect the bonding constant.[42]

Trypsin Inhibitor.—The resolution of the complex of bovine trypsin and bovine pancreatic trypsin inhibitor has been increased to 1.9 Å.[43] The refinement procedures, which involved cycles consisting of phase calculation using the current atomic model, Fourier syntheses using these phases and the observed structure amplitudes, and Diamond's real-space refinement technique plus

[40] R. M. Stroud, L. M. Kay, and R. E. Dickerson, *J. Mol. Biol.*, 1974, **83**, 185.
[41] M. Krieger, L. M. Kay, and R. M. Stroud, *J. Mol. Biol.*, 1974, **83**, 209.
[42] J. L. Chambers, G. G. Christoph, M. Krieger, L. M. Kay, and R. M. Stroud, *Biochem. Biophys. Res. Comm.*, 1974, **59**, 70.
[43] R. Huber, D. Kukla, W. Bode, P. Schwager, K. Bartels, J. Deisenhofer, and W. Steigemann, *J. Mol. Biol.*, 1974, **89**, 73.

difference Fourier syntheses at appropriate points in the analysis, have led to an extremely accurate structure determination as illustrated in Figure 3. This confirms the fact that it is best to use the experimentally determined multiple isomorphous replacement phases only as initial information and to replace them by calculated phase angles in subsequent refinement steps. Using this

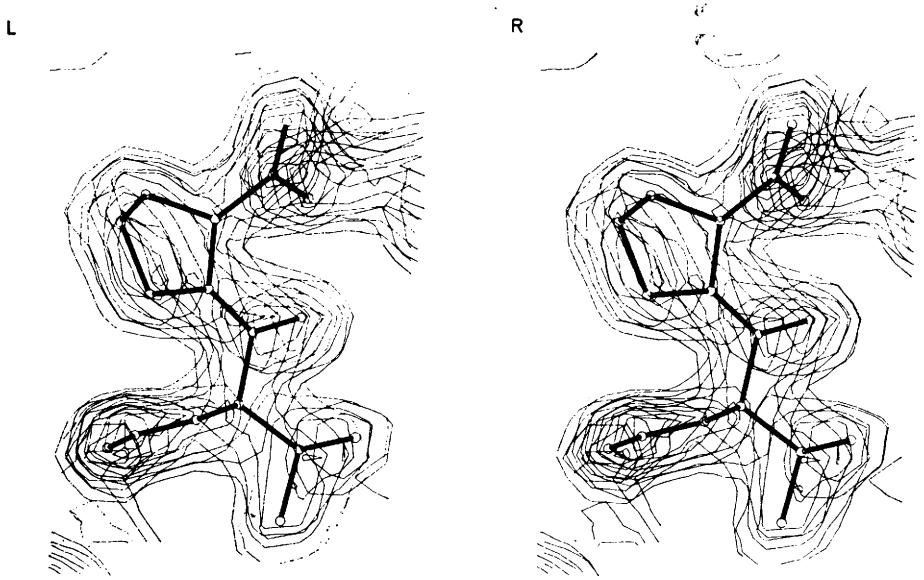

Figure 3 *Electron density and model fit of* Val-27 *to* Pro-28 *of the trypsin–trypsin inhibitor complex*
(Reproduced by permission from *J. Mol. Biol.*, 1974, **89**, 73)

method for the refinement of the complex gives a standard crystallographic *R*-factor of 0.23 for 20 500 significantly measured reflections to 1.9 Å resolution, using an overall temperature factor of 20 Å.[3] The estimated standard deviation of atomic positions was 0.09 Å.

A second group has calculated the crystal structure of the complex of porcine trypsin with soybean trypsin inhibitor at 2.6 Å resolution. This structure confirmed the previous results.[44, 45] The conformation of the active site of the complex should show how the polypeptide chain is oriented at the active site of the enzyme. Co-ordinates for the adjacent parts of the inhibitor and the trypsin molecule were refined by the real-space refinement technique. Calculation of interatomic distances at the active site showed the complex to be in the form of a tetrahedral adduct of the scissile bond to the active serine. The strong binding energy of the inhibitor and the stabilization of the tetrahedral form result from the nature of the active site of the enzyme, which is designed to stabilize the transition state of peptide hydrolysis. The area of contact between the molecules

[44] D. M. Blow, J. Janin, and R. M. Sweet, *Nature*, 1974, **249**, 54.
[45] R. M. Sweet, H. T. Wright, J. Janin, C. H. Chothia, and D. M. Blow, *Biochemistry*, 1974, **13**, 4212.

is not extensive, only including 12 to 18 amino-acids, but it is sufficient to supply 15—20 kcal mol^{-1} of binding energy. These results give further insight into the role of the tetrahedral intermediate in the reaction mechanism and also emphasize the importance of secondary specificity in the hydrolysis of protein and peptide substrates (Figure 4).

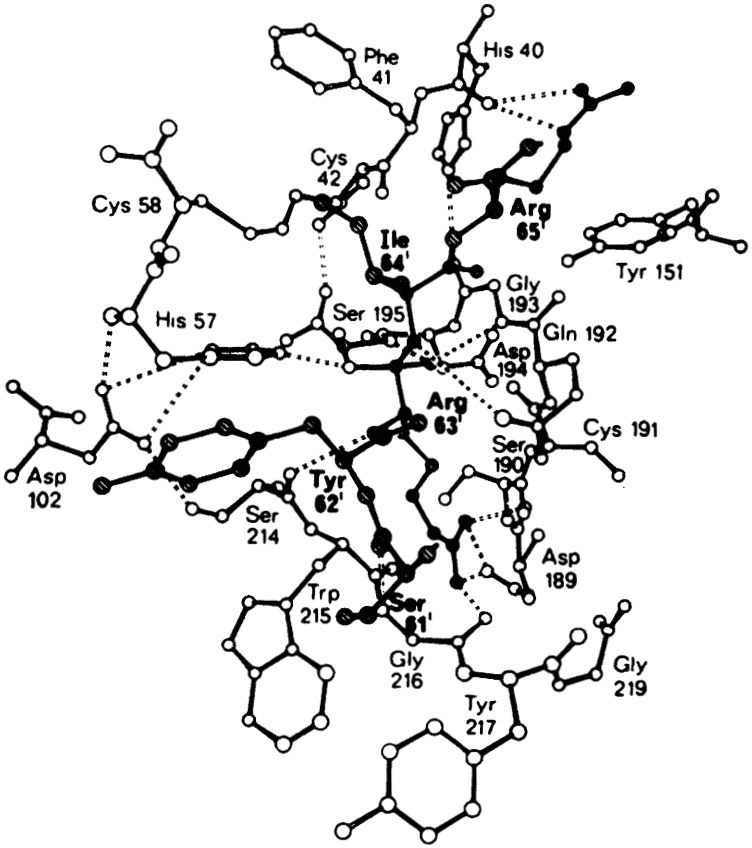

Figure 4 *Residues 61′—65′ of soybean trypsin inhibitor and the parts of the trypsin molecule involved in binding them. Atoms of the inhibitor molecule are shaded. The carbonyl carbon of the scissile peptide bond 63′—64′ is shown tetrahedrally co-ordinated* (Reproduced by permission from *Nature*, 1974, **249**, 54)

Lysozyme.—Several papers have been published describing investigations of the active site of hen egg-white lysozyme. Bivalent copper or cobalt ions are non-competitive inhibitors of lysozyme which inhibit at high concentrations (K_a = 3.8 × 10^2 mol l^{-1}). They both bind to the same location which is *ca.* 2—3 Å from the carboxy-group of Asp-52, *ca.* 5 Å from the carboxy-group of Glu-35, and *ca.* 7 Å from Trp-108. The addition of a saccharide inhibitor increases the association constant. This is related to a change in conformation about residues 35 and 108 which affects the metal-binding properties of

Asp-52.[46, 47] The binding of a proposed transition-state analogue, the δ-lactone derived from tetra-*N*-acetyl-chitotetraose, to crystalline lysozyme at pH 2.6 has been investigated at 2.5 Å resolution.[48] It is bound in sites A, B, C, and D of the active site cleft with sugar residues in sites A, B, and C in similar positions to those observed in the complex with tri-*N*-acetyl-chitotriose. At site D the δ-lactone ring is in a conformation close to that of a sofa or a boat in which C-6 is axial and the other substituents equatorial. These studies provide support for the concept of the distortion of the substrate and the role of strain in the proposed mechanism of lysozyme catalysis.

Snape *et al.* have found conditions under which urea will bind to crystalline lysozyme. High concentrations of urea produce unfolding of proteins and the perturbation should provide useful information and give an insight into the forces involved in the architecture of proteins and the catalytic power of enzymes. The diffraction pattern showed large intensity changes from that of the native protein. These changes were particularly pronounced at high resolution, indicating extensive structural alterations with no loss of crystallinity. The structural changes were confined within the molecular envelope but were so large that difference Fourier techniques could not be used to study them in detail.[49]

Bacteriophage T4 lysozyme is the enzyme produced in cells of *E. coli* after infection with bacteriophage T4. The molecular weight is 18 700, the primary structure is known, and the enzyme has a catalytic activity similar to that of hen egg-white lysozyme. The 2.5 Å resolution structure[50] shows that *ca.* 60% of the molecule is in a helical conformation and that there is one region of anti-parallel β-pleated sheet structure. The polypeptide chain is folded into two distinct lobes, linked in part by a long helix. The carboxy-terminal part of the polypeptide chain lies exclusively in the upper domain (Figure 5), while the lower domain contains most of the amino-terminal portion of the molecule, although the amino-terminal helix interacts extensively with the upper domain and appears to help link the two lobes together. The main connection between the two domains is a long helix of 20 residues which extends almost from one extremity of the molecule to the other. Between the two lobes there is a cleft which deepens into a hole or cavity. This opening is closed off by side-chains and fairly substantial conformational changes would be required in order to allow a substrate to enter. The tertiary structure is quite different from that of hen egg-white lysozyme although it is not clear whether or not the mechanisms of catalysis of the respective enzymes are related. It is possible that the active sites contain similar catalytic residues.

Thermolysin.—An improved electron-density map has been obtained for the heat-stable proteolytic enzyme, thermolysin.[51] The molecular weight is 34 600, the amino-acid sequence is known, and the structure contains one *cis*-proline.

[46] V. I. Teichberg, N. Sharon, J. Moult, A. Smilansky, and A. Yonath, *J. Mol. Biol.*, 1974, **87**, 357.
[47] A. Yonath, A. Smilansky, and N. Sharon, *F.E.B.S. Letters*, 1974, **49**, 178.
[48] L. O. Ford, L. N. Johnson, P. A. Machin, D. C. Phillips, and R. Tjian, *J. Mol. Biol.*, 1974, **88**, 349.
[49] K. W. Snape, R. Tjian, C. C. F. Blake, and D. E. Koshland, *Nature*, 1974, **250**, 295.
[50] B. W. Matthews and S. J. Remington, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 4178.
[51] B. W. Matthews, L. H. Weaver, and W. R. Kester, *J. Biol. Chem.*, 1974, **249**, 8030.

With the possible exception of the four calcium binding sites there is nothing particularly unusual in the thermolysin conformation which could explain its stability. This could be due to a combination of subtle differences in hydrophobic character, metal binding, hydrogen bonding, and ionic interactions. The co-ordination of the calcium ions varies between six- and eight-fold, and groups involved in the co-ordination include the oxygens of glutamic and

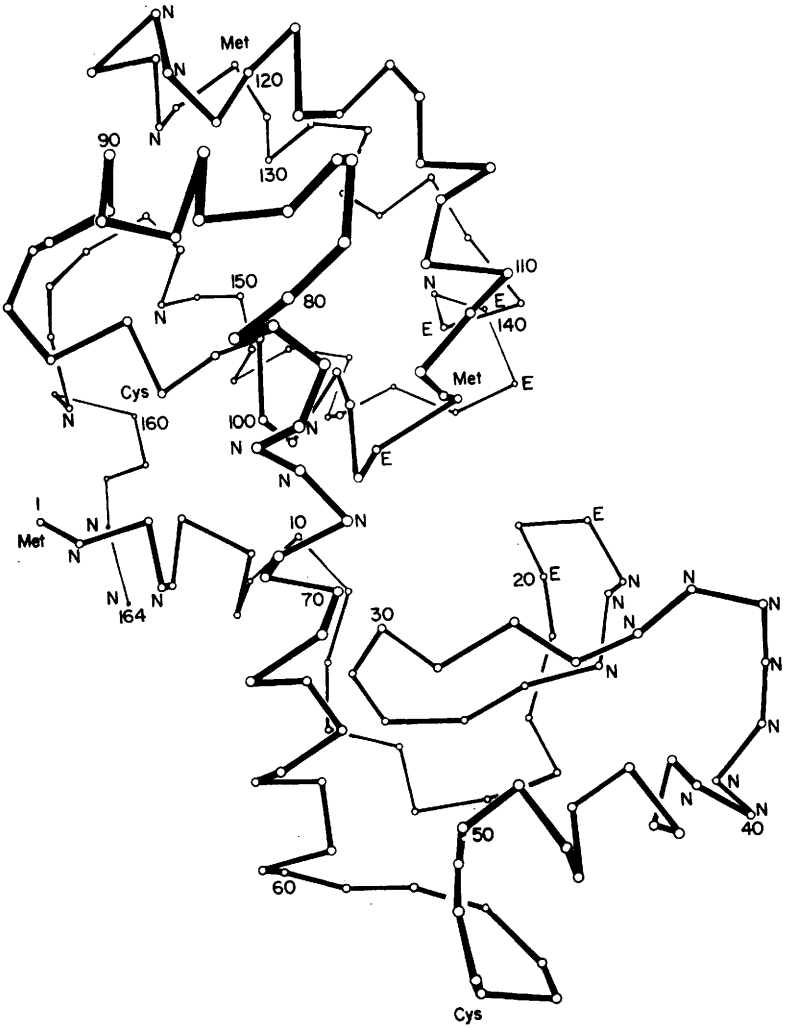

Figure 5 *Perspective drawing illustrating the polypeptide backbone of bacteriophage T4 lysozyme. The approximate position of each alpha carbon atom is indicated by an open circle. Also shown are the methionine and cysteine residues involved in heavy-atom binding. Residues labelled* E *and* N *were respectively designated on the basis of genetic studies as 'essential' and 'non-essential' for full catalytic activity*
(Reproduced by permission from *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 4178)

aspartic carboxy-groups, main-chain carbonyl groups, a threonine hydroxy-group and internal water molecules. The co-ordination of the zinc atom bound at the active site is similar to that found in carboxypeptidase A. A series of lanthanide ions have been bound to thermolysin and the binding of europium has been studied in detail.[52] Europium readily replaces calcium at each of three binding sites and will displace it at a fourth site. There are slight shifts in position due to the higher co-ordination number for europium but very little perturbation of the structure. The rare earths of higher atomic number were found to cause less perturbation of the protein structure than those with lower atomic number, possibly because their ionic radii are less than or equal to that of calcium and their preferred co-ordination numbers closer to those of the substituted ions. The effectiveness of lanthanide ions in replacing calcium function is complicated not only by charge difference and size variation, but also by possible changes in the co-ordination number and the geometry of binding. This is of interest in the use of lanthanide ions as probes of structure and function.

Ribonuclease.—A full description of the structure of bovine pancreatic ribonuclease at a resolution of 2.5 Å and based on four isomorphous heavy-atom derivatives has been published.[53] The three amino-acid residues His-12, His-119, and Lys-41, which are responsible for the catalytic activity, have the same relative positions as in ribonuclease S. Water molecules bound to the surface of the protein can be seen and some structure can be seen in the water lying between molecules.

6 Haemoglobin

The structures of two more monomeric haemoglobins have been determined. Leghaemoglobin is a plant haemoglobin found in the root nodules of leguminous plants; its physiological role is concerned with the fixation of atmospheric nitrogen. The electron-density map at 5 Å resolution[54] shows that leghaemoglobin has a tertiary structure similar to other haemoglobins and myoglobins and suggests that animal and plant haemoglobins have a common origin. The high-resolution (2.5 Å) structure of haemoglobin from the polychaete annelid, *Glycera dibranchiata*, shows the standard myoglobin-type fold.[55] The D helix is absent and the position usually occupied by the distal histidine is occupied by leucine. This explains the vastly reduced affinity for fluoride, azide, and cyanide.

Work on the structure of human haemoglobin continues. A difference map at 3.5 Å resolution confirms that carbon dioxide reacts with *N*-terminal groups of deoxyhaemoglobin to form carbamate ions. The carbamate group is adjacent to the α-amino-group of the β-chains and there is no conformational change. There is also some carbon dioxide binding behind the β-haem group but this is a minor effect.[56] The binding of inositol hexaphosphate to human deoxyhaemo-

[52] B. W. Matthews and L. H. Weaver, *Biochemistry*, 1974, **13**, 1719.
[53] C. H. Carlisle, R. A. Palmer, S. K. Mazumdar, B. A. Gorinsky, and D. G. R. Yeates, *J. Mol. Biol.*, 1974, **85**, 1.
[54] B. K. Vainshtein, E. H. Harutyunyan, I. P. Kuranova, V. V. Borisov, N. I. Sosfenov, A. G. Pavlovsky, A. I. Grebenko, and N. V. Konareva, *Doklady Akad. Nauk S.S.R.*, 1974, **216**, 690.
[55] E. A. Padlan and W. E. Love, *J. Biol. Chem.*, 1974, **249**, 4067.
[56] A. Arnone, *Nature*, 1974, **247**, 143.

globin is similar to that of 2,3-diphosphoglycerate, which it replaces as a regulator of oxygen affinity in avian erythrocytes. One molecule binds per tetramer, in the central cavity between the *N*-termini of the β-chains, such that the cyclohexane ring is parallel to the dyad axis. There are widespread disturbances to the tertiary structure of the β-subunits.[57] The changes in structure in the abnormal human haemoglobin San Diego [β 109(G11) Val → Met] show that its abnormally high oxygen affinity and impaired co-operativity arise from the perturbations of the $\alpha_1\beta_1$ subunit interface.[58] One inter-subunit hydrogen bond is perturbed and this would tend to shift the equilibrium between the oxy and deoxy quaternary structures in favour of the oxy structure, leading to a higher oxygen affinity and lower co-operativity.

A paper by Perutz[59] explains the denaturation of haemoglobin by alkali in terms of the three-dimensional structure. Denaturation by alkali is activated by the ionization of buried, weakly acidic side-chains. Species variations are largely accounted for by the replacement of one buried tyrosine and two cysteines. In general the stability of a three-dimensional structure will decrease on either side of the isoelectric point as the net surface charge changes. In addition the ionization of internal groups will lead to formation of hydration shells which will not fit into the structure and shift the equilibrium towards the unfolded form. The most likely mechanism for the denaturation of haemoglobin is dissociation of the $\alpha\beta$ dimer into monomers, which exposes the cysteines allowing them to become hydrated and shifting the monomer–dimer equilibrium in favour of the monomer form. This would be followed by the unfolding of the chain from the carboxyl end which allows the buried tyrosine to become ionized and hydrated.

A series of three papers by Perutz and co-workers[60–62] interprets various spectral changes observed in haemoglobin in terms of the effect of the globin structure on the state of the haem group. In haemoglobins which have the *R*-type quaternary structure the haems are in the same state as in free α- and β-chains. In the *T* structure the state of the haems is modified so as to affect their spectra and lower their ligand affinities. This modification includes a lengthening of the iron–nitrogen bond distances which leads to an increased displacement of the iron from the plane of the porphyrin ring. Thus the displacement of the iron from the plane of the ring should be greater in tetrameric haemoglobins in the *T* state than in monomeric ones such as myoglobin. All the evidence is consistent with the notion that the allosteric equilibrium of haemoglobin is governed primarily by the displacement of the iron and the proximal histidine from the plane of the porphyrin ring, although in the β-subunits the steric effect of the ligand itself plays a part. The interaction between haem and globin is a reciprocal one. Coboglobin, the cobalt analogue of haemoglobin, also shows co-operativity. Studies of model cobalt–porphyrin systems lead to an estimate of 0.38 Å for the maximum movement of the proximal histidine residue relative to the mean plane

[57] A. Arnone and M. F. Perutz, *Nature*, 1974, **249**, 34.
[58] N. L. Anderson, *J. Clin. Invest.*, 1974, **53**, 329.
[59] M. F. Perutz, *Nature*, 1974, **247**, 341.
[60] M. F. Perutz, J. E. Ladner, S. R. Simon, and C. Ho, *Biochemistry*, 1974, **13**, 2163.
[61] M. F. Perutz, A. R. Fersht, S. R. Simon, and G. C. K. Roberts, *Biochemistry*, 1974, **13**, 2174.
[62] M. F. Perutz, E. J. Heidner, J. E. Ladner, J. G. Beetlestone, C. Ho, and E. F. Slade, *Biochemistry*, 1974, **13**, 2187.

of the porphyrin ring on the oxygenation of coboglobin, which is only half that observed in haemoglobin. Ibers *et al.* suggest that an additional stereochemical basis for co-operativity in coboglobin may be the transition of the initially non-planar porphyrin ring to essential planarity on oxygenation.[63]

7 Structures of other Globular Proteins

The structure of oxidized *Clostridial* flavodoxin, which contains 138 amino-acid residues, has been determined at a resolution of 1.9 Å.[64] The mean figure of merit was 0.81, and the structure contains a central parallel β-pleated sheet plus four helices. The structure is very similar to that of the semiquinone form. The isoalloxazine ring is planar as expected and is in contact with a tryptophan and a methionine. The mutual arrangement of the protein and ribityl phosphate is nearly identical with that found for *Desulfovibrio vulgaris* flavodoxin. However, the orientation and interactions of the flavin ring are different. The structure of oxidized chromatium high potential iron protein at 2.0 Å has been refined to give an *R*-factor of 0.24. The way in which the side-chain of Tyr-19 abuts the central Fe_4S_4 cluster is reminiscent of the structure of bacterial ferredoxin. The difference Fourier synthesis of the oxidized minus reduced structures shows that the reduced Fe_4S_4 cluster shrinks slightly upon oxidation and that the symmetry of the oxidized cluster is closer to tetrahedral than is that of the reduced structure. There is a slight rearrangement of the non-polar side-chains, including Tyr-19, and comparison with the structure of other redox proteins suggests that Tyr-19 plays an active role in electron transport.[65, 66]

Human plasma prealbumin is a tetramer of identical subunits, each of molecular weight 13 500 and containing 124—128 residues. The structure, determined at a resolution of 2.5 Å using three isomorphous heavy-atom derivatives giving a mean figure of merit of 0.65, shows that each subunit contains two extensive β-sheets each consisting of four strands.[67] Half the residues are in these mainly antiparallel sheets. The monomer, which also contains one short α-helix plus loops of extended chain, forms a prolate ellipsoid with the β-sheets forming a large part of the surface. Dimers are formed by further antiparallel β-sheet interactions such that each dimer has two eight-stranded β-sheets. In the tetramer the pleated sheets in each dimer are prevented from coming into contact with each other by a short loop of chain from each monomer that forms the major dimer–dimer interactions. These opposed, but separated sheets form the surface of a large slot that runs through the centre of the molecule. The molecule seems to be built on the same structural principles as concanavalin A and the immunoglobulins in that the monomer–monomer interactions are through the extension of β-sheet structures and dimer–dimer interactions are through the opposition of equivalent β-sheets at right angles. Prealbumin transports both a hormone and a vitamin in serum. Thyroxine is bound directly while retinol (vitamin A alcohol)

[63] J. A. Ibers, J. W. Lauher, and R. G. Little, *Acta Cryst.*, 1974, **B30**, 268.
[64] R. M. Burnett, G. D. Darling, D. S. Kendall, M. E. LeQuesne, S. G. Mayhew, W. W. Smith, and M. L. Ludwig, *J. Biol. Chem.*, 1974, **249**, 4383.
[65] C. W. Carter, J. Kraut, S. T. Freer, N.-H. Xuong, R. A. Alden, and R. G. Bartsch, *J. Biol. Chem.*, 1974, **249**, 4212.
[66] C. W. Carter, J. Kraut, S. T. Freer, and R. A. Alden, *J. Biol. Chem.*, 1974, **249**, 6339.
[67] C. C. F. Blake, M. J. Geisow, I. D. A. Swan, C. Rerat, and B. Rerat, *J. Mol. Biol.*, 1974, **88**, 1.

is bound indirectly through a protein–protein interaction involving its specific carrier molecule. In solution there is one binding site for thyroxine and there are four for retinol binding protein. In the crystal there are two symmetry-related binding sites for thryoxine. These lie on the crystallographic two-fold axis and deep in the central slot. The two sites are *ca.* 10 Å apart and it is not clear whether both sites can be occupied simultaneously. Both concanavalin A and the immunoglobulin fragments form a single binding cavity by opposing the concave faces of their β-sheets; prealbumin appears to form a double binding cavity by opposing the convex faces of the β-sheets.

Thioredoxin is a protein involved in hydrogen transport. It is reduced by NADPH, and contains 108 amino residues of known sequence. The functional part of the molecule is the disulphide bridge in the sequence Cys-X-Y-Cys.

The space group of the crystals of the enzyme from *E. coli* is *C*2, with two molecules in the asymmetric unit, and the structure has been determined at the intermediate resolution of 4.5 Å.[68] The molecules are ellipsoidal in shape (25 × 34 × 35 Å) and form an infinite layer parallel to the *xy* plane. The basic unit is a dimer formed by the interaction of two molecules across the crystallographic dyad, and the cupric ions which are necessary for crystallization are involved in the formation of the layer structure.

Progress is being made with the structure of Met-tRNA synthetase from *E. coli* and two isomorphous heavy-atom derivatives have been reported. They are $Na_3UO_2F_5$, which binds at three sites, and $K_2Pt(CN)_4$, which binds at four sites.[69]

8 Viruses

Preliminary work has been published for two viruses. The cowpea strain of the southern bean mosaic virus crystallizes in the space group *R*32 with $a = 923$ and $c = 299$ Å. There are three virus particles in the rhombohedral unit cell so that the maximum particle diameter is 284 Å. The virus contains 21% RNA by mass and 180 protein subunits each of molecular weight 29 000 ($T = 3$). The diffraction pattern extends to 3 Å resolution.[70] The optical diffraction technique applied to electron micrographs of alfalfa mosaic virus has shown a cylindrical P_6 lattice for the protein coat of the virus.[71]

Considerable progress has been made by Harrison *et al.*[72] who have published a map of tomato bushy stunt virus at 16 Å resolution.

Several papers have been published on the structure of tobacco mosaic virus. The disk of TMV protein is an essential intermediate in the self-assembly process. It consists of two rings of 17 subunits which both point in the same direction. The disk crystallizes in the space group $P22_12_1$ with $a = 228$, $b = 224$, and $c = 174$ Å, and the 17-fold rotation axis of the disk is almost parallel to the

[68] B.-O. Söderberg, A. Holmgren, and C.-I. Brändén, *J. Mol. Biol.*, 1974, **90**, 143.
[69] C. Monteilhet, C. Zelwer, and J.-L. Risler, *F.E.B.S. Letters*, 1974, **46**, 101.
[70] J. E. Johnson, M. G. Rossmann, I. E. Smiley, and M. A. Wagner, *J. Ultrastructure Res.*, 1974, **46**, 441.
[71] J. E. Mellema and H. J. N. van der Berg, *J. Supramol. Structure*, 1974, **2**, 17.
[72] S. C. Harrison, A. Jack, D. Goodenough, and B. M. Sefton, *J. Supramol. Structure*, 1974, **2**, 486.

c-axis. An isomorphous derivative was prepared by treating Cys-27 with methylmercury nitrate, and the heavy atom was found to be at a radius of 60 Å which agrees well with that of 56.2 Å found in the helical structure of the complete virus.[73] A combination of *X*-ray crystallography and electron microscopy showed that the asymmetric unit was one disk of molecular weight 600 000 and that the crystal was composed of a body-centred array of stacks comprising two disks each.[74] The electron density in the three centrosymmetric projections was calculated at 6 Å resolution, and a three-dimensional map calculated by making use of the non-crystallographic symmetry. This showed the disk to be polar so that all the subunits face the same way; the structure can be compared with that of the intact virus produced by interaction with nucleic acid. Certain common features could be identified, including the site of the nucleic acid. The effective resolution of the map is 16 Å in the plane of the disk and 6 Å perpendicular to it.[75] Finch and Klug showed that the *X*-ray diffraction pattern from a well-oriented sol of a stacked disk rod aggregate of TMV had helical parameters consistent with it being a perturbed form of a stacking ring variant of the single helical polymer,[76] and Unwin and Klug have published a three-dimensional image reconstruction of the stacked aggregate at 12 Å resolution.[77] Mandelkow and Holmes[78] have exploited specific chemical modifications of TMV coat protein to produce new heavy-atom derivatives. These labelled the amino-terminus which is on the outside of the particle at a radius of 88 Å and Lys-68 at a radius of 72 Å. These derivatives can be used for phase determination but are also necessary as markers in order to trace the polypeptide chain in an electron-density map of limited resolution. Cys-27, Cys-139, and the carboxy-terminus had been labelled already.

9 Fibrous Proteins

Polypeptides.—An electron-diffraction study of crystals of a β-modification of polyglycine I, monoclinic space group, $a = 9.54, b = 7.04, c = 3.67$ Å, $\beta = 113°$, showed it to have an antiparallel rippled sheet structure.[79] The β-structure of periodic copolypeptides of L-alanine and glycine consists of antiparallel chains which form a good model for group II of silk fibroins.[80] Harget and Krimm[81] have investigated the macromolecular structure and organization of alpha-keratin and have calculated a *z*-axis projection at 21 Å resolution from the small-angle equatorial *X*-ray scattering. This calculation gave a structure with a diameter of 80 Å, corresponding to the 80 Å diameter microfibrils. The core region is surrounded by a peak at a radius of 28 Å.

Collagen.—Piez and Miller have reviewed the structure of collagen fibrils with an emphasis on molecular packing.[82] Two groups have studied the *X*-ray

[73] R. Leberman, J. T. Finch, P. F. C. Gilbert, J. Witz, and A. Klug, *J. Mol. Biol.*, 1974, **86**, 179.
[74] J. T. Finch, P. F. C. Gilbert, A. Klug, and R. Leberman, *J. Mol. Biol.*, 1974, **86**, 183.
[75] P. F. C. Gilbert and A. Klug, *J. Mol. Biol.*, 1974, **86**, 193.
[76] J. T. Finch and A. Klug, *J. Mol. Biol.*, 1974, **87**, 633.
[77] P. N. T. Unwin and A. Klug, *J. Mol. Biol.*, 1974, **87**, 641.
[78] E. Mandelkow and K. C. Holmes, *J. Mol. Biol.*, 1974, **87**, 265.
[79] B. Lotz, *J. Mol. Biol.*, 1974, **87**, 169.
[80] B. Lotz, A. Brach, and G. Spach, *J. Mol. Biol.*, 1974, **87**, 193.
[81] P. J. Harget and S. Krimm, *Biopolymers*, 1974, **13**, 257.
[82] K. A. Piez and A. Miller, *J. Supramol. Structure*, 1974, **2**, 121.

diffraction pattern from collagen. Hosemann *et al.* suggest that the microfibrils in the collagen of native rat-tail tendon are eight-stranded ropes, one-quarter staggered and packed together in an orthorhombic lattice with paracrystalline distortion between adjacent tropocollagen molecules. These octafibrils react under stress like synthetic elastomers with a 135 Å periodicity of hard segments.[83] Fraser *et al.* have indexed the diffraction pattern on the basis of a tetragonal unit cell, space group $P4_1$ or $P4_3$ with $a = 2 \times 38.47$ and $c = 4 \times 670$ Å. All the inter-microfibril contacts are quasi-equivalent and the authors state that the microfibrils are five-stranded ropes with helical symmetry packing as described above.[84]

Knight and Hunt have examined the meridonal and equatorial reflections from a fibril structure of collagen-like protein found in association with other proteins in the inner layer of the egg capsule wall of dogfish.[85]

Muscle.—The *X*-ray pattern obtained when ADP is bound to myosin in glycerol-extracted muscle fibres without causing detachment of the cross-bridges was identical with the rigor pattern, showing that the angle of attachment of the cross-bridges was the same as in rigor muscle.[86] Studies of the cross-bridge arrangement in *Limulus* (horseshoe crab) muscle showed that the structure in rigor was similar to that in insect flight muscle except that the thick filaments are staggered. The myosin filaments in relaxed muscle bear a highly ordered helical array of cross-bridges which is very labile.[87] Study of the *X*-ray diffraction pattern of mammalian heart muscle in the living resting state and in rigor showed the presence of a hexagonal lattice of filaments. The intensity changes, which were similar to those observed in vertebrate skeletal muscle, indicated a similar mechanism for cross-bridge attachment. The poor order in resting muscle compared with that found in skeletal muscle could suggest a small amount of actin–myosin interaction.[88] Shoenberg and Haselgrove investigated the *X*-ray diffraction pattern of a vertebrate smooth muscle (*taenia coli* from adult guinea-pigs) equilibrated at 37 °C after the muscle had been relaxed. Contrary to previous reports they found no evidence for a ribbon structure, and suggest that the observed reflections could have been due to the presence of collagen and actin.[89]

Filamentous Bacterial Viruses.—Marvin and co-workers have studied the structural protein of the filamentous bacterial viruses, which are linear assemblies of protein subunits encapsulating single-stranded circular DNA. These viruses are interesting not only as systems for studying morphogenesis and transport of macromolecules across membranes, but also as models for the structure of more complicated linear assemblies of proteins. Their diffraction patterns contain much information and can be analysed in detail. The diffraction patterns of the Pf1 and Xf strains of filamentous bacterial viruses (Class II) can be interpreted in terms of a simple helix of protein subunits with a 15 Å pitch, having 22 units in

[83] R. Hosemann, W. Dreissig, and Th. Nemetschek, *J. Mol. Biol.*, 1974, **83**, 275.
[84] R. D. B. Fraser, A. Miller, and D. A. D. Parry, *J. Mol. Biol.*, 1974, **83**, 281.
[85] D. P. Knight and S. Hunt, *Nature*, 1974, **249**, 379.
[86] C. D. Rodger and R. T. Tregear, *J. Mol. Biol.*, 1974, **86**, 495.
[87] J. S. Wray, P. J. Vibert, and C. Cohen, *J. Mol. Biol.*, 1974, **88**, 343.
[88] J. Matsubara and B. M. Millman, *J. Mol. Biol.*, 1974, **82**, 527.
[89] C. F. Shoenberg and J. C. Haselgrove, *Nature*, 1974, **249**, 152.

five turns. The subunits are elongated in an axial direction and slope radially, the arrangement being reminiscent of the scales on a fish. The protein helix forms a tube of inner diameter *ca.* 20 Å and outer diameter *ca.* 60 Å. The single-stranded circular DNA is contained within the tube, two strands running the length of the tube. The B protein, which is the major component, contains 50 amino-acids. It is largely α-helical with the long axis of the helix parallel to the long axis of the virion.[90] The *X*-ray diffraction patterns of the fd, If1, and IKe strains (Class I) show the capsid proteins in the virion to be in a left-handed helix, with a pitch of 15 Å and 4.5 units per turn. Again the units are elongated in the axial direction and overlap. They are related to the Class II helix by a small twist about the helix axis.[91] The radial distribution of electron density at a resolution of 15 Å has been calculated for both classes of virions from the fibre diffraction patterns.[92] The map shows a cylindrical molecule with a high-density DNA core of diameter 25 Å surrounded by a shell of protein with inner diameter 40 Å and outer diameter 60 Å. Thus both Class I and Class II virions are helices encapsulating the nucleic acid.

Murein Layer of Bacteria.—Murein, which is the rigid component of almost all bacterial cell walls, consists of polysaccharide chains carrying short peptides of alternating D- and L-amino-acids. These peptides have a 2.2_7 helical conformation. They are linked to the carbohydrate chains through their amino-terminus and form two hydrogen bonds with the sugar residues. There is an angle of *ca.* 150° between the carbohydrate and peptide chains.[93]

Bacterial Flagella.—Bacterial flagella are cylindrical structures a few μm in length and 120 to 300 Å in diameter. They are visible in the light microscope as flagellar bundles along which travel helical waves of displacement, with wavelength 2—2.5 μm. *X*-Ray data from oriented, pulled fibres show that the subunits are arranged in irrational helices with close to 11 subunits in two turns.[94]

Antiparallel cross β-structure is predominant in the *P* filaments from *Salmonella* flagellins. No order exists in the arrangement of the flagellin molecules.[95]

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

edited by R. H. Pain, with contributions by E. A. Carrey, C. E. Johnson, P. Knowles, D. Osguthorpe, G. R. Penzer, H. W. E. Rattle, B. Robson, R. M. Stephens, and E. J. Wood

1 Theoretical Aspects of Protein Conformation

Contributed by B. Robson and D. Osguthorpe

Introduction.—During the past months there has been considerable interest in matters relating to the packing of non-polar side-chains in the interior of

[90] D. A. Marvin, R. L. Wiseman, and E. J. Wachtel, *J. Mol. Biol.*, 1974, **82**, 121.
[91] D. A. Marvin, W. J. Pigram, R. L. Wiseman, E. J. Wachtel, and F. J. Marvin, *J. Mol. Biol.*, 1974, **88**, 581.
[92] E. J. Wachtel, R. L. Wiseman, W. J. Pigram, D. A. Marvin, and L. Manuelidis, *J. Mol. Biol.*, 1974, **88**, 601.
[93] H. Formanek, S. Formanek, and H. Wawra, *European J. Biochem.*, 1974, **46**, 279.
[94] C. Gonzalez-Beltran and R. E. Burge, *J. Mol. Biol.*, 1974, **88**, 711.
[95] K. Wakabayashi, T. Yamaguchi, and H. Kagawa, *J. Mol. Biol.*, 1974, **83**, 545.

globular proteins. Evidence continues to accumulate concerning the overall importance of such packing in the generation and maintenance of the globular conformation. It is therefore pertinent to consider some publications concerned with more general aspects of molecular packing and water structure, and it is notable that many of these come from workers whose ultimate interests are in protein structure.

This review will also cover related topics not covered by the previous review [1] in this series.

Techniques for Calculating Conformational Energies.—Techniques for calculating the conformations of organic molecules in general have been reviewed by Golbiewski and Parczewski.[2] The two principal approaches are the use of quantum mechanical procedures and empirical energy functions. Many groups continue to concentrate on the use of just one of these approaches. The argument in favour of the use of quantum mechanical techniques is that they would be very much more accurate if the proper basis parameters and approximations were used. The arguments in favour of empirical energy functions is that they are more obviously refinable in the light of new experimental evidence, and that the conformational energies of large molecules can be calculated very rapidly. Golbiewski and Parczewski also present a critical discussion of the quantum mechanical PCILO method of Pullman and colleagues, who have carried out an extensive investigation of the conformations of amino-acid residues.[1]

Interactions between the atoms in a molecule may conveniently be classified into covalent (or bonding) interactions and non-covalent (or non-bonding) interactions. Covalent interactions concerned with covalent bond formation generally require high energies for distortions from equilibrium values. Hence most workers retain the concept of a fixed geometry for their molecules, conformational freedom being limited to rotations around single bonds. The highly accurate neutron diffraction studies [3, 4] of amino-acids and their analogues provide assignments of precise bond lengths, valence angles, and those torsion angles which are effectively invariant. Bond lengths involving hydrogen atoms should, however, be treated with caution, because it is the centre of the repulsing electron cloud and not the position of the nucleus which is of interest in conformational energy calculations. Studies of this kind also give some idea of the variations in torsion angles which are normally considered invariant. For example, the partial double bond of the peptide group is normally considered as planar and *trans* with the somewhat less stable *cis* configuration achievable only by the negotiation of a *ca.* 20 kcal barrier. Small distortions can, however, be expected, and Hiltner and Hopfinger [5] have called further attention to some of the consequences of this for polypeptide conformation.

Non-covalent interactions, on the other hand, generally require low energies for distortions from equilibrium values, excepting when there is considerable

[1] A. T. Hagler and B. Robson, in 'Amino-acids, Peptides, and Proteins', ed. R. C. Sheppard (Specialist Periodical Reports), The Chemical Society, London, 1975, Vol. 6, p. 206.
[2] A. Golbiewski and A. Parczewski, *Chem. Rev.*, 1974, **74**, 519.
[3] T. F. Koetzle, L. Golic, M. S. Lehmann, J. J. Verbist, and W. C. Hamilton, *J. Chem. Phys.*, 1974, **60**, 4690.
[4] M. N. Frey, T. F. Koetzle, M. S. Lehmann, and W. C. Hamilton, *J. Chem. Phys.*, 1973, **58**, 2547.
[5] W. A. Hiltner and A. J. Hopfinger, *Biopolymers*, 1973, **12**, 1197.

overlap between atoms. The form of the interactive potential must therefore be treated with care, at points well away from the point of equilibrium as well as close to it. The van der Waal's contribution can generally be represented in the empirical function method by a function of two or three atom-dependent parameters, say A_{ij} and B_{ij}. Here subscripts i and j represent the nature (*e.g.* sp^3 carbon, sp^3 nitrogen) of the pair of interacting atoms. One way of obtaining such parameters is first to derive a set of parameters A_i, A_j which are dependent only on one atom, and then to use a combining rule to obtain A_{ij} from A_i and A_j. Similar considerations apply to B_{ij} and other parameters of the empirical van der Waal's function. Kong [6] has pointed out that a simple combining rule based on the geometric mean, *e.g.* $A_{ij} = \sqrt{(A_{ii}.A_{jj})}$, is inaccurate for atoms which differ considerably in size. He has investigated more suitable combining rules for a number of model potentials, namely the Morse,[7] 6–12,[7] 6-exp,[8] and Dymond–Alder [9] potentials. Cohen and Pack [10] have considered combining rules for higher coefficients of the van der Waal's energy equation which are, however, normally neglected by protein theoreticians. A search for realistic potentials directly applicable to polypeptides and proteins is being undertaken by Hagler and Lifson,[11] Nelson and Hermans,[12] and by Shipman *et al.*[13]

Interaction of Water with Polypeptides and Proteins.—The origin of the hydrophobic interaction lies in the structure of water, and is largely an entropy-driven reaction involving the loss of ordered water when two non-polar groups come into contact. The phenomenon has been discussed by Tanford in his recent book,[14] although the emphasis here is largely on applications to micelle formation and membrane phenomena. The structure of water itself continues to be of extreme interest, because it would seem unlikely that the changes of water structure associated with the hydrophobic effect can be fully understood without a detailed understanding of solute-free water. The simplest possible kind of water structure is a water dimer, and Shipman, Owicki, and Scheraga [15] have studied the structure and energetics of water dimers by empirical energy functions, while Daudey [16] has used *ab initio* quantum mechanical calculations. Lentz and Scheraga [17] have applied LCAO–SCE MO calculations to water polymers, and Abraham [18] has also investigated water clusters of various sizes.

Brickenkamp and Panke [19] have continued their experimental determinations of water structure with an investigation of hydrated n-propylamine. Experimental studies of water in a hydrophobic cavity have been carried out,[20] and

[6] C. L. Kong, *J. Chem. Phys.*, 1973, **59**, 968.
[7] C. L. Kong, *J. Chem. Phys.*, 1973, **59**, 2464.
[8] C. L. Kong and M. R. Chakrabarty, *J. Phys. Chem.*, 1973, **77**, 2668.
[9] C. L. Kong, *J. Chem. Phys.*, 1973, **59**, 1953.
[10] J. S. Cohen and R. T. Pack, *J. Chem. Phys.*, 1974, **61**, 2372.
[11] A. T. Hagler and S. Lifson, *Acta Cryst.*, 1974, **B30**, 1336.
[12] D. J. Nelson and J. Hermans, *Biopolymers*, 1973, **12**, 1269.
[13] L. L. Shipman, A. W. Burgess, and H. A. Scheraga, *Proc. Nat. Acad. Sci. U.S.A.*, 1975, **72**, 543.
[14] C. Tanford, 'The Hydrophobic Effect', Wiley, New York, 1974.
[15] L. L. Shipman, J. C. Owicki, and H. A. Scheraga, *J. Phys. Chem.*, 1974, **78**, 2055.
[16] J. P. Daudey, *J. Quantum Chem.*, 1974, **8**, 29.
[17] B. R. Lentz and H. A. Scheraga, *J. Chem. Phys.*, 1974, **61**, 3493.
[18] F. F. Abraham, *J. Chem. Phys.*, 1974, **61**, 1221.
[19] C. S. Brickenkamp and D. Panke, *J. Chem. Phys.*, 1973, **58**, 5284.
[20] A. Wishnia and S. J. Lappi, *J. Mol. Biol.*, 1974, **82**, 77.

investigations of denaturation of polymers not related to polypeptides should illuminate the complex effects of denaturants on proteins.[21] Increasing attention is being given to the analogy between hydrophobic bonding and water surface effects. Hence Reynolds, Gilbert, and Tanford[22] have explored the empirical correlation between hydrophobic free energy and the aqueous cavity surface formed by the non-polar solute, while Chothia[23] has examined a similar relationship for amino-acid side-chains.

Hydrophobic interactions are, however, not the only way in which polypeptide conformation is affected by solvent. The fact that polypeptide hydrogen bonds can form to water as well as to each other has important consequences for the structure and stability of proteins. It should be recalled that two factors are involved here, (i) the difference in free energy between an intramolecular and a polypeptide–water hydrogen bond, which determines the contribution of the hydrogen bond to the conformational stability of the polypeptide, and (ii) the difference between an intramolecular hydrogen bond and no hydrogen bond, which determines the structuralizing effect which hydrogen bonds have within the interior of a globular protein. Using i.r. spectroscopy, Henson and Swenson[24] have calculated the enthalpies and entropies of interaction of *NN*-dimethylformamide and *NN*-dimethylacetamide with such solvents as water, ethanol, propan-2-ol, and phenol, and have obtained enthalpies in the range -3.2 to -7.6 kcal mol^{-1}. The free energy of transfer from water to ethanol has been of particular interest because the interior of a protein has been considered analogous to an ethanol-like solvent, and this will allow the effects of different kinds of solvent to be compared. Solvents which increase the α-helicity of artificial polypeptides, presumably partly because the free energy of the hydrogen bond to such a solvent is higher than of that to water, have been further studied. Generally the expectation is borne out that the less polar the solvent, the more stable the intramolecular hydrogen bonding.[25] Nevertheless, a certain critical length of polypeptide is required even in a highly non-polar environment, before α-helices will form. Becker and Naider[26] have recently shown that in trifluoroethanol, seven-residue polymethionine is required in order that a c.d. spectrum characteristic of α-helix be produced. Similar results were previously obtained with different side-chains, for example, polyalanine also requires seven residues for stable α-helix formation.[27] Finally, it has been shown that formation of an intramolecular hydrogen bond may replace only some of the water surrounding the hydrogen-bonding group. Pullman and Pullman[28] have studied this problem by quantum mechanical methods. They show that a hydrogen bond in a formamide dimer has an internal energy of -6.4 kcal mol^{-1} in the absence of water, but that in the presence of water two sites are still available for water binding, having internal energies for hydrogen-bond formation of -5.7 and -7.1 kcal mol^{-1}, respectively. In an

[21] P. Dubin and U. P. Strauss, *J. Phys. Chem.*, 1973, **77**, 1427.
[22] J. A. Reynolds, D. B. Gilbert, and C. Tanford, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 2925.
[23] C. H. Chothia, *Nature*, 1974, **248**, 338.
[24] D. B. Henson and C. A. Swenson, *J. Phys. Chem.*, 1973, **77**, 2401.
[25] T. Matsumoto and A. Teramoto, *Biopolymers*, 1974, **13**, 1347.
[26] J. M. Becker and F. Naider, *Biopolymers*, 1974, **13**, 1747.
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[28] A. Pullman and B. Pullman, *Quart. Rev. Biophys.*, 1974, **7**, 505.

α-helical polypeptide the less stable water-binding site is lost for steric reasons, while in the interior of a globular protein all water-binding sites will usually be blocked.

Structure and Folding of Globular Proteins.—The importance of efficient packing of groups in the interior of a globular protein has been emphasized by Richards.[29] Chothia[30] extended this work to show that the loss of accessible surface area by monomeric proteins on folding, and hence approximately the gain in stability due to hydrophobic bonding, is a simple function of molecular weight. Further, his analysis reveals that the proportion of polar groups forming intramolecular hydrogen bonds is roughly constant from protein to protein.

Levitt and Warshel[31] have carried out a computer simulation of the folding of pancreatic trypsin inhibitor, using a simplified model for the structure of a polypeptide chain. The side-chains were represented by single dummy atoms and the interactions between them were computed using, amongst other things, hydrophobic parameters obtained from amino-acid solubilities in ethanol.[32] Backbone hydrogen bonding was neglected, but approximate agreement with the known *X*-ray structure was obtained in some of the computer simulations. Trouble was experienced, however, in forming the α-helical region of trypsin inhibitor, and this region had to be started off in the helical conformation in order that even a rough approximation to an α-helix be obtained. Without treatment of backbone hydrogen bonding, it seems likely that this approach will only work for proteins which, like trypsin inhibitor, have little α-helix.

Local Interactions in Globular Proteins.—Local interactions between groups close together in the primary sequence are partly responsible for the formation of such secondary structure features as α-helices, β-pleated sheets, and reverse turns. Studies emphasizing hydrophobic effects but neglecting backbone hydrogen bonding are not likely to yield satisfactory explanations of such features. The importance of the hydrogen bond between the j'th and $(j+3)$'th peptide unit has been emphasized by the study of Hiltner and Walton[33] on formation of a β-bend. The importance of the hydrogen bond between the j'th and $(j+4)$'th unit has been confirmed by Styme *et al.*[34] in their quantum mechanical CNDO/2 calculations on four linked alanine units.

Analysis of secondary structure in globular proteins can be carried out by a statistical technique using an expected information approach.[35] By means of this approach, the importance of local interactions within[36] and between[37] residues has been investigated. Further, the possibility that local interactions may be important in the formation of a wide variety of kinks and turns in the polypeptide backbone has been examined.[38] The investigation of interactions between

[29] F. M. Richards, *J. Mol. Biol.*, 1974, **82**, 1.
[30] C. H. Chothia, *Nature*, 1975, **254**, 304.
[31] M. Levitt and A. Warshel, *Nature*, 1974, **253**, 694.
[32] Y. Nozaki and C. Tanford, *J. Biol. Chem.*, 1971, **246**, 2211.
[33] W. A. Hiltner and A. G. Walton, *J. Mol. Biol.*, 1975, **92**, 567.
[34] H. Styrne, G. Wettermark, R. Shor, and C. W. David, *J. Phys. Chem.*, 1973, **77**, 3033.
[35] B. Robson, *Biochem. J.*, 1974, **141**, 853.
[36] B. Robson and R. H. Pain, *Biochem. J.*, 1974, **141**, 869.
[37] B. Robson and R. H. Pain, *Biochem. J.*, 1974, **141**, 883.
[38] B. Robson and R. H. Pain, *Biochem. J.*, 1974, **141**, 899.

residues suggests that hydrogen-bonding side-chains can act as helix terminators (in either *N*- or *C*-terminal directions as appropriate) by competing for hydrogen bonding with the backbone. This conclusion is also supported by the analysis by Ptitsyn [39] of invariant features of primary structure in 67 globins.

The current view is that important secondary structure features, particularly right-hand α-helices and β-pleated sheets, are fairly strongly determined by local interactions but that certain requirements concerning their packing into the tertiary structure must be met. These aspects have been considered by Lim in a model [40] and algorithm [41] for the prediction of these secondary structure features. However, many predictive procedures can be applied with considerable success despite that fact that they do not explicitly take account of many of the features which Lim considers necessary for stable tertiary packing.[42]

Studies on Polypeptides Not Related to Globular Proteins.—Many polypeptides which differ from proteins in some important structural aspect still provide an important testing ground for conformational energy calculations. Cyclic oligopeptides continue to attract attention because of their limited degrees of conformational freedom. Ramachandran and his colleagues [43] have carried out calculations on cyclic (Pro-Gly)$_3$, while Grebow and Hooker [44] have investigated L-alanyl-L-histidine diketopiperazine. Polymers of alternating D- and L-amino-acids also do not occur in globular proteins, but provide particularly useful test cases for calculating local interactions. Helical hydrogen bonds between the j'th and $(j+4)$'th peptide units are still possible in such polymers [45] and experimental studies on such helices have been performed.[46] A statistical mechanical study of optical rotation by such polymers has also been carried out.[47]

Homopolymers of L-amino-acids differ from the polypeptide chains of globular proteins only in side-chain homogeneity, and in the past many useful results applicable to globular proteins have been derived from them. This approach continues with an investigation of β-poly-L-lysine as a model for biological self-assembly.[48]

Conclusions.—Theoretical studies on globular proteins are entering a new and exciting phase. While consolidation of techniques and refinement of parameters continues, several publications in recent years show a shift of interest towards not only providing realistic methods, but also providing the simplest and fastest methods which will hopefully achieve realistic results. Although the more ambitious and optimistic investigations require further substantiation, it may be

[39] O. B. Ptitsyn, *J. Mol. Biol.*, 1974, **88**, 287.
[40] V. I. Lim, *J. Mol. Biol.*, 1974, **88**, 857.
[41] V. I. Lim, *J. Mol. Biol.*, 1974, **88**, 873.
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[48] R. Hartman, R. C. Schwaner, and J. Hermans, *J. Mol. Biol.*, 1975, **90**, 415.

possible in the foreseeable future to obtain predictions of unknown protein conformations from their amino-acid sequences, which are not only credible, but calculable in reasonable computer time. Of course, such predictions are not the sole goal of the theoretical approach. Energy refinement of *X*-ray-derived structures is a potentially very powerful tool, and work in this direction continues.[49]

2 Folding of Globular Proteins

Contributed by E. A. Carrey

The Two-state Transition.—Some small globular proteins can unfold co-operatively apparently in the absence of stable intermediates, and many thermodynamic parameters have been obtained which are consistent with this type of transition. Assuming the two-state approximation to hold for ribonuclease, lysozyme, α-chymotrypsin, and β-lactoglobulin with intact disulphide bonds, linear extrapolation of data from the urea- and GuCl-mediated denaturation of these proteins[50] leads to estimates of $\Delta G_{app}^{H_2O}$ for the native structure very much lower than values reported previously from similar work.[51] For lysozyme, for instance, related to both urea- and GuCl-denatured forms, $\Delta G_{app}^{H_2O}$ is apparently 5.8 kcal mol^{-1}, while estimates using Tanford's solubility data are 6.6 and 9.1 kcal mol^{-1}, respectively. The free energy of stabilization (ΔG^0) of lysozyme obtained from scanning microcalorimetric studies of heat denaturation[52] is *ca.* 15 kcal mol^{-1} at 25 °C. The experiments using this technique have been able to confirm the essentially two-state behaviour of the transition in many proteins, with $< 5\%$ deviation from ideality, resulting from highly unstable intermediates. More details of the technique have been summarized by Privalov.[53] The thermodynamics of unfolding of α-lactalbumin in GuSCN have been investigated.[54]

A truly two-state transition is expected to result in superimposable denaturation curves for all parameters observed with respect to the denaturing conditions. This is seldom the case, for instance, when n.m.r. is used to study resonances from several residues in a protein molecule. The temperature-unfolding of ribonuclease[55] appears to involve a 'loosening' of one part of the molecule outside the major unfolding transition, and the urea- and GuCl-denaturation has at least two intermediates in the process.[55] The order in which these residues are exposed to the solvent is similar in most of the unfolding processes in ribonuclease. The behaviour of the arginine residues during heat-denaturation of lysozyme leads to similar conclusions involving a loosening of the surface structure prior to the major transition.[56]

Optical techniques may also be used to separate the equilibria of conformational transitions, as in the case of bovine carbonic anhydrase B, whose denaturation by GuCl can be separated into an initial 'loosening' phase, followed by the

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[55] F. W. Benz and G. C. K. Roberts, *J. Mol. Biol.*, 1975, **91**, 345, 367.
[56] J. H. Bradbury and R. S. Norton, *Internat. J. Peptide Protein Res.*, 1974, **6**, 295.

unfolding of the backbone and exposure of aromatic side-chains to the solvent.[57] The acid-denaturation of this protein,[58] although it does not result in a random coil, also involves two phases. The presence of zinc(II) appears to stabilize the protein and to speed up the folding process. A fluorimetric kinetic study of the acid-denaturation of human carbonic anhydrase B[59] suggests that several ionic bonds are ruptured in an expansion to the activated state in the unfolding process. The rate of unfolding obeys first-order kinetics at low enzyme concentrations. The reversible unfolding step is followed by a slower irreversible step whose rate is reduced by the presence of zinc(II).

Theory of Nucleation and Folding.—A recent computer simulation of the forces involved in protein folding[60] has been used successfully to 'renature' a small protein, bovine trypsin inhibitor, from a disordered structure to a conformation very similar to that of the native protein. The model does not depend on the ability of regions of secondary structure to direct or nucleate subsequent chain folding, but uses the time-average of the short-range forces which act at a longer range to direct folding into a compact approximation of the native globule. Later stages involve interatomic forces in a packing process which has been compared with crystallization, and which, in the computer, involves very complex energy minimization.

The observation of intermediates (reviewed by Baldwin[61]) in experimental studies of protein transitions leads to the concept that a number of 'local' transitions may be taking place, some of which are not more than a slight loosening of surface groups (on larger proteins than trypsin inhibitor), while others contribute to the major co-operative change leading to denaturation. In the reaction leading to the folding of the protein, much theoretical work points to short-range interactions between amino-acid residues being important early in the process, with more long-range effects determining the arrangement of nuclei and the general topology of the polypeptide chain. Nagano[62] has described a sequence in which α- and β-'candidates' are involved in nucleation events, maturing into regions where α-helix and β-sheets are often found adjacent to each other. Ptitsyn's model,[63] which he used to predict the self-organization of helices in myoglobin, visualizes fluctuating nuclei or embryo structures at an early stage, the most stable of which are favoured by long-range interactions and are found in the native structure. The most stable theoretical structure for myoglobin results from independent self-organization from two centres followed by a collapse of the structure into a globular molecule, resembling the known tertiary structure. The existence of globular regions and domains in proteins where the independent folding centres have not annealed together was discussed last year.[64]

[57] K.-P. Wong and C. Tanford, *J. Biol. Chem.*, 1973, **248**, 8518.
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[63] O. B. Ptitsyn, *Studia Biophysica*, 1973, **40**, 159.
[64] E. A. Carrey, in 'Amino-acids, Peptides and Proteins', ed. R. C. Sheppard (Specialist Periodical Reports), The Chemical Society, London, 1975, Vol. 6, p. 214.

The final stage in folding (as described by Nagano [62]) is a close-packing process in which the strong requirement for invariant or conservative primary sequences is especially evident. The computer simulation previously described [60] includes this process, and Lim [65] also describes the 'architectural' constraints on protein structure in terms of interactions between residues and between the polypeptide chain and water. The importance of an interaction in the structure must determine whether its disruption will cause a local movement or a major conformational transition.

The great speed at which polypeptide α-helices have been observed to form in solution suggests that although they are undoubtedly important components of nuclei and of the native structure, the helix-forming reaction is not the rate-limiting process in the folding transition. However, the growth of β-structure in a solution of poly-L-Lys follows so well a process of nucleation and growth that it is suggested as a model of protein self-assembly;[66] the unusual and 'biological' feature of the transition is that the growth stops when a particle size of *ca.* 100 units is obtained. Aggregation paralleling β-sheet formation has been observed in the pH-induced transition of poly-L-Tyr.[67]

Influence of Sequence on Conformation.—*Variations in Protein Sequence.* A mutation which causes an alteration in the primary sequence of a protein is an important biological event, since the structural changes which may result may affect the fitness of the organism to survive. Nevertheless, a wide range of amino-acid sequences is encountered in homologous proteins, some of the most widely studied being haemoglobins [68] and cytochromes.[69, 70] In most cases the amino-acid substitutions are too complex for the origin of differences in stability to denaturation to be pinpointed, although sometimes [70] conformational changes result in well-defined effects on biological activity.

Synthetic peptides which mimic the sequence (111—125) in bovine ribonuclease [71] were combined with the natural (1—118) peptide and the enzymic activity in the complexes were compared. The Tyr-120 substituted peptide was shown to be more active than that from bovine ribonuclease (Phe-120), and similarly, giraffe ribonuclease, which is naturally Tyr-120, is the more active enzyme.

Covalent Breaks in the Primary Sequence. Recombination of fragments obtained from a protein usually involve some overlapping of sequence (as above, or in some staphylococcal nuclease complexes), although ribonuclease S and nuclease T′ are exceptions. The complementation of fragments (6—48) and (49, 50—149) to form nuclease-T′ has been studied by stopped-flow fluorescence measurements.[72] The presence of specific ligands has no effect on the first-order rate

[65] V. I. Lim, *J. Mol. Biol.*, 1974, **88**, 857.
[66] R. Hartman, R. C. Schwaner, and J. Hermans, *J. Mol. Biol.*, 1974, **90**, 415.
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[70] G. W. Pettigrew and A. Schejter, *F.E.B.S. Letters*, 1974, **43**, 131.
[71] R. S. Hodges and R. B. Merrifield, *Internat. J. Peptide Protein Res.*, 1974, **6**, 397.
[72] A. Light, H. Taniuchi, and R. F. Chen, *J. Biol. Chem.*, 1974, **249**, 2285.

constant. Similar rate constants are obtained for combinations of overlapping peptides; the rate-limiting step may be pre-folding of one fragment before combination, or the folding of a disordered complex of the two peptides.

The complementation of pairs of mutant peptides to form the protomers of β-galactosidase has been shown to result in enzymic activity and a tetramer structure [73] and to be aided by interaction with a specific anti-β-galactosidase antibody.

The two products of cyanogen bromide (CNBr) treatment of soybean trypsin inhibitor, one of them a pair of disulphide-linked peptides, can recombine to give nearly total activity at neutral or basic pH.[74] In the case of the much smaller bovine pancreatic trypsin inhibitor, intact disulphide bonds hold the CNBr products into a conformation favouring the formation of a peptide link between the new *N*-terminus and the carbonyl group of homoserine lactone.[75] The resynthesized molecule can be distinguished by the presence of homoserine instead of methionine in the sequence. A similar phenomenon is observed in horse heart cytochrome *c* [76] where Met-65 has been attacked by CNBr.

Carboxypeptidase treatment of haemoglobins, removing one or two *C*-terminal residues from the α- and β-chains, has been shown to result in loss of haem–haem interaction and some of the Bohr effect;[77] some of the normal behaviour is restored by binding inositol hexaphosphate.

Peptide Fragments Obtained from Proteins. It has been confirmed that the small amount (11%) of enzymic activity found in the (1—126) polypeptide of staphylococcal nuclease [78] is due to a 'native-format' in conformational equilibrium in the peptide and not to contamination by intact enzyme. The largest CNBr fragment from growth hormone [79] has a c.d. spectrum resembling that of the native conformation, and possesses one of the two biological activities of the protein. The smaller β-galactosidase peptide used in complementation studies [73] (mol. wt. 45 000) is equivalent to 30% of the sequence and can fold independently to give the native conformation.

The helical regions present in CNBr fragments from cytochrome *c* are stabilized by chloroethanol,[80] the *C*-terminal helix being more stable than an *N*-terminal helix of similar length. It is believed that some chloroethanol-stabilized helices are those which are distorted by interactions with the haem group in the native protein.

Large peptides (mol. wt. 30 000) can be obtained by short non-specific digestion of bovine serum albumin, with most sites of cleavage being found in the *C*-terminal region. The co-operative unfolding of peptides corresponding to this region supports the idea that it is less compact while the transition of the

[73] F. Celada, A. Ullman, and J. Monod, *Biochemistry*, 1974, **13**, 5543.
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[80] C. Toniolo, A. Fontana, and E. Scoffone, *European J. Biochem.*, 1975, **50**, 367.

N-terminal peptides involves isomerization processes before major unfolding.[81] The reversible heat-denaturation of paramyosin and of a large peptide derived from it indicates that the *N*-terminal region is stabilized by a disulphide bond while the *C*-terminal disulphide bond does not appear to have any stabilizing function.[82]

Folding–Unfolding Transitions.—*Probes of Structural Change.* The unfolding of proteins may be probed by changes in the information received from chemical groups in the protein chain (n.m.r., optical activity, intrinsic fluorescence) or attached to it (in fluorescent and spin-label probes and in optical activity induced in an associated molecule).[83]

pH-jump experiments which have used fluorescence measurements include kinetic studies of the acidic transition of δ-chymotrypsin[84] which does not take place in chymotrypsinogen, and of thyroglobulin[85] at very low protein concentrations, whose acid-denatured form can be further unfolded by GuCl. Slow conformational changes after a rapid change of pH result in inactivation of glutamate dehydrogenase,[86] the inactive form having internal fluorescence quenching. The pH-dependent conformational change in glutamate decarboxylase[87] results in a shift of absorption maxima.

It is always important to know whether a probe is an indicator of local or more general conformational changes. The Trp-59 fluorescence of ferricytochrome *c* has been described as a sensitive measure of the overall protein conformation[88] when observing the unfolding transition which leaves some residual structure in GuCl. The fluorescences of two different labels which did not affect the activity, and of one which inhibited nitrogenase[89] were all found to change with temperature and suggested a conformational change in the enzyme at 20 °C.

Spin-label probes have been used to study the reversible conformational changes in leucine aminopeptidase in urea solution,[90] a temperature-dependent 'swelling' of bovine serum albumin,[91] and conformational changes in phosphorylase *a*.[92]

The conformations and conformational equilibria have been investigated for a series of mammalian growth hormones[93] using c.d. spectra and the change of dichroism at selected wavelengths. The unfolding in GuCl has been tentatively described as a three-state process. Reversible conformational transitions in the β-lactamase from *Bacillus cereus* have been demonstrated[94] by comparison of

[81] M. C. Hilak, B. J. M. Harmsen, W. G. M. Braam, J. J. M. Joordens, and G. A. J. Van Os, *Internat. J. Peptide Protein Res.*, 1974, **6**, 95.
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[94] R. B. Davies, E. P. Abraham, and D. G. Dalgleish, *Biochem. J.*, 1974, **143**, 137.

c.d. spectra of renatured and denatured forms in urea, GuCl, and at various pH and temperatures.

Susceptibility to Enzymic Digestion. Proteins are usually more resistant in the native form than when unfolded to attack by proteolytic enzymes, so that accessibility to proteases can be taken as a measure of the amount of unfolding in the protein under attack. An example was given by the different accessibility of various regions of bovine serum albumin to several proteases.[81]

In the analysis of protease digests of lysozyme [95] a mixture of intact lysozyme and small peptides was found, a result of an all-or-none unfolding equilibrium at temperatures between 30 and 40 °C. The folded N form was favoured and protected by complexing with the inhibitor molecule or by internal cross-linking of lysozyme. The glutamine synthetase from *Bacillus stearothermophilus* [96] is susceptible to hydrolysis by thermolysin above 55 °C, but is stabilized by $MgCl_2$.

Disulphide Bond Formation. Some recent studies of the folding of proteins have used the order of disulphide bond formation as an index of correct tertiary interactions in the polypeptide backbone. The regeneration of reduced lysozyme in a mixture of reduced and oxidized glutathione gave evidence in support of a nucleation mechanism since less than the expected random pairings of disulphides were found in two-dimensional chromatography of digested early intermediates. The fractionated digest from an early stage of the regeneration has 20% of the disulphide groups in the peptide (74—96), which is thus proposed as a good candidate for a nucleation region.[97]

Ribonuclease was reoxidized in a similar glutathione mixture, and peptide mapping by diagonal electrophoresis [98] showed that a first phase of incorrect pairing was followed by a gradual series of internal rearrangements of thiol groups; the native conformation here is evidently accessible from a number of 'incorrect' states.

Bovine pancreatic trypsin inhibitor has been renatured in a process which is first order in concentrations of both inhibitor and oxidized dithioreitol.[99] The intermediates at various stages in regeneration have been separated by polyacrylamide gel electrophoresis;[99] three species with one disulphide bond are in rapid equilibrium, but some of the species with two bonds appear to be kinetically trapped away from the main folding pathway. Diagonal electrophoresis was used to isolate the single-disulphide intermediates in the regeneration process; approximately half contain the 'correct' bond Cys-30–Cys-51, and other incorrect bonds include one of these residues and a Cys residue from an α-helix region.[99] The first correct bond is crucial to the folding of the molecule since it forms a loop through which the backbone is threaded; the pairing may be especially favoured by interactions between a region of β-sheet and a long α-helix formation.

In all cases it is probable that the thiol-interchange reactions are slower than the conformational changes required for the bond-making parts of the backbone to be presented to each other. The concentrations of intermediates at each stage

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of disulphide bonding will then be governed by the relative thermodynamic stabilities of each bond, with single-disulphide intermediates being most mobile

The Influence of Intermolecular Interactions on Folding. The proteins which fold up in the laboratory do so in dilute solution; the mixed solutions found in living cells can contain each protein in much higher concentrations, for instance in the area immediately surrounding the ribosomes where they are synthesized. The temperature-unfolding of albumins has been studied with relation to concentration [100] and while the midpoint and range of the transition are independent of protein concentration, the ΔH for the transition increases linearly at low concentrations until a saturation point is reached. A theoretical model for helix–coil transitions in concentrated and dilute protein solutions is proposed which qualitatively accounts for some of the observations.

The full activity of certain proteins may not be attained without the presence of certain interactions which do not stem directly from the primary sequence of amino-acid residues; such interactions may involve small molecules, or protein subunits forming a quaternary structure.

The reversible cold-inactivation of glyceraldehyde-3P dehydrogenase [101] is prevented by coenzymes bound to the tetramer, evidently stabilizing each dimer against denaturation. The association of refolded subunits requires the presence of NAD^+, and it is suggested [102] that the primary sequence alone does not fully direct the correct folding and interactions of the polypeptide chains.

The refolding of human chorionic gonadotropin from acid-denatured subunits α and β is a second-order process which is necessary before the specific biological activity of the β subunit can be expressed,[103] and the unfolding demonstrates that small structural changes can lead to a much decreased binding affinity between the subunits.

Haem Proteins. A recent series of papers by Perutz and his colleagues [104] has emphasized the very close relationship between the haem group and the globin structure in haemoglobin, changes in either being transmitted through the molecule. The kinetics of conformational changes in haemoglobin following laser photolysis of the liganded CO or O_2 demonstrates the linkage of the haem environment with conformational changes throughout the molecule.[105]

It was noted that the ^1H n.m.r. spectrum of apo-cytochrome *c* shows little ordered structure, in comparison with that of ferricytochrome *c* [106] which unfolds with some detectable intermediates. Evidently the binding of the haem group affects the conformation of part of the apo protein; abolition of some of the available helix-forming ability has been suggested [80] by studies of some large fragments of cytochrome *c* in solution.

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The reaction between protoporphyrin IX and apocytochrome *c* peroxidase [107] has been studied by stopped-flow spectrophotometry; a rapid chemical binding is followed by slower conformational changes.

Immunoglobulins. The combination *in vitro* of heavy (H) and light (L) chains of immunoglobulins is well known even in the absence of interchain disulphide bonds. The kinetics are second order and dependent on temperature, and the rates for this non-covalent association are consistent with the known *in vivo* rate of disulphide bond formation.[108] There is substantial variation in the enthalpy of combination [109] and in the c.d. spectra generated by different pairs of H and L chains.[110] The differences in conformational changes on H–L recombination are ascribed largely to differences in the variable regions of the L chain, and the enthalpy differences to changes in the availability of some interactions. The affinity of the combination is so specific that wide variations were found in the preferences of H chains binding to L chains all of the same variable-region subgroup.[111] In all cases the original pairings of the H and L chains were much more stable than heterologous pairing of closely related chains.

Denaturing Conditions.—When α-lactalbumin is denatured by four different salts [112] it resembles lysozyme [113] in forming several denatured states, the action of different types of denaturant resulting in characteristic denatured forms of the proteins. The different degrees of unfolding brought about by different perturbants is illustrated by GuCl-mediated unfolding of many heat-denatured proteins. The effect of temperature in altering the intrinsic viscosities of proteins already presumed to be in a random coil conformation in solutions of urea and GuCl [114] underlines the complexity of such systems. A stabilizing effect of polyhydric alcohols on three different structured polypeptides has been demonstrated [115] and the additivity of the effects of combinations of two different alcohols on the thermal stability of ribonuclease has been investigated.[116] Rapid complexing of butanol with papain is followed by a slower conformational change leading to inactivation;[117] it occurs at low butanol concentrations and is unlikely to be a solvation effect.

The small univalent ion of lithium appears to exert a general electrostatic effect on the surface residues of bovine serum albumin [118] at low concentrations, with preferential binding of LiCl at concentrations above 4 mol l^{-1}. The conformational effect of this ion may be exerted through ion–dipole bonds to internal carbonyl groups, or through disruption of hydrophobic interactions with methyl groups. The solvation of lysozyme and β-lactoglobulin in aqueous GuCl has been shown to involve preferential binding of denaturant at all

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concentrations;[119] when the proteins are completely denatured, three amino-acid residues are involved in binding each denaturant molecule. The interaction of urea with lysozyme has been studied crystallographically.[120] Essentially the same diffraction patterns are obtained from crystals made from a urea–lysozyme solution and from lysozyme crystals soaked in urea.

Egg albumin has been shown to have two classes of binding site for SDS, saturated at 0.4 and 1.4 g SDS/g protein, respectively.[121] The highly co-operative binding has a small associated enthalpy change, suggesting that the reaction is driven by an increase in solvent entropy as the detergent binds and the alkyl chains form parallel hydrophobic arrays. A cationic detergent [122] has been shown to bind to four discrete sites in bovine serum albumin without causing a conformational change and at higher concentrations to bind co-operatively to the protein and cause denaturation in a similar way to SDS. Very much higher concentrations of cationic detergents are required to exert the same effect as an anionic detergent with a similar alkyl chain; the cationic head group apparently has the disadvantage of more electrostatic repulsion as it approaches the less well exposed anionic surface residues. Tanford's group [123] have described methods for estimating the molecular weight and Stokes radius of proteins in detergent solutions.

3 Nuclear Magnetic Resonance

Contributed by H. W. E. Rattle

As the place of the n.m.r. technique in the biological laboratory has moved into perspective, the number of papers dealing with biological applications has levelled off. The relatively few laboratories which specialize in the application of n.m.r. to peptides and proteins have settled down to the hard biochemical labour necessary before the technique can realize its full potential as a window on structures in solution, and the proportion of biologically useful information revealed by their efforts is growing. The field has been reviewed several times in 1974 [124–126] and another useful introduction to the progress made so far will be found in over 1000 pages of the proceedings of a conference on 'e.s.r. and n.m.r. in biology and medicine' published at the end of 1973.[127] At this conference, most of the important laboratories in the field presented reviews of their most recent work, making it a valuable starting-point for any survey of the potential and actual achievements of magnetic resonance methods.

Techniques.—With the increased availability of Fourier transform spectrometers and the consequent prospective increase in carbon-13 resonance work, much

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ground-work on chemical shifts and coupling constants for ^{13}C is being reported. Chemical shifts and ^{13}C–^{13}C coupling constants for amino-acids enriched to 85% ^{13}C have been published,[128, 129] and it is reported that the coupling constant between the α-^{13}C and β-^{13}C of amino-acids depends on the β-substituent.[129] Theoretical values for ^{1}H–^{13}C and ^{13}C–^{13}C spin–spin splittings as a function of torsion angles in peptide systems have been calculated,[130] while the coupling of ^{13}C in amino-acid carboxy-groups to the β-CH_2 protons has been used[131] to determine rotamer populations in valine and aspartic acid, the results confirming previous resonance assignments. Calculations[132] and experimental calibrations[133] of the effect of torsion angles on proton–proton couplings through three or four bonds are of great interest. Another conformation-revealing technique, the use of paramagnetic ions, has been put to a rather unusual use[134] in that it is claimed that the measurable effects of a chromicyanate $[Cr(CN)_6]^{3-}$ ion binding at the *N*-terminus or a gadolinium Gd^{3+} ion at the *C*-terminus of an oligopeptide may be used to sequence at least six residues from each end by noting the fall-off in the perturbations for each succeeding residue. More conventional uses of paramagnetic probes may be rendered less useful by the observation that, for the commonly used Eu(fod)$_3$ ion, there are several competing binding sites on protected dipeptides.[135]

Peptides and Polypeptides.—In dealing with very small peptides, we note a report[136] that in Ala-Pro, Gly-Pro, and Gly-Hypro dipeptides, the *C*-terminal carboxy-group is less acidic, by *ca.* 0.4 pK_a units, in *trans* than in *cis* isomers. Similar dipeptides, together with proline, *cyclo*-(Pro)$_3$, and a series of proline-containing peptide hormones, have been subjected to ^{13}C longitudinal relaxation time (T_1) measurements[137] to investigate the interconversion between the puckered forms of the proline ring; in some peptides there is severe restriction on the movement of the proline carbon atoms. ^{13}C T_1 has also been measured for Gly-Gly-X-Gly-Gly pentamers (X = Met, Pro, Arg, or Lys) and reported along with isomerism data,[138] while a detailed study of the effect of any natural amino-acid Y on the chemical shift of its neighbours in a TFA-Gly-Gly-Y-Ala-OCH_3 oligopeptide has been presented;[139] the effects amount to a few tenths of a part per million.

The possibility of determining bond angles and isomeric forms makes n.m.r. useful in studying cyclic peptides, and synthetic models for such studies have been investigated. Reports have appeared on *cyclo*-(Pro-Gly)$_n$ (n = 2, 3,

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or 4)[140–142] and on the interaction of single amino-acids with the cyclic peptides.[142] The conformation of *cyclo*-(L-Thr-L-His) has also been discussed.[143]

Large synthetic polypeptide molecules appear to have largely outlived their original usefulness as protein models, and fewer investigations into their structure are being reported. Poly-L-lysine in solution has been found to display no long-range order,[144] while changes in the c.d. parameters of poly-(L-Pro-Gly) have been correlated with changes in the ^{1}H n.m.r. spectrum,[145] though without being able to determine whether *cis–trans* isomerism about the Gly-L-Pro bond or isomerism about the α-C—C′ bond of the proline was responsible for them. A study[146] of poly-N^5-(3-hydroxypropyl)-L-glutamine by ^{13}C resonance reveals again the double-peak phenomenon – two separate resonances for the α-carbon which in this case are attributed to fully random–coil and rapidly-exchanging partly helical segments of the molecule. Other polypeptide studies include the binding of poly-L-glutamic acid to lecithin vesicles,[147] the temperature-induced helix–coil transition of poly-(γ-benzyl L-glutamate),[148] and the behaviour of poly-(benzylthio-L-cysteine) in dichloroacetic acid–deuteriochloroform solvent mixtures.[149]

In the field of natural peptides, oxytocin continues to arouse interest. ^{13}C relaxation times reveal[150] that the backbone α-carbon nuclei of the acyclic part of the molecule are, not surprisingly, more mobile than those of the cyclic part. A study of the effect of pH[151] on the carbon resonances of oxytocin attributes spectral changes on protonation of the *N*-terminal amino-group partly to through-bond and through-space charge effects, and partly to a conformational change, possibly involving the disulphide bridge of the molecule. Oxytocin has also been labelled with ^{2}H and ^{15}N for assignments of tyrosine, leucine, and isoleucine resonances.[152] Proton nuclear magnetic relaxation studies on the interaction of bovine neurophysin II with oxytocin reveal rapid exchange between solution and complex, implicating residues 2 and 3 of oxytocin in the binding mechanism.[153]

A complete ^{13}C assignment of the resonances in the spectrum of gramicidin S-A including carbonyl resonances has been made,[154] while gramicidin A has been subjected to a series of studies including proton and carbon magnetic resonance[155–157] which indicated that there may be three possible conformations,

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all of them amenable to the dimerization which is currently favoured for this molecule. Gramicidin S has been the subject of measurements of vicinal NH–α-CH proton–proton couplings with a view to mapping torsion angles down the chain. The same paper [158] discusses valinomycin and the possibility of isotopic substitution of ^{13}C and ^{15}N, whose spin–spin interaction should provide a useful torsion-angle reporter. The complex between valinomycin and alkali cations has been discussed in two papers,[159, 160] one of which [160] indicates that the α-CH–β-CH dihedral angle is predominantly in the *trans* form throughout the molecule. Experiments (reported in five different journals!) on repeating tetra- and penta-peptides of elastin indicate the possibility of a β-turn as a major element of the conformation of the pentapeptide.[161]

Previously suggested conformations for angiotensin II have come under fire following a new study [162] in which the amide NH resonances were assigned by spin-decoupling in aqueous solution and bond angles determined using the Karplus relation. Clearly, more data are needed before a definitive answer can be achieved. More discussion is also needed concerning the conformation of hypothalamic thyrotropin releasing factor: two papers [163, 164] published concurrently propose structures for this small peptide which differ considerably. Less controversy surrounds a study of trifluoroacetyl derivatives of insulin [165] in which the size of aggregates revealed by sedimentation velocity was closely matched by resonance linewidth observations.

Enzymes.—Perhaps the most active area for biological n.m.r. this year has been in enzymes, and many new subjects have been found. Some favourites remain, however, notably ribonuclease with its convenient S-peptide. Carbon-13 atoms have been incorporated into histidine-12 [166] and glycine-6 [167] of synthetic S-peptide, and useful information obtained following reassembly of the active enzyme. In particular, the pK of His-12, which is 6.77 in the peptide, is reduced to $\simeq 4.3$ in the complete enzyme, a phenomenon which is attributed to the titration of aspartic acid-121. The binding of RNase A to 2′-, 3′-, and 5′-AMP [168] and 3′- and 5′-CMP [169] has also been extensively investigated. The presence of a chloroplatinite ion $(PtCl_4)^{2-}$ in a solution of RNase A has been shown [170] to broaden and shift a single methionine *S*-methyl group, probably that of methionine-29. Alkaline phosphatase has been studied, both by ^{35}Cl resonance with and without

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its normal Zn^{2+} ion [171] and by proton relaxation enhancement when in the form of a copper analogue,[172] in which it is found that at least one water molecule or OH^- group has access to the inner co-ordination sphere of the copper ion. Dehydrogenase enzymes to have received attention include yeast alcohol dehydrogenase [173] and glutamate dehydrogenase [174] both studied by the binding of spin-labels, and lactate dehydrogenase studied in its pyruvate–diphosphopyridine nucleotide complex.[175] The resonance lineshape of a proton coupled to a deuterium in the horse liver alcohol dehydrogenase–propan-2-ol complex has been discussed.[176] Complex formation between some pyrimidine nucleosides and *E. coli* aspartate transcarbamylase with a zinc derivative of its regulatory subunit affects the relaxation times of the nucleotides, revealing that while 5′-methyl cytosine is bound rigidly to the enzyme, thymidine is not.[177] A promising newcomer to the ^1H n.m.r. arena is dihydrofolate reductase, investigated with its coenzyme and *p*-aminobenzoyl L-glutamate.[178] It has been found possible [179] to follow the progress of the β-lactamase catalysed hydrolysis of penicillin by watching the fall in area of the penicillin proton resonances and the concurrent rise in the resonances of the product.

An interesting series of papers [180–182] reports investigations into the mechanism of chymotrypsin. A single proton resonance is found to move 3 p.p.m. upfield with titration and is assigned to the proton in a hydrogen bond between His-57 and Asp-102, part of the charge-relay system of the enzyme which is from Asp-102 to Ser-195 *via* His-57. The proton cannot be titrated in the presence of inhibitors. Surprisingly few rare-earth paramagnetic probe studies have been reported (presumably the difficulties in using such probes or interpreting the results have been greater than anticipated) but work has been reported using gadolinium on yeast phosphoglycerate kinase [183] and staphylococcal nuclease [184] in each case with some elucidation of the dimensions of the active site. Other active-site probes used have been the effect of the zinc ion on ^{35}Cl resonance in carboxypeptidase A,[185] manganese in chicken liver pyruvate carboxylase,[186] and the relaxation of ^{205}Tl (a potassium substitute) in adenosine triphosphatase,[187] which indicates that the transport site for univalent cations is very near the catalytic site of the enzyme. A lysyl residue at the active site of creatine kinase

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has been studied by nuclear Overhauser enhancement and inter-nuclear double resonance (INDOR);[188] this is the only example of these powerful but rather problematical techniques found this year.

Iron-binding and Haem Proteins.—The attraction of iron-containing proteins, with their built-in perturbing centres and great biological importance, is again apparent in the 1974 literature. The exchange rates of slowly exchanging amide groups in an algal ferredoxin have been used to investigate its structure,[189] resulting in the proposal that both helical and pleated-sheet structures are present. The susceptibility of *B. polymixa* ferredoxin has also been reported along with its ^{1}H n.m.r. spectra.[190] Studies of cytochromes this year have been restricted to a comparison between the 220 MHz spectra of cytochrome c_2 from *Rhodospirillium rubrum* with horse heart cytochrome *c* [191] and a series of histidine titration experiments on cytochrome *c* samples from various sources.[192] Also, 220 MHz proton spectroscopy was used to detect restricted rotation of one methyl group in a haem side-chain group of horse ferric myoglobin [193] and the increase in mobility subsequent to the addition of various ionic species, leading to the suggestion that a salt bridge near the edge of the haem group may be responsible for hindering the motion of the methyl group. Haem methyl resonances of myoglobin molecules have been extensively studied and assigned by selective deuteriation of haem groups;[194] three of the four are found at -13, -15, and -28 p.p.m. from DSS, with the fourth probably below -43 p.p.m. The relaxation of water protons in the presence of myoglobin, and proposed relaxation and exchange mechanisms, have been reported,[195, 196] while the interaction of CO_2 with myoglobin and the formation of carbamino adducts have been followed by ^{13}C resonance using enriched $^{13}CO_2$.[197] Similar techniques have been used to investigate the binding of ^{13}CO to various haemoglobins [198] and the interaction of cyanate with normal and sickle-cell human haemoglobin.[199]

The 220 MHz spectrum of myoglobin and haemoglobin shows anomalous temperature effects which correspond to magnetic susceptibility changes and may be discussed in terms of spin equilibrium between low- and high-spin states.[200] A theory of the temperature dependence of low-spin iron(III) porphyrin and haemin systems has been presented.[201] The quaternary structures of normal and mutant haemoglobins, and the effects of mutations on them and on the haemo-

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globin oxygen affinity, have been discussed [202] with reference to a high affinity haemoglobin and for a haemoglobin Chesapeake, α, β_2 mutant.[203]

For the first time, several papers on n.m.r. of lipoproteins have appeared, notably two studies by ^{31}P [204] and ^{13}C;[205] the latter suggests that ionic interactions are of only minor importance in the lipid–apolipoprotein complex formation. Another ^{13}C study [206] proposes a model for the high-density human lipoprotein particle.

Miscellaneous.—The binding of small molecules to macromolecules, long a favourite n.m.r. subject, is this year represented by studies on the binding of deuterium-labelled edta to various proteins, measuring relaxation times by pulsed quadrupole relaxation methods,[207] and on the binding of the pesticide bis(*p*-chlorophenyl)acetic acid to serum albumins.[208] Another favourite, the state of water in biological systems, has seen measurements of the relaxation times of water in protein crystals which reveal that most of the water is in the liquid state,[209] and two studies of water in frog muscle,[210, 211] the latter using ^{17}O resonance and enriched $H_2{}^{17}O$. The melting of a lysozyme crystal has been followed using broad-line ^1H n.m.r.[212] It has been pointed out that in a hydrogen–deuterium exchange experiment on protein amide groups, it is useful to monitor the exchange with i.r. spectroscopy as well as n.m.r.; the exchanges in the two may be correlated and the i.r. spectrum will indicate whether or not each amide proton was involved in a hydrogen bond.[213]

4 Spin Labels

Contributed by P. Knowles

In last year's report,[214] it was noted that many workers were deriving quantitative information, particularly on intersite distances and effector binding constants, from protein spin-label studies. This trend has been continued during 1974. With the increasing interest in membrane-bound proteins, the brief coverage of phospholipid membrane spin labelling does not seem so artificial as in the previous report;[214] information on protein–membrane interactions can be obtained whether the probe is located in the lipid matrix or on the protein. For continuity, a similar format to the 1973 literature coverage will be adopted.

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[213] P. H. Von Dreele and I. A. Stenhouse, *J. Amer. Chem. Soc.*, 1974, **96**, 7546.
[214] P. Knowles, in 'Amino-acids, Peptides and Proteins', ed. R. C. Sheppard (Specialist Periodical Reports), The Chemical Society, London, 1975, Vol. 6, p. 229.

