Applications of the Chemical Reactions of Proteins in Studies of their Structure and Function

By R. B. Freedman
DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY COLLEGE LONDON, GOWER ST., LONDON WC1E 6BT

1 Introduction

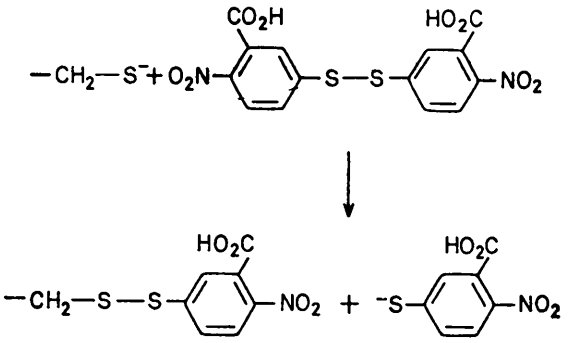
A. Scope.—Proteins are the most versatile of biological molecules, and the diversity of their functions as catalysts, transport systems, and structural components derives from the immense variety of spatial arrangements of functional groups which can arise from different linear sequences of amino-acids. Chemical reactions of proteins can illustrate many aspects of their structure and function. This Review is intended to indicate the range of information which can be obtained by studies of this kind.

Various aspects of this subject are dealt with more fully in recent reviews. Specific protein reagents are discussed by Cohen,[1] while their methodology and applications are dealt with in a volume of 'Methods in Enzymology'.[2] Chemical reactions leading to modification of function in enzymes and other proteins are the subject of other recent reviews.[3] An excellent survey of earlier work is given by Fraenkel-Conrat.[4]

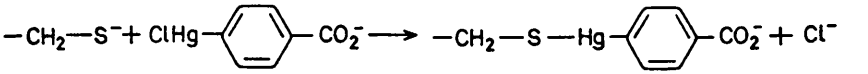
B. Specificity.—The chemical groups in proteins are capable of a wide variety of types of reaction. The side-chains of lysine, tyrosine, cysteine, histidine, arginine, serine, and methionine are all capable of reacting as nucleophiles; at least six of the side-chains are susceptible to oxidation and at least three to electrophilic substitution. This raises the whole question of specificity and selectivity of reaction. A reagent will be said to be 'group-specific' if only side-chain groups of one type of amino-acid are modified under the conditions employed. This is often a requirement in modification studies, but can rarely be guaranteed, and in each case independent checks of the selectivity of the modification must be made. Clearly the outstandingly reactive side-chains are most easy to modify selectively.

Cysteine is a particularly straightforward case. Two characteristic reactions of thiol groups are exchange with disulphide compounds such as 5,5′-dithio-bis-2-

[1] L. A. Cohen, *Ann. Rev. Biochem.*, 1968, **37**, 695.
[2] 'Methods in Enzymology' ed. C. H. W. Hirs, Academic Press, New York, 1967, vol. XI.
[3] (*a*) S. J. Singer, *Adv. Protein Chem.*, 1967, **22**, 1; (*b*) B. L. Vallee and J. F. Riordan, *Ann. Rev. Biochem.*, 1969, **38**, 733; (*c*) E. Shaw, *Physiol. Rev.*, 1970, **50**, 244.
[4] (*a*) H. Fraenkel-Conrat in 'Comprehensive Biochemistry', ed. M. Florkin and E. H. Stotz, Elsevier Publishing Co., Amsterdam, 1963, vol. 7, p. 56; (*b*) H. Fraenkel-Conrat in 'The Enzymes', ed. P. D. Boyer, H. Lardy, and K. Myrbäck, Academic Press, New York, 1959, vol. 1, p. 589.



Scheme 1



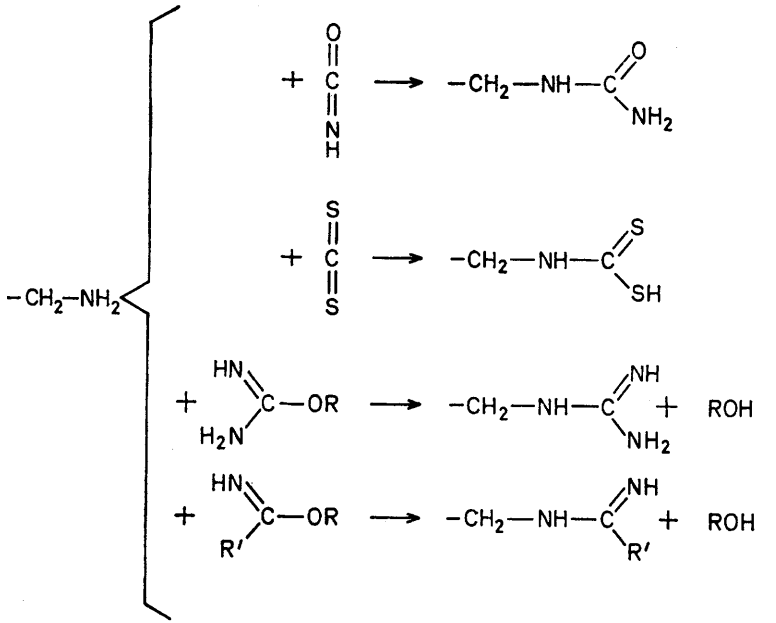
Scheme 2

nitrobenzoic acid (Ellman's reagent, DTNB) (see Scheme 1),[5] and nucleophilic attack on organic mercurial salts such as *p*-chloromercuribenzoic acid (PCMB) (see Scheme 2).[6] Both these reactions are accompanied by changes in u.v. absorption spectra and are quite specific for thiol groups, and so are well suited for quantitative analysis. In addition to these quite characteristic reactions, cysteine is usually the most reactive residue in other nucleophilic reactions such as addition to *N*-alkyl-maleimides[7] or substitution of α-halogeno-acids[8] and nitrated aryl halides.[9]

Thus to obtain specific modification of other nucleophilic groups it is necessary either to choose carefully controlled reaction conditions, or to select reagents which give unstable products on reaction with thiols. The former approach is exemplified by the specific modifications of histidine and methionine by bromo-

[5] G. L. Ellman, *Arch. Biochem. Biophys.*, 1959, **82**, 70.
[6] P. D. Boyer in 'The Enzymes', ed. P. D. Boyer, H. Lardy, and K. Myrbäck, Academic Press, New York, 1959, vol. 1, p. 511.
[7] (*a*) J. R. Heitz, C. D. Anderson, and B M. Anderson, *Arch. Biochem. Biophys.*, 1968, **127**, 627; (*b*) Y. Kanaoka, M. Machida, K. Ando, and T. Sekine, *Biochim. Biophys. Acta*, 1970, **207**, 269.
[8] (*a*) R. N. Perham and J. I. Harris, *J. Mol. Biol.*, 1963, **7**, 316; (*b*) N. Evans and B. R. Rabin, *European J. Biochem.*, 1968, **4**, 548.
[9] (*a*) S. Shaltiel and M. Soria, *Biochemistry*, 1969, **8**, 4411; (*b*) A. Kotaki, M. Harada, and K. Yagi, *J. Biochem.* (*Japan*), 1964, **55**, 553; (*c*) D. J. Birkett, N. C. Price, G. K. Radda, and A. G. Salmon, *F.E.B.S. Letters* 1970, **6**, 346.

or iodo-acetic acid which have been achieved by the use of pH 6 and pH 3 respectively (see Table). At these low pH values, the more reactive nucleophiles (*e.g.* thiol, amine, and phenol) are converted to their unreactive protonated forms. An example of the latter approach is the specific conversion of amino-groups of lysine to urea derivatives by reaction with cyanate,[10] carbon disulphide,[11] *O*-alkyl-ureas,[12] or imidic esters[13] (see Scheme 3). These reactions are carried out at a high pH, in which the corresponding derivatives of cysteine are easily hydrolysed.



