SPECIFIC GROUP REAGENTS FOR PROTEINS

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CONTENTS

I. INTRODUCTION

For some time the biological activity of proteins was considered to be due to non-protein moieties such as occur in hemoglobin, cytochrome, the yellow enzyme, etc. When a concerted effort failed to detect in insulin, urease, or pepsin

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any constituent other than what was regarded as a normal protein component, the hypothesis was accepted that proteins containing only the usual L-amino acids in peptide linkage may possess highly specific biological activities. Investigators next attempted to determine what parts of the protein structure were essential for the various activities. For this purpose a number of reagents were used to modify the proteins. The modified proteins were then assayed and if the activity was found to have been destroyed, it was assumed that the specific group attacked by the reagent played an important rôle in mediating the activity. Since work along these lines is constantly expanding, it appears timely to review the known information concerning the specificity of the various reagents that have been used, and to point out some of the difficulties that may beset an investigator not sufficiently aware of the numerous reactions that may occur.

It now appears to be clearly established that the amino acid components of proteins are bound together by peptide bonds through their α-amino and α-carboxyl groups and that the rest of the amino acid is essentially free. In the case of the monoaminomonocarboxylic acids, such as alanine, leucine, etc., this is a hydrocarbon chain and is therefore chemically inert. However, the more complicated amino acids possess polar groups or other reactive chemical configurations that are available for chemical reactions. Table 1 contains a list of these groups and of their relative abundance in several typical proteins and in a few of unusual composition.

The number and diversity of protein groups would make a discussion of their chemistry lengthy and involved were it not for the fact that the reactions to be considered will be limited to those that (*1*) do not hydrolyze the peptide bonds or the disulfide groups of the protein backbone, and also, preferably, (*2*) do not cause enough disruption of the original configuration to bring about the drastic, often irreversible, structural changes usually lumped under the term "denaturation." The present review will be concerned only with reactions involving primary bonds on the side chains of proteins. Such reactions may render proteins more resistant or more susceptible to denaturation. When a large proportion of the polar groups have been modified, the question whether the protein has become denatured in the process is often one mainly of terminology, since many of the derivatives have properties usually assigned to denatured proteins (e.g., insolubility at the isoelectric point) and yet resemble native proteins in other respects. It should be noted that proteins vary greatly in their sensitivity to various types of denaturing agents and that, therefore, the conditions suitable for the preparation of "native derivatives" of one protein may cause denaturation of another.

Many protein derivatives have been found to be as homogeneous as the protein from which they were prepared (for examples, see 65, 87, 154, 165, 203). These observations indicate that the organic chemical reactions proceed in a straightforward fashion, e.g., a protein molecule in which several of the groups have become substituted is not then more reactive than is the original protein. If, for example, 60 acetyl groups have been introduced, any individual molecule may be expected to contain a number somewhere in the range of 55 to 65 rather than in the range of 20 to 100 introduced groups. On a statistical basis also, it

would be expected that derivatives would be homogeneous whenever an appreciable number of protein groups have participated in the reaction. This homogeneity makes possible the assignment of biological activity to a derivative, and not necessarily to small amounts of unreacted protein in the product obtained after reaction.

If inhomogeneous derivatives are obtained, it appears probable that denaturation changes may have intervened. For reasons not yet thoroughly understood, part of the groups in some native proteins do not appear to be available for reaction but can be made to become so by "denaturation." This phenomenon has recently been reviewed in detail (249). The disruption of the highly organized protein molecule "unmasks," physically or chemically, groups that could not otherwise be detected. Differences in availability have been demonstrated for sulfhydryl, disulfide, and tyrosine groups (reviewed in 10 and 249; see also 200). The possibility that other polar groups may similarly be made more reactive by denaturation has been suggested (10).

Main emphasis will, in this review, be placed on specific protein reagents. These are reagents which under suitable conditions affect only one type of group. A recent comprehensive review of the reactions of native proteins (166) makes unnecessary the inclusion of details concerning many non-specific reagents.[2] Also, since the older literature dealing with the chemical reactions of proteins contains little data of assistance in determining the nature of the reactions observed, it has not been considered in detail. Literature references are available (1, 90, 196).

Criteria for specific reagents

The ideal reagent for the determination of a group specificity should meet the following criteria:

1. The amount of reagent introduced or used up should be measurable.
2. The protein group to be reacted should be measurable.
3. The amount of reagent introduced or used up should be equivalent to the number of protein groups that have reacted.
4. The reaction should proceed under mild conditions and, preferably, be reversible by mild treatment.

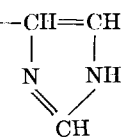
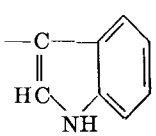
If, under these conditions, the biological activity of a purified protein is first lost and then, after reversal of the reaction, regained, the group in question can be said to play a positive rôle in the biological activity.

Such conditions have been met in only a limited number of cases (e.g., 148, 159, 168, 335, 336). More often investigators have used reagents of doubtful claim to specificity, based mainly on the analogy to their reactions with other proteins. However, instances are now known in which reagents do not react in the same manner with different proteins. It would therefore seem to be necessary to check by reliable analytical techniques the effect of each reagent as it is applied to each new protein.

[2] The authors thank Dr. R. M. Herriott for permitting them to see his manuscript (166) prior to publication.

TABLE 1

Reactive groups of proteins

GROUP	STRUCTURE	ORIGIN	RELATIVE ABUNDANCE*						MODEL SUBSTANCE	RELATIVE ABUNDANCE OF DOMINANT GROUP*
			β-Lactoglobulin	Egg albumin	Insulin	Gliadin	Bovine serum albumin	Edestin		
Amino	$-NH_2$	Lysine Peptide chain end	8.8	5.1	5.0	1.0	9.0	1.8		
Guanidyl	$-NHCNH_2$ (=NH)	Arginine	1.6	3.3	1.7	1.5	3.6	9.3	Salmine sulfate	38
Imidazole		Histidine	1.0	1.5	3.3	1.3	2.5	1.9		
Amide	$-CONH_2$	Glutamine Asparagine	7.7	7.4	9.8	32.0	6.2	12.6	Polyglutamine	62
Aliphatic hydroxyl	—OH†	Serine Threonine	8.2	11.1	6.7	6.4	9.7	9.2	Sericin	35
Indole		Tryptophan	1.0	0.7	0	0.3	0.3	0.6	Gramicidin	20
Thiomethyl	$-SCH_3$	Methionine	2.2	3.5	0	1.1	0.5	1.6		

Disulfide	—S—S—	Cystine	1.0	0.4	5.0	1.1	2.2	0.4	Hair	8
Phenol		Tyrosine	2.1	2.2	7.0	1.8	3.0	2.4		
Sulfhydryl	—SH	Cysteine	0.6	1.0	0	0	0.3	0.25		
Carboxyl	—COOH	Glutamic acid Aspartic acid Peptide chain end	14.8	10.7	10.0	3.7	12.5	8.4	Polyglutamic acid	78

* Gram-equivalents per 10^4 gram of protein.

Amino, indole, SH, and carboxyl group analyses are for the most part based on determinations on the intact protein (114). Other groups are calculated from recent amino acid analyses of hydrolysates (52, 64, 218, 263). The values for insulin and β-lactoglobulin are averages. Polyglutamine and polyglutamic acid (110), sericin (266), and gramicidin (110a) analyses are those of this Laboratory. The data are intended to be illustrative of the relative distribution of groups in several proteins rather than of absolute significance. However, it is doubtful that any one figure is more than 20 per cent in error.