Haemoglobin.—Asakura [215] has attached a spin label to one of the propionic acid groups on the porphyrin ring of haemoglobin. The optical spectrum and oxygen-binding ability were identical with those of native haemoglobin indicating that the spin-labelling had not affected the active centre. Clear spectral differences were observed for the fluoride, cyanide, and azide derivatives of both the spin-labelled haemoglobin and its met form; the changes in lineshape were consistent with different protein conformations in the vicinity of the label. In addition, the amplitude of the nitroxide e.s.r. spectrum was shown to be dependent on the magnetic dipolar interaction with the iron centre; the degree of this dipolar interaction is related to the magnetic moment and the electron spin lattice relaxation time of the iron centre as well as to the distance between the spin label and the iron. Since the low- and high-spin forms of the protein differ in their magnetic moments, one can calculate the ratio of high- to low-spin forms which, for the hydroxide derivative of ferrihaemoglobin, was 53 : 47 at 0 °C; this ratio was temperature dependent.

A second paper [216] compares the properties of haem spin-labelled oxy- and deoxy-haemoglobin. Upon deoxygenation, the spin-label resonance amplitude was decreased to 50% of the original value due to the increased dipolar interaction with the haem in changing from the diamagnetic oxyhaemoglobin form to the paramagnetic deoxyhaemoglobin form. Small but significant changes in lineshape and in the spectral isosbestic points were also observed which suggests that haemoglobin can adopt more than two conformations in the vicinity of the haem during oxy to deoxy transitions. A final observation was that the e.s.r. detectable changes did not parallel optical changes during deoxygenation. These results could not be explained on the basis of the 'sequential model',[217] the 'Allosteric Model',[218] or the 'Generalized Concerted Model' [219] for multi-subunit enzymes but were consistent with a recently proposed 'Tension Model'.[220] The 'Tension Model' considers interactions between subunits in the free as well as ligand bound forms and differs from the sequential model in that the conformation of the *non*-liganded subunit changes in each step as oxygen binds to the partner subunits. In order to explain the lack of correlation between the e.s.r. and optical monitoring of oxygen binding, it must be concluded that the subtle conformational changes reported by the spin label have little effect on the optical absorption spectrum; it may be noted in passing that *X*-ray crystallographic measurements were unable to detect structural differences between acid methaemoglobin and oxyhaemoglobin [221] which are readily detected by spin labelling.[216, 219]

Phosphorylase.—The spin-label method has been successfully used to study conformational transitions in phosphorylase *b*, the inactive form of glycogen phosphorylase.[222, 223] One of the side-achievements of these studies was that

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fully active phosphorylase *a*, with a single spin-label grouping on each subunit, could be prepared. Griffiths, Price, and Radda [224] have now studied the interaction of the effector AMP with spin-labelled phosphorylase *a*. The results were consistent with the 'two state' (concerted) model for allosteric transitions.[218] Extension of these studies to determine the magnitude of conformational changes in both the tertiary and quaternary structure as well as the spatial separation of the active and allosteric sites should be possible. A further publication from this laboratory [225] described studies in which spin-labelled phosphorylase was used to examine the particulate suspension which constitutes 'glycogen particles'; glycogen particles contain phosphorylase *b*, phosphorylase *b* kinase, phosphorylase phosphatase, and other enzymes of glycogen metabolism attached to the polymer glycogen. The authors showed that spin-labelled phosphorylase still undergoes conformational changes when bound in the complex. Although the e.s.r. spectra from spin-labelled phosphorylase *b* bound to the glycogen particle suspension and to one composed solely of glycogen were similar, this was not true of spin-labelled phosphorylase *a*; it was concluded that the phosphorylase *a* is in an unusual environment in the glycogen particle.

Chymotrypsin.—*X*-Ray crystallographic studies [226] have shown that toluene-*p*-sulphonyl fluoride ('tosyl' fluoride) interacts with the active-site serine of several proteases such that the tosyl moiety sits in an aromatic binding pocket in the enzyme. Berliner and Wong [227] have used an extensive series of spin-labelled sulphonyl fluoride derivatives to examine the aromatic binding pockets in α-chymotrypsin and trypsin; if the aromatic pockets are identical in the two enzymes, the amount of immobilization reported by the nitroxide probe should be the same throughout the series. The results clearly showed that the active sites in the two enzymes are different. When indole, a competitive inhibitor for chymotrypsin which has been shown to bind specifically in the aromatic pocket, was added to the various spin-labelled chymotrypsin samples, e.s.r. spectra closely resembling those from trypsin were obtained. This suggests that certain of the spin-labelled sulphonyl fluorides inhibit trypsin other than by occupying the aromatic pocket. A complete analysis of the results for chymotrypsin suggested three binding modes for the spin labels: (i) full occupancy of the aromatic pocket, (ii) partial occupancy of the aromatic pocket, and (iii) binding in a general region of the active sites. Correlation between the *X*-ray diffraction results on these various spin-labelled derivatives, the e.s.r. results, and kinetic inhibitor studies [228] should give interesting information on the question of whether the active-site conformations of these proteases are the same in the crystal as in aqueous solution.

Dehydrogenases.—Sloan and Mildvan [229] have covalently bound 4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidino-oxyl uniquely to the cysteine-43 residue of yeast alcohol dehydrogenase. This modification, which inactivates the enzyme,

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[229] D. L. Sloan and A. S. Mildvan, *Biochemistry*, 1974, **13**, 1711.

allowed a tetramer to be isolated bearing a highly immobilized spin label. The binding of substrates, coenzymes, and inhibitors to the spin-labelled enzyme could be monitored by measuring changes in the longitudinal relaxation rate $1/T_1$ of solvent water protons; on the basis of agreement between the binding constants determined from these data and the K_M or K_I values determined kinetically for the native enzyme, the authors concluded that measurements on the inactivated enzyme were relevant to the native state. From T_1 measurements of six protons, the phosphorus of NADH and the methyl protons of the inhibitor isobutyramide, the distances between the enzyme-bound spin label and these nuclei in the binary (enzyme–NADH) and ternary (enzyme–NADH–isobutyramide) complexes were determined. Using these distances, the geometries of the coenzyme and inhibitor with respect to the nitroxide have been determined. An open conformation for the enzyme-bound coenzyme is indicated with the isobutyramide being located in molecular contact with the face of the dihydropyridine ring and in a suitable orientation for accepting a hydride ion. The validity of this model is supported by n.m.r. and *X*-ray diffraction studies of coenzyme and inhibitors bound to dehydrogenases.[230, 231] The question why the spin-labelled enzyme should be inactive is raised and could perhaps be answered by *X*-ray diffraction studies on the spin-labelled enzyme.

ATPases.—An interesting model for the ATPase mediated transport of Ca^{2+} across the sarcoplasmic reticulum, whereby the enzyme molecule rotates or inverts, has been proposed.[232] This model has now been tested by treating the ATPase located in sarcoplasmic reticulum membrane vesicles with thiol-directed spin labels.[233] The enzyme could be held at different intermediate states of the ATPase cycle or set to catalyse the steady-state process by suitable modification of the metabolic conditions and the amount of spin label exposed to the aqueous medium on the interior or exterior side of the vesicle membrane determined by ascorbate reduction. The results were consistent with the simple rotatory model.[232]

Detailed studies on the reconstitution of the sarcoplasmic reticulum calcium transport pump from defined protein and lipid components are being undertaken.[234–236] It is the authors' intention to introduce spin-label probes into these reconstituted membranes which would allow an analysis of how protein–lipid interactions are involved in the biological process. The procedures developed in these studies for replacing the physiological phospholipid environment by ones of known composition should be applicable to other membrane protein systems.

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Harris, Rosing, and Slater [237] have spin-labelled the SH groups in mitochondrial ATPase and the ATPase inhibitor using maleimide spin labels. No loss of biological activity resulted from the labelling which involved approximately one thiol group per mole of enzyme. The e.s.r. spectra from the labelled ATPase, however, showed two components which indicates non-specific binding. Increased immobilization when the ATPase bound to the mitochondrial membrane was observed; in addition, the inhibitor binding site to the ATPase was affected.

Muscle.—Troponin, the Ca^{2+} receptor in the muscle contractile system, has been shown [238] to be composed of three components designated T, I, and C. Both I and C have been spin-labelled to the extent of *ca.* one mole per mole of protein with maleimide spin label.[239] Slight changes in the spectrum of spin-labelled C (indicated as C*) occur on binding Ca^{2+}; when C* was bound to T, a markedly increased immobilization of the spin label resulted. The changes in the e.s.r. spectrum of the C*–T complex on adding Ca^{2+} were even more dramatic, the spin label being more highly immobilized in the presence of Ca^{2+}. A similar, though less dramatic, change occurred when C was combined with I. The authors concluded that the conformation of C changes on binding to either T or I and that the interaction of Ca^{2+} with C is increased by either of the other components.

Polypeptide Elongation Factor.—The polypeptide elongation factor (E.F.Tu) catalyses a GTP-dependent binding of aminoacyl-tRNA to ribosomes. E.F.Tu has one thiol group essential for GTP (or GDP) binding and one for interaction with aminoacyl-tRNA: the latter thiol has been specifically modified by reaction with maleimide spin label in the presence of GDP and conformational changes in E.F.Tu have been examined.[240] The e.s.r. spectrum of the spin-labelled E.F.Tu GDP complex markedly changed when the GDP was replaced by GTP; these changes demonstrate that a reversible conformational change occurs in E.F.Tu near the active site induced by the GDP into GTP conversion.

Slow Tumbling.—E.s.r. spectra of nitroxides are insensitive to slow rotational motion, *i.e.* with $\tau_R > 10^{-6}$ s. The ability to study slow rotational motion has important biological implications when one is considering large proteins in solution, moderate-sized proteins in fluid membranes or for diffusion within a phospholipid membrane. Hyde and coworkers [241, 242] have described how double-resonance techniques (ENDOR and ELDOR) can be applied to the study of slow tumbling. However, in some cases, more detailed interpretation of the e.s.r. spectrum could provide the required information. Mason and Freed [243] have described a method which involves measuring the linewidth variation in the outer extremes of the spin-label spectrum; the outer extremes near to the rigid limit arise from the few nitroxyl radicals which have their π-orbitals nearly parallel to the applied field direction and are thus resolved from overlap with the spectra from the majority of nitroxides which have orientations intermediate between the parallel

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[238] S. Ebashi, *J. Biochem.* (*Japan*), 1972, **72**, 787.
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[242] M. D. Smirgel, L. R. Dalton, and J. S. Hyde, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 1925.
[243] R. P. Mason and J. H. Freed, *J. Phys. Chem.*, 1974, **78**, 1321.

and perpendicular field directions. Decreased rotational motion increases both the width and separation of the extremes, the width being affected first. It is unlikely that the method would give reliable values for $\tau_R > 10^{-5}$ s: here the double-resonance techniques [241, 242] would be required.

Other work from Freed's laboratory [244] is directed towards the interpretation of e.s.r. spectral lineshapes in the slow tumbling region in terms of various models for molecular reorientation. Although the treatment was sophisticated, satisfactory computer fits to the experimental spectra were obtained from models based on free diffusion, Brownian diffusion, or diffusion by molecular jumps. The authors conclude that considerations other than lineshapes would be required to distinguish between these models.

Mason, Polnaszek, and Freed [245] have developed the approach suggested by Wee and Miller [246] to analyse the motional effects on e.s.r. spectra of spin labels undergoing very anisotropic rotational relaxation in solution. In particular, the case of a nitroxyl undergoing rapid motion about a single bond whilst the macromolecule to which it is attached is reorienting slowly was considered; obviously this case is of great importance for spin labels non-rigidly attached to proteins, since it prevents accurate distance information being derived. Spin-labelled polybenzylglutamate was used as a model during these studies.

Keith, Horvat, and Snipes [247] have shown that the hyperfine splittings and g values for nitroxides containing ^{15}N ($I = \frac{1}{2}$ system) are quite distinct from those measured from the corresponding ^{14}N nitroxide. In principle, this should allow the use of ^{14}N and ^{15}N spin labels for experiments where two events are monitored simultaneously. The paper describes the effects of restricted rotational motion and nitroxide–nitroxide interactions for both ^{14}N and ^{15}N spin labels: it would appear that longer τ_C values could be accurately determined using the ^{15}N nitroxide which would be useful for the study of slow tumbling.

Biradicals.—Mention was made in last year's report [214] of the advantages of spin labels with large hyperfine splittings. Michon and Rassat [248] have considered one type – a biradical which has a large *dipolar* splitting. The authors point out that the large dipolar splitting would simplify the analysis of lineshapes in solution (a problem with clear biological implications as has been discussed with regard to the work of Freed's group [244]) and show how values for τ_C between 10^{-11} and 10^{-7} s can be calculated for the biradical and how information about solvation can be derived through a consideration of the effective volume (including solvation layer) of the biradical in solution.

Metzner, Libertini, and Calvin [249] have studied the effects of solvation and temperature for a series of nitroxide biradicals and commented on spin exchange in relation to the relative orientations of the two nitroxide groupings. Their results suggest that participation by the groupings which link the nitroxide rings is of great importance in determining exchange energies; this effect is in addition to the direct (through-space) exchange mechanism which is the basis for the use of biradicals as 'molecular rulers'.

[244] G. V. Bruno and J. H. Freed, *J. Phys. Chem.*, 1974, **78**, 935.
[245] R. P. Mason, C. F. Polnaszek, and J. H. Freed, *J. Phys. Chem.*, 1974, **78**, 1324.
[246] E. L. Wee and W. G. Miller, *J. Phys. Chem.*, 1974, **77**, 182.
[247] A. Keith, D. Horvat, and W. Snipes, *Chem. Phys. Lipids*, 1974, **13**, 49.
[248] J. Michon and A. Rassat, *J. Amer. Chem. Soc.*, 1974, **96**, 335.
[249] E. K. Metzner, L. J. Libertini, and M. Calvin, *J. Amer. Chem. Soc.*, 1974, **96**, 6515.

Griffith and co-workers [250] have also discussed the use of two spin-label groupings to provide information on the geometries and distances between binding sites in biomolecules. In prototype studies, the biradical 1,4-didoxyl-cyclohexane was trapped in single crystals of the corresponding diamine. Detailed single-crystal orientation studies showed that two conformations of the biradical are present; these have proved to be the equatorial–equatorial and axial–axial chair forms of the *trans*-isomer.

Membranes.—*Membrane-bound Proteins.* Griffith and co-workers have extended their spin-label investigations of boundary lipid in cytochrome oxidase [251] to other components of the respiratory chain;[252] for cytochrome b_5, it was found that the intact detergent-extracted protein bound lipid strongly but that the active segment of protein, released through trypsin treatment of the membrane, did not interact with lipid. This suggests that the hydrophobic part of the protein penetrates into the membrane whilst the polar section, which is removed by the trypsin treatment, protrudes into the aqueous phase.

An interesting example of the restricted action of membrane protein is that of rhodopsin. Rhodopsin is deeply embedded in the photoreceptor membrane and has been shown by high-speed flash photometry [253] to be rotating about an axis perpendicular to the bilayer surface with a rotational relaxation time of *ca.* 20 μs. A similar value for τ_R was deduced indirectly by Trauble and Sackmann.[254] In addition to rotation, rhodopsin has been shown to undergo lateral diffusion. Poo and Cone [255] have calculated a lateral diffusion coefficient for membrane-bound rhodopsin of 3.5×10^{-9} cm^2 s^{-1} from the results of experiments in which a thin strip in the plane of the membrane was photo-bleached and the consequent intermixing of the bleached and unbleached rhodopsin molecules observed spectrophotometrically. Delmelle and Pontus [256] have attached the spin-label 3-maleimide-2,2,5,5-tetramethyl-1-pyrrolinyloxyl to thiol groups in rhodopsin and determined a correlation time $> 2.5 \times 10^{-7}$ s for the membrane-bound protein; the value could be made more precise perhaps by the use of the e.s.r. analysis procedures of Freed [243] or by double-resonance methods.[241, 242] The studies of Delmelle and Pontus [256] have also been handicapped by the presence of both mobile and immobile components in their spin-label spectra; in view of their non-specific labelling technique, this could be due to the location of the spin label in different chemical environments.

Interactions between dimyristoyl lecithin and the apo-protein from high-density lipoprotein have been studied using fatty acid spin-label probes.[257] The results are interpreted in terms of a model for the complex consisting of a lipid bilayer partially coated with protein and with some intercalation of the protein into the bilayer.

[250] O. Rohde, S. P. Van, W. R. Kester, and O. H. Griffith, *J. Amer. Chem. Soc.*, 1974, **96**, 5311.
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[252] P. J. Dehlinger, P. C. Jost, and O. H. Griffith, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 2280.
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Lateral Phase Separations. Fox and co-workers[258] have extended their spin-labelling studies[259] of lateral phase separations in *E. coli* membranes to cytoplasmic membranes isolated from mouse LM cells. The spin label used was a nitroxide derivative of decane which would probably not orient in the bilayer. Four discontinuities at 15, 21, 31, and 37 °C were observed in Arrhenius plots which were interpreted on the basis of lateral phase separations. Arrhenius plots made from physiological data on these membranes (for example, concanavalin binding, hormone-stimulated adenyl cyclase activity, and ATPase activity where the sites of action would be expected to involve the outer monolayer, the inner monolayer, and the whole bilayer, respectively) lead the authors to conclude that the temperatures of 21 and 37 °C define the boundaries for lateral phase separations in the *inner* monolayer and 15 and 31 °C the boundaries for lateral phase separations in the *outer* monolayer. Between the two boundary temperatures, the lipid hydrocarbon chains have enhanced lateral compressibility which can be used to trigger or facilitate membrane protein-mediated processes; in addition, between the boundaries, one has lipid fluidity without loss of bilayer organization.

Similar spin-label studies have been made on mitochondrial membranes[260] where species-specific discontinuities at two temperatures coincided with changes in Arrhenius plots of succinate oxidase activity. Presumably the spin label was protected from biological reduction during these studies, for example by addition of ferricyanide. Baldassare *et al.*[261] have in fact used the rate of spin-label reduction in *E. coli* cytoplasmic vesicles to suggest that spin-labelled stearic acids are reduced *via* thiol groups associated with the succinate–cytochrome b_1 section of the respiratory chain.

Grant, Wu, and McConnell[262] have studied lateral phase separations in binary lipid mixtures of dielaidoyl lecithin (DEL) and dipalmitoyl lecithin (DPL) by spin-labelling and freeze fracture electron microscopy, and discussed their results in terms of a binary phase diagram. Thus a 50 : 50 mixture of the two lipids would be fluid down to 33 °C even though pure DPL gels at 41 °C; quench freezing from a temperature of 36 °C gives smooth-faced freeze fracture patterns whilst freezing from below 33 °C gives a 'ribbed' freeze fracture pattern. The extent of the ribbed features increases down to the gel temperature for DEL (15 °C). At temperatures between 15 and 41 °C, the fluid regions are presumably rich in DEL whilst the gel regions are rich in DPL.

Lateral Expansion. Marsh[263] has used the interacting spin-pair method to demonstrate that lateral expansion occurs in dipalmitoyl lecithin multibilayer films at the phase-transition temperature. The distance of spin-label probe separation (r) rises rapidly as the system is taken through the phase transition. It follows that in the transition region there will be a high *isothermal* compressibility which could, for example, facilitate the insertion of proteins into the

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membrane and enhance the activity of membrane-bound enzymes. The effects of cholesterol on 'lateral expansivity' ($dr/d\tau$) have also been studied,[263] and reveal a co-operativity in the molecular interactions between cholesterol and the membrane; this might also be true of other membrane components, including proteins.

Fluidity Gradients. Seelig and Seelig[264] have prepared selectively deuteriated lipids to investigate membrane structure and dynamics. The deuterium quadrupole splittings of dipalmitoyl lecithin bilayers have been measured, the results suggesting that the order parameters stay constant for most of the hydrocarbon chain length but decrease at the bilayer centre; further, there is evidence that the two fatty acyl chains in dipalmitoyl lecithin are not equivalent. Hubbell and McConnell,[265] using phospholipids selectively spin-labelled down one of the acyl chains, also found that the order parameter decreased towards the bilayer centre, but their results indicated a regular decrease throughout the length of the chain which contradicts the Seeligs' findings. It appears probable that the spin labels are perturbing the system. The constancy of the order parameter found by the Seeligs[266] suggests that *gauche* conformations can occur only in complementary pairs which leave the hydrocarbon chains essentially parallel to each other, *i.e.* well ordered. These *gauche* pairs produce a defect or 'kink' in the bilayer which might be involved in the movement of molecules across membranes.[264] Marsh[266] has calculated the energy separation between *trans* and *gauche* conformations from temperature-variation studies of spin-labelled oriented multibilayers of egg lecithin to provide further evidence for the existence of 'kinks'. Statistical mechanical calculations by this same author[267] also confirm the existence of 'kinks'.

Permeability. The permeability of biological membranes to ions and neutral molecules depends on both the head-group charge and on the hydrophobic barrier presented by the phospholipid alkyl chains. The shape of the hydrophobic barrier across the membrane can be mapped using spin labels in which the nitroxide group is located at different depths in the membrane; measurements of the isotropic splitting factor in a variety of solvents showed a marked polarity dependence which has been used by Griffith and co-workers[268] to estimate the polarity profile both in model membranes and in a liver microsomal preparation. The results also indicated that significant water penetration into the bilayer occurs and this would be expected to participate in permeability processes.

5 Fluorescence

Contributed by G. R. Penzer

The disclaimers listed in the opening paragraph of last year's Report[269] are again appropriate.

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Theory, Methods, and Techniques.—*Technical Developments.* The problem of comparability between the fluorescence results obtained using different uncorrected instruments remains. The general uncertainty is illustrated by the lack of complete agreement over the quantum yield of even one of the most common fluorescence standards, quinine bisulphate. A recent report [270] gives a value of 0.561 ± 7% which agrees with the original work, but does not support a subsequent contention that the earlier value is too high by 20%. It is therefore encouraging that serious attempts are being made to design instruments capable of measuring absolute fluorescence spectra. One such fluorometer, using a pyroelectric detector and incorporating the capacity for measuring phosphorescence, absorption spectra, and polarization of fluorescence as well as fluorescence emission, has been described and evaluated.[271] Other instruments with particular merits (precise detection of small changes in a large total signal,[272] sensitivity to very weak emissions,[273] and use of a tunable laser for excitation [274]) have also been described. A technique for determining the absolute emission quantum yields of powdered samples has been discussed.[275] An article (in two parts) discussing fluorescence and phosphorescence and comparing some of the more common commercial instruments has been published.[276]

The use of sophisticated control systems in fluorometers is becoming more common. For example, computer-linked instruments for the measurement of the polarization of fluorescence have been reported.[277] A microspectrofluorometer has been designed to circumvent the problem of photochemically induced fading by having two illumination systems.[278] One, at low intensity, is used during setting up the sample and the other, at high intensity, is used during the rapid recording of measurements. The instrument incorporates a computer-based system for correcting spectra for variations in instrument sensitivity with wavelength. A number of other studies applying microfluorometry have been described, and applications to the study of single cells are increasing.[279]

A common problem in biochemical fluorometry is that different components of the system have overlapping spectra which cannot be resolved simply by judicious choice of excitation and emission wavelengths. A technique of selective modulation has been described which can help when problems of this kind arise.[280] Imagine two emitters with overlapping absorption spectra. If the excitation wavelength for fluorescence is chosen to be equal to the absorption maximum of one species (λ_0), and is modulated (with frequency F) through an

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amplitude such that at all times $\lambda_0 \pm \Delta\lambda$ is either greater or less than the wavelength of maximum absorption of the second fluorophore, then emission due to the first species is modulated with frequency $2F$ whilst that due to the second has frequency F. The two signals can be distinguished by using a suitable lock-in detection system. When signal-to-noise ratio is high, the recording of derivative luminescence spectra also improves the resolution of different emissions.[281]

The recent interest in insolubilized enzymes and affinity chromatography has prompted the design of a flow cell suitable for the study of fluorometry of proteins bound to agarose.[282] A temperature-jump apparatus with detection by fluorescence intensity and polarization has been described.[283]

Several new instruments capable of measuring fluorescence lifetimes have been reported. Time resolutions in the picosecond range are becoming more common,[284, 285] so that events during the lifetime of the excited state but prior to fluorescence emission can be investigated. Suggestions have been made for ways to correct for variations in photomultiplier response with wavelength[286] and also to overcome drift problems.[287] The use of a cavity-damped argon ion laser as an excitation source has been described,[288] and the advantages of time-resolved spectroscopy (on a nanosecond scale) for separating mixed emissions and filtering out Rayleigh and Raman scatter effects have been shown.[288, 289]

Newer Methods. The year 1974 saw the growth of measurements of circularly polarized luminescence (CPL – the fluorescence analogue of circular dichroism in absorption spectroscopy). Various theoretical discussions have been published.[290] The method has considerable potential in, for example, the evaluation of conformation differences between ground and excited states (see pp. 209 and 211).

Another new method is the detection of c.d. by fluorescence.[291] The technique rests on the fact that for most fluorophores the excitation spectrum parallels the absorption spectrum. The advantage of the method is that it allows study of the dichroism of a single fluorescent chromophore in a mixture of optically active but (under the conditions of the measurement) non-fluorescent species.

The conceptual basis, theory, and experimental realization of a technique called 'fluorescence correlation spectroscopy' have been reported.[292] The method involves studying the rates of decay of spontaneous concentration fluctuations in a defined but open volume of solution (such as the volume illuminated by a

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laser beam during a fluorescence measurement). Rates of diffusion can be mesaured, and the method can also be used to investigate chemical reactions.

The optical detection of magnetic resonance has been used to investigate the triplet states of some flavins and flavoproteins.[293]

Quenching of Fluorescence. It has been known for many years that fluorescent emissions are quenched by many ions and paramagnetic species, but the precise mechanisms of the effects are still subject to research, and a simple universal theory remains elusive. Although the efficiencies of various lanthanide ions as quenchers of indole fluorescence parallel their capacities as electron acceptors [with a 100-fold difference between europium(III) and dysprosium(III)][294] a similar trend is absent in the quenching by inorganic anions.[295] Charge effects are important, and when emitter and quencher carry charges of opposite sign quenching can be 10 times greater than predicted for a diffusion controlled reaction.[296] This suggests that interpretation of quenching results for proteins would usually benefit if the effects of at least two quenchers (bearing opposite charges) were considered. Quenching of protein fluorescence by caesium ions [297] and by *N'*-methylnicotinamide chloride [298] have been documented.

The use of O_2 as a quencher for protein fluorescence was described last year.[269] Its efficiency in quenching various aromatic molecules has now been investigated.[299] Although the interaction can be diffusion controlled with some compounds the rate constant falls to $< 7 \times 10^8\ l\ mol^{-1}\ s^{-1}$ for steric and electronic reasons. Other radicals are also good quenchers, and di-t-butyl nitroxide has been shown to quench both singlet and triplet states indiscriminately.[300]

Concentration quenching of fluorescence is commonly observed, and its various causes have been discussed.[301] Interactions between emitter and solvent can also have profound consequences.[302] Such effects are crucial in determining the properties of the popular fluorescent probes, the *N*-arylnaphthylamine-sulphonates. Members of this class are still sometimes called hydrophobic probes, but more evidence has accrued that solvent polarity is less important than molecular rigidity (see earlier reports in this series for other evidence) in determining the emission characteristics of these (and similar) molecules.[303]

Interest in using the optical properties of triplet states to report on their environments in biological systems has revived. The phosphorescence emissions of Trp and proteins, and their enhancement (at low concentrations) or quenching (at high concentrations) by silver ions, have been studied using aqueous snows

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(containing 10% v/v methanol).[304] In these conditions gross denaturation (such as occurs using organic glasses) is avoided. The singlet and triplet lifetimes of poly-Phe and its model compound, 3-phenylpropionamide, have been reported.[305] Triplet probes offer advantages over fluorescent probes in some circumstances because their greater lifetimes permit the study of slower motions.[306]

Analysis of Results. The rapid increase in the application of emission measurements during the past decade has led to the collection and interpretation of experimental results sometimes moving ahead of a proper theoretical understanding. An example has been the use of resonance energy transfer to measure the separation between a donor and an acceptor. The Förster formula includes an orientation factor, κ^2, which has usually been taken to be $\frac{2}{3}$ (the value expected for random relative orientations) in the absence of any evidence to the contrary. The weakness of this approximation has been exposed,[307, 308] and it has been argued that in order to put sensible limits to the value of κ^2 it is very helpful to measure the time-dependent fluorescence anisotropy of the system. It was nonetheless felt that an attempt to measure the separation between two emitters on a tRNA by energy transfer gave a value which could be assigned no more precisely than in the range 3.4—6.1 nm.[308]

Other developments have concerned the estimation of binding parameters from fluorescence titration curves by curve fitting,[309] a numerical method for calculating the fluorescence polarization of a protein-bound ligand in a mixture of free and bound species,[310] correction for inner filter effects during front surface excitation and emission studies,[311] and improvements to the analyses of fluorescence decay kinetics by the least squares[312] and Laplace transform[313] methods.

General Comments. The impression gained from reading the output of papers on fluorescence during 1974 is that the increased ease with which events on a nanosecond time-scale can be studied is having two main effects. First, the growing capacity to resolve the emissions of mixtures into the contributions of individual components is increasing the detail in which studies by fluorescence of conformation and molecular interactions can be interpreted. Second, there is an improving understanding of the properties of excited states, and the ways in which these properties may differ from those of the ground state. This is important because although it is ground-state properties that are generally biochemically relevant, it is the excited-state behaviour which fluorescence studies detect. Thus, for example, it has been shown that molecular relaxation

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occurs during the excited state lifetime of pepsinogen,[314] and that the folded forms of the model compounds *cyclo*-Gly-Trp and *cyclo*-Ala-Trp are less stable in the excited state than the ground state.[315] CPL has shown that the environment of the dansyl group bound to antidansyl antibodies is asymmetric in the excited state, though no asymmetry was detected by normal circular dichroism.[316]

Fluorescent Probes.—Some of the studies involving fluorescent probes are summarized in Table 1, on p. 214.

Covalent Labels. Fluorescein isothiocyanate (FITC) and dansyl chloride remain two of the most widely used fluorescent labels. A novel application of FITC has been in studying the mobility of proteins in erythrocyte membranes.[317] First the membrane proteins were allowed to react with FITC. Then a single cell was observed and subjected to intense illumination (which photobleached the fluorescein) over about half its area. Subsequently the rate at which unreacted emitters diffused into the bleached area was investigated. Little diffusion was detected over 20 min.

When dansyl chloride is complexed to cyclohepta-amylose prior to reaction, an increased yield of labelling under aqueous conditions can be achieved.[318] The normal specificity of dansyl chloride is for amino-groups, but it can react with Tyr residues which have first been nitrated and then reduced (to amino-tyrosine). Both Tyr residues in soybean trypsin inhibitor were modified in this way, and the two emissions from the labelled product could be distinguished from each other.[319] A number of derivatives have also been reported in which the dansyl group is linked to a reagent with chemical specificity for thiols (Table 1).[320–323] At least one of these (*S*-mercuric-*N*-dansylcysteine) shows a marked environmental sensitivity on immobilization [there is a 20-fold enhancement and a 20 nm shift in the emission maximum on forming mercury-bridged mercaptides with some muscle proteins (tropomyosin, troponin *c*, actin)].[323] Another new —SH reagent is *N*-[*p*-(*S*-benzimidazolyl)phenyl]maleimide.[324] This compound is non-fluorescent, but generates an emitter on reaction with a thiol. The fluorescence is sensitive to the environment. The emission of labelled actin changes on addition of heavy meromyosin and then (further) ATP.[325]

7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-chloride) also reacts with thiols to give a fluorescent product. This has been used to investigate conformation changes in glyceraldehyde-3-phosphate dehydrogenase.[326] It has also

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been applied in a mixed probe study (the second probe being 4-iodoacetamidosalicylic acid) to detect the association of phosphorylase *a* dimers to tetramers.[327] However, the chemical specificity of NBD-chloride for the ATPases of beef heart mitochondria[328] and *Micrococcus dentifricans*[329] is not for thiols but rather for an unusually reactive Tyr hydroxy-group.

Studies with several other covalent labels whose reactive portion is the iodoacetyl group have been described.[330, 331] For example, the cAMP receptor protein of *E. coli* has been modified with both *N*-(iodoacetylaminoethyl)-1-naphthylaminesulphonate (AENS) and dansyl chloride. Each modified protein is fully active. The fluorescence polarization of the dansyl conjugate shows that addition of cAMP does not cause an aggregation change, but the nucleotide does cause enhancement of the fluorescence of the AENS derivative when it binds.[330]

The reactivities of fluorescamine with a variety of model amines[332] and the fluorescence properties of some fluorescamine–protein conjugates[333] have been reported.

Probes with some Biological Specificity. Reports of $1,N^6$-ethenoadenosine and $3,N^4$-ethenocytosine derivatives have enjoyed another good year. The crystal structure of 7-ethyl-$1,N^6$-ethenoadenosine hydrochloride has been described.[334] The three rings are coplanar and the only bond angles which appear strained are the two exocyclic bonds of the purine six-membered ring which are involved in forming the third ring on reaction with chloroacetaldehyde. More new derivatives of various adenine- and cytidine-containing species have been reported.[335–337] It seems that the emissions of dinucleotide derivatives are generally quenched by intramolecular effects[337] which may be reduced on interaction with a macromolecule.[338] It has been strongly argued that the only emitting form of $1,N^6$-ethenoadenine derivatives contains an unprotonated ring system,[339] although if this is true the fact that $(F/F_0)(\tau_0/\tau)$ does not fall to zero below pH 2.0 is surprising.

More $1,N^6$-etheno-2-aza-adenosine derivatives have been reported.[340, 341] These compounds can have advantages over simple etheno-derivatives because their spectra are shifted further into the visible region (and away from common

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competing absorptions and emissions), and because in some cases they retain a higher proportion of the biochemical activity of the underivatized parent compound.[341]

Interest has been shown in using the fluorescence properties of lanthanide ions to probe interaction sites of metal ions in biochemical systems. A number of these studies have involved interactions with tRNA[342] and membranes,[343] but there has also been model work on the optical properties of the ions[344] and their complexes.[345] Terbium(III) has been used to probe high-affinity calcium-binding sites on erythrocyte membrane proteins, and Tyr has been implicated in the interaction.[346] CPL has been used to study terbium(III) : malate and terbium(III) : aspartate complexes,[347] and although the technique is very sensitive to small changes in the environment of the metal ion no difference could be detected between the two metal-binding sites when terbium(III) was added to transferrin.[348]

The emission properties of pyridoxal derivatives are useful in some cases. The interaction between cystathionase and the apoprotein of aspartate aminotransferase has been investigated by studying the fluorescence of 4-pyridoxic-5′-phosphate released when the two proteins interact.[349] (The interactions between pyridoxal-5′-phosphate and aspartate aminotransferase have also been studied by the effects on intrinsic protein fluorescence.[350]) In other instances covalent conjugates can be observed, as when pyridoxamine derivatives of glycogen phosphorylase were used to report on conformation changes.[351] Those planning to use pyridoxal phosphate in fluorescence work should note that its Schiff base with glutamate has fluorescence properties very similar to those of NADH.[352] There may be circumstances in which confusion between the two emissions could arise.

The flavin moiety acts as a built-in fluorescent probe for flavoproteins. There have been studies of free flavins.[353] The effects of aggregation of the apoprotein of *Azobacter* flavodoxin on coenzyme binding have been followed using the emissions of 3-methylFMN and riboflavin.[354] The emission properties of the covalently bound flavin of *Chromatium* cytochrome c_{552} have been studied and interpreted (with other data) to support an interaction through a cysteine thiohemiacetal stabilized by an interaction between a Tyr residue and the flavin.[355]

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An extensive catalogue of the optical and fluorescence properties of reduced flavins and flavoproteins has been published.[356] It is stressed that reduced flavins fluoresce when constrained in a rigid environment. Many flavoproteins provide such surroundings, and those reduced flavoproteins which fluoresce display a wide range of emission characteristics.

Thiochrome diphosphate is a highly fluorescent analogue of thiamine diphosphate.[357] Its interactions with the pyruvate dehydrogenase multienzyme complex have been studied. Excitation energy can be transferred to FAD with 8% efficiency (suggesting a donor–acceptor separation of 3.0—6.0 nm) [285] but in general there is little evidence from fluorescence of interactions between the various active sites of the complex.

Other studies of affinity probes reported during 1974 involved hybrid haemoglobins (using protoporphyrin emission),[358] firefly luciferase (dehydroluciferin fluorescence was used to monitor stopped-flow kinetics),[359] RNA polymerase {6-[*N*-(acetylaminoethyl)-1-naphthylamine-5-sulphonate]thioinosinedicarboxaldehyde is a good inhibitor of the polymerase but does not prevent its binding to DNA},[360] and acetylcholinesterase (various cationic probes).[361–363]

Non-covalent Probes Lacking Biochemical Specificity. The majority of studies in this category have involved use of 1-anilinonaphthalene-8-sulphonate (ANS) or one of its close derivatives. Two other compounds each of which shows 20-fold enhancement on binding to bovine serum albumin are 9-(4′-carboxyanilino)-6-chloro-2-methoxyacridine and *N*-phenylsulphanilate.[364]

In spite of the similarities between ANS, 2-*p*-toluidinyl-naphthalene-6-sulphonate (TNS), and 2-(*N*-methylanilino)naphthalene-6-sulphonate (MNS), their interactions with proteins which cause effects detectable by fluorescence are often quite different. For example, the β-glucosidases A and B from sweet almond emulsin have been studied. ANS forms a 1 : 1 complex with A, while TNS forms a 1 : 4 (approximately) complex with B. Binding the competitive inhibitor D-glucono-δ-lactone enhances the fluorescence of ANS/A, but quenches that of $(TNS)_4$/B.[365] Three (of the many) other studies using ANS have involved pyruvate kinase from *Neurospora* (ANS inactivates this enzyme at 276—277 K but not at 298—308 K),[366] the guanosine nucleotide complexes of bacterial elongation factor tu (EFtu–GTP and EFtu–GDP can be distinguished by their interactions with ANS),[367] and phosphorylase *b* (nanosecond fluorometry detects ANS in two environments, one of which is destroyed by adding the allosteric activator, AMP).[368] TNS has been used to investigate interactions between

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concanavalin A and sugars. There is one TNS site per concanavalin A tetramer. There are no spectral changes on binding a sugar, nor does the emission lifetime alter, implying that no major conformation change occurs when the sugar binds.[369] MNS has been used in a study of phosphofructokinase. The probe fluorescence is shifted and enhanced on binding to the protein, but it is quenched by fructose-6-phosphate and ATP. The number of probe sites increases as the protein dissociates and this has been used to show that substrate binding also depends on the state of aggregation of the enzyme.[370]

Intrinsic Fluorescence.—There have been a few reports of the fluorescence emissions of individual amino-acid residues. Quenching studies have already been mentioned.[294, 297, 298, 304] The fluorescence characteristics of indoles in a variety of solvents have been studied [371, 372] and it was deduced that the lifetime of Trp emission is unlikely to be very sensitive to environment (though very long lifetimes may be diagnostic of Trp in hydrophobic surroundings).[372] Emissions of poly-Phe have been reported,[305, 373] and also the intrinsic fluorescence of horse hepatocuprein (which is devoid of Tyr and Trp and is the first Phe-only protein studied).[374] The emission does not display fine-structure and only changes a little when the molecule is unfolded with 6M guanidine hydrochloride.

The remaining papers in this section are discussed under two subheadings, conformation and ligand binding. The division, however, is not a rigid one, and many studies involve elements of both categories. For example, interactions between various histones [LAK(f2a2), KAS(f2b),[375] GRK(f2a1) [375, 376] and ARE(f3,III) [377]] have been studied by monitoring changes in Tyr emission anisotropy. This parameter is sensitive both to molecular conformation and to intermolecular interactions.

Conformation. Fluorescence emissions can be very sensitive to protein conformation and their observation can provide a simple way to study conformation changes. This is the case for the change associated with the interaction between butan-2-ol and papain which, though 'seen' by fluorescence, can barely be detected by circular dichroism or solvent perturbation difference spectroscopy.[378] Conformation changes in glutamate dehydrogenase (from *Neurospora*) [379] and troponin C [380] have also been investigated using fluorescence.

Fluorescence can be used to compare the conformations of related proteins. Rat lysozyme has emission characteristics almost identical with those of human lysozyme in energy, quantum yield, and sensitivity to pH and binding interactions.[381] Hence it was deduced that there must be a high degree of homology

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Table 1 *Fluorescence probes and their applications*

Probe	*Application*	*Ref.*
6-[*N*-(Acetylaminoethyl)-1-naphthylamine-5-sulphonate]thioinosine-dicarboxaldehyde	RNA polymerase	360
ANS	Bacterial elongation factor tu	367
	cytochrome c_1	*a*
	glutamate dehydrogenase	*b*
	β-glycosidase A	365
	haptoglobin	*c*
	phosphorylase	368, *d*
	pyruvate kinase	366
	troponin	*e*
1-Anilinonaphthyl-4-maleimide	actin	*f*
Aurovertin	ATPase	*g*
N-[*p*-(*S*-Benzimidazolyl)phenyl]-maleimide	actin	325
	myosin, Taka amylase	324
Berberine	alcohol dehydrogenase	*h*
Bis-(3-aminopyridinium)-1,10-decane	acetylcholinesterases	363
Bis(dansyl)cystine	cystathionase	321
Colchine	tubuline	*i*
N-Dansylaziridine	selective reagent for protein thiols	320
Dansyl chloride	cAMP receptor protein	330
	antidansyl antibodies	316
	basic proteins	*j*
	κ and α(S1) casein	*k*
	chymotrypsin	*l*
	high-density lipoprotein	*m*
	ribosomal proteins	*n*
	trypsin inhibitor	319
N-Dansylcysteine	aspartate aminotransferase	322
1-Dansyl-3-*NN*-dimethylaminopropane	acetylcholinesterase	361
Dehydroluciferin	luciferase	359
3,8-Diamino-5,3′-diethylmethylamino-propyl-6-phenylphenanthridinium diiodide	acetylcholinesterase	362
1,N^6-EthenoATP	ATPase	*o*
1,N^6-Etheno-2-azaADP	polynucleotidephosphorylase	340
1,N^6-Etheno-2-azaAMP	cyclic nucleotide phosphodiesterase	341
1,N^6-EthenoNAD	octopine dehydrogenase	338
FITC	aspartate aminotransferase	349
	erythrocyte membrane proteins	317
4-Iodoacetamidosalicylic acid	phosphorylase	327
N-(Iodoacetylamino)-1-naphthalamine-5-sulphonate	cAMP receptor protein	*p*, 330
	myosin	331
N-Methylacridinium	acetylcholinesterase	361, 363
2-(*N*-Methylanilino)naphthalene-6-sulphonate	phosphofructokinase	370
3-MethylFMN	flavodoxin	354
S-Mercuric-*N*-dansylcysteine	actin, tropomyosin, troponin	323
2-Naphthyltriphosphate	heavy meromyosin	*q*
NBD chloride	ATPase	328, 329
	glyceraldehyde-3-phosphate dehydrogenase	326
	phosphorylase	327

Probe	*Application*	*Ref.*
Promazine	bovine serum albumin	*r*
Protoporphyrin	hybrid haemoglobins	358
Pyridoxamine	phosphorylase	351
4-Pyridoxic-5′-phosphate	aspartate aminotransferase	349
Terbium(III)	conalbumin, transferrin	348
	erythrocyte membrane proteins	346
Thiochrome	pyruvate dehydrogenase multi-enzyme complex	285, 357
TNS	concanavalin A	369
	β-glycosidase B	365
	phenylalanine:tRNA ligase	*s*
	S-100 protein	*t*

(*a*) L. S. Kaminsky, Y. L. Chang, and T. E. King, *Biochem. Biophys. Res. Comm.*, 1974, **59**, 688. (*b*) M. Nemat-Gorgani and G. H. Dodd, *Biochem. Soc. Trans.*, 1974, **2**, 969. (*c*) S. F. Russo and W. W. Chen, *Biochemistry*, 1974, **13**, 5300. (*d*) L. Klungsor, *Biochemistry*, 1974, **13**, 1751. (*e*) W. D. McCubbin, R. S. Mani, and C. M. Kay, *Biochemistry*, 1974, **13**, 2689; R. S. Mani, W. D. McCubbin, and C. M. Kay, *ibid.*, p. 5003. (*f*) T. Ohyashiki, T. Sekine, and Y. Kanaoka, *Biochim. Biophys. Acta*, 1974, **351**, 214. (*g*) T. Chang and H. S. Penefsky, *J. Biol. Chem.*, 1974, **249**, 1090. (*h*) J. Kokar, *European J. Biochem.*, 1974, **49**, 179. (*i*) B. Bhattacharyua and J. Wolff, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 2627. (*j*) A. G. Sabel'nikov, T. F. Moiseeva, A. V. Avdeeva, and B. N. Il'yashenko, *Biochim. Biophys. Acta*, 1974, **374**, 304. (*k*) S. Nakai and C. M. Kason, *Biochim. Biophys. Acta*, 1974, **351**, 21. (*l*) J. B. Bridge and P. Johnson, *European Polymer J.*, 1973, **9**, 1327. (*m*) M. D. Barratt, R. A. Badley, R. B. Leslie, C. G. Morgan, and G. K. Radda, *European J. Biochem.*, 1974, **48**, 595. (*n*) F. Pochon, B. Ekert, and M. Perrin, *European J. Biochem.*, 1974, **43**, 115. (*o*) Y. Aizono, J. E. Roberts, M. Sonenberg, and N. I. Swislocki, *Arch. Biochem. Biophys.*, 1974, **163**, 634. (*p*) C. W. Wu and F. Y. H. Wu, *Biochemistry*, 1974, **13**, 2573. (*q*) H. Kawaga, T. Kuwajima, and H. Asai, *Biochim. Biophys. Acta*, 1974, **338**, 496. (*r*) J. D. Keeler and R. P. Sharma, *Biochem. Pharmacol.*, 1974, **23**, 2679. (*s*) P. Bartmann, T. Hanke, B. Hammer-Raber, and E. Holler, *Biochemistry*, 1974, **13**, 4171. (*t*) P. Calissano, S. Alema, and P. Fasella, *Biochemistry*, 1974, **13**, 4553.

in the distribution of aromatic residues throughout the sequence, even though only *ca.* 80% of the total sequence is likely to be identical in the two proteins. The conformations of disuccinyl glucagon and glucagon have been compared by measuring the extent of intramolecular energy transfer in each molecule. The extents are always the same, irrespective of pH.[382]

Intrinsic fluorescence has been used to characterize interactions between proteins and their environments. The emissions of neurotoxins from middle Asian cobra venom in the range pH 2—12 suggest that a single Trp is accessible to freely relaxing water.[383] The association of internal membrane protein from influenza virus with membrane lipid has been studied by measuring energy transfer between protein Tyr and a fluorescence probe in the lipid.[384] The quenching of fluorescence of apolipoprotein-alanine and apolipoprotein-glutamine I by pyridinium and iodide ions has been used to quantitate both protein accessibility and phospholipid binding efficiency.[385] The Tyr and Trp emissions from *Staph. aureus* endonuclease have been distinguished by lifetime measurements, and anisotropy studies suggest a correlation time for the protein

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which means it is strongly hydrated.[386] The fluidity[387] and polarity[388] of the environments of membrane proteins have been investigated, and the intrinsic fluorescence of myelin has been reported.[389]

Specific sites on a protein can be studied providing a fluorescent residue is nearby. Two Trp residues in papain have been identified at the S_2 (Trp-69) and S_1' (Trp-177) substrate binding sites.[390] The site to which eosin binds on lysozyme has been located near to Trp-108.[391]

The fluorescence of a protein is usually sensitive to its unfolding, and the effects of guanidine hydrochloride on various species (*e.g.* paramyosin from *Mercenaria mercenaria*,[392] carbonic anhydrase B,[393] and ferricytochrome *c*[394]) have been followed by monitoring emission properties. In the cytochrome *c* case it was found that residual order remains in the molecule even at guanidine hydrochloride concentrations much higher than are needed to induce the major unfolding. The importance of such residual structures in forming nucleation centres during folding is discussed.[394]

Ligand Binding. In this kind of work there are often advantages in monitoring the emissions of both protein and ligand, if this is possible. For example, in a stopped-flow investigation of the binding of bilirubin to serum albumins three relaxation times were detected. One (protein fluorescence quenching, $\tau_{\frac{1}{2}} < 10$ ms) was attributed to primary binding of the ligand. The second (appearance of bilirubin fluorescence, $\tau_{\frac{1}{2}} \sim 50$ ms) and third (further protein fluorescence quenching, $\tau_{\frac{1}{2}} \sim 300$ ms) were connected with conformation changes subsequent to binding.[395] It is argued that 'multiple relaxation processes in macromolecular binding may prove to be rather general phenomena'. Interactions of reduced pyridine nucleotides with proteins form another common system where dual emissions (nucleotide and protein) can be monitored, and studies of diphtheria toxin[396] and malic dehydrogenase[397] have been made this way.

Some flavoproteins have been investigated. The emission properties of both Trp and FAD residues of lipoamide dehydrogenase have been measured and interpreted in terms of the environments of the emitting groups. Only one of the two Trp residues transfers energy to FAD, and the donor–acceptor separation has been estimated to be ~ 1.5 nm.[398] The intrinsic protein fluorescence of D-amino-acid oxidase shows a blue shift when FAD binds and the emission results (supplemented by o.r.d. studies) have been used to study conformation, conformation changes, and the proximities of Trp, Tyr, and flavin moieties.[399]

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The binding interactions between lactose and several different homogeneous preparations of rabbit antilactose antibody (obtained by isoelectric focusing) have been studied by following Trp emission. With some fractions the fluorescence increased on binding lactose, but with others it decreased.[400] The interactions of various saccharides with wheat germ agglutinin have also been studied by following enhanced Trp emission from the protein.[401] Fluorescence changes when various substrates are added to glutamine synthetase (from *E. coli*) have been correlated with an enzyme reaction mechanism.[402] Interactions between some bis-quaternary ammonium ligands and acetylcholinesterase have been followed by the quenching of Trp fluorescence,[403] and a similar effect has enabled study of the hydrophobic binding sites in bovine serum albumin and erythrocyte ghost proteins.[404] Usually O_2 quenches fluorescence, but a specific binding to lipoxygenase apparently causes a 20% enhance of emission.[405]

6 Infrared Spectroscopy

Contributed by R. M. Stephens

Model Compounds.—The i.r. spectra of the fundamental and overtone regions of *N*-methylacetamide and two other secondary amides have been measured in solution at various temperatures between 22 and −190 °C. At least two hydrogen-bonded species were found and the association between molecules increased as the temperature decreased. The effect of hydrogen-bond formation on the anharmonicity of the N—H stretching and on the N—H stretching/N—H bending coupling constant was examined.[406] Other i.r. studies have been made on methylamides and *N*-acetylamides and conformational changes correlated with the corresponding c.d. spectra.[407] The association of amide groups by hydrogen-bonding and its dependence on chemical structure was examined in a methacrylamide–styrene copolymer (I) and isobutyramide (II). The observed decrease in intensity of the NH_2 absorption bands from the solution of (II) at 3532 and 3415 cm^{-1} with increasing concentration and those at 3498, 3354, 3290, and 3192 cm^{-1} with increasing temperature were attributed to association isobutyramide molecules. Comparison of these data with those obtained from solution of (I) in the NH_2 absorption region indicated similar structures, and it was suggested that dimer formation occurred for solutions of (II) at a concentration of <0.5 mol l^{-1}. The equilibrium constant for the reaction was calculated from the free and dimer concentration of (II) molecules and an estimate of the relative number of amide groups involved in intermolecular hydrogen-bonding was made.[408] The presence of intramolecular hydrogen bonds between the guanidine

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[408] N. A. Kuznetsov and A. R. Smolyanskii, *Zhur. priklad. Spectroskopii*, 1974, **20**, 672.

group and the peptide C=O bond in arginine-containing peptides was established using i.r. spectroscopy.[409] Hydrogen-bonding mechanisms in RCH_2CONH_2 and $RCH_2CONHMe$ (R = H, Cl, EtO, or $EtOCH_2$) have also been studied.[410] Spectra of various amide complexes in $CHCl_3$ solutions have been recorded in the NH_2 stretching region. For 1—3 mol l^{-1} and >6 mol l^{-1} solutions amide–base complexes of 1 : 1 and 1 : 2 stoicheiometry respectively were observed. The 1 : 1 complexes showed two new absorption bands between the ν_{sym} and ν_{asym} bands of the free molecules and a broad doublet below the ν_{sym} band was attributed to free as well as bonded NH groups in the two types of rotational isomeric complex, respectively. The frequencies and relative intensities of the absorption bands depended on the degree of perturbation of the resonance interactions in the NH stretching vibration and due to the complexing.[411]

The interactions between the amide group and water and various salts have been studied in two organic solvents, MeCN and THF. The amides were $MeC(O)NMe_2$ and MeC(O)NHMe and the salts were perchlorates, tetrabutyl ammonium halides, and LiBr. The results indicate anion binding on H_2O and on amide NH groups, and cation binding on amide C=O groups even when a relatively large amount of H_2O is present. Double interactions such as $M^+ \cdots C{=}O \cdots M^+$, $M^+ \cdots C{=}O \cdots H_2O$ and $H_2O \cdots C{=}O \cdots H_2O$ occur. The role of the anions in the formation of these species is discussed. The peptide group as a whole is perturbed by the interactions with one site. A comparison is proposed between the above data and the structural transitions observed in polypeptides in the presence of water and salts.[412] On the basis of their i.r. spectra the assignment of $MeCONRMe/CF_3CO_2H$ associations to the ionic form $(MeCOHNRMe)^+/(CF_3COO)^-$ has been questioned with the conclusion that under the experimental conditions ionic and hydrogen-bonded structures are indistinguishable.[413] Correlation between the i.r. and Raman spectra of many amino-acids has been made in the region 4000—400 cm^{-1} and Raman lines characteristic of NH_3^+ and CO_2^- groups have been assigned.[414] The polarized i.r. spectrum of N-deuteriated alanine has been recorded between 300 and 3200 cm^{-1}. The use of local symmetry models for non-interacting parts has allowed the normal modes and polarization behaviour to be studied. Symmetric and degenerate deformation modes of the NH_3^+ group were reassigned. The polarization behaviour and assignments of the methyl CH stretching bands were also discussed.[415]

A normal co-ordinate analysis of the A_n and B_n fundamentals of glycine anhydride has confirmed the empirical vibrational assignments made from the i.r. spectrum. The calculated potential energy distributions showed that the N—D in-plane bending and ring skeletal stretching vibrations coupled together.[416] The fundamental symmetric (ν_{sym}) and asymmetric (ν_{asym}) NH_2 stretching frequencies of some amines have been measured in dilute CCl_4 solutions. The

[409] R. Vegners, G. Cipein, and I. Dipans, *Khim. prirod. Soedinenii*, 1973, 763.
[410] N. N. Bessonova and I. M. Ginzburg, *Zhur. obshchei Khim.*, 1974, **44**, 384.
[411] I. M. Ginzburg and N. N. Bessonova, *Zhur. obshchei Khim.*, 1974, **44**, 378.
[412] M. H. Baron, C. De Loze, and G. Sagon, *J. Chim. phys. physicochim. biol.*, 1973, **70**, 1509.
[413] P. Coubelas and C. Garrigou-Lagrange, *Spectrochim. Acta*, 1974, **30A**, 550.
[414] R. S. Krishnan, V. N. Sankaranarayanan, and Krishnon, *J. Indian Inst. Sci.*, 1973, **55**, 66.
[415] R. F. Adamowicz and M. L. Sage, *Spectrochim. Acta*, 1974, **30A**, 1007.
[416] M. Asai, K. Nodo, and A. Sado, *Spectrochim. Acta*, 1974, **30A**, 1147.

s character of the hybrid orbitals of the N—H bonds (b^2) has been calculated from ν_{sym} and ν_{asym}. The basicities (pK_{BH+}) of a number of compounds have been obtained. Separate linear relations have been found between the indicated values for each group of compounds. For anilines which have the most highly electron-accepting substituents there are deviations from the linear dependence $b^2 = f(pK_{BH+})$. Differences have been explained by a combination of the variations of the N-atom hybrid state and the population of the orbital base pair under the influence of substituents.[417] The vibrational levels related to the NH_2, NDH, and ND_2 groups of $PhNH_2$ and its N-deuteriated derivative were calculated using a perturbation method on a four-atom model. An out-of-plane type of deformation co-ordinate was used to reduce the anharmonicity constant for the NH_2 wagging mode. The assumed anharmonic potential contains no higher order terms except those derived from the Morse function for the individual bonds. The calculated frequencies agree with the observed values. The observed anharmonicity constants X_{aa} and X_{ss} are so much affected by the Darling–Dennison resonance that the theoretical relationship for the unperturbed constants $(X_{aa}) > (X_{ss})$ is violated.[418]

Polypeptides.—Intensities and other spectral parameters for the amide I and II bands of α-helical polypeptides in solution have been studied. The polypeptides used were poly-(γ-benzyl glutamate), poly-γ-glutamate, poly-(γ-ethyl glutamate), and polymethionine dissolved in chloroform, and poly-lysine, poly-(glutamic acid), and the fibrillar protein tropomyosin in D_2O. The majority of the parameters were characteristic. The half-width of the amide I band varied in the range 15—40 cm^{-1} for different polypeptides in different solutions. The correlation between this parameter of the amide I band and the stability of the α-helix was estimated. A new weak band near 1537 cm^{-1} of an unknown origin was observed for the hydrogen-bonded form of polypeptides in the α-helical state.[419] Measurement of the far-i.r. absorption spectrum of α-helical poly-L-alanine between 4 and 200 cm^{-1} at temperatures between 300 K and 4.2 K showed no characteristic features associated with predicted longitudinal acoustic modes. The intermolecular interactions in solution and in the solid state invalidated the isolated α-helix model which had been used to describe the low-frequency normal modes of the homopolymer chains.[420] Assignment of hydrogen-bonded and non-bonded character to each peptide NH group in a molecule has been made by obtaining the n.m.r. and i.r. spectrum simultaneously at various time intervals throughout the H–D exchange. Resonance frequency shifts arising from the isotropic exchange occur in both spectra. The n.m.r. changes are used to determine the amino-acid residue to which an exchanged NH belongs, and the changes in the i.r. spectrum are used to determine whether the exchanged NH was free or intramolecularly hydrogen-bonded.[421]

The conformations of depsipeptide derivatives dissolved at a low concentration in an inert solvent such as CCl_4 have been studied using i.r. and n.m.r. spectroscopy. Experiments were performed on molecules containing an ester

[417] E. V. Titov, *J. Mol. Structure*, 1973, **19**, 301.
[418] Y. Tanaka and K. Machida, *J. Mol. Spectroscopy*, 1974, **51**, 508.
[419] Yu. N. Chirgadze and E. V. Brazhnikar, *Biopolymers*, 1974, **13**, 1701.
[420] W. J. Shotts and A. J. Severs, *Chem. Phys. Letters*, 1973, **21**, 586.
[421] P. H. Von Dreele and I. A. Stenhouse, *J. Amer. Chem. Soc.*, 1974, **96**, 7456.

group between amide linkages. These molecules adopt a C_{10} conformation, similar to the β-structures in proteins and cyclic peptides, which is stabilized by intramolecular hydrogen bonds between amide groups. Experiments reveal two different C_{10} conformations, the stabilities of which depend on the configuration of the chiral atoms in the molecules.[422] The conformational states of tripeptide compounds containing *N*-terminal prolyl residues and a chiral or non-chiral *C*-terminal moiety, R^1-CO-Pro-Y-NHR^2, have been determined in very dilute solution in inert solvents, *e.g.* CCl_4, $CCl_2{=}CCl_2$. Most of the solute molecules are again present in two different C_{10} structures, labelled C_{10}' and C_{10}''. L–D Stereoisomers exclusively adopt the C_{10}'' conformation, L–L sequences do not fold so easily, and only a few molecules are in the C_{10}' conformation. Most of them are in two other states labelled $C_7^2C_5^3$ and C_7^3. In the $C_7^2C_5^3$ form the prolyl moiety and the *C*-terminal residue are in the C_7 and C_5 arrangement, respectively. The C_7^3 structure is characterized by an equatorial C_7 conformation of the *C*-terminal unit.[423]

The conformations of histones and synthetic polypeptides in different solvents were determined using i.r. and c.d. Thus $(Lys)_n$, $(Lys\text{-}Ala_3)_n$, $(Lys\text{-}Ala)_n$, $(Lys_2\text{-}Ala)_n$, and $(Lys\text{-}Ala_2)_n$ have helical and random-coil structures in $ClCH_2CH_2OH$ and Me_2SO, respectively. In aqueous media, polypeptides with ionized side-chain groups possess a conformation similar to that of charged poly-amino-acids. Histones possess configurations similar to that of the synthetic polypeptides under all conditions.[424]

Proteins.—Crystal structures and thermal properties of fibroin prepared by different methods have shown that fibroin from the posterior section of the silk gland that has been extended in H_2O has a partial β-structure as the amide I bands occurred at 1650 and 1630 cm^{-1}. Fibroin prepared from degummed silk by the copper ethylenediamine method was amorphous and the amide I band was seen at 1630 cm^{-1} only. An α-helical structure was observed from an $(NH_4)_2SO_4$ precipitate preparation with an amide I band at 1645 cm^{-1}. Generally, decomposition caused an increase in i.r. absorption at *ca.* 1700 cm^{-1} and the intensities of the amide II and III bands decreased markedly.[425]

Laser Raman spectra were taken from a single crystal of native insulin in the region 280—1800 cm^{-1}. As expected, the spectra showed more detail than those obtained from polycrystalline powders. Studies have been made on deuteriated insulin from which the contribution of side-group vibrations in the amide III region was determined. In addition, the Raman spectra of insulin fibrils and glucagon fibrils were compared, with the conclusion that both contain anti-parallel β-structures.[426] Laser Raman spectra of native (egg white) lysozyme in aqueous solution were recorded over the temperature range 32—76 °C. Very little change in the spectra was observed, suggesting that conformational changes were confined to side-chains. If the protein solution is heated for 2 h at 100 °C

[422] G. Boussard, M. T. Cung, M. Marraud, and J. Neel, *J. Chim. phys. physicochem. biol.*, 1974, **71**, 842.
[423] G. Boussard, M. Marraud, and J. Neel, *J. Chim. phys. physicochim. biol.*, 1974, **71**, 1081.
[424] L. I. Mar'yash, L. V. Abaturov, and V. A. Shibnev, *Studia Biophys.*, 1974, **43**, 41.
[425] I. Aoki, Y. Ishida, T. Takeuchi, and H. Arimoto, *Nippon Sansigoku Zasshi*, 1974, **93**, 19.
[426] N. T. Yu, C. Chang, and J. D. Huber, *Arch. Biochem. Biophys.*, 1974, **160**, 614.

the resultant irreversible denaturation produces a gel whose Raman spectrum at 32 °C is markedly different in the amide I, II, and disulphide regions. At most only one of the four disulphide bonds has been broken, together with a substantial reorientation of the geometry of the others along with a disordering of the polypeptide backbone.[427] Resonance Raman spectra have been recorded for various haem proteins. Laser excitation in the Soret and in the α–β band regions bring out different sets of Raman bands *via* different resonance scattering mechanisms. This discrimination permits the acquisition of an unusually complete set of vibrational frequencies and leads to reliable correlations amongst the various haem Raman spectra.[428]

7 Mössbauer Spectroscopy

Contributed by C. E. Johnson

The main source of literature information for this report is the half-yearly compilation 'The Index of Publications in Mössbauer Spectroscopy of Biological Materials' by Dr. Leopold May, Department of Chemistry, The Catholic University of America, Washington, D.C. 20017, U.S.A.

During the year 1974 work has been reported on haem proteins, iron–sulphur proteins, ferritin, hormones, and vitamin B_{12}.

Haem Proteins.—Haemin (haemin chloride or ferric protoporphyrin IX chloride) is the small stable planar inorganic molecule which can be produced by removing the protein from the haem groups in haemoglobin. Studies of its Mössbauer spectra are made in order to throw light on the spectra of iron in haem proteins. Measurements of the electric hyperfine interaction (*i.e.* chemical shift and quadrupole splittings) have been reported by Amusa *et al.*[429] using several different commercial sources of the compound. It was concluded that the discrepancies between values of the quadrupole splitting previously reported in the literature by different groups were real, and were ascribed to the different allotropic polycrystalline forms which are supplied by different manufacturers. The same group[430] has studied the magnetic hyperfine splitting which is observed in haemin at low temperatures when it is magnetically diluted in a suitable solvent. They used both frozen solutions and crystals of haemin in $\alpha\beta\gamma\delta$-tetraphenylporphyrin (TPP), tetrahydrofuran (THF), and dimethyl sulphoxide (DMSO). In THF they were able to obtain almost a pure magnetic spectrum with virtually no central quadrupole doublet, showing that there was no aggregation of the haemin. The effective magnetic field at the iron nuclei was found to vary in the range 477—499 kG (47.7—49.9 T), and depended upon the side-chains, the solvent or host crystals, and the concentration used. MO calculations have been performed[431] on an idealized model of an Fe^{3+} ion overlapping with the orbitals of neighbouring atoms to give a value of the chemical shift in haemin. The predicted shift was considerably larger than the measured value, presumably

[427] M. C. Chen, R. C. Lord, and R. Mendelsohn, *Biochim. Biophys. Acta*, 1974, **328**, 252.
[428] T. G. Spiro and C. Streka, *J. Amer. Chem. Soc.*, 1974, **96**, 338,
[429] A. Amusa, P. Debrunner, E. Münck, and H. Frauenfelder, *Phil. Mag.*, 1974, **29**, 915.
[430] A. Amusa, P. Debrunner, H. Frauenfelder, E. Münck, and G. de Pasquali, *J. Phys.* (*C*), 1974, **7**, 1881.
[431] P. Moutsos, J. G. Adams, and T. T. Sharma, *J. Chem. Phys.*, 1974, **60**, 1447.

owing to the neglect of electron transfer. A calculation[432] of the quadrupole splitting using a linear combination of polarized atomic orbitals gave a result in good agreement with the experimental value both in sign and magnitude.

Lang *et al.*[433] have made Mössbauer studies on purified cytochrome *c* oxidase from beef heart. Cytochrome oxidase (cytochrome *c*; O_2 reductase), the terminal enzyme of the respiratory chain, catalyses the oxidation of ferrocytochrome *c* by molecular oxygen, which is reduced to water. The active unit of the enzyme contains two haem *a* groups and a molecular weight of *ca.* 700 000. Though the haem *a* groups are thought to be identical, there has been much debate about whether they are bound in the same way to the protein. The Mössbauer work was done with high concentration samples, enriched in ^{57}Fe. The results indicate that the two haems are bound in different ways to the native protein. One of the components shows a quadrupole splitting of 2.0 mm s^{-1} and an isomer shift of 0.2 mm s^{-1} in the oxidized enzyme at 195 K, and is thus similar to the low-spin Fe^{3+} of oxidized cytochrome *c*. The other component behaves more like a high-spin compound and the data fit reasonably well with the high-spin Fe^{3+} of oxidized cytochrome *c* peroxidase. In the reduced sample, only the first component (*i.e.* low-spin Fe^{2+}) is clearly seen, while there are no spectral features that indicate the presence of high-spin Fe^{2+}.

Baumgartner *et al.*[434] have studied the CN^-, F^-, imidazole, and other complexes of cytochrome *c* obtained from horse heart and also an undecapeptide of the protein. The variation of quadrupole splitting with temperature gave the crystal-field splittings and spin–orbit coupling of the iron and hence permitted an evaluation of the distortions from octahedral symmetry around the iron in the haem group.

Bearden *et al.*[435] have reported measurements on haem complexes of rabbit haemoplexin. Haemoplexin is a porphyrin-binding protein found in mammalian serum, and its spin state when oxidized had not previously been determined. The magnetic hyperfine interaction observed in the Mössbauer spectra at 4.2 K was characteristic of low-spin ($S = \frac{1}{2}$) ferric iron.

Gonser *et al.*[436] have described the determination of the principal axis of the electric field gradient in deoxygenated myoglobin. In myoglobin there is only one haem group per molecule and it crystallizes into a habit where the haem planes are parallel to each other. There are two haems per unit cell, one being transformed into the other by a 180° rotation about the crystallographic *b*-axis followed by a translation. Hence the axis of the electric field gradient may be determined from observations of the relative intensities of the two lines of the quadrupole doublet as a function of the angle between the γ-ray direction and the crystal axis. Measurements were made on a single crystal grown from a solution of sperm whale myoglobin enriched to 80% in ^{57}Fe and subsequently reduced by washing

[432] P. Moutsos and R. R. Sharma, *Bull. Amer. Phys. Soc.*, 1974, **19**, 37; R. R. Sharma and P. Moutsos, *Phys. Rev.*, 1975, **B11**, 1840.
[433] G. Lang, S. J. Lippard, and S. Rosen, *Biochim. Biophys. Acta.*, 1974, **336**, 6.
[434] C. P. Baumgartner, M. Sellers, R. Nassif, and L. May, *European J. Biochem.*, 1974, **46**, 625.
[435] A. J. Bearden, W. T. Morgan, and U. Müller-Eberhard, *Biochem. Biophys. Res. Comm.*, 1974, **61**, 265.
[436] U. Gonser, Y. Maeda, A. Trautwein, F. Parak, and H. Formanek, *Z. Naturforsch.*, 1974, **29b**, 241.

the crystal in a solution of 4M-$(NH_4)_2SO_4$ + $Na_2S_2O_4$ in water. After deoxygenation, crystallographic alignment and Mössbauer measurements were carried out at 77 K. The chemical shift and quadrupole splitting shows that the iron remains in the high-spin Fe^{2+} form. Measurements were made with eight different orientations selected by rotating the crystal about the *b* axis, keeping the *b*-axis and the γ-ray direction perpendicular to each other. The principal component, V_{zz}, of the electric field gradient tensor was found to be positive and orientated not along the haem axis, as might be expected from symmetry, but along one of the four Fe–N directions in the haem plane.

Measurements have been made by Trautwein *et al.*[437] of Mössbauer spectra of the ^{57}Fe-enriched CO complex of sperm whale myoglobin (MbCO) at 4.2 K with and without magnetic fields applied perpendicular to the γ-ray direction. The iron here is in a low-spin ferrous state. The electric field gradient was found to be positive, with $\Delta E_Q = 0.363$ mm s^{-1} and chemical shift $\delta = +0.266$ mm s^{-1}. MO calculations were carried out for several possible arrangements of the CO ligand relative to the haem group, and it was concluded that the data are fitted best if the iron atom lies in the haem plane and if $\angle$FeCO is *ca.* 135°.

Iron–Sulphur Proteins.—Loew and co-workers[438–440] have performed semi-empirical MO calculations on the active site of rubredoxins. These are the simplest of the iron–sulphur proteins, each molecule containing a single iron atom surrounded by a distorted tetrahedral array of four cysteine sulphur atoms. Values for the quadrupole coupling and the anisotropic magnetic hyperfine coupling have been calculated both for the oxidized and the reduced protein for several possible models. A comparison with experimental data has been made to suggest possible conformations at the active site.

Iron–sulphur proteins containing two iron atoms and two labile sulphurs per molecule, *e.g.* plant ferredoxins, have been extensively studied during the past few years.[441] They were initially characterized by their e.p.r. signal in the reduced state which corresponds to a spin of $\frac{1}{2}$ with a mean *g*-value less than 2, and by the absence of an e.p.r. signal in the oxidized state. Mössbauer measurements have shown that in the oxidized state both irons are Fe^{3+} with spin $\frac{5}{2}$, while in the reduced state one is Fe^{3+} and the other is Fe^{2+} with spin 2. The spins are coupled antiferromagnetically together to give in the oxidized molecule a total spin of zero, *i.e.* a non-magnetic ground state, and in the reduced molecule a total spin of $\frac{1}{2}$. Measurements have now been made on more complex proteins.

Spectra of the eight-iron ferredoxin from the bacterium *Clostridium pasteurianum* have been reported by two groups.[442, 443] The structure of a similar protein (the ferredoxin from *Peptococcus aerogenes*) has been determined by *X*-ray

[437] A. Trautwein, Y. Maeda, F. E. Harris, and H. Formanek, *Theor. Chim. Acta*, 1974, **37**, 67.
[438] G. H. Loew, M. Chadwick, and D. A. Steinberg, *Theor. Chim. Acta*, 1974, **33**, 125.
[439] G. H. Loew and D. Lo, *Theor. Chim. Acta*, 1974, **32**, 217.
[440] G. H. Loew and D. Lo, *Theor. Chim. Acta*, 1974, **33**, 137.
[441] C. E. Johnson, *J. Phys.* (*Paris*), 1974, **35**, suppl. 1, C1–57; R. H. Sands and W. R. Dunham, *Quart. Rev. Biophys.*, 1974, **7**, 443.
[442] C. L. Thompson, C. E. Johnson, D. P. E. Dickson, R. Cammack, D. O. Hall, U. Weser, and K. K. Rao, *Biochem. J.*, 1974, **139**, 97.
[443] K. Gersonde, H. E. Schlaak, M. Breitenbach, F. Parak, H. Eicher, W. Zgoralla, M. G. Kalvius, and A. Mayer, *European J. Biochem.*, 1974, **43**, 307.

diffraction, and it is found that the molecule contains two clusters each containing four iron and four labile sulphur atoms arranged at alternate corners of an approximate cube. The iron atoms are co-ordinated along the edges of the cube to four sulphur atoms in the cysteine residues of the amino-acid chain of the protein. Each iron thus has a tetrahedral environment of four sulphur atoms. As for the two-iron proteins (for which the iron has been suggested to have a similar environment) an e.p.r. signal is only observed in the reduced molecule, and it has a mean g-value less than 2. Thompson *et al.*[442] observed Mössbauer spectra of both redox states over a range of temperature and in magnetic fields up to 6 T. At high temperatures (77 K and above) the spectra of both states consist essentially of the superposition of two or more closely similar doublets. The average chemical shift for the oxidized protein led to the proposal that each of the two four-iron active centres consists formally of two Fe^{3+} and two Fe^{2+} atoms. The average chemical shift (δ) and quadrupole splitting (ΔE_Q) increase on reduction, consistent with there being one Fe^{3+} and three Fe^{2+} atoms per centre in the reduced molecule. The spectral changes on reduction show that all the iron atoms are affected when one electron is added to each four-iron centre. Separate Fe^{3+} and Fe^{2+} spectra were not observed (as they were, for instance, in the reduced two-iron plant ferredoxins[441]) suggesting that the d electrons are not localized on particular atoms, but are shared approximately equally by all four atoms in the four-iron centres. At low temperatures (4 K and below) no magnetic hyperfine interaction was observed in the oxidized protein even in an applied magnetic field, confirming the non-magnetic nature of the molecule in the oxidized state, and suggesting that the four iron atoms in each centre are antiferromagnetically coupled together to give zero spin. Magnetic hyperfine interaction produced a broadening of the lines in the spectrum of the reduced protein at low temperatures, and showed that all the iron atoms were magnetic. This demonstrates that one electron goes to each centre on reduction. On application of a large magnetic field to the reduced protein at low temperatures, larger hyperfine fields were observed. Their signs were found to be both positive and negative to the total spin on the molecule, thus directly showing that antiferromagnetic coupling exists between the iron atoms in the reduced state.

Gersonde *et al.*[443] have also studied this protein by e.p.r. as well as Mössbauer spectroscopy. They attribute the absence (or at any rate the small value) of a magnetic hyperfine splitting in the Mössbauer spectrum of the reduced molecule at low temperatures to spin–spin coupling between the two four-iron clusters in the molecule. In a magnetic field of 2 T, magnetic hyperfine splitting appeared which showed general agreement with the data of Thompson *et al.*, and it is suggested that this splitting is observed because the external field overcomes the spin–spin coupling. Eicher *et al.*[444] have developed a cluster orbital theory to explain the Mössbauer spectra of oxidized and reduced *Clostridium* ferredoxin. It was assumed that all four irons are in the ferric state when oxidized, and that they are in an environment of predominantly trigonal symmetry. Agreement with experimental data[443] is good.

[444] H. Eicher, F. Parak, L. Bogner, and K. Gersonde, *Z. Naturforsch.*, 1974, **29c**, 683.

A related protein is the high potential iron protein (HIPIP) from *Chromatium*. This has a high and positive redox potential of +350 mV, which distinguishes it from the ferredoxins (redox potential *ca.* −400 mV) which it resembles in many respects. *X*-Ray crystallographic measurements have shown that the active centre is identical, within the limit of the resolution of the measurements, with the two four-iron centres in the eight-iron bacterial ferredoxins.[445] Mössbauer effect measurements have been made on this protein by Dickson *et al.*[446] Measurements of the reduced protein confirm that it is non-magnetic. Spectra of the oxidized protein showed hyperfine splitting at low temperatures and in applied magnetic fields, clearly indicating that some iron atoms have a positive hyperfine field, which is evidence for antiferromagnetic coupling. The spectra can be interpreted in terms of two types of iron atom with positive and negative hyperfine fields of 9 and 12 T, respectively. A consideration of the chemical shifts and other evidence suggests formal valencies of two Fe^{3+} and two Fe^{2+} atoms in the non-magnetic reduced state, and three Fe^{3+} and one Fe^{2+} atoms in the oxidized state. However, separate Fe^{3+} and Fe^{2+} spectra were not seen, suggesting that the *d* electrons are not localized on particular iron atoms.

In Table 2 the mean chemical shifts in the Mössbauer spectra of the iron–sulphur proteins are listed, together with the assignments of the valence states

Table 2 *Chemical shifts (relative to iron metal at* 290 K*) of iron–sulphur proteins at* 195 K

	δ/mm s^{-1}	*Active centre*
Fe^{3+} in rubredoxin	0.25	Fe^{3+}
Fe^{3+} in adrenodoxin	0.26	
Fe^{3+} in spinach ferredoxin	0.22	
Oxidized *Chromatium* high-potential iron–sulphur protein	0.32	3 Fe^{3+} + 1 Fe^{2+}
Reduced *Chromatium* high-potential iron–sulphur protein	0.42	2 Fe^{3+} + 2 Fe^{2+}
Oxidized *C. pasteurianum* ferredoxin	0.43	
Reduced *C. pasteurianum* ferredoxin	0.57	1 Fe^{3+} + 3 Fe^{2}
Fe^{2+} in rubredoxin	0.65	Fe^{2+}
Fe^{2+} in spinach ferredoxin	0.56	

of the iron atoms. It is believed that in the four-iron centre of *Chromatium* HIPIP two states exist: oxidized (3 Fe^{3+} + 1 Fe^{2+}) and reduced (2 Fe^{3+} + 2 Fe^{2+}). It has been conjectured that a further electron could be added to the reduced molecule to produce a 'super-reduced' state, *i.e.* 1 Fe^{3+} + 3 Fe^{2+} (which is formally equivalent to the active centre in reduced *C. pasteurianum* ferredoxin). Such molecules have been produced by the action of O_2-free DMSO and sodium dithionite though they cannot be isolated from the reduced protein. Their Mössbauer spectra have been measured by Dickson and Cammack.[447] At 77 K the spectrum consists of two quadrupole doublets. One has $\delta = 0.46$ mm s^{-1} and $\Delta E_Q = 1.09$ mm s^{-1} and is, within experimental error, identical with that of

[445] C. W. Carter, J. Kraut, S. T. Freer, N. Xuong, R. A. Alden, and R. G. Bartsch, *J. Biol. Chem.*, 1974, **249**, 4212.

[446] D. P. E. Dickson, C. E. Johnson, R. Cammack, M. C. W. Evans, D. O. Hall, and K. K. Rao, *Biochem. J.*, 1974, **139**, 105.

[447] D. P. E. Dickson and R. Cammack, *Biochem. J.*, 1974, **143**, 763.

reduced HIPIP. The other has $\delta = 0.59$ mm s^{-1} and $\Delta E_Q = 1.28$ mm s^{-1}; the shift is close to that of the reduced state of *C. pasteurianum* ferredoxin. This demonstrates that the iron atoms in super-reduced HIPIP and reduced *C. pasteurianum* ferredoxin are in a similar valence state, *i.e.* 1 Fe^{3+} + 3 Fe^{2+}, though there may be some non-equivalence between the iron atoms within each four-iron centre. The low-temperature spectrum shows a broad magnetic h.f.s. pattern, and shows that there are some magnetic differences between the four-iron centres in these two molecules. Possibly this is due to the effects of spin–spin interactions between the two four-iron clusters in the ferredoxin.

The Mössbauer spectra of the Mo–Fe (Kp1) and the Fe (Kp2) proteins prepared from ^{57}Fe-enriched nitrogenase of *Klebsiella pneumonia* have been measured by Smith and Lang.[448] Nitrogenase from a number of micro-organisms has been separated into two proteins, the larger containing molybdenum, iron, and acid-labile sulphur, and the other iron and acid-labile sulphur. The reduced Mo–Fe protein in the presence of $Na_2S_2O_4$ was found to contain three iron species giving spectra with the following properties (using the authors' nomenclature):

(M4) $\delta = 0.65$ mm s^{-1} and $\Delta E_Q = 3.05$ mm s^{-1} at 4.2 K with an intensity corresponding to two iron atoms per molecule of protein. This was interpreted as being due to a spin-coupled pair of high-spin Fe^{2+} atoms.

(M5) $\delta = 0.61$ mm s^{-1} and $\Delta E_Q = 0.83$ mm s^{-1} at 77 K with intensity corresponding to eight iron atoms per molecule of protein. This was interpreted as being due to Fe_4S_4 or Fe_2S_2 low-spin ferrous iron clusters, though a comparison of the chemical shift with Table 2 suggests that high-spin ferrous is much more likely.

(M6) $\delta = 0.37$ mm s^{-1} and $\Delta E_Q = 0.71$ mm s^{-1} at 77 K also with intensity corresponding to eight iron atoms per molecule of protein, and showing a broad magnetic hyperfine spectrum at 4.2 K. In the presence of the Fe protein, $Na_2S_2O_4$, ATP, and Mg^{2+} this latter species changed to

(M7) $\delta = 0.46$ mm s^{-1} and $\Delta E_Q = 1.04$ mm s^{-1} at 4.2 K. Thus the M6 → M7 transformation was shown to be similar to the change observed [444] on reduction of *Chromatium* HIPIP. On oxidation with the redox dye Louth's Violet these species changed to two states which had (M1) $\delta = 0.37$ mm s^{-1}, $\Delta E_Q = 0.75$ mm s^{-1} at 77 K and magnetic h.f.s. at 4.2 K and

(M2) $\delta = 0.35$ mm s^{-1} and $\Delta E_Q = 0.9$ mm s^{-1} at 4.2 K.

By contrast the Fe protein in the reduced form contained only one type of iron cluster with $\delta = 0.50$ mm s^{-1} and $\Delta E_Q = 0.9$ mm s^{-1} at 195 K, which showed magnetic h.f.s. at 4.2 K. It has not yet been possible to oxidize this protein without damaging the molecule.

Frankel *et al.*[449] have made measurements on synthetic tetrameric dianions $[Fe_4S_4(SR)_4]^{2-}$ where R = CH_2Ph or Ph. These molecules have been shown to be close structural and electronic analogues of the active sites of the four-iron centre of oxidized bacterial ferredoxins or reduced HIPIP. The formal oxidation states of the atoms are two iron(II) and two iron(III), but several techniques show

[448] B. E. Smith and G. Lang, *Biochem. J.*, 1974, **137**, 169.
[449] R. B. Frankel, T. Herskovitz, B. A. Averill, R. H. Holm, P. J. Krusic, and W. D. Phillips, *Biochem. Biophys. Res. Comm.*, 1974, **58**, 974.

that the iron sites are indistinguishable. The Mössbauer spectra gave a doublet with $\delta = 0.36$ mm s^{-1} and $\Delta E_Q = 1.25$ mm s^{-1} at 77 K, showing no hyperfine interaction when a magnetic field is applied at low temperatures. The shift was close to the value expected for 2 Fe^{3+} + 2 Fe^{2+} by extrapolation of the 195 K data of Table 2. One-electron reduction gives the trianions $[Fe_4S_4(SR_4)]^{3-}$ and their spectra gave $\delta = 0.48$ mm s^{-1}, $\Delta E_Q = 1.15$ mm s^{-1} at 77 K, and at 4.2 K showed a broad asymmetric magnetic hyperfine pattern, which agrees with the behaviour of the 1 Fe^{3+} + 3 Fe^{2+} active centres of the proteins.

Ferritin.—The iron-storage protein ferritin is spherical and *ca.* 120 Å diameter, comprising an inner 70 Å diameter core of iron(III) oxyhydroxyphosphate coated by 24 identical protein subunits. The structure of the core, which has the approximate molecular composition $(FeOOH)_8,FeO,PO_4H_2$ and a molecular weight up to *ca.* 400 000, is still unsolved.[450]

Webb and Gray[451] have studied Mössbauer and electronic absorption spectra of the core of ferritin, and also two model compounds isolated from hydrolysed iron(III) nitrate solutions. The chemical shift and hyperfine field for iron in the ferritin core indicate mainly octahedral iron(III) co-ordination.

Hormones.—Work has previously been reported on ^{129}I spectra of iodine in two hormones from the thyroid gland: 3,5-di-iodo-L-tyrosine (DIT) and L-thyroxine (T_4). Now Oberley *et al.*[452] have observed significant Mössbauer absorption ($\frac{1}{2}$%) for these and some similar hormones using the naturally occurring ^{127}I isotope (100% abundant), thus demonstrating the feasibility of using the technique in clinical research.

Vitamin B_{12}.—Vitamin B_{12} and its derivatives contain cobalt in an environment somewhat similar to that of iron in the haem proteins. If ^{57}Co is incorporated into the molecule, Mössbauer emission spectra may be measured. Cardin *et al.*[453] have measured the spectra of hydroxocobalamin, 5′-deoxyadenosylcobalamin (vitamin B_{12} coenzyme), and the holoenzyme system ethanolamine–ammonia lyase. The measurements were made in frozen aqueous solution buffered at pH 7.4 over the temperature range 4—190 K. The spectra gave broad lines and showed no differences attributable to chemical modification of hydroxocobalamin, implying that all the ^{57}Fe-labelled daughter products contain the metal stabilized in the same chemical environment. The most probable interpretation of the results is suggested to be a rapid (on the Mössbauer time-scale) cleavage of the cobalt–carbon bond following the electron-capture process.