Scheme 3

It follows from these considerations that no general methods exist for the specific modification of the weakly nucleophilic hydroxy-groups of serine and threonine. Nevertheless, in a remarkable number of hydrolytic enzymes, halogenophosphate esters[3c,14] and sulphonyl halides[15] are found to react rapidly and specifically with a single serine residue. For example, chymotrypsin is rapidly inactivated by reaction with di-isopropyl fluorophosphate (DFP), as

[10] G. R. Stark, *Biochemistry*, 1965, **4**, 1030.
[11] C. H. Chervenka and P. E. Wilcox, *J. Biol. Chem.*, 1956, **222**, 621.
[12] W. A. Klee and F. M. Richards, *J. Biol. Chem.*, 1957, **229**, 489.
[13] M. J. Hunter and M. L. Ludwig, *J. Amer. Chem. Soc.*, 1962, **84**, 3491
[14] A. K. Balls and E. F. Jansen, *Adv. Enzymol.*, 1952, **13**, 321.
[15] A. M. Gold, in ref. 2, p. 706.

Table *Useful chemical modifications not mentioned in the text*

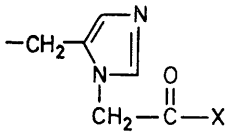
Amino-acid	*Reagent*	*Product*	*Specificity*	*Determination*	*Comments*	*Reference*
Tyrosine	*N*-Acetyl imidazole	*O*-Acetyl tyrosine	+ +	Spectrophotometry	May be reversed by hydroxylamine.	*a*, *b*
Histidine	α-Halogeno-acids, amides	(i)	+ + (at pH 5—7)	Amino-acid analysis	Only unusually reactive histidines are modified.	*c*, *d*
	5-Diazo-1-*H*-tetrazole	(ii)	−	Spectrophotometry	Tyrosine and perhaps other residues are also modified.	*e*
Methionine	α-Halogeno-acids, amides	(iii)	+ + (at pH 2—5)	Amino-acid analysis	Thioether is the only nucleophile which is unprotonated in acid media.	*f*
	H_2O_2 (pH < 4)	Methionine sulphoxide	+	Amino-acid analysis	Alkaline hydrolysis is required before amino-acid analysis. Thiol compounds regenerate methionine. Using H_2O_2, cysteine residues are destroyed.	*g*
	Rose Bengal + $h\nu$	Methionine sulphoxide	+	Amino-acid analysis		*h*

a. R. T. Simpson, J. F. Riordan, and B. L. Vallee, *Biochemistry*, 1963, **2**, 616
b. J. F. Riordan and B. L. Vallee, *Biochemistry*, 1963, **2**, 1460
c. E. A. Barnard and W. D. Stein, *J. Mol. Biol.*, 1959, **1**, 339
d. A. M. Crestfield, W. H. Stein, and S. Moore, *J. Biol. Chem.*, 1963, **238**, 2413
e. H. Horinishi, Y. Hachimori, K. Kurihara, and K. Shibata, *Biochim. Biophys. Acta.*, 1964, **86**, 477
f. H. G. Gundlach, S. Moore, and W. H. Stein, *J. Biol. Chem.*, 1959, **234**, 1761
g. N. P. Neumann, S. Moore, and W. H. Stein, *Biochemistry*, 1962, **1**, 68
h. G. Jori, G. Galiazzo, A. Marzotto, and E. Scoffone, *Biochim. Biophys. Acta*, 1968, **154**, 1

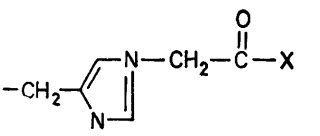
Cystine	2-Mercapto-ethanol	—SH	++	See Section 2A	No other groups in protein are susceptible to mild reduction.	*i*
	Performic Acid	$—SO_3H$	–	Amino-acid analysis	Cysteine is also oxidised to cysteic acid; widely used to determine total cystine + cysteine. Methionine and tryptophan are also attacked.	*j*
Tryptophan	*N*-Bromo-succinimide	(iv)	–	Spectrophoto-metry Spectrofluor-imetry	Tyrosine and histidine residues also oxidised. Reaction can proceed further to break peptide bonds (Section 3A). A more selective analogue was recently described.	*k* *l*
	2-Hydroxy-5-nitrobenzyl bromide	Several cyclised products	++	Spectrophoto-metry	Products fully characterised; contain nitrophenol group with environmentally sensitive spectrum.	*m*
	Sulphenyl halides	(v)	+	Spectrophoto-metry	Cysteine is also modified, but this can be selectively reversed.	*n*
Arginine	1,2-Cyclo-hexanedione	(vi)	+	Remaining arginine by amino-acid analysis	pH 11 required, the mildest conditions known for any arginine reagent. Lysine also modified. Product probably derives from pinacol rearrangement.	*o*, *p*

i. R. Cecil in 'The Proteins', ed. H. Neurath, Academic Press, New York, 1963, vol. 1, p. 379, and ref. 6
j. C. H. W. Hirs in ref. 2, p. 59
k. T. F. Spande and B. Witkop in ref. 2, p. 498
l. G. S. Omenn, A. Fontana, and C. B. Anfinsen, *J. Biol. Chem.*, 1970, **245**, 1895
m. G. M. Loudon and D. E. Koshland, jun., *J. Biol. Chem.*, 1970, **245**, 2247
n. E. Boccu, F. M. Veronese, A. Fontana, and C. A. Benassi, *European J. Biochem.*, 1970, **13**, 188
o. K. Toi, E. Bynum, E. Norris, and H. A. Itano, *J. Biol. Chem.*, 1967, **242**, 1036
p. Woo-Hoe Liu, G. Feinstein, D. T. Osuga, R. Haynes, and R. E. Feeney, *Biochemistry*, 1968, **7**, 2886

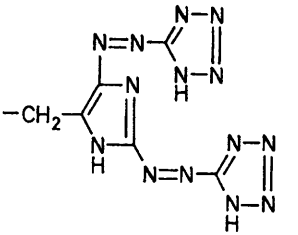
(i)



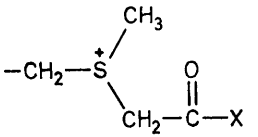
or



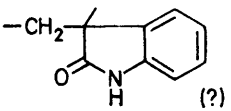
(ii)



(iii)

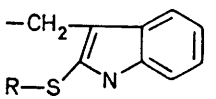


(iv)

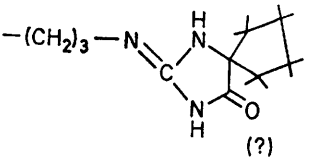


(?)

(v)



(vi)



(?)

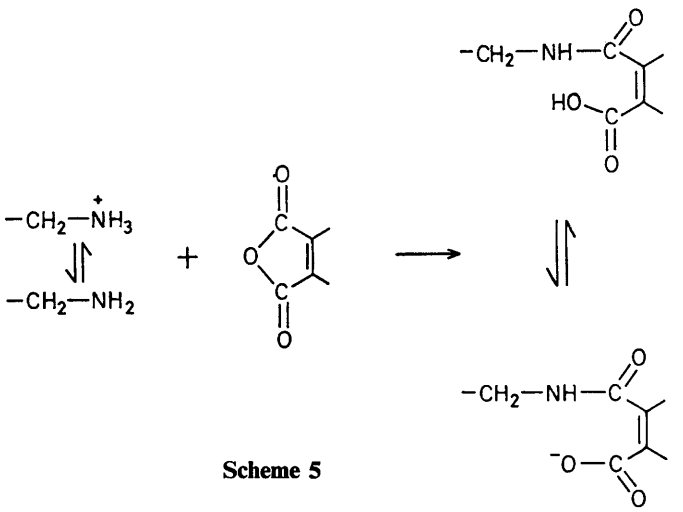
in Scheme 4. This emphasises that reactions occurring at functional groups of proteins may differ from model reactions with simple compounds in rate, extent, sensitivity to changes in pH and solvent, and in the number of side-reactions. Although it is possible to regard a protein as an array of functional groups, it is important to remember that the entire protein molecule can affect the reaction at a particular group.

Scheme 4

The environment of a group may affect its reactivity both sterically and electronically. Some groups may be unreactive simply because they are inaccessible to external reagents (see Section 2B). In other cases the proximity of charged groups, or groups capable of hydrogen-bonding or of charge-transfer interaction, may alter the reactivity of a particular group. The protein environment may also affect the reagent through electrostatic effects or by promoting non-covalent binding of the reagent to the protein. As the result of a combination of these effects, particular residues of a given type in a protein may be markedly reactive or unreactive. For example, the thiol groups of the enzyme L-glutamate dehydrogenase are so unreactive that treatment with an *N*-alkyl-maleimide[16] or with 2,4,6-trinitrobenzenesulphonic acid (TNBS)[17] leads to modification of a specific reactive amino-group. For exceptionally reactive residues, such as the serine mentioned above, modification can in favourable circumstances occur with little modification of other residues, and such a reaction is said to be 'site-specific'.