† Hydroxyproline and hydroxylysine also furnish aliphatic hydroxyl groups, but these amino acids appear to have limited distribution in proteins.

The attention of investigators has been focused almost exclusively on the sulfhydryl, amino, and phenolic groups of proteins as possible active "centers" for the activities of enzymes, hormones, toxins, and viruses. Neglect of the other polar groups stems in part from the fact that they are less active chemically and thus less likely to be involved in the physiological reactions, but probably is due mostly to the lack of analytical techniques adequate to determine whether or not these groups have reacted with the various reagents.

In Section II of this review we shall outline the present knowledge of the specific reactions available for each of the protein polar groups. In Section III we shall discuss some of the reagents in more detail.

II. Protein Groups

A. SULFHYDRYL GROUPS

The sulfhydryl (hereafter shortened to SH) groups of proteins are attributed to cysteine. Considerably more work has been done with the chemical reactions of this group than with those of the others discussed below, stimulated in part by its essential nature for many enzymes and in part by the intriguing differences in its reactivity in native and denatured proteins. In view of the large numbers of papers in the field and the availability of several comprehensive reviews (10, 31, 157, 158, 249, 295), attention will be directed in this summary only to the most significant recent developments.

Practically all protein reagents react with SH groups. Those that have been claimed to react *only* with this group may be classified as follows: (*1*) mild oxidizing agents (ferricyanide, porphyrindin, iodine, iodosobenzoate, etc.); (*2*) alkylating agents such as iodoacetamide and iodoacetate; and (*3*) mercaptide-forming organometallic compounds such as *p*-chloromercuribenzoate and trivalent organic arsenicals.

The use of oxidizing agents has been criticized (19), since it can be shown that, unless the choice of reagents and conditions is ideal, other protein groups may be attacked. Furthermore, since the primary reversible reaction is a conversion of SH to disulfide bonds, it is dependent upon the spatial proximity of pairs of SH groups.

With iodoacetamide and iodoacetate, the lack of reversibility and lack of specificity are disadvantages (19, 295).

Possibly *p*-chloromercuribenzoate (159) and related mercury and arsenic compounds will prove most helpful in future studies (see Section III,H). The advantages of this class of reagents are: (*1*) they have a great affinity for SH groups and are thus effective under mild conditions and in low concentrations, (*2*) they react with single SH groups, (*3*) they react with SH groups that appear masked to many oxidizing agents, (*4*) they appear highly selective in combining with no protein group other than SH, and (*5*) their combination with SH groups is reversible by the addition of an excess of a simple mercaptan.

The following examples may serve to illustrate the different reactivities of SH groups in proteins to different reagents. Hellermann *et al.* (159) identified the SH groups of *urease* as follows: Assuming a unit weight of 21,300, one SH group

was available for reaction with representatives of all three types of reagents (nitroprusside, porphyrindin, iodosobenzoate, chloromercuribenzoate, iodoacetamide) *without* decrease in enzymatic activity. A second SH group reacted rapidly with additional *p*- or *o*-chloromercuribenzoate but was less available to the other reagents mentioned. After this reaction, urease activity was abolished but could be restored by the addition of excess simple SH compounds. Three additional SH groups could be determined in *denatured* urease by means of iodosobenzoate or porphyrindin. Balls and Lineweaver (14, 15) demonstrated that the SH group of *papain* reacted with one equivalent of iodine or iodoacetamide, although it was neither detectable by nitroprusside nor oxidized by porphyrindin. Singer and Barron (296) showed that *p*-chloromercuribenzoate reacted with both free and masked SH groups of a *myosin* preparation to abolish its adenosine triphosphatase activity. On the other hand, porphyrindin or iodosobenzoate oxidized only the free SH groups without causing extensive inactivation. Iodoacetamide, in the low concentrations used, did not react even with the available SH groups.

These detailed studies illustrate two points worthy of emphasis: (*1*) There is no clear line of demarcation between "available" and "unavailable" groups; the activities of protein SH groups may vary from that of simple mercaptans to complete resistance to all SH reagents, and (*2*) the activity of a biologically active protein may depend not only on one kind of group, but even only on a single one of several of the same kind occurring in the molecule. This essential group may be more or less chemically reactive, compared to the other similar groups in the molecule.

Such refinement of approach to the general problem of protein group specificity has not yet been achieved for the SH groups of other enzymes, nor for *any* of the other groups of biologically active proteins.

Bersin (30), Hellermann (157, 158), Greenberg and Winnick (139), and Barron and Singer (19, 297) have subjected many enzymes to reaction with SH reagents. With due consideration for variations in rates of reaction and occasional results difficult of interpretation, their data permit valid conclusions to be drawn concerning the essentiality (or lack of essentiality) of the SH groups. Singer (295) has summarized this information.

The importance of SH groups in those proteins that require them for activity tends to overshadow the possible rôles of other groups in the same molecule. To overcome the technical difficulty due to the reactivity of SH groups, Greenberg and Winnick (139) protected the SH groups of papain by oxidation with dilute hydrogen peroxide and then acetylated with ketene. The product was reactivated in the usual manner by reducing agents before assay (see table 2). *p*-Chloromercuribenzoate might be used in similar manner to mask SH groups specifically and reversibly.

Cavallito and Haskell (61) have suggested that the activity of several antibacterial agents may reside in the great reactivity of the structure,

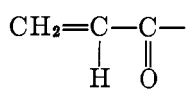


TABLE 2

Essentiality of protein groups for biological activity

PROTEIN	PROTEIN GROUPS									REFERENCES
	Amino		Phenol		Sulfhydryl†		Other			
	Need	Reagent	Need	Reagent	Need	Reagent	Group	Need	Reagent	
Enzymes:										
Amylase (pancreas)	+	K, F, N, P	−(?)		−	CMB, IA	Disulfide	−	Cy	(210, 211) (55)
β-Amylase (barley)	−	K, N	+	N	+	N				(335)
					+	I, CMB, IA, Fe				(336)
Chymotrypsin	−	K, F, N, P	+	K, N	None		Disulfide	−	H_2S	(300)
Lysozyme	+	F, B, P, N			?[e]	I	Carboxyl	+	AA	(5)
							Amide or guanidyl	+	F	(117)
Papain	−	K	+	K	+	Cy, IA[a]				(14, 15) (139)
Pepsin	−	K[a]	+	K[a]	None					(162, 173)
			+	I[a]						(163)
	−	N	+	N						(256)
	−	CSO	+	CSO						(323)
Phosphatase (acid)	+	N, F, P								(12)
Phosphatase (alkaline)			+	Oxid.						(299)
	+	K, F, N, P								(137)
Solanain	−	N	+	K, I, N	−	IA, etc.				(139)
Hormones:										
Adrenocorticotropic (pituitary)	+	K, F, N	+	K, I	None					(207)
Gonadotropins: Follicle-stimulating (FSH)	+	K, N								(205, 206)
							Disulfide	+	Cy, T	(219)