8 Dissociation and Association of Proteins

Contributed by E. J. Wood

Analytical Ultracentrifuge Techniques.—*TV Scanner and Computer Systems.* At least two groups of workers[454] have investigated the possibility of using a

[450] T. G. Hoy, P. M. Harrison, and M. Shabbir, *Biochem. J.*, 1974, **139**, 603.
[451] J. Webb and H. B. Gray, *Biochim. Biophys. Acta*, 1974, **351**, 224.
[452] L. W. Oberley, U. Herskowitz, and J. C. Ehrhardt, *Phys. Letters* (*A*), 1974, **50**, 77.
[453] D. J. Cardin, J. N. Joblin, A. W. Johnson, G. Lang, and M. F. Lappert, *Biochim. Biophys. Acta*, 1974, **371**, 44.
[454] (*a*) E. G. Richards and D. Rockholt, *Arch. Biochem. Biophys.*, 1973, **158**, 864; (*b*) P. H. Lloyd and M. P. Esnouf, *Analyt. Biochem.*, 1974, **60**, 25.

television camera, instead of a photomultiplier tube and moving slit, with the absorption optical system of the analytical ultracentrifuge. Richards and Rockholt [454a] described the use of a commercial optical multichannel analyser as a scanner, with a u.v.-sensitive vidicon tube as the light detector. A reduced image of the cell was projected on to the surface of the vidicon tube which was divided up into 500 channels scanned *ca.* 30 times per second. The signal from the vidicon tube was converted into digital form for each channel so that the number of counts in a given channel was proportional to the light intensity for that radial position. A store was used to accumulate the counts for each channel for any desired number of scans up to 9999. The output of the instrument could be an oscilloscope display, thereby providing real time viewing of the image, or a chart recorder trace, or a digital printout. The accuracy of the instrument was verified by comparison of absorbance values with those determined by means of a spectrophotometer, and finally the analyser was used in the determination of the molecular weight of myoglobin by sedimentation equilibrium. It was concluded that even operating in the single-beam mode the optical multichannel analyser performed better than a double-beam commercial scanner giving greater accuracy and reduced scatter in the experimental points. Also, since the data were already in digital form, direct interfacing with a computer for analysis would be possible. A similar but somewhat less sophisticated system was devised by Lloyd and Esnouf,[454b] again using a vidicon tube as the detector. In this system the images of the cells were arranged to be at right angles to the line-scan of the camera: thus a pair of pulses on each line of the television scan results, one corresponding to the solvent and the other to the solution. The analogue measuring circuits deriving the absorbance of the solution from these pulses are slightly different from those used with a photoelectric scanner system. The pulses are not stored in a holding circuit which stores their peak amplitude, but in an integrator which stores the product of the amplitude and the duration of the pulses. Integration has the advantage over peak detection in that it is less affected by electronic noise in the system. The output from this system was not digital, which may be considered something of a disadvantage, as one of the most exciting developments in ultracentrifuge technique has been the direct interfacing of the ultracentrifuge with a computer. The system described by Crepeau *et al.*,[455a] did not involve a TV scanner but used the standard photoelectric scanner of a Beckman model E ultracentrifuge with an analogue-to-digital converter, the output being fed into a computer. This is a modification of the system originally described [455b] involving the addition of a disc store, an $X-Y$ recorder, and a modified triggering system using pulses from the rotor collar. In an experiment values of absorbance as a function of position in the cell are measured at sedimentation equilibrium, the computer collecting one transmittance point per revolution per cell. Some 5000 transmittance points may be collected from each sample-reference pair, and by making use of the multiplexing system and five six-channel Yphantis centrepieces in a six-hole

[455] (*a*) R. H. Crepeau, C. P. Hensley, and S. J. Edelstein, *Biochemistry*, 1974, **13**, 4860; (*b*) R. H. Crepeau, S. J. Edelstein, and M. J. Rehmar, *Analyt. Biochem.*, 1972, **50**, 213; (*c*) Q. H. Gibson, B. M. Hoffman, R. H. Crepeau, S. J. Edelstein, and C. Ball, *Biochem. Biophys. Res. Comm.*, 1974, **59**, 146.

rotor, one scan can give sufficient data to allow analysis of up to 15 solution-reference pairs. The output could be obtained in several different modes, such as an oscilloscope display, a chart record, or a high-speed printout, and could be in the form absorbance *vs.* radius or log absorbance *vs.* radius squared. In a study of the tetramer–dimer equilibrium for haemoglobin using this instrument the data were averaged in groups of 50 pairs of points and then fitted to an equation of the form

$$\text{absorbance} = \text{baseline} + A \exp(\alpha M_i r^2) + B \exp(2\alpha M_i r^2)$$

where $\alpha = 2RT\omega^2/(1 - \bar{v}\rho)$ and M_i = molecular weight of monomer in a monomer–dimer equilibrium or dimer weight in a dimer–tetramer equilibrium. Eventually the dissociation constant was found from

$$K_{4,2} = [\text{dimer}]^2/[\text{tetramer}]$$

where [dimer] = $A/1000\varepsilon$ and [tetramer] = $B/1000\varepsilon$, and ε is the millimolar extinction for haem. The programme for the least-squares fitting procedure was based on a general multiple linear-regression fit to a function linear in its coefficients. Certain statistical parameters were also calculated to test how well the data were fitted by the equation. It was shown that for carboxyhaemoglobin the data were best described in terms of two exponentials corresponding to dimers and tetramers, and a value for $K_{2,4}$ of 1.1×10^{-6} mol l^{-1} was derived. The system was also used to investigate the effect of inositol hexaphosphate on the dissociation constant as well as to show that the dissociation constant for sickle cell haemoglobin (HbS) in the carbonmonoxy form was identical with that for normal haemoglobin. The tetramer–dimer equilibrium in manganese haemoglobin was also investigated.[455c]

Interferometry. An optical pulse modulation system for laser interference studies in the analytical ultracentrifuge has been described [456] which can also be used in the non-pulsed mode. In the pulsed mode, the duration of the pulses was 0.8 μs at frequencies greater than 100 000 p.p.s. Such systems can be used to perform multiplexing, and fringe definition is improved.[457]

An attempt has been made to relieve the tedium of obtaining accurate fringe measurements using a microcomparator, by automating the procedure and adopting fibre optics to observe the fringes. In the instrument described [458] an enlarged image of the fringes is projected on to the face of a fibre optics analyser fitted with an electronic control unit and stepping motors. The fibre optic array is arranged to be parallel with the *y*-axis, and the instrument finds the edges of fringes by comparing the intensity of illumination at the central fibres with that in peripheral fibres. The positional information is translated into digital pulses and in this form therefore the data can be directly fed into a computer.

A number of technical procedures for the alignment of optical systems have appeared. In addition to a book by Lloyd on optical methods in ultra centrifugation,[459] Rees *et al.*[460] have described in great detail what they claim

[456] J. A. Lewis and J. W. Lyttleton, *Analyt. Biochem.*, 1973, **56**, 52.
[457] C. H. Paul and D. A. Yphantis, *Analyt. Biochem.*, 1972, **48**, 588, 605.
[458] R. M. Carlisle, J. I. H. Patterson, and D. E. Roark, *Analyt. Biochem.*, 1974, **61**, 248.
[459] P. H. Lloyd, 'Optical Methods in Ultracentrifugation, Electrophoresis, and Diffusion', Clarendon Press, Oxford, 1974.
[460] A. W. Rees, E. A. Lewis, and M. S. DeBuysere, *Analyt. Biochem.*, 1974, **62**, 19.

to be a less tedious procedure for rapid alignment of both the schlieren and the interference optical systems of Beckman instruments, and Cox and Ansevin [461] have described a method for the rapid alignment of the interference mask. Precise mask alignment is especially critical for achieving the full potential from difference sedimentation experiments. Difference sedimentation equilibrium has been developed by Springer *et al.*[462] as a method for measuring directly small differences in molecular weight. The measurement of molecular weights by the normal sedimentation equilibrium techniques has a precision of *ca.* 2—3%, thus slight changes in molecular weight resulting from concentration-dependent or ligand-mediated changes in the aggregation state cannot be interpreted unambiguously in terms of association–dissociation equilibria. The technique of difference sedimentation equilibrium offers the possibility of accurately measuring changes in molecular weight as small as 1%. Such measurements will additionally be of great value in interpreting data from difference sedimentation velocity experiments because changes in sedimentation coefficients may be due either to changes in frictional coefficients or to shifts in association–dissociation equilibria. In the difference sedimentation equilibrium method, the difference in effective molecular weight ($\Delta\sigma$) is obtained from the slope of a plot of the difference in concentration (Δc) divided by the average concentration ($\bar{c}$) against the square of the radial position (r^2). It is necessary to use both the interference optical system to measure Δc, and the schlieren optical system to measure, by integration, $\bar{c}$. Springer *et al.*[462] have developed the theory of the method and have tested it both by computer simulation and experimentally. For example, they measured known changes in effective molecular weight of 1—10% produced by adding various quantities of D_2O to one of each sample pair. Despite the presence of imperfections in the ultracentrifuge cells, differences in effective molecular weight could be measured with an absolute error of $<0.2\%$ using concentrations ($\bar{c}$) in the range 2—10 fringes. A special three-compartment cell and corresponding Rayleigh mark permitting the interferometric determination of $\bar{c}$ as well as Δc were also developed for greater accuracy.

Other developments include an ultracentrifuge with an electromagnetic drive which was capable of very low speeds without hunting for sedimentation equilibrium work (600 rev/min) [463]. The instrument was used to determine the molecular weight of a virus (5.55×10^6) at very low concentrations.

A commercial scanner system has been used to investigate the properties of lactate dehydrogenase under the conditions of the assay for enzymic activity. These conditions are usually characterized by the use of concentrations very much lower than those used for the investigation of proteins in the ultracentrifuge.[464] Activity transport and gel chromatography were also used. Below protein concentrations of 0.1 $\mu g\,ml^{-1}$ dissociation to dimer occurred accompanied by loss of activity. Several self-associating systems have been examined by sedimentation equilibrium, including chymotrypsinogen A,[465]

[461] D. J. Cox and A. T. Ansevin, *Analyt. Biochem.*, 1974, **58**, 161.
[462] M. S. Springer, M. W. Kirschner, and H. K. Schachman, *Biochemistry*, 1974, **13**, 3718.
[463] J. H. McGee, D. W. Kupke, W. Godschalk, and J. W. Beams, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 3866.
[464] P. Bartholmes, H. Durchschlag, and R. Jaenicke, *European J. Biochem.*, 1973, **39**, 101.
[465] M. S. Tung and R. F. Steiner, *European J. Biochem.*, 1974, **44**, 49.

papain,[466] lima bean proteinase inhibitor,[467] β-lactoglobulin,[468, 469] and lysozyme.[470] The lysozyme system was used to test a procedure for the analysis of non-ideal systems by sedimentation equilibrium. Another type of association–dissociation that has been investigated by sedimentation equilibrium is that of the non-allosteric enzyme, aldolase. This is in fact a tetramer–dimer–monomer system, but there have been reports in the literature that the enzyme contains three, four, or six subunits. It was shown by sedimentation equilibrium experiments in 10M acetic acid with no added supporting electrolyte that the subunit behaved as a single thermodynamic component, mol. wt. 40 000.[471] Hsu and Neet [472] studied aldolase by sedimentation equilibrium and analysed the data by the methods of Yphantis, of Adams, and of Steiner. It was concluded that a rapid, reversible association–dissociation equilibrium existed with association constants $K_{1,2} = 1.4 \times 10^5\,\mathrm{l\,mol^{-1}}$ and $K_{2,4} = 6.1 \times 10^5\,\mathrm{l\,mol^{-1}}$. It is of interest that the dissociation of myosin has also been studied in 1—10M acetic acid.[473] Many ligand-mediated associations have been studied, including the association of *E. coli* K12 aspartokinase III at high ionic strengths in the presence of lysine,[474, 475] and the association–dissociation of carboxyltransphosphorylase of *Propionibacterium shermanii* mediated by oxaloacetate, malate, and fumarate.[476] Such ligand-induced subunit interactions no doubt have an important role in the control of metabolism in bacteria.

Ker and Aune [477] have suggested a technique for the analysis of sedimentation equilibrium data by a numerical procedure following an experiment of the type they call 'moderate speed sedimentation equilibrium'. The aim was to obtain a distribution throughout the cell with the meniscus concentration neither high nor zero. A meniscus concentration of about half a fringe is suitable. The data were analysed using the method of orthogonal polynomials for curve fitting and the method may be used to study associating–dissociating systems.

Gel Chromatograpy.—The theory of the use of gel chromatography for the study of interacting protein systems has been discussed in a previous Report.[478] Winzor and his co-workers have extended the procedure in several directions. The origin and consequences of concentration dependence in gel chromatography have been investigated,[479] and a procedure that corrects for Donnan effects in studies of ligand binding has been described.[480] A disadvantage of the use of the

[466] M. W. Pandit and M. S. N. Rao, *Biochim. Biophys. Acta*, 1974, **371**, 211.
[467] J. D. Sakura and S. N. Timasheff, *Arch. Biochem. Biophys.*, 1973, **159**, 123.
[468] L. H. Tang and E. T. Adams, *Arch. Biochem. Biophys.*, 1973, **157**, 520.
[469] J. L. Sarquis and E. T. Adams, *Arch. Biochem. Biophys.*, 1974, **163**, 442.
[470] L. A. Holladay and A. J. Sophianopoulos, *Analyt. Biochem.*, 1974, **57**, 506.
[471] S. Szuchet and D. A. Yphantis, *Biochemistry*, 1973, **12**, 5115.
[472] L. S. Hsu and K. E. Neet, *Biochemistry*, 1973, **12**, 586.
[473] S. Szuchet and C. R. Zobel, *Biochemistry*, 1974, **13**, 1482.
[474] C. Richaud, J. P. Mazat, C. Gros, and J. C. Patte, *European J. Biochem.*, 1973, **40**, 619.
[475] J. D. Funkhauser, A. Abraham, V. A. Smith, and W. G. Smith, *J. Biol. Chem.*, 1974, **249**, 5478.
[476] W. E. O'Brien and H. G. Wood, *J. Biol. Chem.*, 1974, **249**, 4917.
[477] E. G. Ker and K. C. Aune, *Analyt. Biochem.*, 1974, **62**, 1.
[478] G. L. Kellett, in 'Amino-acids, Peptides, and Proteins', ed. R. C. Sheppard, (Specialist Periodical Reports), The Chemical Society, London, 1973, Vol. 5, p. 230.
[479] L. W. Nichol, M. Janado, and D. J. Winzor, *Biochem. J.*, 1973, **133**, 15.
[480] D. O. Jordan, S. J. Lovell, D. R. Phillips, and D. J. Winzor, *Biochemistry*, 1974, **13**, 1832.

normal Hummel and Dreyer[481] method is that any Donnan redistribution of small ions resulting from confinement of the macroion to the mobile phase of the gel column is not taken into account. The new method suggested involves arranging for there to be an extended zone of charged macromolecular acceptor on the column pre-equilibrated with a known concentration of ionized ligand. The method was used successfully to study the binding of copper to serum albumin in $I = 0.2$ acetate at pH 4.0 on a column of Sephadex G-25, and also for investigating the DNA-Cu^{2+} system. The binding of Ca^{2+} to apothermolysin was studied by gel chromatography:[482] at pH 9 in the concentration range 10^{-6}—10^{-3} mol l^{-1}, two calcium ions were found to dissociate simultaneously and the slope (n) of the Hill plot was 2.0. The effect of allosteric ligands on the activity and aggregation of rabbit muscle phosphofructokinase was extensively investigated by gel chromatography by Hammes' group.[483] Whereas the Stokes radius of the protein in the presence of the inhibitor citrate (5 mmol l^{-1}) was 37 Å, in the presence of the activator fructose 6-phosphate (0.1—10 mmol l^{-1}) it was 68 Å, thought to correspond to the tetramer. Only the tetramer showed enzymic activity. The association behaviour of human erythrocyte phosphofructokinase has also been studied by gel chromatography.[484] The effect of phosphate on the stability of the tetramer of lactate dehydrogenase (LDH) was studied by Lovell and Winzor.[485] In acetate-Cl at pH 5 ($I = 0.2$) LDH from rabbit muscle completely dissociated to dimers, but this could be prevented by phosphate at concentrations above 30 mmol l^{-1} (or by NAD or by NADH) and activity was then retained. The dissociation to dimers was reversible upon raising the pH to 7.0 but some irreversible changes appeared to take place depending upon how long the protein had been exposed to pH 5. Lovell and Winzor[486] have used a gel chromatography procedure to study rapid equilibria of the type

$$A + B \rightleftharpoons C + D$$

In this method the frontal procedure for the system $V_A = V_C < V_B = V_D$ was used to study electron transfer between reduced cytochrome c and ferricyanide on Sephadex G-25.

Protein–protein interactions have also been studied by many groups. Winzor and co-workers investigated the dimerization of insulin at pH 11.0.[487] In such a system an unusual elution pattern may be obtained in difference gel chromatography experiments involving the transfer of a solute from one solvent system in which it is stable to one in which it undergoes a time-dependent isomerization. The theory employed in this case is based on a set of partial differential equations expressing mass conservation during migration. A value for the first-order rate constant of 0.011 min^{-1} for the dimerization of insulin under these conditions was obtained by this procedure.

[481] J. P. Hummel and W. J. Dreyer, *Biochim. Biophys. Acta*, 1962, **63**, 530.
[482] G. Voerdouw and R. S. Roche, *Biochemistry*, 1974, **13**, 5017.
[483] P. M. Lad, D. E. Hill, and G. G. Hammes, *Biochemistry*, 1973, **12**, 4303.
[484] G. Zimmermann, K. W. Wenzel, J. Gauer, and E. Hofmann, *European J. Biochem.*, 1973, **40**, 501.
[485] S. J. Lovell and D. J. Winzor, *Biochemistry*, 1974, **13**, 3527.
[486] S. J. Lovell and D. J. Winzor, *Arch. Biochem. Biophys.*, 1973, **156**, 640.
[487] S. J. Lovell, L. W. Nichol, and D. J. Winzor, *F.E.B.S. Letters*, 1974, **40**, 233.

The dissociation of haemoglobin continues to interest many investigators. Chiancone *et al.*[488] used Bio-Gel P-100 columns to study the dissociation of normal and carboxypeptidase-digested human haemoglobin by the difference method. In agreement with previous results,[489] the deoxy-derivative of normal haemoglobin was much less dissociated than the oxy- or carbonmonoxy-derivatives. This difference tended to vanish in those proteins in which, as a result of extensive digestion, the conformational change accompanying ligand binding was abolished. Human oxy-haemoglobin at protein concentrations between 0.15 and 0.73% in the presence of 1.0M-KI had a molecular weight of between 34 000 and 36 700 as determined by gel filtration [490] indicating that it was almost wholly dimeric. Deoxygenation caused the molecular weight to return to *ca.* 60 000.

By means of computer simulation procedures, theoretical reaction boundaries have been generated for several reversible, macromolecular association–dissociation reactions under kinetic control.[491] It was shown that for a system initially at equilibrium, kinetic control will be expressed when the half-time of the first-order rate constant is near one order of magnitude of the time the macromolecules have been on the column. Ackers and his collaborators [492] have considered the effects of non-uniform column packing in analytical gel chromatography both for monodisperse solutes and for associating solutes. In the latter case considerable changes can occur in the shape of the reaction boundary profiles although the qualitative feature of the constant column case are retained.

A relatively new development is the use of affinity chromatography for the quantitative study of acceptor–ligand interactions. Andrews *et al.*[493] investigated the effects of *N*-acetyl-D-glucosamine and D-glucose on the elution of the A protein of human lactose synthetase from a column of α-lactalbumin linked to a Sepharose matrix, and Nichol *et al.*[494] have considered the evaluation of equilibrium constants by affinity chromatography. The systems they investigated were Sephadex–lysozyme–glucose and Sephadex–lectin–galactose.

Light Scattering.—A number of light-scattering studies of associating–dissociating protein systems have appeared. The association–dissociation properties of beef liver glutamate dehydrogenase at different ionic strengths were studied in the presence and absence of 5% dioxan and 2—6M ethylene glycol.[495] These reagents appeared to interfere with hydrophobic interactions and cause dissociation. Lipoamide dehydrogenase could be isolated as a dimer in two forms: (a) dissociable and (b) non-dissociable. The interconversion of these and their dissociation to inactive monomers were studied by light scattering and gel chromatography.[496]

[488] E. Chiancone, N. M. Anderson, E. Antonini, J. Bonaventura, C. Bonaventura, M. Brunori, and C. Spagnuolo, *J. Biol. Chem.*, 1974, **249**, 5689.
[489] G. L. Kellett, *J. Mol. Biol.*, 1971, **59**, 401.
[490] S. Tomita, Y. Enoki, T. Ochiai, M. Kawase, and T. Okuda, *J. Mol. Biol.*, 1973, **73**, 261.
[491] J. K. Zimmerman, *Biochemistry*, 1974, **13**, 384.
[492] G. H. Weiss and G. K. Ackers, *Analyt. Biochem.*, 1974, **57**, 569; J. K. Zimmerman and G.K. Ackers, *ibid.*, p. 578.
[493] P. Andrews, B. J. Kitchen, and D. J. Winzor, *Biochem. J.*, 1973, **135**, 897.
[494] L. W. Nichol, A. G. Ogston, D. J. Winzor, and W. H. Sawyer, *Biochem. J.*, 1974, **143**, 435
[495] F. P. Gauper, K. Markau, and H. Sund, *European J. Biochem.*, 1974, **49**, 555.
[496] H. van Muiswinkel-Voetberg, J. Visser, and C. Veeger, *European J. Biochem.*, 1973, **33**, 265.

In the dissociable form there was a reversible monomer–dimer equilibrium (K_{ass} 3—6 μl mol^{-1}). The tetramer–dimer equilibrium of carbonmonoxyhaemoglobin at neutral pH in the concentration range 0.7—12 g l^{-1} was studied by light scattering by Norén *et al.*[497] as an extension of their previous work. At 0.1 mol l^{-1}, the K_{diss} for tetramer to dimer was of the order of 10^{-6} mol l^{-1}, but in 2M-NaCl was *ca.* 10^{-5} mol l^{-1}. This is still smaller than other reported values: removing bound 2,3-diphosphoglycerate (DPG) had no effect on these values. Engelborghs and Lontie[498] studied the dissociation of *Helix pomatia* α-haemocyanin by light scattering and investigated the effect of alkali chlorides in causing dissociation. In contrast to the pH-induced dissociation this process was both completely reversible and rapid. The *in vitro* assembly of neurotubules from porcine tubulin has also been investigated.[499]

Transport Studies.—Several theoretical and practical treatments of the sedimentation velocity analysis of interacting systems have appeared.[500] Haschemeyer and Estis[501] have presented a method for the analysis of simple ideal associating systems from sedimentation velocity data, and the method was used in the study of the ornithine-induced self-association of the glutamine-dependent carbamyl phosphate synthetase of *E. coli*. Several groups have successfully used computer simulation to predict boundary profiles for interacting systems. Holloway and Cox[502] considered solutes undergoing indefinite self-association and computed changes in boundary shape resulting from varying the association constant and the molecular weight of the associating monomer. Both ideal and non-ideal solutes were considered. All of the calculated boundaries were skew with the leading limb of the gradient profile steeper than the trailing limb. No shoulders or subsidiary peaks were observed for any of the models used. Cann and Oates[503] computed theoretical sedimentation (and electrophoretic) patterns for several kinetically controlled macromolecular interactions such as irreversible isomerization, dimerization, dissociation into identical subunits, and reversible and irreversible dissociation of a complex. The results indicated that two peaks would be expected for half-times of reaction ranging from 0.3 to 2.5 times the duration of the experiment, depending on the difference in mobility between product and reactant. An unambiguous method for distinguishing between reaction and inherent heterogeneity is described. In a further paper[504] theoretical patterns were computed for reversible kinetically controlled macromolecular dimerization reactions, both non-mediated and ligand-mediated. The pattern predicted for half-times of 20—60 s was practically the same as that for instantaneous re-equilibration during differential transport of monomer and dimer. When the half-time of dissociation of dimer was < *ca.* 200 s, it was predicted that resolution

[497] I. B. E. Norén, D. A. Bertoli, C. Ho, and E. F. Casassa, *Biochemistry*, 1974, **13**, 1683.
[498] Y. Engelborghs and R. Lontie, *J. Mol. Biol.*, 1973, **77**, 577.
[499] F. Gaskin, C. R. Cantor, and M. L. Shelanski, *J. Mol. Biol.*, 1974, **89**, 737.
[500] L. M. Gilbert and G. A. Gilbert, in 'Methods in Enzymology', ed. C. H. W. Hirs and S. N. Timasheff, 1973, **27**, 273; J. R. Cann and W. B. Goad, *ibid.*, p. 296.
[501] P. P. Trotta, L. F. Estis, A. Meister, and R. H. Hashemeyer, *J. Biol. Chem.*, 1974, **249**, 482; P. P. Trotta, L. M. Pinkus, R. H. Hashemeyer, and A. Meister, *J. Biol. Chem.*, 1974, **249**, 492.
[502] R. R. Holloway and D. J. Cox, *Arch. Biochem. Biophys.*, 1974, **160**, 595.
[503] J. R. Cann and D. C. Oates, *Biochemistry*, 1973, **12**, 1112.
[504] J. R. Cann and G. Kegeles, *Biochemistry*, 1974, **13**, 1868.

of the reaction boundary into two peaks would only be observed in ligand-mediated dimerization. Computer simulation has also been used as the basis for a semi-empirical theory for the evaluation of binding constants from experimental parameters obtained from gel electrophoresis of interacting molecules.[505] Sedimentation patterns contain information on sedimentation and diffusion. McNeil and Bethune[506] have used numerical methods for the simultaneous determination of sedimentation and diffusion coefficients from transient state sedimentation analysis. Though only applied to homogeneous non-associating solutes so far, it is to be expected that this technique, which allows a rapid estimate of M_{app}, should prove useful in studying systems involving time-dependent polymerization or aggregation. On somewhat similar lines, Grievink *et al.*[507] used Gehatia's equation and the least-squares method to compute protein molecular weights from refractive index gradient curves.

Many associating–dissociating systems are pressure dependent, and a recent review discusses the observation and identification of pressure-dependent systems in sedimentation velocity experiments.[508] The possibility of convection is known to lead to instabilities in such systems.[509] Convection is a problem at the very low concentrations that may be studied by the use of scanner optics. It was found that erroneous values for the sedimentation coefficient of glyceraldehyde 3-phosphate dehydrogenase were obtained below *ca.* 0.05 mg ml^{-1} unless a stabilizing gradient of sucrose was added.[510] As the sedimentation coefficient actually appeared to decrease in the low concentration range such systems might be wrongly interpreted as undergoing dissociation.

The technique of reacting-enzyme sedimentation was used to study the monomer–dimer equilibrium of yeast hexokinase,[511] and the dimer–tetramer equilibrium of the threonine-sensitive aspartokinase-homoserine dehydrogenase of *E. coli*.[512] Difference sedimentation was used to investigate multiple aggregation states of phosphoribosyladenosine triphosphate synthetase (hexamer under normal assay conditions).[513] This enzyme was reversibly cold-sensitive between 4 and 7.5 °C and could aggregate or dissociate depending upon the protein concentration. Difference sedimentation was also used to study the binding of successive NAD molecules to the subunits of glyceraldehyde 3-phosphate dehydrogenase.[514]

Electron Microscopy.—Relatively few electron microscopic studies of protein association and dissociation have appeared. The technique as applied to proteins has been reviewed.[515] It has been used largely in addition to other techniques

[505] J. Eisinger and W. E. Blumberg, *Biochemistry*, 1973, **12**, 3648.
[506] B. J. McNeil and J. L. Bethune, *Biochemistry*, 1973, **12**, 3244, 3254.
[507] J. Grievink, R. T. B. Houterman, and K. DeGroot, *Analyt. Biochem.*, 1974, **57**, 137.
[508] W. F. Harrington and G. Kegeles, in 'Methods in Enzymology', ed. C. H. W. Hirs and S. N. Timasheff, 1973, **27**, 306.
[509] M. Johnson, D. A. Yphantis, and G. H. Weiss, *Biopolymers*, 1973, **12**, 2477.
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[512] J. C. Mackall and K. E. Neet, *European J. Biochem.*, 1974, **42**, 275.
[513] S. M. Parsons and D. E. Koshland, *J. Biol. Chem.*, 1974, **249**, 4119.
[514] G. D. Smith and H. K. Schachman, *Biochemistry*, 1973, **12**, 3789.
[515] R. M. Oliver, in 'Methods in Enzymology', ed. C. W. H. Hirs and S. N. Timasheff, 1973, **27**, 616.

as a check on the processes one believes one is observing by other methods. Haga *et al.*[516] studied the polymerization–depolymerization behaviour of microtubules by flow birefringence and electron microscopy. They investigated the effects of temperature, Ca^{2+} concentration, EGTA, and colchicine on this system. Microtubule polymerization and depolymerization in D_2O was studied by Houston *et al.*,[517] who showed that the microtubules formed in D_2O were indistinguishable in the electron microscope from those formed in H_2O.

The inducible lysine decarboxylase of *E. coli* B is composed of subunits of molecular weight 80 000.[518] In the electron microscope the native form of the enzyme appears to be a decamer (mol. wt. 780 000) formed by the cyclic association of five dimers, in the form of two stacked pentameric rings. However, higher aggregates were also observed (*ca.* 60S) resulting from the linear stacking of decamers to form rod-like structures of indefinite length. The glutamine synthetase of *Bacillus stearothermophilus* (19.7S) was shown to have a similar arrangement of subunits except that it was a double hexagonal dodecameric structure.[519] *E. coli* glutamine synthetase had a similar structure [520] and appeared to undergo a zinc-induced paracrystalline aggregation.[521] This two-layer hexagonal arrangement of subunits is reminiscent of that found in many annelid erythrocruorins,[522] though the one from *Oenone fulgida* appears to be anomalous in having an additional, centrally placed subunit as well.

The assembly of viruses or virus capsids is of course an area of study where the electron microscope has great potential. Much effort has been devoted to the study of the assembly of tobacco mosaic virus (TMV) protein. A 20S disc is a species often observed and this is composed of 34 monomers in a two-turn helix which appears to be able to add to partially assembled rods. However, Richards and Williams [523] found that monomers could also be added to the growing rod. They studied addition to partially assembled rods from either 'disc' (a 70 : 30 mixture of 20S disc and 4S 'A protein' complex) or 'A protein' alone. ('A protein' is a multicomponent equilibrium mixture of monomer, trimer, and higher polymers.) The stacked disc aggregate of TMV protein alone was studied by Klug and his associates [524] by means of *X*-ray diffraction and also by means of the technique of 3D-image reconstruction from electron micrographs of aggregates. Srinivasan and Lauffer [525] have compared by electron microscopy and light scattering the polymerization behaviour of normal TMV protein with that of a number of TMV strains such as the Dahlmense strain and the PM2 strain.

The assembly of the capsids of some spherical viruses has also received attention. The capsid of adenovirus is formed by the association of 252 protein

[516] T. Haga, T. Abe, and M. Kurokawa, *F.E.B.S. Letters*, 1974, **39**, 291.
[517] L. L. Houston, J. Odell, Y. C. Lee, and R. H. Himes, *J. Mol. Biol.*, 1974, **87**, 141.
[518] D. L. Sabo, E. A. Boeker, B. Byers, H. Waron, and E. H. Fischer, *Biochemistry*, 1974, **13**, 662.
[519] F. C. Wedler and F. M. Hoffmann, *Biochemistry*, 1974, **13**, 3207.
[520] J. E. Ciardi, F. Cimino, and E. R. Stadtman, *Biochemistry*, 1973, **12**, 4321.
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[524] J. T. Finch and A. Klug, *J. Mol. Biol.*, 1974, **87**, 633; P. N. T. Unwin and A. Klug, *ibid.*, p. 641; P. N. T. Unwin, *ibid.*, p. 657; R. Leberman, J. T. Finch, P. F. C. Gilbert, J. Wits, and A. Klug, *ibid.*, 1974, **86**, 179.
[525] S. Srinivasan and M. A. Lauffer, *Arch. Biochem. Biophys.*, 1973, **158**, 53.

subunits on an icosahedral surface. Pereira and Wrigley[526] have reported conditions for the *in vitro* assembly of parts of this shell (hexon monomers) to form empty virus shells. The protein from a spherical plant virus, cowpea chlorotic mottle virus, could also be reassembled *in vitro* to give a 50S species equivalent to the capsid.[527] The pH was an extremely important factor in the reassembly, and the dependency on protein concentration indicated that capsid formation was a quasi-crystallization.

Siezen and van Bruggen[528] used the electron microscope and the ultracentrifuge to study the dissociation of *Helix pomatia* α-haemocyanin and the conformation of the resulting subunits. In its native form this haemocyanin has a molecular weight of *ca.* 9×10^6 and dissociates first to one-half and then to one-tenth and one-twentieth molecules with increasing pH. The one-half molecules are pentameric rings, and electron microscopy revealed that the one-tenth molecules could exist in two distinct conformations depending upon the ionic strength. At pH values near 8.0 and high ionic strength the one-tenths existed in a compact form, but at low ionic strength at this pH, or at higher pH values, a loose form existed. The compact-to-loose change was characterized by a change in structure to a flexible cluster of globules. Concomitantly there was a decrease in sedimentation coefficient and an increase in intrinsic viscosity and frictional ratio. Stable intermediates (2/10, 3/10, 4/10 molecules) were rarely, if ever, seen in the electron microscope, but they could be stabilized by intramolecularly cross-linking one-half molecules by means of dimethylsuberimidate prior to dissociation. From such data Siezen and van Bruggen have built up a convincing model for the structure of native *H. pomatia* haemocyanin.

Kinetic Studies.—*Protein–Protein Interactions.* Though there is considerable information on the assembly of viruses, flagella, *etc.*, little is known about the assembly processes of oligomeric enzymes. Bothwell and Schachman[529] have made an attempt to rectify this situation by studying the assembly of aspartate transcarbamylase (ATCase). This enzyme is composed of six catalytic and six regulatory polypeptide chains, the former organized into trimers (C) and the latter into dimers (R). Structural studies support the view that the oligomer is a complex of two C subunits bridged by three R subunits, *i.e.* C_2R_3. The association of C and R at protein concentrations of the order of mg ml^{-1} is complete within seconds: this process was studied by spectral changes in stopped-flow experiments. However, Bothwell and Schachman have also studied the process at much lower concentrations (μg ml^{-1}) where assembly is slower (30 min) and where it might be possible to study individual steps and identify and characterize possible intermediates. The method used involved mixing ^{125}I-labelled subunits[530] (*e.g.* ^{125}I-C) with excess R, stopping after suitable times by an appropriate 'chase' (*e.g.* unlabelled C), and identifying and quantitating intermediates by electrophoresis on polyacrylamide gels. Measurements of the rate of combination of regulation-subunit-deficient molecules (*i.e.* C_2R_2) with free regulatory subunits

[526] H. G. Pereira and N. G. Wrigley, *J. Mol. Biol.*, 1974, **85**, 617.
[527] K. W. Adolph and P. J. G. Butler, *J. Mol. Biol.*, 1974, **88**, 327.
[528] R. J. Siezen and E. F. J. van Bruggen, *J. Mol. Biol.*, 1974, **90**, 77.
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[530] J. M. Syvanen, Y. R. Yang, and M. W. Kirschner, *J. Biol. Chem.*, 1973, **248**, 3762.

by this method gave a second-order rate constant of 10^5 l mol^{-1} s^{-1} for the formation of bonding domains between catalytic and regulatory chains. This technique is still in a preliminary state of development but would appear to show considerable promise for the study of assembly processes.

Other workers have followed the kinetics of assembly or association of proteins by different methods. The interaction of Kunitz trypsin inhibitor with 2-chymotrypsin was followed by stopped-flow and proflavine displacement methods.[531] At neutral pH there was a fast pre-equilibrium (diffusion-controlled rate and dissociation equilibrium constant 5×10^4 mol l^{-1}) followed by slow formation of the final complex (rate constant 350 s^{-1}). In the case of many enzymes the rate of reassociation of subunits after removing a denaturant such as urea by dilution may be followed by difference spectra, fluorescence measurements, or regain of enzyme activity. For the L-asparaginase of *E. coli* B (a tetrameric enzyme) it was shown that upon removing urea, refolding of the peptide chain was complete within 30 s after dilution of the denaturant, and that this was followed by a slower aggregation of the subunits into the oligomers.[532] There was no evidence for dimers as intermediates, and both L- and D-asparagine were equally effective in inducing association of monomer to tetramer. The rate of reassociation of the inactive subunits of formyl tetrahydrofolate synthetase from Clostridia to give active tetramer was followed by the rate of regain of enzymic activity.[533] Increasing the ionic strength increased the rate and extent of reassociation, and difference spectroscopy suggested that aromatic amino-acids became buried during the reassociation process.

There has been much interest in the association processes of certain hormones. Many of these are glycoproteins with the structure $\alpha\beta$. The fluorescent probe ANS (1-anilinonaphthalene-8-sulphonate) only binds to the associated forms of certain of these hormones, not to the subunits.[534] The rates of association and dissociation of human luteinizing hormone were studied by this method.[535] The first-order dissociation rate was strongly dependent on temperature, pH, and urea concentration, but the rate of recombination was also dependent upon pH and temperature but independent of the subunit concentrations in the range 10—40 μmol l^{-1}. Bovine thyrotropin was studied by the same method.[536] The kinetics of dissociation and reassociation of ovine pituitary interstitial cell stimulating hormone were studied by c.d. and sedimentation velocity.[537] Dissociation was rapid and first-order, exposing previously buried tyrosyl groups. The reassociation process was more complicated; there appeared to be a rapid, probably second-order combination of α and β subunits followed by a much slower, first-order rearrangement of conformation resulting in the burying of at least two tyrosine residues. Other hormone systems that have been studied

[531] U. Quast, J. Engel, H. Heumann, G. Krause, and E. Steffen, *Biochemistry*, 1974, **13**, 2512.
[532] S. Shifrin and C. L. Parrott, *J. Biol. Chem.*, 1974, **249**, 4175.
[533] J. A. K. Harmony, P. J. Shaffer, and R. H. Himes, *J. Biol. Chem.*, 1974, **249**, 394.
[534] S. M. Aloj, K. C. Ingham, and H. Edelhoch, *Arch. Biochem. Biophys.*, 1973, **155**, 478.
[535] K. C. Ingham, S. M. Aloj, and H. Edelhoch, *Arch. Biochem. Biophys.*, 1973, **159**, 596; S. M. Aloj, H. Edelhoch, K. C. Ingham, F. J. Morgan, R. E. Canfield, and G. T. Ross, *ibid.*, p. 497.
[536] K. C. Ingham, S. M. Aloj, and H. Edelhoch, *Arch. Biochem. Biophys.*, 1974, **163**, 589.
[537] T. A. Bewley, M. R. Sairam, and C. H. Li, *Arch. Biochem. Biophys.*, 1974, **163**, 625.
[538] W. E. Merz, U. Hilgenfeldt, P. Brockerhoff, and R. Brossner, *European J. Biochem.*, 1973, **35**, 297.

by similar methods include the recombination of the subunits of human chorionic gonadotrophin (by c.d.)[538] and human luteinizing hormone (by u.v. spectra).[539]

Changes in the c.d. spectrum were used to follow the reversible aggregation and conformation changes in TMV protein.[540] Double-disc formation was stimulated by a decrease in pH or by an increase in ionic strength, and it was shown that conformational changes preceded the aggregation process.

Protein–Small Molecule Interactions. The fluorescence stopped-flow apparatus described previously by Chen and his co-workers[541] was used to study the binding of bilirubin to serum albumin.[542] There was an initial rapid binding as shown by quenching of protein fluorescence, a moderately fast development of bilirubin fluorescence, and a slower conformational change shown by further quenching of protein fluorescence.

The interaction of CTP and the ATP analogue, 6-mercapto-9-β-D-ribofuranosyl 5′-TP, with ATCase was studied by the temperature-jump method. Two processes were observed with CTP alone, probably reaction with catalytic and regulatory sites, respectively, but in presence of carbamyl phosphate and succinate, a single process was observed.[543]

There have been many studies of the kinetics of the reaction of ligands with oxygen-binding proteins. Van Driel *et al.*[544] used stopped-flow and temperature-jump methods to investigate the reaction of *Helix pomatia* haemocyanin with oxygen, and Ilgenfritz and Schuster[545] used the temperature-jump method in an extensive study of oxygen binding by human haemoglobin. McDonald and Noble[546] used the stopped-flow technique to study the kinetics of the reaction *p*-hydroxymercuribenzoate with thiol groups in human adult and foetal haemoglobins in order to investigate conformational changes occurring upon oxygenation. Cole and Gibson[547] used the temperature-jump method to observe the binding of n-butylisocyanide by human haemoglobin. Two relaxation processes were observed which they attributed to the binding of ligand to α and β subunits.

Protein–Small Molecule Equilibria.—In addition to the kinetic studies already mentioned, protein–small molecule equilibria have been studied extensively from both the theoretical and the experimental standpoints, particularly as applied to the interaction between haemoglobin and oxygen. Imai[548] obtained precise oxygen equilibrium curves for human adult haemoglobins in the presence and absence of DPG, and then analysed the data according to the stepwise oxygenation theory of Adair and the allosteric model of Monod, Wyman, and Changeux. This work was extended in later papers to determine successive association constants for the binding of oxygen.[549] DPG markedly reduced

[539] W. H. Bishop and P. J. Ryan, *Biochem. Biophys. Res. Comm.*, 1974, **59**, 1298.
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[541] R. F. Chen, A. N. Schechter, and R. L. Berger, *Analyt. Biochem.*, 1969, **29**, 68.
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[547] F. X. Cole and Q. H. Gibson, *J. Biol. Chem.*, 1973, **248**, 4998.
[548] K. Imai, *Biochemistry*, 1973, **12**, 798.
[549] I. Tyuma, K. Imai, and K. Shimizu, *Biochemistry*, 1973, **12**, 1491; K. Imai and I. Tyuma, *Biochim. Biophys. Acta*, 1973, **293**, 290.

k_1, k_2, and k_3 without affecting k_4, whereas inositol hexaphosphate enormously reduced all four k values.

Hopfield [550] has presented a theoretical treatment of the relation between structure, co-operativity, and spectra in a model of haemoglobin action, and Herzfeld and Stanley [551] have given a general approach to co-operativity and its application to haemoglobin. This latter model combines the concepts of preferential binding and quaternary constraints (Monod, Wyman, and Changeux), nearest-neighbour subunit interaction (Koshland), and changes in subunit aggregation (Briehl). It is claimed that the general model can explain systems which previous models could not.

Minton and Imai [552] have developed an allosteric model employing three affinity states because they considered that the two-state model did not provide even an approximate description except under defined extreme conditions. Ackers and Halvorson [553] have considered the variation of the saturation function (Y) with total protein concentration, and the variation of the subunit dissociation constant with ligand concentration. The functions were used to obtain correlations between changes in dimer–dimer contact energy and the sequential binding steps.

Edelstein [554] has extended the allosteric model for haemoglobin and has considered the consequences of the well-established functional non-equivalence of the α- and β-chains. This aspect has also been studied by Mansouri and Winterhalter.[555] The equilibrium binding of oxygen and CO to haemoglobin was studied by means of n.m.r. in the presence and absence of DPG.[556] Previously the effects of anions and ligands on the tertiary structure and ligand binding site had been investigated by a similar method involving the ring-current shifted proton resonances in the 250 MHz n.m.r. spectrum.[557] Intermediate states in the ligation of haemoglobin in the presence of DPG were also studied by ^{19}F n.m.r. spectroscopy using the β93-trifluoroacetonylated derivative.[558] Calorimetry has been used to measure the binding of DPG and inositol hexaphosphate to haemoglobin,[559] and the enthalpy change for formation of the DPG-haemoglobin complex was determined.[560] The Bohr effect in the reaction of haemoglobin with CO was investigated by microcalorimetry.[561]

The study of ligand binding by haemoglobins is well developed but both theory and experiment in the case of the bigger multisubunit oxygen-carrying pigments such as haemocyanins and erythrocruorins are less advanced. Nevertheless it has been established that erythrocruorins and haemocyanins bind oxygen co-operatively, and there is a suggestion that in the case of mollusc haemocyanins at least, a closed ring of five one-tenth molecules (*i.e.* a one-half molecule) is a

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[559] D. P. Nelson, W. D. Miller, and L. A. Kiesow, *J. Biol. Chem.*, 1974, **249**, 4770.
[560] B. E. Hedlund and R. Lovrien, *Biochem. Biophys. Res. Comm.*, 1974, **61**, 859.
[561] J. A. Rudolph and S. J. Gill, *Biochemistry*, 1974, **13**, 2451.

necessary condition to constrain the protein in a state of low oxygen affinity.[562, 563] Oxygen binding by *Cancer magister* haemocyanin was studied by resonance Raman spectroscopy.[564]

Other equilibria between small molecules and proteins that have been investigated are the interaction between L-aspartate or acetyl-CoA and the phosphoenolpyruvate carboxylase of *Salmonella typhimurium*,[565] that between fructose 1,6-diphosphate and phosphofructokinase,[566] and that between Ca^{2+} and the various muscle proteins.[567]

Subunit Structure of Proteins.—In the course of the survey of the literature for 1973 and 1974 for this report it was noted that the subunit structure of at least 150 proteins had been determined by SDS gel electrophoresis, or by treatment with guanidine hydrochloride, urea, or other dissociating reagent. No attempt has therefore been made to produce a table of subunit structures, and instead a few selected examples will be considered.

The iron-storage protein ferritin and the corresponding apo-protein have been studied by a number of groups. The iron-free apo-protein has a molecular weight of around half a million, but there is controversy over the number and possible heterogeneity of the subunits. Crichton *et al.*[568] considered that the apo-protein was composed of 24 subunits of molecular weight 18 500 daltons, but Linder *et al.*[569] obtained evidence that subunits of molecular weights 13 000 and 19 500 daltons were present. Niitsu *et al.*[570] found that the major component in SDS gel electrophoresis was one of molecular weight about 19 000 but that a slow dissociation took place resulting in the production of units of molecular weight 10 000—11 000 and 7000—8000.

SDS gel electrophoresis is proving particularly useful in the investigation of proteins which are composed of subunits of different sizes. Rabbit muscle phosphorylase kinase, native molecular weight 1.33×10^6 dalton, appeared to have the composition $A_4B_4C_8$, where the molecular weights of the component subunits were 118 000, 108 000, and 41 000, respectively.[571] Lee *et al.*[572] studied an interesting polyenzyme responsible for the synthesis of the antibiotic tyrocidine. Three enzymic activities were required, corresponding to protein molecular weights 100 000, 230 000, and 440 000. The last of these appeared to bind the last six amino-acids in the tyrocidine sequence and was made up of six subunits of molecular weight 70 000, while the 230 000 component activated and bound proline, L- and D-phenylalanine and appeared to contain three 70 000 dalton subunits. It was suggested that there might be one subunit for each amino-acid activated.

[562] R. van Driel and E. F. J. van Bruggen, *Biochemistry*, 1974, **13**, 4079.
[563] J. S. Pearson and E. J. Wood, *F.E.B.S. Letters*, 1974, **48**, 246.
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[572] S. G. Lee, R. Roskoski, K. Bauer, and F. Lipmann, *Biochemistry*, 1973, **12**, 398.

The NADPH-sulphite reductase of *E. coli* (mol. wt. 700 000) was shown to have the structure $\alpha_8\beta_4$, where both α and β subunits have molecular weights in the region 54 000—60 000,[573] and the nitrite reductase of *E. coli* K12 (mol. wt. 720 000—773 000) appeared to have the structure $\alpha_4\beta_4$, where the molecular weights of the α and β subunits were 142 000 and 58 000, respectively.[574] Certain lectins and other plant proteins have received intensive study and usually appear to be multisubunit proteins. The phytohaemagglutinin from pea was composed of two subunits of molecular weight 18 000 and two of 10 500,[575] and that from lentil had two of molecular weight 18 000 and two of molecular weight 8000.[576] The storage protein of *Vicia faba*, legumin, could be dissociated in two stages depending upon whether 2-mercaptoethanol (ME) was present or not:[577]

$$\alpha_6\beta_6 \xrightarrow{\text{SDS}} 6\alpha\beta \xrightarrow{\text{SDS-ME}} 6\alpha + 6\beta$$

where the molecular weights of the α and β subunits were 36 200 and 22 000, respectively. Ascorbate oxidase underwent a similar dissociation:[578]

$$140\,000 \longrightarrow 2 \times 65\,000 \longrightarrow 2 \times 38\,000 + 2 \times 28\,000$$

The agglutinin of *Ricinus communis* (mol. wt. 125 000) appeared to be composed of components of molecular weights 33 000, 30 000, and 27 500, and the toxin from the same plant (mol. wt. 55 000) appeared to be made up of components of molecular weight 32 000 and 28 000.[579] Caution needs to be exercised when examining glycoproteins on SDS gels because of anomalous binding of SDS, and it may be noted here that many plant proteins, including some of those mentioned above, are glycoproteins. It was shown for example that a human erythrocyte glycoprotein, molecular weight 29 000, of composition *ca.* 55% carbohydrate and 45% protein, bound SDS anomalously and gave an incorrect molecular weight in SDS gel electrophoresis.[580] The α-amylase of *Bacillus subtilis* (molecular weight of native protein 48 000) did not bind as much SDS as bovine serum albumin, and whereas its molecular weight determined in concentrated guanidine hydrochloride solution was 23 500, indicating the presence of two subunits, the molecular weight in SDS-gels appeared to be 154 000.[581] The fact that caeruloplasmin is a glycoprotein may also explain the current difficulties in establishing its subunit structure.[582]

Tanford *et al.*[583] have described procedures suitable for the determination of molecular weights and Stokes radii of proteins in detergent solutions by sedimen-

[573] L. M. Siegel and P. S. Davis, *J. Biol. Chem.*, 1974, **249**, 1587.
[574] C. H. MacGregor, C. A. Schnaitman, D. E. Normansell, and M. G. Hodgins, *J. Biol. Chem.*, 1974, **249**, 5321.
[575] T. Marik, G. Entlicher, and J. Kocourek, *Biochim. Biophys. Acta*, 1974, **336**, 53.
[576] O. Fliegerova, A. Salvetova, M. Ticha, and J. Kocourek, *Biochim. Biophys. Acta*, 1974, **351**, 416.
[577] D. J. Wright and D. Boulter, *Biochem. J.*, 1974, **141**, 413.
[578] K. G. Strothkamp and C. R. Dawson, *Biochemistry*, 1974, **13**, 434.
[579] L. G. Gürtler and H. J. Horstmann, *Biochim. Biophys. Acta*, 1973, **295**, 582.
[580] S. P. Grefrath and J. A. Reynolds, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 3913.
[581] E. D. Mitchell, P. Riquetti, R. H. Loring, and K. L. Carraway, *Biochim. Biophys. Acta*, 1973, **295**, 314.
[582] S. Freeman and E. Daniel, *Biochemistry*, 1973, **12**, 4806.
[583] C. Tanford, Y. Nozaki, J. A. Reynolds, and S. Makino, *Biochemistry*, 1974, **13**, 2369.

tation equilibrium and sedimentation velocity with analysis of the data by equations appropriate for multicomponent systems. The Stokes radius can also be measured independently by gel chromatography and the molecular weight then determined by sedimentation velocity alone. The procedures were intended for the study of proteins, such as membrane proteins, which are only sparingly soluble in simple aqueous media. However, they are equally applicable to other proteins in detergent media and should therefore be useful in investigating, for example, glycoproteins which behave anomalously on SDS-gels. The determination of molecular weights of protein–SDS complexes by controlled pore glass chromatography has also been described.[484]

Allen [585] studied the binding of SDS to bovine serum albumin under a number of conditions, and Katz and his co-workers [586] measured the volume effects produced by reaction of several haem proteins with SDS using a dilatometric procedure. The isotherms obtained of volume change, δV, against molarity of SDS were composite depending on detergent binding, conformational changes, disruption of quaternary structure, and the type and ligand state of the haem group. Van Heyningen [587] found that adding K^+ to protein–SDS complexes precipitated more than 90% of the free SDS but less than 10% of the protein. The results of Nozaki *et al.*[588] with a cationic detergent (tetradecyltrimethylammonium chloride) suggested that such compounds were not suitable as substitutes for SDS in procedures in which the denaturing action of the detergent was an essential feature, *e.g.* SDS-gel electrophoresis. Resistance to denaturation is also a problem in certain instances. Uricase, which appears to be a tetramer of molecular weight 125 000, required heating at over 90 °C in SDS for 2 h, or at 78 °C in 6M guanidine hydrochloride for 1 h in order to dissociate it.[589] Problems have also been encountered in trying to dissociate mollusc haemocyanin (molecular weight of native proteins *ca.* 9×10^6). Brouwer and Kuiper [590] found that the smallest molecular weight they could obtain for *Helix pomatia* haemocyanin by SDS or guanidine hydrochloride treatment was about 265 000, and Wood and Peacocke [591] obtained similar results with *Murex trunculus* haemocyanin. Since these haemocyanins bind one molecule of oxygen per 50 000 molecular weight, the 'subunit' obtained by SDS or guanidine hydrochloride treatment corresponds to five or six active sites. Lontie's group [592] obtained functional subunits of *Helix pomatia* α-haemocyanins of molecular weight *ca.* 50 000 by treatment of the native haemocyanin with a bacterial protease.

Arthropod haemocyanins differ considerably from mollusc haemocyanins in being smaller, in having a lower copper content, and in having a different arrangement of subunits. The subunit structure has been studied by SDS and guanidine hydrochloride treatment. *Cancer magister* haemocyanin appeared to contain

[584] R. C. Collins and W. Haller, *Analyt. Biochem.*, 1973, **54**, 47.
[585] G. Allen, *Biochem. J.*, 1974, **137**, 575.
[586] S. Katz, J. E. Miller, and J. A. Beall, *Biochemistry*, 1973, **12**, 710.
[587] S. van Heyningen, *Biochim. Biophys. Acta*, 1973, **328**, 303.
[588] Y. Nozaki, J. A. Reynolds, and C. Tanford, *J. Biol. Chem.*, 1974, **249**, 4452.
[589] O. M. Pitts, D. G. Priest, and W. W. Fish, *Biochemistry*, 1974, **13**, 888.
[590] M. Brouwer and H. A. Kuiper, *European J. Biochem.*, 1973, **35**, 428.
[591] E. J. Wood and A. R. Peacocke, *European J. Biochem.*, 1973, **35**, 410.
[592] R. Lontie, M. Deley, H. Robberecht, and R. Witters, *Nature*, 1973, **242**, 180.

more than one type of monomer,[593] and the haemocyanin of *Cherax destructor* and *Callianassa californiensis* both appeared to exist as a 17S hexamer.[594] In the case of *Callianassa* haemocyanin 17S and 39S particles were present in the haemolymph and these appeared to be in a monomer–tetramer relationship in which the 17S monomer had a molecular weight of *ca.* 4.3×10^5.

SDS gel electrophoresis with earthworm erythrocruorin revealed the presence of six components with molecular weights 12 000, 14 000, 16 000, 19 000, 31 000, and 36 000.[595] Amino-acid analysis of the isolated subunits suggested the presence of six different types of polypeptide chain. Native earthworm erythrocruorin had a molecular weight of between 3.2 and 3.8 $\times 10^6$ daltons [595, 596] and this dissociated in the alkaline pH region to give species with sedimentation coefficients 10.1, 3.5, and 2.3S. It was proposed that the 10S subunit was a one-twenty-fourth molecule, and it was shown that this subunit was capable of binding oxygen co-operatively.[597]

Many studies of the effects of guanidine hydrochloride and urea on proteins have appeared. A procedure has been described for gel chromatography in 6M guanidine hydrochloride containing 0.1M 2-mercaptoethanol which permits the determination of molecular weights of peptides in the size range 2—146 amino-acids in length from the distribution coefficient of the peptide on the column,[598] and a similar procedure with proteins labelled with dansyl groups allowed protein samples of 0.05 mg to be examined.[599] The determination of molecular weights by electrophoresis in concentrated urea only worked after complete disruption of all disulphide links and even then was inferior in accuracy to the SDS-gel method.[600]

Results on the regain of activity of flounder muscle glyceraldehyde 3-phosphate dehydrogenase after urea treatment suggested that the primary structure of the protein alone did not have sufficient information for proper reconstitution.[601] However, addition of NAD upon removal of urea led to an 80% recovery of enzyme activity indicating that the cofactor played a major role in organizing proper refolding. Elbaum and Herskovits [602] studied the dissociation of human haemoglobin by urea and amides (formamide, acetamide, *etc.*) by osmotic pressure and light-scattering measurements. They hoped to predict what sort of amino-acid and peptide interactions were important for the maintenance of the associated protein. The effectiveness of these compounds in dissociating haemoglobin increased with increasing chain length or hydrocarbon content of the substituent alkyl group. Rabbit muscle phosphorylase *b*, after $NaBH_4$ reduction, could be completely and reversibly dissociated to monomers by treatment with

[593] J. S. Loehr and H. S. Mason, *Biochem. Biophys. Res. Comm.*, 1973, **51**, 741; D. E. Carpenter and K. E. van Holde, *Biochemistry*, 1973, **12**, 2231.
[594] A. C. Murray and P. D. Jeffrey, *Biochemistry*, 1974, **13**, 3667; R. Roxby, K. Miller, D. P. Blair, and K. E. van Holde, *ibid.*, p. 1662; K. Miller and K. E. van Holde, *ibid.*, p. 1668.
[595] J. M. Shlom and S. N. Vinogradov, *J. Biol. Chem.*, 1973, **248**, 7904.
[596] M. M. David and E. Daniel, *J. Mol. Biol.*, 1974, **87**, 89.
[597] M. M. David and E. Daniel, *F.E.B.S. Letters*, 1973, **32**, 293.
[598] A. Protzel and A. J. Morris, *J. Biol. Chem.*, 1974, **249**, 4594.
[599] M. Yoshino and T. Takagi, *Analyt. Biochem.*, 1973, **54**, 290.
[600] T. Poole, B. S. Leach, and W. W. Fish, *Analyt. Biochem.*, 1974, **60**, 596.
[601] P. J. Marangos and S. M. Constantinides, *Biochemistry*, 1974, **13**, 904.
[602] D. Elbaum and T. T. Herskovits, *Biochemistry*, 1974, **13**, 1268.

6.7% formamide.[603] The dissociation of proteins by chaotropic salts has been investigated. Katz *et al.*[604] studied volume effects produced by protein-ion interactions, and Sawyer and Puckridge[605] found that the ability of such chaotropic ions to dissociate proteins followed the Hofmeister series for proteins which self-interact mainly *via* formation of intramolecular hydrogen bonds (β-lactoglobulin A, haemoglobin) but not necessarily for proteins having hydrophobic associations (concanavalin A). Aviram[606] has also studied the interaction of chaotropic anions with proteins. Such studies will be useful in suggesting ways of dissociating multisubunit proteins without necessarily denaturing them in the process.

Examples of Associating–Dissociating Systems.—Only one example of an associating–dissociating protein system will be considered, that of sickle-cell haemoglobin (HbS). There has been a great deal of work on this haemoglobin in recent years because of the clinical interest in sickle-cell anaemia and because of the possibility of using reagents which cause the aggregates to dissociate in the treatment of this hereditary condition. At low partial pressures of oxygen HbS molecules aggregate to form a gel-like material composed of long fibres. These can apparently extend the length of the erythrocyte and deform it. The rate of gel formation is of crucial importance as extensive sickling will only occur if erythrocytes stay in the capillary bed long enough, and likewise unsickling will only occur if the arterial transit time is long in comparison with the half-time for depolymerization. A thermodynamic model for gelation of HbS was proposed by Minton.[607] In the first stage aggregation occurs to produce rod-like microtubular arrays, and then in a second stage the tubules are aligned to form a nematic phase. Wilson *et al.*[608] studied the initial stage of aggregation by means of the technique of quasi-elastic light scattering. The transition from a non-aggregated to an aggregated state was observed in solutions of HbS when the oxygen pressure was reduced below a critical value. The results were consistent with an association process linear with time, in which any size aggregate could combine with any other size aggregate. The gelation process was temperature dependent. Malfa and Steinhardt[609] studied the temperature-dependent latent period in the aggregation process by viscosity measurements. Moffat and Gibson[610] similarly used a temperature change, rather than deoxygenation, to study the rate of polymerization and depolymerization of HbS using turbidity measurements to follow the process. They also found a lag followed by a rapid increase in turbidity which was pseudo first order. The depolymerization showed no lag and was biphasic. There was also gross hysteresis typical of a multi-molecular aggregation process when the temperature jump was cycled with a 2-h period at each temperature. Jones *et al.*[611] compared the kinetic stability of

[603] J.-I. Tu and D. J. Graves, *J. Biol. Chem.*, 1973, **248**, 4617.
[604] S. Katz, J. K. Crissman, and L. C. Roberson, *J. Biol. Chem.*, 1974, **249**, 7892.
[605] W. H. Sawyer and J. Puckridge, *J. Biol. Chem.*, 1973, **248**, 8429.
[606] I. Aviram, *European J. Biochem.*, 1973, **40**, 631.
[607] A. P. Minton, *J. Mol. Biol.*, 1974, **82**, 483.
[608] W. W. Wilson, M. R. Luzzana, J. T. Penniston, and C. S. Johnson, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 1260.
[609] R. Malfa and J. Steinhardt, *Biochem. Biophys. Res. Comm.*, 1974, **59**, 887.
[610] K. Moffatt and Q. H. Gibson, *Biochem. Biophys. Res. Comm.*, 1974, **61**, 237.
[611] D. D. Jones, W. P. McGrath, D. Carroll, and J. Steinhardt, *Biochemistry*, 1973, **12**, 3818.

normal and sickle-cell haemoglobins at the extremes of pH, and Bookchin and Nagel[612] investigated the requirements for polymerization of HbS by preparing half-liganded hybrid tetramers with either the α or the β chain fixed in the cyanmet state. Gelation occurred on deoxygenation of the ferrous chains. Briehl and Ewert[613] investigated the gelation of the met-form of HbS by a sedimentation equilibrium method[614] and found that a phase change indicative of gel formation occurred at higher concentrations than those required for gelation of deoxy-HbS. Several substances have been studied as potential anti-sickling agents. Zinc is one such agent that may have potential as a therapeutic agent or as a molecular probe.[615] In addition to having an anti-sickling action, zinc also interacts with normal human haemoglobin, increasing the oxygen affinity.[616] Waterman *et al.*[617] studied the effect of compounds which disrupt non-covalent quaternary and tertiary structures in inhibiting the aggregation of HbS, and Benesch *et al.*[618] found that substitution of the *N*-terminal amino-groups with pyridoxal derivatives inhibited gelation and increased the solubility of HbS. It had previously been shown that cyanate inhibits sickling by carbamylating the *N*-terminal amino-groups.[619] Several groups have studied the effects of DPG and of inositol hexaphosphate on the stability of HbS as measured by rate of precipitation[620] or by a sedimentation equilibrium method.[614]

[612] R. M. Bookchin and R. L. Nagel, *J. Mol. Biol.*, 1973, **76**, 233.
[613] R. W. Briehl and S. M. Ewert, *J. Mol. Biol.*, 1974, **89**, 759.
[614] R. W. Briehl and S. M. Ewert, *J. Mol. Biol.*, 1973, **80**, 445.
[615] G. J. Brewer and F. J. Oelshlegel, *Biochem. Biophys. Res. Comm.*, 1974, **58**, 854.
[616] F. J. Oelshlegel, G. J. Brewer, C. Knutsen, A. S. Prasad, and E. B. Schoomaker, *Arch. Biochem. Biophys.*, 1974, **163**, 742.
[617] M. R. Waterman, K. Yamaoka, L. Dahm, J. Taylor, and C. L. Cottam, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 2222.
[618] R. Benesch, R. E. Benesch, and S. Yung, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 1504.
[619] A. Cerami and J. M. Manning, *Proc. Nat. Acad. Sci. U.S.A.*, 1971, **68**, 1180.
[620] K. Adachi and T. Asakura, *Biochemistry*, 1974, **13**, 4976.

3
Peptide Synthesis

BY D. J. SCHAFER

1 Introduction

The year under review, while not perhaps the most dramatic 12 months for peptide chemistry, has still provided a few highlights. The most notable is surely the elegant total synthesis of human insulin reported by the Ciba group. Meanwhile the flow of progress reports on ribonuclease and cytochrome *c*, for example, tell us that the mammoth tasks of protein synthesis which have been undertaken are moving inexorably towards completion. The short cut of solid-phase synthesis still fails to provide a completely satisfying alternative to the classical approach, but work continues on all fronts to improve and refine the method and it may yet fulfil its early promise. Much work continues on devising ingenious new ways of carrying out the basic tasks of peptide synthesis. Unfortunately many new developments languish as solutions in search of a problem, but the occasional exception (the dramatic increase in the use of *N*-hydroxybenzotriazole for coupling is a good example) demonstrates the real need for new approaches. Ugi's use of four-component condensations is, in this respect, one of the most exciting developments in recent years, offering the prospect of peptide-bond formation in a step which is both intramolecular and extremely rapid. Future developments will be awaited with great interest.

The format for this chapter is more or less as established in earlier volumes. Appendix II now includes some additional data on the amino-acid derivatives reported during the year. The synthesis of the smaller peptide hormones and analogues and other small peptides continues unabated as can be seen from the diverse list of syntheses achieved during the year given in Appendix I.

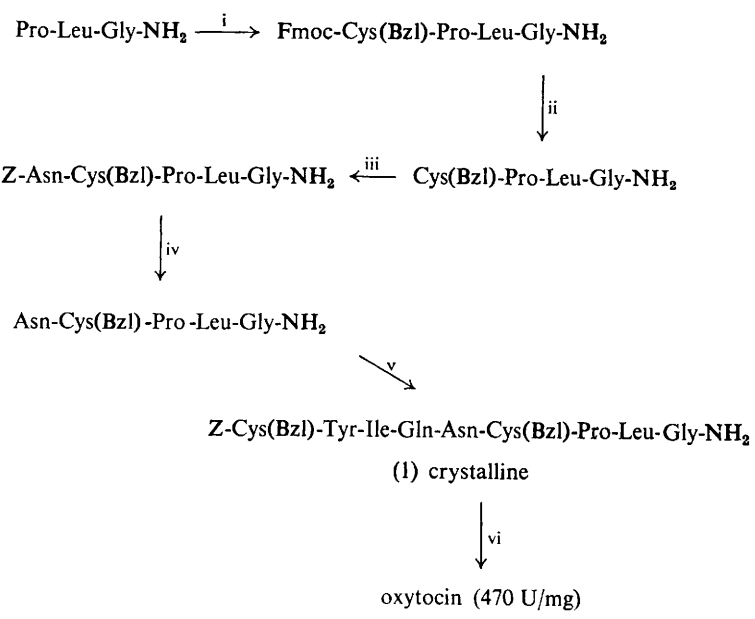
2 Methods

Several reviews have appeared,[1, 2] including one on the tactics of peptide synthesis with minimal side-chain protection.[3e] A book has appeared dealing among other topics with the procedures of peptide synthesis.[4a] A review by Young aimed at advanced undergraduate level provides a superbly clear account of the essentials of the subject.[5]

[1] M. Fridkin and A. Patchornik, *Ann. Rev. Biochem.*, 1974, **43**, 419.
[2] Y. Shimonishi, *Sogo Rinsho*, 1974, **23**, 1296 (*Chem. Abs.*, 1974, **81**, 136 461f).
[3] 'The Chemistry of Polypeptides', ed. P. G. Katsoyannis, Plenum, New York, 1973; (*a*) M. Bodanszky and Y. S. Klausner, p. 21; (*b*) G. T. Young, p. 43; (*c*) I. Photaki, p. 59; (*d*) J. Rudinger, p. 87; (*e*) R. Hirschmann and D. F. Veber, p. 125; (*f*) J. S. Fruton, p. 143; (*g*) E. Wünsch, p. 279; (*h*) K. Medzihradszky, p. 259; (*i*) A. Patchornik, M. Fridkin, and E. Katchalski, p. 315; (*j*) R. B. Merrifield, p. 335; (*k*) P. Fankhauser and M. Brenner, p. 389.
[4] MTP International Reviews of Science: 'Organic Chemistry, Series One', 1973, Vol. 6; (*a*) C. H. Stammer, p. 135; (*b*) R. Wade, p. 161.
[5] G. T. Young, *Essays Chem.*, 1972, **4**, 115.

Protective Groups.—Reviews have appeared on photosensitive protecting groups,[6] and on the use of tosyl and related groups in peptide synthesis.[3d] The present state and perspectives in the synthesis of peptides are the subject of a review which concentrates particularly on protecting groups.[7] A book has appeared dealing with protecting groups in organic chemistry.[8] The sections on amino- and carboxy-protection are not aimed particularly at peptide synthesis and will be of little value to peptide chemists. In contrast, the chapter on thiol protection [8a] reviews the cysteine thiol group particularly thoroughly.

Established Methods of Amino-group Protection. The inability to use catalytic hydrogenolysis generally for the removal of such groups as *N*-benzyloxycarbonyl in the presence of amino-acids which contain sulphur has been a particularly painful thorn for the peptide chemist. The news [9, 10] that the benzyloxycarbonyl group can be removed by catalytic hydrogenolysis in liquid ammonia solution in the presence of such amino-acids is therefore extremely welcome. The technique has been examined in a range of amino-acid derivatives [9] and its value has been demonstrated in a synthesis of oxytocin (Scheme 1).[10] Unfortunately only



Conditions: i, Fmoc-Cys(Bzl)–DCCI–HOBt; ii, 8 h in refluxing liquid NH_3; iii, Z-Asn-ONp; iv, Pd black–H_2–liquid NH_3, 6—8 h; v, stepwise as for iii and iv with four other ONp esters; vi, Na–liquid NH_3

Scheme 1

[6] B. Amit, U. Zehavi, and A. Patchornik, *Israel J. Chem.*, 1974, **12**, 103.
[7] K. Jost, *Chem. Listy*, 1974, **68**, 145 (*Chem. Abs.*, 1974, **80**, 146 493m).
[8] 'Protective Groups in Organic Chemistry', ed. J. F. W. McOmie, Plenum, London 1973; (*a*) R. G. Hiskey, p. 235.
[9] J. Meienhofer and K. Kuromizu, *Tetrahedron Letters*, 1974, 3259.
[10] K. Kuromizu and J. Meienhofer, *J. Amer. Chem. Soc.*, 1974, **96**, 4978.

incomplete removal occurred with (2) in spite of the lack of problems with closely related compounds such as (3). The cysteine residue was therefore introduced

Z-Cys(Bzl)-Pro-Leu-Gly-NH_2 (2) Z-Cys(Bzl)-Gly-NH_2 (3)

with the base-labile 9-fluorenylmethyloxycarbonyl (Fmoc) group as amino protection, subsequently removed by refluxing in liquid ammonia for 8 h. The benzyloxycarbonyl group was removed from the other intermediates of the stepwise synthesis completely and without problems by hydrogenolysis over palladium black. The crystalline protected nonapeptide (1) was converted in the usual way into highly active oxytocin. Despite the evident occasional failure the method seems to be quite widely applicable and should prove extremely useful.

The unwanted loss of side-chain benzyloxycarbonyl and other benzyl-based side-chain protecting groups is a recently identified problem particularly in solid-phase synthesis (see p. 253). The satisfactory removal of the t-butoxycarbonyl group may be carried out with trifluoroacetic acid–acetic acid 7 : 3 leaving such groups intact, as shown in a synthesis of vasoactive intestinal peptide (VIP).[11] In this same synthesis the final removal of benzyloxycarbonyl groups with hydrogen bromide in trifluoroacetic acid was unsatisfactory even in the presence of scavengers, and removal of these protecting groups was accomplished more suitably by reaction with trifluoroacetic acid alone for 2.5 days. The *p*-phenylazobenzyloxycarbonyl group, being more stable than the benzyloxycarbonyl group, has been used for the side-chain protection of lysine during the solid-phase synthesis of a protected myoglobin sequence.[12]

Selective removal of the *N*-benzyloxycarbonyl group from (4) can be achieved by hydrogenolysis in the presence of cyclohexylamine (Scheme 2). Hydro-

Ser (Bzl) $\xleftarrow{\text{i}}$ Z-Ser(Bzl) (4) $\xrightarrow{\text{ii}}$ Ser

Conditions: H_2–Pd in the presence of (i) 4 mol $C_6H_{11}NH_2$–MeOH; (ii) 1 mol HCl–MeOH

Scheme 2

genolysis under acidic conditions removes also the *O*-benzyl group.[13] The catalytic hydrogenolysis of benzyl esters has been studied using the homogeneous catalyst tris(triphenylphosphine)rhodium chloride.[14]

The selective removal of *N*-trityl in the presence of an *N*-(2-*p*-biphenylisopropoxycarbonyl) group has been used in an elegant total synthesis of insulin.[15] The procedure involves reaction at a controlled 'pH' (using a glass electrode without taking into account the non-aqueous solvent) using hydrogen chloride in 90% trifluoroethanol. This now establishes a remarkable range of four groups

[11] M. Bodanszky, Y. S. Klausner, C. Y. Lin, V. Mutt, and S. I. Said, *J. Amer. Chem. Soc.*, 1974, **96**, 4973.
[12] R. Uhmann and E. Bayer, *Annalen*, 1974, 1955.
[13] H. Medzihradszky-Schweiger, *Ann. Univ. Sci. Budapest. Rolando Eotvos Nominatae, Sect. Chim.*, 1972, 35 (*Chem. Abs.*, 1974, **80**, 83 584u).
[14] H. Sugiyama, S. Tsuchiya, S. Seto, Y. Senda, and S. Imaizumi, *Tetrahedron Letters*, 1974, 3291.
[15] P. Sieber, B. Kamber, A. Hartmann, A. Jöhl, B. Riniker, and W. Rittel, *Helv. Chim. Acta*, 1974, **57**, 2617.

(trityl, 2-*p*-biphenylisopropoxycarbonyl, t-butoxycarbonyl, and benzyloxycarbonyl) all removable by acidolysis but whose rates of removal differ sufficiently to give mutually selective removal for each, under appropriate conditions. The use of hydrogen chloride in 90% trifluoroethanol at a lower (0.5—1) controlled 'pH' also served for the selective removal of the 2-*p*-biphenylisopropoxycarbonyl group in the presence of t-butyl-based side-chain protection.[15]

The $\beta\beta\beta$-trichloroethyloxycarbonyl group which can be removed reductively (using zinc) has been used for the protection of the ε-amino-group of lysine and the terminal hydrazide group of a fragment in the synthesis of dogfish melanotrophin.[16]

t-Butoxycarbonylamino-acids can be prepared in high yields from t-butoxycarbonyl azide and amino-acids in dioxan–water (1 : 1) in the presence of triethylamine without the need for a pH meter.[17] Di-t-butyl pyrocarbonate, $Bu^tO\cdot CO\cdot O\cdot CO\cdot OBu^t$ (5), has been used to introduce the t-butoxycarbonyl group into amino-esters (see Vol. 5, p. 260) and its use has now been extended to the synthesis of t-butoxycarbonylhydrazide derivatives from the corresponding hydrazide (Scheme 3).[18] The t-butoxycarbonyl group can be determined

$$Z\cdot NH\cdot CHR\cdot CO\cdot NHNH_2 \xrightarrow{(5)} Z\cdot NH\cdot CHR\cdot CO\cdot NHNHBoc$$

Conditions: pyridine–DMF

Scheme 3

quantitatively in amino-acid and peptide derivatives by a simple non-aqueous titration.[19] An excess of perchloric acid in acetic acid removes all of the t-butoxycarbonyl groups and the excess of perchloric acid not forming a salt with the liberated amine is back-titrated against sodium methoxide using preferably a cresol red indicator.

An improved method of preparing (6) and a number of related derivatives involving the use of benzyl pentachlorophenyl mixed carbonate (7) in the presence of *N*-trimethylsilylacetamide (8) has been described.[20] Full practical details have

$Z\text{-}Arg(Z_2)$	$Bzl\cdot O\cdot CO\cdot OPcp$	$MeCONHSi(Me)_3$	$Bu^tO\cdot CO\cdot OPh$
(6)	(7)	(8)	(9)

been published for the synthesis of t-butoxycarbonyl amino-acids (exemplified by proline) using the mixed carbonate (9) and the amino-acid tetramethylguanidinium salt.[21]

N-Trifluoroacetyl groups may be introduced in the presence of acid-labile groups by the action of trifluoroacetic anhydride on *N*-trimethylsilylamino-acid trimethylsilyl esters (10) (Scheme 4).[22] The co-product (11) is volatile and the

[16] H. Watanabe, M. Kubota, H. Yajima, A. Tanaka, M. Nakamura, and T. Kawabata, *Chem. and Pharm. Bull. (Japan)*, 1974, **22**, 1889.
[17] Z. Grzonka and B. Lammek, *Synthesis*, 1974, 661.
[18] S. I. Dolinskii, V. F. Pozdnev, and E. S. Chaman, *Khim. prirod. Soedinenii*, 1974, 266 (*Chem. Abs.*, 1974, **81**, 49 995q).
[19] S. Ehrlich-Rogozinski, *Israel J. Chem.*, 1974, **12**, 31.
[20] E. L. Smithwick, jun. and R. T. Shuman, *J. Org. Chem.*, 1974, **39**, 3441.
[21] U. Ragnarsson, S. M. Karlsson, B. E. Sandberg, and L. E. Larsson, *Organic Syntheses*, 1973, 53.
[22] H. R. Kricheldorf and M. Fehrle, *Synthesis*, 1974, 420.

$$(CF_3CO)_2O + Me_3Si{\cdot}NH{\cdot}CHR{\cdot}CO{\cdot}O{\cdot}SiMe_3$$
(10)

$$\xrightarrow{i}$$

$$CF_3CO{\cdot}O{\cdot}SiMe_3 + CF_3CO{\cdot}NH{\cdot}CHR{\cdot}CO{\cdot}O{\cdot}SiMe_3$$
(11) (12)

$$\xrightarrow{ii} CF_3CO{\cdot}NH{\cdot}CHR{\cdot}CO_2H$$

Conditions: i, room temp.; ii, H_2O

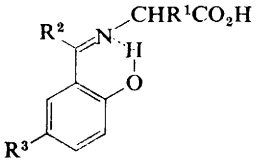
Scheme 4

esters (12) may be distilled before hydrolysis to the *N*-trifluoroacetylamino-acids. The *o*-nitrophenylsulphenyl group may be introduced analogously using *o*-nitrophenylsulphenyl chloride and the trimethylsilyl derivative (10).[23]

Trimethylsilyl isothiocyanate, Me_3SiNCS (13), may be used in conjunction with mercaptoethanol to remove *o*-nitrophenylsulphenyl groups.[23] The approach is similar to the established use of isothiocyanate salts for this purpose but has the advantage of being usable in organic solvents in which the salts are insoluble.

A selective removal of the t-butoxycarbonyl group in the presence of a t-butyl ester has been described.[24] Since it relies on the removal of unchanged t-butoxycarbonyl derivative it is only of limited value.

New Methods of Amino-group Protection. A full paper has appeared on the ketimine derivatives (14) described earlier (see Vol. 5, p. 270).[25] They are more

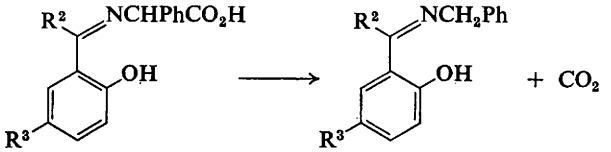


(14) R^2 = Ph, R^3 = H, Cl, or Me
R^2 = Me, R^3 = H

stable than would be expected from their Schiff-base type of structure (although they can be removed acidolytically leaving t-butoxycarbonyl groups intact) and the hydrogen bond between the nitrogen atom and the 2-hydroxy-group is suggested as an explanation. One side-reaction which occurred with phenylglycine derivatives (15) was the spontaneous decarboxylation (Scheme 5) to give (16).

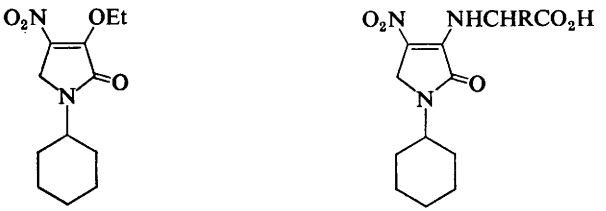
Amino-derivatives (18) derived from the nitroenol ether 4-nitro-1-cyclohexyl-3-ethoxy-2-oxo-3-pyrroline (17) (Scheme 6) have been studied for possible use in

[23] H. R. Kricheldorf and M. Fehrle, *Synthesis*, 1974, 422.
[24] H. Kinoshita and H. Kotake, *Chem. Letters*, 1974, 631.
[25] B. Halpern and A. P. Hope, *Austral. J. Chem.*, 1974, **27**, 2047.



(15) (16)

Scheme 5



(17) (18)

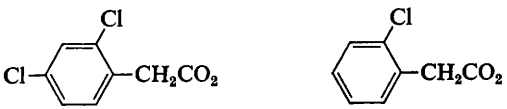
Conditions: $NH_2CHRCO_2^-$–borate buffer pH 10–MeCN

Scheme 6

peptide synthesis.[26] The reagent (17) may also be useful for modifying the amino-groups of proteins and resembles other compounds which have been used in this way.

Further details have appeared of a wide range of β-dicarbonyl derivatives of amino-acids (see Vol. 4, p. 324). Side-reactions of glutamine and asparagine protected in this way are claimed to be less than the other N^{α}-protecting groups.[27] The use of the 2-(methylsulphonyl)ethyloxycarbonyl group has been described in a synthesis using the Merrifield t-alkoxycarbonylhydrazide resin.[28] The group (*cf.* ref. 29) is stable to the conditions of cleavage from the resin.

A number of side-chain protecting groups have been proposed to overcome the partial cleavage of benzyl-based side-chain protecting groups during the removal of t-butoxycarbonyl protection in solid-phase synthesis, including the 2,4-dichlorobenzyloxycarbonyl group (19). The corresponding 2-chlorobenzyl-oxycarbonyl group (20) has now been found to be equally satisfactory.[30] Synthesis



(19) (20)

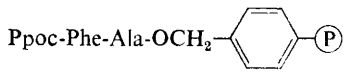
[26] P. L. Southwick, R. F. Dufresne, and J. J. Lindsey, *J. Org. Chem.*, 1974, **39**, 3351.
[27] A. Balog, E. Vargha, D. Breazu, F. Gonczy, and L. Beu, *Rev. Roumaine Chim.*, 1974, **19**, 689 (*Chem. Abs.*, 1974, **81**, 37 793j).
[28] E. M. T. Wolters, G. I. Tesser, and R. J. F. Nivard, *J. Org. Chem.*, 1974, **39**, 3388.
[29] P. M. Hardy, H. N. Rydon, and R. C. Thompson, *Tetrahedron Letters*, 1968, 2525.
[30] B. W. Erickson and R. B. Merrifield, *Israel J. Chem.*, 1974, **12**, 79.

of decalysylvaline by solid-phase synthesis with each of the three intermediates in Scheme 7 gave satisfactory products with both substituted benzyloxycarbonyl derivatives but not with the unsubstituted group.

$Lys_{10}Val$ using:	(a) Boc-Lys(Z)	30% branching
	(b) Boc-Lys Z(2,4-Cl)	0.2% branching
	(c) Boc-Lys Z(2-Cl)	0.2% branching

Scheme 7

An alternative to the use of less easily removed side-chain protection is of course more easily removed α-amino protection, and the 2-*p*-biphenylisopropoxycarbonyl group has been considered for this purpose. However, the group was selected to be removable in the presence of t-butoxycarbonyl groups and might be unnecessarily acid labile when used in conjunction with benzyl-based side-chain protection. The 2-phenylisopropoxycarbonyl (Ppoc) group, looked at briefly during the original work of Sieber and Iselin,[31] has now been re-examined for this new role.[32] The derivatives can be prepared by the phenyl carbonate/amino-acid tetramethylguanidine salt method. They are generally crystalline, have improved storage characteristics compared with 2-*p*-biphenylisopropoxycarbonyl derivatives, and it should be possible to prepare them sufficiently economically as not to preclude their general use. The rate of deprotection of the resin-bound dipeptide (21) was studied using a range of concentrations of

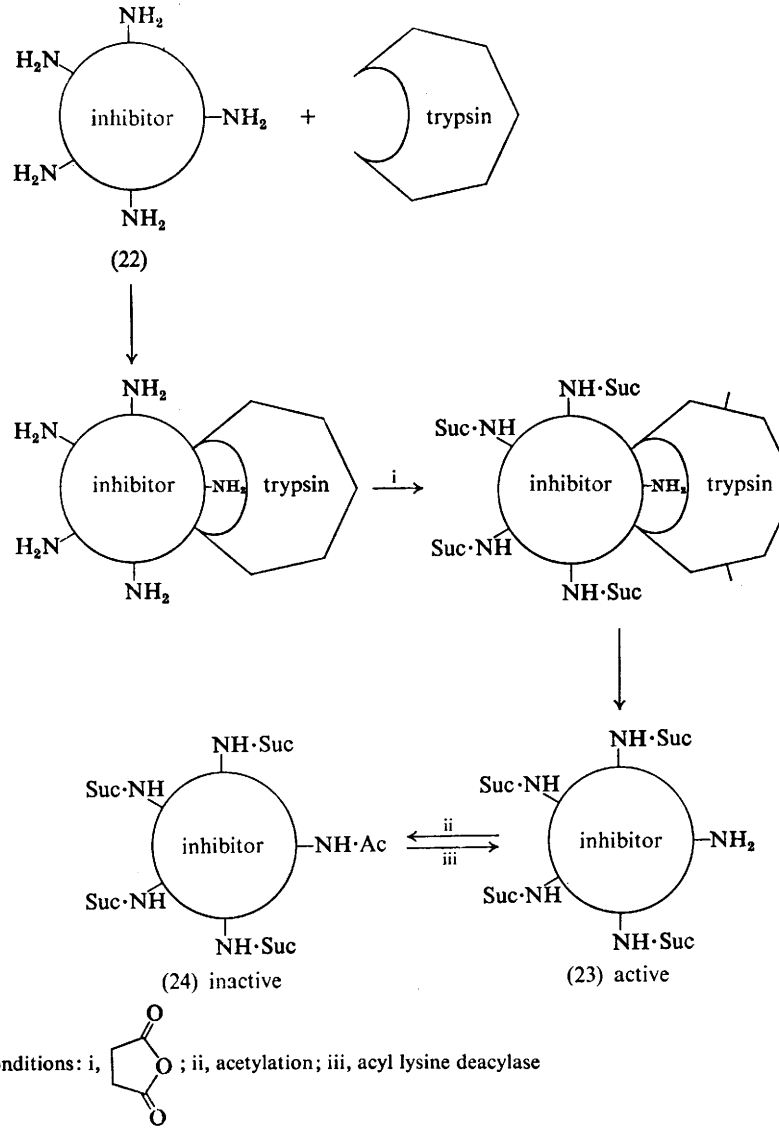


(21)

trifluoroacetic acid in dichloromethane. Suitably rapid removal was found with a 1—2% concentration of acid which should certainly leave benzyl-based side-chain protection untouched. The derivatives (for a list see Appendix II) were used in three parallel syntheses of bradykinin using different concentrations of trifluoroacetic acid (0.5, 1, and 2%, respectively) for deprotection. Using the latter two concentrations, pure hormone was synthesized in 41 and 42% yield. Competition experiments demonstrated no extra steric hindrance compared with the t-butoxycarbonyl group.

The simple acetyl group has been given a new role in protection of the lysine side-chain.[33] The novelty lies in the use of the enzyme acyl lysine deacylase for its specific removal and the new approach promises to be useful not only in studies with modified native proteins but also in totally synthetic products, particularly where a minimal side-chain protection approach is used. Its usefulness was demonstrated by a study on the Kunitz trypsin-kallikrein inhibitor as shown schematically in Scheme 8. By combining the inhibitor (22) with trypsin (thereby protecting the active-site lysine) and succinylating the complex, the soluble active succinyl inhibitor (23) was obtained [acetylation of (22) gave an

[31] P. Sieber and B. Iselin, *Helv. Chim. Acta*, 1968, **51**, 622.
[32] B. E. B. Sandberg and U. Ragnarsson, *Internat. J. Peptide and Protein Res.*, 1974, **6**, 111.
[33] H. Jering, G. Schorp, and H. Tschesche, *Z. physiol. Chem.*, 1974, **355**, 1129.



Scheme 8

insoluble derivative]. Its activity in inhibiting trypsin was destroyed on acetylation to give (24) but was fully regenerated by the action of acyl lysine deacylase.

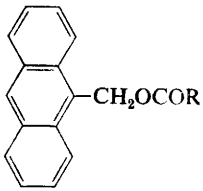
Platinum complexes have been examined as agents for the protection of amino-groups.[34]

Protection of Carboxy-groups. The removal of methyl and ethyl esters by boron tribromide in dichloromethane (see p. 258) may prove important in allowing the

[34] B. Purucker and W. Beck, *Chem. Ber.*, 1974, **107**, 3476.

use of these esters without the necessity for an alkaline hydrolysis step, with its dangers of racemization, to regenerate the acid.[35]

9-Anthrylmethyl esters (25) have been found to be a useful new way of protecting carboxy-groups.[36] The esters are stable to base and some nucleophiles



(25)

(*e.g.* ethylamine in DMF) and to trifluoroacetic acid in oxygenated solvents such as dioxan. They may be removed by trifluoroacetic acid in dichloromethane or preferably by sodium methyl mercaptide in HMPA. They have not yet been explored for peptide synthesis but may well have a useful role in this field. The 9-anthrylmethyl group can also be used for protecting phenols, thiophenols, and mercaptans.

The saponification of methyl esters can be conveniently monitored by the disappearance of the CH_3 peak in the proton magnetic resonance spectrum. At the same time the ratio of phenyl to t-butyl peaks can provide a check on the lack of removal of t-butyl esters.[37]

Protection of the Arginine Guanidino Group. The usual method of preparing benzyloxycarbonyl N^{ω}-tosylarginine gives rise to lactam and ditosyl by-products,[38] and improvements have been suggested.

Ammonolysis of peptides containing N^{ω}-nitroarginine can lead to extensive formation of ornithine peptides as a by-product. This side-reaction occurred in a synthesis of LH-RH [39] and has been studied further in model compounds. The lesson is to remove the nitro protection before carrying out ammonolysis reactions, and this can limit the tactics available in a synthesis particularly on solid-phase.