C. Choice of Reagent.—The emphasis which has been laid on selectivity may seem to suggest that this is a criterion which is always paramount in applications of chemical reactions of proteins. This is far from the case. To alter the electrophoretic mobility or solubility of a protein, modification reactions should be selected which will introduce the greatest number of charged groups. The reaction of nucleophilic groups with cyclic anhydrides[18] is particularly useful in that the reaction converts neutral thiol groups and positively charged amino-groups into derivatives which are negatively charged at pH 7(see Scheme 5). In some applications it is essential that the chemical reactions involve mild conditions so that no generalised structural alteration occurs. This is unimportant if the study is a determination of the number of residues of a given kind in a protein.

[16] J. J. Holbrook and R. Jeckel, *Biochem J.*, 1969, **111**, 689.
[17] R. B. Freedman and G. K. Radda, *Biochem. J.*, 1969, **114**, 611.
[18] P. J. G. Butler, J. I. Harris, B. S. Hartley, and R. Leberman, *Biochem. J.* 1969, **112**, 679.



Scheme 5

The criteria in this instance are that the reaction is rapid, complete, and easily quantitated; in such circumstances the protein may well be denatured before reaction. The criteria by which a reagent is to be assessed will, therefore, depend entirely on the particular application involved.

Another relevant property is the stability of the reaction product. It is sometimes valuable if the group introduced by a modification reaction can be selectively removed, rather like a protecting group in organic synthesis, but often it is essential that the group should not be displaced in subsequent handling. In the case of the cyclic anhydrides mentioned above, a series of analogous reagents has been developed which differ in product stability. In mild acid conditions, when the carboxy-group is protonated, all are susceptible to hydrolysis at the amide, facilitated by the neighbouring carboxy-group, but the ease of hydrolysis is dependent on steric restrictions between the amide and carboxy-group. ε-*N*-Maleyl-lysine has a half-life of 12 h at pH 3·5,[18] while the derivatives from 2-methylmaleic anhydride and 2,3-dimethylmaleic anhydride are much more labile.[19] More recently, a further anhydride, giving products of intermediate stability, has been introduced.[20]

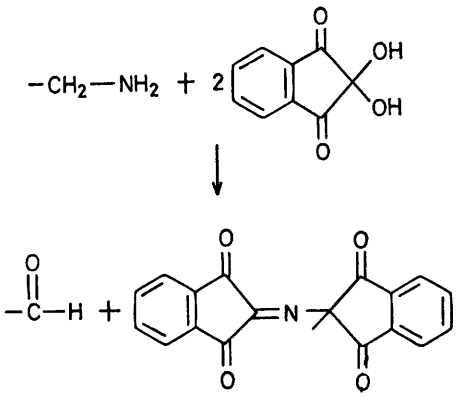
Ease of quantitation is an asset even in cases where determination of the number of a given kind of residue is not the main purpose. Any protein derivative should be characterised in terms of the extent of reaction, and this is very easily done if the reaction introduces a group which can be detected spectroscopically or by amino-acid analysis. Otherwise, the reagent must be isotopically labelled so that the incorporation of radioactivity into the protein can be monitored. Spectroscopic methods have the advantage that continuous monitor-

[19] H. B. F. Dixon and R. N. Perham, *Biochem. J.*, 1968, **109**, 312.
[20] M. Riley and R. N. Perham, *Biochem. J.*, 1970, **118**, 733.

ing of the modification reaction may be possible, and much information can be derived from the kinetics of reaction (see Sections 2B, 3C, and 3D).

2 Applications in the Study of Protein Structure

A. Primary Structure.—Simple reactions for the detection of particular functional groups were among the earliest known reactions of proteins. Most were 'colour tests', which were rarely quantitative, and the actual chemistry of the reactions was frequently uncertain.[21] A few of these reactions are still used for analysis. The biuret reaction of peptide bonds gives a blue copper complex, and is a widely used and reproducible method for the determination of total protein.[4a] Sensitivity is increased if this is used together with the Folin phosphomolybdotungstate reagent, which oxidises phenols.[22] Reaction of ninhydrin with amino-groups to yield a coloured product (Scheme 6) is another of the old tests still



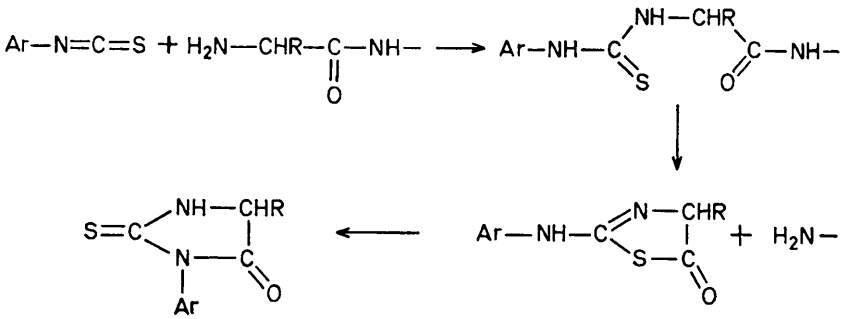
Scheme 6

used analytically,[4a] and it is employed in the automatic amino-acid analysers which have replaced the group tests.[23]

An important application of chemical modification is the labelling of terminal groups in the determination of amino-acid sequence in small peptides. Sanger introduced fluoro-dinitrobenzene to label α-NH_2 groups of insulin.[24] A number

[21] E. G. Young in 'Comprehensive Biochemistry', ed. M. Florkin and E. H. Stotz, Elsevier Publishing Co., Amsterdam, 1963, vol. 7, p. 1.
[22] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 1951, **193**, 265.
[23] D. H. Spackman, W. H. Stein, and S. Moore, *Analyt. Chem.*, 1958, **30**, 1190.
[24] F. Sanger, *Biochem. J.*, 1945, **39**, 507.

of side-chain groups also react, but after acid hydrolysis the yellow α-dinitrophenylamino-acids are easily separated and identified by their chromatographic properties. This method is still used in sequence work, but labelling with 1-dimethylaminonaphthalene-5-sulphonyl chloride ('dansyl' chloride) is an alternative.[25] This reagent has the advantages that the dansyl group is strongly fluorescent so that smaller quantities of label can be detected, and the products are slightly more stable to acid hydrolysis.[25,26] An alternative sequencing procedure is to label the terminal α-NH_2 by reaction with phenyl isothiocyanate.[27] The product cyclises, splitting the terminal peptide bond to give an identifiable derivative and a new terminal α-NH_2 group (Scheme 7). Thus the reaction can occur to remove amino-acids sequentially from the α-NH_2 terminus, and an automated procedure has been developed.[28]



Scheme 7

The sensitivity of these last two procedures is exemplified by some recent work on the enzyme methionyl-tRNA synthetase from *E.coli*.[29] Using 50 μg of enzyme, the active site was labelled with radioactive methionine and, after enzymic hydrolysis, radioactive peptides were isolated. Their compositions were determined by hydrolysis and conversion of the amino-acids to dansyl derivatives; 20 pmol of each peptide were required for this. The sequence was determined by a micro-method employing the stepwise treatment above (Edman degradation) followed by identification as dansyl derivatives, and less than 1 nmol was required to determine the sequence of an octapeptide. Terminal α-CO_2H groups are not usually identified by chemical modification.