Interstitial-cell stimulating (ICSH).....	+	K, N							(205, 206)
						Disulfide	+	Cy, T	(115)
Prolan.....	−	K[a]	+	K[a]					(205, 206)
	−	N	+	N					(37, 201, 206)
						Disulfide	+	Cy, T	(115)
Hormones:									
Pregnant mare serum (PMS).....	+	K, N							(201, 205, 206)
	+	N							(37)
						Disulfide	+	Cy	(95)
	+	A, P							(96)
						Disulfide	+	Cy, T	(115)
Insulin[b].....					None	Disulfide	+	Cy	(330)
						Disulfide	+	Cy, T	(341)
			+	I[a]					(148)
						Disulfide	+	T	(311, 337)
						Disulfide	+	Na–NH_3	(268)
	−	K[a]	+	K[a]					(312)
	−	K	+	K					(12)
	−	GCT							(179)
						Hydroxyl	−	H_2SO_4	(130)
						Carboxyl	+	AA	(58, 195)
						Amide	−	F	(117)
						Guanidyl	−	F	(117)

TABLE 2—*Continued*

PROTEIN	PROTEIN GROUPS									REFERENCES
	Amino		Phenol		Sulfhydryl†		Other			
	Need	Reagent	Need	Reagent	Need	Reagent	Group	Need	Reagent	
Hormones—Continued										
Lactogenic	+	N								(205, 206)
	+	P								(47)
			+	I[a]						(204)
	+	K[a]								(203)
					None		Disulfide[a]	+	Cy, T	(115)
							Carboxyl	+	AA	(202)
Parathyroid							Carboxyl	+	AA	(326)
	+	K[a]								(342)
							Disulfide	−	H_2S, etc.	(325)
							Disulfide	−	$Na\text{-}NH_3$	(269)
Pitocin							Disulfide	−	Cy	(293)
Virus:										
Tobacco mosaic[e]	−	K[a], P[a], N	+?	K[a]						(286, 287)
	−	K[a], P[a]	?	K[a], P[a]						(236)
			+	K	−	I				(11)
	−	B	?	B						(4)
Toxins:										
Diphtheria	+	K[a], F[a]			None					(251)
Scarlet fever	?	K, N	+	I, K, N						(18)
Shiga antigen[d]	+	K								(317)
	+	A								(324)
Crotoxin	+	A	+	I	None		Disulfide	+	Cy	(304)
							Hydroxyl	+	H_2SO_4	(117)
							Carboxyl	+	AA	(117)

* *Symbols:*

K	= ketene	I	= iodine
F	= formaldehyde	T	= thioglycolate
N	= nitrous acid	A	= acetic anhydride
P	= phenyl isocyanate	GCT	= *O*-glucosido-*N*-carbobenzoxytyrosyl azide
Cy	= cystine or cysteine	AA	= acid alcohol
CMB	= chloromercuribenzoate	CSO	= carbon suboxide
IA	= iodoacetamide or iodoacetate	B	= benzoyl chloride
Fe	= ferricyanide	Oxid.	= oxidizing agents

[a] Analytical data available.

[b] Derivatives of insulin prepared with phenyl isocyanate (175) and carbobenzoxy chloride (128) were inactive, presumptive evidence that amino groups were involved. However, more recent experiments indicate that the phenol groups may have reacted (see text).

[c] Extensive modification of tobacco mosaic virus with carbobenzoxy chloride, chlorobenzoyl chloride, and benzenesulfonyl chloride caused inactivation (236), but the degree of inactivation differed with each derivative depending upon the plant used for the assay.

[d] Both ketene and acetic anhydride may have reacted with the carbohydrate component of the antigen complex.

[e] Although lysozyme can be inactivated by iodine and partially reactivated by reducing agents (5, 227), it has not been possible to demonstrate SII groups by any technique now available (114).

[f] Numerous enzymes have been examined for SH group essentiality. Unless information concerning other groups is available, they have not been included. A table complete to 1945 has been compiled by Singer (295).

for SH groups. It is of interest that maleic acid, proposed by Morgan and Friedman (242) as a SH reagent, has the same basic structure. These investigators indicated that the reaction involved addition to the double bond.

$$RSH + HOOCCH{=}CHCOOH \rightarrow RSCH(COOH)CH_2COOH$$

However, Greenberg and Winnick (139) found that inactivation of SH enzymes by maleic acid was reversible by cysteine and cyanide, and suggested that maleic acid acted as an oxidizing agent.

B. DISULFIDE GROUPS

The only chemical effect of reducing agents on unmodified proteins so far observed is the conversion of disulfide to SH groups. Those most commonly used are cysteine, glutathione, thioglycolic acid, and hydrogen sulfide. The extent and rate of reduction of the disulfide linkage under the same conditions varies for different proteins and is greatly affected by the degree of denaturation (174, 260, 333).

All enzymes that require free SH groups are activated in the presence of low concentrations of reducing agents, which, therefore, do not disturb the basic structure. However, proteins that contain relatively high amounts of cystine appear to be particularly sensitive to, and to be adversely affected by, small amounts of reducing agents. Thus, insulin (12.5 per cent cystine) (233), crotoxin (13.2 per cent cystine) (304), lysozyme (5 per cent cystine) (114), and lactogenic hormone (3.1 per cent cystine) (116) upon reduction become insoluble, possibly through the formation of new hydrogen bonds involving the SH groups. Reduced insulin (18b, 311, 330, 337, 341) and reduced crotoxin (304) are biologically inactive.[3] Miller and Andersson (233, 234) showed that reduced insulin, in the presence of a detergent, had an apparent molecular weight smaller than that of insulin, but that, in the absence of detergent, the reduced insulin was inhomogeneous and highly aggregated.

Conclusions regarding the disulfide groups of other proteins have sometimes been drawn from their biological activities after treatment with reducing agents under the conditions used for inactivating insulin (325). Since it is known that disulfide groups may differ widely in their reactivity toward reducing agents, reinvestigation with adequate analytical controls appears indicated in these cases.

Controlled reduction of the lactogenic hormone by cysteine or thioglycolic acid caused a loss of solubility, but activity could still be demonstrated if suitable means were used for dissolving the reduced protein (116). More severe reducing

[3] The reactivation of reduced insulin by careful oxidation has been reported from one laboratory (see 121), but other workers have been unable to duplicate these experiments (330, 337, 341). If the disulfide linkages of insulin are important structural elements for the integrity of the molecule, it is understandable that reduction would cause internal dislocation. The probability that the correct pairs of SH groups could then recombine during oxidation would be low. However, alternate reduction and oxidation with *retention* of activity might be achieved in the *solid state* with experimental methods similar to those used by Patterson *et al.* (252) with wool.

conditions caused a true loss of activity. Similarly, all types of gonadotropic hormones were inactivated under sufficiently severe conditions of reduction with cysteine or thioglycolic acid (115). However, these hormones are remarkably resistant to reducing agents compared to insulin, which is rapidly inactivated by low concentrations. It seems plausible that drastic reducing conditions would destroy the activity of most biological proteins that contain cystine, including even those enzymes that require one or more free SH groups for activity, but this does not appear to have been demonstrated, although Scott and Sandstrom (292) and Sizer and Tytell (302) showed that increasing concentrations of reducing agents, or decreasing oxidation–reduction potentials, ultimately decreased the activities of papain and urease, respectively. On the other hand, the posterior pituitary hormones (293) and the cobra venom neurotoxin (303) have withstood inactivation by reduction even though they do contain cystine. The extent of reduction was not determined analytically, but in the case of the posterior pituitary hormones, alkylation of the reduced proteins caused inactivation, indicating that the cystine (or cysteine) residues were essential regardless of their being in the oxidized or reduced state.