Protection of Thiol Groups and the Synthesis of Cystine Peptides. A book has been published on the chemistry and biochemistry of the thiol group in amino-acids, peptides, and proteins.[40] Cystine and cysteine peptides have been reviewed.[3c]

The selective removal of *S*-trityl groups (with simultaneous formation of a disulphide bond) can be carried out in the presence of *S*-acetamidomethyl protection using iodine in trifluoroethanol, and has been used in the total synthesis of human insulin.[15] The comment in last year's Report (Vol. 6, pp. 295–297) that iodine oxidation of mixtures of *S*-tritylcysteine peptides gave higher than statistical yields of unsymmetrical disulphides is incorrect. In the experiments

[35] A. M. Felix, *J. Org. Chem.*, 1974, **39**, 1427.
[36] N. Kornblum and A. Scott, *J. Amer. Chem. Soc.*, 1974, **96**, 590.
[37] R. M. Cook, D. Stevenson, and B. Weinstein, *Internat. J. Peptide and Protein Res.*, 1974, **6**, 55.
[38] J. R. Bell, J. H. Jones, D. M. Regester, and T. C. Webb, *J.C.S. Perkin I*, 1974, 1961.
[39] H. Künzi, M. Manneberg, and R. O. Studer, *Helv. Chim. Acta*, 1974, **57**, 566.

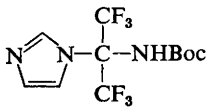
discussed,[40a] the yield of unsymmetrical product was 96% of that expected for statistical coupling. The selective removal of *S*-ethylcarbamoyl groups in the presence of *S*-tetrahydropyranyl and *S*-trityl groups has been used in the synthesis of a protected ovine insulin A chain fragment.[41]

The use of *p*-methylbenzyl rather than *p*-methoxybenzyl for protecting cysteine during solid-phase synthesis has been recommended [30] in view of the loss of side-chain protection during removal of *N*-t-butoxycarbonyl groups with the latter.

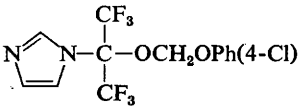
Thiol protection by means of the 2-picolyl-1-oxide group has been studied.[42]

Protection of the Histidine Imidazole Group. The use of the 2,4-dinitrophenyl group for histidine side-chain protection and its other applications in protein chemistry have been reviewed.[43]

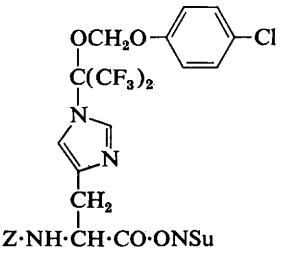
The 1,1,1,3,3,3-hexafluoro-2-(*p*-chlorophenoxymethoxy)propyl (HF-PA) group (27) has been examined as a protecting group for the imidazole side-chain of histidine.[44] Having two electron-withdrawing trifluoromethyl groups, compounds of type (26) and (27) should be more effective at decreasing the basicity of the



(26)



(27)



(28)

imidazole ring than the 2,2,2-trifluoro-1-alkoxycarbonylaminoethyl groups also used for histidine protection, in addition to avoiding the creation of a second asymmetric centre. Derivatives (26) were found to hydrolyse spontaneously on contact with water but the *p*-chlorophenyl acetal (27) was found to offer a suitable combination of stability with reasonable acid lability. The group is stable to alkaline and hydrogenolytic conditions and the *N*-hydroxysuccinimido ester (28) could be prepared and was used in a successful synthesis of TRH.

The search for a suitable histidine derivative for use in the 'rapid excess mixed anhydride' procedure has provided an interesting comparison of a number of imidazole protecting groups.[45] Racemization occurred particularly during the coupling of (29a), (29b), and (29d). During the hydrolysis of excess mixed anhydride, some loss of side-chain occurred with (29e) and (30). Side-reactions of

[40] M. Friedman, 'The Chemistry and Biochemistry of the Sulfhydryl Group in Amino-acids, Peptides, and Proteins', Pergamon, New York, 1973.
[40a] B. Kamber, *Helv. Chim. Acta*, 1973, **56**, 1370. (We are grateful to Dr. Werner Rittel for drawing our attention to this misunderstanding.)
[41] A. Wittinghofer, *Annalen*, 1974, 290.
[42] Y. Mizuno and K. Ikeda, *Chem. and Pharm. Bull. (Japan)*, 1974, **22**, 2889.
[43] S. Shaltiel, *Israel J. Chem.*, 1974, **12**, 403.
[44] H. H. Seltzman and T. M. Chapman, *Tetrahedron Letters*, 1974, 2637.
[45] H. C. Beyerman, J. Hirt, P. Kranenburg, J. L. M. Syrier, and A. van Zon, *Rec. Trav. chim.*, 1974, **93**, 256.

Boc-His(X) Z-His(Z)

(29) a; X = Bzl, d; X = Dnp (30)
b; X = Ztf, e; X = Boc
c; X = H, f; X = Tos

(29d) gave unidentified coloured impurities. A major side-reaction occurred with (29b) involving transfer of the Ztf group to the N^{α}-position during coupling. The tosyl protection could be removed completely leaving N^{α}-benzyloxycarbonyl and N^{α}-t-butoxycarbonyl groups intact, by treating with 10 equivalents of pyridine hydrochloride in DMF for 2 h.

A useful paper has appeared on the tosyl group giving a comparison of its stability under a range of conditions with a number of other protecting groups (Scheme 9).[46] The tosyl group has the advantage of causing less racemization

N^{im}-protecting group	Conditions								
	i	ii	iii	iv	v	vi	vii	viii	ix
Bzl	B	A	S	S	S	S	S	S	S
Z	A	A	A	A	B	B	B	A	B
Trt		A	S		B	B	A		
Boc-tf	S				A	A	A		
Tos	S	A	A	B	C	C	S	A	A

Conditions: i, H_2–Pd; ii, Na–NH_3; iii, 1M-NaOH; iv, 25% NH_3, room temp., 1h; v, 2M–HBr–HOAc, room temp., 1 h; vi, 4M-HCl–dioxan, room temp., 1 h; vii, Anhydrous TFA, 15 °C 30 min; viii, Anhydrous HF, 0 °C, 1 h; ix, HOBt, room temp., 1 h. A = removed, B = slowly removed, C = partially removed, S = stable

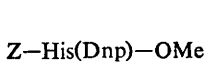
Scheme 9

than is found with, for example, the benzyl derivative. It is stable to triethylamine in DMF and to trifluoroacetic acid, does not interfere with catalytic hydrogenolysis, is more stable towards nucleophiles than, for example, the N^{im}-benzyloxycarbonyl group, and is probably the most generally useful histidine side-chain protecting-group. Removal by nucleophiles still takes place however. The group is completely removed by 1M-NaOH but aqueous ammonia gives only slow cleavage. More disturbing is the suggestion of removal by attack with amino-groups generated by deprotection of Z-His(Tos) derivatives. Its complete removal by *N*-hydroxybenzotriazole must clearly be borne in mind in any intended use, and the authors claim to have taken advantage of this effect in a synthesis (to be published). *N*-Protected amino-acid derivatives are preferably stored as dicyclohexylamine salts.

The position of protecting groups on the imidazole ring of histidine is a subject which has received remarkably little attention in the past. This has been put right at least in one case by a careful study of the position of the Dnp group.[47] The action of benzylchloroformate on (31) gives (32), *via* a Bamberger-type ring-opening, rather than (33), as shown by proton magnetic resonance studies of the product and related imidazole models. Consequently the Dnp group is in the τ position shown (34).

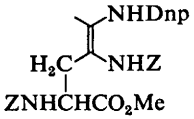
The imidazole Dnp group is stable to 50% trifluoroacetic acid in dichloromethane for more than 200 h at 20 °C.[30]

[46] T. Fujii and S. Sakakibara, *Bull. Chem. Soc. Japan*, 1974, **47**, 3146.
[47] J. R. Bell and J. H. Jones, *J.C.S. Perkin I*, 1974, 2336.

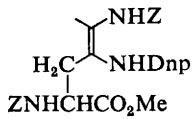


Z—His(Dnp)—OMe

(31)



(32)



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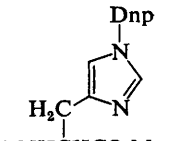


(34)

Protection of Other Functional Side-chains and General Deprotection of Peptides. Boron tribromide appears to have advantages for the deprotection of peptides.[35] Use of 1M tribromide in dichloromethane led to complete removal of a wide range of protecting groups from amino-acid and dipeptide derivatives. Groups removed included the t-butoxycarbonyl and benzyloxycarbonyl groups, benzyl and t-butyl esters and ethers, the dichlorobenzyl ether from tyrosine, and the *p*-nitrobenzyl ester. The method also succeeds in removing methyl and ethyl esters and the ability to do this under non-basic conditions is an extremely valuable addition to the armoury of methods available to the peptide chemist. Side-chain tosyl protection on arginine, and benzyl protection on cysteine and on histidine are unaffected by the reagent. Remarkably, no alkylation was observed in deprotecting (35), (36), or (37). No detectable amino-acid formation took

Z-Met	Z-Trp	Boc-Tyr(Bzl)	Z-Ala-Leu-OBzl	Z-Ala-Leu-OEt
(35)	(36)	(37)	(37a)	(37b)

place by the action of the reagent on (37a) or (37b) from which it was concluded that the peptide bond is quite stable under these conditions. Disadvantages include the partial conversion of glutamine and asparagine into the free acids (but without any transpeptidation) and an incomplete breakdown of N^{ω}-nitro-arginine residues to a mixture of arginine and ornithine residues. This procedure clearly holds great promise provided it can be extended satisfactorily to larger and more complex peptides, and future developments will be awaited with interest.

Trifluoromethanesulphonic acid has been proposed as a convenient reagent for removing a number of protecting groups.[48] A wide range of groups were removed from amino-acid derivatives using the reagent in trifluoroacetic acid or dichloromethane at room temperature with anisole as a scavenger. N^{α}-*o*-Nitrophenylsulphenyl, t-butoxycarbonyl, benzyloxycarbonyl, and *p*-methoxybenzyloxycarbonyl groups, benzyl and t-butyl esters and benzyl ethers, as well as N^{ω}-arginine and N^{im}-histidine tosyl groups were removed. The *S*-benzyl group was removed from cysteine in 30 min at 40 °C, while the *S*-*p*-methoxybenzyl group could be removed more quickly at room temperature. N^{ω}-Nitro protection

[48] H. Yajima, N. Fujii, H. Ogawa, and H. Kawatani, *J.C.S. Chem. Comm.*, 1974, 107.

was incompletely removed from arginine. N^{α}-Benzyloxycarbonyl groups were removed from tyrosine and tryptophan without side-reactions but the ring substitution side-reaction found with other methods of removing the *O*-benzyl ether from tyrosine was still encountered.

The 2-chlorobenzyl ether is stable to the conditions of removal of the t-butoxycarbonyl group during solid-phase synthesis.[30]

Colloidal palladium has been reported to be useful in the hydrogenolysis of benzyl groups.[49]

Formation of the Peptide Bond.—A very useful review of the scope and limitations of the azide method in peptide synthesis has appeared.[50] Recent developments on the formation of peptide bonds[51] and the use of active esters in peptide synthesis[3a] have been reviewed.

Kemp has analysed the reaction rates of the general peptide-forming reaction (Scheme 10) and derived the formula for the second-order rate constant given

$$\text{Z-A-ONp} + \text{H-B-OEt} \longrightarrow \text{Z-A-B-OEt}$$

$$k_{\text{A-B}} = (k_{\text{A-Gly}})(k_{\text{Gly-B}})(1/k_{\text{Gly-Gly}})$$

Scheme 10

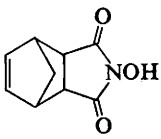
in the scheme which is generally applicable except in the case of proline.[52] The remarkable implication of this is that the steric effect of each amino-acid on the coupling rate is exerted independently of the other amino-acid. The effects of each amino-acid depend on whether it provides the amino- or carboxy-group but the result allows the prediction of some 400 rate constants by measuring only 39. The exception is proline where, particularly in the prolylproline case, there appears to be an interaction between the two amino-acids. The results have been interpreted by a careful analysis of the many conformational possibilities available to the transition state for the reaction. Further studies are needed but the possibility which this work raises of predicting the rates of peptide fragment couplings is exciting and potentially very important.

The nucleophilic activity of amino-acid methyl esters towards toluene-*p*-sulphonyl chloride has been studied and interpreted as a function of the combined inductive and steric effects of the amino-acid side-chain.[53]

Activated Esters. Esters of *N*-hydroxy-5-norbornene-2,3-dicarboximide (38) have been used as an alternative to the *N*-hydroxysuccinimido esters which they resemble.[54] Unlike *N*-hydroxysuccinimide, (38) is a rigid structure and as a result, it is suggested, gives esters which do not suffer the side-reactions (particularly formation of β-alanine derivatives) associated with *N*-hydroxysuccinimido esters. The imide (38) is soluble in both water and organic solvents and can be

[49] S. Sugasawa, S. Hatsuno, K. Shigezane, and T. Mizoguchi, Japan Kokai 74 66 605 (*Chem. Abs.*, 1974, **81**, 152 637y).
[50] Y. S. Klausner and M. Bodanszky, *Synthesis*, 1974, 549.
[51] J. H. Jones, *Chem. and Ind.*, 1974, 723.
[52] D. S. Kemp, S.-L. Hsia Choong, and J. Pekaar, *J. Org. Chem.*, 1974, **39**, 3841.
[53] L. M. Litvinenko, A. I. Bilobrova, A. F. Popov, and Y. A. Sharanin, *Zhur. org. Khim.*, 1973, 1833 (*Chem. Abs.*, 1974, **80**, 60 168).
[54] M. Fujino, S. Kobayashi, M. Obayashi, T. Fukuda, S. Shinagawa, and O. Nishimura, *Chem. and Pharm. Bull. (Japan)*, 1974, **22**, 1857.

readily prepared from the corresponding anhydride. The compound can be used as an 'additive' with dicyclohexylcarbodi-imide and, used in this way, in the Anderson test and in the synthesis of (39) (detecting allo-isoleucine by amino-



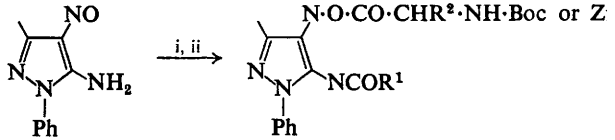
(38)

Z-Phe-Ile-Gly-OBzl

(39)

acid analysis after acidic hydrolysis) gave $<1\%$ racemization. The method was used in a successful synthesis of LH-RH and a number of benzyloxycarbonyl-amino-acid esters have been described (see Appendix II).

Esters derived from substituted 5-amino-4-nitrosopyrazoles, *e.g.* (40), have been used for peptide synthesis (see Vol. 4, p. 342). They have a number of useful features including a colour change from red to green on aminolysis. A new series of acetyl (41) and benzoyl (42) derivatives of 5-amino-3-methyl-4-



(40)

(41) R^1 = MeCO
(42) R^1 = PhCO

Conditions: i, $(MeCO)_2O$ or PhCOCl; ii, Boc(or Z)NHCHR^{2}CO$_2$H–DCCI or mixed anhydride

Scheme 11

nitroso-1-phenylpyrazole have now been described.[55] The benzoyl derivatives were preferred, being more readily prepared and having superior coupling properties. The co-product (43) of aminolysis could be removed by alkaline washing but the suggestion of yellow impurities might indicate possible problems. A number of t-butoxycarbonyl- and benzyloxycarbonyl-amino-acid esters are tabulated in Appendix II.

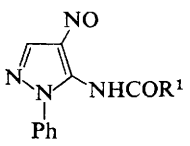
4-Pyridinaldoxime (44) has been used to form esters of benzyloxycarbonyl-amino-acids which were used for the synthesis of Leu-Ala-Gly-Val.[56] The basic property of the esters facilitates their removal from the coupling reaction.

The 4-nitroguaiacyl ester (45) couples to benzylamine three times faster than (46). The *o*-methoxy-group is claimed to hinder base-catalysed racemization.[57]

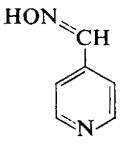
A paper has appeared on the formation of active esters using diaryl sulphites in pyridines.[58]

N-*Carboxy-andydrides.* The synthesis of *o*-nitrophenylsulphenylamino-acid *N*-carboxy-anhydrides is achieved without racemization by the action of phosgene

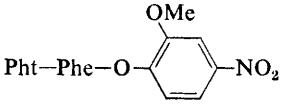
[55] P. Giori, M. Guarneri, D. Mazzotta, and C. A. Benassi, *Gazzetta*, 1974, **104**, 707.
[56] P. Y. Romanovskii, I. K. Liepkaula, and G. I. Cipens, *Zhur. obshchei Khim.*, 1974, **44**, 1193.
[57] K. Bankowski, A. W. Lipkowski, and S. Drabarek, *Roczniki Chem.*, 1974, **48**, 715.
[58] N. Yamazaki, F. Higashi, and M. Niwano, *Tetrahedron*, 1974, **30**, 1319.



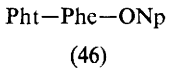
(43)



(44)

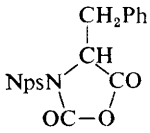


(45)



(46)

on *o*-nitrophenylsulphenylamino-acids, albeit in low yield.[59] The alternative route from the sulphenyl chloride and *N*-carboxy-anhydrides in the presence of base has been reported to give the derivatives without racemization [60] but in other hands this route led to extensive racemization.[59] The observation that the phenylalanine derivative (47) was completely racemized in less than an hour by



(47)

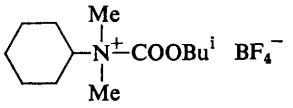
treatment with 1% triethylamine in benzene would seem to indicate that considerable problems are likely in the use of these derivatives for peptide synthesis.

A study of the c.d. spectrum at different stages in the polymerization of mixtures of D- and L-γ-benzylglutamate *N*-carboxy-anhydride suggests that the secondary structure of the polymer plays a part in the stereoselection of the monomer being incorporated.[61] The changes depend on whether sodium methoxide or n-butylamine is used as initiator, suggesting a different mechanism of polymerization in each case. In a somewhat similar study, the rate of polymerization of phenylalanine *N*-carboxy-anhydride (with for example poly-[DL-*N*-methylalanine]diethylamide) was greater with either the L or the D anhydride than with the DL (or a mixture of equal amounts of D and L, eliminating differences in purity as a cause), again indicating stereoselectivity during polymerization.[62]

Other Coupling Methods. The use of *N*-hydroxybenzotriazole in peptide synthesis has been reviewed.[63]

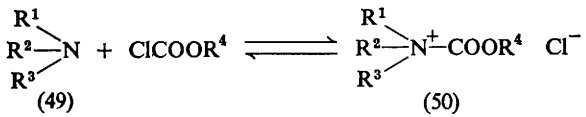
[59] J. Halstrom, K. Brunfeldt, and K. Kovacs, *Z. physiol. Chem.*, 1974, **355**, 82.
[60] H. R. Kricheldorf and M. Fehrle, *Chem. Ber.*, 1974, **107**, 3533.
[61] T. Akaike, T. Makino, S. Inoue, and T. Tsuruta, *Biopolymers*, 1974, **13**, 129.
[62] Y. Imanishi, K. Kugimiya, and T. Higashimura, *Biopolymers*, 1974, **13**, 1205.
[63] E. Munekata and S. Sakakibara, *Yuki Gosei Kagaku Kyokai Shi*, 1973, **31**, 853 (*Chem. Abs.*, 1974, **80**, 83 560h).

Carbonic mixed anhydride couplings require careful control of the reaction conditions to ensure the best results when using alkyl chloroformates to form the anhydride. A series of *N*-alkoxycarbonyl-*NNN*-trialkylammonium fluoroborates, *e.g.* (48), have now been prepared and studied as improved reagents for



(48)

the formation of carbonic mixed anhydrides.[64] The fluoroborates were prepared from the corresponding chlorides (50) by treating with hydrogen fluoride–boron trifluoride in ether at −78 °C and were relatively stable highly crystalline derivatives. Owing to its nucleophilicity, the chloride ion can liberate the free base (49) (Scheme 12) but the corresponding non-nucleophilic fluoroborate salt



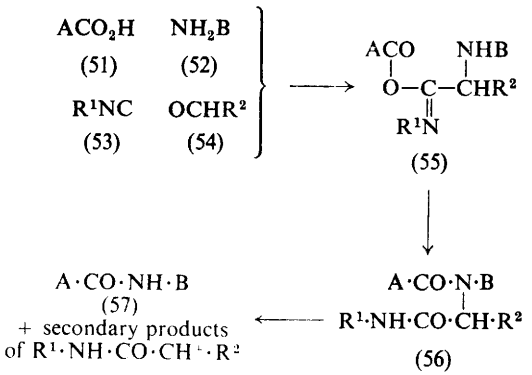
(49) (50)

Scheme 12

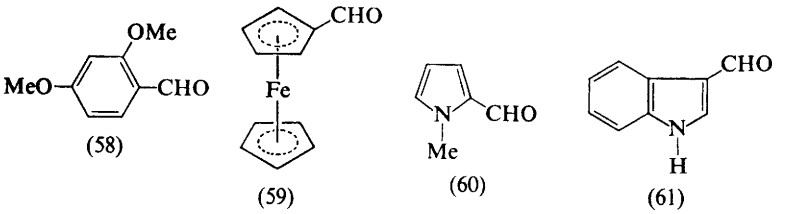
can be used for the formation of a mixed anhydride under non-basic conditions. The use of salts such as (48) in a series of peptide syntheses has shown that yields parallel those of conventional mixed anhydride couplings but relatively little racemization (approximately 3% in the Anderson test) was observed. This will need to be checked in a more sensitive test but the fluoroborates, which can be stored for months at room temperature if kept dry, promise to be very convenient reagents for routine mixed anhydride couplings. Their use in introducing urethane protecting groups is also being investigated.

Further developments of the Ugi four-centre condensation approach to peptide synthesis have been described.[65] The danger of racemization during fragment coupling is exacerbated because coupling is a second-order reaction while racemization is effectively first order. The advantage of the four-centre condensation approach shown in Scheme 13 is that the actual formation of the peptide link (55) → (56) is first order and extremely rapid. The critical step is (56) → (57) which must go without decomposition or racemization, and a search has been made for aldehydes such that R^2 assists this cleavage reaction. Model couplings in which (51) is phenylacetic acid, (52) is benzylamine, and (53) is t-butyl isocyanide have been carried out with aldehydes (58)—(61), giving products (56) which were readily converted into (57) by cold trifluoroacetic acid. With chloral as the aldehyde, spontaneous formation of (57) takes place. The use of *o*-nitrobenzaldehyde [with (53) as cyclohexyl isocyanide] gives a derivative which can be converted photolytically into (57). Model reactions are presently

[64] J. V. Pankstelis and M. Kim, *J. Org. Chem.*, 1974, **39**, 1499.
[65] H. von Zychlinski, I. Ugi, and D. Marquarding, *Angew. Chem. Internat. Edn.*, 1974, **13**, 473.

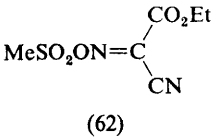


Scheme 13

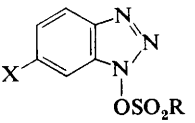


being carried out on peptides, hopefully to establish suitable racemization-free conditions for peptide synthesis.

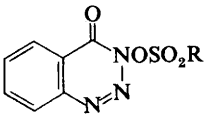
A preliminary report has appeared on the use of sulphonates of strongly acidic *N*-hydroxy-compounds as coupling agents.[66] The derivatives (62)—(64) could be used either directly or to pre-activate the carboxy-component; in both



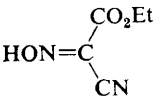
(62)



(63) X = H, R = Me or Ph
X = Cl, R = Bu^n, Ph, or Ph (4-Cl)



(64) R = Me or Ph

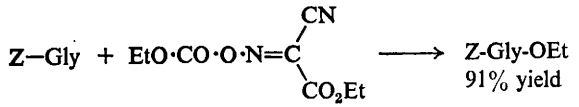


(65)

cases tertiary amine had to be added. In a preliminary study there was less racemization than with dicyclohexylcarbodi-imide. Further work will be needed to establish whether racemization is a problem and to investigate the possibility of sulphonamide formation as a side-reaction.

[66] M. Itoh, H. Nojima, J. Notani, D. Hagiwara, and K. Takai, *Tetrahedron Letters*, 1974, 3089.

The use of strongly acidic oximes such as (65) in conjunction with di-imide to suppress racemization during coupling has been described previously. Mixed carbonates of a range of oximes related to (65) have now been found to provide a generally applicable esterification of acylamino-acids, as in the example given in Scheme 14.[67] Esters with 2,4,5-trichloro-, pentachloro-, and *p*-nitro-phenol have

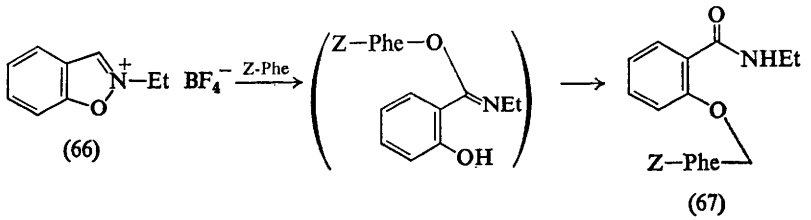


Conditions: *N*–Et–morpholine–CH_2Cl_2

Scheme 14

also been used to prepare protected amino-acid active esters. One side-reaction, observed in some cases, is formation of the oxime ester.

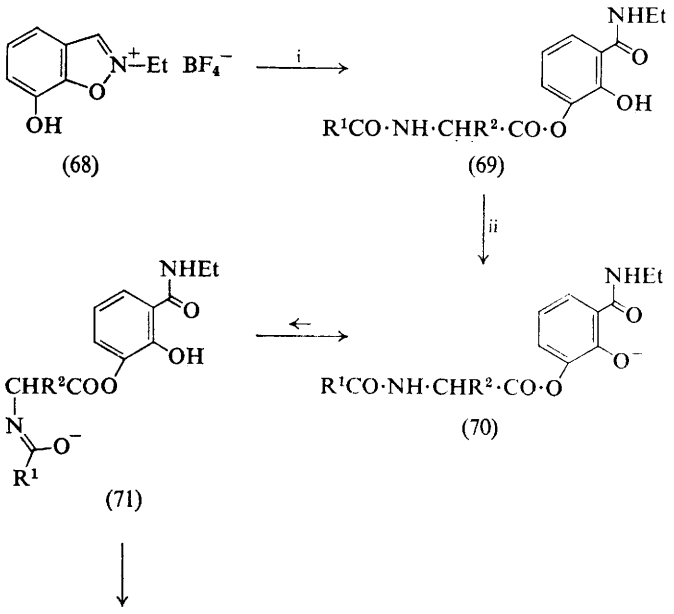
Three full papers have appeared on the use of benzisoxazolium salts in peptide synthesis.[68–70] 2-Ethylbenzisoxazolium fluoroborate (66) gives crystalline esters, *e.g.* (67), with protected amino-acids or it can be used as a direct coupling agent. From a study of the effect of pH on oxazolone formation, ideal coupling conditions were established involving a two-phase system of pyridine acetate



Scheme 15

buffer and organic solvent. Disadvantages of esters (67) include a slow coupling rate with significant racemization and a tendency to a Brenner type of rearrangement. Nevertheless, the method was used for a number of syntheses including oligomers of Gly-Leu-Gly.[68] The derivatives formed from 2-ethyl-7-hydroxybenzisoxazolium fluoroborate (68) are more suitable for peptide synthesis. Coupling is catalysed by tetramethylguanidine, and in model systems racemization takes place *via* an oxazolone and its extent is relatively low (see Vol. 3, p. 238). Excess tetramethylguanidine has no effect on racemization over a broad pH range and this is explained as an 'internal buffering effect' (Scheme 16) in which the equilibrium between (70) and (71) lies largely towards the form (70) but only form (71) is able to give rise to oxazolone. The second function of the 2-hydroxyl is to provide catalysis for the aminolysis of an otherwise unreactive phenyl

[67] M. Itoh, *Bull. Chem. Soc. Japan*, 1974, **47**, 471.
[68] D. S. Kemp, S.-W. Wang, R. C. Mollan, S.-L. Hsia, and P. N. Confalone, *Tetrahedron*, 1974, **30**, 3677.
[69] D. S. Kemp, S.-W. Wang, J. Rebek, jun., R. C. Mollan, C. Banquer, and G. Subramanyam, *Tetrahedron*, 1974, **30**, 3955.
[70] D. S. Kemp, S. J. Wrobel, jun., S.-W. Wang, Z. Bernstein, and J. Rebek, jun., *Tetrahedron*, 1974, **30**, 3969.



Conditions: i, $R^1CO\cdot NH\cdot CHR^2\cdot CO_2^-$; ii, tetramethylguanidine

Scheme 16

ester (*cf.* catechol esters[71]). The scope and limitations of the esters in peptide synthesis were examined in a range of peptides. The esters are considered most suitable for the synthesis of relatively small peptides.[70]

Further details have been published on the applications of diphenylphosphorazidate in peptide synthesis (see Vol. 5, p. 290),[72] and the method has been reviewed.[73] Further and fuller details have appeared on the use of adducts of phosphorus compounds and tetrahalogenomethanes (see Vol. 4, p. 344),[74] and on the applications of *N*-phosphonium salts of pyridines (see Vol. 5, p. 289)[75–78] in peptide synthesis. The use of anode oxidation for the synthesis of peptides by diaryl disulphides and triethylphosphite has been described.[79]

The kinetics of peptide bond formation by symmetrical anhydrides has been studied by use of the stopped-flow technique, and by following the changes of peptide and anhydride bands in the i.r.[80]

[71] J. H. Jones and G. T. Young, *J. Chem. Soc.* (*C*), 1968, 436.
[72] T. Shioiri and S. Yamada, *Chem. and Pharm. Bull.* (*Japan*), 1974, **22**, 849, 855, 859.
[73] T. Shioiri and S. Yamada, *Yuki Gosei Kagaku Kyokai Shi*, 1973, **31**, 666 (*Chem. Abs.*, 1974, **80**, 60 160p).
[74] T. Takeuchi and S. Yamada, *Chem. and Pharm. Bull.* (*Japan*), 1974, **22**, 832, 841.
[75] N. Yamazaki and F. Higashi, *Bull. Chem. Soc. Japan*, 1974, **47**, 170.
[76] N. Yamazaki and F. Higashi, *Tetrahedron*, 1974, **30**, 1323.
[77] N. Yamazaki, F. Higashi, and S. A. Kazaryan, *Synthesis*, 1974, 436.
[78] N. Yamazaki, F. Higashi, and Y. Saito, *Synthesis*, 1974, 495.
[79] Y. V. Mitin and N. P. Zapevalova, *Zhur. obshchei Khim.*, 1974, **44**, 2074.
[80] M. Mutter and H. Hagenmaier, *Angew. Chem. Internat. Edn.*, 1974, **13**, 149.

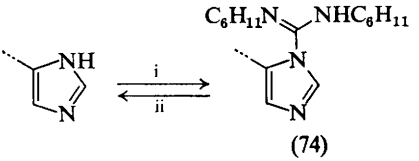
The use of two equivalents of (72) with one of dicyclohexylcarbodi-imide – presumably proceeding through the symmetrical anhydride – has been shown to avoid formation of nitrile (73).[81] The procedure was used for the solid-phase

Z-Gln	$Z\text{-}NHCH(CO_2H)(CH_2)_2CN$
(72)	(73)

synthesis of two *C*-terminal secretin sequences and no nitrile could be detected by amino-acid analysis (after enzymolysis) or by i.r. or mass spectral studies with either the crude or purified peptide.

EEDQ may be used advantangeously for the introduction of glutamine and asparagine derivatives during solid-phase peptide synthesis.[82]

Reaction of the imidazole side-chain of histidine with dicyclohexylcarbodi-imide during coupling reactions has been found to give derivatives (74) as shown (Scheme 17).[83] The side-reaction was observed during a synthesis of human



(74)

Conditions: i, DCCI; ii, warm MeOH

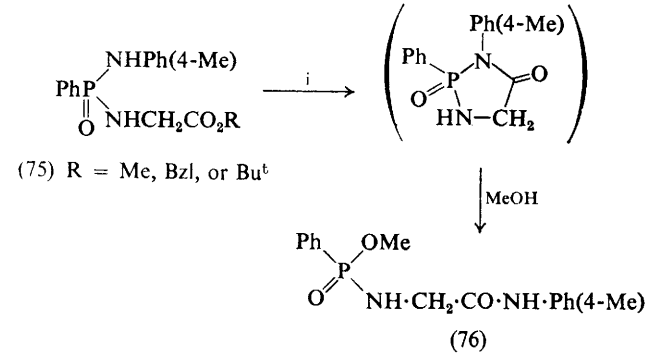
Scheme 17

parathyroid hormone, and was investigated and confirmed in model compounds. It occurs particularly at higher temperatures with an excess of di-imide and, a point especially important to note, is markedly catalysed by *N*-hydroxybenzotriazole. The reaction fortunately is readily reversed by warming in methanol.

The observation that (75) can be converted into (76) as in Scheme 18 was used as the basis for a proposed method of peptide synthesis (Scheme 19).[84] Although the concept is appealing, the method is unfortunately as yet far from being a practical reality.

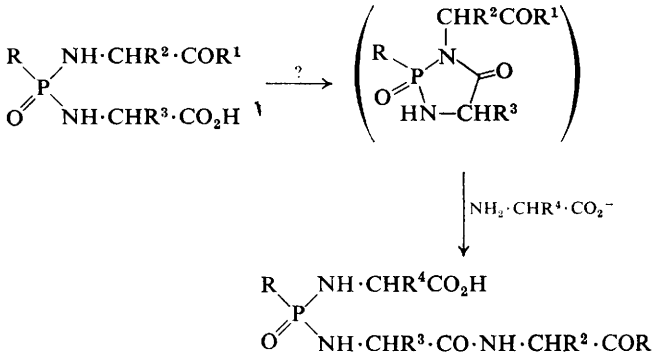
Racemization.—Kemp has applied his racemization test to the *N*-hydroxybenzotriazole–dicyclohexylcarbodi-imide method, using a range of solvents and concentrations of reactants.[85] The degree of racemization is similar to that of the di-imide–*N*-hydroxysuccinimide and *p*-nitrophenyl ester methods but is an order of magnitude greater than that of the azide method. The degree of racemization in DMF is not much greater than in other solvents (unlike the *N*-hydroxysuccinimide case) which is a particular advantage in view of the widespread use of this solvent for peptide work. The degree of racemization was found to depend little on changes in concentration of *N*-hydroxybenzotriazole, confirming earlier work.

[81] B. Hemmasi and E. Bayer, *Z. physiol. Chem.*, 1974, **355**, 481.
[82] C. T. Wang, I. D. Kulesha, P. L. Stefko, and S. S. Wang, *Internat. J. Peptide and Protein Res.*, 1974, **6**, 59.
[83] H. Rink and B. Riniker, *Helv. Chim. Acta*, 1974, **57**, 831.
[84] M. Mulliez, *Tetrahedron Letters*, 1974, 2351.
[85] D. S. Kemp, H. Trangle, and K. Trangle, *Tetrahedron Letters*, 1974, 2695.



Conditions: i, NaOMe or NaOPh–MeOH

Scheme 18



Scheme 19

The dangers of racemization during the coupling of N^{im}-benzylhistidine derivatives are now well known. The use of ethyl-2-hydroxy-2-cyanoacetate to prevent racemization during such couplings has been described and applied to the synthesis of angiotensin analogues.[86] This racemization danger has been illustrated further by the results of a synthesis of TRH.[87] The tripeptide methyl ester (77) when originally prepared by solid-phase synthesis, coupling Boc-His(Bzl)

Glp-His-Pro-OMe (77) Glp-D-His-Pro-OMe (78)

by means of dicyclohexylcarbodi-imide alone, had an unsatisfactory melting point (115 – 160 °C). The compound was characterized by recrystallization of an analytical sample. A second synthesis in which Boc-His(Bzl) was coupled with the addition of *N*-hydroxybenzotriazole gave, however, a sample with different physical properties. The optical purity of the various preparations was studied by L-amino-acid oxidase degradation after catalytic hydrogenolysis and acid

[86] M. C. Khosla, M. M. Hall, R. R. Smeby, and F. M. Bumpus, *J. Medicin. Chem.*, 1974, **17**, 431.
[87] J. L. M. Syrier and H. C. Beyerman, *Rec. Trav. chim.*, 1974, **93**, 117.

hydrolysis. The 'analytical sample' gave no less than 95% of D-histidine and was clearly the enantiomer (78). The crude product from which it was crystallized gave 26% D-histidine, and the product from the second synthesis gave essentially no D-histidine. None of the three samples was distinguished on thin-layer chromatography. TRH prepared from the first methyl ester had *ca.* 65% of the biological activity of that prepared from the second. Apart from establishing the value of *N*-hydroxybenzotriazole for coupling Boc-His(Bzl), this work demonstrated rather well the difficulty of establishing the purity of a peptide product, even one as small as a tripeptide, and points to the need both for simple reliable methods of determining optical purity in final peptides and for more powerful methods to establish homogeneity of peptides. In the interim a critical approach is clearly vital; for example, the use of 'analytical samples' should be avoided.

Repetitive Methods of Peptide Synthesis.—*Solid-phase Synthesis.* A number of reviews have appeared [3j, 3k] including a particularly useful comprehensive account surveying the literature to June 1971.[88]

Fragment condensation on a solid support is a technique being used increasingly as the methods for monitoring the outcome of coupling reactions improve and as techniques for coupling fragments without racemization become more reliable. Bradykinin has been synthesized by coupling the fragments (79), (80), and

Z(OMe)-Ser(Bzl)-Pro-Phe (79) Z(OMe)-Pro-Gly-Phe (80) Z-Arg(NO_2)-Pro (81)

(81) successively to an ω-nitroarginine resin, using dicyclohexylcarbodi-imide/*N*-hydroxysuccinimide for coupling.[89] Completion of each coupling was demonstrated using 2-hydroxy-1-naphthaldehyde and the crude yield of bradykinin was 81%. Racemization of phenylalanine was estimated by acid hydrolysis, reaction with L-leucine *N*-carboxy-anhydride and separation of the diastereoisomers, giving a figure of 3.76% D-Phe against a control value of 2.91%.

Similarly, the human corticotrophin-like intermediate lobe peptide (CLIP), corresponding to the ACTH sequence 18—39, has been synthesized by coupling four fragments.[90] The first fragment (82) was attached to a bromomethyl resin by reaction of the acid in the presence of dicyclohexylamine, and three further fragments (83)—(85) were added using EEDQ for coupling. Yields at each

Z(OMe)-(37—39)-OH (82) Z(OMe)-(27—36)-OH (83)

Z(OMe)-(20—26)-OH (84) Z-(18—19)-OH (85)

stage were based on percentage incorporation of a new amino-acid and approached 90%. The synthesis of the porcine pancreatic trypsin inhibitor using this approach

[88] J. Meienhofer, in 'Hormonal Proteins and Peptides', ed. C. H. Li, Academic Press, New York, 1973, Vol. 2, p. 45.
[89] S. M. Karlsson and U. Ragnarsson, *Acta Chem. Scand.*, 1974, **28B**, 376.
[90] H. Kawatani, F. Tamura, and H. Yajima, *Chem. and Pharm. Bull. (Japan)*, 1974, **22**, 1879.

is described later. The approach has also been used to add the final t-butoxycarbonyl pentapeptide unit in a synthesis of the oligomeric elastin model peptide (86),[91] and to add two and three t-butoxycarbonyl tetrapeptide units to a tetrapeptide resin to give (87), used for the synthesis of the corresponding cyclic oligomers.[92]

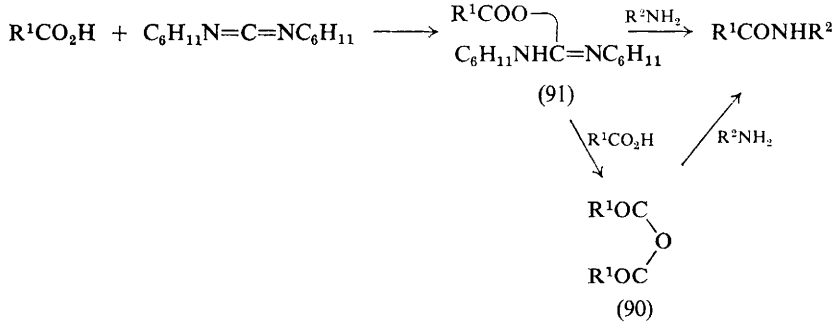
HCO-(Val-Pro-Gly-Val-Gly)$_3$-OMe (86)

Boc-(Val-Pro-Gly-Gly)$_n$-O-resin (87) n = 3 or 4

[^{3}H]Z-Gly (88)

[^{14}C](Z-Gly)$_2$O (89)

The outcome of competition experiments where limited amounts of (88) and (89) compete for a range of amino-components has been used to study the mechanism of dicyclohexylcarbodi-imide couplings (see Vol. 6, p. 320). This approach has now been applied to solid-phase synthesis.[93] Unlike the case in solution, coupling takes place entirely through the symmetrical anhydride (90) (Scheme 20) and not at all through the *O*-acylisourea (91), since the outcome with



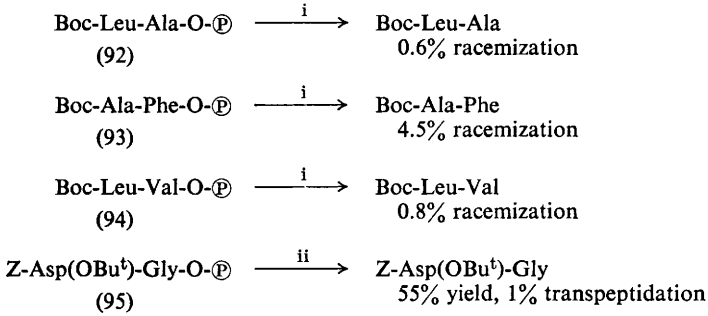
Scheme 20

(88) and (89) is identical. This is presumably because the rate of diffusion of reagents to amino-component is slow compared with the rate of formation of (90). The implication for solid-phase synthesis is that excess of carbodi-imide above that required to form (90) is superfluous and may even be detrimental, the ideal ratio of acid to carbodi-imide being 2 : 1. The results also help explain the effectiveness of symmetrical anhydrides as found in earlier work.

Fuller details have been published [94a] of the use of thallous 2-dimethylaminoethoxide as a catalyst in the removal of peptides from the solid-phase resin by transesterification with 2-dimethylaminoethanol (see also ref. 94*b*). This has been studied using the model dipeptidyl resins (92)—(94) whose diastereoisomeric free peptides were separable on an amino-acid analyser, and using (95) to examine

[91] D. W. Urry, W. D. Cunningham, and T. Ohnishi, *Biochemistry*, 1974, **13**, 609.
[92] D. W. Urry and T. Ohnishi, *Biopolymers*, 1974, **13**, 1223.
[93] J. Rebek and D. Feitler, *J. Amer. Chem. Soc.*, 1974, **96**, 1606.
[94] (a) J. Y. Savoie and M. A. Barton, *Canad. J. Chem.*, 1974, **52**, 2832; (*b*) M. A. Barton, R. U. Lemieux, and J. Y. Savoie, *J. Amer. Chem. Soc.*, 1973, **95**, 4501.

possible α-β transpeptidation. Rates were considerably faster than in the absence of catalyst and the potential of the method is therefore extended to more sterically hindered cases than before. Racemization varied with the *C*-terminal amino-acid (Scheme 21) and clearly could be a problem in unfavourable cases. Trans-



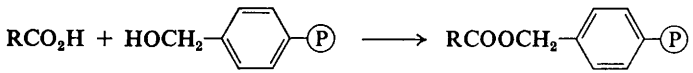
Conditions: i, $(Me_2NCH_2CH_2O)_2Tl$–$Me_2NCH_2CH_2OH$; ii, after 1 h reaction

Scheme 21

peptidation appears to be manageable and the t-butyl ester was stable to the reaction conditions. The toxic properties of thallium compounds will no doubt have to be considered in some applications where the method would otherwise be suitable.

The use of ultrasonic waves has been claimed to increase the rate of diffusion of reagents into the solid-phase resin matrix and thereby enhance the reaction. This approach was tried in the synthesis of some model tripeptides and bradykinin and gave a purer sample of the latter than a control experiment with mechanical stirring.[95] Whether the conditions of mechanical agitation, which are known to be rather important, were optimal in this control experiment is not entirely clear. The approach is, however, an unusual one and further studies will be awaited with interest.

Protected amino-acids and peptides can be attached to hydroxymethylated resin using the 'oxidation–reduction' method.[96] The use of tri-n-butylphosphine was necessary to give a sufficiently rapid reaction (Scheme 22) and the corre-



Conditions: PBu^n_3–2,2′-dithiodipyridine–CH_2Cl_2

Scheme 22

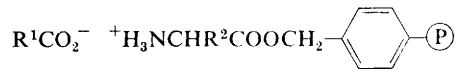
sponding esterifications using triphenylphosphine/2,2′-dithiodipyridine or using 2-pyridylthiol esters were much slower, as was the reaction when DMF was added to the dichloromethane reaction solvent.

The formation of imides and the consequent α → β transpeptidation is a serious problem with Asp-Gly and Asp-Ser sequences, particularly with the acid

[95] S. Takahashi and Y. Shimonishi, *Chem. Letters*, 1974, 51.
[96] M. Ueki and S. Ikeda, *Chem. Letters*, 1974, 25.

side-chain esterified during solid-phase synthesis when strongly acidic conditions are used for the removal of protecting groups. An effective way of circumventing this problem has now been described.[97] By using 2-*p*-biphenylisopropoxycarbonyl groups for N-protection, side-chains can be protected with t-butyl-based groups and the peptide attached to the resin with an alkoxybenzyl ester so that removal from the resin and removal of side-chain protection is achieved under mild conditions (50% trifluoroacetic acid in dichloromethane). Under these conditions no transpeptidation occurs. The peptide derivative can subsequently be treated with hydrogen fluoride (to remove the arginine nitro-protection for example) without transpeptidation since the acid does not undergo this reaction under these conditions, unlike the ester. The method was used in a synthesis of a bovine growth hormone nonapeptide sequence.[97] The advantages of the 2-*p*-biphenylisopropoxycarbonyl group for the synthesis of peptides such as gastrin have been pointed out and a synthesis of pentagastrin described.[82]

Further competition studies have been described on the coupling of various derivatives to amino-acyl resins.[98] The relative rates depend on the nature of the amino-acid being coupled and are slower but acceptable with a number of peptide derivatives. As previously established, the nature of R in ROCO-amino-acid derivatives does not affect the coupling rate. Formation of ion-pairs (96)

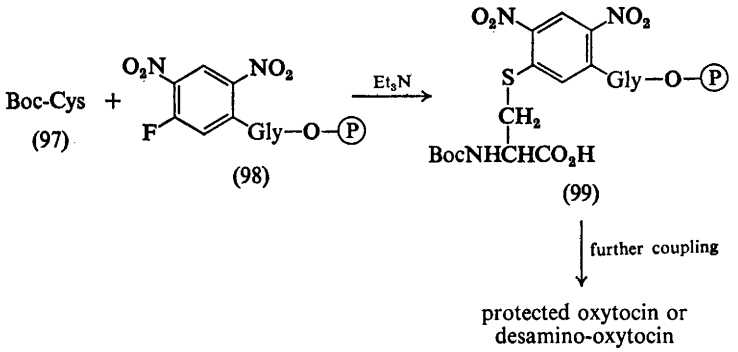


(96)

has been studied as a function of solvent by attempting to wash R^1CO_2H off the resin with the solvent followed by the addition of dicyclohexylcarbodi-imide and measuring the amount of R^1CO_2H coupled.[98] In non-polar solvents a strong ion-pair formation takes place.

A bidirectional approach to solid-phase synthesis, in which histidine was attached to the resin by its side-chain through a modified dinitrophenyl group, was used in a synthesis of TRH (see Vol. 5, p. 310). This approach has now been extended to cysteine (Scheme 23).[99] t-Butoxycarbonylcysteine (97) reacted in the presence of triethylamine with *N*-(2,4-dinitro-5-fluorophenyl)glycyl resin (98) to give the side-chain protected derivative (99). By further coupling, first with Pro-Leu-Gly-NH_2 and then stepwise from the amino end of the tetrapeptide, it was possible to synthesize oxytocin and desamino-oxytocin sequences. The peptides were removed from the resin with 2-mercapto-ethanol. Unfortunately the dinitrophenyl protection on cysteine leads to β-elimination in the presence of base, giving peptides containing dehydroalanine. This side-reaction was avoided by carrying out couplings in the presence of acetic acid–*N*-methylmorpholine-DMF mixtures but the use of dicyclohexylcarbodi-imide directly for coupling was precluded as a result, and active esters or pre-activated acids were used instead.

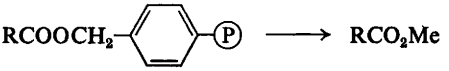
[97] S. S. Wang, C. C. Yang, I. D. Kulesha, M. Sonenberg, and R. B. Merrifield, *Internat. J. Peptide and Protein Res.*, 1974, **6**, 103.
[98] U. Ragnarsson, S. M. Karlsson, and B. E. B. Sandberg, *J. Org. Chem.*, 1974, **39**, 3837.
[99] J. D. Glass, A. Talansky, Z. Grzonka, I. L. Schwartz, and R. Walter, *J. Amer. Chem. Soc.*, 1974, **96**, 6476.



Scheme 23

A comparative study of the synthesis of Val-Gly-Phe-Ala-Leu-Ala on solid phase using di-imide, carbonic mixed anhydride, and Woodward's reagent K respectively for coupling has concluded that the efficacy of the three coupling methods is in that order (see ref. 391).

Diazomethane catalysed transesterification (Scheme 24) has been used for removing peptide from solid-phase resin.[93]



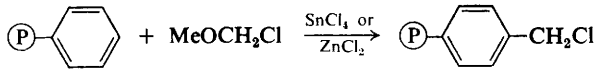
Conditions: CH_2N_2–MeOH

Scheme 24

Other topics studied include the steric hindrance occurring with benzhydryl resins,[100] the use of *S*-acetamidomethylcysteine in solid-phase synthesis (along with an improved procedure for the synthesis of Cys[Acm]),[101] and the use of 3-nitrophthalic anhydride for the suppression of deletion sequences during the synthesis of [5-leucine] gastrin I-(5—13)-nonapeptide and ACTH-(11—14)-tetrapeptide.[102] In a study of the use of activated esters for solid-phase synthesis, using a model peptide, the 5-chloro-8-quinolyl ester was considered the most suitable.[103]

Supports used for Solid-phase Synthesis. The usual methods of preparing chloromethylated polystyrene resins for solid-phase synthesis involve reaction with chloromethyl methyl ether in the presence of stannic chloride (Scheme 25). The reaction must be carefully cooled and efficiently mixed to give a reasonably controlled outcome and can be difficult to scale-up or to control when low levels of substitution are required for the synthesis of larger peptides. An improved procedure has now been described (Scheme 25) using zinc chloride as catalyst.[104]

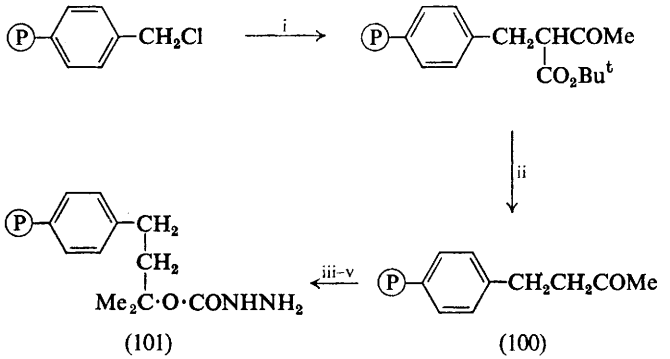
[100] G. Losse and B. Meisegeier, *J. prakt. Chem.*, 1974, **316**, 629.
[101] P. Hermann and E. Schreier, *J. prakt. Chem.*, 1974, **316**, 719.
[102] B. Penke and C. Birr, *Annalen*, 1974, 1999.
[103] H.-D. Jakubke and A. Baumert, *J. prakt. Chem.*, 1974, **316**, 241.
[104] R. S. Feinberg and R. B. Merrifield, *Tetrahedron*, 1974, **30**, 3209.



Scheme 25

A solution of the catalyst in dry peroxide-free THF is stable for at least several weeks and the substitution rate is proportional to the concentration of chloromethyl methyl ether and can therefore be controlled. A trial synthesis of Leu-Ala-Gly-Val using a resin prepared in this way and 2-*p*-biphenylisopropoxycarbonylamino-acids gave a product free (< 0.05%) of failure sequences.

A new method has been described (Scheme 26) for preparing the modified Merrifield resin (101) in which the peptide is attached through an alkoxycarbonyl-



Conditions: i, $CH^-(CO_2Bu^t)COMe$; ii, TFA–CH_2Cl_2; iii, MeMgBr; iv, PhOCOCl; v, N_2H_4

Scheme 26

hydrazide link to the resin (see Vol. 2, p. 162).[28] This was prepared as before from the ketone (100) but the ketone was derived by a two-stage process from a chloromethyl resin. The advantages are that the extent of chloromethylation can be used to determine the extent of substitution, and that the use of hydrogen fluoride (in the addition of methyl vinyl ketone) is avoided.

An investigation of eight different chloromethylated polystyrene supports for the synthesis of the model peptide (102) has shown a strong dependence of the

Gly-Val-Gly-Ala-Pro

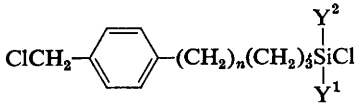
(102)

product purity and coupling yields on the support used.[105] The intermediate peptidyl-resins were deprotected and the products studied by an ion-exchange chromatography procedure which separated a variety of peptide sequences expected as by-products.

A full paper has appeared describing the use of benzhydrylamine polymers for solid-phase synthesis. Syntheses of TRH, eledoisin, and a calcitonin-(26—32)-heptapeptide are described.[106]

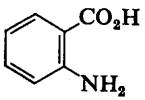
[105] H. Frank and H. Hagenmaier, *Tetrahedron*, 1974, **30**, 2523.
[106] P. G. Pietta, P. F. Cavallo, K. Takahashi, and G. R. Marshall, *J. Org. Chem.*, 1974, **39**, 44.

Silicone matrices for solid-phase synthesis have been prepared by polymerizing compounds (103) on to porous glass. The support, in the form of a column, was used for the synthesis of (104).[107]



(103) a; n = 0, Y^1, Y^2 = Cl
b; n = 1, Y^1 = Cl, Y^2 = Me
c; n = 1, Y^1, Y^2 = Me

Gln-Gln-Gly-Gly-Tyr-NH_2
(104)



(105)

The usefulness of phenyl ester supports for the synthesis of peptide amides has been explored further in the synthesis of ACTH-(1—20)-amide analogues.[108]

A series of peptide derivatives of anthranilic acid (105) have been prepared.[109] The neighbouring-group effect of the *o*-carboxy-groups makes possible the cleavage of bonds close to it under mild conditions and this is being investigated as a possible basis of a new method for attaching peptide to resin in solid-phase synthesis.

Techniques for Monitoring Solid-phase Synthesis. High-pressure liquid chromatography has been used to separate phenylthiohydantoins derived by Edman degradation of samples of peptidyl-resin during solid-phase synthesis. The method provides a quantitative check on couplings and the increased sensitivity and convenience allow this approach to be used to analyse the side-reactions over several steps of the synthesis.[110]

In designing procedures for coupling during solid-phase synthesis it is clearly important to know how the availability of the protected amino-acid which is being coupled varied with time. This is particularly so when dicyclohexylcarbodiimide is used and there are known side-reactions which can remove the coupling component such as the formation of *N*-acyl urea. One technique which has been described [111] for determining the availability of di-imide activated protected amino-acids involves the addition of an excess of glycine t-butyl ester to couple with all the remaining activated component after a particular period of time. The resulting dipeptide derivative is deprotected using trifluoroacetic acid and the free dipeptide measured accurately using a suitably calibrated amino-acid analyser. As an example, in the coupling of t-butoxycarbonylalanine to a glycyl resin, 61% of the expected amount of acid component could still be coupled after 8 h reaction.

The yield in coupling 2-*p*-biphenylisopropoxycarbonylamino-acids to the solid-phase resin has been determined by deprotection of a weighed sample and spectrophotometric determination of the 2-(*p*-biphenyl)propene liberated.[28]

Side-reactions in Solid-phase Synthesis. Despite the advances made in the techniques of the Merrifield method in recent years, the attempt to achieve a

[107] W. Parr, K. Grohmann, and K. Hägele, *Annalen*, 1974, 655.
[108] J. Blake and C. H. Li, *J. Medicin. Chem.*, 1974, **17**, 233.
[109] J. Noguchi, M. Kawai, and M. Hamada, *Israel J. Chem.*, 1974, **12**, 87.
[110] G. Frank and W. Strubert, *Chromatographia*, 1973, **6**, 522 (*Chem. Abs.*, 1974, **80**, 71 070k).
[111] L. C. Dorman, *Biochem. Biophys. Res. Comm.*, 1974, **60**, 318.

synthesis of many stages without any intermediate purification still places great demands on the reactions and procedures involved and not surprisingly is not universally successful. It is reported to have given a particularly unsatisfactory result in an attempted synthesis of porcine proinsulin C-chain.[112] It is clearly vital that workers in this field be able to identify those situations where the method is likely to be most useful and to identify areas needing the greatest improvement. It is therefore refreshing to find accounts of syntheses with more than the usual detail of problems encountered and impurity patterns obtained. In a synthesis of the oligopeptides (106), the product obtained after coupling and deprotecting each additional Boc-Glu(Bzl) residue was increasingly heterogeneous on ion-exchange chromatography.[113] The extent of side-reactions was less when a resin of lower percentage of cross-links with milder N^{α}-deprotection conditions and more efficient washing. With compounds like (106) the number

$(Glu)_n$-Phe(NO_2)-Phe
(106) $n = 0$—4

Ser-Ser-Thr-His-Pro-Val-Phe-His
(107)

of by-products explainable as failure sequences is less than usual since the same amino-acid is added repeatedly. The authors propose a number of side-reactions to account for the products, including inter-chain aminolysis, formation of γ-glutamyl peptides, incomplete removal of γ-benzyl groups, formation of methyl esters during cleavage, and formation of pyroglutamyl peptides.

In another case, the solid-phase synthesis of the *C*-terminal octapeptide of mouse nerve growth factor (107) gave a product which on ion-exchange chromatography was resolved into a major component together with a large number of significant impurities which were studied by amino-acid analysis and chromatography. It should be noted that this was only a first synthesis with no attempt at optimization but the result is revealing and should be widely studied.[114]

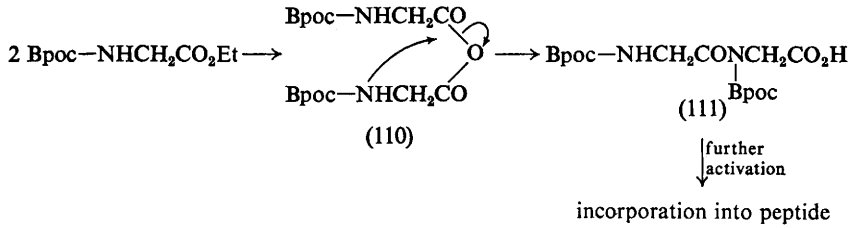
The simultaneous incorporation of two glycine residues during the coupling of urethane-protected glycine previously observed in solution couplings has now been identified as a problem in solid-phase synthesis.[115] The reaction (Scheme 27) was found to lead to an impurity of Leu-Ala-Gly-Gly-Val in a synthesis of Leu-Ala-Gly-Val using carbonic mixed-anhydrides for coupling. Although two residues of (108) were incorporated on coupling to a valyl resin, the attempted

Bpoc-Gly
(108)

Bpoc-Gly-Val-resin
(109)

acylation of (109) by (108) did not give such a product. The mechanism proposed therefore involves intramolecular formation of the dipeptide (111) from the symmetrical anhydride (110) and its subsequent activation and incorporation into the peptide. The smaller tendency of symmetrical anhydrides themselves

[112] A. Baumert and H.-D. Jakubke, *Die Pharmazie*, 1974, **29**, 419.
[113] G. M. Bonora, C. Toniolo, A. Fontana, C. Di Bello, and E. Scoffone, *Biopolymers*, 1974, **13**, 157.
[114] R. Hogue-Angeletti, R. A. Bradshaw, and G. R. Marshall, *Internat. J. Peptide and Protein Res.*, 1974, **6**, 321.
[115] R. B. Merrifield, A. R. Mitchell, and J. E. Clarke, *J. Org. Chem.*, 1974, **39**, 660.



Scheme 27

to undergo this reaction is explained by the effect of triethylamine (used in forming the mixed anhydride) on the intramolecular reaction.

Other side-reactions which may be involved in solid-phase synthesis have been studied further.[116] Intra-chain aminolysis of the resin benzyl ester leading to formation of cyclic peptide by-products is most important at the dipeptide stage (leading to dioxopiperazines). The latter can also be formed from longer peptides with loss of the *N*-terminal dipeptide. Intermolecular aminolysis can occur leading to a lengthened peptide. The hydroxymethylpolystyrene groups liberated by aminolysis in principle can be esterified during the coupling step but in practice this reaction is slow in the absence of base.

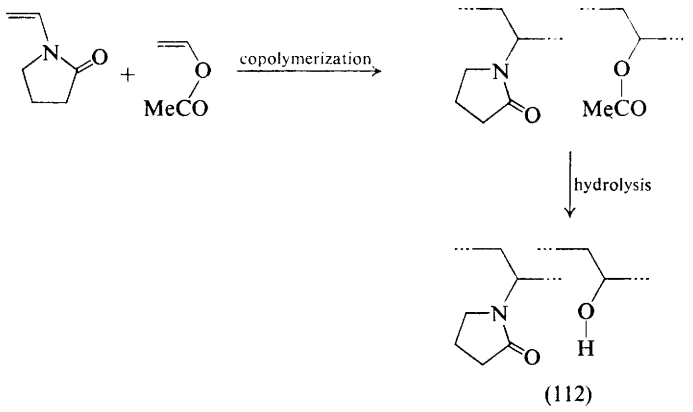
Other Repetitive Methods. The picolyl ester method [3b] and the use of polymeric reagents [3i,39] have been reviewed.

Bayer's liquid-phase method of peptide synthesis uses a soluble polyethyleneglycol ester as a support and ultrafiltration to separate the growing peptidyl-polymer from co-products. An extension to this method [117] stems from the observation that such peptidyl-polymer esters are precipitated in highly crystalline form from dichloromethane on adding ether. This provides a simple effective method of separating the non-polymeric co-products and by-products and the approach has been used in a synthesis of an ACTH (14—20) sequence [117] and of substance P.[118] In the latter synthesis the peptide was attached to a polyethyleneglycol of 15 000 molecular weight and 2—3-fold excesses of symmetrical anhydrides were used for couplings. A continuous-flow method has been developed for the rapid quantitative determination of coupling yield on soluble polymer supports, using ninhydrin or fluorescamine,[119] and analysis of coupling by this method indicated > 99.5% completion at each step with reaction times of 10—60 min. Cleavage from the resin by ammonolysis followed by hydrogen fluoride–anisole deprotection and purification by paper and continuous-flow electrophoresis gave substance P of high biological activity. It should be noted that although the crystallization process is effective for removing low molecular weight substances, impurities which carry the polymeric ester group will not be separated and the process should not be confused with the crystallization of non-polymeric compounds of lower molecular weight where separation of structurally very similar molecules may be achieved.

[116] M. Rothe and J. Mazanek, *Annalen*, 1974, 439.
[117] M. Mutter and E. Bayer, *Angew. Chem. Internat. Edn.*, 1974, **13**, 88.
[118] E. Bayer and M. Mutter, *Chem. Ber.*, 1974, **107**, 1344.
[119] H. Hagenmaier and M. Mutter, *Tetrahedron Letters*, 1974, 767.

The rates of coupling of t-butoxycarbonylglycine *p*-nitrophenyl ester to a variety of glycine esters, including polyethyleneglycol esters of varying molecular weight, have been compared.[120] The rates for the polymeric esters (mol. wts. 2000—20 000) follow a classical linear second-order kinetic pattern and show no sign of lowered rate constant as the reaction proceeds 60—80% to completion. This contrasts with results for solid-phase where rates show a noticeable falling off after 50% completion of reaction. The rates for triglycyl polymeric esters were essentially the same as those for the amino-acid esters and surprisingly the rates for ethyl and 2-methoxyethyl esters were slower (*ca.* 1 : 1.7) than for the polymeric esters. Tautomeric catalysis (as found with imidazole and 1,2,4-triazole) was suggested as an explanation. Clearly there is nothing in these results to preclude the use of the polyethyleneglycol esters in peptide synthesis.

As an alternative support to polyethyleneglycol, a copolymer (112) of 1-vinyl-2-pyrrolidinone and vinyl alcohol has been prepared (Scheme 28) and used in a synthesis of the model peptide Ala-Gly-Leu-Ala.[121]



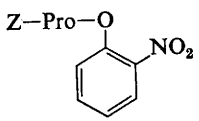
(112)

Scheme 28

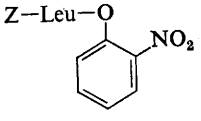
The use of *o*-nitrophenyl esters for coupling has been developed into a repetitive *in situ* technique in which the growing peptide is kept within one reaction vessel and treated with various solvents to remove co-products and for N^{α}-deprotection. The method has now been applied to the synthesis of *SS'*-dibenzyloxytoceine[122] and of [Nle][17]-vasoactive intestinal polypeptide (VIP)-(14—28)-*C*-terminal sequence.[123] The former paper also gives details of a number of new benzyloxycarbonylamino-acid *o*-nitrophenyl esters. The esters lend themselves particularly well to this approach because in comparison with, for example, *p*-nitrophenyl esters they are more readily aminolysed, their aminolysis is less solvent dependent, and *o*-nitrophenol is very soluble in organic

[120] E. Bayer, M. Mutter, R. Uhmann, J. Polster, and H. Mauser, *J. Amer. Chem. Soc.*, 1974, **96**, 7333.
[121] E. Bayer and K. Geckeler, *Annalen*, 1974, 1671.
[122] M. Bodanszky, M. Kondo, C. Y. Lin, and G. F. Sigler, *J. Org. Chem.*, 1974, **39**, 444.
[123] M. Bodanszky, C. Y. Lin, and S. I. Said, *Bio-organic Chem.*, 1974, **3**, 320.

solvents. The reasons for this behaviour have been studied.[124] The *o*-nitro substituent is expected to exert a greater electron-withdrawing effect than the *p*-nitro, explaining the increased activity of the esters, but this is not reflected in the measured pK_a values of the phenols (*ortho* 7.17, *para* 7.15) because the effect of intramolecular hydrogen-bonding in *o*-nitrophenol just compensates for an expected greater acidity. The high optical rotation of the esters together with its pronounced temperature dependence points to a cyclic conformation in the esters, explaining the relatively low solvent dependence of coupling rate. This cyclic conformation is apparently not brought about by the hydrogen-bonded structures which immediately suggest themselves, since there were no differences between the i.r. spectra of amino-acid derivatives (113) and (114) which differed in the solvent dependence of the rate of their coupling with benzylamine.

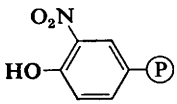


(113)

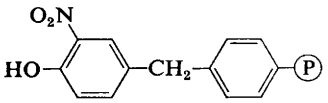


(114)

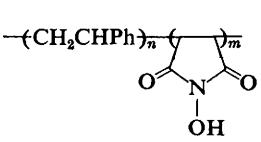
Drawbacks to the insoluble active-ester approach using poly-(4-hydroxy-3-nitrostyrene) (115) include the contamination by 'fines', the severe steric effects (showing as a greater reactivity for benzyloxycarbonyl than for t-butoxycarbonyl derivatives) and a restriction to DMF as solvent for swelling the resin. Some of the disadvantages are eliminated in a new support (116), prepared from 4-hydroxy-3-nitrobenzyl chloride and a polystyrene resin, with a substitution rate of *ca.* 30%, in which the site of attachment of the protected amino-acid is



(115)



(116)



(117)

further from the polymer backbone.[125] The new support has been used to synthesize a range of amino-acid derivatives and from them a number of model peptides.

The copolymer (117) of styrene and *N*-hydroxymaleimide has been used to prepare resin-bound active esters for peptide synthesis.[126] The aminolysis of polymeric *p*-nitrophenyl esters has been studied.[127]

The picolyl ester approach has been applied to the repetitive synthesis of 4-picolyloxycarbonylhydrazides, and several peptides including (118) have been prepared.[128]

[124] M. Bodanszky, M. L. Fink, K. W. Funk, M. Kondo, C. Y. Lin, and A. Bodanszky, *J. Amer. Chem. Soc.*, 1974, **96**, 2234.
[125] R. Kalir, M. Fridkin, and A. Patchornik, *European J. Biochem.*, 1974, **42**, 151.
[126] S. V. Rogozhin, Yu. A. Davidovich, S. M. Andrev, and A. I. Yurtanov, *Doklady Akad. Nauk S.S.S.R.*, 1973, **212**, 108 (*Chem. Abs.*, 1974, **80**, 60 192a).
[127] V. Böhmer, H.-E. Sauerbrey, and H. Kämmerer, *Makromol. Chem.*, 1974, **175**, 2505.
[128] R. Macrae and G. T. Young, *J.C.S. Chem. Comm.*, 1974, 446.

Boc-Leu-Ala-Gly-Val-NHNHCO$_2$Pic
(118)

A repetitive method has been described which relies on the use of *NN*-dialkylethylamines to react with excess active ester after the completion of coupling (Scheme 29).[129] All co-products, including (120), are then either basic or acidic

$$\underset{(119)}{\text{Boc—NHCHR}^1\text{CO}_2\text{Pcp}} \xrightarrow{\text{NH}_2\text{R}^2} \text{Boc—NH·CH·CHR}^1\text{·CO·NH·R}^2$$

$$\text{excess (119)} + \text{R}^3\text{R}^4\text{N(CH}_2)_2\text{NH}_2 \longrightarrow \underset{(120)}{\text{Boc—NH·CHR}^1\text{·CO·NH·(CH}_2)_2\text{·NR}^3\text{R}^4}$$

Scheme 29

and the neutral peptide can be isolated in any of the usual ways. The repetitive cycle is estimated to take 65—85 min.

The possible use of the coupling of free peptide fragments (for example Gly-Leu-Gly) as the basis of a repetitive method of peptide synthesis has been explored in the synthesis of the oligomers (121).[130] In general, the variations

(Gly-Leu-Gly)$_n$
(121) n = 1, 2, 4, or 8

between each particular coupling were too great for any general procedure to apply.

Synthesis of Polymeric Models for Studies in Protein Chemistry.—A detailed comprehensive review of the synthesis, structure, and biological properties of sequential polypeptides has appeared which will clearly be of great use to workers in this area.[131]

In the synthesis of poly-(Arg-Ala-Ala) and poly-(Arg-Gly-Pro) the protection of the arginine side-chain by the N^{ω}-tosyl group, in conjunction with catechol or pentachlorophenyl esters for polymerization, gave a satisfactory product. Neither bis-adamantyloxycarbonyl nor nitro protection gave satisfactory products in this case.[38]

Studies on the structural requirements for antigenicity in sequential polypeptides continue to reward the synthetic chemist with interesting results. The polypeptide (122) induced both immediate and delayed immunological reactions, while the closely related polypeptide (123), although almost incapable of

poly-(Leu-Glu-Lys-Ala-Glu-Ser-Gly) (122) poly-(Leu-Glu-Lys-Ala-Glu-Ala-Gly) (123)

producing the immediate response, was more effective in producing the delayed response.[132]

[129] L. Kisfaludy, I. Schön, T. Szirtes, O. Nyeki, and M. Löw, *Tetrahedron Letters*, 1974, 1785.
[130] D. S. Kemp, Z. W. Bernstein, and G. N. McNeil, *J. Org. Chem.*, 1974, **39**, 2831.
[131] B. J. Johnson, *J. Pharm. Sci.*, 1974, **63**, 313.
[132] P. C. Brown, R. D. Cowell, L. E. Glynn, and J. H. Jones, *Immunology*, 1974, **27**, 479.

A series of oligomers containing the Tyr-Glu-Ala-Gly sequence has been synthesized by solid-phase to investigate further the structural requirements for antibody binding in this and related polymeric models. Full binding requires the structure (124). The lack of activity of (125) shows that a simple repeating Tyr-Glu sequence is insufficient for binding.[133]

(Tyr-Glu-Ala-Gly)$_4$-Gly-OEt (124)

poly-(Tyr-Glu-Ala-β-Ala)-Gly-OEt (125)

HCO-(Val-Pro-Gly-Val-Gly)$_n$-OMe (126) n = 1—3

HCO-(Val-Pro-Gly-Val-Gly)$_n$-Val-OMe (127) average n = 10—15

The oligomeric (126)[91] and polymeric (127)[134] elastin models have been synthesized. The derivative (127) exhibits (with an almost identical temperature profile) the characteristic property shown by tropoelastin known as co-acervation. This is a reversible phase separation observed on raising the temperature of an aqueous solution to *ca.* 37 °C in which the lower phase is enriched in protein (or peptide).

An alternating diastereoisomeric poly-(γ-benzyl glutamate) has been synthesized.[135] In order to avoid racemization (which is a crucial consideration if a perfectly alternating sequence is to be obtained) the two tetrapeptide catechol esters (128) and (129) were polymerized and the reaction products derived in this way provided a cross-check on the optical purity of each other.

D-Glu(OBzl)-L-Glu(OBzl)-D-Glu(OBzl)-L-Glu(OBzl)-OCat (128)

L-Glu(OBzl)-D-Glu(OBzl)-L-Glu(OBzl)-D-Glu(OBzl)-OCat (129)

Bonora and Toniolo have synthesized a variety of protected oligopeptides (130) in order to examine the critical size required for the development of secondary structure in various solvents.[136—140]

Boc-(X)$_n$-OMe
X = Met, Phe, Leu, or Val; n = 2—7
(130)

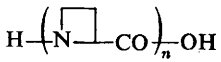
Gly-Gly-Ala-OTcp (131)

Gly-Gly-Lys(Tos)-OTcp (132)

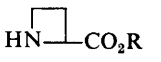
Gly-Gly-Glu(OMe)-OTcp (133)

[133] B. J. Johnson and C. Cheng, *J. Medicin. Chem.*, 1974, **17**, 320.
[134] D. W. Urry, M. M. Long, B. A. Cox, T. Ohnishi, L. W. Mitchell, and M. Jacobs, *Biochim. Biophys. Acta*, 1974, **371**, 597.
[135] A. Caille, F. Heitz, and G. Spach, *J.C.S. Perkin I*, 1974, 1621.
[136] G. M. Bonora and C. Toniolo, *Biopolymers*, 1974, **13**, 2179.
[137] C. Toniolo and G. M. Bonora, *Bio-organic Chem.*, 1974, **3**, 114.
[138] C. Toniolo and G. M. Bonora, *Makromol. Chem.*, 1974, **175**, 1665.
[139] C. Toniolo and G. M. Bonora, *Makromol. Chem.*, 1974, **175**, 2203.
[140] C. Toniolo, G. M. Bonora, and A. Fontana, *Internat. J. Peptide and Protein Res.*, 1974, **6**, 371.

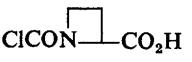
The polymerization of (131), (132), and (133) has been studied.[141] The molecular weight of the product increases with increasing triethylamine concentration in the case of (131) and (132) but not (133). Synthesis of poly-L-azetidine-2-carboxylic acid (134) has been achieved by polymerization of the



(134)



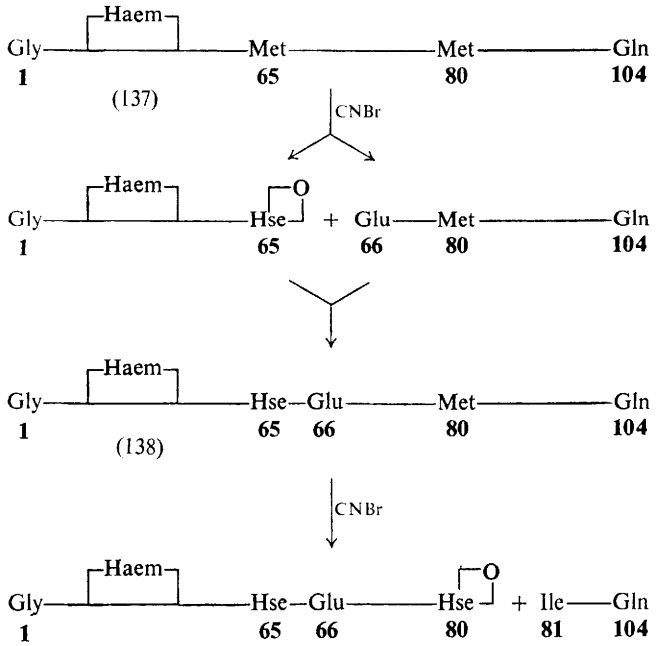
(135) a; R = NSu
b; R = Pcp



(136)

amino-acid *N*-hydroxysuccinimido (135a) and pentachlorophenyl (135b) esters.[142] The *N*-carboxy-anhydride procedure could not be used in this case since even (136) failed to cyclize to the presumably strained bicyclic anhydride.

Synthetic Operations with Peptides of Biological Origins.—As discussed in last year's Report (p. 351), the action of cyanogen bromide on basic pancreatic trypsin inhibitor (Trasylol) gives an unstable chain-cleaved product which on standing reverts to a single-chain 58 amino-acid analogue containing homoserine in place of methionine.[143a] Similar observations have now been recorded for cytochrome *c* (137).[143b] The fragments comprising residues 1—65 and 66—104 can be separated after cyanogen bromide treatment of the protein (Scheme 30).



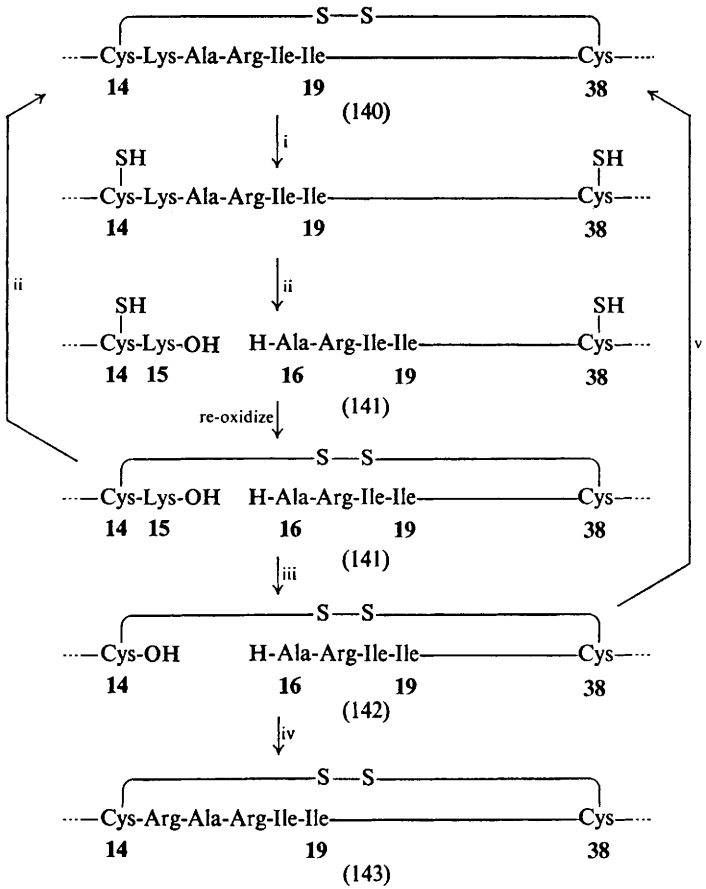
Scheme 30

[141] M. Berdiev, V. K. Burichenko, A. B. Zegelman, and V. A. Shibnev, *Khim. prirod. Soedinenii*, 1974, **10**, 206 (*Chem. Abs.*, 1974, **81**, 50 002v).

[142] R. Boni and A. S. Verdini, *J.C.S. Perkin I*, 1974, 2173.

[143] (*a*) D. F. Dyckes, T. Creighton, and R. C. Sheppard, *Nature*, 1974, **247**, 202; (*b*) G. Corradin and H. A. Harbury, *Biochem. Biophys. Res. Comm.*, 1974, **61**, 1400.

On admixture in aqueous solution, these form a complex which slowly reverts to a single-chain molecule (138) with properties nearly identical with those of the starting cytochrome. The second methionine is unchanged by the initial cyanogen bromide treatment, possibly because the sulphur atom functions as a ligand to the haem group. This haem–ligand interaction between the two fragments may aid the resynthesis reaction by stabilizing the tertiary structure in the same way that a disulphide bond spanning the two fragments of basic trypsin inhibitor is thought to act. Further treatment of the resynthesized cytochrome analogue with a larger excess of cyanogen bromide causes cleavage at this second methionine providing firm proof of chain resynthesis. Both groups of authors have pointed out the potential of these reactions for the partial synthesis of a range of analogues of the respective proteins, and these possibilities are being pursued.[143a, b]



Conditions: i, $NaBH_4$; ii, trypsin; iii, carboxypeptidase B; iv, large excess of arginine–carboxypeptidase B–trypsin; v, large excess of lysine–carboxypeptidase B–trypsin

Scheme 31

Binding of the protein inhibitors to their enzyme usually leads to at least partial cleavage of the bond between the active-site lysine and the amino-acid which follows. This is not the case for the Kunitz trypsin-kallikrein inhibitor for reasons which have been made clear by an interesting series of experiments (Scheme 31) (see also Chapter 5).[144] The chain-cleaved molecule in question was prepared by selective reduction of the Cys^{14}–Cys^{38} disulphide bond with sodium borohydride, followed by the action of trypsin. This cleaves the Lys^{15}–Ala^{16}, Arg^{39}–Ala^{40}, and Arg^{17}–Ile^{18} bonds at different rates and by controlling the reaction conditions the first can be made the only major point of cleavage. After removal of trypsin (Sephadex G50) and reoxidation, the chain-cleaved inhibitor (141) was purified by ion-exchange chromatography. It was an effective inhibitor but in the process completely reverted to (140), even in the presence of only a catalytic amount of enzyme. Thus the equilibrium must lie predominantly in the direction of the intact rather than the cleaved Lys-Ala bond, unlike the situation with other inhibitors. This trypsin-catalysed peptide-bond formation was used to prepare three analogues of the inhibitor. Carboxypeptidase B removed Lys^{15} from (141) giving the inactive molecule (142). In the presence of trypsin and a large excess of lysine together with carboxypeptidase B, the reversal of this cleavage [to give (141)] could be driven to completion since (141) is removed continuously giving (140). Similarly if arginine is used in place of lysine, the Arg^{15} analogue (143) is produced. This was found to be equiactive with (140). Using carboxypeptidase A with (142) and a large excess of phenylalanine or tryptophan, the corresponding Phe^{15} and Trp^{15} analogues were prepared which were better inhibitors of chymotrypsin than trypsin.

Two varieties of ferredoxin have been converted into the corresponding [Leu^2] analogues. In the case of ferredoxin from *Clostridium acidi-urici*, two successive Edman degradations removed the *N*-terminal Ala-Tyr sequence, and coupling with t-butoxycarbonylleucine and with t-butoxycarbonylalanine in aqueous DMF gave the modified sequence.[145] This molecule still has one tyrosine residue (Tyr^{30}) and to establish whether this plays a role a new natural ferredoxin containing arginine in place of tyrosine in position 30 was modified. The presence of lysine precluded the use of Edman degradation, but controlled chymotrypsin proteolysis followed by reaction with Boc-Ala-Leu-ONSu gave the required modified sequence.[146] Both analogues were active, demonstrating that tyrosine does not play a crucial role in the activity of the ferredoxins.

The aspartic acid residue 52 of hen egg-white lysozyme has been converted into a homoserine residue.[147] After specific esterification using as an affinity label the 2′,3′-epoxypropyl β-glycoside of di-(*N*-acetyl-D-glucosamine), the enzyme was reduced with dithiothreitol, the ester reduced to a homoserine residue with borohydride and the molecule reoxidized to the modified enzyme. This binds substrates but has little catalytic activity.

[144] H. Jering and H. Tschesche, *Angew. Chem. Internat. Edn.*, 1974, **13**, 660, 661, 662, 663.
[145] E. T. Lode, C. L. Murray, W. V. Sweeney, and J. C. Rabinowitz, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 1361.
[146] E. T. Lode, C. L. Murray, and J. C. Rabinowitz, *Biochem. Biophys. Res. Comm.*, 1974, **61**, 163.
[147] Y. Eshdat, A. Dunn, and N. Sharon, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 1658.

3 Syntheses Achieved

The chemical synthesis of biologically active peptides has been the subject of several reviews.[148, 149, 150, 45] Other reviews have covered the synthesis of proteins [151] and of phosphatidylpeptides.[152]

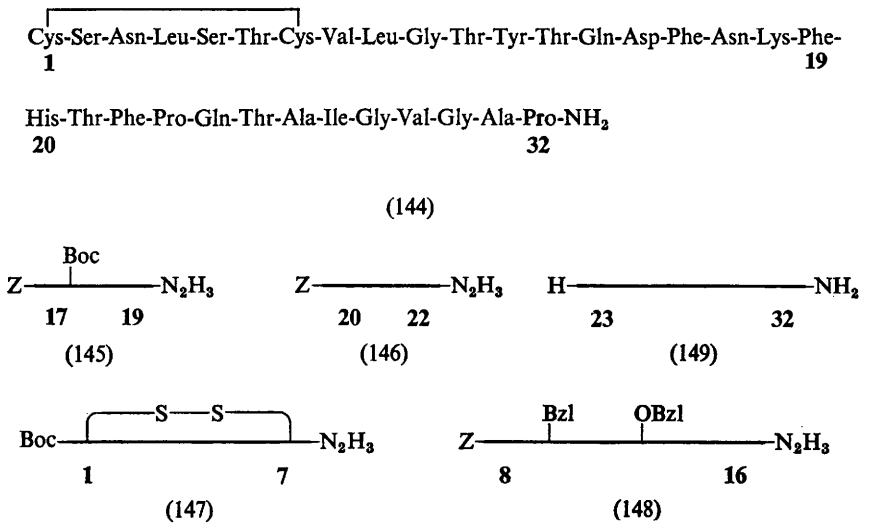
Calcitonin.—The synthetic procedure established for the synthesis of human calcitonin (see Vol. 3, p. 253) has been applied to the synthesis of two analogues, [Ser^{29},Thr^{31}] and [Ala^{29},Val^{31}] human calcitonin.[153] The analogues were designed to resemble the highly active salmon calcitonin in the *C*-terminal region (Scheme 32). The former analogue was more active than human calcitonin and the

-Ser-Gly-Thr-Pro-NH_2	-Val-Gly-Ala-Pro-NH_2
29 30 31 32	**29 30 31 32**
salmon	human

Scheme 32

C-terminal region no doubt contributes to the increased activity of salmon calcitonin.

Tackling the problem from the other end, Nozaki and Muramatsu have described in a preliminary communication their synthesis of the hybrid molecule (144) with the same 1—10 sequence as salmon calcitonin and an 11—32 human calcitonon sequence.[154] The synthesis employed the stepwise coupling of a series

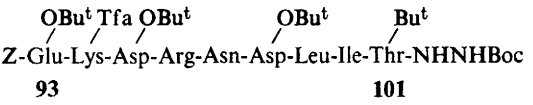


[148] R. Geiger, *Chem. Unserer Zeit*, 1973, **7**, 131.
[149] N. Izumiya and Y. Mitsuyasu, *Farumashia*, 1973, **9**, 117 (*Chem. Abs.*, 1974, **80**, 27 456s).
[150] W. Lutz and L. Klyszejko-Stefanowicz, *Postepy Biochemii*, 1974, **20**, 35.
[151] M. Waki and N. Izumiya, *Kobunshi*, 1973, **22**, 12 (*Chem. Abs.*, 1974, **80**, 3776v).
[152] S. Mel'nik, M. A. Miropol'skaya, and G. I. Samokhvalov, *Vitam. Vitam. Prep.*, 1973, 280 (*Chem. Abs.*, 1974, **80**, 60 159v).
[153] R. Maier, B. Riniker, and W. Rittel, *F.E.B.S. Letters*, 1974, **48**, 68.
[154] S. Nozaki and I. Muramatsu, *Chem. Letters*, 1974, 417.

of azide fragments [from the hydrazides (145)—(148)] starting with the decapeptide (149) as amino-component. The fragments were chosen so that the carboxy-component was of lower molecular weight than the amino-component to facilitate separation. A minimal side-chain protection approach was adopted to assist in chromatographic separation. The final deprotected product was purified by carboxymethylcellulose ion-exchange chromatography and had the same order of activity as human calcitonin – indicating that the *N*-terminal part of the molecule does not significantly contribute to the difference in activity between the human and salmon hormones.