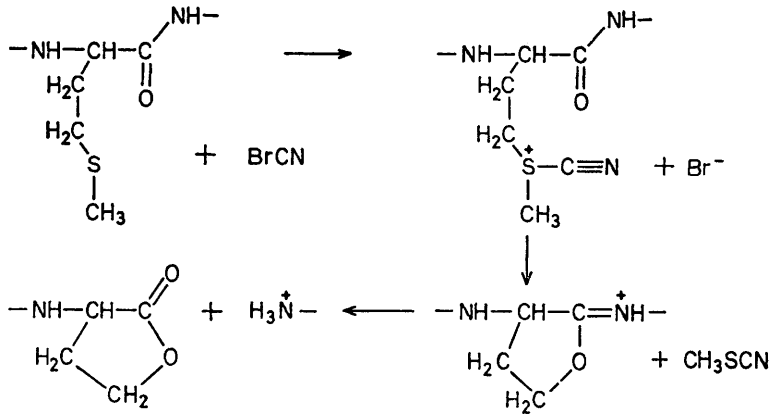
[25] W. R. Gray in ref. 2, p. 139.
[26] C. Gros and B. Labouesse, *European J. Biochem.*, 1969, **7**, 463.
[27] P. Edman, *Acta Chem. Scand.*, 1950, **4**, 283.
[28] P. Edman and G. Begg, *European J. Biochem.*, 1967, **1**, 80.
[29] C. J. Bruton and B. S. Hartley, *J. Mol. Biol.*, 1970, **52**, 165.

Chemical methods for selective cleavage of disulphide bonds are mentioned in the Table. An important application of these methods is in the location of disulphide bonds linking different regions of amino-acid sequence in a protein. In the method of Brown and Hartley,[30] the protein is digested with proteolytic enzymes and the resulting peptides are submitted to electrophoresis on paper. The paper is then exposed to performic acid vapour to oxidise disulphide linkages, and electrophoresis is applied under the same conditions as before but at right angles to the original direction. Peptides containing no disulphide bonds finally lie along a diagonal, but those containing bonds split by the performic acid lie as pairs of peptides displaced from the diagonal. These split peptides can then be identified and the linking pattern determined.

Enzymic digestion of proteins is an essential tool in sequence determination. The enzymes used (pepsin, trypsin, papain, *etc.*) have differences in specificity (that is, different enzymes preferentially split different peptide bonds) so that each produces a different set of peptides, but methods of extending the range of possible products have also been studied. Trypsin is widely used because it selectively hydrolyses peptide links in which the —CO— group is adjacent to a positively charged residue. Natural proteins, therefore, are split after every lysine or arginine residue. To obtain the order in which these peptides are linked in the original protein, it is convenient to 'block' the lysine residues, converting them to uncharged derivatives so that proteolysis only occurs at the arginine residues. Acylation with the reactive ester ethyl trifluoroacetate is favoured because reaction is rapid and the resultant —NH—CO—CF_3 group is easily hydrolysed at a later stage.[31] Alternatively, if a greater number of peptides is required than that given by tryptic digestion of the native protein, the bonds adjacent to cysteine may be made susceptible by reaction with ethyleneimine,[32] to convert the side-chain to —CH_2—S—CH_2—CH_2—$\overset{+}{N}H_3$.

Specific chemical methods of hydrolysis of proteins are alternatives to enzymic methods. Cleavage by cyanogen bromide (Scheme 8) is very useful in that the reaction can be made to occur specifically at methionyl residues,[33] of which there are usually only a few in the protein. Thus in a complete immunoglobulin molecule containing 1320 amino-acid residues there are only 18 methionines,[34] and cyanogen bromide cleavage at these was an essential step in the determination of the complete primary structure.[34,35] A less selective reagent for chemical cleavage of peptide chains is *N*-bromosuccinimide, which hydrolyses bonds adjacent to tryptophan residues.[36]

[30] J. R. Brown and B. S. Hartley, *Biochem. J.*, 1966, **101**, 214.
[31] R. F. Goldberger and C. D. Anfinsen, *Biochemistry*, 1962, **1**, 401.
[32] R. D. Cole in ref. 2, p. 315.
[33] (*a*) E. Gross and B. Witkop, *J. Biol. Chem.*, 1962, **237**, 1856; (*b*) E. Gross in ref. 2, p. 238.
[34] G. M. Edelman, B. A. Cunningham, W. E. Gall, P. D. Gottlieb, U. Rutishauser, and M. J. Waxdal, *Proc. Nat. Acad. Sci. U.S.A.*, 1969, **63**, 78.
[35] M. J. Waxdal, W. H. Konigsberg, W. L. Henley, and G. M. Edelman, *Biochemistry*, 1967, **7**, 1959.
[36] A. Patchornik, W. B. Lawson, E. Gross, and B. Witkop, *J. Amer. Chem. Soc.*, 1960, **82**, 5923.



Scheme 8

B. Tertiary Structure.—Chemical reactions can be used to obtain information on the tertiary structure of proteins in a number of ways, and these will be treated in order of increasing definition of the information.

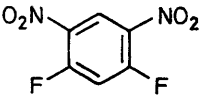
The overall dimensions of a protein in solution can be derived from the rotational relaxation time, a parameter which can be determined if the protein is made strongly fluorescent by reaction with dansyl chloride or fluorescein isothiocyanate. The 'fluorescent conjugate' is excited with polarised light and the extent of depolarisation of the emission yields information about the rotation of the chromophore.[37] Determination of molecular structural parameters from such information involves a number of assumptions, and recent studies suggest that these are frequently unjustified.[38]

Before complete protein structures were available from *X*-ray crystallography, considerable effort was expended in determining whether particular groups in proteins were on the surface or interior of the molecule. Of the methods of differentiating 'buried' and 'exposed' groups, determination of the extent and rate of reaction with group-specific reagents in native and denatured proteins was among the most widely used.[39] Large numbers of the reagents mentioned in this Review have been used for this purpose.[9c,40,41; Table refs. *a*,*e*] 'Buried' and 'exposed' are imprecise terms and simple titration of residues in proteins in various states does not give detailed information about their location, but the method is useful in observing conformational *changes* in proteins. Thus in the

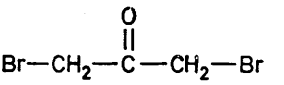
[37] G. Weber, *Adv. Protein Chem.*, 1953, **8**, 415.
[38] (*a*) R. F. Chen, *Arch. Biochem. Biophys.*, 1968, **128**, 163; (*b*) F. W. J. Teale and R. A. Badley, *Biochem. J.*, 1970, **116**, 341.
[39] S. N. Timasheff and M. J. Gorbunoff, *Ann. Rev. Biochem.*, 1967, **36**, 13.
[40] H. Horinishi, K. Nayaka, A. Tani and K. Shibata, *J. Biochem.* (*Japan*), 1968, **63**, 41.
[41] T. F. Spande and B. Witkop in ref. 2, p. 528.

enzymes phosphorylase[9c] and fructose-1,6-diphosphatase,[42] the number of 'exposed' —SH groups is diminished in the presence of the substrate or other ligands. This could arise either from a generalised conformational change in the protein or through specific 'masking' of some groups by the ligand. In the haemoprotein cytochrome *c*, differences in reactivity of methionyl[43] and lysyl[44] residues between the ferri- and ferro-states can only derive from a conformational change.

Chemical reactions can be employed to determine distances between specific groups and reveal some details of protein topology. Bifunctional reagents have been employed to cross-link groups within a given molecule and many such reagents have been known for some years.[45] Most reagents contain the same functional group twice, rather than different groups, and the most common reagents are alkyl, aryl, or sulphonyl halides.[46] In the main, intramolecular reactions have been employed for determining details of tertiary structure. Thus difluorodinitrobenzene (1) has been used to cross-link amino-groups in ribonuclease,[47] and 1,3-dibromoacetone (2) has been found to link a cysteine residue to a histidine in the active sites of several plant proteases.[48]



(1)



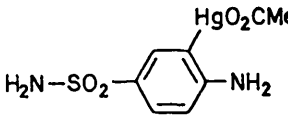
(2)

Spectroscopic methods are also available. The efficiency of energy transfer from an excited to a ground-state chromophore by the resonance mechanism[49] is sensitive to the distance between the chromophores. This has been used in several applications such as determining the distance from tryptophan residues to the active site in both pepsin and carboxypeptidase by labelling the active site with substrate analogues which could be acceptors of resonance energy.[50] In the case of ribonuclease it has been demonstrated that a covalent label can act