Considerable attention has been paid to the effect of reducing agents on keratins but the conditions used are more drastic than would usually be applied to bioactive proteins. Several keratins can be dissolved by the use of reducing agents in dilute alkaline media or in neutral solutions of denaturing agents such as urea, guanidine, detergents, etc. (131, 186). The disulfide bonds of wool can be reduced *in situ*, then reacted with bifunctional halogen compounds to form derivatives containing stable bisthioether cross-links (252).

The disulfide bonds of proteins appear to be specifically attacked by two reagents which are often incorrectly regarded as simple reducing agents. Cyanides and sulfites (or bisulfites) add to the disulfide bond to give one SH and one thiocyanate or thiosulfate group, respectively (67, 222). Surprisingly, these agents have proven almost as effective as reducing agents in activating enzymes (32, 139, 340), from which it must be concluded that the unnatural groups thus introduced do not interfere with the specificity of enzymes. In this connection it appears noteworthy that the sulfite–disulfide reaction has been shown to be reversible under mild conditions (41). The nature of enzymes activated by either sulfite or cyanide (or thiosulfate (181)) appears to call for further study.

In alkaline solution protein disulfide bonds are readily hydrolyzed. The lability of the resulting SH and sulfenic acid (—SOH) groups (284) in alkaline solution leads to further complicated changes that have now been studied in detail with wool and hair (77, 209, 239, 285, 315). Lanthionine is one product of the reaction (73, 77, 177). Biologically active proteins rich in cystine, e.g. insulin, are markedly susceptible to the action of alkali (*cf.* 147), but the complexity of the reactions that occur and the possible lability of other types of protein groups under the same conditions have precluded the use of dilute alkali as a specific agent. However, sensitivity of a protein to alkali may suggest that intact disulfide linkages are essential for its activity.

C. AMINO GROUPS

The primary amino groups of proteins are the ϵ-amino groups of lysine (and hydroxylysine where it occurs) and the α-amino groups at the ends of the peptide chains. In attempting to determine whether amino groups are essential for the activity of proteins, investigators have so far not attempted to differentiate between the two types. In most proteins, the ϵ-amino groups dominate in number. Insulin is an exception (280).

The availability of three methods for determining amino groups (Van Slyke (327), formol titration (118), ninhydrin color (145)) has made possible correlations between the number of amino groups reacted and the loss of biological activity in those cases where amino groups are essential. The reagents most commonly used to react with the amino groups of proteins have been ketene, nitrous acid, phenyl isocyanate, and formaldehyde. The effects of these reagents will be discussed in detail in Section III. In each case secondary reaction may occur depending upon the conditions used. Fortunately, most investigators have chosen to use two or more of these reagents in studying the essentiality of amino groups (see table 2), and the agreement in results of such multiple experiments appears to be reasonable proof that they may be reliable.

It has recently been observed (114) that acetic anhydride under certain conditions is a highly specific reagent for amino groups. Details are given in Section III. Possibly this reagent will now displace ketene, the use of which has several disadvantages.

Numerous other acylating agents attack the amino group, but these have not been studied in sufficient detail to indicate whether or not they may have useful properties as specific reagents. Mellon *et al.* (226) substituted the amino groups of casein almost exclusively by reaction with very limited amounts of benzoyl chloride. When a slight excess was used, groups other than amino were benzoylated.

Dinitrofluorobenzene has been used to advantage by Sanger to identify the free amino groups of insulin (280) and gramicidin-S (281). The reaction is not specific for amino groups, since the phenol, SH, and, possibly, imidazole groups also react. Its advantage lies in the relative resistance of the introduced bonds to hydrolysis by acid, and in the fact that the dinitrophenyl-substituted products of hydrolysis are colored only if the substituent is on an amino group. These substituted amino acids can be separated and identified by chromatographic techniques.

Since ninhydrin has been used in the determination of amino groups, it appeared likely that it might be a specific reagent for these groups (83, 84, 92). However, the conditions necessary to achieve extensive deamination are quite vigorous, and experiments in this Laboratory indicate that SH and possibly other groups are affected (114).

Several investigators have studied (178, 271, 291) and used (66) the transformation of the amino groups of proteins to guanidyl groups by reaction with *S*-methylisothiourea, or *O*-methylisourea. The possibility that other groups may react has not been investigated in detail but appears to be unlikely. The utiliza-

tion of this reaction for the modification of biological proteins might be expected to give interesting derivatives.

D. PHENOL GROUPS

The phenolic groups of tyrosine have received considerable attention. Whenever studied, they have been claimed to be essential for activity.[4] The only reagent that has been considered to react *only* with phenolic groups (in proteins containing no SH groups) is iodine. The reaction is one of substitution on the ortho positions (see Section III for conditions used).

Phenolic groups are attacked by acylating agents but usually at slower rates than are the amino groups. Evidence for the extent of reaction has often been based on the color obtained with Folin's phenol reagent (103). Recent observations indicate that such data must be interpreted with caution.[5] The reaction

[4] Little and Caldwell (210) suggest that the phenol groups of pancreatic amylase are not essential, but the evidence is insufficient to establish the point.

[5] Measurement of protein phenol groups by the Folin phenol reagent has the following disadvantages:

(*1*) The phenol reagent is not specific, since it measures not only phenol, but also indole, SH, and other reducing groups.

(*2*) Tyrosine peptides develop less color than tyrosine (232, 322); hence the color developed by proteins can not be expected to reflect the total tyrosine plus tryptophan contents.

(*3*) Maximal color values are obtained only after proteins are completely denatured (164, 231). This observation suggests that the chromogenic property of tyrosine in the native protein is decreased still further by either hydrogen bonds or steric effects. Actually, the color obtained with proteins varies from 43 to 75 per cent of the calculated values, even lower than the peptide values would suggest (114).

(*4*) The introduction of new groups into a native protein molecule complicates the picture even further, for the new groups may, even without reacting with either phenol or indole groups, so influence their "availability" or their "reactivity" that lower Folin color values are obtained. In one case it has been shown that treatment of tobacco mosaic virus with formaldehyde at pH 7 causes a decrease in this value (188, 275), though neither phenol nor indole groups appear to react under these conditions (108). Furthermore, even where tyrosine groups are acylated, maximal color development depends upon "denaturation" as explained above, and the rates of denaturation differ with different protein derivatives (231).

(*5*) The Folin color cannot quantitatively reflect the extent of reaction of tyrosine residues in proteins when substitution on the carbon atoms of the phenol ring is involved, since all derivatives of tyrosine in which the hydroxyl group is free give some color with the Folin reagent, varying with the kind of groups introduced and the extent of substitution.

Despite these drawbacks, the Folin reaction has been found useful in interpreting the results of ketene acetylation. Herriott (162) developed a method for determining the extent of participation of the phenolic groups in this reaction, based on the observation that phenol acetates are quite unstable in dilute alkali. Even this method cannot be applied indiscriminately to other protein derivatives, since acylated phenol groups differ markedly in their lability toward alkaline hydrolysis (232).

Some of the various disadvantages mentioned above have been overcome in recent work (108) by hydrolyzing protein derivatives by pancreatin (or pepsin) prior to the application of the Folin reagent. By this technique the Folin color

with nitrous acid is discussed in detail in Section III. It has been used by Philpot and Small (256) to study the essential nature of the tyrosine groups of pepsin, by Weill and Caldwell (336) with β-amylase, and by others (table 2).