Cytochrome *c*.—The synthetic work has been reviewed.[155]

An improved synthesis has been described for the protected fragment (150) spanning the 93—101 sequence of baker's yeast iso-1-cytochrome *c*.[156] During an

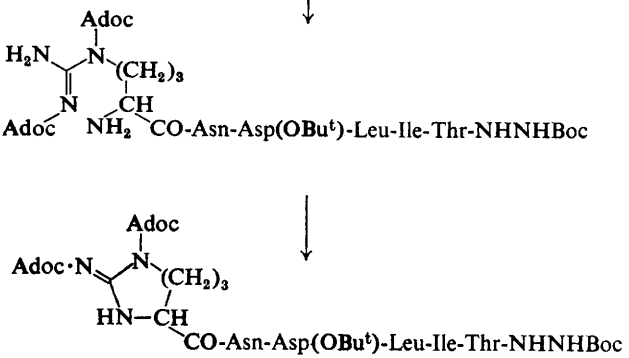


(150)

earlier attempt to synthesize this sequence, hydrogenation of (151) gave a product which could not be purified owing to formation of an *N*-terminal 4-(adamant-1-yl-oxycarbonylimino)-2,4-diazacycloheptylcarbonyl residue, as shown in Scheme 33 (see Vol. 3, p. 228). An improved product resulted by coupling arginine as its N^{ω}-nitro-derivative and after hydrogenolysis coupling the hexapeptide

Z-Arg(Adoc)$_2$-Asn-Asp(OBu^t)-Leu-Ile-Thr-NHNHBoc

(151)



Conditions: H_2–Pd

Scheme 33

[155] S. Sano, *Wenner-Gren Cent. Internat. Symp. Ser.*, 1970, **18**, 35 (*Chem. Abs.*, 1974, **80**, 83 558p).
[156] L. Moroder, G. Borin, F. Marchiori, and E. Scoffone, *Annalen*, 1974, 213.

(protected by protonation on the arginine) to a protected tripeptide with dicyclohexylcarbodi-imide–*N*-hydroxybenzotriazole.

Enzyme Substrates.—The study of peptide substrates continues to give valuable if indirect information about the active sites of some of the many different proteolytic enzymes. A list of such substrates whose synthesis was reported during 1974 is included in Appendix I.

The specificity, size, and stereospecificity of the active site of porcine pancreatic elastase have been studied using a series of synthetic alanine-containing substrates, indicating an active site extending over five amino-acid residues from the cleaved bond towards the *N*-terminus and at least two amino-acid residues towards the *C*-terminus.[157]

Chloromethylketone derivatives of lysine have been used as affinity labels for enzymes with trypsin-like specificity. By using appropriate peptide derivatives, this approach has been made more selective and the synthetic substrate (152) has been found to inactivate plasma kallikrein but not thrombin.[158]

p-Azidophenylalanine peptides have been used as photo-affinity labels for enzymes (ref. 159 and earlier references). The more simply synthesized nitrophenylalanine derivatives have now been shown also to be effective, although the mechanism of photo-activation is not clear.[160]

A series of peptide substrates (153) of papain have been synthesized, which contain an *N*-terminal 6-(*N*-methylanilino)-2-naphthalenesulphonyl (mansyl,

(152) (153) $n = 0$, 1, or 2

Mns) group. Only the Glu-Leu bond is susceptible to cleavage. The mansyl group does not play a role in binding but is drawn into a less polar part of the enzyme on binding.[161]

Trypsin hydrolyses the Arg-Gly and Lys-Ala bonds of insulin at quite different rates. That this is largely due to the influence of the primary sequence has been shown by the synthesis of substrates (154)—(156) containing appropriate partial

Gly-Glu-Arg-Gly (154) Gly-Glu-Arg-Gly-Phe (155) Thr-Pro-Lys-Ala (156) Thr-Pro-Lys-Ala-Phe (157)

sequences, and the modified substrate (157).[162] The hydrolysis rates for (155) and (157) were similar but both were considerably faster than (154) and (156), and therefore the phenylalanine following the Arg-Gly sequence in insulin (but not the Lys-Ala sequence) strongly influences the reaction rate.

Substrates of renin including (158) have been synthesized and attached to agarose for use in the affinity purification of the enzyme.[163]

[157] D. Atlas, *Israel J. Chem.*, 1974, **12**, 455.
[158] J. R. Coggins, W. Kray, and E. Shaw, *Biochem. J.*, 1974, **137**, 579.
[159] E. Escher, R. Jost, H. Zuber, and R. Schwyzer, *Israel J. Chem.*, 1974, **12**, 129.
[160] E. Escher and R. Schwyzer, *F.E.B.S. Letters*, 1974, **46**, 347.
[161] J. Lowbridge and J. S. Fruton, *J. Biol. Chem.*, 1974, **249**, 6754.
[162] N. Nishino, N. Mitsuyasu, and N. Izumiya, *J. Biochem.*, 1974, **75**, 979.
[163] J. Majstoravich, jun., D. A. Ontjes, and J. C. Roberts, *Proc. Soc. Exp. Biol. Med.*, 1974, **146**, 674.

Leu-Leu-Val-Phe-OMe
(158)

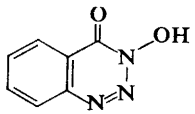
Gastrointestinal Hormones.—A useful review has appeared.[164]

Vasoactive Intestinal Peptide (VIP). The stepwise approach which has been used so successfully in the case of secretin [165] was unsuccessful in the synthesis of VIP[11] owing to solubility problems from as early a stage as the tripeptide. It was necessary to use long reaction times for coupling, with the reaction mixture in the form of a viscous gel, and the stepwise approach was abandoned in favour of a fragment approach after the completion of the synthesis of an 18—28 sequence. The choice of fragments (spanning positions 1—6, 7—13, 14—17, and 18—28) was to some extent determined by their usefulness in confirming the structure of enzyme cleavage products of natural VIP. The fragments were coupled by the azide method and the final product again presented solubility problems, having a tendency to aggregation, and it was characterized by a careful comparison of the products of enzymic and chemical cleavage of the natural and synthetic octacosapeptides.

Secretin. Two syntheses have been reviewed.[166]

A new synthesis of secretin makes full use of acidic *N*-hydroxy racemization-suppressing agents, particularly *N*-hydroxybenzotriazole,[167] and takes advantage of the freedom of choice of strategy offered by these reagents. Fragments were chosen as carboxy-component which had, as in Wünsch's glucagon synthesis, an *N*-terminal Z-Arg(Z)$_2$ residue, catalytic hydrogenolysis of the coupled product leaving free arginyl peptides (protected by protonation) as amino-components.

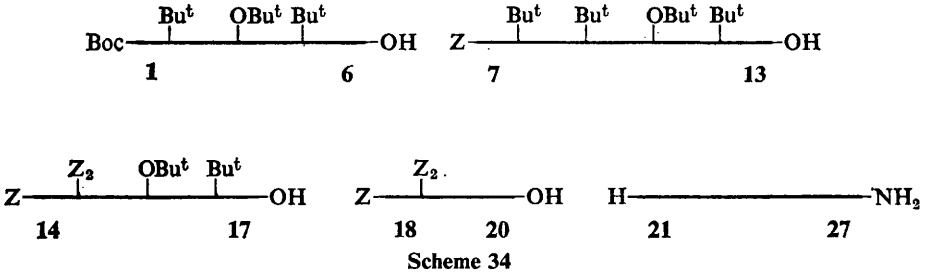
In addition to *N*-hydroxybenzotriazole, the original studies on these reagents [168] identified 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine (159) as an equally



(159)

effective suppressor of racemization, particularly useful for pre-activation when coupling to an amino-component which contained a free carboxy-group or when after catalytic hydrogenolysis in acetic acid all of the acetate was not removed and presented a danger of acetylation. It was found to be particularly useful for coupling some of the carboxy-components (shown in Scheme 34) by pre-activation in the secretin synthesis. *N*-Hydroxybenzotriazole is also useful for increasing the rate of active ester couplings and this approach was used widely for the synthesis of the various fragments.

[164] M. Bodanszky in 'Hormonal Proteins and Peptides', ed. C. H. Li, Academic Press, New York, 1973, Vol. 2, p. 29.
[165] M. Bodanszky, M. A. Ondetti, S. D. Levine, and N. J. Williams, *J. Amer. Chem. Soc.*, 1967, **89**, 6753.
[166] M. Bodanszky, *Handbook Exp. Pharmakol.*, 1973, **34**, 180 (*Chem. Abs.*, 1974, **80**, 48 341r).
[167] G. Jaeger, W. König, H. Wissmann, and R. Geiger, *Chem. Ber.*, 1974, **107**, 215.
[168] W. König and R. Geiger, *Chem. Ber.*, 1970, **103**, 2024, 2034.



Scheme 34

Others. A synthesis has been described of a bovine trypsinogen activation peptide (160) and some related peptides. The inhibition of gastric acid secretion by the peptides was considered too weak to be physiologically significant.[169]

Val-Asp$_4$-Lys

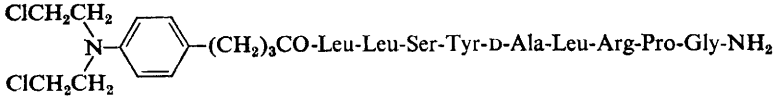
(160)

Hypothalamic Releasing Hormones.—*Growth Hormone Releasing Hormone (GH-RH).* It is now clear that the decapeptide reported earlier (see Vol. 4, p. 366) is not the physiological releasing hormone and is inactive in the more specific tests of growth hormone release. Further syntheses have been described of the decapeptide, which is identical with the *N*-terminal decapeptide of porcine haemoglobin β-chain, and of the corresponding human haemoglobin sequence.[170]

Luteinizing Hormone Releasing Hormone (LH-RH). Syntheses of the hormone have been reviewed.[171] This is an active area of synthesis and a number of particularly interesting compounds have been prepared. Perhaps the most surprising finding was the very high activity found in analogues containing a D-amino-acid in position 6.[172] The replacement of *C*-terminal glycineamide by an ethylamido group is known to give analogues with higher activity in some tests than LH-RH itself,[173] and when this change is combined with the substitution of D-alanine in position 6, an analogue of exceptionally high activity results.[174, 175]

Baker's concept of active-site-directed irreversible inhibitors has not been widely applied in the design of peptide analogues. Nitrogen mustards have been incorporated into bradykinin [176] and angiotensin,[177] and this approach has now been applied to LH-RH with the synthesis [178] of the 1-chlorambucil derivative of

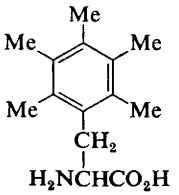
[169] J. M. Bower, C. F. Hayward, I. S. Morley, and J. S. Morley, *Biochem. Biophys. Res. Comm.*, 1974, **60**, 820.
[170] I. Fauszt and S. Bajusz, *Acta Chim. Acad. Sci. Hung.*, 1974, **82**, 471.
[171] N. Yanaihara, *Horumon To Rinsho*, 1973, **21**, 885 (*Chem. Abs.*, 1974, **80**, 23 635b).
[172] M. Fujino, T. Fukuda, S. Shinagawa, S. Kobayashi, I. Yamazaki, R. Nakayama, J. H. Seely, W. F. White, and R. H. Rippel, *Biochem. Biophys. Res. Comm.*, 1974, **60**, 406.
[173] M. Fujino, S. Kobayashi, M. Obayashi, S. Shinagawa, T. Fukuda, C. Kitada, R. Nakayama, and I. Yamazaki, *Biochem. Biophys. Res. Comm.*, 1972, **15**, 697.
[174] A. Arimura, J. A. Vilchez-Martinez, D. H. Coy, E. J. Coy, Y. Hirotsu, and A. V. Schally, *Endocrinology*, 1974, **95**, 1174.
[175] D. H. Coy, E. J. Coy, A. V. Schally, J. Vilchez-Martinez, Y. Hirotsu, and A. Arimura, *Biochem. Biophys. Res. Comm.*, 1974, **57**, 335.
[176] R. J. Freer and J. M. Stewart, *J. Medicin. Chem.*, 1972, **15**, 1.
[177] T. B. Paiva, A. C. M. Paiva, R. J. Freer, and J. M. Stewart, *J. Medicin. Chem.*, 1972, **15**, 6.
[178] C. Y. Bowers, Y.-P. Wan, J. Humphries, and K. Folkers, *Biochem. Biophys. Res. Comm.*, 1974, **61**, 698.



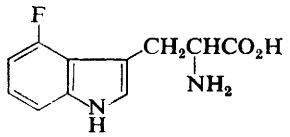
(161)

[Leu^{2,3},D-Ala^{6}]LH-RH, itself a reversible inhibitor of LH-RH. Surprisingly, the 1-chlorambucil analogue (161) had slight LH-RH-like activity unlike [Leu^{2,3},D-Ala^{6}]LH-RH, but by studying LH and FSH responses to the compound at different times it was possible to find evidence for specific irreversible inhibition of LH-RH.

The concept of charge transfer has been invoked to explain the interaction of hormones with their receptors, despite those cases such as [5,8-β-cyclohexylalanine]bradykinin [179] where it is ruled out. Important evidence in favour of this concept in the case of LH-RH derives from the synthesis of an interesting series of analogues with tryptophan replaced by several amino-acids, including pentamethylphenylalanine (162) and 5-fluorotryptophan (163).[180] Remarkably,



(162)



(163)

the 5-fluorotryptophan analogue possesses only one-tenth the activity of the pentamethylphenylalanine analogue (which is almost as active as LH-RH) despite a much closer structural resemblance of the former to the natural hormone. This can be explained by assigning an important role to the expected ability of pentamethylphenylalanine to act as an effective electron donor in a charge-transfer complex. That the tyrosine at position 5 is not involved in charge-transfer complex formation is shown by the high activity of the 5-β-cyclohexylalanine analogue.[181]

Somatostatin (*Growth Hormone Release Inhibiting Hormone, GH-RIH*). Further reports have appeared on the synthesis of this tetradecapeptide [182, 183] and some analogues.[184] If other releasing hormones are a guide, and in view of the clinical interest in the compound, then much more work in this area is likely to be reported in the future. The most intriguing question raised so far is whether the

[179] D. J. Schafer, G. T. Young, D. F. Elliott, and R. Wade, *J. Chem. Soc.* (*C*), 1971, 46; G. A. Fletcher and G. T. Young, *J.C.S. Perkin I*, 1972, 1867.

[180] D. H. Coy, E. J. Coy, Y. Hirotsu, J. A. Vilchez-Martinez, A. V. Schally, J. W. van Nispen, and G. I. Tesser, *Biochemistry*, 1974, **13**, 3550.

[181] H. Künzi, D. Gillessen, A. Trzeciak, R. O. Studer, B. Kerdelhue, M. Jutisz, and W. Lotz, *Helv. Chim. Acta*, 1974, **57**, 2131.

[182] H. U. Immer, K. Sestanj, V. R. Nelson, and M. Gotz, *Helv. Chim. Acta*, 1974, **57**, 730.

[183] J. E. F. Rivier, *J. Amer. Chem. Soc.*, 1974, **96**, 2986.

[184] P. Brazeau, W. Vale, J. Rivier, and R. Guillemin, *Biochem. Biophys. Res. Comm.*, 1974, **60**, 1202.

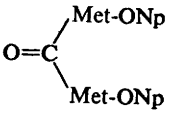
activity found in the reduced peptide [185] is caused by re-oxidation to the disulphide form. This appears to be the most likely explanation in view of the low activity found in derivatives (replacing cysteine by alanine or *S*-methylcysteine) which are unable to cyclize.[186]

Thyrotropin Releasing Hormone (TRH). The application of the conformations proposed for peptide hormones to the design of analogues is an exciting possibility which has been explored for example in the cases of oxytocin [186, 187] and vasotocin.[188] However, the Reporter was surprised to note a correction [189] to a study on the conformation of TRH which suggested that the conformations of [D-Glp]TRH and of TRH were so similar that the biological activity of the former was likely to be as great as that of the natural hormone. The actual results published so far do not bear this out and perhaps serve as a warning against the simple interpretation of biological properties in terms only of the conformation of the hormone.

A synthesis of TRH has been described which proceeds through a number of crystalline intermediates and gives the final hormone in the form of a crystalline tartrate monohydrate.[190] This will no doubt be of interest for *X*-ray crystallographic structure determination.

A [Tyr(Me)^2] analogue of a hormone with the intriguing name of thyroliberin turned out to be none other than the corresponding TRH derivative.[191]

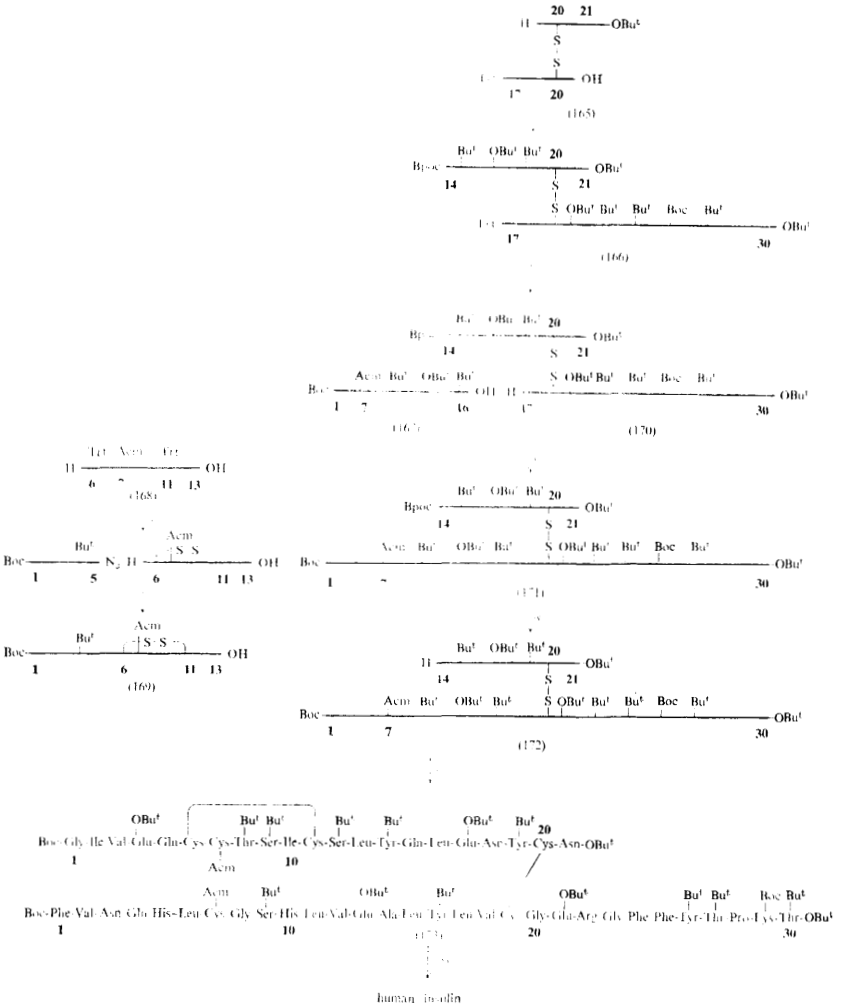
Insulin.—The effective synthesis of insulin and related molecules has remained one of the most important objectives of the synthetic peptide chemistry both because of the challenging synthetic problems which have still needed to be overcome and because of the great importance of the hormone in medicine. One overriding difficulty, the formation of the correct arrangement of disulphide bonds, came much closer to a satisfactory solution with the discovery that a variety of cross-linking agents between the A1 and B29 amino-groups led to a high degree of correct disulphide bond formation (as in the proinsulin molecule) on re-oxidation.[192] The diaminosuberic acid cross-linking agent was particularly suitable since it could be subsequently removed by Edman degradation to regenerate insulin. Another suitable cross-linking agent, carbonylbis-(methionine *p*-nitrophenyl ester) (164), has now been used for the same purpose.[193] Reaction



(164)

[185] P. Brazeau, W. Vale, J. Rivier, and R. Guillemin, *Life Sci.*, 1974, **15**, 351.
[186] Z. Grzonka, J. D. Glass, I. L. Schwartz, and R. Walter, *J. Medicin. Chem.*, 1974, **17**, 1294.
[187] R. Walter, T. Yamanaka, and S. Sakakibara, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 1901.
[188] D. Surovec, P. L. Hoffman, and R. Walter, *Experientia*, 1974, **30**, 823.
[189] A. W. Burgess, F. A. Momany, and H. A. Scheraga, *Proc. Nat. Acad. Sci. U.S.A.*, 1973, **70**, 1456; corrected 1974, **71**, 4640.
[190] C. Hatanaka, M. Obayashi, O. Nishimura, N. Tonkai, and M. Fujino, *Biochem. Biophys. Res. Comm.*, 1974, **60**, 1345.
[191] W. Voelter and H. Hurn, *Z. physiol. Chem.*, 1974, **355**, 1466.
[192] D. Brandenburg, W. Schermutzki, and H. Zahn, *Z. physiol. Chem.*, 1973, **354**, 1521; R. Geiger and R. Obermeier, *Biochem. Biophys. Res. Comm.*, 1973, **55**, 60.
[193] W.-D. Busse and F. H. Carpenter, *J. Amer. Chem. Soc.*, 1974, **96**, 5947.

with insulin gave the A1–B29 cross-linked derivative and the linkage could be removed by cyanogen bromide cleavage. After conversion into the fully reduced peptide (by sulphitolysis followed by dithiothreitol reduction), re-oxidation gave



Conditions: i, fragment condensation, DCCI–HOBt; ii, HCl in 90% CF_3CH_2OH, 'pH' 3.5—4.0; iii, I_2–CF_3CH_2OH; iv, HCl in 90% CF_3CH_2OH, 'pH' 0.5—1; v, 95% CF_3CO_2H, 30 min, 25 °C; vi, 100 mol I_2–60% AcOH (10^{-4} mol l^{-1}), 5—10 min, 25 °C

Scheme 35

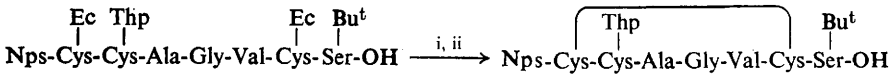
75—86% yields of cross-linked insulin which could be cleaved to give pure insulin in 68% yield.

A further range of cross-linking agents has been studied to determine the effect of the length and nature of the link on the kinetics of the re-oxidation

reaction.[194] It appears that various cross-linking agents are effective provided that they are compatible with the conformation of native insulin.

Perhaps the outstanding synthesis of 1974, described in a preliminary communication,[15] is the total synthesis of human insulin with the disulphide bonds preformed in the appropriate positions on the two chains (Scheme 35). Fragment condensations starting from the key disulphide (165) using dicyclohexylcarbodiimide–*N*-hydroxybenzotriazole for coupling gave (166), in which the two *N*-terminal positions were blocked with trityl and Bpoc groups, respectively. The trityl group was selectively removed using a new acidolytic procedure which leaves the Bpoc group unchanged. This involved a pH-controlled reaction with hydrogen chloride in 90% trifluoroethanol at a 'pH' (using a glass electrode without any correction for solvent) of 3.5—4.0. The selectively protected amino-component (170) was then coupled with the B-chain (1—16) fragment (167) in which the cysteine at position 7 was protected by an acetamidomethyl group. Selective removal of the Bpoc group in (171) leaving the t-butyl-based protecting groups intact was again carried out with hydrogen chloride in 90% trifluoroethanol but at the lower 'pH' of 0.5—1. The resulting monoamino-compound (172) was coupled with the final fragment, the A chain (1—13) sequence in which the 6—11 disulphide was preformed, with the cysteine at position 7 protected by an acetamidomethyl group. Formation of the 6—11 disulphide link was made possible by another innovation, in the form of the selective removal (with simultaneous disulphide formation) of the *S*-trityl groups of the octapeptide (168) leaving the acetamidomethyl group intact. The conditions for this reaction involved the use of iodine in trifluoroethanol. The various t-butyl-based protecting groups were removed from the final protected insulin (173) by 95% trifluoroacetic acid and the acetamidomethyl groups removed with simultaneous formation of the last disulphide bridge by means of iodine in acetic acid. The reverse procedure (*i.e.* removing the t-butyl-based protection after forming the final disulphide bond) has also been used and will be described in a subsequent publication. The final product was purified (as were most of the intermediate fragments) by counter-current distribution, giving some 40 mg of insulin which had full biological activity *in vitro*, crystallized under conditions identical with those under which the natural material crystallizes, and was homogeneous and similar to the natural hormone in a range of studies including thin-layer chromatography, electrophoresis, enzyme 'finger-printing', and amino-acid analysis.

A different approach to the selective protection of cysteine was used in the synthesis of fragments of the A chain of ovine insulin with an intact 6—11 disulphide link (Scheme 36).[41] Cleavage of the *S*-ethylcarbamoyl groups was



Conditions: i, NH_3–MeOH; ii, $C_2H_4I_2$

Scheme 36

[194] A. Wollmer, D. Brandenburg, H.-P. Vogt, and W. Schermutzki, *Z. physiol. Chem.*, 1974, **355**, 1471.

achieved with ammonia in methanol followed by oxidation, with the cysteine in position 7 protected by a tetrahydropyranyl group.

The truncated des-(pentapeptide B26—B30) human (porcine) insulin has been synthesized by combining the A chain which is common to both species with a synthetic B chain-(1—25)-peptide *S*-sulphonate.[195] The synthesis demonstrates the lack of importance of the B chain (26—30) sequence and confirms the results obtained by degradation of the natural molecule.

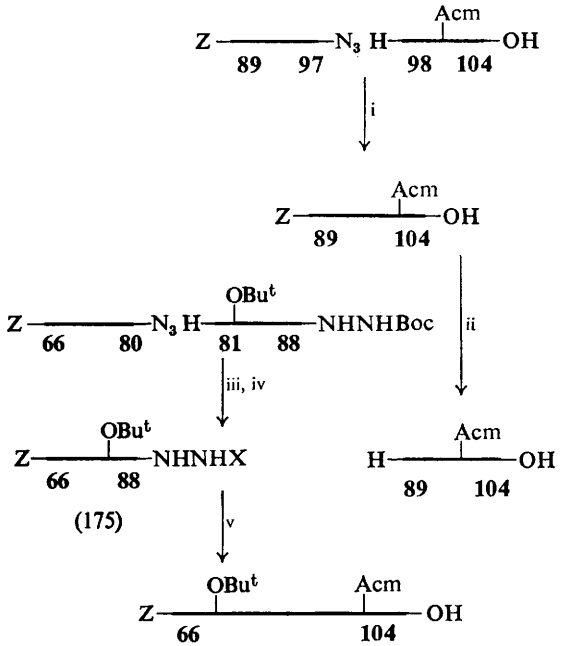
Zahn has outlined the Aachen group's approach to the synthesis of proinsulin in a lecture which is reported briefly.[196]

The solid-phase synthesis of the ovine A chain-(14—21)-octapeptide has been described.[28] The introduction of Boc-Cys(Trt) could not be made to go to completion, and a modified approach was adopted. The protected (14—19) hexapeptide was synthesized on the modified Merrifield t-alkoxycarbonyl-hydrazide resin (see Vol. 2, p. 162) adding amino-acids as their Bpoc derivatives except for the final amino-acid, which was protected by the 2-(methylsulphonyl)-ethyloxycarbonyl group because the latter is stable during cleavage from the resin. The hexapeptide hydrazide was converted into the azide and coupled to the dipeptide Cys(Trt)-Asn (174).

Ribonuclease.—A further detailed paper has appeared on Hofmann's ribonuclease T_1 approach (see previous Volumes), describing the synthesis of a protected (66—104) *C*-terminal nonatriacontapeptide (Scheme 37).[197] Ethylcarbamoyl protection of cysteine suffers the disadvantage that the group is unstable at pH >8.5 and basic solvents cannot be used during purification. In this synthesis, therefore, acetamidomethyl protection was used in preference to the ethyl-carbamoyl used in earlier work. Buffered 8M urea was found to be an excellent solvent for purifying by gel filtration the ribonuclease T_1 fragments, which in this synthesis are minimally protected. The urea (aqueous triethylamine–urea mixtures were used, for example) was easily removed by dialysis, often with precipitation of the peptide. The use of aqueous formic acid as a solvent for purifying the peptide hydrazide (175) led to formation of an *N*-formyl hydrazide (X = HCO) which failed to give an azide on diazotization. The side-reaction was demonstrated by formation of (176) from the corresponding hydrazide and aqueous formic acid. α-Amino-groups were not affected under the same conditions.

The solid-phase synthesis of ribonuclease T_1 and the corresponding Trp-59 → Tyr analogue has been reported.[198] A manual stepwise method was used starting from Boc-Thr(Bzl)-resin. The approach was generally similar to that described in earlier publications; lysine and histidine were protected as the N^{ε}-di-isopropyl-methoxycarbonyl and N^{im}-t-butoxycarbonyl derivatives, respectively. The two syntheses were carried out in parallel and the addition of the 103 amino-acid derivatives took 47 days, giving a 50—54% yield of peptidyl-resin based on

[195] P. G. Katsoyannis, J. Ginos, G. P. Schwartz, and A. Cosmatos, *J.C.S. Perkin I*, 1974, 1311.
[196] H. Zahn, H. Berndt, J. Föhles, D. Brandenburg, W. Schermutzki, and V. K. Naithani, *Angew. Chem. Internat. Edn.*, 1974, **13**, 419.
[197] H. Romovacek, S. Drabarek, K. Kawasaki, S. R. Dowd, R. Obermeier, and K. Hofmann, *Internat. J. Peptide and Protein Res.*, 1974, **6**, 435.
[198] M. Waki, N. Mitsuyasu, S. Terada, S. Matsuura, T. Kato, and N. Izumiya, *Biochem. Biophys. Res. Comm.*, 1974, **61**, 576.



Conditions: i, Et_3N–DMSO–DMF; ii, HBr–TFA; iii, couple, TFA, purification in 45% HCO_2H (X = H + HCO); iv, N_2H_4 (X = H); v, Bu^tONO–HCl, DMF–DMSO

Scheme 37

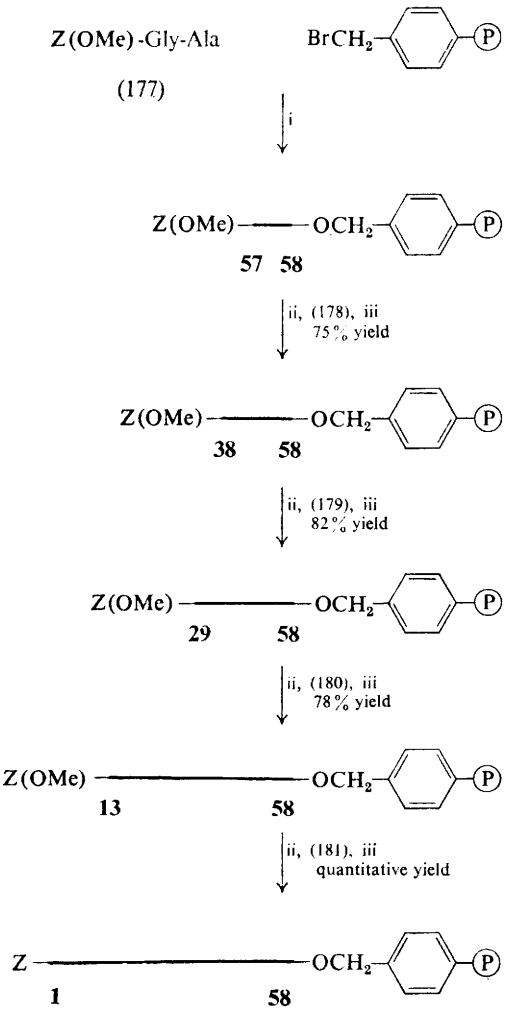
Z-Phe-NHNHCHO
(176)

weight and amino-acid analysis. In the ribonuclease T_1 case, the product of deprotection with hydrogen fluoride–anisole weighed 200 mg and gave 51 mg with the same elution volume as native ribonuclease T_1 on Sephadex G50 gel filtration (after an ultrafiltration step). This material had 0.5% of the activity of the native enzyme. After further extensive purification (G75, DEAE cellulose, and affinity chromatography) some 0.2 mg of material with 49—59% specific activity was obtained in two fractions from a final affinity chromatography column (the same behaviour being exhibited by native ribonuclease T_1). A similar result leading to active material was obtained with the Tyr-59 analogue. In the T_1 case, although the final product had similar characteristics to the native ribonuclease T_1, the similarity in 'finger-printing' experiments between this and a material at an earlier stage of purification with only 3% specific activity throws considerable doubt on the value of the comparisons made.

The general conclusion from the study must be that although a material related to ribonuclease T_1 has been produced and has high specific activity, it is open to question whether this is a single chemical entity and if so whether it is identical with native ribonuclease T_1. The study also provides limited evidence that tryptophan at position 59 can be replaced by tyrosine with retention of activity.

Trypsin Inhibitors.—The chemistry of protein inhibitors of proteolytic enzymes has been reviewed.[199]

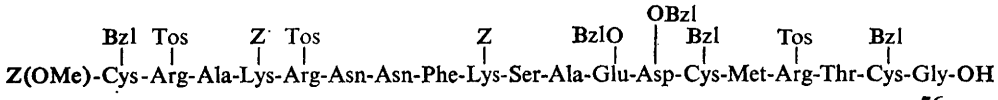
The synthesis has been described of the bovine basic trypsin inhibitor (Kunitz) by fragment condensation on solid-phase (Scheme 38).[200, 201] The first dipeptide [as the free acid (177)] was attached to a bromomethyl resin by direct substitution in the presence of dicyclohexylamine, and four fragments (178)—(181) were



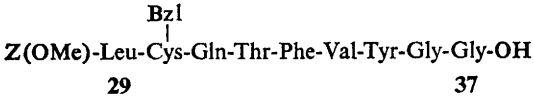
Conditions: i, Dcha; ii, TFA; iii, DCCI–HONSu, 4 days

Scheme 38

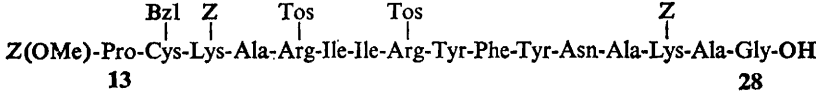
[199] G. E. Means, D. S. Ryan, and R. E. Feeney, *Accounts Chem. Res.*, 1974, **7**, 315.
[200] H. Yajima, Y. Kiso, Y. Okada, and H. Watanabe, *J.C.S. Chem. Comm.*, 1974, 106.
[201] H. Yajima and Y. Kiso, *Chem. and Pharm. Bull. (Japan)*, 1974, **22**, 1087.



(178)



(179)



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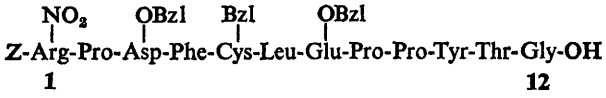

(181)

coupled successively using *N*-hydroxysuccinimide–dicyclohexylcarbodi-imide, each coupling being allowed to proceed for 4 days. Unreacted amino-components were blocked by acetylation and the coupling yield was estimated by amino-acid analysis. After cleavage from the resin by hydrogen fluoride (with anisole and methionine as scavengers) the product was purified by gel filtration and affinity chromatography. It had some 82% of the biological activity of the native inhibitor, using tosylarginine methyl ester as substrate for the trypsin. The fragments (178),[202] (179),[203] (180),[204] and (181)[205] were synthesized by solution methods.

Other Syntheses.—Reviews have appeared on the chemistry of parathyroid hormone[206] and the chemistry of selenium-containing amino-acids and peptides.[207] Ramachandran has reviewed the structure–function relations of ACTH[208, 209] and Medzihradszky the synthesis of ACTH and its biologically active fragments.[3h]

The synthesis has been described of a pentacontapeptide corresponding to positions 42—91 of ovine β-lipotrophin.[210] The corresponding bovine 41—58 sequence contains the complete sequence of β-MSH which may arise naturally from it by degradation and the 48—91 sequence has substantial β-lipotrophin activity. The synthesis of the 42—91 sequence was by solid-phase and was attempted first by the procedure used successfully in the synthesis of ACTH. Tryptophan was protected by the formyl group and coupling was carried out using an acid : carbodi-imide ratio of 2 : 1 (*i.e.* effectively using the symmetrical anhydride), except for asparagine and glutamine which were coupled as *p*-nitrophenyl esters. However, incorporation of some amino-acids (Thr, Val, Ile, Leu) in the 66—83 region was incomplete. Apparently traces of trifluoroacetic acid not removed by washing prevent complete coupling from taking place. A modified procedure was therefore used in which a first coupling was followed by a second in the presence of three equivalents of di-isopropylethylamine. Presumably, since the coupling would be largely complete at this stage, the overall risk of racemization should not be much greater than usual. Nevertheless the possibility was carefully evaluated by the synthesis of the model tripeptides (182) and (183) where enzyme digestion established $< 1\%$ racemization. Cleavage

Glu-Phe-Gly	Glu-D-Phe-Gly
(182)	(183)

of the pentacontapeptide from the resin with hydrogen fluoride–anisole gave the formyl peptide, which was deformylated in liquid ammonia. Purification on

[202] H. Yajima, Y. Kiso, and K. Kitagawa, *Chem. and Pharm. Bull. (Japan)*, 1974, **22**, 1079.
[203] H. Yajima, N. Mizokami, M. Kiso, T. Jinnouchi, Y. Kai, and Y. Kiso, *Chem. and Pharm. Bull. (Japan)*, 1974, **22**, 1075.
[204] H. Yajima, Y. Okada, H. Watanabe, and Y. Kiso, *Chem. and Pharm. Bull. (Japan)*, 1974, **22**, 1067.
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[208] J. Ramachandran, *Israel J. Chem.*, 1974, **12**, 675.
[209] J. Ramachandran, in 'Hormonal Proteins and Peptides', ed. C. H. Li, Academic Press, New York, 1973, Vol. 2, p. 1.
[210] D. Yamashiro and C. H. Li, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 4945.

carboxymethylcellulose and partition chromatography on G-50 Sephadex gave the product, with six times the activity of β-lipotrophin itself (on a weight basis). Purity was established by a variety of procedures including polyacrylamide gel electrophoresis. The u.v. spectrum was similar to that of ACTH as anticipated from the structural similarity.

Further details have been published of the solid-phase synthesis of human parathyroid hormone-(1—34)-amide, and the [Ala^1]-analogue.[211] The synthesis was based on a sequence proposed by Niall,[212] and the structure differs from that proposed by Brewer[213] on which another recent synthesis[214] was based. A comparison of the two synthetic partial sequences and the natural material may contribute to resolving this disparity.

The decapeptide (184) has even more catalytic effect than the previously reported [Ala^7]-decapeptide on the hydrolysis of chitin (though still considerably

Glu-Phe-Ala-Ala-Glu-Glu-Phe-Ala-Ser-Phe

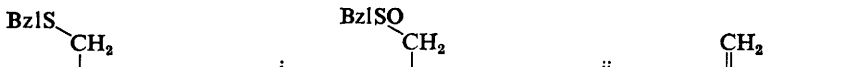
(184)

less than lysozyme which it is designed to mimic), and the authors suggest that it should be designated as an enzyme.[215]

The synthesis of sequences of sperm-whale myoglobin spanning positions 15—23 has allowed the accurate identification of the regions on which the antigenicity towards a variety of antisera depends.[216] The positions vary slightly for each antiserum but each sequence occupies part of a highly accessible surface 'corner' of the native molecule.

Two tryptic peptides corresponding to positions (1—5) and (69—73) of hen egg-white lysozyme previously reported to inhibit the action of histamine on guinea-pig ileum and some related structures have been synthesized and shown to be inactive.[217]

Peptides containing dehydroamino-acids have been prepared by oxidation of sulphides to sulphoxides followed by thermal elimination. A typical example is shown in Scheme 39.[218] Peptide derivatives of aminophosphonic acids



Conditions: i, $NaIO_4$–H_2O–MeOH or Ph(3-Cl)CO_3H; ii, refluxing xylene, 6—10 h, N_2

Scheme 39

[211] G. W. Tregear, J. van Rietschoten, E. Greene, H. D. Niall, H. T. Keutmann, J. A. Parsons, J. L. H. O'Riordan, and J. T. Potts, jun., *Z. physiol. Chem.*, 1974, **355**, 415.

[212] H. D. Niall, R. T. Sauer, J. W. Jacobs, H. T. Keutmann, G. V. Segre, J. L. H. O'Riordan, G. D. Aurbach, and J. T. Potts, jun., *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 384.

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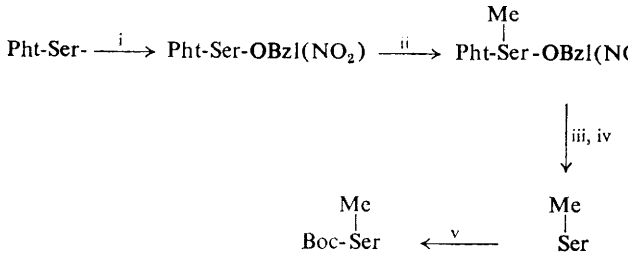
[217] K. Susuki, N. Endo, R. Tani, and H. Kikuchi, *Chem. and Pharm. Bull. (Japan)*, 1974, **22**, 2462.

[218] D. H. Rich, J. Tam, P. Mathiaparanam, J. A. Grant, and C. Mabuni, *J.C.S. Chem. Comm.*, 1974, 897.

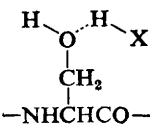
have been prepared.[219] Ideal procedures for such synthesis do not yet appear to have been established. Details of the synthesis of mucopeptide fragments of bacterial cell wall containing diaminopimelic acid have appeared in a series of papers.[220]

Among the more interesting compounds synthesized for structure–activity studies are those in which an amino-acid residue is replaced with a closely similar structure in which one potentially important feature is changed. The most suitable amino-acids for use in this way are often not readily available. Hofmann's studies with histidine replacement by β-pyrazolyl alanine is a good example. The preparation of *O*-methyl serine, which could be used to replace serine and examine the role of hydrogen bonding, has been described (Scheme 40).[221] The

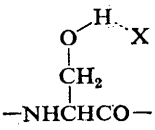


Conditions: i, $Br \cdot CH_2 \cdot Ph(NO_2)$–$Et_3N$; ii, CH_2N_2; iii, H_2–Pd–$BaSO_4$; iv, N_2H_4; v, Boc-N_3–MgO

Scheme 40



(185)



(186)

hydrogen-bonded structure (185) is also possible with *O*-methyl serine but this is not true of (186).

4 Appendix I: A List of Syntheses Reported during 1974

Natural Peptides, Proteins, Analogues and Partial Sequences.—Syntheses are listed under the name of the peptide or protein to which they relate.

Peptide	*Ref.*
Adrenocorticotrophic hormone (ACTH)	
ACTH-(11—14)-tetrapeptide	102
ACTH-(14—20)-heptapeptide	117
[Ala[1]], [D-Ala[1]], and [(^{14}C)Ala[1]]-ACTH-(1—20)-NH_2	108

[219] W. F. Gilmore and H. A. McBride, *J. Pharm. Sci.*, 1974, **63**, 1087.

[220] A. Kolodziejczyk, A. Arendt, T. Sokolowska, and B. Gzella, *Roczniki Chem.*, 1974, **48**, 453; A. Kolodziejczyk, A. Arendt, and T. Sokolowska, *ibid.*, pp. 627, 1305; A. Arendt, A. Kolodziejczyk, T. Sokolowska, and E. Szulfer, *ibid.*, p. 1501; A. Arendt, A. Kolodziejczyk, and T. Sokolowska, *ibid.*, pp. 1707, 1921.

[221] R. S. Hodges and R. B. Merrifield, *J. Org. Chem.*, 1974, **39**, 1870.

Peptide	*Ref.*
[2-, 3-, and 3,4-δ-aminovaleric acid, and 4-ω-aminocaprylic acid]-ACTH-(3—19)-heptadecapeptide	222
[(3,5-^{3}H)Tyr^2]-ACTH-(1—10)-decapeptide	223
Amelitin (Glp-Ala-Gly-Tyr-Ser-Lys)	224
Angiotensin II	
Analogues	225, 226, 227, 228
Analogues of [Ile^8]-angiotensin II substituted in position 1	86
Analogues of [α-phenylglycine^8]-angiotensin II substituted in position 1	229
Analogues substituted in position 1	230
Analogues substituted in positions 1 and 8	231
A cysteinyl derivative of angiotensin II and a spin-labelled derivative of it	232
[Phe(Me)^8]-angiotensin II analogues	233
1-Substituted [Leu^8]-angiotensin II analogues	234
[D-Tyr^8]-angiotensin II and fragments, and the corresponding aza-analogues	235
Val-Tyr-Ile-His-Pro-Phe acylated with a wide range of acids and amino-acids	236
Antamanide	
Analogues	237
Cyclo-(Pro$_2$-Gly$_n$-Pro$_2$-Gly$_m$) n, m = 1,1; 1,2; 2,2; 2,3; 3,3	238
Bacterial cell-wall peptides	
Mucopeptide fragments of bacterial cell walls containing diaminopimelic acid	220
Blanching hormone	239
Bradykinin	32, 89, 95
Di- and tri-peptide fragments containing pipecolic acid	240
[5-, 8-, and 5,8-L-*erythro*-α-amino-β-phenylbutyric acid (Phabu*)]-bradykinins	241
[Har^1]-, [Har^9]-, and [Har^{1,9}]-bradykinin†	242
[Hypro^2]-, [Hypro^3]-, and [Hypro^7]-bradykinin	243
[Phe(4-NO$_2$)^5, Tyr(Me)^8]- and [Tyr(Me)^5, Phe(4-NO$_2$)^8]-bradykinin	244

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* This amino-acid occurs naturally in bottromycin.
† Har = L-Homoarginine.

Peptide	*Ref.*
[Val[1], Thr[6]]-bradykinin	245
Calcitonin	
Calcitonin-(26—32)-heptapeptide amide	106
[Ser[29],Thr[31]] and [Ala[29],Val[31]]-human calcitonin	153
[Ser[2],Val[8]]-human calcitonin	154
Carbonic anhydrase	
Human carbonic anhydrase-(59—68)-decapeptide and its [Ala[65]]-analogue	246
Colistin	
Acylpentapeptides related to colistin	247, 248
n-Octanoyl-, n-decanoyl-, and n-dodecanoyl-amino-acyl colistins	249
Corticotrophin-like intermediate lobe peptide (CLIP)	
Bovine CLIP	250
Human CLIP	90
Cytochrome	
Baker's yeast iso-1-cytochrome *c*-(93—101)-protected nonapeptide	156
Cytochrome B5 fragments	251
Heptapeptide cytochrome *c* fragment attached to a haem group	252
Tetra- and penta-peptide sequences containing Gln and Asn (and [^{14}C] Gly) as models of biological deamidation	253
Z-cytochrome *c*-(14—18)-pentapeptide-OMe coupled to a porphyrin group	254
Eisenine (Glp-Gln-Ala)	
Eisenine and amide	255
Eledoisin	106
[Asn[5], NHAla[6]] eledoisin*	256
[Azala[6]] eledoisin *C*-terminal hexa- and heptapeptides†	257
Eledoisin and analogues attached to dextran	258
Encephalitogenic protein	
[Asn[121]]-human encephalitogenic protein-(113—122)-decapeptide	259
Glycine analogues of human encephalitogenic protein-(113—122)-decapeptide	260
Human encephalitogenic protein-(105—109)-pentapeptide and smaller fragments containing Arg-107	261
Ferrichrome	262

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* NHAla = L-α-hydrazinopropionic acid.
† Azala = —NH·N(Me)·CO—.

Peptide	*Ref.*
Ferredoxin	
A [Leu^2]-analogue of a clostridial-type ferredoxin with Arg in position 30	146
[Leu^2]- and [Gly^2]-analogues of a clostridial-type ferredoxin with Tyr in position 30	145
Fibrinopeptides	
Bovine fibrinopeptide B-(12—21)-decapeptide	263
An Nps-protected-(5—10)-sequence of human fibrinopeptide A	264
The *N*-terminal tetrapeptide of human fibrinopeptide A	265
A protected green monkey fibrinopeptide B-(6—14)-sequence	266
Gastrin	
The *C*-terminal tetrapeptide (using triphenyl phosphite)	267
[Leu^5]-gastrin I-(5—13)-nonapeptide	102
Pentagastrin and other peptide amides	82
Ph(4-I)SO_2-β-Ala derivatives of *C*-terminal gastrin tetra- and penta-peptides	268
Substituted benzoyl-Gly-Trp-Met-Asp-Phe-NH_2 and the corresponding (C2-^{3}H)Gly derivatives as photoaffinity labels	269
Glutathione	
Analogues	270, 271
Gramicidin S	
Isogramicidin S and its [Lys$^{2,2'}$]-analogue	272
MeLeu analogues	273
Orn-Leu-D-Phe-Pro-Val	274
Growth hormone (GH)	
Bovine GH-(125—133)-nonapeptide	97
Growth hormone releasing hormone (GH-RH): see haemoglobin	
Haemoglobin	
The *N*-terminal decapeptide of porcine* and human haemoglobin β-chain	170
Protected human β-chain-(71—77)-pentapeptide	275
A protected human β-chain-(95—100)-sequence	276
Tetra- and hepta-peptide fragments of a human haemoglobin β-chain	277
Histones	
Pentapeptide sequences containing Gln and Asn (and [^{14}C]Gly) as models of biological deamidation of histone IV	278

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* Previously identified as GH-RH.

Peptide	*Ref.*
Insulin	
Human insulin	15
Analogues of des(B-chain-*C*-terminal hexapeptide)-insulin	279, 280
B-chain-(1—14)-sequence	281
$CO(Met)_2$-cross-linked insulin	193, 282
Des-(A-chain-Ala^{30})-bovine insulin	283
Des-(pentapeptide B26—B30)-human (porcine) insulin	195
[Gly^5]-B-chain-(1—8)-octapeptide	284
Peptide fragments related to insulin A-chain incorporating alanine β-sulphonyl chloride	285
[Phe^{16}]- and [Ala^{16}]-protected B-chain-(15—20) sequence	286
A protected (disulphide) ovine A-chain-(10—21) sequence	287
A protected porcine B-chain-(21—30) sequence	288
Protected sheep insulin A-chain fragments with an intact 6—11 disulphide link	41
Sheep insulin A-chain-(14—21)-octapeptide	28
Three tetra- and penta-peptide fragments containing bonds cleaved by trypsin	162
Isariin	289, 290
Isariic acid	291
Lipotrophin	
Ovine β-lipotrophin-(42—91)-*C*-terminal peptide	210
Luteinizing hormone realeasing hormone (LH-RH)	39, 54, 292, 293, 294
Acetylated *C*-terminal partial sequences	295
Acidic analogues	296
Analogues	297, 298, 381
Analogues of des-GlyNH_2^{10}-LH-RH-ethylamide substituted in positions 5 and 6	299
Analogues of des-GlyNH_2^{10}-LH-RH-ethylamide substituted in positions 6 and 10	172

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Peptide	*Ref.*
Analogues substituted in position 2	300
Analogues substituted in position 8	301
[Chlorambucil[1], Leu[2,3], D-Ala[6]]-LH-RH: an irreversible inhibitor	178
[Cit[8]]-, [Har[8]]-, and [Lys[8]]-LH-RH*	302
Des-GlyNH_2[10]-[D-Ala[6]]-LH-RH ethylamide	175
Des-GlyNH_2[10]-LH-RH ethylamide and its des-His[2] and des-Trp[3] derivatives	303
[Gly[5]]-, [Phe[5]]-, [Tyr(Me)[5]]-, and [Cha[5]]-LH-RH	181
[Har[8]]-LH-RH*	304
[Ile[2]]-, [Tyr[3,5]]-, and [Leu[2,3]]-LH-RH	305
[D-Leu[6]]-LH-RH and des-GlyNH_2[10]-[DLeu[6]]-LH-RH†	306
[Phe(Me_5)[3]]-, [Trp(5-F)[3]]-, [Phe(NO_2)[3]]-, [Phe(NH_2)[3]]-, and [His[3]]-LH-RH	180
[2-L-β-(Pyrazolyl-3)alanine]-LH-RH	307
Stereoisomeric analogues	308
Lysozyme	
Hen egg-white lysozyme (1—5) and (69—73) tryptic peptides and related peptides	217
[Homoserine[52]]-hen egg-white lysozyme	147
Malformin A	309
Melanocyte-stimulating hormone (MSH)	
Dogfish MSH	16
[(^{14}C)Gly[10]]-MSH	310
α-MSH hydrazide	311
[(^{3}H)Tyr[2]]-MSH	312
Myoglobin	
A protected myoglobin-(58—73)-hexadecapeptide	12
Sperm-whale myoglobin-(15—21), -(16—23), and -(15—23) sequences	216
Nerve growth factor (NGF)	
The *N*-terminal octapeptide of mouse NGF	114
Oxytocin	10
Analogues	313, 314
[($\alpha\beta\beta$-^{2}H)Cys[1]]- and [($\alpha\beta\beta$-^{2}H)Cys[6]]-oxytocin	315

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* Har = L-homoarginine.

† It is not entirely clear to the Reporter which analogues this paper describes.

Peptide	*Ref.*
[1,6-αα′-Diaminosuberic acid]-oxytocin ('dicarba-oxytocin')	316
S,S′-Dibenzyloxytoceine by the *in situ* technique	122
Desamino-1-carba-[Phe^2]-, -[Tyr(Me)^2]-, and -[Ile^2]-oxytocin	317
[Gly^7]-oxytocin and dicarba-[Gly^7]-oxytocin	187
Heterodetic cyclic pentapeptides with the isotocin and glumitocin ring	318
[D-2-Hydroxy-3-mercaptopropanoic acid^1]-oxytocin	319
[(α-^{2}H)Leu^8]-, [(^{15}N)Ile^3]-, and [(^{15}N)Tyr^2]-oxytocin	320
[β-Mercapto-ββ-diethylpropionic acid^1,Leu^4]-oxytocin	321
Mercury mercaptide derivative of oxytocin	322
[Met^4]-oxytocin	323
Oxytocin and desamino-oxytocin using the 'bidirectional solid-phase' approach	99
[Phe^7] and [Val^7]-oxytocin-(6—9) dimer	324
[Phe(4-F)^2]-oxytocin and -desamino-oxytocin	325
[Phe^3]-, [Val^4]-, and [Gly^7]-desaminodicarba-oxytocin	325*a*
[Pro^9]- and [Glu^9]-oxytocin and [Lys^8]-oxytocin methylamide	186
Protected [Met^4]- and [Cys(Me)^4]-oxytocin	324
[Tyr(2-I)^2]-oxytocin	326
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Parathyroid hormone (PTH)	
The *N*-terminal PTH-(1—34)-peptide	328
The *N*-terminal PTH-(1—34)-peptide and its [Ala^1]-analogue	211
Proparathyroid hormone (Lys-Ser-Val-Lys-Lys-Arg-PTH) *N*-terminal sequences (no data)	329
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Norphalloin analogues	330
Physalaemin	
A (^{3}H) acetyl hexapeptide sequence	331
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Proinsulin	
[(U-^{14}C)Arg^{31}]-porcine proinsulin-(28—34)-heptapeptide	334

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Peptide	*Ref.*
Human proinsulin-(33—63) ('C-peptide'), [Lys(HCO)[64]]-human proinsulin-(31—65), and [Glu[4],Gln[43],Lys(HCO)[64]]-human proinsulin-(31—65)-peptides	335
An unsuccessful attempt to synthesize porcine proinsulin C-chain by solid-phase	112
Ribonuclease	
Carbohydrate substituted bovine ribonuclease B-(34—37)-, and -(34—38)-peptides	336
[His(4-F)[12]]-ribonuclease S-(1—15)-pentadecapeptide	337
[(im-C2-[13]C)His[12]]-ribonuclease S-(1—15)-pentadecapeptide	338, 339
[Leu[11]]- and [Lys[11]]-ribonuclease A-(1—20) and the corresponding [Orn[10]]-peptide	340
A protected ribonuclease T_1-(66—104)-nonatriacontapeptide	197
Ribonuclease T_1 and [Tyr[59]]-ribonuclease T_1	198
[Tyr[120]]- and [Ala[120]]-ribonuclease-(111—124)	341
Rubredoxin	
A protected rubredoxin-(A28—A37)-decapeptide	37
Scotophobin	
Protected scotophobin *C*-terminal-(8—15)-octapeptide amide	342
Secretin	
A [Nle[17]]-secretin-(14—28)-pentadecapeptide	123
C-terminal hexa- and nona-peptide fragments	343
Somatostatin (growth hormone release inhibiting hormone – GH-RIH)	182, 183
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Fragments by shortening successively from the *N*-terminus	348
Tentoxin	349
Thyrotrophin releasing hormone (TRH)	44, 87, 106, 293, 350
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Peptide	*Ref.*
Crystalline TRH tartrate	190
[D-His^2]-TRH	354
[Phe^2]-TRH	355
[Tyr(Me)^2]-TRH	191
Triosephosphate isomerase	
A protected hexapeptide sequence (10—15)	356
A protected nonapeptide sequence (1—9)	357
Trypsinogen activation peptide	
The bovine peptide (Val-Asp$_4$-Lys) and analogues modified in position 1	169
Trypsin inhibitors	
An active site hexapeptide (Cys-Lys-Ala-Gly-Gly-Cys) and its N^{α}-acetyl amide	358
Bovine basic pancreatic trypsin inhibitor by fragment condensation on solid-phase	200—205
Des-Lys^{15}-, des-Ala^{16}-[Arg^{15}]-, -[Phe^{15}]-, and -[Trp^{15}]-trypsin-kallikrein inhibitor (Kunitz)	144
[52-Homoserine]-basic pancreatic trypsin inhibitor	143*a*
Protected peptides spanning the entire sequence of porcine pancreatic trypsin inhibitor II (Kazal)	359
Valinomycin	
Analogues	360, 361, 362
Vasoactive intestinal peptide (VIP)	11
[Nle^{17}]-VIP-(14—28)-pentadecapeptide	123
Vasopressin	
Arginine vasotocin analogues	188
[Har^8]-vasopressin and [Har^8]-desaminovasopressin	363
[8-ε-Hydroxynorleucine]-vasopressin	364
[Leu^4,D-Arg^8]-vasotocin and [Leu^4,D-Arg^8]-desaminovasotocin	365
[Lys^8]-vasopressin-(6—9)-tetrapeptide dimer	366
[α-Mercapto-acetic acid^1,Lys^8]- and [α-mercaptobutyric acid^1,Lys^8]-vasopressin	367
[β-Mercapto-$\beta\beta$-diethylpropionic acid^1,Leu^4,Lys^8]-vasopressin	322
[β-Mercapto-$\beta\beta$-diethylpropionic acid^1,Lys^8]-vasopressin	368
[Orn^8]-, [Arg^8]-, and [D-Arg^8]-6-carba-desaminovasopressin	369

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Enzyme Substrates.—Substrates are listed under the general name of the principal enzyme which acts on them.

Peptide	*Ref.*
D-Alanine peptidase	
A carbohydrate linked pentapeptide containing (U-^{14}C) D-Ala	370
Carboxypeptidase A	
Depsipeptide substrates	371
Pentapeptide substrates containing D- and L-Ala	372
Chymotrypsin	
N-peptidyl derivatives of 2-phenylethylamine	373
Phe(*p*-N_3)-containing di- and tri-peptide photoaffinity ligands	159
Phe(*p*-N_3)-, Phe(*p*-NO_2)-, Phe(*m*-N_3)-, and Phe(*o*-NO_2,*p*-N_3)-containing photoaffinity ligands	160
Elastase	
Acylated-Ala_3-*p*-nitroanilides	374
A series of substrates	157
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A tetrapeptide substrate coupled to agarose and dextran	375
Microbial acid proteinases	
Z-aminoacyl-Leu-Ala_2 and Z-Phe-aminoacyl-Ala_2	376
Microbial alkaline proteinases	
Z- or Ac-$(Ala)_{0-3}$-(Phe, Ala, or Lys)-OMe	377
Papain	
N-substituted derivatives of Val-Glu-Leu-Gly	161
Pepsin	
Peptides containing the Phe-Phe sequence	378
Z-Gly_3-Phe_2, and Z-Gly_4-Phe_2 3-(4-pyridyl)propyl esters	379
Plasmin	
Lysine peptide substrates of plasmin and trypsin	380
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$(Gln)_n$-Phe(NO_2)-Phe (n = 1—4)	113
Renin	
A series of di- to tetra-peptide substrates two of which were attached to agarose for affinity chromatography	163
Transglutaminase	
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Trypsin	
Di- and tri-peptide substrates for affinity labelling containing a Lys-CH_2Cl terminus	158
Synthetic insulin fragments as trypsin substrates	162

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Ala-Gly-, Glu-Ala-Gly-, and Tyr-Glu-Ala-Gly-$(Tyr-Glu-Ala-Gly)_3$-Gly	133

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Peptide	*Ref.*
Boc-(Gly-Cys-Gly)$_{3 \text{ or } 4}$-NH_2	383
Boc-(Nva-Gly-Pro)$_n$-OMe (n = 1—6)	384
Cyclo-(Pro-Gly)$_n$ (n = 2—4) and *cyclo*-(D-Pro-Gly-L-Pro-Gly)	385
Gly-Leu-Gly oligomers	130
HCO-(Val-Pro-Gly-Val-Gly)$_n$-OMe (n = 1—3)	91
HCO-(Val-Pro-Gly-Val-Gly)$_n$-Val-OMe polymer (n = *ca.* 10—15)	134
(Nle-Arg)$_{27}$-NH_2	386
Poly-(Ala-Lys-Pro), -(Ala-Pro-Lys), -(Ala-Lys-Pro-Lys), and -(Ala-Lys$_2$-Pro-Lys)	387
Poly-(Ala-Orn-Ala, Gly or Glu)	388
Poly-(Arg-Ala-Ala)	38
Poly-[Asp(OMe)-Gly], -[Gly-Asp(OMe)], -[Gly-Orn(Tos)], and -[Orn(Tos)-Gly]	389
Poly-(Glu-Ala$_3$-Ser) and -(Ala$_2$-Glu-Ala-Ser)	390
Poly-(L-Glu-D-Glu-L-Glu-D-Glu) and -(D-Glu-L-Glu-D-Glu-L-Glu)	135
Poly-(Glu-Tyr)	392
Poly-[Gly$_2$-Ala], -[Gly$_2$-Lys(Tos)], and -[Gly$_2$-Glu(OMe)]	141
Poly-(Gly-Orn-Gly) and -(Orn-Orn-Gly) and their guanidination products	393
Poly-(Gly-Val-Gly-Val-Pro)	394
Poly-(Leu$_3$-Gly), -(Leu$_2$-Gly), -(Leu-Gly), and -(Leu-Gly$_2$)	395
Poly-(Leu-Glu-Lys-Ala-Glu-[Ser or Ala]-Gly)	132
Poly-(Lys-Ala$_3$)	395*a*
Poly-(Lys-Ala-Gly)	396
Poly-(Lys-Ala-Pro)	397
Poly-(Lys-Gly), -(Lys-Gly$_2$), and -(Gly-Lys-Gly$_2$)	398
Poly-(Lys$_3$-Gly) and -(Orn$_3$-Gly)	399
Poly-[Lys (oligo-amino-acyl)]	400
Poly-(Lys-Pro), -(Lys$_2$-Pro), -(Lys$_3$-Pro), -(Lys-Pro$_2$), and -(Lys-Ala)	401
Poly-(Phe-Lys-Phe-Lys)	402

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Peptide	*Ref.*
Polytripeptides	403
Poly-(Tyr-Glu-Ala-β-Ala)-Gly-OMe	133
$(Pro\text{-}Pro\text{-}Gly)_n\text{-}(Ala\text{-}Pro\text{-}Gly)_m\text{-}(Pro\text{-}Pro\text{-}Gly)_n$ ($2n + m = 15$; $m = 1$, 3, and 5) and $(Pro\text{-}Pro\text{-}Gly)_n$ ($n = 10$, 12, 14, and 15)	404
A series of arginine-containing polydi- and polytri-peptides	405
Substituted sequential polymers of Gly, Glu, Lys, and Ala	406

Miscellaneous Peptides

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Acetylated peptide models for mass spectrometry	408
Ala-Ala-Ala	409
Ala-Gly-Leu-Ala using a new soluble polymeric support	121
Arg- and Lys-containing tripeptides	410
Aspartyl-hydroxyamino-acid esters	411
Boc-Arg(NO_2)-Pro derivatives	412
Boc-Leu-Ala-Gly-Val-$NHNH_2$ *via* the 4-picolyloxycarbonyl hydrazide	128
$Boc\text{-}(Leu)_n\text{-}OMe$ ($n = 2$—7)	138
$Boc\text{-}(Met)_n\text{-}OMe$ ($n = 2$—7)	136
$Boc\text{-}(Phe)_n\text{-}OMe$ ($n = 3$—9)	137
$Boc\text{-}(Val)_n\text{-}OMe$ ($n = 2$—7)	139, 140
Cyclic hexapeptides containing the Pro-D-Phe sequence	413
Cyclic tetrapeptides	414
Cyclo-(Leu-Trp-Leu-Trp)	415
Cyclo-([^{14}C]Trp-Pro) and cyclo-([^{14}C]Trp-[^{3}H]Pro)	416
Cyclo-(Val-D-hexahydromandelic acid)$_3$	417
Cys-Ala-Gly ⌜——⌝ Cys-aminoacyl-Cys-OMe	101 418
⌜——⌝ $Cys\text{-}(Gly)_n\text{-}Cys\text{-}OMe$ ($n = 0$—5), -OHC ($n = 0$—4), and related peptides	419
Diaminobutyric acid- and glycine-containing peptides	420

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Peptide	*Ref.*
Di- and tri-peptides of L-Dopa*	421
Di- and tri-peptides of His and Gly	422
Di- and tri-peptides synthesized using a polymeric HONSu derivative	126
Di- and tri-peptides synthesized using phosphines and CCl_4 or CBr_4	74
Di- and tri-peptidyl derivatives of amino-indane	423
Dipeptide esters (and their interaction with DNA)	424
Dipeptide esters (and their interaction with human serum albumin)	425
Dipeptides containing proline and a dehydro-amino-acid	426
Dipeptides of aminophosphonic acids	219
Dipeptide stationary phases for g.l.c.	427
DNA-stabilizing peptides	428
[^{13}C]-Enriched peptides of Val, Pro, and Gly	429
Estrone-Tyr_3-OMe	430
Four tetrapeptides containing Tyr and Gly	431
Gln-Gln-Gly-Gly-Tyr-NH_2	107
$(Glu[Bzl(NO_2)])_n$ (n = 2 or 3)	432
Glu-Phe-Ala-Ala-Glu-Glu-(Phe or Ala)-Ala-Ser-Phe as substitute glycosidase 'enzymes'	214
γ-Glutamyl dipeptides of methionine and S-substituted cysteine	433
Glycopeptides	434
Gly-Gly substituted in a variety of ways with ^{15}N and ^{2}H	435
Gly_2-(Met, Pro, Arg, or Lys)-Gly_2	436
Gly-Val-Gly-Ala-Pro and related 'failure' sequences	105
Gly-Val-Ser-DL-Phe-Val-Leu	437
Hydroxyamino-acid glycodipeptides	438
Leu-Ala-Gly-Gly-Val (by-product in the synthesis of Leu-Ala-Gly-Val by solid-phase)	115
Leu-Ala-Gly-Val using 4-pyridinaldoxime esters	56
Lys_n-Ala_3-Lys(Dnp) (n = 1—10)	439
Lys-Ala-Lys-OH, -OMe, and Lys-Trp-OMe	440
Lys-Phe(4-F) and Lys-Lys-Phe(4-F)	441

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* β-(3,4-Dihydroxyphenyl)alanine.

Peptide	*Ref.*
D- and L-Methionine and alanine di- and tri-peptide esters	442
Nucleoside dipeptide	443
Peptides containing allothreonine	444
Peptides containing Lys, Ser, and Gly	445
Peptides containing Met and Gly	446
Peptides incorporating anthranilic acid	109
Peptides incorporating $\alpha\beta$-dehydroamino-acids	218
Peptides of serine and purinyl- and pyrimidyl-DL-alanine	447
Peptides synthesized by solid-phase	119
Peptides with Trp and Tyr separated by Ala and Gly 'spacers'	448
Poly-(azetidine-2-carboxylic acid)	142
Poly-[Lys(Me)], -[Orn(Me)], and -[Orn(Et)]	449
Poly-(DL-Lys)-poly-(Val)-poly-(DL-Lys) 'sandwich' polymer	450
Poly-γ-1-naphthylmethyl-L-glutamate	451
Poly-[Orn(Bzl)]	452
Prolyl-amino-acids with partial asymmetric induction of the LD form	453
Protected Leu-Ala-Gly-Val and related peptides	454
Protected Met oligomers and some related diastereoisomeric di- and tri-peptides	455
Radiolabelled peptides as possible precursors of the ergotocine alkaloids	456
N^{ε}-(α-11-E-retinylidene) lysine peptides	457
A series of hexapeptides with Gly_4, Ala_4, and Pro_4 central sequences	458
A series of peptides synthesized using new polymeric active esters	125
Ser-Phe-Pro, Gly-Phe-Ser-Pro-Phe, and Glp-Lys-Trp-Ala-Pro	459
Ser-Pro-Cys-Ser-Glu-Thr-Tyr as an esterase model	460
Tetrapeptides of Tyr and Glu attached to multichain poly-Ala and poly-Pro	461
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Val-Gly-Phe-Ala-Leu-Ala	103, 391

5 Appendix II: A List of Some Useful Synthetic Intermediates Described during 1974

The list has been divided into two sections. The first includes derivatives of the 20 amino-acids of the genetic code which are therefore commonly found in

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natural proteins. The second includes a variety of others which seemed of potential interest to the synthetic peptide chemist, although the selection of compounds for inclusion in both sections – particularly the second – is necessarily subjective.

Melting points and optical rotation (when available) have generally been included in the list. Inclusion of compounds should certainly not be taken as evidence of novelty, although for the most part the compounds described are new.

Abbreviations (see also Vol. 4, p. 450, and the beginning of this volume for abbreviations in common usage).

Fmoc	9-Fluorenylmethyloxycarbonyl
HF-PA	1,1,1,3,3,3-Hexafluoro-2-(*p*-chlorophenoxymethoxy)propyl
Msc	2-(Methylsulphonyl)ethyloxycarbonyl
NOPY	3-(4-Nitro-1-cyclohexyl-2-oxo-3-pyrrolin)yl
OEbz	2-(*N*-Ethyl)carboxamidophenyl (from 2-ethylbenzisoxazolium salt)
OHbz	2-Hydroxy-3-(*N*-ethyl)carboxamidophenyl (from 2-ethyl-7-hydroxybenzisoxazolium salt)
ONBor	*N*-Hydroxy-5-norbornene-2,3-dicarboximido ester
ONg	4-Nitroguiacyl ester
OPAO	4-Pyridinaldoxime ester
OPmp(Ac)	1-Phenyl-3-methyl-4-nitroso-5-acetylaminopyrazole ester
OPmp(Bz)	1-Phenyl-3-methyl-4-nitroso-5-benzoylaminopyrazole ester
OQ(5-Cl)	5-Chloroquinolyl-8-ester
Ppoc	2-Phenylisopropoxycarbonyl
Troc	βββ-Trichloroethyloxycarbonyl

Coded Amino-acids

Compound	*m.p./°C*	$[\alpha]_D$*	*Conc.*	*Solvent*	*Ref.*
Alanine					
Boc-Ala-OPh(2-phenacyloxy)	100—102	−45.2	1	DMF	395*a*
Boc-Ala-OPmp(Ac)	oil	—	—	—	55
Boc-Ala-OPmp(Bz)	121—123	—	—	—	55
Nps-Ala *N*-carboxy-anhydride	170—173	+20.2	2	dioxan	60
Nps-Ala *N*-carboxy-anhydride	147—148	+73.8†	1	C_6H_6	59
[Ph], [Ph(2-OH,4-Cl)]C=Ala	182	−24	0.5	MeOH	25
Z-Ala-OHbz	116—117	−65.4	2	AcOEt	70
Z-Ala-ONp(*o*)	94	−51	2	DMF–AcOH 99 : 1	122
Z-Ala-OPAO	90—97	−31.4	1	EtOH	56
Z-Ala-OPmp(Ac)	oil	—	—	—	55
Z-Ala-OPmp(Bz)	‡	—	—	—	55
Arginine					
Boc-Arg(NO_2)-OPmp(Bz)	108—110	—	—	—	55
Bz-Arg$(Z)_2$	172—173	+20.0	1.5	DMF	463
Tos-Arg(NO_2)	167—169	+23.6	1	MeOH	464
Z-Arg(NO_2)-OPAO	not characterized				230

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* Generally in the temperature range 20—25° C.
† 578 nm.
‡ Given as 144—116.

Coded Amino-acids (*cont.*)

Compound	*m.p.*/°C	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
Z-Arg(Tos) by an improved procedure					38
Z-Arg(Tos)-OPcp	*ca.* 80—90	−2.1	1	$CHCl_3$	38
Z-Arg(Tos)$_2$O$^-$Na$^+$	161—166	—	—	—	38
Z-Arg(Z)$_2$ by an improved procedure					463
Z-Arg(Z)$_2$-OTcp	124—126	−8.6	1	THF	167
Z-D-Arg(NO_2)-OPAO	no data				225
Z(OMe)-Arg(Z)$_2$ by an improved procedure	139—141	+16.6	1.5	$CHCl_3$	463
Z(OMe)-Arg[Z(OMe)]$_2$	125—128	+1.9	1	DMF	463
Asparagine					
A series of β-dicarbonyl N^α-protected derivatives					27
[Ph], [Ph(2-OH,4-Cl)]C=Asn	161	−48	0.5	MeOH	25
Z-Asn-OEbz	137—139	−30.0	3.1	Me_2CO	68
Z-Asn-OHbz	170—171	−32.5	2	DMF	70
Z-Asn-ONg	175—176	−22	2	DMF	57
Z-Asn-ONp(*o*)	162	−42	2	DMF–AcOH 99 : 1	122
Aspartic acid					
Nps-Asp(OBzl) *N*-carboxy-anhydride	132—134	+156.6	2	dioxan	60
Pht-Asp(OMe)-NH_2	167—168	−81	1	DMF	465
Z-Asp(OBzl)-ONp(*o*)	74—75	−32	2	DMF–AcOH 99 : 1	122
Z-Asp(OMe)-NH_2	120—121	+5.3	1	AcOH	465
Z-Asp(OMe)-ONg	92—93	−37	2	DMF	57
Cysteine					
Cys(Acm),H_2O (improved method)	193—195	−43.0	1	H_2O	101
Cys[Bzl(4-Me)]	211—213	—	—	—	30
Fmoc-Cys(Bzl)	118—120	−45.6	1	DMF	10
Nps-Cys(Bzl) *N*-carboxy-anhydride	110—112	+148.5	2	dioxan	60
Nps-Cys(Ec),Dcha	165—166	−22.7	0.5	DMF	41
Nps-Cys(Ec)-ONSu	152	−70.4	1	DMF	41
[Ph], [Ph(2-OH,4-Me)]C=Cys(Bzl)	59	−136	0.5	MeOH	25
Trt-Cys(Thp),Dcha	165—166	−22.7	0.5	DMF	41
Z-Cys(Acm),Dcha	158—161	−25.0	2	MeOH	101
Z-Cys(Bzl)-OHbz	101—103	−48.8	2.3	AcOEt	70
Z-Cys(Bzl)-ONg	109—111	−39	2	dioxan	57
Z-Cys(Bzl)-ONp(*o*)	98—99	−105	2	DMF–AcOH 99 : 1	122
Glutamic acid					
Boc-Glu(OBzl)-OTcp	104—105	—	—	—	388
Glu(OBzl)-OTcp,HCl	—	—	—	—	388
Glu(OMe) *N*-carboxy-anhydride	100	−20.2	1.8	MeCN	255
Nps-Glu(OMe) *N*-carboxy-anhydride	100—102	−27	2	dioxan	60
Z-Glu(OBu^t)-OHbz	159—160	−35.2	2	AcOEt	70

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Coded Amino-acids (*cont.*)

Compound	*m.p./°C*	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
Z-Glu(OBu^t)-ONBor	120—121	+32.0	2	MeOH	54
Z-Glu(OBu^t)-OPmp(Bz)	oil	—	—	—	55
Z-Glu(OBzl)-ONp(*o*)	83—84	−43	2	DMF–AcOH 99 : 1	122
Z-Glu(OMe)-ONg	73—74	−25	2	DMF	57
Z(OMe)-Glu(OBzl)-NHNHTroc	85—88	−11.2	0.8	DMF	16
Glutamine					
Boc-Gln-OQ(5-Cl)	200—202	−45	1	DMF	112
[Ph], [Ph(2-OH,4-Cl)]C=Gln	107	−43	0.5	MeOH	25
A series of β-dicarbonyl N^{α}-protected derivatives					27
Z-Gln-OHbz	145—146	−43.5	1.7	AcOEt	70
Z-Gln-ONg	154—156	+71	1	DMF	57
Z-D-Gln-ONg	155—156	−72	1	DMF	57
Z-Gln-ONp(*o*)	135—136	−39	2	DMF–AcOH 99 : 1	122
Glycine					
Boc-Gly-OPmp(Ac)	135—136	—	—	—	55
Gly-O$(CH_2)_2$OMe,HCl	107—108	—	—	—	120
Gly-O-(esters with polyethyleneglycol of mol. wt. 2000—20 000)	—	—	—	—	120
NOPY-Gly	185—186	—	—	—	26
Nps-Gly-*N*-carboxy-anhydride	168—170	—	—	—	60
[Ph], [Ph(2-OH,4-Cl)]C=Gly	160	—	—	—	25
Ppoc-Gly	71.5—72.5	—	—	—	32
Ppoc-Gly,Dcha	158.5—159.5	—	—	—	32
Z-Gly-OHbz	121—122	—	—	—	70
Z-Gly-ONBor	114—116	—	—	—	54
Z-Gly-ONp(*o*)	75	—	—	—	122
Z-Gly-OPAO	116—117	—	—	—	56
Z-Gly-OPmp(Bz)	129—130	—	—	—	55
Histidine					
Aoc-His(Tos)	109—111	+10.0	1	pyridine	46
Boc-His(Bzl)-ONp	145	—	—	—	246
Boc-His(Bzl)-ONSu	*ca.* 60	—	—	—	246
Boc-His(Dnp)-ONp	157—158	—	—	—	246
Boc-His(Tos)	*	+15.1	1	MeOH	46
Boc-His(Ztf)-ONp	not cryst.	—	—	—	246
Boc-His(Ztf)-ONSu	*ca.* 85—90	—	—	—	246
Nps-His(Tos)	141—142	+39.9	1	DMF	46
Nps-His(Tos)-OBzl	102—104	+24.8	1	DMF	46
Nps-His(Tos)-OMe	101—103	+34.1	1	DMF	46
[Ph], [Ph(2-OH,4-Cl)]C=His(Bzl)	133	−84	0.5	MeOH	25
Z-His(Boc)-NHNHBoc	65—70	—	—	—	18
Z-His(HF-PA)	no data				44
Z-His(HF-PA)-OMe	56—58	—	—	—	44
Z-His(HF-PA)-ONSu	oil	—	—	—	44
Z-His(Z)-OTcp	109—112	−6.46	1	$CHCl_3$	350
Z(OMe)-His(Tos),Dcha	179.5—180.5	+20.0	1	DMF	46

* Given as 123—115.

Coded Amino-acids (*cont.*)

Compound	*m.p./°C*	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
Isoleucine					
Ile-O-cyclohexyl,HCl	—	—	—	—	427
Ile-ONg,HBr	202—204	−15	1	DMF	57
Nps-Ile *N*-carboxy-anhydride	109—111	+51.3	2	dioxan	60
[Ph], [Ph(2-OH,4-Me)]C=Ile	133	−68	0.5	MeOH	25
Z-Ile-ONBor	105—107	−19.8	2	dioxan	54
Z-Ile-ONg	90.5—92	−16	1	DMF	57
Z-Ile-ONp(*o*)	oil	—	—	—	122
Leucine					
Dnp-Leu *N*-carboxy-anhydride	126—128	+31.3	2	dioxan	60
NOPY-Leu	86—87	+22.4	2	95% EtOH	26
Nps-DL-Leu *N*-carboxy-anhydride	102—104	—	—	—	60
[Ph], [Ph(2-OH,4-Me)]C=Leu	130	−88	0.5	MeOH	25
Z-Leu-ONBor	85—86	−33.2	2	dioxan	54
Z-D-Leu-ONBor	68—71	+31.7	2	dioxan	54
Z-Leu-ONg	68—69.5	−23	2	DMF	57
Z-Leu-ONp(*o*)	87—89	−47	2	DMF–AcOH 99 : 1	122
Z-Leu-OPmp(Bz)	117—119	—	—	—	55
Lysine					
Boc-Lys[Z(2-Cl)]	oil	—	—	—	30
Boc-Lys[Z(NO_2)]-OQ(5-Cl)	125—126	−41.5	1	DMF	112
Bz-Lys(Me,Tos)	118	+1.6	1	AcOH	449
Bz-Lys(Tos)	85	−4.6	1	AcOH	449
Lys(Me,Z)	215—217	+14.0	0.5	AcOH	449
Lys(Z)-OPcp,HCl	146—148	+12.5	—	—	387
Nps-Lys(Z) *N*-carboxy-anhydride	147—149	+50.3	2	dioxan	60
Nps-Lys(Z)-OPcp	118—119	−32.8	—	—	387
Z-Lys(Boc)-ONp(*o*)	76—78	−32	2	DMF–AcOH 99 : 1	122
Z-Lys-OEt,Tos-OH	113—115	−7.8	2	DMF	272
Z(OMe)-Lys(Troc)-OTcp	—	—	—	—	16
Methionine					
Boc-Met-OPmp(Ac)	135—138	—	—	—	55
$CO(Met)_2$	167—168	+4.8	1	MeOH	193
$CO(Met\text{-}ONp)_2$	183—184	−41	0.5	DMF	193
[Ph], [Ph(2-OH,4-Me)]C=Met	120	−93	0.5	MeOH	25
Z-Met-OHbz	106—107	−33.8	2	AcOEt	70
Z-Met-ONp(*o*)	65—67	−56	2	DMF–AcOH 99 : 1	122
Phenylalanine					
Boc-Phe-ONBor	157—158	−20.1	2	dioxan	54
Boc-Phe-OPmp(Ac)	140—142	—	—	—	55
Boc-Phe-OPmp(Bz)	133—135	—	—	—	55
NOPY-Phe	155—156	−164	2	95% EtOH	26
NOPY-DL-Phe	148—149.5	—	—	—	26
Nps-Phe *N*-carboxy-anhydride	162	+130.6*	1	C_6H_6	59

* 578 nm.

Coded Amino-acids (*cont.*)

Compound	*m.p./°C*	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
Nps-Phe *N*-carboxy-anhydride	151—153	+101	2	dioxan	60
Nps-DL-Phe *N*-carboxy-anhydride	143—145	—	—	—	60
Nps-Phe-OTcp	133—135	−70.1	0.5	DMF	317
[Ph], [Ph(2-OH,4-Me)]C=Phe	80	−240	0.5	MeOH	25
Ppoc-Phe	*ca.* 30	—	—	—	32
Ppoc-Phe,Dcha	152.5—153.5	+23.9	1	MeOH	32
Z-Phe-NHNHFor	192—194	−21.7	1	MeOH	197
Z-Phe-OEbz	133—134	−55.5	2.1	DMF	68
Z-Phe-OHbz	93—94	−47.0	2	AcOEt	70
Z-Phe-ONBor	77—79	−17.7	2	dioxan	54
Z-Phe-ONp(*o*)	109—110	−63	2	DMF–AcOH 99 : 1	122
Z-Phe-OPmp(Ac)	oil	—	—	—	55
Z-Phe-OPmp(Bz)	133—135	—	—	—	55
Proline					
Boc-Pro-OPmp(Ac)	oil	—	—	—	55
Boc-Pro-OPmp(Bz)	121—124	—	—	—	55
NO-Pro-ONg	100—102	−51	1	DMF	57
Ppoc-Pro	107—108.5	−33.8	1	MeOH	32
Pro-OMe, bis-(4-tosyl) imide salt	157—158	−12.6	—	MeOH	387
Z-Pro-ONBor	119—121	−53.6	2	dioxan	54
Z-Pro-ONg	110—111	−22	1	DMF	57
Z-Pro-ONp(*o*)	oil	—	—	—	122
Z-Pro-OPmp(Bz)	115—117	—	—	—	55
Serine					
Boc-Ser(Me),Cha	153.4—154.5	+19.8	1	MeOH	221
Boc-Ser-OPmp(Bz)	94—98	—	—	—	55
[Ph], [Ph(2-OH,4-Cl)]C=Ser	154	−32	0.5	MeOH	25
[Ph], [Ph(2-OH,4-Cl)]C=Ser(Bzl)	120	−40	0.5	MeOH	25
Pht-Ser(Me)-OBzl(NO_2)	126.2—126.8	−48.4	1	CH_2Cl_2	221
Pht-Ser-OBzl(NO_2)	179.5—180.8	−46.1	0.8	DMF	221
Ppoc-Ser(Bu^t),Cha	167—169	+37.8	1	MeOH	32
Ser(Me)	197	+17.9	0.4	MeOH	221
Z-Ser(Bu^t)-OPmp(Bz)	112—114.5	—	—	—	55
Z-Ser(Bzl)-ONp(*o*)	55—57	−18	2	DMF–AcOH 99 : 1	122
Z-Ser-OHbz	116—117	−40.2	—	—	70
Z-Ser-OPmp(Bz)	94—98	—	—	—	55
Threonine					
[Ph], [Ph(2-OH,4-Cl)]C=Thr	95	−40	0.5	MeOH	25
Thr(Bzl) by an improved procedure	201—202	−33.29	1	AcOH	97
Z-Thr-OHbz	112—113	−45.7	1.5	AcOEt	70
Z-Thr-OPcp	162—164	−9.3	1	DMF	170
Z(OMe)-Thr-OPcp	142—145	—	—	—	202
Tryptophan					
Boc-Trp-ONBor	159—161	−21.9	2	dioxan	54
[Ph], [Ph(2-OH,4-Cl)]C=Trp	162	−364	0.5	MeOH	25
Z-Trp-ONp(*o*)	116—118	−65	2	DMF–AcOH 99 : 1	122
Tyrosine					
Boc-Tyr(Me),Dcha	151—152	+34.2	0.5	DMF	317

Coded Amino-acids (*cont.*)

Compound	*m.p./°C*	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
Boc-Tyr(Me)-OTcp	109—110	−31.8	0.5	DMF	317
Msc-Tyr(Bu^t)	130	+4.0	1	MeOH	28
		+10.3	1	90% AcOH	
Nps-Tyr(Bzl) *N*-carboxy-anhydride	187—189	+84.5	2	dioxan	60
[Ph], [Ph(2-OH,4-Cl)]C=Tyr(Bzl)	75	−174	0.5	MeOH	25
Tyr(Z)-OBzl,HCl	196–197	−12.23	1	MeOH	421
Z-Tyr(Bzl)-ONg	131	−10	2	dioxan	57
Z-Tyr(Bzl)-ONp(*o*)	137	−55	2	DMF–AcOH 99 : 1	122
Valine					
Boc-Val-OPmp(Bz)	140—141	—	—	—	55
Nps-Val *N*-carboxy-anhydride	147—149	+7.9*	1	C_6H_6	59
Nps-Val *N*-carboxy-anhydride	152—155	+41.5	2	dioxan	60
[Ph], [Ph(2-OH,4-Cl)]C=Val	146	−52	0.5	MeOH	25
Z-Val-OHbz	77—79	−48.5	2	AcOEt	70
Z-Val-ONBor	99—101	−25.2	2	dioxan	54
Z-Val-ONp(*o*)	oil	—	—	—	122
Z-Val-OPmp(Bz)	132—135	—	—	—	55

* 578 nm.