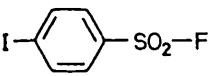
[42] G. J. S. Rao, S. M. Rosen, and O. M. Rosen, *Biochemistry*, 1969, **8**, 4904.
[43] K. Ando, Y. Orii, S. Takemori, and K. Okunuki, *Biochim. Biophys. Acta*, 1965, **111**, 540.
[44] D. J. Birkett, R. B. Freedman, N. C. Price, and G. K. Radda in 'Chemical Reactivity and Biological Role of Functional Groups in Enzymes', ed. R. M. S. Smellie, Academic Press, London and New York, 1970; Biochemical Society Symposia, No. 31, p. 147.
[45] P. Alexander, M. Fox, K. A. Stacey, and L. F. Smith, *Biochem. J.*, 1952, **52**, 177.
[46] F. Wold in ref. 2, p. 617.
[47] P. S. Marfey, H. Nowak, M. Uziel, and D. A. Yphantis, *J. Biol. Chem.*, 1965, **240**, 3264.
[48] S. S. Husain and G. Lowe, *Biochem. J.*, 1968, **110**, 53.
[49] (*a*) Th. Förster, *Discuss. Faraday Soc.*, 1959, No. 27, p. 1; (*b*) L. Stryer and R. P. Haugland, *Proc. Nat. Acad. Sci. U.S.A.*, 1967, **58**, 719.
[50] (*a*) R. A. Badley and F. W. J. Teale, *J. Mol. Biol.*, 1969, **44**, 71; (*b*) S. A. Latt, D. S. Auld, and B. L. Vallee, *Proc. Nat. Acad. Sci. U.S.A.*, 1970, **67**, 1383.

to photosensitize oxidation of neighbouring residues,[51] and this method might yield topological information.

Finally, chemical modification is important in producing heavy-atom derivatives of proteins, which are essential for complete structural analyses by *X*-ray diffraction.[52] Mercury atoms can be introduced covalently by reaction at sulphydryls; in haemoglobin, *p*-chloromercuribenzoate and other reagents have been used.[52] With carbonic anhydrase and chymotrypsin, heavy atoms were introduced by reaction with inhibitor analogues such as (3) and (4) respectively.[53, 54] It has been suggested that when suitable reactive groups are not present, acetoacetylation of a protein could be followed by reaction of mercurials at the activated methylene group.[55]



(3)



(4)

It has been suggested that advances in *X*-ray crystallographic techniques have made chemical modification studies of protein structure entirely superfluous. While it is clear that no chemical modifications can give the complete detailed molecular picture provided by an electron density map, they still retain some value. It is noteworthy that in the two recent papers in which Perutz presented a detailed stereochemical mechanism for co-operative effects in haemoglobin,[56] chemical modification studies were quoted extensively. These studies provided information on functional alterations in chemically modified haemoglobins (Section 3D), on the reactivity of particular groups in haemoglobin (Section 2B), and on changes in structure recorded by spin labels (Section 3E).

3 Applications in the Study of Protein Function

A. Functional Sites of Proteins.—In considering proteins which act on small molecules, such as enzymes, antibodies, and transport proteins, it is reasonable to assume that the small molecule or 'substrate' interacts with only a portion of the protein surface and that only a few groups of the protein act directly on the substrate. The term 'active-site' will be used to describe the entire region of protein involved in substrate binding, specificity, and catalysis. Particular side-

[51] E. Scoffone, G. Galiazzo, and G. Jori *Biochem. Biophys. Res. Comm.*, 1970, **38**, 16.
[52] C. C. F. Blake, *Adv. Protein Chem.*, 1968, **23**, 59.
[53] B. Tilander, B. Strandberg, and K. Fridborg, *J. Mol. Biol.*, 1965, **12**, 740.
[54] P. B. Sigler, B. A. Jeffery, B. W. Matthews, and D. M. Blow, *J. Mol. Biol.*, 1966, **15**, 175.
[55] D. G. Lindsay and S. Shall, *Biochem. J.*, 1969, **115**, 587.
[56] M. F. Perutz, *Nature*, 1970, **228**, 726, 734.

chain groups may be referred to as 'binding groups' or 'catalytic groups' respectively if they are directly involved in forming non-covalent bonds to the substrate or if they participate in the bond-breaking and bond-forming of the catalytic reaction.

Many factors have been supposed to contribute to the extraordinary catalytic properties of enzymes,[57] including special juxtapositions of functional groups, and particularly favourable microenvironments for reaction. Precisely these factors influence the reactivity of functional groups in chemical modification reactions, so it is not surprising that groups showing unusual reactivity in modification reactions are often implicated in enzymic catalysis. Vallee and Williams[58] have proposed that this reactivity may arise from particular electronic properties of functional groups which are characteristic of enzymes even in the absence of substrate – they define such groups as being in an 'entatic' state. This property allows the catalytic groups to be selectively modified by chemical reagents, which was how these groups were first identified.

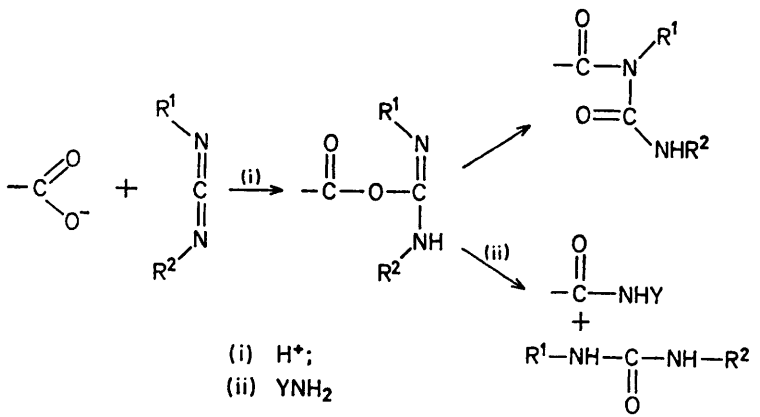
In the case of the 'active serine' of chymotrypsin[3b, 3c, 14] (Section 1B), the unusual chemical properties arise from the hydrogen-bonding of the serine hydroxy-group to the imidazole of a histidine residue and thence to an aspartate located in an unusually non-polar region. These residues act as a 'charge-relay' system[59] and effectively reduce the pK_a of the serine hydroxy-group in some reactions.[60] The enzyme's inactive precursor, chymotrypsinogen, lacks this feature of tertiary structure, which accounts for its lack of activity as a hydrolytic enzyme, and its failure to react with DFP.

Another well-characterised case is Lys-41 of ribonuclease. This group is particularly reactive to activated aryl halides[61] because the pK_a is unusually low[61b] and so more of the reactive, unprotonated species is present. The lower pK is probably caused by the large number of positive groups near to Lys-41 in the native molecule.[62]

Chemical modification of functional proteins has generated the concept of 'essential' residues – residues whose modification leads to loss of the protein's function. 'Essential' is a neutral term, involving no assumptions about how the protein's function depends on the 'essential' residue. Less equivocal information derives from studies in which a chemical modification is shown to have *no* effect on the protein's activity.[63] Ideas on which residues can be 'essential' to the activity of enzymes have tended to be determined by the availability of specific reagents. Thus the large number of specific modification reactions for

[57] (*a*) H. Gutfreund and J. R. Knowles in 'Essays in Biochemistry,' vol. 3, ed. P. N. Campbell and G. D. Grenville, Academic Press, London, 1967, p. 25; (*b*) D. E. Koshland jun., and K. E. Neet, *Ann. Rev. Biochem.*, 1968, **37**, 359.
[58] B. L. Vallee and R. J. P. Williams, *Proc. Nat. Acad. Sci. U.S.A.*, 1968, **59**, 498.
[59] D. M. Blow, J. J. Birktoft, and B. S. Hartley, *Nature*, 1969, **221**, 337.
[60] M. R. Holloway, *Ann. Reports* (*B*), 1968, **65**, 601.
[61] (*a*) C. H. W. Hirs, *Brookhaven Symp. in Biology*, 1962, **15**, 154; (*b*) R. P. Carty and C. H. W. Hirs, *J. Biol. Chem.*, 1968, **243**, 5244; 5254; (*c*) R. B. Freedman and G. K. Radda, *Biochem. J.*, 1968, **108**, 383.
[62] G. Kartha, J. Bello, and D. Harker, *Nature*, 1967, **213**, 862.
[63] C. J. Epstein, C. B. Anfinsen, and M. Sela, *J. Biol. Chem.*, 1962, 237, 3458.