Sizer (301) has claimed that the enzyme, tyrosinase, can oxidize a few tyrosine groups of some proteins, but his conclusions have been questioned (91). The possibility of using enzymes to modify the side chains of intact proteins had not previously been demonstrated, although Harris (150) has since identified a phosphatase in frog's eggs capable of hydrolyzing the phosphate groups of intact phosphoproteins.

Wormall (344) used tetranitromethane as a mild nitrating agent for serum proteins. No chemical studies were made of the derivative, but its similarity in immunological properties to proteins treated with nitric acid is evidence that the phenolic groups were affected. The action of tetranitromethane on simple phenols results in the introduction of nitro groups ortho to the hydroxyl group (283). Further studies of this reagent appear indicated.

Oxidizing agents, when used in excess, attack the phenol (and indole) groups of proteins (6, 299). The ease of oxidation of the phenolic group of tyrosine is enhanced by increases in temperature, pH, and phosphate concentration (49). Herriott (166) has suggested that the Folin reagent might be used to advantage to study the phenol (plus indole) groups of proteins.

The phenolic groups of proteins also react with alkylating agents, diazomethane, diazonium compounds, epoxides, various oxidizing agents, and concentrated sulfuric acid, but not specifically (see Section III).

E. INDOLE GROUPS

Although the indole group is a fairly reactive one, few direct studies have been made concerning its importance in various biological activities. Tryptophan itself reacts readily with a number of reagents, but these reactions are generally dependent upon the free amino group, since acylated derivatives are less reactive.

Lately it has been shown that gramicidin reacts rapidly with formaldehyde in alkaline solution to form a derivative containing methylol (hydroxymethyl) groups on the indole nitrogen atoms (108). The tryptophan residues in typical proteins react similarly, but guanidyl, amino, and amide groups (all absent from gramicidin) also combine with formaldehyde under the same conditions. The introduction of the methylol groups into gramicidin required an alkaline medium and resulted in a loss of toxicity. Since the preparation of formaldehyde toxoids from some toxins also requires, or is favored by, an alkaline medium (82, 88, 97),

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value of untreated proteins has approximated their total tyrosine plus tryptophan contents, and substituents on the phenolic hydroxyl groups can be estimated. Acetylated proteins cannot be analyzed by this technique, owing to the instability of the phenol acetate linkage under the conditions of incubation used.

In contrast to the Folin test, the Lugg and other colorimetric methods are suitable for determining the degree of reaction of the phenolic groups in proteins when substituents stable to acid hydrolysis are introduced in the carbon ring. Even in this case, exceptions exist. Monoiodotyrosine gives an intense Millon test (149, 165). Diodotyrosine gives a positive test by the Thomas procedure (319).

the possibility was expressed that the preparation of some toxoids may involve reactions of the tryptophan residues (108).

The destruction of the indole (and phenol) groups of proteins by oxidizing agents, including the Folin reagent, has already been mentioned. The indole groups of gramicidin are known to be attacked by acylating reagents (110a). In all likelihood, the indole groups of proteins react with nitrous acid and iodine, but the evidence is scattered and requires substantiation (see Section III). In none of these cases would the reaction be likely to be useful in specificity studies.

F. IMIDAZOLE GROUPS

The possible rôle of the imidazole groups of histidine in the biological activities of proteins has received no attention. Of the reagents that are known to react with these groups, diazonium compounds may have promise. Numerous protein derivatives have been prepared by coupling with diazonium compounds (196), but the protein groups involved are not known with certainty. At one time, the reaction was considered to involve only the imidazole and phenol groups, but more recently it has been shown that more of the diazotized compound can be introduced than can be accounted for on the basis of these only (50, 51, 154, 155, 187). The work of Eagle and Vickers (87) suggests that indole and amino groups are also involved. Kapeller-Adler and Boxer (187) believed that coupling occurs with *all* ring compounds, including the pyrrolidine ring.

Histidine gives a strong color upon reaction with diazotized sulfanilic acid (Pauly reaction), the reaction with tyrosine is weaker, and other amino acids do not give colored products (218). It appears possible that the imidazole groups of proteins might be the *only* ones affected by reaction with *limited* amounts of diazo compounds. For example, Reiner and Lang (264) were able to introduce up to 3.8 radicals (per 10^4 gram) of several different diazotized aromatic compounds into insulin without loss of activity and concluded that unsubstituted *phenolic* groups were not essential, in contrast to the results of other investigators (148, 312). However, there are sufficient imidazole residues in insulin (3.3 per 10^4 gram) to account for the added groups (two diazonium molecules may react with each imidazole group). The possibility that limited amounts of diazotized compounds may act as specific reagents for the imidazole ring is worthy of further study.

A number of other reagents are known to attack the imidazole ring, but only under conditions where the reactions of other groups are of first importance. Among these may be mentioned nitrous acid (313, 338), benzoyl chloride (81, 172), and benzenesulfonyl chloride (172). Bauer and Strauss (20) demonstrated the simultaneous iodination of the imidazole and phenolic groups of globin, but in other proteins the former do not appear to react with iodine (20, 148, 163, 200). Mustard gas and the nitrogen mustards react non-specifically with the imidazole groups of proteins (78, 125, 190).

G. GUANIDYL GROUPS

The guanidyl groups of proteins are those of arginine. Although it would seem likely that this highly charged group, most basic of those in proteins, might

be of importance in various biological activities, its rôle has not yet been studied, nor are there available useful specific reagents for such studies.

Experiments in this Laboratory indicate that formaldehyde reacts rapidly with the guanidyl and amide groups of proteins at pH 11–12 at room temperature (107). The derivative of egg albumin, after isolation by dialysis, contained almost the same number of amino and SH groups as the original protein. In tryptophan-free proteins, this technique may prove useful for the preparation of guanidyl- and amide-substituted proteins. A sample of insulin treated by this procedure was found to retain its original activity (117). The guanidyl groups of proteins also react with formaldehyde in neutral and acid solution but the reaction is slow, and amino and amide groups are also involved (112, 316b).

Acylating agents and nitrous acid in acetic acid react with guanidyl groups, but at rates considerably slower than those observed with amino and phenol groups, and under conditions where several other types of groups are also attacked (see Section III). Indications have been obtained (114, 144) that phenyl or chlorophenyl isocyanate may react slowly with the guanidyl groups of protamines, but these reagents like the others mentioned are non-specific. The introduction of nitro groups on the guanidyl residues of proteins by reaction with concentrated nitric acid in the presence of fuming sulfuric acid (191) is obviously of little use for the type of research covered in this review.

H. ALIPHATIC HYDROXYL GROUPS

The free aliphatic hydroxyl groups of proteins are those of serine, threonine, hydroxyproline, and hydroxylysine. The latter two amino acids appear to be of importance only in proteins related to collagen (263).

At low temperatures, concentrated sulfuric acid reacts rapidly with proteins to form acid sulfate esters of the aliphatic hydroxyl groups (266). Since the amino and many other polar groups of proteins do not react, sulfuric acid appears to be more selective in this respect than other reagents so far investigated.

The only other groups that are affected are the SH groups, which give substituted *S*-thiosulfuric acids, and some of the phenol groups which give substituted sulfuric acids after short treatment or sulfonic acids if exposure to the acids is prolonged (108, 130, 266). The phenolic groups react more slowly than do the aliphatic hydroxyl groups. Partial destruction of some other amino acids, particularly arginine, appears to occur if the reaction is allowed to proceed for 24 hr. (216).