Other Amino-acids

Compound	*m.p./°C*	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
β-Alanine					
β-Ala-ONp,HBr	195—197	—	—	—	268
Pht-β-Ala-ONg	182—184	—	—	—	57
Z-β-Ala-ONBor	125—126	—	—	—	54
α-Amino-isobutyric acid (Aib)					
Nps-Aib *N*-carboxy-anhydride	170—173	—	—	—	60
L-*erythro*-α-Amino-β-phenylbutyric acid (Phabu)					
Z-Phabu	69—72	−16.7	1.8	DMF	241
Azetidine-2-carboxylic acid (Aze)					
Aze-ONSu,HCl	127—129	−44.9*	1	MeOH	142
Aze-OPcp,HCl	134—135	−9.5*	1	MeOH	
Boc-Aze	104—106	−124.6*	1	AcOEt	
Boc-Aze-ONSu	115—116	−117.2*	1	AcOEt	
Boc-Aze-OPcp	98—99	−72*	1	AcOEt	
3,4-Dihydroxyphenylalanine (Dopa)					
Dopa (3-Me,4-Z)-OBzl,HCl	76—80	−12.93	2	MeOH	421
Dopa (Z_2)-OBzl,HCl	114—117	−10.36	1	MeOH	
D-Dopa (Z_2)-OBzl,HCl	107—113	+10.35	1	MeOH	
Z-Dopa (3-Me,4-Z)	126—129	+39.55	2.1	$CHCl_3$	
Z-Dopa (Z_2)	82—84	−0.67	5.5	MeOH	
Z-Dopa (Z_2)-ONp	115—117	−16.80	1	DMF	
Z-D-Dopa (Z_2)	80—85	+1.35	3.3	MeOH	
p-Fluorophenylalanine					
Boc-Phe(F),Dcha	190	+30.0	0.9	DMF	325

* 578 nm.

Other Amino-acids (*cont.*)

Compound	*m.p./°C*	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
5-Fluorotryptophan					
Boc-Trp(5-F)	157—158	−6.1	2.1	AcOH	180
Homoarginine (Har)					
Z-Har(Tos)	55—65	−2.3	2	MeOH	363
Z-Har(Tos),Dcha	155—157	+4.6	4	MeOH	363
ε-Hydroxynorleucine					
Nle(ε-OAc)	amorphous	—	—	—	364
Z-Nle(ε-OAc)	amorphous	—	—	—	364
N-Methylisoleucine					
Boc-MeIle	gum	—	—	—	86
N-Methyl-*allo*-isoleucine					
Boc-D-Me-*a*-Ile	oil	—	—	—	231
Boc-D-Me-*a*-Ile-ONp	oil	—	—	—	231
p-Nitrophenylalanine					
Phe(NO_2)-OMe,HCl	199—202	+20.0	1	MeOH	375
Norleucine					
Boc-Nle-ONp(*o*)	90—92	−61.5	2	DMF–AcOH 99 : 1	123
Nps-Nle,Dcha	196—198	—	—	—	197
Ornithine					
Boc-Orn(=CHPh)-OBu^t	102—103	−17.8	1.90	MeOH	466
Boc-Orn[=CHPh(4-OMe)]-OBu^t	96—97.5	—	—	—	466
Boc-Orn(Z)-OBu^t	oil	−14.66	1.03	MeOH	466
Bz-Orn(Tos)	182	+2.1	1	AcOH	449
Nps-Orn(Z) *N*-carboxy-anhydride	142—144	+37.2	2	dioxan	60
Orn(=CHPh)	200	−8.4	1	H_2O	452
Orn(Z)-OBu^t	no data				466
Z-Orn-OEt, Tos-OH	oil	—	—	—	272
Z-Orn(Pht)-ONg	108.5—109.5	−25	2	DMF	57
Pentamethylphenylalanine					
Boc-Phe(Me_5)	163—165	−9.6	2	MeOH	180
		−27	2	AcOH	
α-Phenylglycine					
Nps-DL-phenylglycine *N*-carboxy-anhydride	132—134	—	—	—	60
Phenylglycine-OBu^t, AcOH	110—112	+55	1	MeOH	229
Z-phenylglycine	128—129	+106	1	MeOH	229
Z-phenylglycine-OBu^t	47—48	+66.7	1	MeOH	229
Pyroglutamic acid					
Glp-ONg	172—176	+13	3	DMF	57
Me-Glp	150—151	−33.6	1.1	H_2O	381
Me-Glp-ONSu	no data				381
Z-Glp-ONBor	144—145	−41.9	0.2	EtOH	54

[466] J. Widmer and W. Keller-Schierlein, *Helv. Chim. Acta*, 1974, **57**, 657.

4

Peptides with Structural Features not Typical of Proteins

BY B. W. BYCROFT

1 Introduction

An increasing number of peptides with novel features continue to be isolated from both micro-organisms and plants. The past year has seen a considerable increase in the number of the so-called peptide alkaloids isolated and characterized. The diversity of structural units, found predominantly in cyclic dipeptides, which arise from the interaction of a C_5 unit with tryptophan has been further demonstrated. The occurrence of such a unit in the cyclic heptapeptide ilamycin B_1 is of considerable interest.

Once again considerable effort has centred on conformational studies of both natural and synthetic homodetic and heterodetic peptides both in solution and in the crystal lattice. The most notable synthetic achievement in terms of biologically active peptides has been that of malformin A. In addition, the important synthesis of tentoxin offers a new approach to naturally occurring peptides containing a dehydroamino-acid residue.

The penicillin–cephalosporin field has witnessed a major development, namely the application of total synthesis as a viable alternative to modification of naturally occurring β-lactams for the formation of useful antibiotics. Investigations on the structural and biosynthetic aspects of peptidoglycan from bacterial cell walls have consolidated the general structural theories relating to these complex molecules.

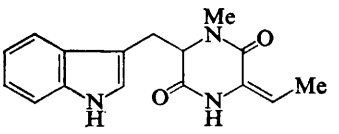
2 Cyclic Peptides (Homodetic Peptides)

2,5-Dioxopiperazines (Cyclic Dipeptides).—Formation of 2,5-dioxopiperazines on the enzymatic hydrolysis of proteins is well established, and it now appears that some of these compounds may be responsible for the bitter taste of aged beverages and foodstuffs. *cyclo*-L-Prolyl-L-leucine is responsible for the bitterness of aged sake,[1] while the bitter principle of casein hydrolysate has been identified as *cyclo*-L-leucyl-L-tryptophan.[2] A number of cyclodipeptides containing either L-leucine or L-tryptophan have been prepared and all shown to possess varying degrees of bitterness.

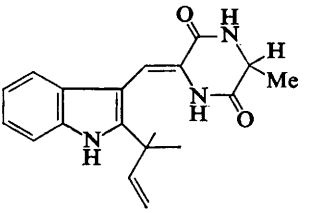
The tryptophan-containing dioxopiperazine (1) is a minor component of the streptovaricin complex[3] and shows weak activity against RNA-directed DNA

[1] K. Takahashi, M. Tademima, K. Kitamoto, and S. Sato, *Agric. and Biol. Chem. (Japan)*, 1974, **38**, 927.
[2] T. Shita and K. Nunami, *Tetrahedron Letters*, 1974, 509.
[3] K. Kakinuma and K. L. Rinehart, jun., *J. Antibiotics*, 1974, **27**, 733.

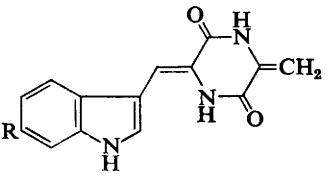
polymerase (reverse transcriptase). Three new prenylated cyclodipeptides (2)—(4) all containing dehydrotryptophan residues have been isolated from *Aspergillus amstelodamis*.[4] The diprenylated compound (4) was isolated independently from the same organism during a biosynthetic investigation on the



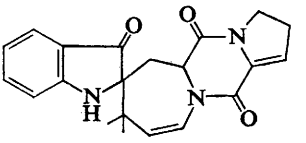
(1)



(2)



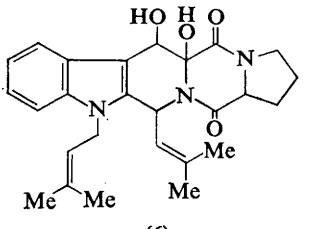
(3) R = H
(4) R = $Me_2C{=}CHCH_2$



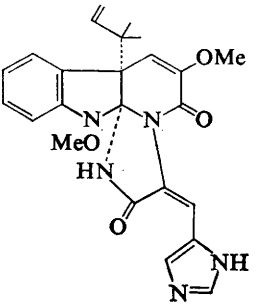
(5)

echinulin group.[5] There is strong evidence to suggest that introduction of the C_5 units and subsequent oxidation steps occur on a preformed cyclodipeptide both for the echinulin group[6] and the related brevianamides. It has been demonstrated[7] that *cyclo*-L-tryptophyl-L-proline is incorporated efficiently into brevianamide A (5) by *Penicillium brevicompactum*.

The tremorgenic toxin fumitremorgin A (6) represents a further example of this interesting group of metabolites. The gross structure was proposed on the analysis of spectral data, but no stereochemical assignments have yet been



(6)

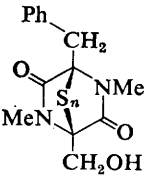


(7)

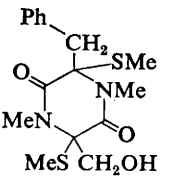
[4] A. Dossena, R. Marchelli, and A. Pochini, *J.C.S. Chem. Comm.*, 1974, 771.
[5] R. Cardillo, C. Fuganti, G. Gatti, D. Ghiringhelli, and P. Grasselli, *Tetrahedron Letters*, 1974, 3163.
[6] C. M. Allen, jun., *Biochemistry*, 1972, **11**, 2154.
[7] J. Baldas, A. J. Birch, and R. A. Russell, *J.C.S. Perkin I*, 1974, 50.

advanced.[8] The structure of oxaline (7) from *P. oxalicum* rests firmly on an *X*-ray crystallographic analysis,[9] and although it is not strictly a cyclodipeptide the relationship to the echinulin and brevianamide groups, as well as the occurrence of a dehydrohistidine residue, is noteworthy. Model chemical reactions for the introduction of a 1,1-dimethylallyl group into the 2-position of a tryptophan system which may be significant in relation to the biogenesis of these compounds have been reported.[10] The presence of a *N*-1,1-dimethylallyltryptophan residue in the cyclohexapeptide ilamycin (see below) appears to be the only example of this system.

Two further epithiodioxopiperazines (8) and (9) with antiviral activity have been isolated from *P. turbatum*.[11] The structure of (8) has been defined by an



(8) $n = 2$
(9) $n = 4$



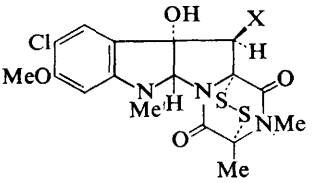
(10)

X-ray analysis and is enantiomeric with hyalodendrin (described in last year's report). In addition, the organism produces (10) which is biologically inactive. Gliovictin is a metabolite of *Helminthosporium victoriae*, and has been reported, on the basis of instrumental analysis, to possess the same structure.[12] A direct comparison of the two compounds has not been made but the physical data described strongly suggest that they are identical.

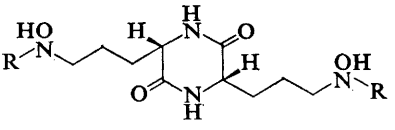
Total syntheses of racemic hyalodendrin (8)[13] and sporidesmin B (11)[14] have extended the utility of the general route already developed for the synthesis of epidithiodioxopiperazine antibiotics. The addition of thioacetic acid across the double bonds of dehydrocyclodipeptides affords the β-addition products, but in the presence of strong acid the α-adducts are formed in quantitative yield,[15] thus providing a convenient alternative for introduction of sulphur into cyclodipeptides. Biosynthetic investigations have so far indicated that antibiotics of this general type are formed by modification of the corresponding cyclodipeptide. The feeding of tryptophan stereoselectively labelled in the β-methylene group has shown that the side-chain hydroxylation in the biosynthesis of sporidesmin A (12) proceeds with overall retention of configuration.[16]

[8] M. Yamazaki, K. Sasago, and K. Miyaki, *J.C.S. Chem. Comm.*, 1974, 408.
[9] D. W. Nagel, K. G. R. Pachler, P. S. Steyn, P. L. Wessels, G. Gafner, and G. J. Kruger, *J.C.S. Chem. Comm.*, 1974, 1021.
[10] G. Casnati, R. Marchelli, and A. Pochini, *J.C.S. Perkin I*, 1974, 754.
[11] K. H. Michel, M. O. Chaney, N. D. Jones, M. M. Hoehn, and R. Nagarajan, *J. Antibiotics*, 1974, **27**, 57.
[12] F. Dorn and D. Arigoni, *Experientia*, 1974, **30**, 135.
[13] G. M. Strunz and M. Kakushima, *Experientia*, 1974, **30**, 719.
[14] S. Nakatsuka, T. Fukuyama, and Y. Kishi, *Tetrahedron Letters*, 1974, 1549.
[15] P. J. Machin and P. G. Sammes, *J.C.S. Perkin I*, 1974, 698.
[16] G. W. Kirby and M. J. Varley, *J.C.S. Chem. Comm.*, 1974, 833.

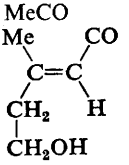
Two syntheses [17, 18] of the bacterial growth factor rhodotorulic acid (13) have appeared. The first employs a benzyl group for protecting the hydroxamic acid function,[17] whereas the second starts from 5-nitro-L-norvaline and has been extended [18] to include a synthesis of dimerumic acid (14).



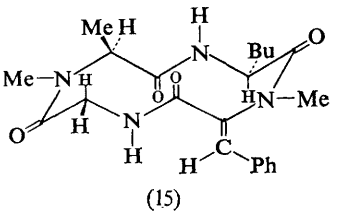
(11) X = H
(12) X = OH



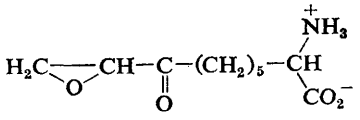
(13) R = MeCO
(14) R = Me



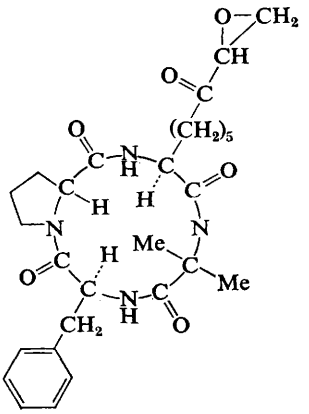
Naturally Occurring Cyclic Peptides.—In this section only those peptides are included for which firm structural assignments, or corrections to already proposed structures, have been made. The amino-acid sequence of the phytotoxic cyclic tetrapeptide from *Alternaria tenuis* has been revised [19] and the new structure (15) confirmed by an *X*-ray analysis of the dihydro-derivative [20] and by a total



(15)



(17)



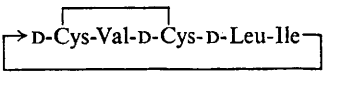
(16)

synthesis.[21] Chlamydocin (16), a metabolite of *Diheteraspora chlamydosporia* with pronounced cystostatic activity, is also a cyclic tetrapeptide containing the unusual amino-acids L-α-aminoisobutyric and D-proline, as well as the previously

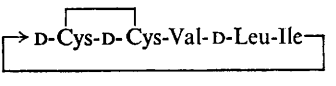
[17] T. Fujii and Y. Hatanaka, *Tetrahedron*, 1973, **29**, 3825.
[18] J. Widmer and W. Keller-Schierlein, *Helv. Chim. Acta*, 1974, **57**, 1904.
[19] W. L. Meyer, L. F. Kuyper, R. B. Lewis, G. E. Templeton, and S. H. Woodhead, *Biochem. Biophys. Res. Comm.*, 1974, **56**, 234.
[20] W. L. Meyer, L. F. Kuyper, D. W. Phelps, and A. W. Cordes, *J.C.S. Chem. Comm.*, 1974, 339.
[21] D. H. Rich and P. Mathiaparanam, *Tetrahedron Letters*, 1974, 4037.

unknown L-amino-acid (17). The structure of this compound has been confirmed by synthesis.[22]

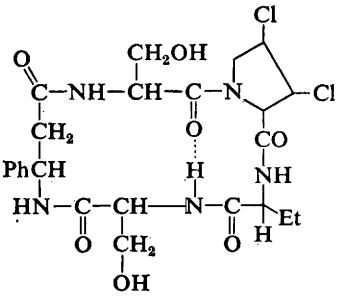
The structure (18) proposed for malformin A some 10 years ago has been critically re-examined in an important investigation which offers a salutary lesson for all those working on biologically active peptides. On the basis of



(18)



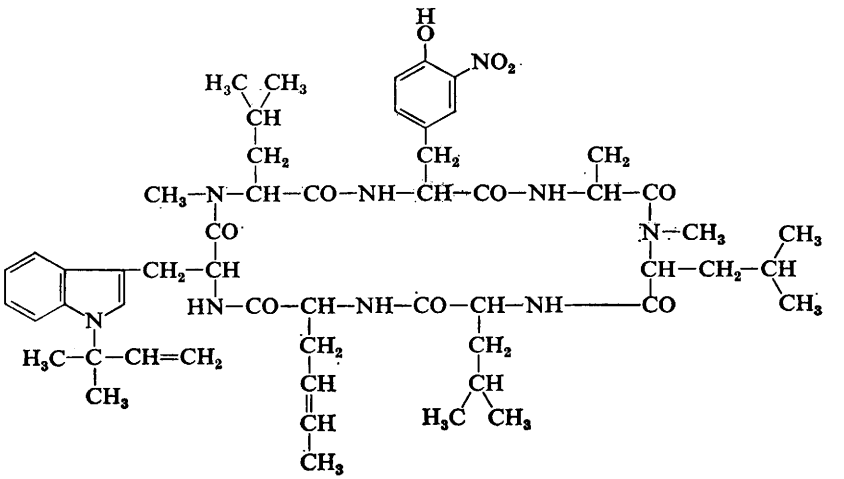
(19)



(20)

chemical degradation, an alternative structure (19) was established and confirmed by total synthesis. The synthetic product had physical, spectral, and biological properties identical with those of the natural compound.[23]

X-Ray crystallographic analyses have established the structures of the cyclic pentapeptide cyclochlorotine (20), which contains a L-dichloroproline residue,[24] and of the cyclic heptapeptide antibiotic ilamycin B_2 (21) (Figure 1).[25] The structure (21), in which all the amino-acids possess the L-configuration, accords



(21)

[22] A. Closse and R. Huguenin, *Helv. Chim. Acta*, 1974, **57**, 533.
[23] M. Bodanszky and G. L. Stahl, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 2791.
[24] H. Yoshioka, K. Nakatso, M. Sato, and T. Talsumo, *Chem. Letters*, 1973, 1319.
[25] Y. Iitaka, H. Nakamura, K. Takada, and T. Takita, *Acta Cryst.*, 1974, **B30**, 2817.

with that originally proposed on the basis of chemical investigations.[26] The presence of the 1-(1,1-dimethylallyl)-tryptophan residue is unique to this class of peptide antibiotics. The crystal conformation is also significant with two *cis* amide linkages associated with the *N*-methyl amino-acid residues and two

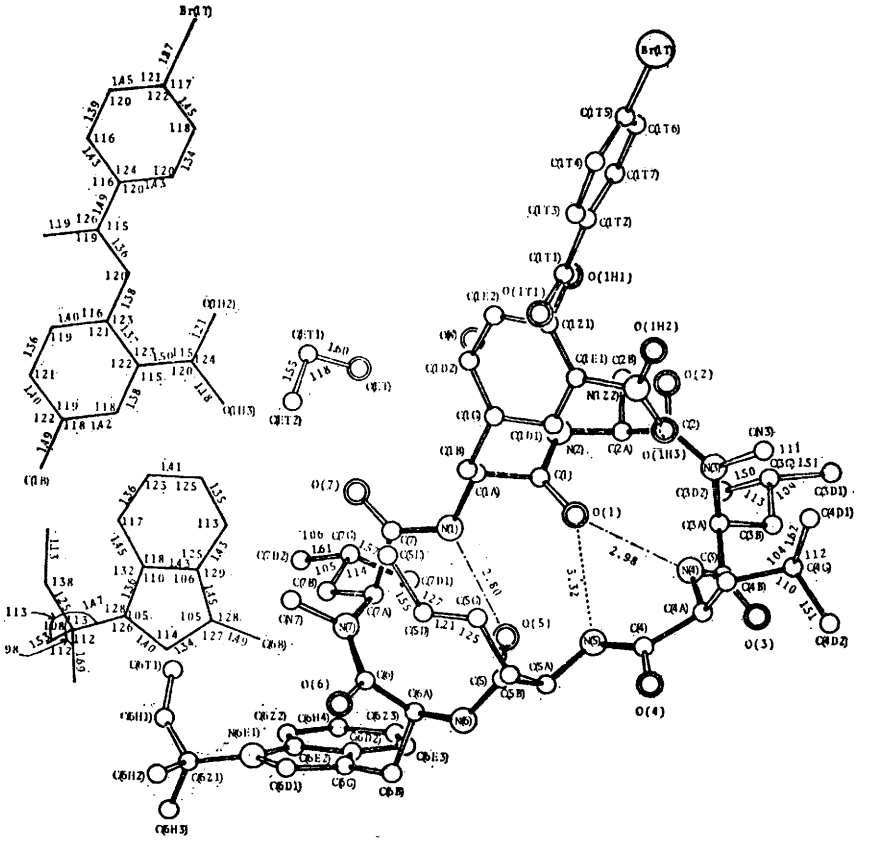
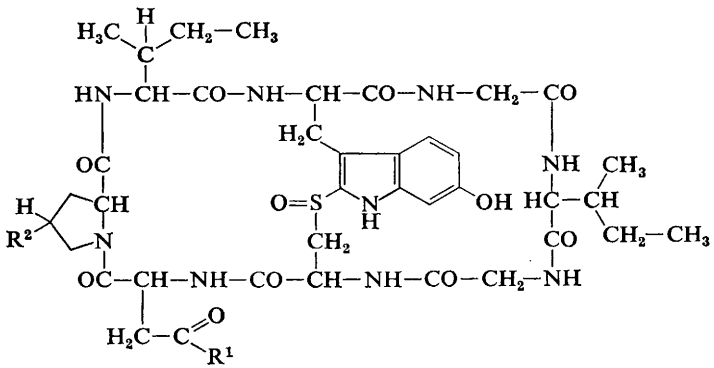


Figure 1 *The crystal structure of ilamycin* p*-bromobenzoate. The numbering of the atoms and the bond lengths and angles in the side-chains are shown*
(Reproduced by permission from *Acta Cryst.*, 1974, **B30**, 2817)

transannular hydrogen bonds. These observations compare favourably with the data obtained from ^{1}H n.m.r. studies on the solution conformation.

Cyclic Peptides from *Amanita phalloides*.—Once again the toxic and non-toxic peptides from the green deathcap toadstool have attracted considerable attention. Chromatography of a methanolic extract of *Amanita phalloides* on Sephadex LH-20 has led to the isolation of two minor components, proamullin (22) and amanullinic acid (23). The structures of these non-toxic compounds are based on spectral and degradative evidence, in addition to comparisons with the

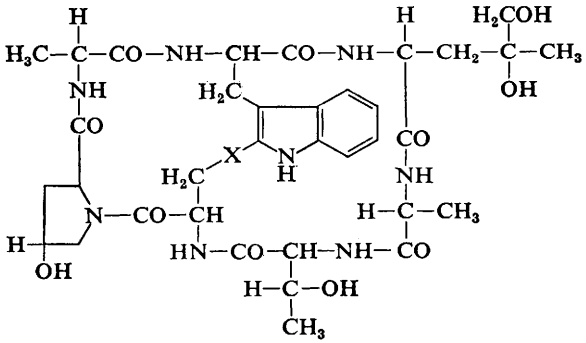
[26] T. Takita, H. Naganawa, and H. Umezawa, *J. Antibiotics*, 1964, **A17**, 264.



(22) $R^1 = NH_2$, $R_2 = H$
(23) $R^1 = OH$, $R^2 = OH$
(24) $R^1 = NH_2$, $R^2 = OH$

known peptide amanullin (24). Two further neutral toxic derivatives, probably esters of β-amanitin, were isolated in insufficient quantity for complete characterization.[27]

Oxidation of phalloidin (25) with hydrogen peroxide in acetic acid yields two diastereoisomeric sulphoxides (26) and (27). One of the sulphoxides is as toxic



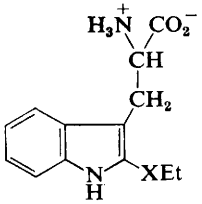
(25) X = S
(26) X = SO(*R*)
(27) X = SO(*S*)

as the parent compound, whereas the other is considerably less toxic. In order to determine the relative chirality at the sulphur atom the model sulphoxides (28) and (29) were prepared and the diastereoisomers separated. An *X*-ray analysis of (28) established the *R*-configuration at the chiral sulphur, and by comparison of the o.r.d. curves the toxin phalloidin sulphoxide (26) can be assigned the same configuration.[28] Similar o.r.d. comparisons with the amanitins allowed assignments of the *R*-configuration to this series of toxins. The *S*-sulphoxide of

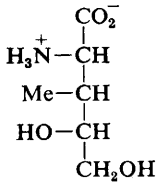
[27] A. Buku and T. Wieland, *Annalen*, 1974, 1587.
[28] T. Wieland, M. P. Jordan de Urries, H. Indest, A. Gieren, M. Sturm, and W. Hoppe, *Annalen*, 1974, 1570.

O-methyl-α-amanitin shows virtually no toxicity but on further oxidation is converted into the toxic sulphone.[29]

Further fine detail of the stereochemistry of the amanitin group stems from an *X*-ray analysis on the lactone of the γ-hydroxyisoleucine residue from γ-amanitin. The structure (30), which shows both the absolute and relative chirality, has



(28) X = SO(*R*)
(29) X = SO(*S*)



(30)

revealed a different configuration at C-4 than formerly assumed.[30] Since the corresponding dihydroxyisoleucine of α- and β-amanitin has been converted into (30) the same stereochemical conclusions can be drawn for this residue.

Antamanide readily forms complexes with alkali-metal ions. The crystal and solution structures of the lithium complex and the sodium complex of [Phe[4],Val[6]]-antamanide have been compared.[31] The details of the former structure (Figure 2) reveal two intra-ring hydrogen bonds, as well as four Li—O ligands.[32] The two Pro-Pro amide linkages are in a *cis* conformation and the polar residues of the molecule are turned towards the interior, while the exterior surface contains the hydrophobic side-groups. Details of the solution conformation of antamanide metal complexes have been deduced using temperature jump relaxation techniques and ultrasonic absorption.[33]

Synthetic analogues of antamanide and retroantamanide (see Table 1, p. 333) have provided further valuable information on the conformational requirements for biological activity. It appears that the sequence of two prolines in positions 7 and 8 is a prerequisite for biological activity.[34]

Gramicidins and Related Peptides.—The general sequence and mechanism of non-ribosomal polypeptide synthesis are now fairly well understood and the present state of knowledge has been reviewed.[35, 36] Microbiological studies on the formation[37] and properties[38] of the gramicidin S synthetases are advancing well and it is of considerable general interest that high productivity fermentations

[29] A. Buku, R. Altmann, and T. Wieland, *Annalen*, 1974, 1580.
[30] A. Gieren, P. Narayanan, W. Hoppe, M. Hasan, K. Michl, T. Wieland, H. O. Smith, G. Jung, and E. Breitmaier, *Annalen*, 1974, 1561.
[31] D. J. Patel and A. E. Tonelli, *Biochemistry*, 1974, **13**, 788.
[32] I. L. Karle, *J. Amer. Chem. Soc.*, 1974, **96**, 4000.
[33] W. Burgermeister, T. Wieland, and R. Winkler, *European J. Biochem.*, 1974, **305**, 311.
[34] T. Wieland, C. Birr, W. Burgermeister, P. Trietsch, and G. Rohr, *Annalen*, 1974, 24.
[35] S. G. Laland and T. L. Zimmer, *Essays in Biochemistry*, 1973, **9**, 31.
[36] F. Lipmann, *Accounts Chem. Res.*, 1973, **11**, 361.
[37] C. C. Matteo, M. Glade, A. Tanaka, J. Piret, and A. L. Demain, *Biotechnol. and Bioeng.*, 1975, **17**, 129.
[38] K. Shimura, M. Iwaki, M. Kanda, K. Hori, E. Kaji, S. Hasegawa, and Y. Saito, *Biochim. Biophys. Acta*, 1974, **338**, 577.

for gramicidin S synthetases have been developed.[39] The object of the investigation is to provide biocatalyst for preparative scale production of gramicidin. The implications of this work to the production of antibiotics in general are obvious. Further details of the mechanism have been clarified using mutant strains of *Bacillus brevis*,[40] and the transfer of the thioester-bound D-phenylalanine from the light enzyme of the synthetase to the acceptor site on the heavy

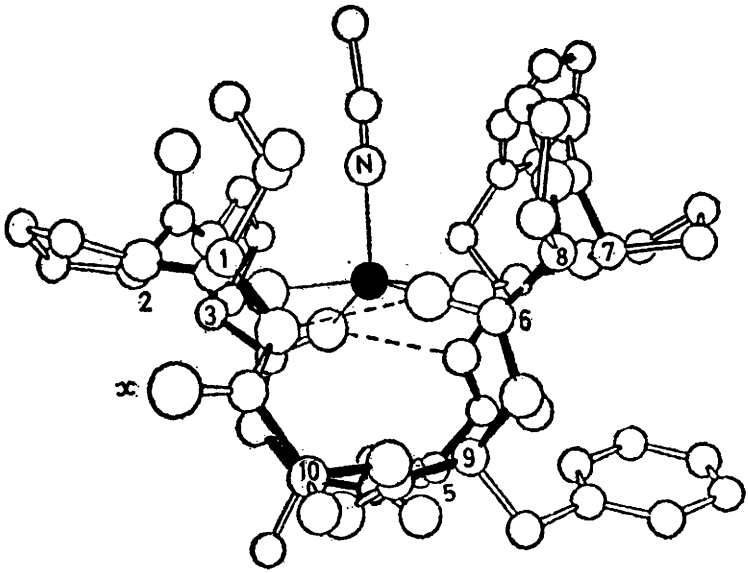


Figure 2 *Diagram of the lithium antamamide complex*
(Reproduced by permission from *Biochemistry*, 1974, **13**, 788)

chain has been investigated by means of affinity chromatography.[41] The isolation of a peptidyl pantotheine protein has established the involvement of pantotheine in the tyrocidine biosynthesis.[42]

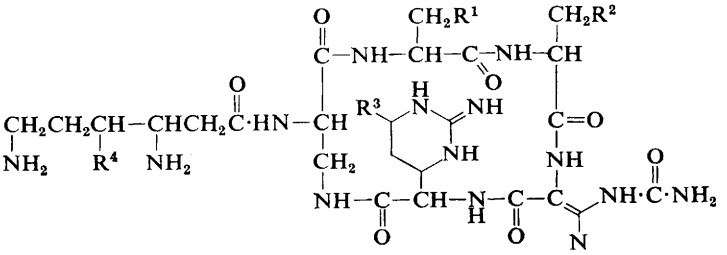
The first completely unambiguous assignment of the ^{13}C n.m.r. spectrum of a cyclic peptide has been achieved with gramicidin S-A by selective biosynthetic enrichment. The assignments accord well with those previously made based on Grant's rule and comparisons with the individual amino-acids. In addition the amide carbonyls can be assigned, a task difficult to accomplish by other methods.[43] Phosphorescence studies on the related antibiotics tyrocidine B and C taking advantage of the presence of the tryptophan residues has led to useful data concerning the emission characteristics of these residues which probably reflects their microenvironment.[44]

[39] C. H. Tzeng, K. D. Thrasher, J. P. Montgomery, B. K. Hamilton, and D. I. C. Wang, *Biotechnol. and Bioeng.*, 1975, **17**, 143.
[40] M. Kambe, Y. Imae, and K. Kurahashi, *J. Biochem. (Japan)*, 1974, **75**, 481.
[41] L. Pass, T. L. Zimmer, and S. G. Laland, *European J. Biochem.*, 1974, **47**, 607.
[42] S. G. Lee and F. Lipmann, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 607.
[43] J. A. Sogn, L. C. Craig, and W. A. Gibbons, *J. Amer. Chem. Soc.*, 1974, **96**, 3306.
[44] C. F. Beyer, W. A. Gibbons, L. C. Craig, and J. W. Longworth, *J. Biol. Chem.*, 1974, **249**, 3204.

The linear polypeptide gramicidin A facilitates the passive diffusion of alkali cations and hydrogen ions through natural and artificial lipid bilayer membranes. The temperature dependence of these channels[45] and the cation-binding properties[46] have been studied. Extensive spectroscopic investigations[47–50] on gramicidin A have provided considerable information on the conformers in both the crystal lattice and in solution, but it is suggested that the molecule exists predominantly as helical dimer in methanol solution.

Synthetic investigations (see Table 1, p. 333) in the gramicidin field continue to explore the relationships between antimicrobial activity and the general structural features of the molecule. Large ring analogues designated 'iso-gramicidins' in which the ω-amino-group of ornithine or lysine forms the ring amide linkage have been prepared[51] and shown to be biologically inactive. Other studies have attempted to define the contribution of the intramolecular hydrogen bonds to the antimicrobial activity and analogues containing *N*-methyl-leucine instead of leucine exhibit activity comparable with that of the parent compound.[52] An alternative approach has involved systematically replacing the valine and leucine residues with L-α-hydroxyisovaleric acid to give depsipeptide analogues.[53] These molecules, which lack the intramolecular hydrogen bonds in varying degrees, were either weakly active or totally inactive.

Viomycin and Related Cyclic Peptides.—It now appears that a closely related family of tuberculostatic antibiotics with the general structure illustrated (31)



(31)

viomycin	$R^1 = OH, R^2 = OH, R^3 = OH, R^4 = H$
tuberactinomycin A	$R^1 = OH, R^2 = OH, R^3 = OH, R^4 = OH$
tuberactinomycin N	$R^1 = OH, R^2 = OH, R^3 = H, R^4 = OH$
capreomycin 1A	$R^1 = OH, R^2 = NH_2, R^3 = H, R^4 = H$
capreomycin 1B	$R^1 = H, R^2 = NH_2, R^3 = H, R^4 = H$

[45] E. Bamberg and P. Laeager, *Biochim. Biophys. Acta*, 1974, **367**, 127.
[46] S. R. Byrn, *Biochemistry*, 1974, **13**, 5186.
[47] K. J. Rothschild and H. E. Stanley, *Science*, 1974, **185**, 616.
[48] W. R. Veatch, E. T. Fossel, and E. R. Blout, *Biochemistry*, 1974, **13**, 5249.
[49] W. R. Veatch and E. R. Blout, *Biochemistry*, 1974, **13**, 5257.
[50] E. T. Fossel, W. R. Veatch, Yu. A. Ovchinnikov, and E. R. Blout, *Biochemistry*, 1974, **13**, 5264.
[51] H. Takiguchi and Izumiya, *Bull. Chem. Soc. Japan*, 1974, **47**, 221.
[52] H. Sugano, H. Abe, M. Miyoshi, T. Kato, and N. Izumiya, *Bull. Chem. Soc. Japan*, 1974, **47**, 698.
[53] A. L. Zhuze, G. A. Kogan, N. A. Krit, T. M. Andronova, M. P. Filatova, L. B. Senyavina, E. A. Meshcheryakova, I. D. Ryabova, G. A. Ravdel, and L. A. Shchukina, *Mol. Biol. Moscow*, 1974, **8**, 84.

are produced by various *Streptomyces*. The Reporter must declare his involvement in this area and point out that the capreomycin group also probably possesses this general structure.[54]

The absolute configuration of the γ-hydroxy-β-lysine present in tuberactinomycin A and N has been assigned the L-threo configuration[55] and a total synthesis of this unusual amino-acid reported.[56] Biosynthetic studies on viomycin indicate that the β-lysine carbon skeleton is derived from lysine without randomization[57] and that the basic guanidine amino-acid is derived from arginine.[58] As yet there is no information relating to the origin of the chromophore.

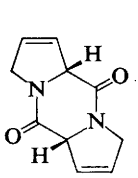
Conformational Studies on Homodetic Peptides.—A general paper offers a review of the integrated conformational studies conducted on cyclopeptides by the Russian school over the past 10 years.[59] A further review from the same group is limited to the application of n.m.r. techniques and represents a valuable résumé of the present state of the art.[60] N.m.r. methods still dominate as the most generally useful technique for the determination of solution conformation, and yet another refinement of the Karplus relation for the dependence of peptide vicinal $NH—CH_\alpha$ coupling constant on the corresponding dihedral angle has been reported.[61] ^{13}C N.m.r. studies continue to increase in number and relevant articles on amino-acids,[62] peptide carbonyl assignment,[63] lanthanide shifts,[64] and the use of ^{13}C–^1H spin coupling for side-chain conformations[65] have appeared.

It is now generally assumed that for cyclodipeptides containing an amino-acid with an aromatic side-chain, such as phenylalanine, tyrosine, or tryptophan, the preferred solution conformation is the one in which the aromatic ring is folded over the dioxopiperazine ring. Conformational energy calculations for *cyclo*-(L-Ala-L-His) indicate that when the imidazole ring is uncharged the most stable conformation is that already described. However, on protonation electrostatic effects become significant and the folded conformer is destabilized relative to other possible conformations.[66] A recent ^1H n.m.r. study on *cyclo*-(L-Thr-L-His) suggests that when the imidazole is uncharged the folded form of the L-histidine residue predominates but is not unique.[67] The folded form of the tryptophan-containing cyclodipeptides *cyclo*-(Gly-L-Trp) and *cyclo*-(L-Ala-L-Trp) appear,

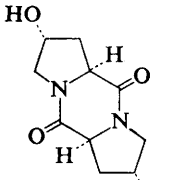
[54] B. W. Bycroft, D. Cameron, L. R. Croft, A. Hassanali-Walji, A. W. Johnson, and T. Webb, *Nature*, 1971, **231**, 301.
[55] T. Wakamiya and T. Shiba, *J. Antibiotics*, 1974, **27**, 901.
[56] T. Wakamiya, T. Teshima, I. Kubota, and T. Shiba, *Bull. Chem. Soc. Japan*, 1974, **47**, 2292.
[57] J. H. Carter, R. H. Du Bus, J. R. Dyer, J. C. Floyd, K. C. Rice, and P. D. Shaw, *Biochemistry*, 1974, **13**, 1227.
[58] J. H. Carter, R. H. Du Bus, J. R. Dyer, J. C. Floyd, K. C. Rice, and P. D. Shaw, *Biochemistry*, 1974, **13**, 1221.
[59] Yu. A. Ovchinnikov and V. T. Ivanov, *Tetrahedron*, 1974, **30**, 1871.
[60] V. F. Bystrov, S. L. Portnova, T. A. Balashoya, S. A. Koz'min, Y. D. Gavrilov, and V. A. Afanasev, *Pure Appl. Chem.*, 1973, **36**, 19.
[61] M. T. Lung, M. Marraud, and J. Well, *Macromolecules*, 1974, **7**, 606.
[62] D. A. Torchia, J. R. Lyerla, jun., and C. M. Deber, *J. Amer. Chem. Soc.*, 1974, **96**, 5009.
[63] J. Feeney, P. Partington, and G. C. K. Roberts, *J. Magn. Resonance*, 1974, **13**, 268.
[64] H. Kessler and M. Molter, *Angew. Chem.*, 1974, **86**, 553.
[65] J. Feeney, P. E. Hansen, and G. C. K. Roberts, *J.C.S. Chem. Comm.*, 1974, 465.
[66] P. E. Grebow and T. M. Hooker, jun., *Biopolymers*, 1974, **13**, 2349.
[67] M. Ptak and A. Heitz, *Org. Magn. Resonance*, 1974, **6**, 358.

on the basis of time-resolved u.v. spectroscopic studies, to be much less stable in the excited state than in the ground state.[68]

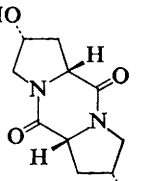
The amino-acid 3,4-dehydro-DL-proline readily cyclizes to give only the *cis*-dioxopiperazine (32). The structure and crystal conformation of this compound follow from an *X*-ray analysis.[69] The dioxopiperazine ring occurs in a folded conformation with a dihedral angle of 139°. The relative thermodynamic stabilities of the isomers (33) and (34), which are presumably dependent on



(32)



(33)



(34)

conformation factors, have been exploited [70, 71] in a conversion of D-aHyp into L-Hyp.

Previous n.m.r. studies on cyclic tripeptides have provided evidence that the peptide units are *cis* planar with the exception of proline which appears to be *cis* but non-planar. Recent crystallographic analysis of *cyclo*-(L-Pro)$_3$ and *cyclo*-(L-Pro-L-Hyp-L-Hyp) (Figure 3) have established that all three peptide bonds are *cis* and that there is no excessive deviation from planarity.[72] In addition, new conformers were observed for the proline rings different from any of those reported before in *X*-ray or n.m.r. studies.

Solution n.m.r. studies on *cyclo*-(L-Pro-Gly)$_2$ and related cyclotetrapeptides are in accord with a *cis-trans-cis-trans* peptide backbone, but there is also evidence for conformers due to slow rotation about the proline C_α—C=O single bond.[73] Vibrational analysis of *cyclo*-(Gly)$_4$ employing the Urey–Bradley force field approach provides conformation data comparable with those available from related molecules.[74]

A number of cyclic hexapeptides containing the L-Pro-D-Phe sequence have been prepared.[75] Detailed conformational studies suggest two preferred conformers both with average C_2 symmetry and containing *trans*- and *cis*-proline peptide bonds, respectively. The capacity for enantiomeric differentiation between D- and L-amino-acid salts with *cyclo*-(L-Pro-Gly)$_n$ peptides (n = 3 or 4) has been observed. A structure for the complexes based on ^{13}C n.m.r. data and model building is shown schematically in Figure 4. The alkylammonium NH protons of the salt are positioned into a peptide-binding cavity consisting of four glycine carbonyl groups.[76]

[68] B. Donzel, P. Gauduchon, and Ph. Wahl, *J. Amer. Chem. Soc.*, 1974, **96**, 801.
[69] I. L. Karle, H. C. J. Ottenheym, and B. Witkop, *J. Amer. Chem. Soc.*, 1974, **96**, 539.
[70] C. Eguchi and A. Kakuta, *Bull. Chem. Soc. Japan*, 1974, **49**, 2277.
[71] C. Eguchi and A. Kakuta, *J. Amer. Chem. Soc.*, 1974, **96**, 3985.
[72] G. Kartha, G. Ambady, and P. V. Shankar, *Nature*, 1974, **247**, 205.
[73] C. M. Deber, E. T. Fossel, and E. R. Blout, *J. Amer. Chem. Soc.*, 1974, **96**, 4015.
[74] H. S. Randhawa, N. V. R. Reddy, and C. N. Rao, *Biopolymers*, 1974, **13**, 2565.
[75] K. D. Kopple, T. J. Schampel, and A. Go, *J. Amer. Chem. Soc.*, 1974, **96**, 2597.
[76] C. M. Deber and E. R. Blout, *J. Amer. Chem. Soc.*, 1974, **96**, 7566.

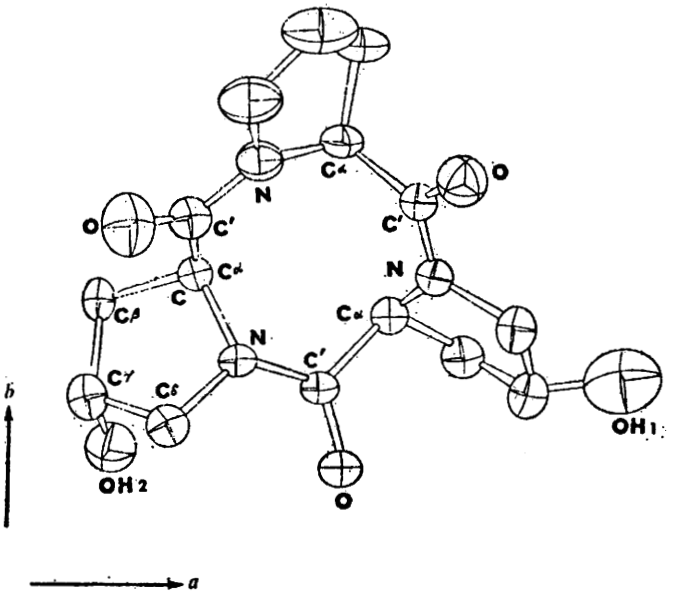


Figure 3 *The crystal structure of* cyclo-(L-*Pro*-L-*Hyp*-L-*Hyp*)
(Reproduced by permission from *Nature*, 1974, **247**, 205)

Further n.m.r. studies on the naturally occurring antibiotics polymycin B_1 [77] and telomycin [78] have led to the conclusion that the former is not stabilized by internal hydrogen bonds, whereas the latter is significantly stabilized by three intramolecular hydrogen bonds.

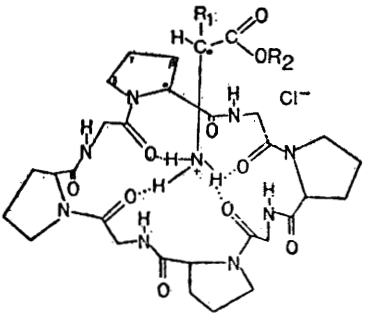


Figure 4 *Schematic representation of amino-acid complexes*
(Reproduced by permission from *J. Amer. Chem. Soc.*, 1974, **96**, 7566)

Synthesis of Homodetic Cyclic Peptides.—Table 1 summarizes the details of cyclic peptides synthesized in 1974. It is evident that dicyclohexylcarbodi-imide-

[77] R. E. Galardy, L. C. Craig, and M. P. Printz, *Biochemistry*, 1974, **13**, 1674.
[78] N. G. Kumar and D. W. Urry, *Biochemistry*, 1973, **12**, 3811, 4392.

N-hydroxysuccinimide method for cyclization leads the list in popularity, followed by the active ester and azide methods.

In an interesting synthesis of the phytotoxic tentoxin, a convenient method for generating *N*-methyldehydroamino-acid residues in an already constructed linear peptide has been developed (Scheme 1).[79] The peptide (**35**) was prepared

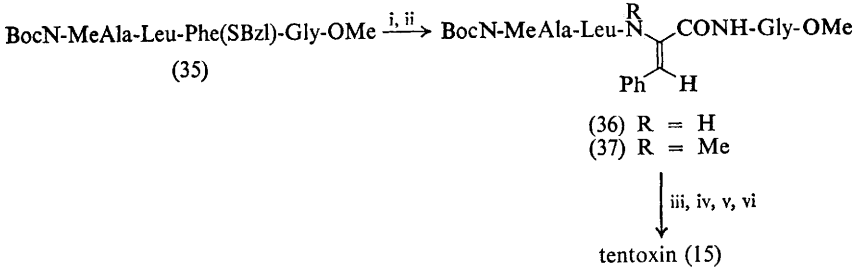
Table 1 *Syntheses of cyclic peptides achieved in* 1974 *(derived from linear analogues unless otherwise stated)*

Peptide	*Bond formed in cyclization step*	*Method for cyclization*	*Ref.*
Tentoxin	MeAla-Gly	*a*	21
cyclo-(D-Cys-D-Cys-Val-D-Leu-Ile) malformin A	Ile-D-Leu	*b*	23
cyclo-(D-Cys-Val-D-Cys-D-Leu-Ile)	Ile-D-Leu	*b*	23
[Pro^6-Phe^7]-antamanide	Pro-Phe	*c*	34
[Gly^7]-antamanide	Phe-Phe	*c*	34
[*Des*-Pro^7]-antamanide	Phe-Phe	*c*	34
[Glu^1]-antamanide	Pro-Glu	*c*	34
[Asp^4]-antamanide	Phe-Asp	*c*	34
[Asp^1-Val^4]-antamanide	Phe-Phe	*c*	34
[L-*retro*-*O*-Carboxymethyl-Tyr^6]-antamanide	Tyr-Phe	*c*	34
Isogramicidin S	Val-Pro	*c*	51
Iso-[2,2′-Lys]-gramicidin S	Val-Pro	*c*	51
[3-*N*-Methyl-leucine]-gramicidin S	Val-Pro	*c*	52
[3,3′-Di-*N*-methyl-leucine]-gramicidin S	Val-Pro	*c*	52
cyclo-(D-Phe-Orn-Pro)$_2$	D-Phe-Pro	*b*	75
cyclo-(D-Phe-Ala-Pro)$_2$	D-Phe-Pro	*b*	75
cyclo-(D-Phe-His-Pro)$_2$	D-Phe-Pro	*b*	75
cyclo-(D-Pro-Gly-Pro-Gly)$_2$	Pro-Gly	*d*	80
cyclo-(Pro-Sar)$_2$	Pro-Gly	*d*	80
cyclo-(Lys-Glu-Lys-Glu)	Lys-Glu	*b*	81
cyclo-(Lys-Asp-Lys-Asp)	Lys-Asp	*b*	81
cyclo-(Ala-Lys-Ala-Glu)	Ala-Glu	*b*	81
cyclo-(Ala-Ala-Lys-Glu)	Ala-Glu	*b*	81
cyclo-(Pro-Pro-Gly-Pro-Pro-Gly)	Pro-Gly	*c*	82
cyclo-(Pro-Pro-Gly-Pro-Pro-Gly-Gly)	Pro-Gly	*c*	82
cyclo-(Pro-Pro-Gly-Gly-Pro-Pro-Gly-Gly)	Pro-Gly	*c*	82
cyclo-(Pro-Pro-Gly-Gly-Pro-Pro-Gly-Gly-Gly)	Pro-Gly	*c*	82
cyclo-(Pro-Pro-Gly-Gly-Gly-Pro-Pro-Gly-Gly-Gly)	Pro-Gly	*c*	82

a) 2,4,5-trichlorophenyl ester; (*b*) azide method; (*c*) DCC–*N*-hydroxysuccinimide; (*d*) *p*-nitrophenyl ester.

by a solid-phase procedure and oxidized with periodate to the corresponding sulphoxide which on heating afforded the dehydropeptide (**36**). Methylation of (**36**) with methyl iodide in DMF occurred in good yield only on the dehydrophenylalanine residue (**37**). The subsequent cyclization of this linear peptide was achieved by the active ester method in modest yield.[21] Unfortunately, only the preliminary details of the important synthesis of malformin A, the outline of which is shown in Scheme 2, have appeared.[23]

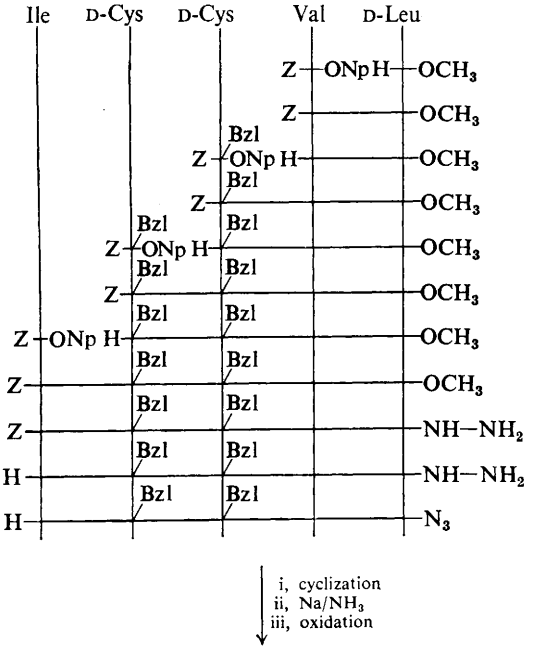
[79] D. H. Rich, J. Tam, P. Mathiaparanam, J. A. Grant, and G. Mabuni, *J.C.S. Chem. Comm.*, 1974, 897.
[80] C. M. Deber and E. R. Blout, *Israel J. Chem.*, 1974, **12**, 15.
[81] S. Rishi, K. B. Mathar, and M. M. Dhar, *Indian J. Chem.*, 1974, **12**, 458.
[82] T. Wieland and M. Hollosi, *Annalen*, 1974, 1596.



Reagents: i, $NaIO_4$; ii, MeI–K_2CO_3–DMF; iii, HO^-; iv, DCC–2,4,5-trichlorophenol; v, HCl–HOAc; vi, $EtNPr^i_2$

Scheme 1

Various analogues of antamanide listed in Table 1 have been synthesized following routes already developed. The still favoured dicyclohexylcarbodi-imide–*N*-hydroxysuccinimide process has been successfully applied to linear peptide precursors derived either by fragment synthesis or by automated solid-phase procedures.[34, 82] A number of cyclic hexapeptides synthesized for conformational studies were prepared by stepwise synthesis of tripeptide fragments, which were coupled to form the linear hexapeptides. The cyclizations were achieved by the azide method and it is noticeable that the yields using this method are con-



malformin A (19)

Scheme 2

siderably higher [75] than other methods. Similarly, good yields (65—75%) were obtained for a number of cyclic tetrapeptides also using the azide method.[81]

3 Depsipeptides (Heterodetic Peptides)

Series One of the MTP International Review of Science includes a volume with a general review chapter on depsipeptides,[83] partly overlapping earlier Reports in this series. General review articles on the conformational aspects of cyclic peptides which include both natural and synthetic depsipeptides have already been cited.

Actinomycin.—The importance of actinomycin and its analogues as biochemical tools for inhibiting DNA transcription and as potential chemotherapeutic agents is reflected in two recent review articles.[84, 85] Further aspects of the biological activity of the novel series of natural actinomycins which contain *N*-methyl-leucine instead of proline,[86] together with the structure of a new actinomycin D containing glycine in place of sarcosine,[87] have been reported. Speculation on the origin of the actinomycin chromophore continues; it is known that kynurenine and 3-hydroxy-4-methylkynurenine are precursors of the chromophore whereas 3-hydroxykynurenine does not appear to be involved. A proposal that 3-hydroxy-4-methylkynurenine is derived directly from methionine and kynurenine has been made.[88]

Detailed analysis of actinomycin D nucleotide interactions using ^{1}H, ^{13}C, and ^{31}P n.m.r. techniques [89] has provided further support for the Sobell–Jain [90] proposal that the phenoxazone ring of actinomycin D incalates between guanosine–cytidine base pairs in the double helix.

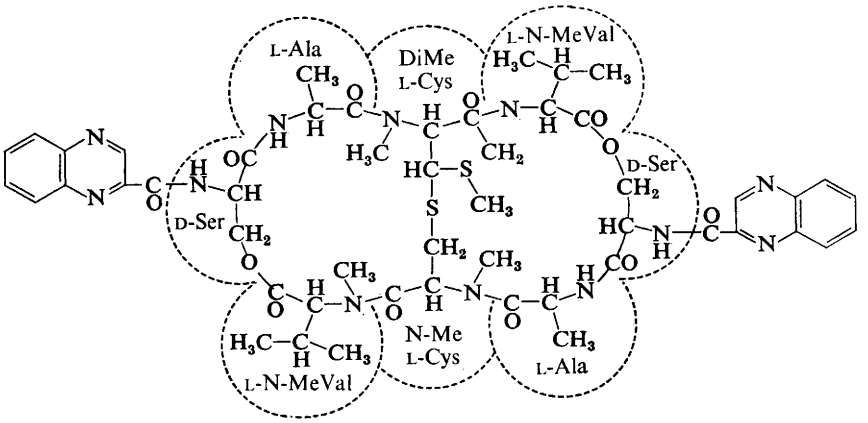
Valinomycin.—The details of the solution conformation of the valinomycin–potassium ion complex have generated a great deal of interest over the last several years. The general consensus of opinion is in agreement on the secondary structure, but examination [91] of high-resolution n.m.r. data has led to a revision of the proposed α-CH—β-CH orientation of the valyl side-chains. The fluorescence of the thallium complex [92] and the e.s.r. spectrum of the sodium tetracyanoethylene complex [93] have been employed to study the nature of complexation.

An alternative synthesis of valinomycin using the phosphite method for ring-closure proceeds in moderate yield.[94] Several analogues of valinomycin have been synthesized by fragment condensation (classical and solid-phase methods) followed by cyclization using the original acid chloride method.[95, 96] This

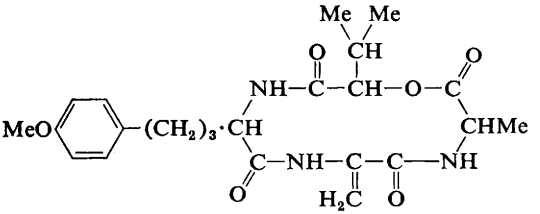
[83] H. A. James, 'Depsipeptides', MTP International Review of Science, Organic Chemistry, Series One, Vol. 6, ed. D. H. Hey and D. I. John, 1973, p. 213.
[84] H. Brockmann, *Cancer Chemotherapy Reports*, 1974, **58**, 9.
[85] J. Meienhofer, *Cancer Chemotherapy Reports*, 1974, **58**, 21.
[86] K. Mason, E. Katz, and A. B. Mauger, *Arch. Biochem. Biophys.*, 1974, **160**, 402.
[87] M. L. Dewan, T. I. Orlova, and A. B. Silaev, *Antibiotiki*, 1974, **19**, 107.
[88] R. B. Herbert, *Tetrahedron Letters*, 1974, 4525.
[89] D. J. Patel, *Biochemistry*, 1974, **13**, 1476, 2388, 2396.
[90] H. M. Sobell and S. C. Jain, *J. Mol. Biol.*, 1972, **68**, 21.
[91] D. W. Urry and N. G. Kumar, *Biochemistry*, 1974, **13**, 1829.
[92] G. Cornelius, W. Gartner, and D. H. Haynes, *Biochemistry*, 1974, **13**, 3052.
[93] M. P. Eastman, *J.C.S. Chem. Comm.*, 1974, 789.
[94] M. Rothe and W. Kreiss, *Angew. Chem.*, 1974, 1012.
[95] E. I. Vinogradova, L. A. Fonina, A. A. Sanasaryan, I. D. Ryabova, and V. T. Ivanov, *Khim. prirod. Soedinennii*, 1974, **10**, 233.
[96] E. I. Vinogradova, L. A. Fonina, I. D. Ryabova, and V. T. Ivanov, *Khim. prirod. Soedinennii*, 1974, **10**, 278.

program has also included syntheses of valinomycin and enniatin B analogues containing charged spin labels for subsequent conformational investigations.[97]

Other Depsipeptides.—A preliminary communication has reported [98] the revised structure (38) for the interesting antibiotic echinomycin in an article primarily



(38)



(39)

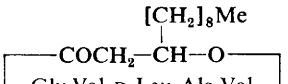
related to the intercalation properties of the antibiotic with DNA. The similarities to actinomycin with respect to the pseudo-symmetry of the peptide rings were noted. The structure (39) has been assigned [99] on the basis of physical and spectral data to alternariolide from *Alternaria mali* Roberts, which is responsible for a certain disease of apples.

The linear peptide (40) prepared by a stepwise procedure proved [100] to be identical with isariic acid obtained on base hydrolysis of isariin (41). Attempts to lactonize (40) to the natural depsipeptide by a variety of methods failed, owing no doubt to the severe steric hindrance at the carboxy-group of the *C*-terminal valine residue. A successful synthesis was subsequently achieved [101] through D-isoisariic acid (42) which was converted in modest yield into (41).

[97] V. T. Ivanov, L. V. Sumskaya, I. I. Mikhaleva, M. A. Laine, I. D. Ryabova, and Yu. A. Ovchinnikov, *Khim. prirod. Soedinennii*, 1974, **10**, 346.
[98] M. J. Waring and L. P. G. Wakelin, *Nature*, 1974, **252**, 653.
[99] T. Okuno, Y. Ishita, K. Sawai, and T. Matsumoto, *Chem. Letters*, 1974, 635.
[100] P. M. Hardy, R. A. Prout, and H. N. Rydon, *J.C.S. Perkin I*, 1974, 796.
[101] P. M. Hardy, R. A. Aubrey, and H. N. Rydon, *J.C.S. Perkin I*, 1974, 802.

D-Hydodec-Gly-Val-D-Leu-Ala-Val-OH

(40)



(41)

H_2N-Gly-Val-D-Leu-Ala-Val-D-hydodec-OH

(42)

H_2N-Val-D-Leu-Ala-Val-D-hydodec-Gly-OH

(43)

A further synthesis, again in moderate yield, from the linear precursor (43) prepared by the solid-phase procedure has been reported.[102] Cyclization was achieved using dicyclohexylcarbodi-imide–*N*-hydroxysuccinimide. An improved synthesis of the calcium- and barium-binding dipsipeptide beauvericin has been described.[103]

It is proposed[104] that natural enniatin A from *Fusarium sambucinum* is a mixture of at least two isomers, since *N*-methyl-leucine was isolated as a mixture of *erythro*-L and *threo*-L epimers on hydrolysis. Radiotracer studies have established the primary precursors of the peptidolactone antibiotic etamycin.[105] The base-catalysed addition of thioglycolic acid to the dehydrobutyrine residue in stendomycin is partially stereospecific, giving rise to optically active *S*-carboxymethyl-β-methylcysteine.[106]

4 Peptide Alkaloids

The Rhamnaceae continue to be a fruitful source of peptide alkaloids with a further 25 new cyclopeptides having been isolated over the past year. The structure of frangulanine (44) has been confirmed by an *X*-ray analysis of the trimethyl methiodide.[107] The stereochemistry of the β-hydroxyleucine residue was confirmed to be the *erythro* form and a twisted relationship between the benzene ring and the adjacent double bond demonstrated (Figure 5). Both ring amide linkages are *trans* but because of the *N*-methylation little can be deduced about the conformation of the native peptide. However, an initial high-resolution ^1H n.m.r. investigation of frangulanine and discarine B, which included studies of solvent and temperature dependence and of deuterium exchange, has given data on the solution conformation.[108]

Approximately a third of the new peptide alkaloids reported belong to the frangulanine group and contain the characteristic *p*-alkoxystyrylamino residue. The highly distinctive mass spectrometric fragmentation pattern, together with chemical degradation, has been used to elucidate the structures of these

[102] K. Okada, Y. Kurosawa, and M. Hiramoto, *Chem. and Pharm. Bull. (Japan)*, 1974, **22**, 2136.
[103] R. W. Roeske, S. Isaac, T. E. King, and L. K. Steinrauf, *Biochem. Biophys. Res. Comm.*, 1974, **57**, 554.
[104] T. K. Audhya and D. W. Russell, *J.C.S. Perkin I*, 1974, 743.
[105] D. J. Hook and L. C. Vining, *Canad. J. Biochem.*, 1974, **51**, 1630.
[106] M. Bodanszky and A. Bodanszky, *J. Antibiotics*, 1974, **27**, 312.
[107] M. Takai, K. Kawai, Y. Ogihara, Y. Iitaka, and S. Shibata, *J.C.S. Chem. Comm.*, 1974, 653.
[108] C. Chang, E. W. Hagaman, E. Wenkert, M. G. Sierra, O. A. Mascaretti, V. M. Merkuza, and E. A. Ruveda, *Phytochemistry*, 1974, **13**, 1273.

compounds as (44) and (45).[109–114] The majority differ only in the amino-acid residues, but scutianine D and E from *Scutia buxifolia* contain the same amino-acid residues. Scutianine D contains L-*erythro*-β-hydroxyleucine and L-*threo*-β-phenylserine, while scutianine E has the corresponding D-isomers; D-*erythro*-β-hydroxyleucine had not previously been encountered in natural products. An

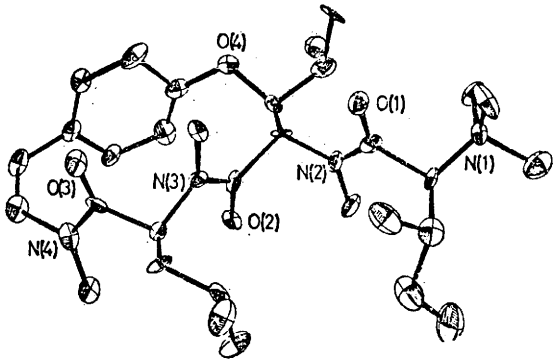


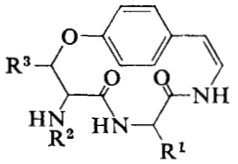
Figure 5 *The crystal structure of frangulanine trimethyl methiodide* (Reproduced by permission from *Phytochemistry*, 1974, **13**, 1273)

independent investigation[111] on the same plant resulted in the isolation of an alkaloid with apparently the same physical properties as scutianine D and for which the same structural formula was proposed, although no stereochemical assignments were made to the amino-acid residues. Unfortunately, this compound was named scutianine C, a name already assigned to another alkaloid.[110]

The structures of further members of a group of cyclopeptides (46) and (47) isolated from various *Zizyphus* species and possessing a 13-membered ring, but still containing an ether linkage within the ring, have been elucidated by the general procedures outlined above.[114–117] In addition, several cyclopeptides (48), with a 15-membered ring which has a carbon bridge instead of an ether linkage, have been found to co-occur with both the previously mentioned groups.[117, 118]

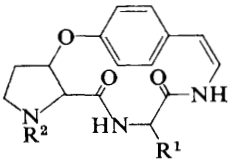
Crude alkaloid extracts from *Ziziphus amphibia* contain, as well as cyclopeptide alkaloids, the isoquinoline peptide amphibine-I (49), the structure of which was elucidated by spectroscopic and degradative procedures.[118]

[109] R. Tschesche and E. Ammermann, *Chem. Ber.*, 1974, **107**, 2274.
[110] V. M. Merkuza, S. M. Conzalez, O. A. Mascaretti, E. A. Ruveda, C. Chang, and E. Wenkert, *Phytochemistry*, 1974, **13**, 1279.
[111] M. Silva, D. S. Bhakuni, P. G. Sammes, M. Pais, and F. X. Jarreau, *Phytochemistry*, 1974, **13**, 861.
[112] R. Tschesche, H. Wilhelm, E. U. Kaussmann, and G. Eckhardt, *Annalen*, 1974, 1694.
[113] R. Tschesche, C. Spilles, and G. Eckhardt, *Chem. Ber.*, 1974, **107**, 686.
[114] R. Tschesche, G. A. Miana, and G. Eckhardt, *Chem. Ber.*, 1974, **107**, 3180.
[115] R. Tschesche, I. Khokhar, C. Spilles, G. Eckhardt, and B. K. Cassels, *Tetrahedron Letters*, 1974, 2941.
[116] B. K. Cassels, G. Eckhardt, E. U. Kaussmann, and R. Tschesche, *Tetrahedron*, 1974, **30**, 2461.
[117] R. Tschesche, S. T. David, R. Zerbes, M. von Radloff, E. U. Kaussmann, and G. Eckhardt, *Annalen*, 1974, 1915.
[118] R. Tschesche, C. Spilles, and G. Eckhardt, *Chem. Ber.*, 1974, **107**, 1329.



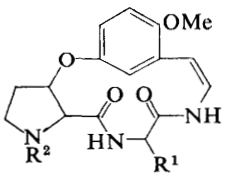
(44)

Frangulanine[107]	R^1 = Bu^i, R^2 = Me_2Ile, R^3 = Pr^i	*Hovenia dulcis* Thumb
Scutianine C[109]	R^1 = CH_2Ph, R^2 = Me_2Ile, R^3 = Pr^i	*Scutia buxifolia* Reiss
Scutianine D[110, 111]	R^1 = CHOHPh, R^2 = Me_2Phe, R^3 = Pr^i	
Scutianine E[110, 111]	R^1 = CHOHPh, R^2 = Me_2Phe, R^3 = Pr^i	
Crenatine A[112]	R^1 = CH_2Ph, R^2 = Me_2Leu, R^3 = Ph	*Discaria crenata* Regel



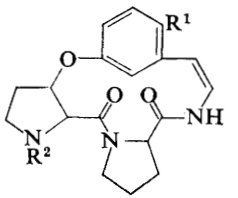
(45)

Mauritine C[113]	R^1 = CH_2Ph, R^2 = MeVal	*Ziziphus mauritiana* Lam.
Mauritine D[113]	R^1 = Bu^s, R^2 = Leu-Me_2Ile	
Mauritine E[113]	R^1 = CHOHPh, R^2 = Val-Me_2Ala	
Mauritine F[113]	R^1 = CH_2Ph, R^2 = Val-MeAla	
Amphibine F[114]	R^1 = CH_2Ph, R^2 = MeIle	*Ziziphus amphibia* A. Cheval
Amphibine G[114]	R^1 = Bu^i, R^2 = Me_2Trp	



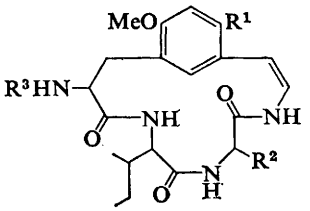
(46)

Amphibine H[114]	R^1 = CH_2Ph, R^2 = Val-Me_2Ala	*Ziziphus amphibia* A. Cheval
Mummularine A[115]	R^1 = Bu^s, R^2 = Ile-Me_2Phe	*Zizyphus nummularia*
Mummularine B[115]	R^1 = CH_2Ph, R^2 = Val-MeAla	
Mummularine C[115]	R^1 = Bu^i, R^2 = Me_2Phe	



(47)

Zizyphine F[116]	R^1 = OH R^2 = Ile-Me_2Ile	*Zizyphus oenoplia* Mill
Zizyphine C[117]	R^1 = OMe, R^2 = Ile-Me_2Phe	
Zizyphine G[116]	R^1 = OH, R^2 = Ile	

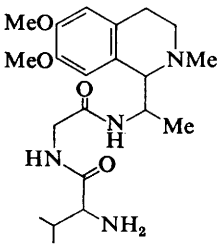


(48)

Zizyphine D[117] R[1] = H, R[2] = COHMeEt, R[3] = Me
Zizyphine E[117] R[1] = H, R[2] = COHMeEt, R[3] = H
} *Zizyphus oenoplia*

Mucronine E[118] R[1] = OMe, R[2] = Bu[i], R[3] = Me
Mucronine F[118] R[1] = OMe, R[2] = Bu[i], R[3] = H
Mucronine G[118] R[1] = OMe R[2] = Bu[s], R[3] = H
Mucronine H[118] R[1] = H, R[2] = CH_2Ph, R[3] = H
} *Zizyphus mucronata* Willd.

Abyssenine A[118] R[1] = H, R[2] = Bu[i], R[3] = Me
Abyssenine B[118] R[1] = H, R[2] = Bu[s], R[3] = Me
Abyssenine C[118] R[1] = H, R[2] = Bu[s], R[3] = H
} *Zizyphus abyssinica* Hochst.

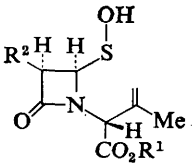


(49)

5 Penicillins and Cephalosporins

The policy adopted in previous years of outlining only the more significant developments relating to the chemistry and origin of these important antibiotics has been maintained. The major endeavour has once again been devoted to the structural modification of the penam and cepham skeleton. Aspects of the chemical interconversions of β-lactam antibiotics through penicillin sulphoxides have been reviewed.[119] The intermediacy of sulphenic acids (50) in the thermal rearrangements of penicillin sulphoxides is well established; although they had not previously been isolated a variety of reagents had been used to trap them. Arene sulphinic acids,[120] acetylenic esters,[121] and trimethylsilyl chloride[122] represent further examples of reagents capable of trapping these intermediates to give compounds which can subsequently be employed for further skeletal

[119] R. D. G. Cooper, *Accounts Chem. Res.*, 1973, **6**, 33; 'Penicillins and Cephalosporins', MTP International Review of Science, Organic Chemistry, Series One, Vol. 6, ed. D. H. Hey and D. I. John, 1973, p. 247.
[120] R. D. Allan, D. H. R. Barton, M. Girijavallabhan, and P. G. Sammes, *J.C.S. Perkin I*, 1974, 1456.
[121] D. H. R. Barton, I. H. Coates, P. G. Sammes, and C. M. Cooper, *J.C.S. Perkin I*, 1974, 1459.
[122] T. S. Chou, *Tetrahedron Letters*, 1974, 725.

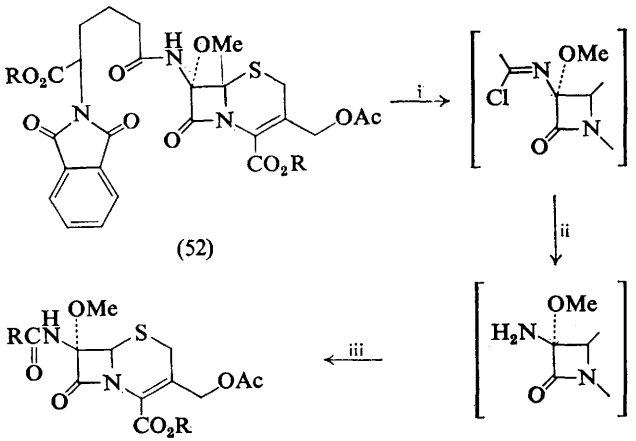


(50)

(51) $R^1 = CH_2C_4H_6\text{-}p\text{-}NO_2$, R^2 = phthalimido

modification. In view of the enormous effort expended on these rearrangements over the past 10 years, it is surprising that the isolation of a pure crystalline sulphenic acid intermediate (51) has only been reported in the past year.[123]

The 6(7)-substituted derivatives of penams and cephams still continue to stimulate considerable interest. The cleavage procedure outlined in Scheme 3



(52)

(53)

Reagents: i, PCl_5–py; ii, MeOH; iii, RCOCl–py

Scheme 3

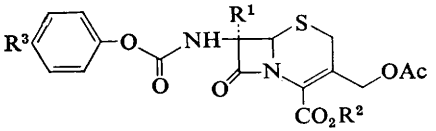
provides a convenient route for obtaining 7α-methoxy-compounds (53) from the naturally occurring 7α-methoxy-7β-aminoadipamidocephalosporins (52) in acceptable yield.[124] Alternatively, 7β-aminocephalosporin can be converted into the *p*-nitrobenzylcarbonate (54) and oxidized with t-butylhypochlorite and methanol to (55). The carbonate protecting group is removed on hydrogenation and the resulting amino-compound acylated in the normal manner.[125]

Various 6α-substituted penicillins have been prepared from the isocyanide (56) on treatment with alkyl halides and base or with methyl methoxycarbonyl

[123] T. S. Chou, J. R. Burgtorf, A. L. Ellis, S. R. Lammert, and S. P. Kukolja, *J. Amer. Chem. Soc.*, 1974, **96**, 1610.

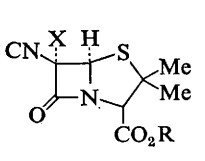
[124] W. H. W. Lunn, R. W. Burchfield, T. K. Elzey, and E. V. Mason, *Tetrahedron Letters*, 1974, 1307.

[125] W. H. W. Lunn and E. V. Mason, *Tetrahedron Letters*, 1974, 1311.

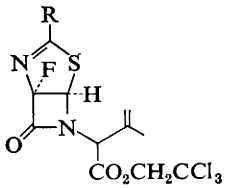


(54) R^1 = H, R^2 = $CHPh_2$ R_3 = NO_2
(55) R^1 = OMe, R^2 = $CHPh_2$, R^3 = NO_2

disulphide. Conversion of the isocyanide into acylamino was achieved by treating with toluene-*p*-sulphonic acid followed by acylation.[126] Attempts to prepare 6-fluoropenicillin derivatives by fluorination of the anion derived from the corresponding imino chloride with perchloryl fluoride led to formation of the rearrangement product (57) as the major component.[127] Reaction of the



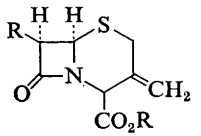
(56)



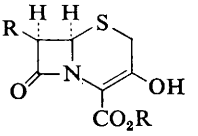
(57)

imino chloride of penicillin G pivaloylmethyl ester with base afforded the corresponding ketenimine which had almost completely epimerized at C-6, thus providing a further method for the preparation of penicillin C-6 epimers.[128]

Considerable emphasis relating to the chemistry of cephalosporins has centred on modification at C-3, since previous variations at this position have been particularly fruitful, resulting in clinically useful drugs. Full details of the electrochemical reduction of cephalosporanic acids to the corresponding 3-methylene cepham derivatives (58) have appeared.[129] These compounds on ozonolysis afford the 3-hydroxycephems (59) which have been used to prepare



(58)

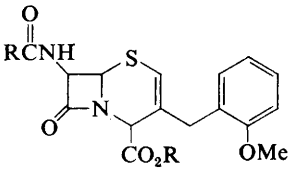


(59)

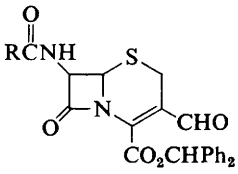
a variety of 3-substituted antibiotics.[130, 131] The acetoxy and carbamoyl groups at C-3 in cephalosporins and cephamycins, respectively, can be replaced by a variety of nucleophiles, including *C*-nucleophiles, in the presence of strong

[126] P. H. Bentley and J. P. Clayton, *J.C.S. Chem. Comm.*, 1974, 278.
[127] W. A. Spitzer, T. Goodson, jun., M. O. Chaney, and N. D. Jones, *Tetrahedron Letters*, 1974, 4311.
[128] R. D. Carroll, E. S. Hamanaka, D. K. Pirie, and W. M. Welch, *Tetrahedron Letters*, 1974, 1515.
[129] M. Ochiai, O. Aki, A. Morimoto, T. Okada, K. Shinozaki, and Y. Asahi, *J.C.S. Perkin I*, 1974, 258.
[130] R. R. Chauvette and P. A. Pennington, *J. Amer. Chem. Soc.*, 1974, **96**, 4986.
[131] R. Scartazzini and H. Bickel, *Helv. Chim. Acta*, 1974, **57**, 1919.

Lewis acids.[132, 133] The products are of the Δ^2 series, *e.g.* (60), which can be returned to the biologically important Δ^3 series by the usual oxidative–reductive isomerization method. New series of C-3-modified antibiotics have been derived from the formyl compounds (61), either by decarbonylation in the presence of tris(triphenylphosphine)rhodium chloride to give the unsubstituted series[134] or by oxidation to the corresponding carboxylic acids.[135]

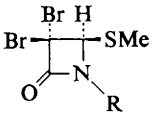


(60)

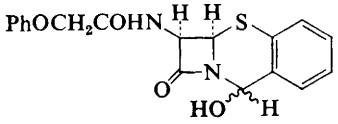


(61)

Further routes directed at preparing the basic azetidinone system from penicillins continue to be explored. The action of methyl iodide and base on methyl 6,6-dibromopenicillanate afforded the β-lactam (62), which on oxidation with osmium tetraoxide gave the simple azetidinone (63). A similar reaction with 6-acylaminopenicillanates yields products in which the β-lactam has opened.[136] The details of the conversion of penicillanic acids into monocyclic



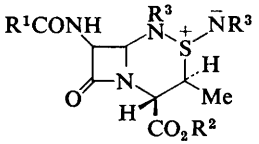
(62) R = $C(CO_2Me)=CMe_2$
(63) R = H



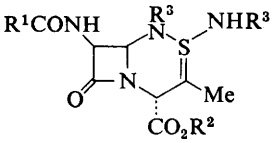
(64)

azetidinones with mercury(II) acetate,[137] as well as their subsequent transformation into 4-alkylthio-derivatives,[138] have been described. The thiazolidine ring of penicillin V has been removed by standard procedures and replaced by the unusual cephem system (64)[139] and several fused oxazoline–azetidinones have been prepared from anhydropenicillin.[140] An attempt to modify the thiazolidine ring of penicillins with chloramine T led to the novel ylide (65), the structure of which has been established by an *X*-ray crystallographic analysis.[141] The ylide on thermolysis afforded the β-lactam (66) and this was subsequently converted

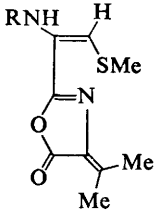
[132] S. Karady, T. Y. Cheng, S. H. Pines, and M. Sletzinger, *Tetrahedron Letters*, 1974, 2629.
[133] H. Peter, H. Rodriguez, B. Muller, W. Sibral, and H. Bickel, *Helv. Chim. Acta*, 1974, **57**, 2024.
[134] H. Peter and H. Bickel, *Helv. Chim. Acta*, 1974, **57**, 2044.
[135] D. O. Spry, *J.C.S. Chem. Comm.*, 1974, 1012.
[136] J. P. Clayton, J. H. C. Nayler, M. J. Pearson, and R. Southgate, *J.C.S. Perkin I*, 1974, 22.
[137] R. J. Stoodley and N. R. Whitehouse, *J.C.S. Perkin I*, 1974, 181.
[138] D. F. Corbett and R. J. Stoodley, *J.C.S. Chem. Comm.*, 1974, 438.
[139] J. C. Sheehan, H. C. Dalzell, J. M. Greenwood, and D. R. Ponzi, *J. Org. Chem.*, 1974, **39**, 277.
[140] S. Wolfe, S.-L. Lee, J. Ducep, G. Kannengiesser, and W. S. Lee, *Canad. J. Chem.*, 1974, **53**, 497.
[141] M. M. Campbell, G. Johnson, A. F. Cameron, and I. R. Cameron, *J.C.S. Chem. Comm.*, 1974, 868.



(65) $R^1 = CH_2OPh$, $R^2 = Me$, $R^3 = p\text{-}MeC_6H_4SO_2$



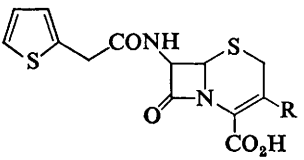
(66)



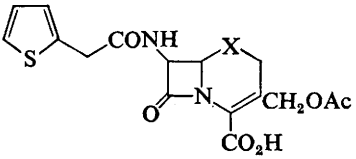
(67)

into a number of didehydrodipeptides lacking the sulphur atom.[142] The structure and stereochemistry of the rearrangement product (67) of penicillins with methyl chloroformate have been established by a total synthesis.[143]

The total synthesis of β-lactam systems, as opposed to chemical modification of naturally occurring compounds, now appears to be a viable alternative for the investigation of structure–activity relationships. This is exemplified by the syntheses of racemic 3-methylcephalothin (68)[144] and 3-arylcephalosporins



(68) R = CHMeOAc
(69) R = aryl



(70) X = O
(71) X = CH_2

(69)[145] following the route described in last year's report. In addition, the method has been further extended to include the syntheses of the racemic antibiotics oxacephalothin (70) and carbacephalothin (71) in which the sulphur atom has been replaced by an oxygen atom and methylene group, respectively.[146, 147] Both compounds, which would be extremely difficult to prepare by modification of known antibiotics, show antimicrobial activity comparable to sodium cephalothin.

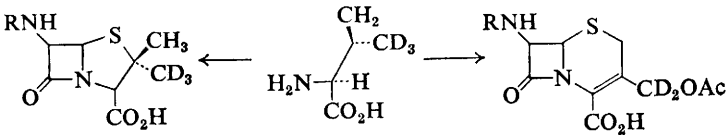
The syntheses described above, as well as a number of others discussed below, use the cycloaddition of a masked glycine to a suitably substituted imine for the construction of the β-lactam. Normally, the newly created stereochemistry of the azetidinone hydrogens is *trans* instead of the required *cis*. The epimerization of *trans* to *cis* can be achieved by protonation of the intermediate enolate anion under kinetically controlled conditions.[148] Cycloaddition has been used to

[142] M. M. Campbell and G. Johnson, *J.C.S. Chem. Comm.*, 1974, 974.
[143] C. J. Veal and D. W. Young, *J.C.S. Chem. Comm.*, 1974, 266.
[144] N. G. Steinberg, R. W. Ratcliffe, and B. G. Christensen, *Tetrahedron Letters*, 1974, 3567.
[145] R. A. Firestone, N. S. Maciejewicz, and B. G. Christensen, *J. Org. Chem.*, 1974, **39**, 3384.
[146] L. D. Cama and B. G. Christensen, *J. Amer. Chem. Soc.*, 1974, **96**, 7582.
[147] R. N. Guthikonda, L. D. Cama, and B. G. Christensen, *J. Amer. Chem. Soc.*, 1974, **96**, 7584.
[148] R. A. Firestone, N. S. Maciejewicz, R. W. Ratcliffe, and G. B. Christensen, *J. Org. Chem.*, 1974, **39**, 437.

synthesize a variety of azetidinones which have served as starting materials for more elaborate structures.[149–153]

An extension of the recently developed photolytic Wolff rearrangement of 3-diazopyrrolidine-2,4-diones for the synthesis of β-lactams,[154] and a total synthesis of bis-norpenicillin V following the original Sheehan method,[155] have been reported.

The biochemistry of penicillin and cephalosporin fermentations has been authoritatively reviewed in an article which also summaries the present state of our knowledge concerning the biosynthesis of β-lactam antibiotics.[156] The details of the preparation and incorporation of chirally labelled ^{13}C-valine, first communicated last year, have now been described in full.[157] As a sequel to this work the synthesis of chirally labelled C^2H_3-valines was performed and the compounds fed to washed cells of *Cephalosporium acremonium*. The deuterium atoms were located by mass spectrometry [158] and the results of these experiments are outlined in Scheme 4. $[C^2H_3]_2$-DL-valine has also been synthesized and



Scheme 4

incorporated into penicillin V. This experiment demonstrates the retention of all six hydrogen atoms of the valine C—Me groups in biosynthesis of penicillins.[159]

Further syntheses of dehydropeptides related to the supposed biosynthetic intermediates have so far failed to yield β-lactams under a variety of conditions.[160–162] Deacetylcephalosporin C, not previously observed as a natural product, has been shown to be a metabolite of a large number of *Streptomyces* and fungi.[163] Preparations of the important penicillin N and

[149] R. Lattrell, *Annalen*, 1974, 1937.
[150] M. D. Bachi and O. Goldberg, *J.C.S. Perkin I*, 1974, 1184.
[151] A. K. Bose, J. C. Kapar, S. G. Amin, and M. S. Manhas, *Tetrahedron Letters*, 1974, 1917.
[152] A. K. Bose, A. Lai, B. Dayal, and M. S. Manhas, *Tetrahedron Letters*, 1974, 2633.
[153] J. A. Edwards, A. Guzman, R. Johnson, P. J. Beeby, and J. H. Fried, *Tetrahedron Letters*, 1974, 2031.
[154] J. R. Hlubucek and G. Lowe, *J.C.S. Chem. Comm.*, 1974, 419.
[155] J. Hougmartens, P. J. Claes, and H. Vanderhaeghe, *J. Medicin. Chem.*, 1974, **17**, 388.
[156] A. L. Demain, *Lloydia*, 1974, **37**, 147.
[157] D. J. Aberhart and L. J. Lin, *J.C.S. Perkin I*, 1974, 2320.
[158] H. Kluender, F. C. Huang, A. Fritzberg, H. Schnoes, C. J. Sih, P. Fawcett, and E. P. Abraham, *J. Amer. Chem. Soc.*, 1974, **96**, 4054.
[159] J. Aberhart, J. Y.-R. Chu, N. Neuss, C. H. Nash, J. Occolowitz, L. L. Huckstep, and N. DeLa Higuera, *J.C.S. Chem. Comm.*, 1974, 564.
[160] J. Cheney, C. J. Moores, J. A. Raleigh, A. I. Scott, and D. W. Young, *J.C.S. Chem. Comm.*, 1974, 47.
[161] J. Cheney, C. J. Moores, J. A. Raleigh, A. I. Scott, and D. W. Young, *J.C.S. Perkin I*, 1974, 986.
[162] R. B. Morin, J. R. Lake, and E. M. Gordon, *Tetrahedron Letters*, 1974, 2979.
[163] R. Nagarajan, L. D. Boeck, R. L. Hamill, C. E. Higgens, and K. S. Yang, *J.C.S. Chem. Comm.*, 1974, 321.

isopenicillin N which are suspected of being the precursors of cephalosporins and other penicillins, respectively, have been described.[164]

6 Peptides Linked to Carbohydrates

This important biological field which also encompasses many aspects of carbohydrate chemistry has been restricted to glycopeptides isolated from or involved in the biosynthesis of cell walls. The reason for this decision is partly lack of space, but also to avoid digressing too far from the central theme of this chapter. The emphasis has remained on the peptide constituents; in particular those containing unusual amino-acids and peptide linkages.

Glycopeptides from Bacterial Cell Walls.—The cell wall peptidoglycan presents a remarkably uniform structure throughout the bacterial world. The basic glycan consists of linear strands of alternating β-1,4-linked pyranosides, *N*-acetylglucosamine, and *N*-acetylmuramic acid residues. The carboxy-groups of the muramic acid residues are linked to the peptide units having the general sequence (72). The peptide units of adjacent glycan strands are in turn cross-linked through bridges which extend from the *C*-terminal D-Ala of one tetrapeptide either to the ω-amino-group of the L-R_3 residue or through a Lys or Orn to the α-carboxy-group of D-glutamic acid of another peptide unit.[165–167] The major effort throughout the year has been aimed at clarifying the mechanism of formation of these complex structures.