sulphydryl groups led to the early definition of 'essential' sulphydryl groups, and other residues were frequently neglected. This is demonstrated by the recent surge of interest in carboxy-groups in enzymes. Early methods for the modification of enzyme carboxy-groups required either strenuous conditions, such as HCl in methanol, or very vigorous reagents, such as diazomethane or mustard gas.[64] Then Riehm and Scheraga noted that water-soluble carbodi-imides modify carboxy-groups in ribonuclease,[65] and Hoare and Koshland extended this observation and derived a general method of modification (Scheme 9).[66] The initial



Scheme 9

reaction produces an *O*-acyl-isourea, which then rearranges slowly to give an *N*-acyl-urea. If a nucleophile is present, this can displace the carbodi-imide as a substituted urea, and this reaction is usually much more rapid than the rearrangement.[40, 66] Hence a nucleophilic group can be specifically coupled to carboxyl by carbodi-imide activation. This method (together with an analogous procedure involving activation by isoxazolium salts[67]) has led to vigorous investigation of the role of carboxy-groups in proteins,[67–70] and to the identification of 'essential' carboxy-groups in lysozyme,[68] ribonuclease,[69] and trypsin.[67]

The interpretation of functional changes following chemical modification is discussed in Section 3C. It should be pointed out that not all chemical modifications of a given group produce the same result. If five $-NH_2$ groups of cyto-

[64] H. Fraenkel-Conrat and H. S. Olcott, *J. Biol. Chem.*, 1945, **161**, 259.
[65] J. P. Riehm and H. A. Scheraga, *Biochemistry*, 1966, **5**, 99.
[66] D. G. Hoare and D. E. Koshland jun., *J. Biol. Chem.*, 1967, **242**, 2447.
[67] P. Bodlaender, G. Feinstein, and E. Shaw, *Biochemistry*, 1969, **8**, 4941.
[68] Tsau-Yen Lin and D. E. Koshland jun., *J. Biol. Chem.*, 1969, **244**, 505.
[69] M. Wilchek, A. Frensdorff, and M. Sela, *Biochemistry*, 1967, **6**, 247.
[70] (*a*) A. Eyl and T. Inagami, *Biochem. Biophys. Res. Comm.*, 1970, **38**, 149; (*b*) A. Bezkorovainy and D. Grohlich, *Biochim. Biophys. Acta*, 1970, **214**, 37.

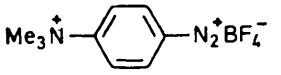
chrome *c* are modified with TNBS the protein cannot interact with its substrate, cytochrome *a*, but this interaction is enhanced after guanidination of the amino-groups of cytochrome *c*.[71] Thus it is the positive charge, not the —NH_2 group itself, which is 'essential' for activity in this system.

B. Methods of Ensuring Specificity.—To derive useful information from functional changes in proteins following chemical modification, the modification must be minimal and easily characterised. Specificity is therefore most important, and several methods are available to help achieve it.[3a] It is possible to isolate enzymes covalently labelled with substrate or with substrate analogues when the enzymes are involved in phosphate transfer[72] or hydrolysis[8a, 73] and there is an acyl- or phosphoryl-intermediate in the enzyme reaction. In cases in which a carbonyl group is bound to an enzyme amino-group in the catalytic process, this complex may be 'trapped' by borohydride reduction,[74] and the site of attachment determined.

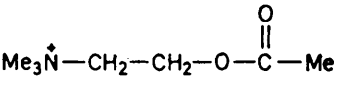
Specificity may also be achieved by 'affinity labelling'. This requires the synthesis of a molecule containing a reactive group capable of reacting chemically with groups of a protein, but also containing characteristic molecular structures resembling the protein's substrate so that the 'affinity label' preferentially binds non-covalently in the active-site region before forming the covalent link.[75] Affinity labels for the hydrolytic enzymes were among the earliest developed,[3c] but reagents have subsequently been synthesised [(5),[76] (6),[77] (7)[78]] to label active sites and receptors for really interesting biological molecules such as acetylcholine (8), oestradiol (9), and adenosine triphosphate (10).

In the absence of such a 'handle' for obtaining specific modification, it is advantageous to use an unreactive reagent for modification so that any intrinsic difference in reactivity between different groups is clearly expressed in a kinetic selectivity of reaction.[11,61,79,80] Even with a very slow reaction, though, the reacting groups have to be sufficiently distinct before selectivity can be obtained.[81] The specific modification of 'active-site' serine groups mentioned above is an example of kinetic selectivity. For small proteins it is possible to ensure that a particular labelled protein is a single chemical entity, by allowing the reaction

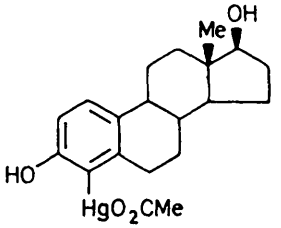
[71] S. Takemori, K. Wada, K. Ando, M. Hosokawa, I. Sekuzu, and K. Okunuki, *J. Biochem. (Japan)*, 1962, **52**, 28.
[72] E. P. Kennedy and D. E. Koshland jun., *J. Biol. Chem.*, 1957, **228**, 419.
[73] H. F. Noller and S. A. Bernhard, *Biochemistry*, 1965, **4**, 1118.
[74] (*a*) E. H. Fischer, A. B. Kent, E. R. Snyder, and E. G. Krebs, *J. Amer. Chem. Soc.*, 1958, **80**, 2906; (*b*) S. Warren, B. Zerner, and F. H. Westheimer, *Biochemistry*, 1966, **5**, 817.
[75] B. R. Baker, 'Design of Active-site Directed Irreversible Enzyme Inhibitors', John Wiley and Sons, New York, 1967.
[76] J.-P. Changeux, T. R. Podleski, and L. Wofsy, *Proc. Nat. Acad. Sci. U.S.A.*, 1967, **58**, 2063.
[77] Chang-Chen Chin and J. C. Warren, *J. Biol. Chem.*, 1968, **243**, 5056.
[78] (*a*) A. J. Murphy, J. A. Duke, and L. Stowring, *Arch. Biochem. Biophys.*, 1970, **137**, 297; (*b*) A. J. Murphy and M. F. Morales, *Biochemistry*, 1970, **9**, 1528.
[79] Choh Hao Li, *Nature*, 1956, **178**, 1402.
[80] Table, ref. *j*.
[81] (*a*) G. Bailin, *Biochim. Biophys. Acta*, 1969, **181**, 328; (*b*) H. Ozawa, *Biochemistry*, 1970, **9**, 2158.



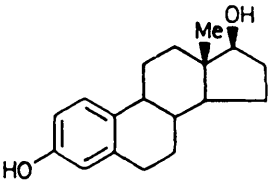
(5)



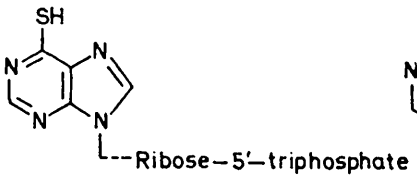
(8)



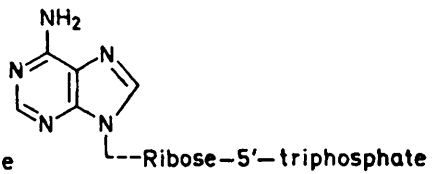
(6)



(9)



(7)



(10)

to proceed with a non-specific reagent and separating the products by column chromatography.[61a,61b,82]

In the last resort, specificity of a sort may be obtained by 'differential labelling', which is useful for labelling antibody and transport protein binding sites which are not distinguished by particular reactivity. The protein together with its substrate is allowed to react first, the presence of the substrate ensuring that groups in the active site are not modified. The excess reagent and substrate are then removed and the protein is then reacted with a radioactively labelled sample of the same reagent. Only residues originally 'protected' by the substrate are radioactively labelled in the resultant protein. The method and some caveats about its use are discussed by Singer.[3a]

C. Interpretation of Functional Changes.—After ensuring that chemical modification occurs specifically, it is then necessary to show that no gross structural change has occurred in the protein by determining the hydrodynamic, optical rotary dispersion, and other properties of the modified protein. Gross structural

[32] M. J. Ettinger and C. H. W. Hirs, *Biochemistry*, 1968, **7**, 3374.

changes can be definitely excluded if the modification reaction can simply be reversed and the biological activity regained.