This reaction should be of value in studies of the aliphatic hydroxyl groups of biological proteins. Glendenning *et al.* (130; *cf.* 39) showed that insulin that had been sulfated with sulfuric acid retained its activity, indicating that the reaction conditions are not necessarily destructive to the protein molecule.

Repeated treatment with methyl sulfate in cold aqueous alkaline solution has been found to methylate about one-half of the aliphatic hydroxyl groups in silk fibroin (135). Aliphatic hydroxyl groups of proteins are attacked by acylating agents, but generally more slowly than are amino or phenolic groups (see Section III,D,1).

I. CARBOXYL GROUPS

The free carboxyl groups of proteins are those δ- and ε-carboxyl groups of aspartic and glutamic acids not present as amides and those occurring at the ends of the peptide chains.

Felix and coworkers (81, 98, 99, 100) allowed proteins to react with methyl alcohol *saturated* with hydrogen chloride and considered that they had accomplished quantitative esterification of the carboxyl groups. Waldschmidt-Leitz *et al.* (332) and Kiesel and Znamenskaja (189) indicated that these conditions also caused other reactions, such as loss of amide groups, destruction of tryptophan, and hydrolysis of peptide linkages. For example, salmine sulfate after treatment by this technique contained 1.2 per cent amino nitrogen (as per cent of total nitrogen) (332), although the untreated protamine contains no detectable end groups (113).

Carr *et al.* (58) studied the inactivation of insulin with alcohol containing small amounts of acid and suggested that esterification of the carboxyl groups had occurred. The activity was recovered by exposure to dilute alkali. Charles and Scott (63), on the basis of methoxyl and *N*-methyl determinations, expressed doubt that the reaction was a simple esterification. However, their data are at least partly interpretable as showing only the presence of difficultly removable alcohol (*cf.* 130, 182, 225). The inactivation of insulin with acid-alcohol and complete regeneration of activity by mild alkaline hydrolysis has been duplicated recently (195).

When proteins were treated with 0.02–0.1 *N* hydrochloric acid in methanol, their carboxyl groups were found to be almost quantitatively esterified (111). The number of methoxyl groups that could be introduced was in good agreement with the number of carboxyl groups present. Amino, phenolic, indole, and amide groups were shown not to be affected. Esterification occurred with higher primary alcohols, but was progressively (with the molecular weight of the alcohol) slower and less complete. This procedure should prove useful in investigating the rôle of carboxyl groups in those proteins *that will not be denatured under these conditions.*[6] However, the inability of several workers to reactivate esterified proteins, other than insulin, by mild alkaline hydrolysis (117, 202, 326) requires further investigation.

At pH 5.5–6.0, mustard gas [bis(2-chloroethyl) sulfide] appears to esterify a certain proportion of the carboxyl groups of several proteins (167). The amount of mustard introduced is closely equivalent to the carboxyl groups reacted (as determined by titration) (78). Although the tyrosine Folin color is reduced

[6] Egg albumin was 60 per cent methylated even at −5°C. (2 days) but appeared to be completely denatured. On the other hand, with serum albumin, 45 per cent of the carboxyl groups were methylated under the same conditions, and the product was soluble and heat-coagulable at its isoelectric point (pH 7.3). A negative nitroprusside test (in 8 *M* guanidine hydrochloride) indicated that at some time the SH groups may have been available for autoxidation. In a control experiment, serum albumin, temporarily "denatured" by exposure to 8 *M* guanidine hydrochloride for 30 min. and then recovered by dialysis, was still soluble and heat-coagulable, but the nitroprusside test was considerably decreased.

(167), it appears probable that the phenolic groups are not involved, since no appreciable change in titration between pH 8.5 and 11.0 was observed (78). The difficulty in interpreting Folin color reactions has already been mentioned (see footnote 5). At any rate, there is a possibility that mustard gas may be useful as a specific reagent for carboxyl groups in proteins containing no available SH (or thioether) groups (Section III).

In addition to these reagents, protein carboxyl groups have been esterified by diazomethane, methyl sulfate, methyl iodide, methyl *p*-toluenesulfonate, and 1,2-epoxides, but these reagents generally also attack other groups in the protein molecule (see Section III).

J. AMIDE GROUPS

The amide groups of proteins occur on the ϵ- and δ-carboxyl groups of glutamic and aspartic acids. The proportion of such carboxyl groups that are present as amide differs from one protein to another. Whether part of the ammonia liberated by mild acid hydrolysis might arise also from carboxyl groups at the ends of the peptide chains is not known.

The amide group is comparatively inert. Hydrolysis occurs more rapidly than with peptide bonds (328), but acceptable methods for quantitatively hydrolyzing amide groups *without some hydrolysis of the main chain* have not been described.

The amide groups of proteins react slowly with formaldehyde in neutral or acid solution (110, 345), but under the same conditions amino, guanidyl, and perhaps other groups also react. It has recently been found that protein amide (and guanidyl) groups react rapidly with formaldehyde at room temperature in alkaline solution (107) (see Section II,G). The retention of insulin activity after such treatment (117) suggests that unsubstituted amide (and guanidyl) groups are not essential for the activity of this protein.

K. THIOETHER LINKAGE

The methylthioether linkage of methionine has received no attention in specificity studies. The possibility that hydrogen peroxide may be a useful reagent for the modification of this group is suggested by the observations of Jaques and Bell (184), who correlated roughly the consumption of hydrogen peroxide (0.04 *N*) at pH 6.6 with the methionine contents of fibrin and fibrinogen. Toennies and Callan (320) had previously shown that under certain conditions methionine reacted even more rapidly than cysteine with hydrogen peroxide. The product is the sulfoxide. Methionine in proteins reacts non-specifically with mustard gas at pH 7 and 9, as indicated by nutrition experiments with mustard gas-treated proteins (190). The work of Stein and Moore (310) suggests that *in acid medium* mustard gas may act as a somewhat more specific reagent for the thioether linkage in proteins. The nitrogen mustards also react with methionine (124).

Iodine adds to the thioether linkage of methionine to form a colorless periodide. *N*-Acylated methionine periodides are unstable (197); hence this reaction probably does not occur with proteins.

III. Protein Reagents

A. OXIDIZING AGENTS

The effect of oxidizing agents on the SH groups of proteins has received much attention (10, 19, 31, 139, 157, 158, 159, 297). The following generalizations appear valid. The number of SH groups oxidized depends upon the reagent, the conditions of the reaction, and the relative "availability" of the SH groups. Iodine appears to be able to oxidize some SH groups which are not available to other oxidizing agents (7, 8) and in acid solution the oxidizing action of iodine may be specific (8, 171). In neutral and alkaline solution other reactions occur (see Section III,F). A number of other oxidizing agents have been carefully studied, particularly for the purpose of determining quantitatively the number of sulfhydryl groups in proteins. Ferricyanide (6, 8, 9), porphyrindin (53, 140, 141, 192), iodosobenzoate (159, 160), tetrathionate, and Folin's uric acid reagent (9) have been found to be useful reagents, providing the concentration of reagent, and time, temperature, and pH of the reaction are carefully controlled.