The structure (73) of the peptide isolated from *Pseudomonas aeruginosa* has been established by standard chemical and enzymatic procedures.[168] The biosynthesis of peptidoglycan is catalysed by a series of cytoplasmic and membrane-associated enzymes. The inhibition of one of these enzymes by certain

-Ala-D-Glu-(L-R_3-D-Ala) (72) H_2N-Ala-D-Glu-A_2pm-Ala-OH (73)

UDP-NAc-D-muramyl
|
L-Ala-D-Glu-A_2pm-D-Ala-D-Ala (74)

antibiotics results in the accumulation of nucleotide-activated peptidoglycan precursors which can subsequently be used for further studies. Cells of *Bacillus megaterium* can use the externally added precursors UDP-*N*-acetyl-D-glucosamine and (74) to synthesis peptidoglycan if the bacteria are first treated with toluene.[169]

A particulate enzyme system isolated from *B. stearothermophilus* which catalyses the synthesis of peptidoglycan from uridine nucleotide precursors has been studied. The nature of the enzymatically synthesized peptidoglycan was elucidated by comparison with similar material obtained after lysozyme digestion of

[164] H. Vanderhaeghe, A. Vlietinck, M. Claesen, and G. Parmentier, *J. Antibiotics*, 1974, **28**, 169.
[165] K. H. Schleifer and O. Kandler, *Bacteriol. Rev.*, 1972, **36**, 407.
[166] J. C. Sadoff, *J. Infect. Diseases*, 1974, **130**, 61.
[167] J. Rotta, *J. Hyg. Epidemiol. Microbiol. Immunol.*, 1974, **18**, 353.
[168] H. D. Heilman, *European J. Biochem.*, 1974, **43**, 35.
[169] W. P. Schrader and D. P. Fan, *J. Biol. Chem.*, 1974, **249**, 4815.

isolated vegetative cell walls of this organism.[170] Incubation of *Micrococcus luteus* cells in the presence of penicillin G leads to an accumulation in the culture medium of a linear uncross-linked peptidoglycan. The product was digested with lysozyme to yield mainly the disaccharide (75) and the hexapeptide (76). It was

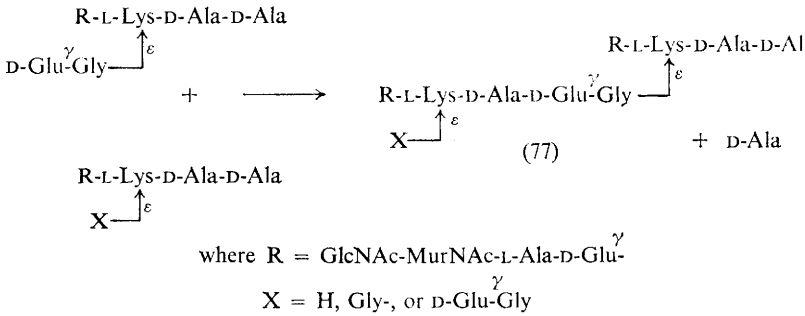
GlcNAc-β-(1 → 4)-MurNAc
(75)

Ala-D-i-Glu-L-Lys-D-Ala-D-Ala
|
Gly
(76)

concluded that the secretion of the peptidoglycan is due to the inhibition of the attachment of newly synthesized peptidoglycan strands by transpeptidation to the pre-existing cell wall.[171] Similar investigations have been conducted on *Bacillus megaterium*.[172]

The synthesis of peptidoglycan by cell-free membrane preparations from β-lactamase-negative mutant of *B. licheniformis* afforded uncross-linked polymer, the formation of which was not inhibited by β-lactam antibiotics. The release of D-alanine by the action of D-alanine carboxypeptidase was inhibited by the antibiotic.[173] Studies on D-alanine carboxypeptidase[174] from *Streptococcus faecalis* and on an exocellular DD-carboxypeptidase–transpeptidase[175] from *Streptomyces* R39 have been reported.

The means of biosynthesis of the unusual peptidoglycan (77) and a further *in vitro* demonstration of transpeptidation are outlined in Scheme 5.[176] The



Scheme 5

specificity profile of the translocase from *Staphylococcus aureus* towards the peptide subunit (78) has been investigated.[177] On the basis of glycine substitution, the enzyme has a high specificity for L-alanine in position 1 and D-alanine in

[170] P. E. Linnett and J. L. Strominger, *J. Biol. Chem.*, 1974, **249**, 2489.
[171] D. Mirelman, R. Bracha, and N. Sharon, *Biochemistry*, 1974, **13**, 5045.
[172] W. P. Schrader, B. E. Beckman, M. M. Beckman, J. S. Anderson, and D. P. Fan, *J. Biol. Chem.*, 1974, **249**, 4807.
[173] J. B. Ward, *Biochem. J.*, 1974, **141**, 227.
[174] B. Oppenheim, R. Koren, and A. Patchornik, *Biochem. Biophys. Res. Comm.*, 1974, **57**, 562.
[175] J.-M. Ghuysen, P. E. Reynolds, H. R. Perkins, J.-M. Frère, and R. Moreno, *Biochemistry*, 1974, **13**, 2539.
[176] P. E. Linnett, R. J. Roberts, and J. L. Strominger, *J. Biol. Chem.*, 1974, **249**, 2497.
[177] W. P. Hammes and F. C. Neuhaus, *J. Biol. Chem.*, 1974, **249**, 3140.

position 4, and low specificity for D-alanine in position 5. The translocase has a low specificity for the diamino-acid at position 3.

The peptidoglycan of mycobacteria is bridged by an unusual type of inter-peptide linkage extending from a free carboxy-group of a *meso*-diaminopimelic acid residue of one peptide unit to the amino-group of the same residue of another peptide unit. The occurrence of these linkages in *Mycobacterium smegmatis* was established by isolation from partial hydrolysates and characterization by mass spectral analysis of the di- and tri-peptides (79) and (80).[178] Synthetic work in the field of bacterial cell wall chemistry has been reviewed.[179]

UDP-MurNAc-R_1-D-Glu-Lys-R_4-R_5	A_2pm-A_2pm	A_2pm-A_2pm-A_2pm
(78)	(79)	(80)

7 Linear Peptides containing Unusual Structural Features

γ-Glutamyl peptides are widely distributed throughout the plant kingdom and several new derivatives, together with their sources, are listed in Table 2.[180–183]

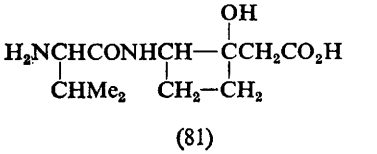
Table 2

Peptide	*Source*	*Ref.*
γ-L-Glutamyl-D-alanine	*Piscum sativum*	180
γ-L-Glutamyl-3-(1-uracil)-L-alanine	*Fagus silvatica*	181
γ-L-Glutamyl-L-phenylalanyl-3-(1-uracil)-L-alanine	*F. silvatica*	181
γ-L-Glutamyl-L-pipecolic acid	*Gleditsia caspica*	182
γ-L-Glutamyl-S-(*trans*-1-propenyl)-L-cysteine sulphoxide	*Santalum album*	183

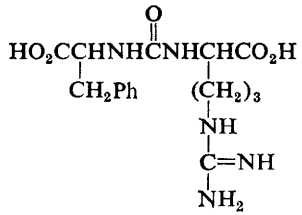
The preparation of several γ-glutamyl dipeptides of sulphur-containing amino-acids have been described.[184] Fenugreekine is a steroidal sapogenin-dipeptide ester from the seeds of *Trigonella foenum graecum* which on acid hydrolysis affords diosgenin and a mixture of three isomers of 4-hydroxyisoleucine. The structure of the dipeptide moiety has not yet been formulated.[185]

The isolation and structures of anti-metabolites derived from micro-organisms, about a dozen of which are di- or tri-peptides, have been reviewed.[186] In addition, a number of new peptide anti-metabolites all with interesting structural features have been isolated: L-arginyl-D-*allo*-threonyl-L-phenyl-alanine,[187, 188] L-2-amino-4-methylphosphinobutyryl-L-alanine-L-alanine,[189] and

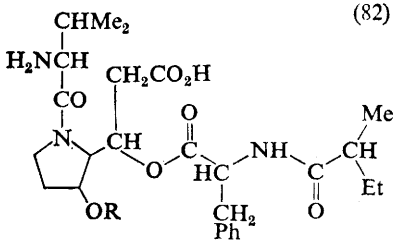
[178] J. Wietzerbin, B. C. Das, J.-F. Petit, E. Lederer, M. Leyh-Bouille, and J.-M. Ghuysen, *Biochemistry*, 1974, **13**, 3471.
[179] E. Bricas, *Chem. Polypeptides*, 1973, 205.
[180] T. Ogawa, M. Fukuda, and K. Sasaoka, *Biochim. Biophys. Acta*, 1973, **297**, 60.
[181] I. Kristensen and P. O. Larsen, *Phytochemistry*, 1974, **13**, 2799.
[182] G. Dardenne, J. Casimir, and H. Soresen, *Phytochemistry*, 1974, **13**, 1515.
[183] R. Kuttan, N. G. Nair, A. N. Radhakrishnan, T. F. Spande, H. J. Yeh, and B. Witkop, *Biochemistry*, 1974, **13**, 4394.
[184] J. F. Carson and F. F. Wong, *J.C.S. Perkin I*, 1974, 685.
[185] S. Ghosal, R. S. Srivastava, D. C. Chatterjee, and S. K. Dutta, *Phytochemistry*, 1974, **13**, 2247.
[186] D. L. Pruess and J. P. Scannell, *Adv. Appl. Microbiol.*, 1974, **17**, 19.
[187] W. A. Konig, H. Kneifel, E. Beyer, G. Muller, and H. Zahner, *J. Antibiotics*, 1973, **26**, 44.
[188] W. A. Konig, W. Loeffler, W. H. Meyer, and P. Uhmann, *Chem. Ber.*, 1973, **106**, 816.
[189] Y. Ogawa, T. Tsuruoka, S. Inoue, and T. Niida, *Meiji Seika Kenkyu Nempo*, 1973, 42.



(81)

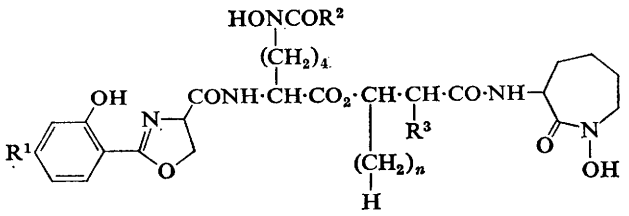


(82)



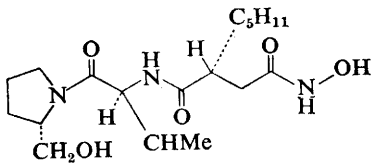
(83)

the novel cyclobutane derivative (81).[190] Further examples of biologically active small peptides are (82) produced by *Streptomyces filipinensis*[191] and the minor components of the detoxin D group which are simple fatty acid esters of the parent compound (83).[192]



(84)

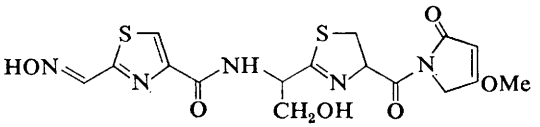
(85) R^1 = Me, R^2 = $CH{=}CH(CH_2)_{14}Me$, R^3 = Me, n = 2
(86) R^1 = H, R^2 = Me, R^3 = Me, n = 15 and 17



(87)

[190] D. L. Pruess, J. P. Scannell, J. F. Blount, H. A. Ax, M. Kellett, T. H. Williams, and A. Stempel, *J. Antibiotics*, 1974, **27**, 754.
[191] K. Fujimoto, K. Tatsuta, T. Tsuchiya, S. Umezawa, and H. Umezawa, *J. Antibiotics*, 1974, **27**, 685.
[192] N. Otake, K. Furihata, K. Kakinuma, and H. Yonehara, *J. Antibiotics*, 1974, **27**, 484.

The general structure (84) for the mycobactin group of iron-complexing hydroxamic acid derivatives has been confirmed[193] by an *X*-ray analysis of mycobactin P (85). Nocobactin NA from *Nocardia asteroides* resembles mycobactin M (86) in structure except that it has an oxazole ring in place of an oxazoline ring.[194] The antibiotic actinonin (87) which is also a hydroxamic acid derivative and contains an unusual L-prolinol residue has been synthesized.[195] A revised structure (88) for the antibiotic althiomycin has been reported by two



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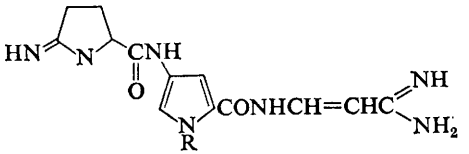
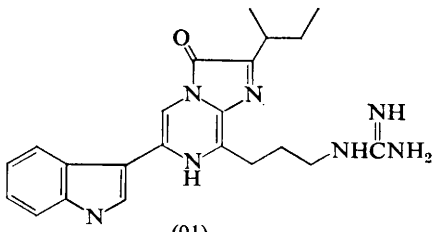


(89) R = H
(90) R = Me

groups.[196, 197] The molecule contains the interesting oxime system as well as a modified D-cysteine residue. Kikumycin A and B, which can probably be regarded as modified peptides, are represented by (89) and (90), respectively.[198]

8 Miscellaneous

The structure (91) of the luminescent substance *cypridina* luciferin is now well established and structurally can be considered as a modified peptide. Recent work on the source of luminescence of the luminous squid *Watasenia scintillans*

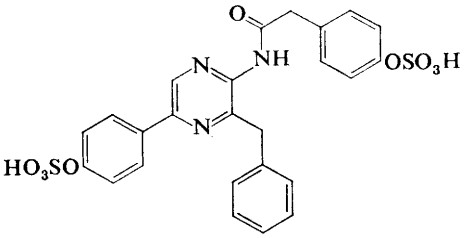


(91)

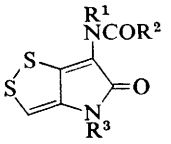
[193] E. Hough and D. Rogers, *Biochem. Biophys. Res. Comm.*, 1974, **57**, 73.
[194] C. Ratledge and G. A. Alan, *Biochem. J.*, 1974, **139**, 407.
[195] N. H. Anderson, W. D. Ollis, J. E. Thorpe, and A. D. Ward, *J.C.S. Chem. Comm.*, 1974, 420, 421.
[196] H. Sakakibara, H. Naganawa, M. Ohno, K. Maeda, and H. Umezawa, *J. Antibiotics*, 1974, **27**, 899.
[197] B. W. Bycroft and R. Pinchin, *J.C.S. Chem. Comm.*, 1975, 121.
[198] T. Takaishi, M. Suzuki, and A. Tatematsu, *Org. Mass Spectrometry*, 1974, **9**, 635.

has led to the isolation and characterization of (92), which is considered as the direct oxidation product of the luciferin.[199, 200]

Attempts to incorporate intact peptides into ergot peptide alkaloids have been unsuccessful. The precursors are degraded to the component amino-acids before incorporation.[201] The holomycin group (93) of antibiotics are of interest for



(92)



(93)

their biological activity and because they are formally derivable from a cysteine-containing dipeptide. A general synthesis of these compounds has been described.[202] Syntheses of the unusual amino-acids streptolidine [203, 204] from the streptothricin antibiotics and β-(2-thiazolyl)-β-alanine [205] from bottromycin, as well as syntheses of a number of peptides containing purines and pyrimidines,[206] have been reported.

[199] F. McCapra, *Pure Appl. Chem.*, 1970, **24**, 611.
[200] T. Goto, H. Iio, S. Inoue, and H. Kakoi, *Tetrahedron Letters*, 1974, 2321.
[201] H. G. Floss, M. Tcheng-Lin, H. Kabel, and P. Stadler, *Experientia*, 1974, **30**, 1369.
[202] K. Hagio and N. Yoneda, *Bull. Chem. Soc. Japan*, 1974, **47**, 1489.
[203] S. Kusumoto, S. Tsuji, and T. Shiba, *Bull. Chem. Soc. Japan*, 1974, **47**, 2690.
[204] T. Goto and T. Ohgi, *Tetrahedron Letters*, 1974, 1413.
[205] K. Seto, K. Torii, K. Bori, K. Inabata, S. Kuwata, and H. Watanabe, *Bull. Chem. Soc. Japan*, 1974, **47**, 151.
[206] M. T. Doel, A. S. Jones, and R. T. Walker, *Tetrahedron*, 1974, **30**, 2755.

5
Chemical Structure and Biological Activity of Enzymes

BY A. R. FERSHT

1 Introduction

Scope and Direction of this Chapter.—There are 1500 known enzymes. Just to catalogue them and the reactions which they catalyse would take more space than allocated. It is clearly impossible to list every paper published during the year and in any case this is conveniently done in *Chemical Abstracts*, Biochemistry Section 7. What has been attempted is a review of some of the more important or provocative papers in the context of the development of ideas and principles and to point out experimental methods of interest in certain areas. As this is the first review of the subject in this series, topics are prefaced by an introduction mentioning earlier work and, where possible, statements of the current problems.

2 General Principles Concerning Binding and Catalysis

It has long been postulated that the active site of an enzyme is complementary to the structure of the substrate in its transition state rather than in its initial form.[1, 2] Earlier attempts to prove [3] this from thermodynamics have shown only that the *dissociation constants* of transition states from enzymes are lower than those for the original substrate. However, the dissociation constant just measures the equilibrium constant between the transition state (or substrate) in water and on the enzyme. The difference in binding is due in the main to the inherent instability of transition states in water rather than forces distorting the substrate towards its transition state; therefore nothing can be said concerning enzyme substrate complementarity from this result.[4] A proof concerning the complementarity has now been presented.[4] It is shown that when the enzyme is complementary to the transition-state structure it is the term k_{cat}/K_M that is maximized. However, this is not a sufficient criterion for the optimization of reaction rates. If an enzyme encounters *in vivo* a substrate concentration generally at the level of [S] then in order to optimize the rate it should have evolved to a K_M value that is greater than [S] (whilst keeping the value of k_{cat}/K_M at its optimum value). A corollary of this is that intermediates that occur on the reaction pathway should not accumulate but exist at low steady-state concentrations, *i.e.* where their breakdown rates are faster than their formation. Although these are not hard and fast rules since they refer only to optimization, and optimization may not be possible or necessary, they do provide a framework in which to interpret

[1] J. B. S. Haldane, 'Enzymes', Longmans, Green, and Co., London, 1930, p. 182.
[2] L. Pauling, *Chem. Eng. News*, 1946, **24**, 1375.
[3] R. Wolfenden, *Accounts Chem. Res.*, 1972, **5**, 10.
[4] A. R. Fersht, *Proc. Roy. Soc.*, 1974, **B187**, 397.

phenomena and design experiments. For example, it is not surprising that it has been difficult to detect intermediates in the reactions of pepsin, carboxypeptidase, *etc.* with their physiological substrates since the above rule suggests they should not accumulate.

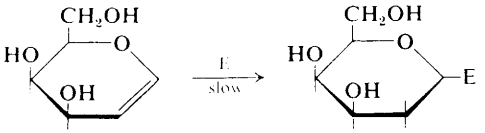
It seems that the biological roles of 'strain' and 'induced fit' are to increase the K_M for reactions to attempt to satisfy the criterion of $K_M > [S]$. It is also discussed how 'strain' may be induced in enzymatic reactions in light of recent results from crystallography and theoretical work.[4] Specificity is shown to be independent of strain, induced fit, and non-productive binding.

Schray and Klinman[5] have also pointed out how the dissociation constant of a transition state relative to that of a substrate or transition-state analogue from an enzyme contains other factors than the actual binding forces, in this case emphasizing the effects of entropy.

3 Transition State Analogues

Some 18 years ago Pauling[2] announced that he and the late Carl Niemann were attempting to synthesize transition-state analogues to test the hypothesis that enzymes bind transition states tightly. This has now turned into a growth industry in terms of both terminology and examples. Some are not true transition-state analogues but depend on mimicking other factors.

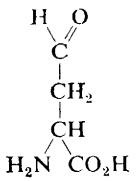
Wentworth and Wolfenden[6] have coined the term 'Reversible Inhibitor' for the interaction of D-galactal (1) with β-galactosidase. The slow inhibition of the



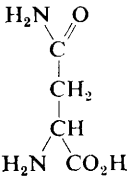
(1)

enzyme by the D-galactal is presumably due to formation of an ester bond. This supports the idea of the hydrolytic reaction occurring *via* a double-displacement pathway.

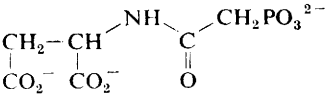
Another attempt from the same laboratory[7] was to use aspartic β-semialdehyde (2) an analogue for asparagine (3) as a probe for the mechanism of asparaginase. The rationale of this is that if the β carbonyl carbon becomes tetrahedral during



(2)



(3)

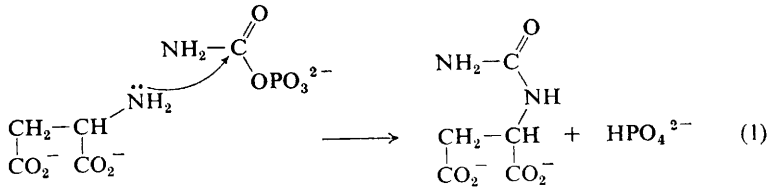


(4)

[5] K. Schray and J. P. Klinman, *Biochem. Biophys. Res. Comm.*, 1974, **57**, 641.
[6] D. F. Wentworth and R. Wolfenden, *Biochemistry*, 1974, **13**, 4715.
[7] J. O'Connor, J. O'C. Westerik, and R. Wolfenden, *J. Biol. Chem.*, 1974, **249**, 6351.

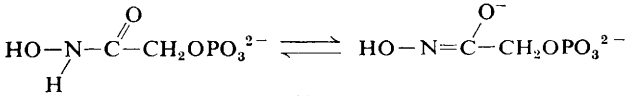
the reaction then the aldehyde should be a potent inhibitor (*cf.* aldehyde inhibitors of elastase).[8] The experiment was unfortunately inconclusive as the K_I for (2) is 3.6×10^{-6} mol l^{-1} as opposed to a K_M of 1.2×10^{-5} mol l^{-1} for (3).

More successful was the use of (*N*-phosphonacetyl)-L-aspartate (4) as an activated complex analogue in aspartate transcarbamylase.[9] This mimics the transition-state complex for reaction (1). Compound (4) is a potent inhibitor of *de novo* pyrimidine synthesis in cell cultures with $K_I = 10^{-4}$ mol l^{-1}. It should be noted that (4) is not necessarily a true transition-state analogue since the strong binding may be attributable to having the binding sites of both the carbamyl phosphate and aspartate on the one molecule.

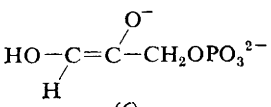


(1)

Another ingenious inhibitor is Collins'[10] enolate analogue *p*-glycolohydroxamate (5). This is analogous to the enolate intermediate (6) from glyceraldehyde 3-phosphate in the reactions of triose phosphate isomerase and yeast aldolase.

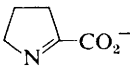


(5)

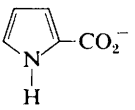


(6)

It was hoped by the authors[11] that Δ-1-pyrolline-2-carboxylate (7) would be an even better inhibitor than pyrrole-2-carboxylate (8), the classic transition-state analogue inhibitor of proline racemase, introduced by Cardinale and Abeles.[12] However, in both cases $K_I \sim K_M/160$.



(7)



(8)

[8] R. Thompson, *Biochemistry*, 1974, **13**, 5495.
[9] E. A. Swyryd, S. S. Seaver, and G. R. Stark, *J. Biol. Chem.*, 1974, **249**, 6945.
[10] K. D. Collins, *J. Biol. Chem.*, 1974, **249**, 138.
[11] M. V. Keenan and W. L. Alworth, *Biochem. Biophys. Res. Comm.*, 1974, **57**, 500.
[12] G. J. Cardinale and R. H. Abeles, *Biochemistry*, 1968, **7**, 3970.

The borinic, boronic acid inhibitors of serine proteases saga continued with examples for acetylcholine esterase and chymotrypsin.[13, 14]

4 Entropy *versus* Orbital Steering

The final blast in this controversy has now perhaps come. The reader is referred to Jencks and Page's article [15] where the death knell appears to have been tolled on orbital steering. It is clear that there is no problem in accounting in general for the magnitude of enzymic catalysis.

5 Enzyme Evolution

Hartley [16] has reviewed recent work on 'enzyme families' and strongly advocates the role of gene doubling where one gene carries through the original protein with only non-lethal mutations and the other can mutate through which would be lethal to single genes, eventually to give a new protein. It appears that the mammalian enzymes chymotrypsin, trypsin, elastase, and thrombin could have evolved from a common precursor, *i.e.* by divergent evolution. On the other hand, the bacterial serine protease subtilisin [17] has evolved to have (i) the same Asp-His-Ser charge-relay system, (ii) the same set of binding subsites for the acyl portion, and (iii) the same set of >NH backbone hydrogen-bond donors for the O^- of the tetrahedral intermediate as the mammalian enzymes, despite there being no sequence homologies, *i.e.* convergent evolution. There is one oddity in that there is a bacterial serine protease, α-lytic protease from *Streptomyces griseus*, which is 43% homologous with bovine trypsin!

Binding Domains.—From an analysis of the structural homologies of several enzymes that bind nucleotides, Rossman *et al.*[18] and Ohlsson *et al.*[19] suggest that there is a common 'fold' for the binding of NAD, ATP, ADP, *etc.* It is suggested that sophisticated enzymes have evolved by fusion of primordial genes coding for smaller, mono-functional units, *e.g.* it is predicted that the polypeptide chains of aminoacyl-tRNA synthetases should fold in at least three different domains, one for ATP binding, one for the amino-acid, and the third for the tRNA. If this theory is correct then the evolution of enzymes could be much simpler than previously thought. But Fletterick *et al.*[20] cannot fit the proposed nucleotide binding fold into the electron density for the ATP binding site of hexokinase. Either not all nucleotide binding proteins have evolved from the same primordial gene or, as Fletterick *et al.*[20] suggest, the similarities found by Rossman *et al.*[18] and Ohlsson *et al.*[19] may have arisen through a combination of divergent evolution and a limitation on the number of ways of forming certain supersecondary structures.

[13] J. D. Rawn and G. E. Lienhard, *Biochemistry*, 1974, **13**, 3124.
[14] K. A. Koehler and G. P. Hess, *Biochemistry*, 1974, **13**, 5345.
[15] W. P. Jencks and M. I. Page, *Biochem. Biophys. Res. Comm.*, 1974, **57**, 887.
[16] B. S. Hartley, 'Symposia of the Society for General Microbiology', 1974, Number XXIV, 151.
[17] J. Kraut, J. D. Robertus, J. J. Birktoft, R. A. Alden, P. E. Wilcox, and J. C. Powers, 'Cold Spring Harbor Symposia on Quantitative Biology,' 1971, **36**, 117.
[18] M. G. Rossman, D. Moras, and K. W. Olsen, *Nature*, 1974, **250**, 194.
[19] I. Ohlsson, B. Nordström, and C.-I. Brändén, *J. Mol. Biol.*, 1974, **89**, 339.
[20] R. J. Fletterick, D. J. Bates, and T. A. Steitz, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **72**, 36.

6 Methods for Examining Enzyme–Substrate Interaction

The difficulty in examining enzyme–substrate interactions is that the (active) enzyme–substrate complex generally transforms into products in the millisecond time region, thus precluding study by n.m.r., *X*-ray diffraction, and other techniques requiring the slow acquisition of data. One way of overcoming this is to work at extremely low temperatures. Douzou and co-workers [21, 22] have pioneered this technique and have examined the complexes of horse radish peroxidase in 70% DMF at −65 °C. In the past two years this has been taken up by Fink [23–27] who has been attempting to show that the reactions of chymotrypsin at low temperatures in DMSO solutions may be extrapolated to physiological conditions. This does appear to be the case. For example, the Arrhenius plot for the acylation of chymotrypsin by *N*-acetyltryptophan *p*-nitrophenyl ester was found to be linear from −45 to 0 °C and extrapolated to the expected value at 25 °C.[24] The acylenzyme *N*-acetyltryptophan chymotrypsin could be isolated from the system by chromatography at −40 °C. Fink and Wildi point out that a change from 25 to −100 °C for a reaction of an activation energy of 11.5 kcal leads to a 3×10^6-fold change in rate. They were able to separate out three phases in the hydrolysis of acetylphenylalanine methyl ester by α-chymotrypsin at −80 to −90 °C in 65% DMSO. The enzyme–substrate complex has similar fluorescence characteristics to those of the free enzyme but the acylenzyme has a lower quantum yield and a blue shift in λ_{max}. Fink and Good [28] were also able to show a burst of *p*-nitrophenol release in the reaction of β-glucosidase with *p*-nitrophenyl-β-glucoside at temperatures below which turnover occurs.

The Equilibrium Method.—An alternative technique which may be used in some cases [29] involves setting up an equilibrium system which favours the enzyme substrate complex, *e.g.*

$$R^1CONHR^2 \rightleftharpoons R^1CO_2^- + R^2NH_2$$

The addition of enzyme to this system does not disturb the overall equilibrium constant for the reaction; the system is time independent and measurements may be made at leisure. To set up a system of this type for chymotrypsin the free energies of hydrolysis of a wide series of amides were determined.[30] The semicarbazide derivatives (9) are particularly suitable; they are similar in stereochemistry to the glycinamide derivatives, very soluble, of suitable hydrolytic

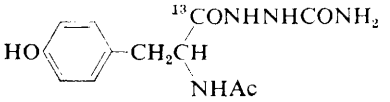
$$\text{chymotrypsin.AcPhe-NHNHCONH}_2 \rightleftharpoons \text{AcPhe} + \text{NH}_2\text{NHCONH}_2 + \text{chymotrypsin}$$

(9)

[21] P. Douzou and F. Leterrier, *Biochim. Biophys. Acta*, 1970, **220**, 330.
[22] P. Douzou, R. Sireix, and F. Travers, *Proc. Nat. Acad. Sci. U.S.A.*, 1970, **66**, 787.
[23] A. L. Fink, *Arch. Biochem. Biophys.*, 1973, **155**, 473.
[24] A. L. Fink, *Biochemistry*, 1973, **12**, 1736.
[25] A. L. Fink, *Biochemistry*, 1974, **13**, 277.
[26] A. L. Fink, *J. Biol. Chem.*, 1974, **249**, 5027.
[27] A. L. Fink and E. Wildi, *J. Biol. Chem.*, 1974, **249**, 6087.
[28] A. L. Fink and N. E. Good, *Biochem. Biophys. Res. Comm.*, 1974, **58**, 126.
[29] A. R. Fersht and M. Renard, *Biochemistry*, 1974, **13**, 1416.
[30] A. R. Fersht and Y. Requena, *J. Amer. Chem. Soc.*, 1971, **93**, 3499.

equilibrium constant, and subsequently found to be good substrates of chymotrypsin with low K_M values.[31] Fortunately, the K_M for (9) is much less than its solubility, acetylphenylalanine binds poorly and semicarbazide weakly if at all. By soaking a crystal of α-chymotrypsin in a mixture of (9) and semicarbazide at pH 4.2 and using *X*-ray diffraction methods, the binding site of (9) was located and found to be in a non-productive mode.[29] This is consistent with observations that the dimeric form of the enzyme which is in solution at low pH is inactive and the crystalline form of the enzyme appears to be this dimer.

Robillard, Shaw, and Shulman[32] applied this technique to n.m.r. methods using (10) and produced two important results. They found from ^{13}C n.m.r.



(10)

that (i) there is no strain induced in the amide linkage on binding to the enzyme and (ii) no tetrahedral intermediate accumulates.

7 Allosteric Interactions

Herzfield and Stanley[33] have presented a generalized approach to co-operativity. It combines the concepts of preferential binding and quaternary constraints (Monod, Wyman, and Changeux[34]), nearest neighbour subunit interactions (induced fit theory of Koshland, Nemethy, and Filmer[35]), and changes in subunit aggregation (Briehl[36]). Their model can explain negative co-operativity (whereas the Monod, Wyman, and Changeux model cannot) and can treat situations where quaternary changes and conformational changes are coupled with ligand binding (whereas the Koshland model cannot). It requires a lot of computing, however, to solve so many equations in so many unknowns.

Goldbeter[37] has pointed out that the Monod, Wyman, and Changeux model can account for negative *kinetic* co-operativity, *i.e.* when the two pre-existing conformational states postulated in the theory differ in their catalytic activity. Only negative co-operativity of catalysis can be explained on the model – not binding. Similarly half-of-the-sites reactivity[38] cannot be explained in terms of the concerted model.

8 Half-of-the-sites Reactivity

The phenomenon of certain enzymes with $2n$ apparently identical subunits reacting only at n of these is now well established. New examples continue to be

[31] A. R. Fersht and Y. Requena, *J. Amer. Chem. Soc.*, 1971, **93**, 7079.
[32] G. Robillard, E. Shaw, and R. G. Shulman, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 2623.
[33] J. Herzfield and H. E. Stanley, *J. Mol. Biol.*, 1974, **82**, 231.
[34] J. Monod, J. Wyman, and J.-P. Changeux, *J. Mol. Biol.*, 1965, **12**, 88.
[35] D. E. Koshland, G. Nemethy, and D. Filmer, *Biochemistry*, 1966, **5**, 365.
[36] R. W. Briehl, *J. Biol. Chem.*, 1963, **238**, 2361.
[37] A. Goldbeter, *J. Mol. Biol.*, 1974, **90**, 185.
[38] A. Levitzki, W. B. Stallcup, and D. E. Koshland, *Biochemistry*, 1971, **10**, 3371.

found, *e.g.* superoxide dismutase.[39] Two reviews have appeared during the year.[40, 41] The two questions are: why is this so and how does it arise? Four examples will be discussed.

Alkaline Phosphatase.—The usually studied enzyme is a dimer of 2 × 43 000 mol. wt. from *E. coli*. It has a broad specificity and is induced when phosphate becomes limiting for growth. The intestinal enzyme is a dimer of 2 × 69 000 mol. wt.[42] The current controversies centre on whether this enzyme does in fact exhibit half-of-the-sites reactivity and if so what is the mechanism for this. (Unless otherwise stated it is the *E. coli* enzyme that is discussed.)

The reaction proceeds through a phosphoryl enzyme:

$$E + ROPO_3^{2-} \rightleftharpoons E.ROPO_3^{2-} \xrightarrow{\text{slow}} E'.ROPO_3^{2-} \longrightarrow E'OPO_3^{2-} + ROH$$

The finding that the enzyme catalysed the hydrolysis of wide ranges of phosphate esters and anhydrides with the same V_{max} led Wilson, Dyan, and Cyr[43] to postulate that the hydrolysis of the phosphorylenzyme was rate determining. However, various rapid measuring and sampling pre-steady-state studies[44–48] show that dephosphorylation is not rate determining. It is suggested by Trentham and Gutfreund[47] that there is a rate-determining rearrangement of the enzyme or enzyme–substrate complex. It is a classical, and previously considered reliable, argument that a common V_{max} for various substrates implies a common rate-determining step and hence a common intermediate. Pre-steady-state studies must always be used in conjunction with such a steady-state work.

At low pH the rate constant for dephosphorylation decreases and becomes rate determining. Burst experiments, also at low pH, using 2,4-dinitrophenyl and 4-methylumbelliferyl phosphates reveal that only one of the two sites is acylated.[45–49] Lazdunski and co-workers have elaborated this half-of-the-sites reactivity into the 'flip-flop' mechanism.[40, 50, 51] The 'heart' of the flip-flop mechanism is a step which involves the two subunits alternating roles in a rigidly maintained reciprocating manner. One site phosphorylates, while the other dephosphorylates and binds a new substrate molecule.

First, there is evidence against the enzyme exhibiting half-of-the-sites reactivity. Bloch and Schlesinger[52] claim that the enzyme as normally isolated contains up to two molecules of tightly bound inorganic phosphate. On purging the enzyme

[39] E. M. Fielden, P. B. Roberts, R. C. Bray, D. J. Lowe, G. M. Mautner, G. Rotilio, and L. Calabrese, *Biochem. J.*, 1974, **139**, 49.
[40] M. Lazdunski, *Progr. Bio-org. Chem.*, 1974, **3**, 81.
[41] F. Seydoux, O. P. Malhotra, and S. A. Bernhard, *Crit. Rev. Biochem.*, 1974, 227.
[42] M. Fosset, D. Chappelet-Tordo, and M. Lazdunski, *Biochemistry*, 1974, **13**, 1783.
[43] I. B. Wilson, J. Dyan, and K. Cyr, *J. Biol. Chem.*, 1964, **239**, 4182.
[44] W. N. Aldridge, T. E. Barman, and H. Gutfreund, *Biochem. J.*, 1964, **92**, 23c.
[45] H. N. Fernley and P. G. Walker, *Nature*, 1966, **212**, 1435.
[46] H. N. Fernley and P. G. Walker, *Biochem. J.*, 1969, **111**, 187.
[47] D. R. Trentham and H. F. Gutfreund, *Biochem. J.*, 1968, **108**, 455.
[48] T. W. Reid and I. B. Wilson, *Biochemistry*, 1971, **10**, 380.
[49] S. H. D. Ko and F. J. Kezdy, *J. Amer. Chem. Soc.*, 1967, **89**, 7139.
[50] M. Lazdunski, C. Petitclerc, D. Chappelet, and C. Lazdunski, *European. J. Biochem.*, 1971, **20**, 124.
[51] M. Lazdunski, *Current Topics Cell Reg.*, 1972, **6**, 267.
[52] W. Bloch and M. J. Schlesinger, *J. Biol. Chem.*, 1973, **248**, 5794.

of endogenous phosphate they find that they can then bind 1.5—2.2 mol of phosphate per mol of enzyme. The purged enzyme also reacts with 2,4-dinitrophenyl phosphate to give a 'burst' of 1.5 mol of phosphate per mol of enzyme.

In a second set of ingenious experiments, Bloch and Schlesinger [53] prepared the enzyme from a mutant which gives a catalytically inactive enzyme. They then hybridized the inactive mutant with the wild-type enzyme to give a dimer consisting of one inactive and one active subunit. This hybrid gives half the burst amplitude and half the steady-state rate of the native enzyme. The authors conclude that 'it is most important now to re-examine whether any functional subunit interaction occurs in the enzyme rather than to continue the search for mechanisms to explain an interaction that may not exist'.

Further evidence against the flip-flop mechanism has been supplied by Kelly, Sperow, and Butler [54] for both the *E. coli* and intestinal enzymes. They find that unreactive analogues of pyrophosphate bind to enzyme and stimulate the hydrolysis of *p*-nitrophenyl phosphate. This is consistent with the second site in the dimer having a binding rather than a catalytic role and not the alternating effect required by 'flip-flop'.

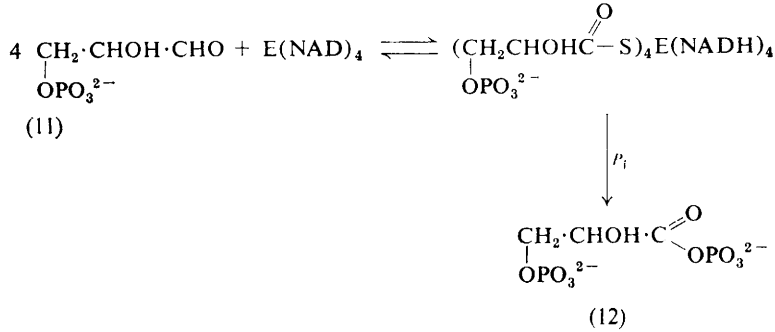
Against all this, further evidence in favour of subunit interactions has been presented from Lazdunski's laboratory for the enzymes from *E. coli* [55] and the intestine.[56] For this latter enzyme it was found from equilibrium dialysis that there is one strong site and one weak site for phosphate binding at pH 8.0. Stopped-flow experiments at acid pH with 2,4-dinitrophenyl phosphate show a biphasic release of phosphate, one at $>1000\ s^{-1}$ and the other at $100\ s^{-1}$. Furthermore, quenching of the phosphoenzyme formed at pH 7.5—8 with [^{32}P]AMP gives only one site acylated. Similarly only one mol of 2,4-dinitrophenol is released in the 'burst' from 2,4-dinitrophenyl phosphate at this pH. This system is complicated in that there appear to be two forms of the enzyme and the equilibrium between the two is affected by phosphate or substrates.[48, 52] This may lead to artefacts in the pre-steady-state kinetics.

It seems clear that the flip-flop mechanism does not hold. Further evidence is required before it is definitely known whether the negative co-operativity of substrate binding and half-of-the-sites reactivity are artefactual or not.

Glyceraldehyde-3-phosphate Dehydrogenase.—This is a ubiquitous enzyme, a tetramer of *ca.* 150 000 mol. wt. consisting of four identical chains. It catalyses the oxidative phosphorylation of glyceraldehyde-3-phosphate (11) to 1,3-diphosphoglycerate (12) *via* an acyl(thio)enzyme intermediate.

Steady-state and Pre-steady-state Kinetics and the Reaction Mechanism. Until this year steady-state and pre-steady-state studies did not really tally. However, Duggleby and Dennis [57–59] have purified the enzyme from *Pisum sativum* and have

[53] W. Bloch and M. J. Schlesinger, *J. Biol. Chem.*, 1974, **249**, 1755.
[54] S. J. Kelly, J. W. Sperow, and L. G. Butler, *Biochemistry*, 1974, **13**, 3503.
[55] D. Chappelet-Tordo, M. Iwatsubo, and M. Lazdunski, *Biochemistry*, 1974, **13**, 3754.
[56] D. Chappelet-Tordo, M. Fosset, M. Iwatsubo, C. Gache, and M. Lazdunski, *Biochemistry*, 1974, **13**, 1788.
[57] R. G. Duggleby and D. T. Dennis, *J. Biol. Chem.*, 1974, **249**, 162.
[58] R. G. Duggleby and D. T. Dennis, *J. Biol. Chem.*, 1974, **249**, 167.
[59] R. G. Duggleby and D. T. Dennis, *J. Biol. Chem.*, 1974, **249**, 175.



(11) (12)

produced very satisfactory data. The difficulty of non-linear initial rate plots is apparently due to (i) the instability of glyceraldehyde-3-phosphate under the conditions of the assay and (ii) the product inhibition by 1,3-diphosphoglycerate. The latter problem is not overcome by replacing phosphate by arsenate in the assay mixture as is usually done, since the 1-arseno-3-phosphoglyceric acid formed is not rapidly hydrolysed and is a potent inhibitor of the enzyme.[60] The first difficulty is overcome by initiating the reaction with the glyceraldehyde-3-phosphate and the second by removing the 1,3-diphosphoglyceric acid using 3-phosphoglycerate kinase and ADP. The results obtained are consistent with the mechanism of Trentham,[61, 62] given the effect of the difference in assay conditions. Both authors agree that the free enzyme is not part of the reaction cycle.

Subunit Interactions. Current problems concern whether or not there is half-of-the-sites reactivity and whether the negative co-operativity of NAD binding is due to a pre-existing asymmetry in the apoenzyme or is induced by coenzyme binding. Although high-resolution *X*-ray diffraction data have been published,[63, 64] these are on the holoenzyme. (It should be noted that the interaction between Lys-183 of one subunit and the pyrophosphate moiety of NAD bound in another mentioned in ref. 63 is retracted in the section added in proof at the end.)

It appears that with the physiological substrate all four sites are reactive with apparently equal reactivity.[61] It is only with bulky artificial substrates such as β-(2-furyl)acryloyl phosphate[65] that half-of-the-sites reactivity occurs with only two of the sites reacting. Levitzki[66, 67] suggests that the interactions of the alkyl (or acyl) group of an alkylating (or acylating) reagent on the reactive thiol with the nicotinamide or glyceraldehyde-3-phosphate binding sites are not transmitted to the other subunits. But interactions of the alkyl group with the adenine subsite induce a conformational change that is transmitted to a vacant

[60] J. Teipel and D. E. Koshland, jun., *Biochim. Biophys. Acta*, 1970, **198**, 183.
[61] D. R. Trentham, *Biochem. J.*, 1971, **122**, 71.
[62] P. J. Harrigan and D. R. Trentham, *Biochem. J.*, 1974, **143**, 353.
[63] M. Buehner, G. C. Ford, D. Moras, K. W. Olsen, and M. G. Rossmann, *J. Mol. Biol.*, 1974, **82**, 563.
[64] M. Buehner, G. C. Ford, D. Moras, K. W. Olsen, and M. G. Rossmann, *J. Mol. Biol.*, 1974, **90**, 25.
[65] R. A. MacQuarrie and S. A. Bernhard, *Biochemistry*, 1971, **10**, 2456.
[66] J. Schlessinger and A. Levitzki, *J. Mol. Biol.*, 1974, **82**, 547.
[67] A. Levitzki, *J. Mol. Biol.*, 1974, **90**, 451.

subunit across one of the three sets of isologous intersubunit binding domains so inducing half-of-the-sites reactivity. (The proposed interaction *via* Lys-183 has to be amended – see above.) The authors [66] also argue in favour of an induced asymmetry on NAD binding. Using a fluorescent analogue of NAD, N^6-etheno-adenine dinucleotide, and amongst other techniques, the circular polarization of luminescence technique of Steinberg, it is found that the greatest change in the structure of the tetramer occurs on the binding of the first NAD. The fluorescent analogue of NAD is useful not only as a spectral probe but also, since it binds more weakly than NAD, the otherwise experimentally inaccessible tight binding constants may be measured. The results are given in Table 1.

Table 1 *Negative co-operativity in the binding of NAD and its fluorescent analogue, εNAD with rabbit muscle glyceraldehyde-3-phosphate dehydrogenase* [66]

	Dissociation constant (M)	
	εNAD	NAD
K_1	$< 10^{-6}$	$< 10^{-11}$
K_2	$< 10^{-6}$	$< 10^{-9}$
K_3	3.1×10^{-6}	$0.3—4 \times 10^{-6}$
K_4	1.75×10^{-5}	$2—3.5 \times 10^{-5}$

The other point of view, *i.e.* a pre-existing asymmetry, is taken by Bernhard and his group.[68, 69] From an analysis of the reactivity of the four fast reacting thiols in the sturgeon enzyme, Seydoux and Bernhard [69] postulate a rather complex model for the holoenzyme, involving two sets of sites with C_2 symmetry but involving intramolecular transconformational isomerization of tight and loose sites.

Hexokinase.—With regard to the last points, *X*-ray diffraction studies on the B II form of hexokinase,[70] which is probably the physiologically important form, show that the subunits in the dimer are identical but the dimer is asymmetric. That is, there is a pre-existing asymmetry in the molecule which accounts for the negative homotropic interactions, *etc.*

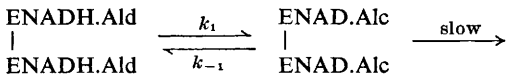
Liver Alcohol Dehydrogenase.—This dehydrogenase is a symmetric dimer. The high-resolution *X*-ray diffraction structure of the enzyme was solved last year.[71] The NAD binds in an open conformation as found for the glyceraldehyde phosphate dehydrogenase.

Again it is disputed whether the oxidation of alcohols or the reverse reaction, the reduction of carbonyl groups, involves half-of-the-sites reactivity.[72–78] The

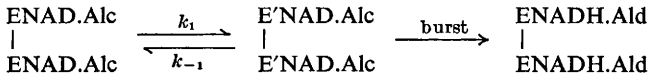
[68] F. Seydoux, S. Bernhard, O. Pfenninger, M. Payne, and O. P. Malhotra, *Biochemistry*, 1973, **12**, 4290.
[69] F. Seydoux and S. Bernhard, *Bioorg. Chem.*, 1974, **1**, 161.
[70] W. F. Anderson, R. J. Fletterick, and T. A. Steitz, *J. Mol. Biol.*, 1974, **86**, 261.
[71] C. I. Brändén, H. Eklund, B. Nordström, T. Boiwe, G. Soderlund, E. Zeppezauer, I. Ohlsson, and A. Akeson, *Proc. Nat. Acad. Sci. U.S.A.*, 1973, **70**, 2439.
[72] S. A. Bernhard, M. F. Dunn, P. L. Luisi, and P. Schack, *Biochemistry*, 1970, **9**, 185.
[73] M. F. Dunn and S. A. Bernhard, *Biochemistry*, 1971, **10**, 4569.
[74] J. T. McFarland and S. A. Bernhard, *Biochemistry*, 1972, **11**, 1486.
[75] P. L. Luisi and E. Bignetti, *J. Mol. Biol.*, 1974, **88**, 653.
[76] H. Theorell and K. Tatemoto, *Arch. Biochem. Biophys.*, 1971, **142**, 69.
[77] J. J. Holbrook and H. F. Gutfreund, *F.E.B.S. Letters*, 1973, **31**, 157.
[78] N. H. Hijazi and K. J. Laidler, *Biochem. Biophys. Acta*, 1973, **315**, 209.

Bernhard school[72–75] claims kinetically non-equivalent sites as in Scheme 1 (where Ald = aldehyde and Alc = alcohol). Other workers[76–78] claim that there is no subunit interaction and that half-of-the-sites reactivity is due to unfavourable equilibria between enzyme-bound intermediates, *e.g.* in Schemes 2 and 3, $k_{-1}/k_1 \sim 1$.*

Scheme 1



Scheme 2



Scheme 3

Luisi and Bignetti[75] now claim a 'flip-flop' type mechanism.[40, 79] They show that the half-of-the-sites reactivity in the oxidation of benzyl alcohol is independent of reagent concentrations, pH, temperature, and the substitution of analogues for NAD and using other alcohols. This makes Schemes 2 and 3, where $k_1 \sim k_{-1}$, seem unlikely. Using isobutyramide, which binds tightly with NADH at the active site, the reaction stops when one mol of NADH is produced per enzyme dimer. The authors claim that the only mechanism that fits all their experimental data is one in which the two subunits have distinct and alternating functions during each enzyme cycle, *e.g.* triggering of the chemical transformation and the transformation itself.

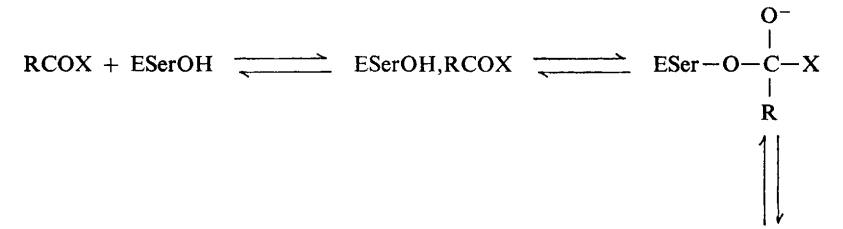
The conclusion concerning the occurrence of half-of-the-sites reactivity is that although it does appear to widespread many of the examples may be artefactual. To quote Tartakover, the chess grandmaster, the mistakes are all there waiting to be made. Assuming the phenomenon does exist, the problem is now why and what is its physiological role?

9 Chymotrypsin and the Serine Proteases

The currently accepted mechanism for the hydrolysis of amides and esters by the serine proteases is shown in Scheme 4. Chymotrypsin is specific for both the acyl portion R (tryptophan, phenylalanine, and tyrosine) and in its physiological substrates, the leaving group X.[80]

[79] K. Harada and R. G. Wolfe, *J. Biol. Chem.*, 1968, **243**, 4123.
[80] A. R. Fersht, D. M. Blow, and J. Fastrez, *Biochemistry*, 1973, **12**, 2035.

* Schemes 1—3 represent possible interpretations of half-of-the-sites reactivity. In Scheme 1 the two subunits are non-equivalent. In Schemes 2 and 3 there is an equilibrium constant of unity either between reagents and products bound to the enzyme or between two isomeric complexes.[75]

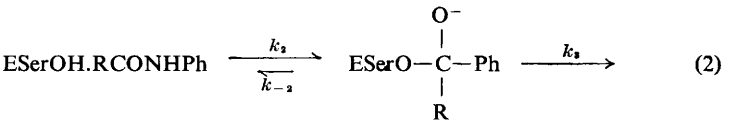


Scheme 4

Evidence for Intermediates on the Reaction Pathway.—The enzyme–substrate complex is indicated from dye displacement studies and the use of chromophoric substrates.[81] The first evidence for the acyl-enzyme, ESerOCOR, was first presented over 20 years ago [82] and subsequently confirmed several times using reactive ester substrates where the acyl-enzyme actually accumulates as the major solution species. The ultimate proof of acylation on Ser-195 is the solution of the structures of crystalline indoleacryloyl- and carbamoyl-chymotrypsin by *X*-ray diffraction techniques.[83, 84]

The acyl-enzyme mechanism for peptide and amide substrates was only established beyond doubt last year.[85] The acyl-enzyme does not accumulate in this case so the detection has to be indirect. It was shown that (i) the generation of a known acyl-enzyme from an ester substrate partitioned in the same ratio between water and added amine and peptide nucleophiles as did the hydrolysis of peptide substrates, (ii) the attack of the nucleophile occurred after the rate-determining step in the hydrolysis of amides, and (iii) the overall rate constants for the hydrolytic reaction calculated from the individual rate constants measured for the acyl-enzyme mechanism accounted for the observed value. The current problem concerns the existence of the tetrahedral intermediate.

The Search for the Tetrahedral Intermediate.—*Amides and Anilides.*—The first evidence for a tetrahedral intermediate came from a study of the pH dependence of the hydrolysis of acetyl-L-tryptophan- and acetyl-L-tyrosine-anilides and from the variation of k_{cat} and K_M with anilide structure.[86, 87] It was furthermore proposed that the substrate was actually bound as the tetrahedral intermediate, which was suggested to be the predominant species.



(2)

$$k_2 > k_{-2}, \quad k_3 < k_{-2}$$

[81] G. P. Hess, J. McConn, E. Ku, and G. McConkey, *Phil. Trans. Roy. Soc.*, 1970, **B257**, 89.
[82] B. S. Hartley and B. A. Kilbey, *Biochem. J.*, 1954, **56**, 288.
[83] R. Henderson, *J. Mol. Biol.*, 1970, **54**, 341.
[84] G. T. Robillard, J. C. Powers, and P. E. Wilcox, *Biochemistry*, 1972, **11**, 1773.
[85] J. Fastrez and A. R. Fersht, *Biochemistry*, 1973, **12**, 2025.
[86] M. Caplow, *J. Amer. Chem. Soc.*, 1969, **91**, 3639.
[87] E. C. Lucas and M. Caplow, *J. Amer. Chem. Soc.*, 1972, **94**, 960.

These arguments proved to be illusory as the variations in k_{cat}, K_M, and the pH dependence of the hydrolysis are due to non-productive binding artefacts.[88] Both the anilide and the indole ring of tryptophan or the phenolic ring of tyrosine compete for the aromatic binding pocket of the enzyme. Hydrophobic substituents on the anilide ring favour the non-productive binding (see Figure 1).

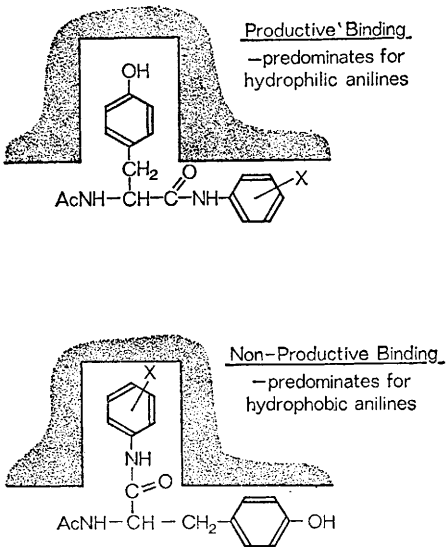


Figure 1 *Illustration of the productive and non-productive binding modes of tyrosine-anilide substrates and chymotrypsin*
(Reproduced by permission from *Biochemistry*, 1973, **12**, 2035)

Unfortunately, some of the common substituents used in structure reactivity studies, *e.g.* MeO, Cl, have correlated Hammett σ-values and Hansch[89] (hydrophobic) π-values, so, in general, care has to be taken not to confuse substituent effects on non-productive binding/productive binding ratios with inductive and mesomeric electronic effects on the actual reaction. There is also spectrophotometric evidence against the accumulation of a tetrahedral intermediate in anilide hydrolysis using a chromogenic substrate.[88] N.m.r. experiments have also failed to detect the accumulation of an intermediate in the hydrolysis of a hydrazide.[32]

Another approach has been the attempt to detect the intermediates kinetically by the effect of pH on the partition of their breakdown to reagents and products. The pH dependence of k_{cat} for formylhydrazide and semicarbazide substrates and of $^{14}N/^{15}N$ kinetic isotope effects in amide hydrolysis has led to the suggestion that there is a (tetrahedral) intermediate on the reaction pathway and at low pH its formation is (partially) rate determining but at high pH its breakdown is (partially) rate determining in these cases.[31] A very detailed re-examination

[88] J. Fastrez and A. R. Fersht, *Biochemistry*, 1973, **12**, 1067.
[89] C. Hansch and E. Coats, *J. Pharm. Sci.*, 1970, **59**, 731.

of the pH dependence of the kinetic parameters show that there is a *small* effect consistent with the occurrence of a tetrahedral intermediate whose partitioning is pH dependent in the case examined.[29]

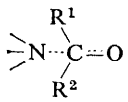
Esters. Hirohara *et al.*[90] have put forward an argument based on the 'element effect' in favour of rate-determining formation of a tetrahedral intermediate in the acylation of chymotrypsin by specific ester substrates. The rate constant for the acylation of chymotrypsin by acetyltryptophan *p*-nitrophenyl ester is similar to that by the *p*-nitrothiophenyl ester, and that by the ethyl ester is similar to that by the thioethyl ester, despite the thio-groups being better leaving groups. This evidence is inadequate on two points: (i) enzymes require a precise stereochemical fit with their substrates so the substitution of a large sulphur atom for a smaller oxygen may lead to an unfavourable interaction compensating for the enhanced reactivity (as found on substitution of sulphur for oxygen in the nucleophilic serine of subtilisin); and (ii) the 'element effect' has been shown to be unreliable as a guide to the rate-determining step in non-enzymatic ester hydrolysis.[91]

To summarize, the evidence for a tetrahedral intermediate is not yet conclusive, although the Reporter, for one, believes it to be on the reaction pathway. This lack of firm evidence is not surprising in view of the prediction that intermediates should not accumulate on enzymatic reaction pathways.

Trypsin (Chymotrypsin)–Protein Inhibitor Complexes.—There are many naturally occurring polypeptides that bind specifically to trypsin and/or chymotrypsin and other serine proteases with dissociation constants as low as 10^{-13} mol l^{-1}. Apart from their biological interest they have become important to our understanding of the serine proteases. The scene was first set when the structure of the pancreatic trypsin inhibitor (solved at Münich) was fitted into the active site of chymotrypsin (solved at Cambridge).[92] This interaction was later used in conjunction with solution studies to deduce the conformation of a peptide substrate bound at the active site of chymotrypsin and to give the most complete stereochemical description of the hydrolytic mechanism of the enzyme yet.[80] The mechanism by which 'strain' is introduced is also described. In the past year the structures of the crystallized complexes of the soyabean and pancreatic inhibitors with trypsin have been solved.[93–95] The complexes appear to exist as the tetrahedral adduct between the hydroxy-group of Ser-195 of the enzyme and the reactive carbonyl group of the peptide backbone of the inhibitor. However, the bond distances around the tetrahedral carbon are not quite correct,[93a] the distance between the $^{\gamma}O$ of Ser-195 and the supposedly tetrahedral carbon is 2 Å.

[90] H. Hirohara, M. L. Bender, and R. S. Stark, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 1643.
[91] A. R. Fersht and W. P. Jencks, *J. Amer. Chem. Soc.*, 1970, **92**, 5442.
[92] D. M. Blow, C. S. Wright, D. Kukla, A. Rühlmann, W. Steigemann, and R. Huber, *J. Mol. Biol.*, 1972, **69**, 137.
[93] R. Huber, D. Kukla, W. Bode, P. Schwager, K. Bartels, J. Deisenhofer, and W. Steigemann, *J. Mol. Biol.*, 1974, **89**, 73.
[93a] A. Rühlmann, D. Kukla, P. Schwager, K. Bartels, and R. Huber, *J. Mol. Biol.*, 1973, **77**, 417.
[94] D. M. Blow, J. Janin, and R. M. Sweet, *Nature*, 1974, **249**, 54.
[95] R. M. Sweet, H. T. Wright, J. Janin, C. H. Chothia, and D. M. Blow, *Biochemistry*, 1974, **13**, 4212.

It may be that a true tetrahedral intermediate is not formed. Work from Dunitz's laboratory[96] on the structure of small cyclic carbonyl compounds shows that a whole range of intermediate structures (13) exist, where the N···C distance shortens, the C=O bond lengthens and the carbon moves out of the



(13)

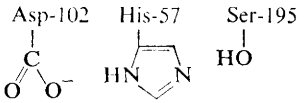
R^1R^2O plane. However, Quast *et al.*,[97] in a rapid kinetics study, have shown that association of the pancreatic trypsin inhibitor with chymotrypsin is a two-step process.

Is the Tetrahedral Intermediate Important for the Stability of the Complex?—The tight binding of the complexes was at first puzzling, and the hypothesis that the complex is an acyl enzyme[98] (of the cleaved inhibitor and Ser-195) was put forward. However, a dissociation constant of 10^{-13} mol l^{-1} is not difficult to account for. The small molecule isoleucine binds to the isoleucyl-tRNA synthetase with a dissociation constant of 5×10^{-6} mol l^{-1}: three such interactions combined would give a dissociation constant of 1.25×10^{-16} mol l^{-1} (even ignoring the favourable entropy terms)! The problem in accounting for enzyme–substrate interactions is not why are they so strong, since there is plenty of binding energy available, but why observed K_M values for enzymes are so high. That is, how is the available intrinsic binding energy of the enzyme and substrate used for catalysis rather than binding? Quast *et al.*[97] showed that the first step in the association of the pancreatic trypsin inhibitor with trypsin accounted for 30% of the total binding energy. Ako *et al.*[99] and Vincent *et al.*[100] have determined the binding of the above inhibitor to anhydrotrypsin (where the OH of Ser-195 has been chemically removed). K_{diss} increases from 6×10^{-14} mol l^{-1} with the native enzyme to 1.1×10^{-13} mol l^{-1} for the anhydro form. Similarly the Kazal inhibitor binds with dissociation constants of 3.3×10^{-11} and 3.4×10^{-10} mol l^{-1}, respectively.[100] Clearly, the major forces in the associations are non-covalent.

The crystallographic studies are important models for protein–protein interactions as well as for the specific mechanisms in this case. The folding of the basic pancreatic trypsin inhibitor investigated by the trapping of the mixed disulphide bridge intermediates on renaturation of the denatured reduced protein has proved to be an important entry into the general problem of folding.[101–103]

[96] J. D. Dunitz and F. K. Winckler, *Acta Cryst.*, 1975, **B31**, 251.
[97] U. Quast, J. Engel, H. Neumann, G. Krause, and E. Steffen, *Biochemistry*, 1974, **13**, 2512.
[98] M. Laskowski and R. W. Sealock, 'The Enzymes', ed. P. D. Boyer, Academic Press, New York, 1971, Vol. 3, p. 376.
[99] H. Ako, R. J. Foster, and C. A. Ryan, *Biochemistry*, 1974, **13**, 132.
[100] J.-P. Vincent, M. Peron-Renner, J. Pudles, and M. Lazdunski, *Biochemistry*, 1974, **13**, 4205.
[101] T. E. Creighton, *J. Mol. Biol.*, 1974, **87**, 563.
[102] T. E. Creighton, *J. Mol. Biol.*, 1974, **87**, 579.
[103] T. E. Creighton, *J. Mol. Biol.*, 1974, **87**, 603.

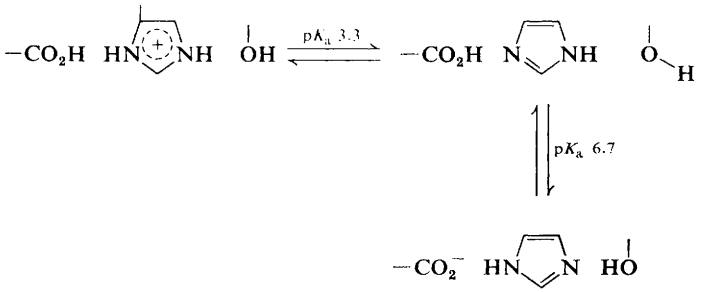
The Charge Relay System.—The most unexpected result to emerge from the *X*-ray diffraction work on α-chymotrypsin was the buried Asp-102 (so surprising in fact that the sequence was revised from Asn-102). Blow, Birktoft, and Hartley hypothesized [104] that this residue was ionized at physiological pH as in (14) to



(14)

give the so-called charge relay system. It is not possible to detect if this group is ionized by directly titrating the enzyme since this would require measurements at pH < 2 where the 'background' of protons in solution is too high. This difficulty was overcome by converting the surface carboxyls into amides by carbodi-imide coupling with semicarbazide and then determining the proton uptake on denaturation.[105] In this way it was shown that the active form of the enzyme contains the Asp-102 ionized.

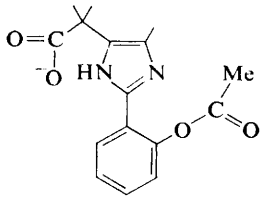
Where is the Proton at low pH?—Hunkapiller *et al.*[106] interpret their n.m.r. data on the coupling constant between the ^{13}C-2 carbon and its directly bonded hydrogen of the His-57 of α-lytic protease (assumed to be identical functionally with chymotrypsin) to indicate that the microscopic ionizations of the enzyme are as in reaction (3). They postulate that the effective base in the system is the



(3)

Asp-102 and not the His-57. The pK_a of 3.3 for the first ionization of the system does not appear to hold in chymotrypsin as the proton uptake experiments mentioned above [105] and the pH dependence of catalysis [29] suggest that this ionization occurs at pH < 2. Robillard and Shulman [107] have assigned the proton between Asp-102 and His-57 in several serine proteases at −15 to −18 p.p.m. in their ^1H n.m.r. experiments. However, they assign the location of the proton at low pH to the imidazole thus:

[104] D. M. Blow, J. J. Birktoft, and B. S. Hartley, *Nature*, 1969, **221**, 337.
[105] A. R. Fersht and J. Sperling, *J. Mol. Biol.*, 1973, **74**, 137.
[106] M. W. Hunkapiller, S. H. Smallcombe, D. P. Whitaker, and J. H. Richards, *Biochemistry*, 1973, **12**, 4732.
[107] G. Robillard and R. G. Shulman, *J. Mol. Biol.*, 1974, **86**, 519.



(15)

Rogers and Bruice[108] have made a model for the charge relay system (15) where the $-CO_2^-$ is locked against the imidazole NH. Interestingly, they find that at low pH, even in a 94 : 6 mixture of MeCN : H_2O, the low pH form is the zwitterionic $CO_2^-ImH^+$ and that the presence of the $-CO_2^-$ contributes no more than a three-fold increase in the rate of the intramolecularly catalysed deacylation of the phenolic ring by the imidazole. The role of the charge relay system in the enzyme is not clear. Fersht and Sperling[105] and Fastrez and Fersht[85] suggest that the functions of Asp-102 are (i) to constrain the position of the His-57 imidazole to provide a rigid active site so that stereochemical contacts are precisely defined and 'strain' effects may be exerted, and (ii) to polarize and raise the effective pK_a of His-57. Hunkapiller *et al.*[106] suggest that charge separation is minimized by Asp-102 acting as the accepting base for the proton that is being transferred.

Conformational Equilibria.—The activation of chymotrypsinogen by trypsin involves the cleavage of the Arg-15—Ile-16 peptide bond. The newly released α-amino-group of Ile-16 then moves to form a buried salt bridge with the carboxylate of Asp-194. In doing so this forms the binding pocket for the aromatic acyl portion of the substrate and positions the backbone NH groups of Gly-193 and Ser-195 for binding the tetrahedral intermediate oxyanion.[109–111] These changes may be mimicked in solution by the effect of pH on the protonation state of the Ile-16. At high pH the salt bridge between Ile-16 and Asp-194 becomes broken as the α-NH_3^+ of Ile-16 discharges its proton. Studies of these equilibria have enabled the strength of the salt bridge to be calculated and the effect on the pK_a's of the group to be determined. This approach showed that the pK_a of Ile-16 is 7.85 when free but increases to 9.96 when in the salt bridge.[112]

This approach has now been applied to the acidic transition at low pH due to the titration of Asp-194.[113] When this is protonated the salt bridge is again broken. The pK_a of Asp-194 is 1.5 when in the salt bridge and 4 when free.

Catalytic Activity of the Zymogen.—It was shown by Fersht and Sperling[105] that the charge relay system is intact in the zymogen. It has now been shown that chymotrypsinogen does have some residual activity.[114] Towards *p*-guanidobenzoic

[108] G. A. Rogers and T. C. Bruice, *J. Amer. Chem. Soc.*, 1974, **96**, 2473.
[109] S. T. Freer, S. Kraut, J. D. Robertus, H. T. Wright, and Ng H. Xuong, *Biochemistry*, 1970, **9**, 1997.
[110] H. T. Wright, *J. Mol. Biol.*, 1973, **79**, 1.
[111] H. T. Wright, *J. Mol. Biol.*, 1973, **79**, 13.
[112] A. R. Fersht, *J. Mol. Biol.*, 1972, **64**, 497.
[113] J.-R. Garel, S. Epely, and B. Labouesse, *Biochemistry*, 1974, **13**, 3117.
[114] A. Gertler, K. A. Walsh, and H. Neurath, *Biochemistry*, 1974, **13**, 1302.

acid *p*-nitrophenyl ester the zymogen is 10^6—10^7 times less reactive than chymotrypsin. However, an acyl-zymogen is formed that deacylates only 70 times more slowly than the acyl chymotrypsin. It should be noted that this is a very poor substrate and that the activity towards specific substrates is virtually non-existent. However, results such as these emphasize the importance of stereochemical fit in these reactions, and that the catalytic activity cannot be localized in any one function, *e.g.* the charge relay system.[115]

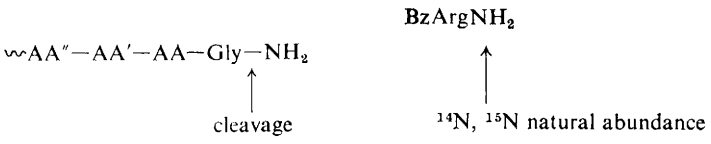
Miscellaneous.—An interesting artefact has been discovered in the deacylation of non-specific acyl-enzymes.[116] When the deacylation rate constant of α-benzamido-*trans*-cinnamoyl-chymotrypsin is measured directly using substrate quantities and first-order kinetics the rate constant is 0.102 s^{-1}. Using zero-order plots and catalytic quantities of enzyme the value falls to 0.033 s^{-1}. The difference is due to impurities in the chymotrypsin which are proportional to the amount of enzyme used. This presumably has led to artefacts in some well-known studies of the pH dependence of deacylation.[116]

Interesting crystallographic studies have been performed on trypsin.[117–119]

10 Papain and the Thiol Proteases

Lowbridge and Fruton [120] have investigated the active site of papain using polypeptides with *C*-terminus Glu-Leu-Gly (which cleave between the Glu and Leu). The results are consistent with there being an extended active site [121] and with the main specificity site being a hydrophobic amino-acid, AA in (16).[122]

On the other hand, a systematic investigation [123] of the specificity of ficin and bromelain shows that both enzymes readily cleave glycyl-, alanyl-, and leucyl-bonds. Under more vigorous conditions valyl-, phenylalanyl-, tyrosyl-, and certain other bonds will also be cleaved.



(16) (17)

Following up the $^{14}N/^{15}N$ kinetic isotope effect work on the chymotrypsin-catalysed hydrolysis of amides,[124] O'Leary's group [125] has determined this isotope effect for *N*-benzoylarginamide (17) with papain. Hydrolysis of the

[115] A. R. Fersht, *F.E.B.S. Letters*, 1973, **29**, 283.
[116] J. De Jersey, D. T. Keough, J. K. Stoops, and B. Zerner, *European J. Biochem.*, 1974, **42**, 237.
[117] R. M. Stroud, L. M. Kay, and R. E. Dickerson, *J. Mol. Biol.*, 1974, **83**, 185.
[118] M. Krieger, L. M. Kay, and R. M. Stroud, *J. Mol. Biol.*, 1974, **83**, 209.
[119] J. L. Chambers, G. G. Christoph, M. Krieger, L. M. Kay, and R. M. Stroud, *Biochem. Biophys. Res. Comm.*, 1974, **59**, 70.
[120] J. Lowbridge and J. S. Fruton, *J. Biol. Chem.*, 1974, **249**, 6754.
[121] I. Schechter and A. Berger, *Biochem. Biophys. Res. Comm.*, 1967, **27**, 157.
[122] O. K. Behrens and M. Bergmann, *J. Biol. Chem.*, 1939, **129**, 587.
[123] A. A. Kartt, J. A. Hinds, and B. Zerner, *Biochemistry*, 1974, **13**, 2029.
[124] M. H. O'Leary and M. D. Kluetz, *J. Amer. Chem. Soc.*, 1972, **94**, 3505.
[125] M. H. O'Leary, M. Urberg, and A. P. Young, *Biochemistry*, 1974, **13**, 2077.

lighter isotope compound proceeds 2.5% faster, the isotope effects being 1.025, 1.024, and 1.025 at pH 4, 6, and 8, respectively. These are considerably larger than with chymotrypsin.[124] It is suggested that there is either a rate-determining breakdown of a tetrahedral intermediate (reaction 5) or a concerted reaction

$$\text{E.RCONH}_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{ES}-\overset{\overset{\text{O}^-}{|}}{\underset{\underset{\text{R}}{|}}{\text{C}}}-\text{NH}_2 \xrightarrow[k_2]{\text{slow}} \text{ESCOR} \qquad (5)$$

where RCO_2H = BzArg

involving no discrete intermediate. For chymotrypsin $k_2 \sim k_1$,[30, 124] but here $k_2 \ll k_{-1}$ (and $k_{-1} > k_1$ presumably).

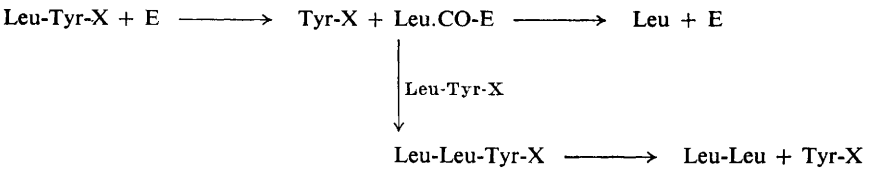
11 Pepsin and the Acid Proteases

Pepsin has a broad specificity for hydrophobic residues on either side of the bond which is cleaved. Extensive studies from Fruton's laboratory [126] have shown that the specificity of the enzyme is manifested in k_{cat} and not in K_M, *i.e.* the additional binding energy in the specific substrates is being used for strain-induced fit (or to overcome non-productive binding). These results have now been extended to another acid proteinase, cathepsin D.[127] It is also shown that the extended active site of pepsin can accommodate at least seven peptide residues.

There are considerable problems concerning the mechanism.[128] The main difficulty is the uncertainty of what, if any, intermediates occur on the reaction pathway. Evidence for an amino-enzyme, ECONHR, has come from transpeptidation experiments using product analysis techniques. Russian workers [129] have now produced a direct spectrophotometric technique for determining the kinetics of transpeptidation by using *N*-benzyloxycarbonyl-*p*-nitro-L-phenylalanine as the carboxyl acceptor, and *N*-acetyl-L-phenylalanine-L-tyrosine and *N*-acetyl-L-tyrosine-L-tyrosine, *i.e.* two substrates that should generate the same amino-enzyme, ECOTyr. They found that (i) both substrates gave the same transpeptidation rate constants, (ii) the ratio between transpeptidation and hydrolysis is the same for both, and (iii) the K_M values for transpeptidation are consistent with the amino-enzyme theory. This is good evidence for there being a common ECOTyr amino-enzyme intermediate. They further point out that some earlier results [130] where transpeptidation was not found are probably due to the transpeptidation/hydrolysis ratio being low.

Until now the only evidence in favour of an acyl-enzyme intermediate, EOCOR, has come from ^{18}O exchange experiments between a virtual substrate and water. Direct evidence for this has now been produced.[131] Both pepsin and penicillopepsin catalyse the hydrolysis and transpeptidation of Leu-Tyr-X (where

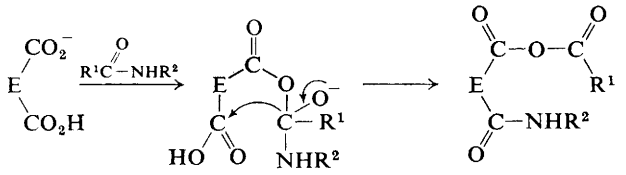
[126] J. S. Fruton, 'The Enzymes', ed. P. D. Boyer, Academic Press, New York, 1971, Vol. 3, p. 120.
[127] P. S. Sampath-Kumar and J. S. Fruton, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 1070.
[128] G. E. Clement, *Progr. Bio-org. Chem.*, 1973, **2**, 177.
[129] V. Antonov, L. D. Rumsh, and A. G. Tikhodeeva, *F.E.B.S. Letters*, 1974, **46**, 29.
[130] M. S. Silver and M. Stoddard, *Biochemistry*, 1972, **11**, 191.
[131] M. Tahashi, T. T. Wang, and T. Hofmann, *Biochem. Biophys. Res. Comm.*, 1974, **57**, 39.



Scheme 5

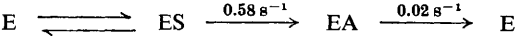
X = Leu or NH_2), according to Scheme 5. Although these experiments do not exclude the possibility of transpeptidation occurring *via* an amino-enzyme intermediate, the authors feel that an acyl enzyme is more likely since with Leu-Leu-Tyr-Leu (i) 3—4 times as much Tyr-Leu as Leu-Tyr is formed and (ii) Leu-Leu-Tyr-Leu is produced. Also with Leu-Tyr-NH_2, Leu-Leu can only arise *via* an acyl-enzyme.

If both observations are correct then only mechanisms which give both an amino- and an acyl-enzyme can be considered; *e.g.*



12 Carboxypeptidase

The first direct evidence for an acyl-enzyme intermediate with yeast carboxypeptidase has now been provided.[132] *p*-Nitrophenyl trimethylacetate as a substrate gives a burst thus:



However, in view of the inherent reactivity of the substrate but the low rate constants, and, as the authors point out, the lack of known relationships between the esterase and exopeptidase activities, this does not imply such an intermediate in the physiologically important reaction.

In an investigation from Neurath's laboratory[133] of the intrinsic activities of zymogens, bovine procarboxypeptidase AS_5 was shown to be active towards *small* ester and peptide substrates (*cf.* ref. 114). For example, the k_{cat} for the hydrolysis of Bz-GlyPhe is 3 s^{-1}, which is $\frac{1}{40}$ of that for catalysis by the enzyme, whilst K_M at 2.7 mmol l^{-1} is very similar to the value of 1.9 mmol l^{-1} for the enzyme. The catalytic apparatus at the active site and the peptide binding site pre-exist in the zymogen.

In conclusion, most of the outstanding difficulties concerning the mechanism of the proteases are related to the question of detection of intermediates. In view of the theoretical work of Fersht[4] which predicts that intermediates should not,

[132] Y. Nakagawa and E. T. Kaiser, *Biochem. Biophys. Res. Comm.*, 1974, **61**, 730.
[133] J. R. Uren and H. Neurath, *Biochemistry*, 1974, **13**, 3512.

as a rule, accumulate with physiological substrates, these difficulties will not be easy to resolve.

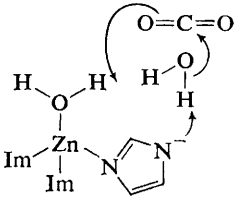
13 Carbonic Anhydrase

This is a zinc metallo-enzyme of molecular weight *ca.* 30 000 which catalyses the dehydration of bicarbonate and the hydration of carbon dioxide. The zinc is co-ordinated by three histidine residues. The turnover numbers are some of the highest known, *e.g.* for hydration[134] $k_{cat} = 10^6\ s^{-1}$, dehydration[135] $4 \times 10^5\ s^{-1}$ (bovine enzyme at 25 °C).

There are three outstanding problems with the enzyme: (i) what is the ionizing group, with $pK_a \sim 7$, responsible for activity? (ii) what is the bound substrate species in the dehydration reaction (H_2CO_3 or HCO_3^-)? (iii) are the high turnover numbers compatible with the rates of proton transfer?

The classical mechanism involves a zinc-bound water molecule which ionizes at higher pH to become the nucleophilic zinc-bound hydroxide. In the reverse reaction $H_2CO_3^-$ binds to the enzyme and is dehydrated.[136–138] On the other hand, König *et al.*[139, 140] argue that at high pH the fourth zinc ligand is water and at low pH this is lost and the zinc is liganded only by the protein. In the dehydration reaction the bound species is then H_2CO_3. There are strong arguments against this.[137, 138] In references 136—138 the problem of H^+ transfer rates is overcome by postulating that these occur *via* buffer species in solution.

Two conflicting papers concerning this have been published this year. One group[141] using ^{13}C n.m.r. claims to have located the C-2 carbons of the histidines co-ordinated to the zinc (His-64, His-67, His-200). None of their pK_a values followed the pH dependence of the activity-linked ionization (pK_a 7.45 in D_2O, 9.4 in D_2O + 10 mmol l^{-1} I^-). The ionization must represent that of the fourth zinc ligand. On the other hand, Appleton and Sarkar,[142] using the pK_a values of model compounds, suggest that it is the NH of an imidazole ring that is ionizing and propose a new mechanism shown in (18).



(18)

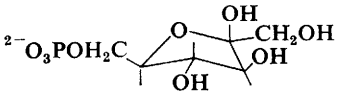
[134] J. C. Kernohan, *Biochim. Biophys. Acta*, 1964, **81**, 346.
[135] J. C. Kernohan, *Biochim. Biophys. Acta*, 1965, **96**, 304.
[136] R. G. Khalifah, *Proc. Nat. Acad. Sci. U.S.A.*, 1973, **70**, 1986.
[137] S. Lindskog and J. E. Coleman, *Proc. Nat. Acad. Sci. U.S.A.*, 1973, **70**, 2505.
[138] R. H. Prince and P. R. Woolley, *Bio-org. Chem.*, 1973, **2**, 337.
[139] S. H. Konig and R. D. Brown, *Proc. Nat. Acad. Sci. U.S.A.*, 1972, **69**, 2422.
[140] S. H. Konig, R. D. Brown, T. E. Needham, and N. A. Matwiyoff, *Biochem. Biophys. Res. Comm.*, 1974, **53**, 624.
[141] I. D. Campbell, S. Lindskog, and A. I. White, *J. Mol. Biol.*, 1974, **90**, 469.
[142] D. W. Appleton and B. Sarkar, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 1686.

14 Phosphofructokinase

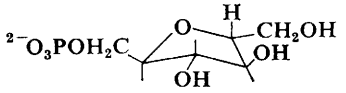
fructose-6-phosphate + ATP ⟶ fructose-1,6-diphosphate + ADP

Three groups have reported this year on the anomeric specificity of this enzyme. It is interesting to see how each used different techniques to obtain the same answer. D-Fructose-1-phosphate exists as *ca.* 80% the β anomer and 20% the α. If the interconversion of the two anomers is slow compared with the phosphorylation reaction then a rapid reaction study searching for biphasic traces of 80% and 20% should decide which anomer is the reactive species. The problem is how to monitor the reaction as there are no spectral changes. Wurster and Hess [143] solved this by using a classic trick of metabolic studies by using a coupled enzyme system. The formation of ADP was coupled with the formation of NAD. Monitoring the NAD by stopped-flow spectrophotometry they found a fast phase of 76% and a slow phase of 24%. Fishbein *et al.*[144] using rapid quenching techniques to analyse the reaction products point by point in the tens of milliseconds time-scale found an 80% burst. Furthermore, they showed that the enzyme phosphorylated only the β anomer of methyl-$\alpha\beta$-D-fructofuranoside-6-phosphate.

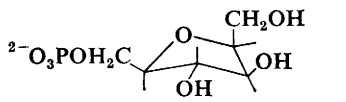
In a more extensive study [145] using the novel locked substrates shown, it was found that it is essential for the sugar to be in the β anomeric form for catalysis but the other anomers still bind.



D-fructose-1-phosphate
K_M = 0.043 mmol l^{-1}
V_{max} = 100%



2,5-anhydro-D-mannitol-1-phosphate
K_M = 0.41 mmol l^{-1}
V_{max} = 87%



2,5-anhydro-D-glucitol-1-phosphate
K_i = 0.34 mmol l^{-1}
competitive inhibitor

15 Aminoacyl-tRNA Synthetases

These enzymes charge the correct tRNA with the correct amino-acid. As the overall fidelity of protein synthesis is better than one part in 10^4 the specificity

[143] B. Wurster and B. Hess, *F.E.B.S. Letters*, 1974, **38**, 257.
[144] R. Fishbein, A. Benkovic, K. J. Shray, I. J. Siewers, J. A. Steffens, and S. J. Benkovic, *J. Biol. Chem.*, 1974, **249**, 6047.
[145] T. A. W. Koener, jun., E. S. Younathan, A.-L. E. Ashour, and R. J. Voll, *J. Biol. Chem.*, 1974, **249**, 5749.

requirements are high.[146] Historically the charging reaction was found as a two-step process [147, 148]

$$E + AA + ATP \rightleftharpoons EAA \sim AMP + PP$$

$$EAA \sim AMP + tRNA \longrightarrow AA\text{-}tRNA + AMP + E$$

$$AA + ATP + tRNA \longrightarrow AA\text{-}tRNA + AMP + PP$$

The enzymes are diverse in structure. There is a large class consisting of single polypeptide chains of molecular weight *ca.* 110 000 (Ile, Leu, Val from *E. coli* and *B. stearothermophilus*), a second class of dimers (Trp, 2 × 37 000; Tyr, 2 × 45 000; Met, 2 × 85 000), and a third class of tetramers, $\alpha_2\beta_2$ of molecular weight *ca.* 200 000—300 000.[149] This year the molecular weight of the methionyl activating enzyme from *E. coli* has been reported to be 2 × 85 000;[150] the glycyl, 2 × 80 000 + 2 × 33 000;[151] the phenylalanyl, 2 × 39 000 + 2 × 94 000 [152] or 2 × 37 000 + 2 × 98 000;[153] and the phenylalanyl from yeast, 2 × 61 500 + 2 × 72 500.[154]

Some problems concerning these enzymes are: (i) why this structural diversity and are there any simplifying factors? (ii) what is the reaction mechanism? (iii) how is the high specificity attained?

Structural Diversity.—Last year Kula [155] showed that only eight distinct peptides containing cysteine could be isolated from various digests of the isoleucyl-tRNA synthetase from *E. coli* MRE 600 (molecular weight 102 000, single polypeptide chain) despite there being 14 cysteine residues in the protein. This was interpreted as being consistent with 'the asymmetric unit of isoleucyl-tRNA synthetase appears to be roughly half the molecular weight'. A rigorous study by Koch, Boulanger, and Hartley [156] on several aminoacyl-tRNA synthetases comparing the monomeric leucyl- and valyl-tRNA synthetases with the symmetrically dimeric tyrosyl-tRNA synthetase (all from *B. stearothermophilus*) found only half the expected number of certain peptides. Similar deficiencies were found with the methionine activating enzyme (see Table 2). Three lines of evidence suggested that the monomeric enzymes consist of two large repeating units which have been covalently joined: (i) each monomer contains more than one copy of each tryptic peptide; (ii) widely separated regions of each contain the same tryptic peptide; (iii) far fewer unique tryptic peptides can be isolated than expected for a non-repetitive sequence. Similar strong evidence has been produced for the leucyl-enzyme from *E. coli*.[157] Each individual chain of the

[146] R. B. Loftfield, *Biochem. J.*, 1963, **89**, 82.
[147] M. B. Hoagland, *Biochim. Biophys. Acta*, 1955, **16**, 288.
[148] P. Berg, *J. Biol. Chem.*, 1956, **222**, 1025.
[149] R. B. Loftfield, *Progr. Nucleic Acid Res. Mol. Biol.*, 1972, **12**, 87.
[150] G. L. E. Koch and C. J. Bruton, *F.E.B.S. Letters*, 1974, **40**, 180.
[151] D. L. Ostrem and P. Berg, *Biochemistry*, 1974, **13**, 1338.
[152] T. Hanke, P. Bartmann, H. Hennecke, H. M. Kosakowski, R. Jaenicke, E. Holler, and E. Böck, *European J. Biochem.*, 1974, **43**, 601.
[153] G. Fayat, S. Blanquet, P. Dessen, G. Batelier, and J.-P. Waller, *Biochimie*, 1974, **56**, 35.
[154] J. M. Berthe, P. Mayer, and H. Dutler, *European J. Biochem.*, 1974, **151**, 1474.
[155] M.-R. Kula, *F.E.B.S. Letters*, 1973, **35**, 299.
[156] G. L. E. Koch, Y. Boulanger, and B. S. Hartley, *Nature*, 1974, **249**, 316.
[157] R. M. Waterson and W. H. Konigsberg, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 376.