It is then essential to ascertain whether the modification has occurred at the 'active-site'. This cannot be assumed, even for affinity labelling. Firstly, the loss of activity should show a stoicheiometric relation with the extent of modification,[3a,83] or, better still, the rate of modification of a given group should be correlated with the rate of loss of biological activity.[84] Secondly, a substrate (or inhibitor known to bind at the active site) must decrease the extent of modification of the protein and the extent of inactivation, while related molecules which do not bind to the active site should have no effect.[3a] (This effect provides an indirect method of determining enzyme–substrate dissociation constants.[85])

If the inactivation is closely correlated with modification in these ways, then the group modified may well be a catalytic or binding group. Nevertheless, there remains the possibility that the group modified is in the active-site region, but not directly involved in either catalysis or binding. Modification of such a group might perturb the delicate structural arrangement of the active site through the introduction of a charge or a bulky group, and hence cause inactivation. Only a high resolution structural study can eliminate this possibility.

D. Modification of Functional Sites.—This is the most common application of chemical reactions of proteins, and the reviews[3,4] give numerous examples of the identification of 'essential' residues. The significance of the information derived from such studies can be improved in a variety of ways. The protein, modified at the essential residue, can be isolated and subjected to intense structural study[82] or, less directly, factors influencing the rate of modification can be studied. Thus in yeast alcohol dehydrogenase, the rate of inactivation by *N*-alkyl-maleimides increases with the size of the alkyl group, suggesting the presence of a hydrophobic binding site near to the catalytic site.[7a] The variation in rate of inactivation with pH can demonstrate that the group reacting has an unusual pK_a,[61b] or implicate another ionising group in the mechanism of modification.[8b] The difference in modification behaviour between iodoacetate and iodoacetamide yields information about the presence of charged residues near the group which has been modified.[8b]

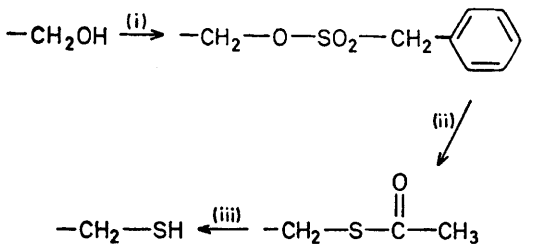
Further detail can be derived from studies of modification by optical isomers of α-iodopropionic acid and its amide.[86] Thus in papain, the relative rates of reaction of the D-(+)- and L-(−)-stereoisomers of α-iodopropionic acid and its amide with a reactive thiol group at the active site allow some predictions to be made about the steric distribution of charged groups about this thiol group. These predictions were confirmed by the complete *X*-ray structure analysis. In those dehydrogenases which contain a reactive thiol group, a correlation was

[83] Tsou Chen-Lu, *Scientia Sinica*, 1962, **11**, 1535.
[84] W. J. Ray jun., and D. E. Koshland jun., *J. Biol. Chem.*, 1961, **236**, 1973.
[85] M. C. Scrutton and M. F. Utter, *J. Biol. Chem.*, 1965, **240**, 3714.
[86] K. Wallenfels and B. Eisele in 'Chemical Reactivity and Biological Role of Functional Groups in Enzymes', ed. R. M. S. Smellie, Academic Press, London and New York, 1970; Biochemical Society Symposia, No. 31, p. 21.

obtained between the selectivity in reaction with the D- and L-antipodes of α-iodopropionic acid, and the steric selectivity in transfer of hydrogen from substrates to the coenzyme NAD^+.[86,87] Those dehydrogenases which transfer hydrogen to the A-side of the nicotinamide ring react more rapidly with the D-(+)-stereoisomer, while those which transfer hydrogen specifically to the B-side react more rapidly with the L-(−)-isomers.

An alternative approach to the elucidation of the stereochemistry of an active site has been employed for the enzyme staphylococcal nuclease. Cross-linking reagents were used to define the distance between particular residues[88] and then affinity labels were prepared which were different *p*-diazophenyl-phosphate derivatives of the substrate analogue deoxythymidine.[89] The sites at which these diazo-derivatives reacted with nucleophilic groups of the enzyme recorded the disposition of these groups about the ribose ring when the substrate analogue was bound to the enzyme.

In some cases chemical modification can enhance an enzyme's activity; in liver alcohol dehydrogenase, modification of amino-groups weakens the binding of the enzyme–product complex and hence accelerates dissociation, the rate-determining step.[90] When an enzyme can use alternate substrates, modification can lead to inactivation towards one substrate but retention of activity towards the other.[91] In enzymes whose activity may be regulated by compounds unrelated to the substrate, chemical modification can lead to 'desensi-



(i) $C_6H_5-CH_2-SO_2F$;

(ii) $CH_3-CO-S^-K^+$;

(iii) H^+

Scheme 10

[87] B. Eisele and K. Wallenfels, *European J. Biochem.*, 1968, **6**, 29.
[88] P. Cuatrecasas, S. Fuchs, and C. B. Anfinsen, *J. Biol. Chem.*, 1969, **244**, 406.
[89] (*a*) P. Cuatrecasas, M. Wilchek, and C. B. Anfinsen, *J. Biol. Chem.*, 1969, **244**, 4316; (*b*) P. Cuatrecasas, *J. Biol. Chem.*, 1970, **245**, 574.
[90] B. V. Plapp, *J. Biol. Chem.*, 1970, **245**, 1727.
[91] C. W. Long, A. Levitzki, and D. E. Koshland jun., *J. Biol. Chem.*, 1970, **245**, 80.

tization', *i.e.* loss of response to the regulator without loss of actual enzymic activity.[92] This has been interpreted as evidence that the 'regulator' binds to the enzyme at a site distinct from the active site, and this intrepretation is implicit in the 'allosteric' theories of enzyme control.[93]

Perhaps the most minute chemical modification of an active site ever attempted was the synthesis of 'thiol-subtilisin'; in this, the 'active' serine of the enzyme subtilisin was converted to a cysteine residue, as shown in Scheme 10.[94] 'Active' cysteines are also found in proteolytic enzymes, and the difference in size of the —OH and —SH groups is less than 1Å. Nevertheless, in this case the synthetic 'thiol' enzyme was almost entirely inactive[94] although no major structural change had occurred. This has led Koshland to propose[95] that the angular requirements of reactions may be very precise, and that enzyme active-sites are finely adjusted to maximise reactivity.

A significant use of chemical modification was the demonstration that an enzyme's activity could be the same in solution as in a crystal. Bernhard synthesized a coloured acyl-chymotrypsin whose self-catalysed hydrolysis resembled the enzyme's natural action.[96] The rate of this reaction could be followed spectrophotometrically and was the same in the solid and solution phases.

In the case of transporting proteins, differential labelling first allowed the galactoside-transporting protein of *E. coli.* to be identified[97] and the use of reagents of different permeabilities has shown that the binding-site of this protein moves through the cell membrane from 'outward' to 'inward' facing.[98] In antibodies, affinity labelling has allowed the antigen binding site to be located.[99]

E. Reporter Groups.—Covalent attachment of a chemical group which has a spectral property sensitive to its environment permits the investigation of regions of a protein through their influence on the 'reporter' group. The environmental factors most easily studied are polarity and local viscosity. Although it is rarely possible to measure these, an empirical scale correlating them with the absorbance, fluorescence, or e.p.r. spectral properties of a 'reporter' group can often be constructed.