Oxidation of proteins by disulfide compounds such as cystine and oxidized glutathione would appear to be a specific reaction for SH groups. No others in proteins are known to react (174, 237). Since this is an equilibrium reaction, an excess of the reagent is required. Balls and Lineweaver (15) inactivated papain with cystine. It has been suggested (250, 333) that the disulfide obtained from monothioglycol would be a useful reagent of this type.

Other oxidizing agents not yet mentioned that have been used with SH enzymes are hydrogen peroxide, potassium permanganate, and perbenzoic acid (139, 157, 158, 340). Conditions are chosen with proteins such that at least part of the activity can be regenerated by the action of reducing agents. Air oxidation, particularly when catalyzed by heavy metal salts (*cf.* 29), attacks SH groups. The catalytic effect of the cupric ion extends to anaerobic oxidations as well (8, 152). Weill and Caldwell (336) oxidized the SH groups of β-amylase with ferricyanide and cupric ions. Ferricyanide alone was without effect. The catalytic effect of heavy metals is inhibited by small amounts of cyanide (9). The reaction of quinone with the SH group of cysteine is an addition and not a simple oxidation to cystine (128a, 192).

Despite the fact that much useful information has been obtained, the disadvantages of the oxidizing agents outweigh their advantages as specific agents for SH groups. The weaker reagents often do not attack all of the SH groups; the stronger ones attack other groups besides. Even when oxidation of SH groups is complete, as judged by disappearance of the nitroprusside test, and other groups escape detectable reaction, complete reversal of the oxidation (measured by enzyme reactivation) is often not achievable. In these cases, it may be assumed that oxidation of SH groups has proceeded to the sulfonate or to some other intermediate, but non-reversible, stage of oxidation (10, 11).

Other groups are attacked by oxidizing agents when the conditions are more severe than those required for SH groups. Those generally affected seem to be the phenol and indole groups (10, 53, 237). Sizer (298, 299, 300, 302) followed the inactivation of enzymes by oxidizing agents of varying potentials and con-

cluded, from spectrographic evidence, that the phenol groups were oxidized. The effect of oxidizing agents on thioether groups has not been studied in detail, but the known reaction of hydrogen peroxide (see Section II,K) suggests that other oxidizing agents might also react with this group. Lyman *et al.* (217) removed quantitatively the methionine, cystine, tyrosine, and tryptophan from a peptone preparation by oxidation with hydrogen peroxide in acid solution (*cf.* 321). Cysteic acid has been isolated from oxidized keratins (73). The action of hydrogen peroxide (5 per cent) on tobacco mosaic virus resulted in a 60 per cent drop in amino nitrogen (308). The Folin reagent oxidizes phenol and indole (and SH) groups under conditions where most proteins are not denatured. Smetana and Shemin (305) found that exposure of egg albumin to light in the presence of hematoporphyrin caused reversible oxidation of SH groups and progressive destruction of tryptophan and histidine. Tyrosine was apparently unaffected. This is in contrast to the earlier work of Harris (151), who found the amino acids tryptophan and tyrosine, but not histidine, susceptible to such oxidation (*cf.* 59, 208).

Desnuelle and Antonin (79) reacted proteins with periodate. The only groups affected appeared to be side chains of hydroxylysine. If serine or threonine occurred at the amino end of the peptide chain it would also be expected to react under these conditions. Alloxan was suggested as an oxidizing reagent for SH groups by Labes and Freisberger (194), but this compound may also attack the amino (62) and other groups. The toxicities of both alloxan and tetrathionate are ascribed to their SH-combining property (254).

Air oxidation destroys the biological activity of prolan, even though SH groups appear to be absent (37, 48, 142). Several viruses can be stabilized against air inactivation by cysteine (16, 34, 244, 346). The rôle of SH groups in these proteins is not known; however, at least one virus appears to require SH groups for its activity (183). The possibility that easily oxidizable groups other than SH may play important rôles in the biological activities of proteins cannot be dismissed.

B. REDUCING AGENTS

The reagents most commonly employed to reduce the disulfide bonds of proteins have been cysteine, glutathione, thioglycolic acid, monothioglycol, and hydrogen sulfide. They are generally used in excess, and in neutral or alkaline solution (89, 104, 129, 131, 174, 186, 233, 234, 252, 258, 259, 330, 341), but Stern and White (311) used acid media for the partial reduction of insulin. Although Mirsky and Anson (237) and Fraenkel-Conrat (104) found thioglycolate the most effective reducing agent for protein disulfide bonds, Wintersteiner (341) indicated that cysteine, thioglycolate, and thiolactate were equally effective as reducing agents for insulin. This is in accord with the fact that the SH compounds have very similar oxidation–reduction potentials (33, 124, 278). The absence of ionizing groups in thioglycol is an advantage in some studies (250). 1,2-Dithiopropanol (British Anti-Lewisite, BAL) was more effective than other SH compounds for reactivating enzymes that had been inactivated with Lewisite (18a) (see Section III,H).

The action of cyanides on the disulfide linkage is one of addition (222):

$$P—S—S—P + CN^- \rightarrow P—SCN + P—SH$$

This reaction has found utility in the qualitative test for disulfide bonds by the nitroprusside reagent (7, 8, 10, 333), as a supplementary reagent for SH enzyme activation (139), and in the preparation of reduced keratins (131, 314).

The action of bisulfite ions on the disulfide linkage was shown by Clarke (67; *cf.* 214) to occur as follows:

$$P—S—S—P + HSO_3^- \rightarrow P—S—SO_3^- + P—SH$$

Bisulfite has been used mostly in studies with keratins (41, 93, 94, 186, 230). Maximum reduction of wool occurs at pH 5 (93, 230, 316), but only half of the disulfide bonds are available for the reaction (209, 230). When bisulfited wool is rinsed with water, approximately half of the SH and *S*-cysteinyl sulfonate groups revert to disulfide groups, and the reaction can be reversed completely by treatment with pH 8 buffer for 1 hr. at 50°C.(41).

Phenylhydrazine has been found to activate SH enzymes (13, 340), but the mechanism is disputed (13, 27, 157).

Catalytic hydrogenation has been used to remove iodine from iodinated insulin (148). Removal of approximately two-thirds of the iodine resulted in a corresponding recovery of biological activity. Since the activity of insulin is so dependent upon its intact disulfide groups, these were apparently not reduced under the conditions used. Tests for SH groups were negative. Clutton, Harington, and Yuill (69) and Humphrey and Yuill (179) removed carbobenzoxy groups from *O*-glucosidyl-*N*-carbobenzoxytyrosyl insulin by catalytic hydrogenation in alkaline solution, but by the same technique Gaunt and Wormall (128) could not remove carbobenzoxy groups from insulin that had been treated with carbobenzoxy chloride. Freudenberg, Dirscherl, and Eyer (120) inactivated insulin by catalytic hydrogenation. In no case has the formation of SH groups been demonstrated.

Sodium in liquid ammonia was used by Clutton, Harington, and Mead (68) to remove carbobenzoxy groups from the gelatin derivative obtained with *O*-glucosidyl-*N*-carbobenzoxytyrosyl azide. This reaction was not applicable to the analogous insulin derivative. Roberts (268) found that insulin was rapidly inactivated by sodium in liquid ammonia. In contrast, the parathyroid hormone was fairly stable to this type of reduction (269). A number of other reducing agents (zinc–acid, tin–acid, sodium amalgam, activated magnesium) have been applied to proteins, mostly to insulin (119, 120; *cf.* 116). No specific utility has as yet been demonstrated.