Table 2 *Repeating units in aminoacyl-tRNA synthetases* [156]

Aminoacyl-tRNA synthetase	*Subunit structure*/ mol. wt. × 10^{-3}	*Number of peptides observed* / *Number of peptides expected*			
		Total	*Arg*	*His*	*Cys*
Tyrosyl-	2 × 44	0.85	0.79	0.9	1.0
Valyl-	1 × 110	0.38	0.46	0.39	0.38
Leucyl-	1 × 110	0.38	0.51	0.37	0.42
Methionyl-	2 × 66 [a]	0.53	0.56	0.5	0.6

[a] R. S. Mulvey (unpublished data) has shown that the molecular weight is 2 × 82 000. The ratios of peptides isolated relative to those expected are closer to 0.43 than the values quoted.

dimeric methionyl-tRNA synthetases also contains repeating sequences.[156, 158] Similar observations have been found for phosphofructokinase.[159] Clearly, when comparing the classes of aminoacyl-tRNA synthetases the substructure has to be taken into account. The diversity is lowered in some ways in view of this since the monomers appear to be functionally similar to the dimers.

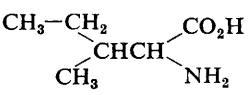
Mechanism.—The current controversy concerns whether or not the classical two-step mechanism holds. The mechanism of the aminoacylation of tRNA by aminoacyl-tRNA synthetases has recently been extensively reviewed by Loftfield.[149] He points out that 'a consensus has developed in favour of a mechanism in which enzyme reacts, probably first with ATP, then with amino-acid to form aminoacyladenylate; then sequentially to release pyrophosphate, bind tRNA, transfer the aminoacyl group to the tRNA, and finally, in what might be a rate-determining step, release the esterified tRNA'. However, Loftfield himself strongly advocates a mechanism in which tRNA first binds to the enzyme and then reacts with amino-acid and ATP in a concerted reaction, *i.e.* where there is no formation of an aminoacyladenylate. Some of the evidence in favour of this is that the concentrations of enzymes and tRNA *in vivo* are such that the enzyme–tRNA complexes are the predominant species; certain enzymes under all conditions and other enzymes under special conditions will undergo the pyrophosphate exchange reaction only in the presence of tRNA; and the rate of transfer of the aminoacyl radical from the aminoacyladenylate complexes to the tRNA is calculated to be too slow to account for the known turnover numbers and also the extent of transfer is too low.[149, 160, 161]

For the valyl- and isoleucyl-enzyme of *E. coli* it is claimed [160, 161] that the overall rate of tRNA charging in the absence of Mg^{2+}, but in the presence of spermine, is faster than the rate of formation of aminoacyladenylate in the absence of tRNA. Further support for a concerted mechanism, in the presence of spermine, has been provided by Takeda and Matsuzaki [162] from an analysis of steady-state kinetics. It is claimed that in the absence of Mg^{2+} and in the presence of spermine the reaction is 'concerted', there being a random addition of ATP, Ile, and $tRNA^{Ile}$ before the release of products. In the presence of

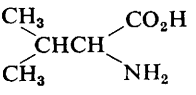
[158] C. J. Bruton, R. Jakes, and G. L. E. Koch, *F.E.B.S. Letters*, 1974, **45**, 26.
[159] C. J. Coffee, R. P. Aaranson, and C. Frieden, *J. Biol. Chem.*, 1973, **248**, 1381.
[160] K. Matsuzaki and Y. Takeda, *Biochim. Biophys. Acta*, 1973, **308**, 339.
[161] R. B. Loftfield and A. Pastuszyn, *Biochem. Biophys. Res. Comm.*, 1972, **47**, 775.
[162] Y. Takeda and K. Matsuzaki, *Biochem. Biophys. Res. Comm.*, 1974, **59**, 1302.

Mg^{2+} the reaction is ping-pong Bi Uni Uni Bi, *i.e.* consistent with the stepwise mechanism.

Specificity.—*The Amino-acid.* The problem is exemplified by the isoleucyl-tRNA synthetase and valine. An enzyme cannot exclude a smaller substrate from its



isoleucine



valine

active site. All that can happen is that the smaller substrate makes fewer interactions so that less binding energy is available for catalysis or binding. In the case of valine, the one methylene group difference between it and isoleucine can contribute at the most a factor of 150—200 towards catalysis. Yet protein synthesis is faithful to better than 1 part in 10^4. Two mechanisms have been put forward to account for this. First, Baldwin and Berg [162a] found experimentally that the complex between the isoleucyl-tRNA synthetase and valyladenylate would not transfer valine to tRNA but instead was hydrolysed. Secondly, two independent groups [163, 164] found that the isoleucyl-tRNA synthetase rapidly hydrolysed Val-$tRNA^{Ile}$. It was suggested that the first mechanism could in fact be due to the second. Several groups have reported this year on the latter mechanism, the AMP and pyrophosphate independent deacylation of misacylated and correctly acylated tRNA's.[165–167] Bonnet [166] suggests that the deacylation is non-specific; the amino-acid side-chain is not bound to the enzyme but the ester bond is somehow labilized. For example, (i) the valyl-tRNA synthetase from yeast catalyses the deacylations of Ser-$tRNA^{Ser}$, Ala-$tRNA^{Ala}$, Pro-$tRNA^{Pro}$, Arg-$tRNA^{Arg}$, and Trp-$tRNA^{Trp}$; (ii) the free amino-group is not essential, hydroxyvalyl-$tRNA^{Val}$ is deacylated slowly; (iii) high concentrations of cognate amino-acid do not inhibit the deacylation reaction; (iv) the enzymatic and non-enzymatic pH rate profiles for hydrolysis are identical. Bonnet and Ebel [165] have pointed out that the rates of deacylation of misacylated tRNA's are insufficient to affect significantly the steady-state concentration of a misacylated tRNA. The mechanism for specificity is therefore still not clear. Hopfield [168] has suggested how the specificity may be increased by involving sequential steps. Specificity is manifested at the formation of the aminoacyladenylate and then on rates of diffusion of the aminoacyladenylate from the enzyme.

tRNA Specificity. There is a vast literature on this and only three examples will be mentioned. The first two illustrate the power of the genetic approach using

[162a] A. N. Baldwin and P. Berg, *J. Biol. Chem.*, 1966, **241**, 831.
[163] E. W. Eldred and P. R. Schimmel, *J. Biol. Chem.*, 1972, **247**, 2961.
[164] M. Yarus, *Proc. Nat. Acad. Sci. U.S.A.*, 1972, **69**, 1915.
[165] J. Bonnet and J. P. Ebel, *F.E.B.S. Letters*, 1974, **39**, 259.
[166] J. Bonnet, *Biochimie*, 1974, **56**, 541.
[167] A. Sourgotchov, S. Blanquet, G. Fayat, and J.-P. Waller, *European J. Biochem.*, 1974, **46**, 431.
[168] J. J. Hopfield, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 4135.

mutants. Ghysen and Celis[169] have characterized six mis-suppressing mutants of *E. coli sup*^{3} tyrosine transfer tRNA. Just a single base change in the amino-acid acceptor step may cause a change in the specificity from glutamine to tyrosine and glutamine. Another set of studies[170, 171] using amber suppressors shows that a single base change in the anticodon of a $tRNA^{Trp}$, besides altering its coding specificity, also alters its amino-acid accepting specificity in accepting glutamine rather than tryptophan.

Another approach of potentially general importance has been introduced by Schoemaker and Schimmel.[172] Tyrosyl-tRNA synthetase was linked to its tRNA by direct u.v. irradiation. By digesting the tRNA and finding the regions protected by the enzyme it was found that pieces of the dihydrouracil arm, the anticodon loop, and the extra loop are joined to the enzyme.

16 Lysozyme

Lysozyme hydrolyses the polysaccharide component of bacterial cell walls. The polymer is generally of the form $(NAG\text{-}NAM)_n$ where NAG = *N*-acetyl-glucosamine and NAM = *N*-acetylnuramic acid. The elucidation of the mechanism for this hydrolysis is one of the triumphs of *X*-ray crystallography.[173–175] The enzyme has six subsites, A—F, all of which can accommodate NAG residues. NAM fits only into B, D, and F. It was proposed that the bond that is to be cleaved is located between sites D and E. The oxygen of the leaving group is protonated by Glu-35 whilst the carbonium ion that is formed in site D is stabilized by the negative charge on Asp-52. Furthermore, Blake *et al.*[174] found, from model building, bad contacts between the enzyme and the saccharide in site D when it is in the full-chair conformation; however, these are relieved on forming the half-chair conformation which is taken up by the carbonium ion. They proposed that the substrate is bound in the energetically unfavourable half-chair conformation, *i.e.* it is strained towards the transition state.

Levitt[176] has re-examined the binding of hexa-NAG to lysozyme using sophisticated calculations of energy functions rather than wire models. The co-ordinates of both the enzyme and the substrate were energy-refined by expressing all the bond lengths, bond angles, torsion angles, and non-bonded interatomic distances as empirical energy functions and minimizing the energy by computation. His results for the D-sugar ring and D subsite are given in Figure 2, where the torsion and angle strain in the ring as well as the non-bonded interactions between the enzyme and substrate are plotted. Three approaches used are illustrated. First (○) the substrate is assumed to be flexible and it is fitted into a rigid enzyme; second (△) both the enzyme and substrate are assumed

[169] A. Ghysen and J. Celis, *J. Mol. Biol.*, 1974, **83**, 333.
[170] L. Soll, *J. Mol. Biol.*, 1974, **86**, 233.
[171] M. Yaniv, W. R. Folk, P. Berg, and L. Soll, *J. Mol. Biol.*, 1974, **86**, 243.
[172] H. J. P. Schoemaker and P. R. Schimmel, *J. Mol. Biol.*, 1974, **84**, 503.
[173] D. C. Phillips, *Scientific American*, 1966, **215**, 78.
[174] C. C. F. Blake, L. N. Johnson, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *Proc. Roy. Soc.*, 1967, **B167**, 1967.
[175] C. A. Vernon, *Proc. Roy. Soc.*, 1967, **B167**, 389.
[176] M. Levitt, 'Peptides, Polypeptides, and Proteins', ed. E. R. Blout, F. A. Bovey, M. Goodman, and N. Lotan, Wiley, New York, 1974, p. 99.

to be flexible, and the pair are convergently energy refined to give a mutual best fit; and third (□) the enzyme is assumed to be partially flexible. It is seen that in all cases the most favourable situation is on the left-hand side of the Figure

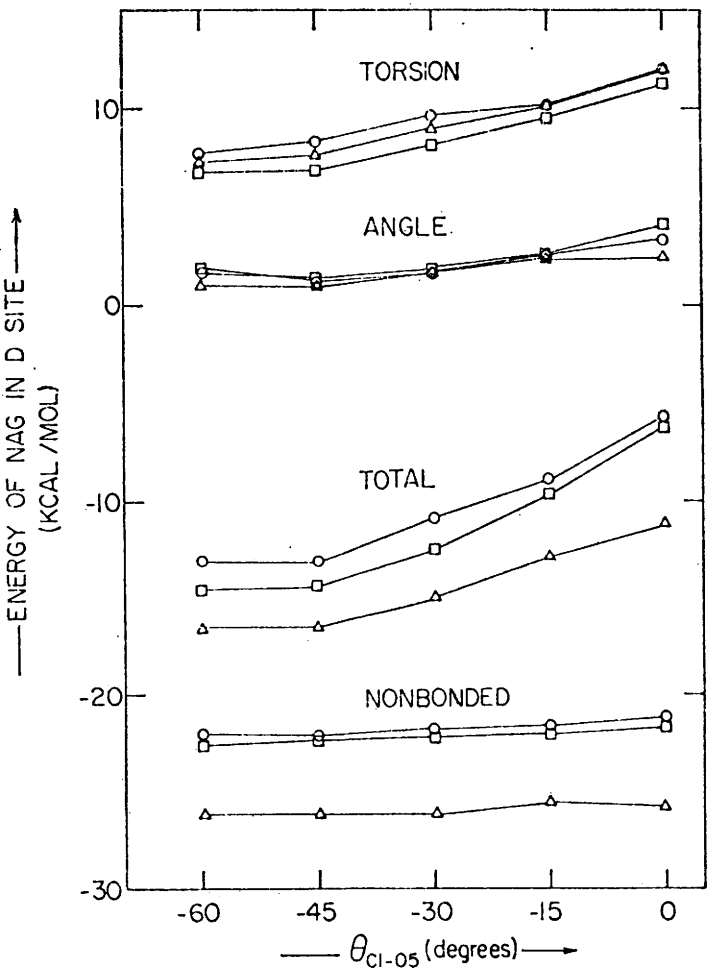
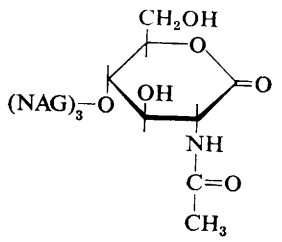


Figure 2 *The dependence of the different energy contributions of the* D-*sugar ring on the torsion angle* $\theta_{C\text{-}1-O\text{-}5}$ *for various artificially locked* $(NAG)_6$ *substrates bound to lysozyme (see text).* $\theta_{C\text{-}1-O\text{-}5} = -60°$ *for a full-chair and* 0° *for a half-chair*
(Reproduced by permission from 'Peptides, Polypeptides, and Proteins', ed. E. R. Blout, F. A. Bovey, M. Goodman, and N. Lotan, Wiley, New York, 1974, p. 99)

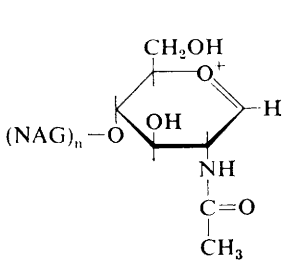
where the substrate is bound as the full-chair rather than the half-chair on the right-hand side. This suggests then that the substrate binds in its usual full-chair conformation at residue D and that there is little gain in non-bonded energy on going to the half-chair in the transition state. Levitt concludes that

transition states are often stabilized by electrostatic forces rather than van der Waals steric effects.

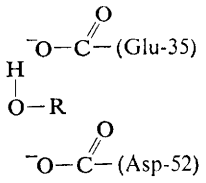
Set against this is the work [177] using the transition-state analogue (19) for the transition state (20). The authors conclude that the tight binding of (19) (K_{diss} = 3.3 × 10^6 l mol^{-1}) to the enzyme shows that the affinity of subsite D



(19)



(20)



is 6 × 10^3 times greater for the half-chair than for the full chair. However, this figure is estimated indirectly and may be on a false assumption. (20) binds only 32 times more tightly than $(NAG)_4$.[177] But Secemski *et al.*[177] assume that this is a bad basis for comparison on the grounds that $(NAG)_4$ avoids the D site by binding in A,B,C, with the fourth residue (proximal to the A site) unbound. They then compare the binding of (20) to that of NAG-NAM-NAG-NAM, which binds weakly (K_{diss} = 2 × 10^3 l mol^{-1}). Levitt suggests that the weak binding of the latter is a consequence of the bulky NAM in D and that this does not arise for NAG. In support of this it has been found [178] that NAG-NAM-NAG-NAG (which must bind in A,B,C,D, due to the NAM having to bind in B) has a K_{diss} of *ca.* 2 × 10^5 l mol^{-1}. Using this as the reference compound in the calculation of Secemski *et al.*, it can be shown that the transition state analogue binds only 16 times more tightly than the 'substrate'. Support for Levitt's contention that $(NAG)_4$ may bind at A,B,C,D comes from various binding studies.[179–182]

Ford *et al.*[183] have bound (19) to crystalline lysozyme. An *X*-ray diffraction study shows that it does bind in A,B,C,D but the lactone in D may take up a *sofa* conformation. Interestingly enough they found in a companion study on the binding of $(NAG)_4$ that this also binds in A,B,C,D thus proving Levitt's contention. It seems then that the binding of (19) provides little evidence for the strain mechanism. Much of the interpretation of binding data must also be re-evaluated in the light of this. The observed poorer binding in the D site could just be a consequence of the concomitant expulsion of two bound water molecules

[177] I. I. Secemski, S. S. Lehrer, and G. E. Lienhard, *J. Biol. Chem.*, 1972, **247**, 4740.
[178] J. J. Pollock, Ph.D. Thesis, The Weizmann Institute of Science, 1969.
[179] S. K. Banerjee and J. A. Rupley, *Arch. Biochem. Biophys.*, 1973, **155**, 19.
[180] S. K. Banerjee and J. A. Rupley, *J. Biol. Chem.*, 1973, **248**, 2117.
[181] E. Holler, J. A. Rupley, and G. P. Hess, *F.E.B.S. Letters*, 1974, **40**, 25.
[182] R. S. Mulvey, R. J. Gualtieri, and S. Beychok, *Biochemistry*, 1974, **12**, 2683.
[183] L. O. Ford, L. N. Johnson, P. A. Machin, D. C. Phillips, and R. Tjian, *J. Mol. Biol.*, 1974, **88**, 349.

from Asp-52.[176] It is speculated that this increases the electrostatic potential of the negatively charged oxygen.[176]

The rest of the original lysozyme mechanism has stood the test of time. Reduction of the $-CO_2^-$ of Asp-52 to $-CH_2OH$ (to give homoserine) removes the catalytic activity,[184] proving the importance of this residue. The carbonium ion mechanism is confirmed by a secondary tritium isotope effect of 19%[185] (although the method of synthesis of the tritiated substrates may shock conservatively minded organic chemists).

[184] Y. Eshdat, A. Dunn, and N. Sharm, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 1658.
[185] L. E. H. Smith, L. H. Mohr, and M. A. Raffery, *J. Amer. Chem. Soc.*, 1973, **95**, 7497.

6

Metal Derivatives of Amino-acids, Peptides, and Proteins

BY R. D. GILLARD, R. W. HAY, and D. R. WILLIAMS

1 Introduction

This chapter describes work published during the years 1973 and 1974 in the general field indicated by the title. Attention is concentrated on those aspects which relate most directly to the modification of the reactivity and other properties of the organic species, produced by their attachment to metal ions. While there has been little in the way of startling developments, there have been some extremely elegant and ingenious applications of known chemistry to more and more complicated systems. Consolidation of existing ideas and methods has been very evident.

A large number of valuable and interesting reviews have appeared. Many of these relate to metallo-enzyme, or metallo-protein systems, and their major emphasis tends to be on investigations, by the methods of physical inorganic chemistry, of the nature of the binding site of the metal. Among these, a recent volume of 'Progress in Inorganic Chemistry' is concerned [1] with current research topics in bio-inorganic chemistry. This contains reviews of metallo-protein redox reactions, the detailed chemistry of vitamin B_{12} and related inorganic systems which can be described as models for the vitamin, the transport of alkali-metal ions, and the increasingly important topic of the use of lanthanide ions as probes in biological systems, in this case with specific reference to their use in evaluating chemical shifts in n.m.r. experiments.

There is some debate concerning the proper name of the field of activity which is concerned in general with the effects and importance of inorganic species in biological systems. A plethora of names is current, and many of the reviews and books which have appeared show the variants on the names which are commonly accepted by current users. The titles of some books and reviews are given, in order to convey an impression of the depth and breadth of coverage of this field.

They include: 'Inorganic Biochemistry';[2] 'Metal Ions in Biological Systems (Studies of some Biochemical and Environmental Problems)';[3] 'Techniques and Topics in Bioinorganic Chemistry';[4] which contains articles on structural and electronic aspects of metal ions in proteins, the principles of catalysis by metallo-enzymes, the biochemical function of molybdenum, polynuclear proteins

[1] 'Progress in Inorganic Chemistry', ed. S. J. Lippard, Wiley, New York, 1973, Vol. 18.
[2] 'Inorganic Biochemistry', ed. G. L. Eichhorn, Elsevier, Amsterdam, 1973, Vols. 1 and 2.
[3] 'Metal Ions in Biological Systems', ed. S. K. Dhar, Plenum Press, New York, 1973.
[4] 'Techniques and Topics in Bioinorganic Chemistry', ed. C. A. McAuliffe, Macmillan, London, 1974.

containing iron(III), and metal ions as probes in n.m.r. studies in biochemistry. The latest volume of 'Structure and Bonding' is concerned with metal bonding in proteins,[5] and contains a number of interesting articles. A new and useful multi-volume work has begun to appear; this is 'Metal Ions in Biological Systems', edited by Sigel, which has several volumes[6] concerned successively with simple complexes, mixed ligand complexes, and complexes of high molecular weight.

As an example of the depth of coverage in a specialized field, we might consider the non-haem proteins containing iron. Although, during the period concerned, only one review of ferritin has appeared,[7] there has been a great deal of attention given to the iron–sulphur proteins.[8–12] This is one field where the enormous and rapid increase of our knowledge concerning the biochemical function for the iron–sulphur low-potential proteins has generated a large increase in the amount of literature.

Another field of which this is true is the use of transition-metal compounds in various chemotherapeutic approaches. The realization that the presence of metal ions in thermodynamically and/or kinetically stable compounds may well lead to important or attractive new biological activities has been many years in coming, although there has been a steady trickle of experiments since the time of Ehrlich. However, the deliberate screening of large numbers of transition-metal co-ordination compounds as potential therapeutic agents is a new departure and, as such, there has been a sharp increase in the volume of literature devoted to studies of this kind. Among the available reviews, three are concerned with the application of co-ordination compounds of platinum in cancer chemotherapy.[13–15] One of these[15] is by Rosenberg, who made the observations which have generated so much interest among inorganic co-ordination chemists, in the regression of certain tumours brought about by *cis*-dichlorodiammineplatinum(II) and related compounds.

We describe some of the areas which have been the subject of numerous reviews at the most relevant section in this chapter. The Report is broken up into rather arbitrary divisions, which in general follow the practice of previous volumes.

2 Amino-acids

Binding.—*Diffraction Studies.* A good deal of work has been reported in the past 10 years concerning the ways in which copper ions interact with amino-acids,

[5] 'Structure and Bonding', ed. J. D. Dunitz, P. Hemmerich, J. A. Ibers, C. K. Jorgensen, J. B. Neilands, D. Reinen, and R. J. P. Williams, Springer Verlag, Berlin, 1974.
[6] 'Metal Ions in Biological Systems', ed. H. Sigel, Marcel Dekker, New York, Vols. 1—3, 1974.
[7] R. R. Crichton, *Angew. Chem. Internat. Edn.*, 1973, **12**, 57.
[8] S. J. Lippard, *Accounts Chem. Res.*, 1973, **6**, 282.
[9] R. Mason and J. A. Zubieta, *Angew. Chem. Internat. Edn.*, 1973, **12**, 390.
[10] C. A. McAuliffe and S. G. Murray, *Inorg. Chim. Acta Rev.*, 1972, **6**, 103.
[11] 'Iron–Sulphur Proteins', ed. W. Lovenberg, Academic Press, New York, 1973. Volume 1 concerns biological properties, and Volume 2 molecular properties.
[12] 'Nomenclature of Iron–Sulphur Proteins (corrections)', *Biochemistry*, 1973, **12**, 3582.
[13] D. R. Williams, *Inorg. Chim. Acta Rev.*, 1972, **6**, 123.
[14] M. J. Cleare, *Coordination Chem. Rev.*, 1974, **12**, 349.
[15] B. Rosenberg, *Naturwiss.*, 1973, **60**, 399.

peptides, and proteins. Some of this work has now been reviewed,[16] in an account of models for interaction between copper ions and proteins, based on studies both in solution and from crystal structures. Although a number of structures of copper-containing species have again been reported, there is not the dominance by this metal which has been characteristic of the past decade.

The crystal and molecular structure of *O*-(β-D-xylopyranosyl)-L-serine and of its copper complex have been determined, and some of the reactions have been considered. Each molecule of the ligand [17] is firmly attached to a copper ion at three points, and the conformation of the side-chain seems to be determined by the co-ordination to copper. Similarly, as has been quite commonly found with other amino-acidato complexes of copper(II), a new structure of the bis-complex with *NN*-dimethylglycinatocopper(II) has shown that the two amino-acid ligands are in the *trans*-configuration.[18]

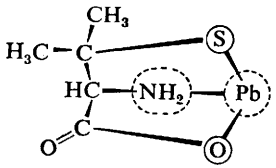
Among structures determined for complexes of amino-acids with other metal ions, that of the dimeric complex with chromium(III) of glycine is noteworthy.[19] This compound, di-(μ-hydroxo)tetraglycinatodichromium(III), $[Cr(gly\text{-}O)_2OH]_2$, contains two hydroxo-groups, bridging two chromium ions, with the unit of four atoms CrOCrO strictly planar. Another remarkable finding from *X*-ray crystal structure analysis is that bis-(L-ornithinato)palladium(II) contains two chelate rings, each of seven members, which is an unusual situation for a system which could equally well form a five-membered ring.[20] Both the chelate rings take up a twist-chair conformation, and the carboxylate group is in a quasi-equatorial position. It should perhaps be remembered that the form of the ornithine ligand found in this crystallized complex is not necessarily the only form to be present in solution, but the result is still striking.

In keeping with the fact that complexes of amino-acids substituted at the nitrogen atom seem to be attracting proportionately more attention, there has been a report of the crystal structure of bis(sarcosinato)nickel(II) dihydrate. Here, Guha has found [21] that, with octahedral chelation around the nickel ion, the molecule is centrosymmetric. Twinning was something of a problem in this study.

In continuing his extremely valuable study of model compounds for interaction between metal ions and proteins, using *X*-ray diffraction studies of the crystal structures of metal complexes of amino-acids and peptides, Freeman has reported the structures of a number of complexes of cadmium ion with amino-acids and peptides. These include [22] bis-(L-methioninato)cadmium(II), and the corresponding asparagine complex, and complexes with glutamate, glycyl-L-glutamate, and glycylglycine. In the one complex which was obtained at a low pH, the terminal amino-group of the peptide was protonated, and metal binding was at the O(peptide) and O(carboxy). Freeman has also contributed a structure

[16] R. Osterberg, *Coordination Chem. Rev.*, 1974, **12**, 309.
[17] L. T. J. Delbaere, M. Hihham, B. Kamenar, P. W. Kent, and C. K. Prout, *Biochim. Biophys. Acta*, 1972, **286**, 441.
[18] T. S. Cameron, C. K. Prout, F. J. C. Rossotti, and D. Steele, *J.C.S. Dalton*, 1973, 2626.
[19] J. T. Veal, W. E. Hatfield, D. Y. Jeter, J. C. Hempel, and D. J. Hodgson, *Inorg. Chem.*, 1973, **12**, 342.
[20] Y. Nakayama, K. Matsumoto, S. Ooi, and H. Kuroya, *J.C.S. Chem. Comm.*, 1973, 170.
[21] S. Guha, *Acta Cryst.*, 1973, **B29**, 2167.
[22] R. J. Flook, H. C. Freeman, C. J. Moore, and M. L. Scudder, *J.C.S. Chem. Comm.*, 1973, 753.

of D-penicillaminatolead(II), in a comment [23] on the application of D-penicillamine to the treatment of lead poisoning. The crystals were obtained from a solution of lead nitrate and of D-penicillamine, each of which were 0.01 mol l^{-1}. The structure (1) has some interesting features. The amino-, carboxy-, and thiolate



(1)

groups from a single molecule of the penicillamine provide the base of a trigonal pyramid, whose apex is filled by the ion of lead. Interestingly, this same 1 : 1 compound is the solid form isolated under a surprising range of conditions.

A valuable review of the absolute configurations of metal complexes which have been determined by *X*-ray methods has been given by Saito.[24] This list includes a number of complexes of amino-acids, with particular attention to those which involve a metal showing octahedral co-ordination, notably cobalt(III).

In view of the increasing importance of the methyl-mercury moiety in studies of the effects of mercury in natural systems, largely as an environmental hazard, it is pleasing to see that a number of reports are concerned with the interaction of methyl-mercury with proteins and amino-acids. The structure of DL-penicillaminatomethylmercury(II) has been given.[25] The amino-acid in its zwitterionic form, $SC(Me)_2CHN^+H_3CO_2^-$, is attached to the methyl-mercury unit through a deprotonated thiol group. The characteristic co-ordination number of two is preserved for mercury, the mean C—Hg—S angle being 178°. The same group has shown [26] that the methyl-mercury moiety is attached to L-cysteine again through a deprotonated thiol group, whereas methionine co-ordinates through an amino-group in its 1 : 1 complex with methyl-mercury.

Finally, the crystal structure of a derivative of copper(II) cysteine has been studied.[27] Both penicillamine disulphide (PDS) and penicillamine cysteine disulphide (PCDS) have been found in the urine of patients undergoing therapy with penicillamine for Wilson's disease (hepatolenticular degeneration). The authors therefore relate their findings to the ease of removal of copper ions from the body.

Solution Studies. Applications of magnetic resonance to the determination of molecular conformation of metal complexes in solution have been increasing in the period which is covered by the Report. For example, when a labile complex is formed in solution between a ligand which is diamagnetic and a metal ion

[23] H. C. Freeman, G. N. Stevens, and I. F. Taylor, *J.C.S. Chem. Comm.*, 1974, 366.
[24] Y. Saito, *Coordination Chem. Rev.*, 1974, **13**, 305.
[25] Y. S. Wong, P. C. Chieh, and A. J. Carty, *J.C.S. Chem. Comm.*, 1973, 741.
[26] Y. S. Wong, N. J. Taylor, P. C. Chieh, and A. J. Carty, *J.C.S. Chem. Comm.*, 1974, 625.
[27] J. A. Thick, D. Mastropaolo, J. Potenza, and H. J. Schugar, *J. Amer. Chem. Soc.*, 1974, **96**, 726.

which is paramagnetic, the nuclear resonance signals from those nuclei on the ligand which are closest to the site of interaction are preferentially broadened. This, of course, implies a direct and quantitative relationship between the broadening of a given resonance line and the distance of the relevant nucleus from the paramagnetic centre. Natusch has discussed [28] the conditions where values of this distance can be obtained from paramagnetically broadened nuclear resonance lines. The complexes investigated included those between the bivalent ions manganese, copper, cobalt, or nickel, and tervalent iron or chromium, and the ligands α-alanine, α-aminobutyric acid, norvaline, asparagine, glutamine, aspartic acid, and a variety of other amino-acids and similar cell constituents. The method was evaluated by comparing experimental values for internuclear distances with those measured from Dreiding models, where the complexes are sufficiently rigid for this to be meaningful. This seems likely to be a very useful method for evaluating conformations in solution, in an attempt to overcome the now familiar problem of the relationship between solid-state structures determined by diffraction methods and those of the reactive species in solution.

A further attempt to establish the structure of a complex in solution concerns [29] the copper(II) 1 : 2 complex of (*S*)-β-(2-pyridylethyl)-L-cysteine. Here, a battery of physical techniques, notably resonance spectroscopy and circular dichroism, showed that, in solution, the co-ordination of the copper ion was like that in the bis-complex with glycinate.

Optical activity has continued to attract some attention as a potential means of commenting on solution structures of metal complexes of amino-acids. For example, the rotatory strength of the *d–d* transitions of copper(II) complexes with α-amino-acids has been studied again.[30] In view of the fact that these are the very complexes which were used by Pfeiffer in his original assignment of the relative configurations of the amino-acids, it may seem surprising that this question should still attract interest. However, they have been such useful compounds that it would seem desirable to know why the copper bands become optically active, and this paper addresses itself to that question. The conclusion is that the optical activity of the *d–d* transitions in these bis-(α-amino-acid) complexes is governed by the electronic characteristics of the vicinal groups. Similarly,[31] quantitative c.d. spectra have been measured for a series of bis(amino-acid) complexes of nickel(II).

Other solution studies have been concerned with the control of the binding mode of amino-acids with metal ions, and one of the more striking studies concerns the copper complexes of 3,4-dihydroxyphenylalanine, L-dopa, which of course is used in the treatment of Parkinson's disease. In the new report,[32] the first solid complexes of dopa with the copper ion have been isolated, and a number of different modes of binding between the amino-acid and the metal ion observed. At a pH of 5.0, the blue-purple complex which is isolated from the solution is formulated as containing binding through the amino-acid moiety as

[28] D. F. S. Natusch, *J. Amer. Chem. Soc.*, 1973, **95**, 1688.
[29] R. H. Fish, J. J. Windle, W. Gaffield, and J. R. Scherer, *Inorg. Chem.*, 1973, **12**, 855.
[30] B. C. Verma and Y. P. Myer, *Bioinorganic Chemistry*, 1972, **1**, 141.
[31] R. A. Haines and M. Reimer, *Inorg. Chem.*, 1973, **12**, 1482.
[32] W. L. Kwik, E. Purdy, and E. I. Stiefel, *J. Amer. Chem. Soc.*, 1974, **96**, 1638.

in the glycinate complexes. A second complex, obtained under more alkaline conditions, is formulated as containing copper bonded through the 3,4-dihydroxy function.

Histidine continues to attract a great deal of attention as a metal-binding agent in chemical and biological systems, and a review of this subject has appeared.[33] A most interesting and novel observation has been made in that linkage isomerism[34] of histidine has been demonstrated in ruthenium complexes. The linkage isomerism concerns the two modes of attachment of the imidazole ring of histidine to ruthenium, both of which have now been characterized, one through the imidazole-N-3, and the other, obtained by rearrangement in acidic solution, through the imidazole-C-2 atom. The potential significance of this N–C linkage isomerism in biochemistry is briefly discussed.

The system copper(II)–L-histidine continues to attract attention, because of the possibility of numerous isomers, and the current uncertainty as to which are the dominant species. For example, the equilibria in this system have been studied[35] and the results given for the pH range from 2 to 11. The detailed knowledge of the species present obtained in this paper was used as a starting point for a consideration[36] of the structure, which employed visible spectra and some i.r. results, in addition to the more usual thermodynamic quantities derived from stability studies, to make structural assignments. Finally, the equilibria involved in the ternary systems containing copper(II) and L-histidine with either L-serine or L-glutamine have been investigated by potentiometric titration in aqueous solution.[37]

Ternary complexes have also formed the subject of a wide-ranging study by Sigel and his group.[38] They have reported the influence of the size of the chelate rings on stabilities of mixed-ligand complexes of copper(II), and found that, in general, mixed-ligand complexes containing a five- and six-membered chelate ring are more stable than those containing either two five- or two six-membered rings. Among the ligands studied were glycinate and β-alaninate. In a study of some ternary mixed complexes of copper(II) with diethylenetriamine, the c.d. spectra of the compounds containing optically active amino-acids were used to draw structural conclusions.[39]

The stability constants of cupric complexes with amino-acids have again proved a popular area for research, perhaps because it is still not common knowledge that structural deductions concerning solutions of cupric compounds from spectroscopic properties are unlikely to be rigorous. The mixed complexes are particularly well studied because of their importance in biological systems. For example, Gergely and Sovago have reported[40] the stability constants of the parent and mixed complexes of cupric ion with histidine and a number of aliphatic amino-acids, including glycine, alanine, and norvaline. The thermo-

[33] R. J. Sundberg and R. B. Martin, *Chem. Rev.*, 1974, **51**, 471.
[34] R. J. Sundberg and G. Gupta, *Bioinorganic Chemistry*, 1973, **3**, 39.
[35] T. P. A. Kruck and B. Sarkar, *Canad. J. Chem.*, 1973, **51**, 3549.
[36] T. P. A. Kruck and B. Sarkar, *Canad. J. Chem.*, 1973, **51**, 3563.
[37] T. P. A. Kruck and B. Sarkar, *Canad. J. Chem.*, 1973, **51**, 3555.
[38] H. Sigel, R. Caraco, and B. Prijs, *Inorg. Chem.*, 1974, **13**, 462.
[39] T. Murakami, T. Nozawa, and M. Hatano, *Bull. Chem. Soc. Japan*, 1973, **46**, 2456.
[40] A. Gergely and I. Sovago, *J. Inorg. Nuclear Chem.*, 1973, **35**, 4355.

dynamic values have been obtained by direct calorimetry. Similarly, Martin and his collaborators have reported their recent work [41] on the interactions of cupric ions with the α-amino-β-hydroxy-acids, L-threonine, L-allo-threonine, and L-serine. From measurements of both visible and c.d. spectra, they conclude that, at neutral pH, the ligands are bonded to the copper in a 'glycine-like' configuration. However, at pH > 9, one proton from each bound ligand dissociates, and the mode of bonding to the copper ion alters. The mixed ligand complexes of aspartate, with cupric or nickel ions, and glycine, alanine, valine, or leucine, have been studied.[42] A similar study relates to the parent and mixed complexes of aspartic acid, glutamic acid, and glycine with cupric ions.[43]

Other studies of stability constants of amino-acid systems relate to: complexes of L-proline and L-hydroxyproline, with cobalt, nickel, or copper ions;[44] L-glutamine with ions of the first transition series;[45] asparagine and glutamine with ions of the first transition series;[46] glutaminate and serinate with the proton and a number of metal ions;[47] and L-tyrosine with copper, cobalt, and nickel ions.[48]

Relatively little has appeared in the period under consideration concerning the kinetics of formation of amino-acid complexes of metal ions. This is in striking contrast to earlier years, when this question of the rate of binding provided a dominant field of research in co-ordination chemistry. One interesting paper which has appeared concerned the kinetics and thermodynamics of formation of monoglycinate complexes of the later first-row transition-metal ions, at pressures ranging from 1 bar to 3 kbar. This work [49] gives volumes of activation for formation of the monoglycinate complexes. The spectrochemical properties of later first-row transition-metal ions with glycine have been summarized.[50] A further spectroscopic study [51] concerns the mode of binding of the ions cobalt, nickel, copper, and zinc to substituted derivatives of L-alanine, notably 3-[(carboxymethyl)thio]-L-alanine (*S*-carboxymethyl-L-cysteine). This, which has been found in the urine of cystathioninuric patients and in hydrolysed samples of hair, is an effective agent against acne. This work seems to be the first which refers to this particular ligand.

The interaction of the nickel ion seems to have attracted a good deal of attention this year, and reports of solution studies include those relating to cysteine,[52] to the simple amino-acids glycine, DL-alanine, and DL-valine,[53] and to asparagine.[54] This last report also describes the complexes of asparagine with palladium(II).

[41] P. Grenouillet, R.-P. Martin, A. Rossi, and M. Ptak, *Biochim. Biophys. Acta*, 1973, **322**, 185.
[42] M. K. Singh and M. N. Srivastava, *J. Inorg. Nuclear Chem.*, 1973, **35**, 2433.
[43] I. Nagypal, A. Gergely, and E. Farkas, *J. Inorg. Nuclear Chem.*, 1974, **36**, 699.
[44] K. Kustin and Sung-Tsuen Liu, *J.C.S. Dalton*, 1973, 278.
[45] R. C. Tewari and M. N. Srivastava, *J. Inorg. Nuclear Chem.*, 1973, **35**, 2441.
[46] A. C. Baxter and D. R. Williams, *J.C.S. Dalton*, 1974, 1974.
[47] D. R. Williams, *J.C.S. Dalton*, 1973, 1064.
[48] M. L. Barr, E. Bavingartner, and K. Kustin, *J. Coordination Chem.*, 1973, **2**, 263.
[49] M. W. Grant, *J.C.S. Faraday I*, 1974, 560.
[50] V. Bhagwat, P. V. Khadikar, M. G. Kekre, and V. Sharma, *J. Inorg. Nuclear Chem.*, 1974, **36**, 942.
[51] R. Nakon, E. M. Beadle, and R. J. Angelici, *J. Amer. Chem. Soc.*, 1974, **96**, 719.
[52] E. K. Srivastrava, E. V. Raju, and H. B. Mathur, *J. Inorg. Nuclear Chem.*, 1973, **35**, 253.
[53] M. Morin and J.-P. Scharff, *Bull. Soc. chim. France*, 1973, 2198.
[54] R. D. Graham and D. R. Williams, *J.C.S. Dalton*, 1974, 1123.

Among the numerous studies of interactions between metal ions and amino-acids in solution by means of magnetic resonance, there is a novel paper concerning the use of the ^{23}Na resonance in aqueous sodium ion solutions containing cysteine and aspartic acid.[55] Another study [56] refers to the use of both proton and ^{13}C magnetic resonance results in establishing the solution structures of a variety of cysteine derivatives with mercury ions. In acidic solution, the bonding from mercury is to sulphur only, and the resultant complexes are thought to have an extended molecular configuration. Snyder and Angelici have used [57] proton resonance to comment on the structures of complexes between α-amino-acids and nickel ions, even though these species are paramagnetic. The effects of conformation on the three-bond and four-bond coupling constants between platinum and hydrogen have been studied [58] in platinum complexes of methyl-glycines.

In other studies of structure using magnetic resonance, zinc has figured prominently. Thus, a study of the L-aspartic acid complexes of zinc in aqueous solution employed [59] both magnetic resonance measurements and studies of stability constants. Similar measurements of stability constants have been carried out [60] by Rabenstein and Blakney for some cadmium and zinc polyamine and amino carboxylate mixed-ligand complexes: the notable feature in this work is the implied precision of the measured stability constants, which appears to equal that obtainable from the more standard potentiometric methods. Other work on cadmium includes a potentiometric determination of formation constants,[61] this paper including an interesting summary of the occurrence of cadmium in both essential and polluting roles.

A large number of reports have concerned complexes of amino-acids with elements of the lanthanide series, perhaps due to the increasing use of the lanthanide elements as probes for biological structures, and in particular for the replacement of calcium ions. A general description has been given [62] of the various methods which have been employed for studying these complexes. A study of the lanthanide–histidinate complexes has been reported, in which the point of interest concerns the location of the proton in the protonated complex.[63] Other studies relate to a europium–DL-tryptophan complex,[64] complexes of neodymium,[65] and the stabilities of some lanthanide complexes of L-asparagine and L-glutamine.[66]

Other points of interest arising from the solution studies include the observations [67] of a ready deprotonation of the amine in the reaction of a

[55] T. L. James and J. H. Noggle, *Bioinorganic Chem.*, 1972, **2**, 69.
[56] G. A. Neville and T. Drakenberg, *Canad. J. Chem.*, 1974, **52**, 616.
[57] R. J. Snyder and R. J. Angelici, *Inorg. Chem.*, 1974, **13**, 14.
[58] L. E. Erickson, M. D. Erickson, and B. L. Smith, *Inorg. Chem.*, 1973, **12**, 412.
[59] H. Ishizuka, T. Yamamoto, Y. Arata, and S. Fujiwara, *Bull. Chem. Soc. Japan*, 1973, **46**, 468.
[60] D. L. Rabenstein and G. Blakney, *Inorg. Chem.*, 1973, **12**, 128.
[61] M. D. Walker and D. R. Williams, *J.C.S. Dalton*, 1974, 1186.
[62] R. Prados, L. G. Stadtherr, H. Donato, and R. B. Martin, *J. Inorg. Nuclear Chem.*, 1974, **36**, 689.
[63] A. D. Jones and D. R. Williams, *Inorg. Nuclear Chem. Letters*, 1972, **8**, 1009.
[64] S. Lai, *Bull. Chem. Soc. Japan*, 1973, **46**, 2232.
[65] E. R. Birnbaum and D. W. Darnall, *Bioinorganic Chem.*, 1973, **3**, 15.
[66] R. C. Tewara and M. N. Srivastava, *J. Inorg. Nuclear Chem.*, 1973, **35**, 3044.
[67] C. A. McAuliffe, L. M. Vallarino, and J. V. Quagliano, *Inorg. Nuclear Chem. Letters*, 1973, **9**, 625.

zwitterionic amino-acid with nickel chloride, with a more detailed treatment[68] of the amino-acid derived from DL-methylsulphonium methioninate by deprotonation in forming transition-metal complexes. The complex with the cobaltous ion of this same cationic ligand is described in detail.[69] I.r. absorption spectra for phenylalanine complexes have been given,[70] and trialkyl tin derivatives of amino-acids, and indeed of dipeptides, have been described[71] by Ho and Zuckerman.

Stereoselectivity.—Brookes and Pettit have recently shown[72] that stereoselectivity occurs in formation of mixed complexes of cupric ion with histidine and simple amino-acids containing aromatic substituents. Some typical values are given in the Table, which represents a very significant contribution to the

Table *Stability constants for some species* [*copper*(D- *or* L-*His*)(L-α-*amino-acidate*)][72]

Amino-acidate	D-His	L-His	$\Delta \log \beta$ [a]
Tryptophan	18.475	18.003	0.47
Phenylalanine	17.699	17.504	0.20
Valine	17.546	17.603	−0.06
Proline	18.105	18.105	0
Leucine	17.662	17.692	−0.03

[a] $\Delta \log \beta = \log \beta$[Cu(D-His)(L-amino-acidate)] $- \log \beta$[Cu(L-His)(L-amino-acidate)].

vexed question of the sizes and origins of stereoselective effects in the mixed complexes of transition-metal ions. A similar paper[73] relates to mixed complexes of cupric ion containing, *e.g.* *N*-carboxymethyl-L-valine and L-threonine or D-threonine. Similar stereoselective effects occur in the behaviour of complexes of *N*-(2-pyridylmethyl)-L-aspartic acid towards simpler amino-acids. Angelici and his colleagues showed[74] that the metal complexes of this derivative of L-aspartic acid would co-ordinate the L-isomers of alanine, phenylalanine, tryptophan, threonine, leucine, or valine in preference to their enantiomers. A related observation[75] concerns the stereospecific complexation in forming the cupric derivatives of the salicylaldehyde Schiff base with poly-L-ornithine or poly-L-lysine. Blackburn and Jones have discussed the factors involved in the stereoselective effects occasionally observed in hydrolysis of amino-acid esters catalysed by co-ordination complexes.[76]

An old principle has been given an ingenious new application,[77] in a method for determining the optical purity of amino-acids by formation of complexes. When optically active alanine, valine, or leucine is added to an alkaline solution of an anionic cobalt complex of a Schiff base, the enhancement of the optical

[68] C. A. McAuliffe and W. D. Perry, *Inorg. Chim. Acta*, 1974, **10**, 215.
[69] C. A. McAuliffe, W. D. Perry, and K. A. West, *J. Coordination Chem.*, 1974, **4**, 77.
[70] Y. Inomata, T. Inomata, T. Moriwaki, and J. L. Walter, *Spectrochim. Acta*, 1973, **29A**, 1933.
[71] B. Y. K. Ho and J. J. Zuckerman, *Inorg. Chem.*, 1978, **12**, 1552.
[72] G. Brookes and L. D. Pettit, *J.C.S. Chem. Comm.*, 1974, 813.
[73] R. V. Snyder and R. J. Angelici, *J. Inorg. Nuclear Chem.*, 1973, **35**, 523.
[74] R. Nakon, P. R. Rechani, and R. J. Angelici, *Inorg. Chem.*, 1973, **12**, 2431.
[75] M. Dentini, P. DeSantis, and M. Savino, *J.C.S. Chem. Comm.*, 1974, 86.
[76] J. R. Blackburn and M. M. Jones, *J. Inorg. Nuclear Chem.*, 1973, **35**, 1597, 1605, 2421.
[77] Y. Fujii and H. Yoneda, *Chem. Letters*, 1974, 43.

rotation of the amino-acid is very large indeed, suggesting that this method may well find some application. Studies of stereoselective effects in the tris complexes of octahedral metal ions with bidentate amino-acids continue to attract attention. For example, the mixed complexes between L-proline and L- or D-aspartic acid with cobalt(III) have been prepared [78] and separated into numerous stereoisomers using anion exchange chromatography. While stereoselective effects have been found, their interpretation remains uncertain, owing to the fact that these systems are so rarely at equilibrium. The differing interactions between the various isomers of tris(L-alaninato)cobalt(III) and poly-L-lysine in aqueous media have been studied by means of c.d. spectra.[79]

A description of the configuration, preparations, and reactivities of some complexes of amino-acids with cobalt(III) has been given:[80] a number of tris-chelated and dimeric bis-chelated complexes of chromium(III) have also been described, and these latter complexes form the subject [81] of a report by McAuliffe and Perry. Some of the dimeric, hydroxo-bridged, species are also described [82] in a paper which considers the elimination of water from solid complexes.

For studies of the reactivity of co-ordinated amino-acids, it may well be advantageous to prepare an optically active complex of a kinetically inert metal ion, in a form which will lend itself to ready solubility in organic solvents. For this reason, the several papers on such complexes are certainly timely. For example, the synthesis, structure, and stereochemistry of the isomers of pentane-2,4-dionatobis(*S*-aminoacidato)cobalt(III) have been reported,[83] and a later extension relates to the analogous compounds containing chromium.[84] A further paper [85] shows that the absolute configurations of the mixed complexes containing amino-acids are consistent, whether they are assigned from circular dichroism, or from the analysis of anisotropic shielding in their ^{1}H n.m.r. spectra.

The properties of asymmetrically co-ordinated nitrogen atoms have continued to be of interest, and in a typical study of this kind, the isomers of triammine-(sarcosinate-*N*-monopropionato)cobalt(III) were made.[86] Further experiments on the cations $[Co(en)_2\{N\text{-Me-}(S)\text{-Ala}\}]^{2+}$ and its prolinate analogue have been reported.[87] The closely related systems with the quadridentate tetramine, 5(*S*)-methyl-1,4,7,10-tetra-azadecane have been reported,[88] in which the isomers were identified by means of their ^{1}H n.m.r. spectra.

The stereoisomers of the mixed complexes containing cobalt(III) and aspartate with ethylenediamine have been studied.[89] Legg and Neal have reported [90] a

[78] T. Matsuda and M. Shibata, *Bull. Chem. Soc. Japan*, 1973, **46**, 3104.
[79] M. Barteri, M. Branca, and B. Pispisa, *J.C.S. Dalton*, 1974, 543.
[80] R. D. Gillard, S. H. Laurie, D. C. Price, D. A. Phipps, and C. F. Weick, *J.C.S. Dalton*, 1974, 1385.
[81] C. A. McAuliffe and W. D. Perry, *Inorg. Nuclear Chem. Letters*, 1974, **10**, 367.
[82] R. Tsuchiya, A. Uchara, and E. Kyuno, *Bull. Chem. Soc. Japan*, 1973, **46**, 3737.
[83] D. J. Seematter, L. A. Wingert, J. G. Brushmiller, G. W. Everett, and K. S. Finney, *Inorg. Nuclear Chem. Letters*, 1974, **10**, 75.
[84] L. A. Wingert, D. J. Seematter, and J. G. Brushmiller, *J.C.S. Chem. Comm.*, 1974, 976.
[85] G. W. Everett, K. S. Finney, J. G. Brushmiller, D. J. Seematter, and L. A. Wingert, *Inorg. Chem.*, 1974, **13**, 536.
[86] K. I. Okamoto, J. Hidaka, and Y. Shimura, *Bull. Chem. Soc. Japan*, 1973, **46**, 3134.
[87] D. A. Buckingham, J. Dekkers, A. M. Sargeson, and M. Wein, *Inorg. Chem.*, 1973, **12**, 2019.
[88] M. Saburi, M. Homma, and S. Yoshikawa, *Inorg. Chem.*, 1973, **12**, 1250.
[89] Y. Yojima and M. Shibata, *Inorg. Chem.*, 1973, **12**, 1009.
[90] J. I. Legg and J. A. Neal, *Inorg. Chem.*, 1973, **12**, 1805.

cobalt(III) complex of a new stereospecifically binding ligand which occupies all six co-ordination sites of the metal ion and which contains the aspartic acid unit. The very readily obtained stereospecifically formed product of combining three molecules of L-cysteine with cobalt(III) may be oxidized [91] to a sulphinato-complex, which, being a triply charged anion, is an extremely convenient agent for the optical resolution of cations which bear a triple positive charge.

Reactivity.—A most useful review [92] of some aspects of the reactivity of amino-acids when co-ordinated to metal ions has appeared. The chief emphasis is upon the formation and the hydrolysis of esters and peptides, on some reactions of complexes of Schiff bases made from amino-acids, on condensations of the aldol type, and on the activation of hydrogen at the α-carbon atom, as manifested by isotopic exchange with heavy water or by racemization. Pasini and Casella outline the factors which underlie the modification of reactivity of amino-acids when they bind to metal ions, and also provide some general discussion of the possible importance of these concepts in the role of metal ions in cellular processes. Japanese work on the use of complexes of amino-acids with the cupric ion as synthetic precursors has continued.[93] A base-catalysed condensation of either bis(glycinato)copper(II) or *N*-pyruvylideneglycinatoaquocopper(II) with aldehyde sugar derivatives led to the isolation, in good yields, and with some stereoselectivity, of 2-amino-2-deoxyaldonic acids. A useful experimental procedure [94] outlines an easy way of isolating amino-acids from their complexes with the cupric ion by means of a cation exchange resin in the H^+ form. The resin recommended is Amberlite IR-120B.

In continuing their studies of the condensation of co-ordinated amino-acids to form dipeptides, Buckingham, Dekkers, and Sargeson have identified [95] a stabilized amino-alcohol intermediate, in the reaction between isopropylglycinatebis(ethylenediamine)cobalt(III) and glycine-ethyl ester in DMSO. The hydrolysis by base of amino-acid esters and amines in the co-ordination sphere of cobaltic ions has also been studied.[96] In connection with the hydrolysis of amino-acid esters, a recent paper [97] on the specific catalysis of the hydrolysis of *p*-nitrophenylalkoxycarbonylglycinates by hydroxo-complexes of mercury(II) is of interest. The complexes studied contained the mercuric ion bonded to 2,2-bipyridyl and to 1,10-phenanthroline.

The redox effects of metal complexes have not received so much attention this year. Watters and Wilkins have studied [98] the interaction of dioxygen with the cobaltous histidine complex in strongly alkaline conditions. In particular, the work distinguishes between the oxygenated adducts of the strongly alkaline blue solution, and those from less alkaline conditions. Copper catalyses [99] the oxidation of cysteine and similar thiols by hexacyanoferrate(III). This observation

[91] L. S. Dollimore and R. D. Gillard, *J.C.S. Dalton*, 1973, 933.
[92] A. Pasini and L. Casella, *J. Inorg. Nuclear Chem.*, 1974, **36**, 2133.
[93] S. Ohdan, T. Okamoto, S. Maeda, T. Ichikawa, Y. Araki, and Y. Ishido, *Bull. Chem. Soc. Japan*, 1973, **46**, 981.
[94] S. Ohdan, T. Ichikawa, Y. Araki, and Y. Ishido, *Bull. Chem. Soc. Japan*, 1973, **46**, 1019.
[95] D. A. Buckingham, J. Dekkers, and A. M. Sargeson, *J. Amer. Chem. Soc.*, 1973, **95**, 4173.
[96] K. B. Nolan, B. R. Coles, and R. W. Hay, *J.C.S. Dalton*, 1973, 2503.
[97] M. M. Werber and Y. Shalitin, *Bioinorganic Chem.*, 1973, **2**, 275.
[98] K. L. Walters and R. G. Wilkins, *Inorg. Chem.*, 1974, **13**, 752.
[99] G. J. Bridgart, M. W. Fuller, and I. R. Wilson, *J.C.S. Dalton*, 1973, 1274.

has greatly clarified a number of discrepancies which have existed in the literature concerning the use of this oxidation as an analytical procedure. Waldmeier and Sigel have shown [100] that amino-acids inhibit catalase-like activity of the cobaltic complex of hematoporphyrin.

The use of metal-containing reagents in reactions or syntheses of α-amino-acids has not received great attention. However, one report [101] describes the synthesis of piperazine-2,5-diones from α-amino-acids and metal alkoxides. The metals concerned are the earlier transition metals, such as titanium and zirconium, and a typical reaction, of zirconium isopropoxide with glycine, gave a good yield of piperazine-2,5-dione. The rate and mechanism of the decarboxylation, which appears to be oxidative in nature, of cupric complexes of aminomalonic acid have also received attention.[102] The electron transfer from the aminomalonate to the cupric ions seems to be rate-determining. The catalytic efficiency of copper(II) is increased by co-ordination to glycinate.

The chemistry of the Schiff base complexes of metal ions has received far less attention than has been the recent custom. Among the few papers during the period of interest, the β-elimination of *O*-phosphothreonine has been studied [103] in the presence of pyridoxal and a number of metal ions. Of the metal ions studied, only cupric and vanadyl had noteworthy catalytic effects. The presence of organic bases such as piperidine had a further promotional effect on the rate. In another attempt to comment on the mechanism of the reactions of pyridoxylidene amino-acid Schiff base complexes of metal ions, the aluminium complexes have been studied.[104] The proton magnetic resonance spectra of the solution in the region of pD from 3 to 5.5 change rapidly with time, and the intermediate spectra were interpreted in terms of an intermediate species resulting from dissociation of the alpha proton of the amino-acid residue in the Schiff base. Abbott and Martell suggest that this species may be a general intermediate in the metal-catalysed reactions of model systems for vitamin B_6.

A major development this year concerns the increase in the amount of effort devoted to the biochemical role of molybdenum. Schrauzer and his co-workers have reported [105] the reduction of a number of substrates by borohydride and catalysts containing molybdenum and thiolic ligands such as L-cysteine. The dinuclear complex of cysteine with molybdenum(V) was used as the catalyst, although the active form is derived from this by dissociation. The chemistry of this dimeric substance, di-μ-oxo-bis[oxo(L-cysteinato)]molybdate(V), has received some attention this year. Kroneck and Spence have described the characterization of the complex,[106] and given further details of its structure, and a few of its reactions.[107] A kinetic study of the reaction with cysteine is also available,[108] and a survey of some reactions of the dimeric ion has been given by Kay and

[100] P. Waldmeier and H. Sigel, *J. Inorg. Nuclear Chem.*, 1973, **35**, 1741.
[101] N. N. Yoshino and T. Yoshino, *Bull. Chem. Soc. Japan*, 1973, **46**, 2899.
[102] J. H. Fitzpatrick and D. Hopgood, *Inorg. Chem.*, 1974, **13**, 568.
[103] Y. Murakami, H. Kondo, and A. E. Martell, *J. Amer. Chem. Soc.*, 1973, **95**, 7138.
[104] E. H. Abbott and A. E. Martell, *J. Amer. Chem. Soc.*, 1973, **95**, 5014.
[105] G. N. Schrauzer, G. W. Kiefer, P. A. Doemeny, and H. Kisch, *J. Amer. Chem. Soc.*, 1973, **95**, 5582.
[106] P. Kroneck and J. T. Spence, *Inorg. Nuclear Chem. Letters*, 1973, **9**, 177.
[107] P. Kroneck and J. T. Spence, *J. Inorg. Nuclear Chem.*, 1973, **35**, 3391.
[108] R. F. Stephenson and F. A. Schultz, *Inorg. Chem.*, 1973, **12**, 1762.

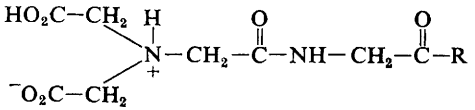
Mitchell.[109] Much of this work, and some related studies on the reduction of flavines, has been summarized.[110]

3 Peptides

During the period under consideration, the work on metal complexes of peptides seems to have been more concerned with consolidation than with new developments, although there have been one or two interesting novel papers. The work to be considered here will be divided, once again rather arbitrarily, into structural aspects, and those concerned with reactivity.

Structural Aspects.—Work on stability constants of peptides with metal ions is now more commonly done with an expressed purpose. For example, in connection with a study of the hydrolysis of amino-acid esters, Nakon and Angelici have studied [111] the stabilities of the cupric complexes of glyclyglycine and glycylsarcosine and their methyl esters, by potentiometric titration, and also by i.r. spectrophotometry. Similarly, the stabilities of cupric and nickel complexes of the Schiff bases made from salicylaldehyde and dipeptides containing glycine and/or β-alanine have been studied.[112] In general, in this series of compounds, the stabilities of the complexes are a function of ring size, and the inclusion of a six-membered ring in the fused ring structure tends to enhance stability. A similar conclusion was reached [113] from a study of the stability constants of the cupric complexes of tripeptides alone, the tripeptides being those of glycine and/or β-alanine. Related work [114] refers to the stability of the fused rings in complexes of cupric ion with dipeptide amides.

A detailed study has been made of the multidentate ligands (2), including syntheses and quantitative equilibria.[115] Stability constants were obtained



(2) R = OH, $NHCH_2CO_2H$, $NHCH_2CONHCH_2CO_2H$

potentiometrically, and some structural comments relating to the metal complexes in water have been made from an analysis of the C=O region of the i.r. spectrum, the spectra being obtained in heavy-water solution. Spectroscopic studies have also been made [116] on the mode of binding by glutathione of the heavy metals cadmium, zinc, lead, and mercury. Here the spectroscopic method employed was the use of ^{13}C n.m.r. At more alkaline conditions, the chemical shifts for the carbons in the carbonyl groups of the two peptide linkages suggest that zinc ions may promote ionization of the peptide protons, and subsequently bind to

[109] A. Kay and P. C. H. Mitchell, *J.C.S. Dalton*, 1973, 1388.
[110] P. Kroneck and J. T. Spence, *Biochemistry*, 1973, **12**, 5020.
[111] R. Nakon and R. J. Angelici, *Inorg. Chem.*, 1973, **12**, 1269.
[112] Y. Nakao and A. Nakahara, *Bull. Chem. Soc. Japan*, 1973, **46**, 187.
[113] O. Yamauchi, Y. Nakao, and A. Nakahara, *Bull. Chem. Soc. Japan*, 1973, **46**, 2119.
[114] O. Yamauchi, Y. Nakao, and A. Nakahara, *Bull. Chem. Soc. Japan*, 1973, **46**, 3749.
[115] R. J. Motekaitis and A. E. Martell, *Inorg. Chem.*, 1974, **13**, 550.
[116] B. J. Fuhr and D. L. Rabenstein, *J. Amer. Chem. Soc.*, 1973, **95**, 6944.

the then-ionized nitrogen of the peptide group. This represents a contrast with the other metal ions, which show no such behaviour. Law and Wood have studied [117] the way in which reduced glutathione co-ordinates to vitamin B_{12} coenzymes. The bonding to cobalt is through sulphur. These authors report that the bond between carbon and cobalt in the two enzymes, 5-deoxyadenosylcobalamine and methylcobalamine, is less stable to light when glutathione co-ordinates rather than 5,6-dimethylbenzimidazole. Reduced glutathione does not form a particularly stable complex with cobaltous ion, and this of course is reminiscent of the old observation that vitamin B_{12} is able to catalyse the oxidation by dioxygen of thiolate groups.

Peptides which contain histidine have again received attention, in respect of their metal-binding properties. For example,[118] the stability constants of a number of metal ions with glycyl-L-histidine, L-histidylglycine, glycylglycyl-L-histidine, glycyl-L-histidylglycine, and L-histidylglycylglycine have been determined. In the same field, the cupric complexes of L-histidylglycine and L-histidylglycylglycine have been studied,[119] the stability constants being determined by potentiometric titration, and the structures of the complexes formed being discussed using spectroscopic information. The binding in the acid pH region seems to be from the metal ion to the nitrogen atoms of amino and imidazole groups, whereas in the neutral region the peptide amide group becomes deprotonated, the resulting complex having three of its co-ordination sites occupied by nitrogen atoms of the deprotonated amide, an amino-group, and an oxygen atom of a carboxyl or carbonyl group in one molecule, the fourth position being occupied by a nitrogen atom from imidazole of another molecule. More details, particularly relating to formation of polymeric species, are given [120] in a later paper from the same laboratory.

Cupric complexes continue to attract the most attention, and a typical report concerns the use of the so-called rule of average environment in peptide complexes of the cupric ion.[121] Attaching a spectroscopic weight to each type of ligand atom likely to be encountered in proteins, including the donor atoms N of amino, N of peptide, N of imidazole, O of peptide, carboxylate, water, or hydroxide, Billo has shown that the position of maximum absorption of a square cupric complex of a peptide can be predicted surprisingly accurately. As he points out, in favourable cases, the nature of the co-ordination site can be identified. Martell and Kim have employed i.r. absorption spectra in aqueous (D_2O) solution to establish the nature of the binding of cupric and nickel chelates of peptides.[122] The i.r. frequencies observed for the peptide carbonyl and the terminal carboxylate frequencies change as the peptide nitrogen group becomes deprotonated. The e.s.r. spectrum of single crystals of glycylglycine, doped with cupric ions, has been studied by two groups.[123, 124] The doped crystals are

[117] P. Y. Law and J. M. Wood, *J. Amer. Chem. Soc.*, 1973, **95**, 914.
[118] A. Yokoyama, H. Aiba, and H. Tanaka, *Bull. Chem. Soc. Japan*, 1974, **47**, 112.
[119] H. Aiba, A. Yokoyama, and H. Tanaka, *Bull. Chem. Soc. Japan*, 1974, **47**, 136.
[120] H. Aiba, A. Yokoyama, and H. Tanaka, *Bull. Chem. Soc. Japan*, 1974, **47**, 1437.
[121] E. J. Billo, *Inorg. Nuclear Chem. Letters*, 1974, **10**, 613.
[122] A. E. Martell and M. K. Kim, *J. Coordination Chem.*, 1974, **4**, 9.
[123] R. L. Lancione and H. C. Allen, *J. Coordination Chem.*, 1974, **3**, 273.
[124] M. Fujimoto, S. Saito, and Y. Tomkiewicz, *Bioinorganic Chem.*, 1973, **2**, 341.

susceptible to irradiation by u.v. light (with wavelength around 250 nm), resulting in an internal conversion which is terminated by the free radicals, $NH_3^+CH_2$-$CONH\dot{C}HCO_2^-$ and $NH_3\dot{C}HCONHCH_2CO_2^-$.[124]

Among peptide complexes of other metals, a systematic study of the dipeptide complexes with palladium(II) has appeared.[125] This employed c.d., ^1H n.m.r., and absorption spectra to establish the nature of the binding sites for palladium. Another study using magnetic resonance[126] is that, using europium, of the conformations in solution of the cyclic peptides, *cyclo*-(L-Pro-L-Pro) and *cyclo*-(L-Pro-D-Pro). There is a flattening of the diketopiperazine ring from a boat form in the former cyclic peptide to a nearly planar form in the latter.

Reactivity.—Several topics of continuing interest have received further contributions during the period of this Report. For example, Stadtherr and Martin have studied the stereoselectivity in dipeptide complexes of the cobaltic ion.[127] In these systems, while there are stereoselective effects discriminating between the possible isomeric products, it is, in general, possible to obtain all the possible stereoisomers. In contrast, the ferrichromes have been thought to bind to and wrap up metal ions in a stereospecific fashion, dictating that one hand only of the octahedron can be stable. Neilands and his collaborators have now reported[128] on the solution conformation of the ferrichromes and related compounds, by studying in particular the dependence upon pH of the individual slow exchange of deuterium for hydrogen in the amide groups of alumichrome. This, the aluminium analogue of ferrichrome, contains four individual protected amide groups. Within the range $3 < pD < 7$, the rates of exchange are relatively constant. Conformational deductions are made, and it is suggested that at neutral pH the exchange of some amide groups may proceed without significant exposure to the solvent.

Another long-standing possibility of employing metal complexation as a means of altering the reactivities of peptides through changes either in stereochemistry or electronic density upon co-ordination concerns the possibility of activating methylene or methine groups selectively in metal complexes. Recent reports concern the selective activation in nickel complexes of tripeptides, made up of glycine and/or DL-alanine.[129] Study of the ^1H n.m.r. spectra, in heavy water, showed that methylene protons of the *C*-terminal glycine residues exchange most effectively in the co-ordination compound, compared with those of the other amino-acid residues. This finding is reminiscent of those for cobaltic complexes of the free peptides. In contrast, and pointing up the possibility of synthetic utilization of the selectivity of activation of individual amino-acid residues, a second paper refers to the co-ordinated dipeptide Schiff bases, where[130] it is no longer the *C*-terminal residues which become more activated, but conversely the *N*-terminal groups which now exchange more readily.

In keeping with the increased interest in molybdenum catalysis in other areas of amino-acid chemistry, there is a report on a glutathione complex of

[125] L. E. Nance, A. F. Schreiner, and H. G. Frye, *Bioinorganic Chem.*, 1974, **3**, 135.
[126] P. E. Young, V. Madison, and E. R. Blout, *J. Amer. Chem. Soc.*, 1973, **95**, 6142.
[127] L. G. Stadtherr and R. B. Martin, *Inorg. Chem.*, 1973, **12**, 1810.
[128] M. Llinas, M. P. Klein, and J. B. Neilands, *J. Biol. Chem.*, 1973, **248**, 915.
[129] Y. Nakao, O. Uyama, and A. Nakahara, *J. Inorg. Nuclear Chem.*, 1974, **36**, 685.
[130] O. Uyama, Y. Nakao, and A. Nakahara, *Bull. Chem. Soc. Japan*, 1973, **46**, 496.

molybdenum as a catalyst.[131] This complex, which was crystallized, but not fully characterized, catalyses the reduction of acetylene to ethylene in the presence of borohydride. The activity of the catalyst, in this system, is said to be 4% of the activity of that quantity of nitrogenase which contains an equivalent amount of molybdenum. The same molybdenum–glutathione complex also acts as a catalyst in the reduction of hydrazine to ammonia in the presence of borohydride. This reaction, which is enhanced considerably by ATP, is rather slow, having a rate described by 2 mol of ammonia produced per minute per mol of molybdenum bound in the catalyst.

The hydrolysis of small peptides has continued to be perhaps the most commonly studied reaction catalysed or promoted by metal ion. The mechanism and a comparison of calculated and experimental rates have been given[132] for the hydrolysis of dipeptides in the presence of the bivalent ions of zinc, cobalt, or nickel. The catalysis of the hydrolysis of glycylglycine methyl ester by cupric ion has been further investigated,[133] with the finding that in the pH region 6.5—8.0, the ester is bound *via* the terminal amino-group, the deprotonated amide nitrogen, and possibly the ester group. It is the co-ordination through the deprotonated nitrogen of the amide group which is thought to move the ester group sufficiently for it to bind to the cupric ion, this promoting the hydrolysis of the ester. The rate shows a first-order dependence on the concentration of hydroxide ion, and the authors suggest a mechanism whereby rate-determining attack by hydroxide occurs at the co-ordinated ester carbon atom. The enhancement of rate is about one thousand-fold. Very similar results were obtained for the corresponding ethyl ester, and for ethyl glycyl-β-alaninate and for ethyl glycyl-L-leucinate.[134] Here, it is pointed out that the rate accelerations for metal-promoted hydrolyses of esters arises chiefly from the highly positive entropy of activation. Margerum and his collaborators have studied effects of changing the stereochemistry within the peptide upon the rates[135] of reactions between the tripeptide cupric complexes and incoming ligands. When glycine is replaced by either L-leucine or L-alanine in these complexes, there is a marked increase in the rates for the attack by nucleophiles.

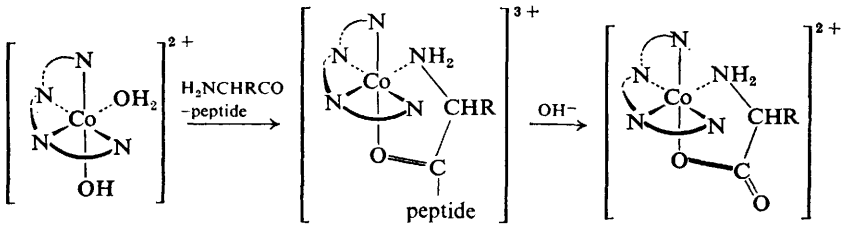
One striking feature of the employment of metal complexes as reagents in peptide chemistry has been the serious attempt during the past two years to use the known reactivity of cobaltic complexes as hydrolytic agents in peptide-sequencing studies. The basic reaction is of the type shown in the equation,

$$cis\text{-}[Co(N)_4(OH)(OH_2)]^{2+} + NH_2\cdot CHR^1\cdot CO\cdot NH\cdot R^2 \longrightarrow cis\text{-}[Co(N)_4(H_2NCHR^1CO_2)]^{2+} + H_2N\cdot R^2 + H_2O$$

where N is a nitrogenous ligand, and the product, where the fixed ligand on the cobalt is triethylenetetramine, would be $[Co_2(trien)(gly)]^{2+}$. This ion has a large number of configurational isomers, and one study this year refers to the

[131] D. Werner, S. A. Russell, and H. J. Evans, *Proc. Nat. Acad. Sci. U.S.A.*, 1973, **70**, 339.
[132] K. Ohkubo and H. Sakamoto, *Bull. Chem. Soc. Japan*, 1973, **46**, 2579.
[133] R. Nakon and R. J. Angelici, *J. Amer. Chem. Soc.*, 1973, **95**, 3170.
[134] R. W. Hay and K. B. Nolan, *J.C.S. Dalton*, 1974, 2542.
[135] H. Hauer, G. R. Dukes, and D. W. Margerum, *J. Amer. Chem. Soc.*, 1973, **95**, 3515.

characterization of these.[136] A single-crystal *X*-ray diffraction study gives the structures, within the same crystals, of two diastereoisomers, and an account of their typical properties is also given. Bentley and Creaser have reported on the qualitative determination[137] of the *N*-terminal amino-acids of peptides and proteins using this general method, involving the β-triethylenetetramine complex of cobalt(III). The method involves treating one micromole of peptide or protein with the reagent at a pH of 8.0, at 45 °C for 3 h. Addition of phosphate buffer (pH 10.5) at 45 °C for 10 min then cleaves the *N*-terminal bidentate amino-acid-cobaltic complex, whose identification can be achieved directly; 22 small peptides and 10 proteins were studied, and the procedure was found to be essentially satisfactory. In a later paper by the same authors, the conclusions are extended[138] and the remark is made that 'the results show that quantitative total hydrolysis of peptides and proteins using cobalt(III)-activated intermediates is a feasible procedure'. It does seem that cysteine residues may give rise to problems, probably through the reduction of metal ion to oxidation state two. Other difficulties are summarized by Oh and Storm, who found[139] that (i) the cobalt complex may undergo side-reactions during the peptide-bond hydrolysis, giving multiple peaks in the detection system; (ii) the chromatographic separation of the cobaltic amino-acid complexes is not simple, because of the double positive charge on the complex, and because the charge tends to smear out the differences in amino-acid structure in the complex. For this and other reasons, these workers have used a complex of cobalt(III) with a lower overall charge, *i.e.* the complex with ethylenediaminediacetic acid. The selective *N*-terminal hydrolysis appears to occur equally well with this complex, and reports of its use will be awaited with interest. Kimura has also reported[140] on the use of the triethylenetetramine cobaltic complex for the sequential hydrolysis of peptides, following the scheme shown, linking (3), (4), and (5). This scheme has been slightly revised here to



(3) (4) (5)

remove an apparent redox change in the original. He concludes that the hydrolysis of the species shown as (4) occurs reasonably well with the majority of the peptides which he tested. However, several complexes of peptides which

[136] D. A. Buckingham, P. J. Cresswell, R. J. Dellaca, M. Dwyer, G. J. Gainsford, L. G. Marzilli I. E. Maxwell, W. T. Robinson, A. M. Sargeson, and K. R. Turnbull, *J. Amer. Chem. Soc.* 1974, **96**, 1713.

[137] K. W. Bentley and E. H. Creaser, *Biochem. J.*, 1973, **135**, 507.

[138] K. W. Bentley and E. H. Creaser, *Inorg. Chem.*, 1974, **13**, 1115.

[139] S. K. Oh and C. B. Storm, *Bioinorganic Chem.*, 1973, **3**, 89.

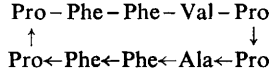
[140] E. Kimura, *Inorg. Chem.*, 1974, **13**, 951.

contained proline or amino-acids such as glutamic acid, with a free carboxy-group, were not so readily hydrolysed, and Kimura feels that this might present a serious problem.

4 Proteins

The possible modification of specific reactivities in sections of protein structures by metal complexation has not attracted attention in the period of this Report. There has, as was true in our earlier Reports, been an enormous expansion of work on metal–protein systems, and in this chapter we can hope only to mention but a few of the more recent developments. New metal-containing proteins and enzymes continue to accumulate rapidly, and an example[141] concerns the so-called 'violet phosphoproteinphosphatase', which has now been shown to be an acid phosphatase of low molecular weight, containing iron. The highly purified enzyme has a constant ratio of enzymatic activity and of absorbance at 550 nm to iron concentration, and the ratio of iron to enzyme seems to be 1 : 1. Perhaps because of the recent interest in the use of compounds of platinum as chemotherapeutic agents, there has been some interest in the involvement of platinum ions with biopolymers. For example, platinum compounds have been found to inhibit the catalytic activity of cytoplasmic malate dehydrogenase from pig heart. It has now been shown[142] by *X*-ray diffraction that, in the crystallized enzyme, there are eight sites which bind platinum. These sites are in pairs.

The chemistry of cation carriers and binders has received some attention, particularly on the stereochemical side. For example,[143] the conformations of the lithium complex with antamanide and of a substituted antamanide complex of sodium have been studied in the crystalline form. Antamanide is a cyclic decapeptide isolated from the mushroom *Amanita phalloides* and forms rather strong complexes with the sodium ion in aqueous environments. The structure of the antamanide is shown as (6). However, in solvents less polar than water,



(6)

for example acetonitrile, it will also form complexes with lithium or potassium ions. In the structure determined by *X*-ray diffraction, two of the peptide linkages are in the *cis*-conformation, these being Pro^2-Pro^3 and Pro^7-Pro^8. Interestingly, the conformation found in the solid state for the sodium complex differs from all of the conformations which have been proposed for the complex in solution, these latter structures having been based on n.m.r. studies.

The use of the rare-earth ions as substitutes for the alkaline earth ions in enzyme systems has continued. For example, Valentine and Cottam have studied[144] the gadolinium ion in the enzyme muscle pyruvate kinase.

[141] H. D. Campbell and B. Zerner, *Biochem. Biophys. Res. Comm.*, 1973, **54**, 1498.
[142] M. Wade, D. Tsernoglon, E. Hill, L. Webb, and L. Banaszak, *Biochim. Biophys. Acta*, 1973, **322**, 124.
[143] I. L. Karle, J. Karle, T. Wieland, W. Burgermeister, H. Faulstich, and B. Witkop, *Proc. Nat. Acad. Sci. U.S.A.*, 1973, **70**, 1836.
[144] K. M. Valentine and G. L. Cottam, *Arch. Biochem. Biophys.*, 1973, **158**, 346.

A new twist has been given to the use of heavy-metal atoms in crystallographic studies of protein molecules, by the incorporation [145] of a mercury atom between the sulphur atoms in the interchain disulphide bond of a Bence-Jones λ-chain dimer. This dithiolatomercury(II) derivative was made both in solution and crystalline form by reduction of the protein with 2-mercaptoethanol, followed by reaction with mercury(II) acetate. The crystals were isomorphous with those of the native protein. An interesting fact is that these crystals were less readily damaged by radiation than other mercurial derivatives of the same protein.

The nature of proteins involving copper ions has again been a popular field of endeavour. One of the more remarkable papers on this theme is a molecular orbital treatment [146] of cupric proteins, by Nickerson and Phelan. This concerns the binding of cupric ion to deprotonated peptide groups of the backbone of proteins. The assumption is made that the local environment of the metal ion is planar, which seems optimistic. The model is sufficiently useful to provide some interpretative power. For example, the strong c.d. bands in some proteins at 250, 350, and 345 nm are assigned to charge transfer between copper and peptide. This paper concludes that the role of deprotonated peptides in binding copper to proteins is dominant. An approach more in line with the vast amount of accumulated experience in this field is that [16] of Osterberg. He discusses models for the interaction between proteins and copper based on studies both in solution and in the solid state, and has collected together a mass of information of great use in attempting to relate observed properties of copper proteins with the likely nature of the binding sites. One of the more active areas concerned with copper-binding proteins is in the field of treatment and understanding of Wilson's disease. In an elegant piece of work,[147] the metal-binding protein metallothionein from the livers of patients with Wilson's disease was isolated, and compared with the same protein from control subjects. Using the apoproteins, the binding constants for copper were determined, and it was found that the apoprotein from patients with Wilson's disease had a stability constant four times greater than that of the control protein. This is taken to suggest that the alterations in copper homeostasis in hepatolenticular degeneration arise because of the synthesis of an abnormal metal-binding protein, which has a higher affinity for copper.

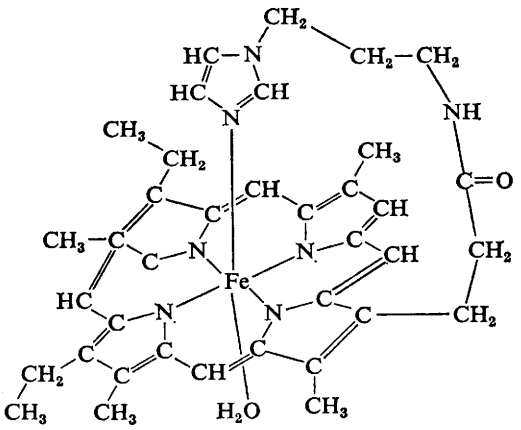
Other work on the interesting metallothioneins comes from Vallee's laboratory.[148] This offers an improved and rather quick isolation procedure for the protein, using gel filtration and chromatography on DEAE-cellulose. The molecular weights of the protein from both liver and kidney seem to be *ca.* 6600, and the amino-acid compositions of both proteins are also similar. The cysteine content is remarkably high, around one-third. A further finding of interest is that, on average, three cysteine residues are available for each metal atom which is bound. The binding of metals to proteins in human serum has also received

[145] K. R. Ely, R. L. Girling, M. Schiffer, D. E. Cunningham, and A. B. Edmondson, *Biochemistry*, 1973, **12**, 4233.
[146] K. W. Nickerson and N. F. Phelan, *Bioinorganic Chem.*, 1974, **4**, 79.
[147] G. W. Evans, R. S. Dubois, and K. M. Hambridge, *Science*, 1973, **181**, 1175.
[148] J. H. R. Kägi, S. R. Himmelhoch, P. D. Whanger, J. L. Bethune, and B. L. Vallee, *J. Biol. Chem.*, 1974, **249**, 3537.

attention[149] from a consideration of how readily metal–protein complexes of copper, zinc, and cadmium are dissociated when histidine or glycine is added.

Studies have continued of the ways in which metal ions are bonded to protein sites. For example, the removal of zinc from thermolysin results[150] in a metal-free apoenzyme. The cobaltous ion will generate 200% of the activity of the native enzyme! The structure of this enzymatically active cobalt thermolysin has been studied by means of absorption and Cotton effects, measured in circular dichroism. The suggestion is that the cobalt ion has an unusual environment. This is thought to be a distorted tetrahedron. Another study on the use of a substituted metal ion in an apoprotein is that[151] on the vanadyl derivative of insulin. This work was done in order to see whether vanadyl ion, VO^{2+}, was potentially useful as a probe for metal-binding sites in protein. The increase of detail available because of the unpaired electron on the vanadyl ion seems likely to be highly useful in establishing the nature of binding sites, particularly where vanadyl can be shown to replace the natural metal ion isomorphously. It seems a little strange that the vanadyl insulin should parallel the zinc insulin so closely, in view of the usually lop-sided nature of the binding around the vanadyl ion. However, this does seem a most fruitful new type of probe for this increasingly popular requirement.

A remarkable development concerns the synthesis of a section of the active site of myoglobin.[152] The compound ferropyrroporphyrin-*N*-3-(1-imidazolyl)-propylamide, whose structure is shown as (7), has been synthesized. This binds dioxygen reversibly, both in the solid state, and when it is dissolved in a film of polystyrene. The behaviour in solution has been further described.[153]



(7)

[149] E. L. Girouse and R. I. Henkin, *Bioinorganic Chem.*, 1972, **2**, 125.
[150] B. Holmquist and B. L. Vallee, *J. Biol. Chem.*, 1974, **249**, 4601.
[151] N. D. Chasteen, R. J. DeKoch, B. L. Rogers, and M. W. Hanna, *J. Amer. Chem. Soc.*, 1973, **95**, 1301.
[152] C. K. Chang and T. G. Traylor, *Proc. Nat. Acad. Sci. U.S.A.*, 1973, **70**, 2647.
[153] C. K. Chang and T. G. Traylor, *J. Amer. Chem. Soc.*, 1973, **95**, 5810, 8475, 8477.

Jensen and his collaborators have published further details concerning the structure of the ferredoxin isolated from *Peptococcus aerogenes*. This non-haem iron protein contains eight iron and eight sulphur atoms, each equally distributed between two units, which are 12 Å apart. Each unit contains four iron atoms, four sulphide groups, and four cysteine sulphur atoms. An ingenious small-scale method for obtaining crystals is described, which has also been applied to other proteins.[154] The synthesis of analogues of the active sites of such proteins has proceeded apace. For example, tetrameric clusters containing the unit Fe_4S_4 have been obtained,[155] with properties very similar to those of the iron clusters in the active sites of the reduced high-potential iron protein from chromatium, and the oxidized ferredoxin from the *Peptococcus* just described. Similarly,[156] synthetic analogues of the active sites of the Fe_2S_2 proteins have also been made. These serve as models for the ferredoxin from spinach, for adrenodoxin, and for putidaredoxin.

The binding site of iron in another non-haem iron protein, transferrin, has been investigated[157] by means of resonance Raman scattering. Four resonance-enhanced bands were observed in the Raman spectra, which were assigned to vibrational modes of phenolate groups, by comparison with a model compound.

The mechanism of action of the enzyme carbonic anhydrase has been the subject of renewed attention. Prince and Woolley have summarized[158] the two views of the mechanism, and have presented arguments against the hypothesis which describes the active alkaline form of the enzyme as containing an undissociated water molecule, which is lost on protonation of a protein group. The more standard view has, of course, been that the acidic group (involved in the ionization to give the alkaline active form of the enzyme) is a water molecule bound to the zinc of the active site. Lindskog and Coleman have also reviewed[159] the evidence for and against a mechanism involving a hydroxozinc species. Finally, and demonstrating that the subject is not yet closed, the third paper to discuss this question comes to the opposite conclusion, assuming that the acid dissociation of the enzyme to give its active form is associated with the ionization of a proton from nitrogen, and not with the ionization of water bound to the zinc.[160] This suggestion rested on results from model systems, including measurement of the acid dissociation constant of water attached to zinc, which also was complexed by a number of imidazoles or substituted imidazoles. A kinetic study[161] of the binding of sulphonamides to carbonic anhydrase suggests that the ionized sulphonamides react with that form of the enzyme in which the water ligand bound to the zinc ion is not ionized.

A study of the zinc-enzyme carboxypeptidase A has appeared[162] which uses the cobalt(II) and nickel derivatives of the protein. Their magnetic susceptibilities

[154] E. T. Adman, L. C. Sieker, and L. H. Jensen, *J. Biol. Chem.*, 1973, **248**, 3987.
[155] B. A. Averill, T. Herskovitz, R. H. Holm, and J. A. Ibers, *J. Amer. Chem. Soc.*, 1973, **95**, 3523.
[156] J. J. Mayerle, R. B. Frankel, R. H. Holm, J. A. Ibers, W. D. Phillips, and J. F. Weiher, *Proc. Nat. Acad. Sci. U.S.A.*, 1973, **95**, 3531.
[157] B. P. Gaber, V. Miskouski, and T. G. Spiro, *J. Amer. Chem. Soc.*, 1974, **96**, 6868.
[158] R. H. Prince and P. R. Woolley, *Bioinorganic Chem.*, 1973, **2**, 337.
[159] S. Lindskog and J. E. Coleman, *Proc. Nat. Acad. Sci. U.S.A.*, 1973, **70**, 2505.
[160] D. W. Appleton and B. Sarkar, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 1686.
[161] J. Olander, S. F. Bosen, and E. T. Kaiser, *J. Amer. Chem. Soc.*, 1973, **95**, 1616.
[162] R. C. Rosenberg, C. A. Root, R.-H. Wang, M. Cerdonio, and H. B. Gray, *Proc. Nat. Acad. Sci. U.S.A.*, 1973, **70**, 161.

over a wide range of temperature have been interpreted in terms of the geometry of the ground state, leading to the conclusion that the properties of the nickel protein are consistent only with an octahedral structure, whereas the cobaltous protein probably has a five-co-ordinate structure. Interestingly, both metal derivatives exhibit full activity as peptidases. In the area of oxidation and reduction, Stiefel has described[163] his proposed mechanism at the molecular level for the action of molybdenum in enzyme systems. In another study of oxidation and reduction catalysis, selenium has been shown to catalyse reduction of cytochrome *c* by glutathione,[164] when in the form of selenite. This paper has an interesting introduction on the catalytic effects of selenium in general, and in biochemistry in particular, reminding us that, whatever we may choose to call it, the area concerned with the effects and functions in cells of species classically regarded as 'inorganic' is expanding rapidly to the point that the need for a good knowledge of inorganic properties among biochemists is becoming urgent.

[163] E. I. Stiefel, *Proc. Nat. Acad. Sci. U.S.A.*, 1973, **70**, 988.
[164] O. A. Levander, V. C. Morris, and D. J. Higgs, *Biochemistry*, 1973, **12**, 4591.

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