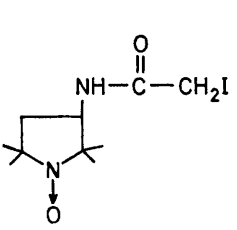
The nitrophenol group can function as a useful reporter group because the spectrum of nitrophenol is very sensitive to its state of ionisation, which is itself sensitive to the environment. The group can be introduced into proteins in a number of ways, the simplest of which is reaction with tetranitromethane, $C(NO_2)_4$, which converts tyrosine residues to *o*-nitrotyrosine.[100] This lowers the

[92] N. C. Price and G. K. Radda, *Biochem. J.*, 1969, **114**, 419.
[93] J. Monod, J.-P. Changeux, and F. Jacob, *J. Mol. Biol.*, 1963, **6**, 306.
[94] (*a*) K. E. Neet and D. E. Koshland jun., *Proc. Nat. Acad. Sci. U.S.A.*, 1966, **56**, 1606; (*b*) K. E. Neet, A. Nanci, and D. E. Koshland jun., *J. Biol. Chem.*, 1968, **243**, 6392.
[95] D. R. Storm and D. E. Koshland jun., *Proc. Nat. Acad. Sci. U.S.A.*, 1970, **66**, 445.
[96] G. L. Rossi and S. A. Bernhard, *J. Mol. Biol.*, 1970, **49**, 85.
[97] C. F. Fox and E. P. Kennedy, *Proc. Nat. Acad. Sci. U.S.A.*, 1965, **54**, 891.
[98] J. Yariv, A. J. Kalb, E. Katchalski, R. Goldman, and E. W. Thomas, *F.E.B.S. Letters*, 1969, **5**, 173.
[99] N. O. Thorpe and S. J. Singer, *Biochemistry*, 1969, **8**, 4523.
[100] M. Sokolovsky, J. F. Riordan, and B. L. Vallee, *Biochemistry*, 1966, **5**, 3582.

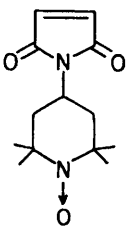
pK_a of the phenol group and so allows study of the role of tyrosine[101, 92] as well as introducing an environmentally sensitive group. Chloromercury derivatives of nitrophenols were recently introduced as sulphydryl reagents;[102] they have advantages common to all mercurials in that the reaction may be reversed by excess of a thiol compound, and in addition, the reporter group is introduced. Finally, the group may be introduced by the reagent 2-bromoacetamido-4-nitrophenol, and in glyceraldehyde-3-phosphate dehydrogenase, labelled with this reagent, the group is sensitive to changes induced by addition of substrates or changes in pH.[103] The azo chromophore is less sensitive in its absorption spectrum, but the derivative formed by reaction of *p*-azobenzene-arsonate with carboxypeptidase allows structural transitions to be followed in the circular dichroism spectrum of the azobenzene chromophore.[104]

Fluorescent 'reporter' groups are especially useful in that fluorescence produces numerous parameters – emission wavelength, lifetime, quantum yield, and polarisation – all of which are sensitive to environment.[105] Reductive coupling of the pyridoxal group to enzymes provides a useful fluorescent probe bound to the active site[106] and labelling with dansyl chloride is a more generally useful method. The latter reagent has been used to label the specialised excitable membrane of the electric eel, to determine the polarity of the membrane interior and the rotational mobility of the labelled component.[107]

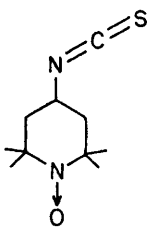
Free-radical covalent labels are usually derivatives of di-t-alkyl nitroxides such as (11)—(13)[108] and (14).[109] The very stable nitroxide radical provides an e.p.r. signal which is easily interpreted and can provide information on the orientation and motion of the label and on conformational changes in the labelled protein.[108] In addition, the radical can influence the relaxation rate of a para-



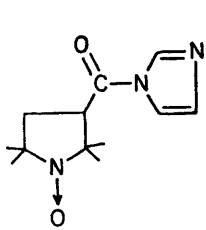
(11)



(12)



(13)



(14)

[101] M. Sokolovsky, J. F. Riordan, and B. L. Vallee, *Biochem. Biophys. Res. Comm.*, 1967, **27**, 20.
[102] C. H. McMurray and D. R. Trentham, *Biochem. J.*, 1969, **116**, 913.
[103] M. E. Kirtley and D. E. Koshland jun., *J. Biol. Chem.*, 1970, **245**, 276.
[104] H. M. Kagan and B. L. Vallee, *Biochemistry*, 1969, **8**, 4223.
[105] L. Stryer, *Science*, 1968, **162**, 526.
[106] M. Cortijo and S. Shaltiel, *Biochem. Biophys. Res. Comm.*, 1970, **39**, 212.
[107] M. Kasai, T. R. Podleski, and J.-P. Changeux, *F.E.B.S. Letters*, 1970, **7**, 13.
[108] H. M. McConnell and B. G. McFarland, *Quart. Rev. Biophysics*, 1970, **3**, 91.
[109] M. D. Barratt, G. H. Dodd, and D. Chapman, *Biochim. Biophys. Acta*, 1969, **194**, 600.

magnetic nucleus through an electron–nuclear dipolar interaction and hence affect the n.m.r. spectrum of protons in bound water or of phosphorus nuclei in other bound species. This is a most useful method of reporting on enzyme–substrate interactions.[110]

F. Subunit Interactions.—Many enzymes are composed of a number of subunits, and the interaction between subunits is of considerable interest. Chemical modification may demonstrate that particular groups are 'essential' for subunit association,[111] or it may explore the dependence of function on subunit interactions. Lactate dehydrogenase has four subunits and four active sites per molecule, but if a single active site is modified the entire 'tetramer' is inactivated.[112] Conversely, with aldolase[113] and glyceraldehyde-3-phosphate dehydrogenase[114] it was found that 'hybrids' of 'active' and 'inactive' subunits were active, the activity of the unmodified subunits being quite unaffected.

The modification of subunit interactions can also throw light on the mechanism of 'allosteric' regulation in oligomeric enzymes.[91, 115] In the case of haemoglobin, the e.p.r. spectrum of a spin-label can be used to monitor a co-operative protein conformational change which is dependent on the state of oxygenation. This has been used to explore the molecular mechanisms of subunit interactions in this protein.[56, 116]

4 Further Applications

Some entirely different applications can be mentioned only briefly. Great advances in immunology have derived from the development of small synthetic antigens called 'haptens' obtained by attachment of groups such as 2,4-dinitrophenyl to protein carriers.[3a] The interaction of antibody and antigen is then analogous to enzyme–substrate binding, and the chemistry of the antibody binding-site and of the interaction can be easily studied.[3a, 99] Reagents which do not penetrate cell membranes will only react with protein components at the external surface, and hence studies of the structural organisation of membranes can be pursued.[117] An application of great technological importance is the intermolecular cross-linking of enzymes,[118] or their chemical attachment to solid supports,[119] which allows industrial exploitation of stable, easily handled enzymes.

[110] M. Cohn, *Quart. Rev. Biophysics*, 1970, **3**, 61.
[111] I. Gibbons and R. N. Perham, *Biochem. J.*, 1970, **116**, 843.
[112] M. Rosenberg, *Fed. Proc.*, 1969, **28**, 535.
[113] E. A. Meighen and H. K. Schachman, *Biochemistry*, 1970, **9**, 1163.
[114] (*a*) E. A. Meighen and H. K. Schachman, *Biochemistry*, 1970, **9**, 1177; (*b*) G. M. L. Spotorno and M. R. Holloway, *Nature*, 1970, **226**, 756.
[115] (*a*) A. D. B. Malcolm and G. K. Radda, *Nature*, 1968, **219**, 947; (*b*) J. H. Wang and Jan-I Tu, *J. Biol. Chem.*, 1970, **245**, 176.
[116] S. Ogawa and H. C. McConnell, *Proc. Nat. Acad. Sci. U.S.A.*, 1967, **58**, 19.
[117] (*a*) H. C. Berg, *Biochim. Biophys. Acta*, 1969, **183**, 65; (*b*) A. H. Maddy and P. H. Kelly, *F.E.B.S. Letters*, 1970, **8**, 341.
[118] (*a*) A. Schejter and A. Bar-Eli, *Arch. Biochem. Biophys.*, 1970, **136**, 325; (*b*) F. A. Quiocho and F. M. Richards, *Proc. Nat. Acad. Sci. U.S.A.*, 1964, **52**, 833.
[119] I. H. Silman and E. Katchalski, *Ann. Rev. Biochem.*, 1966, **35**, 873.

It is hoped that these and previous examples have given some indication of the enormous scope of the method of chemical modification of proteins, the diversity of information which can be gained from it, and the experimental controls and interpretational reservations which arise necessarily from the complexity of proteins as chemical reactants.

I wish to thank Drs A. D. B. Malcolm and G. K. Radda for helpful comments on the manuscript, and Professor B. R. Rabin for some suggestions and for the facilities of his laboratory. I am grateful to the Salters' Company for the award of a Research Fellowship.