C. ALKYLATING AGENTS

1. Iodoacetate and iodoacetamide

These reagents have been used extensively since Rapkine (261) noted that iodoacetate abolished the nitroprusside test for the SH groups of denatured egg albumin. A thioether is formed (80).

$$P—SH + ICH_2COO^- \rightarrow P—S—CH_2COO^- + I^-$$

Iodoacetamide was used after Smythe (306) reported that it was somewhat more reactive than iodoacetate. The specificity of these reagents for the SH group, originally considered to be complete, has been questioned. Michaelis and Schubert (288) noted that they reacted with the amino groups of amino acids (*cf.* 289), but Goddard and Michaelis (131) and Pillemer *et al.* (258) found no reduction in the amino nitrogen content of kerateins that had been treated with iodoacetate. Rosner (273) obtained no reaction of iodoacetate with native egg albumin, but, after denaturation, there was an immediate rapid reaction, ascribable to the SH groups, followed by a slow continuing reaction. Barron and Singer (19) treated SH enzymes that had previously been reacted with *p*-chloromercuribenzoate, with iodoacetate and iodoacetamide. The activities observed, after reactivation with glutathione, indicated that irreversible damage had been caused by these reagents, presumably at positions in the enzyme other than the SH groups. However, since the reaction with chloromercuribenzoate, unlike that with iodoacetamide, is reversible and therefore continually in equilibrium, the possibility remains that iodoacetamide may have reacted with SH groups even under these conditions. In this Laboratory, marked losses have been observed in the amino nitrogen content of serum albumin and insulin treated with 0.05 *M* iodoacetamide at pH 7.5 or 8.5 for 3 days at 40°C.; *p*-cresol, but not the phenolic groups of these proteins, reacted under the same severe conditions (114). There is thus considerable evidence that iodacetate and iodoacetamide may react with groups other than SH.

In addition, they do not *always* react with SH groups. Maver *et al.* (223) found iodoacetamide relatively ineffective for inactivating a lymphosarcoma cathepsin, the activity of which was dependent upon SH groups, and Anson and Stanley (11) report that 0.05-0.1 *M* iodoacetamide, at pH 8 and 37°C., inactivated tobacco mosaic virus without affecting the SH groups.

The lack of reversibility makes this reaction with SH groups usually less advantageous than that with the mercaptide-forming agents, but does provide an analytical method for determining the SH content of reduced proteins. The total cystine plus cysteine content of the reduced *and* alkylated protein is compared with that of the original (104, 237, 252, 258). The difference represents the amount of cystine in the reduced protein. Reaction with other groups would not defeat this purpose.

In general, it may be concluded that if iodoacetate and iodoacetamide rapidly inactivate a protein at neutrality and room temperature, it is probable that the reaction involves SH groups. In any case, analytical determinations of original and residual SH groups should be made.

2. Miscellaneous alkylating agents

A number of other alkylating agents react with SH groups, particularly of reduced proteins. Among these may be mentioned α-halogen acids other than iodoacetate (258), iodoethanol (132), methyl iodide (41, 293), and other alkyl halogen compounds (252). Evidence for specificity is lacking.

The methylation of proteins by methyl iodide, methyl bromide, and dimethyl

sulfate has been studied extensively (*cf.* 1, 40, 166). Methyl iodide and bromide were found by Blackburn *et al.* (40, 41) to introduce amounts of methoxyl into several proteins corresponding roughly to their content of free carboxyl groups, but some *N*-methylation also occurred. Charles and Scott (63) found insulin to be inactivated by intensive treatment with methyl iodide. Thirty per cent of the activity was recoverable after dilute alkaline hydrolysis. From the course and extent of reaction and reactivation, and from the fact that iodine was introduced into the protein, they concluded that esterification and subsequent hydrolysis of the carboxyl groups were *not* the primary cause of the observed inactivation. However, it is possible that the technique used (refluxing with methyl iodide in alcohol solution) might have caused esterification of the carboxyl groups by the alcohol. Hydriodic acid, released during the reaction of methyl iodide with other groups, would have acted as catalyst (see Section II,I).

Dimethyl sulfate is similarly non-specific. It has been found to react with phenol, SH, aliphatic hydroxyl, amino, guanidyl, and other nitrogenous groups (1, 40, 42, 98, 99, 135). However, Christensen (66) observed that treatment of tyrocidine with dimethyl sulfate produced extensive methylation of the phenol groups but little, if any, *N*-methylation.

Diazomethane has been used by several investigators. Rutherford, Patterson, and Harris (277) treated silk fibroin with diazomethane and found that the alkali-stable methoxyl groups corresponded in number to those of tyrosine. The alkali-labile ones were presumably esters of the free carboxyl groups. Esterification was also observed by Matula (221). (For previous papers on diazomethane, see references 2, 3, 169.)

D. ACYLATING AGENTS

1. Ketene

This reagent has frequently been used in studies of protein group specificity (table 2). Herriott and Northrop (162, 168) treated pepsin at pH 4–5.5 with ketene gas and found that reaction with amino groups was considerably more rapid than that with phenol groups. Activity was retained during acetylation of the amino groups but decreased proportionately with the acetylation of the phenol groups. Upon prolonged ketenization, more acetyl groups were introduced than could be accounted for by amino and phenol groups only. In a study of insulin, Stern and White (312) observed that ketene attacked amino groups more rapidly than phenol groups. Activity was retained during acetylation of the amino groups but disappeared during prolonged ketenization. These studies were considered by other workers to indicate that rapid inactivation of a biologically active protein with ketene meant that the amino groups were essential and slow inactivation meant that the phenolic groups were essential.

The difficulty in such a generalization is well illustrated by recent work. For example, Li and Kalman (203) have shown that the phenol groups of the lactogenic hormone may be attacked by ketene even more rapidly than the amino groups, and in the case of the parathyroid hormone, Wood and Ross

(342) found both types of groups to become only about 40 per cent acetylated. Selective acetylation of the amino groups has, however, been achieved by Herriott (162) and several subsequent workers, by the expedient of exposing the ketenized preparations to conditions of acidity or alkalinity which have been shown to cause hydrolysis of the labile phenyl acetate but not of the acetylamino or of true peptide or amide linkages.[7]

While the action of ketene on the amino and phenolic groups of proteins has often been studied analytically, determinations of the total number of acetyl groups introduced, which alone could assure non-participation of other types of groups, have been performed in only a few instances. The work of Herriott and Northrop (168), already mentioned, indicated that more acetyl groups were introduced upon prolonged ketenization of pepsin than were accounted for by the amino plus phenolic groups reacted. Sandor and Goldie (279) also found considerable more acetyl groups in ketenized serum proteins than corresponded to the number of amino groups reacted; phenolic groups did not appear to react in this case. Herriott and Northrop (168) suggested that the slow acetylation of aliphatic hydroxyl groups might be responsible for their findings with pepsin. This was in agreement with the known reactivity of ketene (309). Other authors have generally assumed that the aliphatic hydroxyl groups of proteins are not acetylated on the basis of model experiments with glucosamine (27), the carbohydrate constituent of egg albumin (247), and hydroxyglutamic acid (248). However, it has lately been found possible to acetylate by prolonged ketenization some of the hydroxyl groups of sericin and polyvinyl alcohol (114; *cf.* 57, 334). There is thus still a possibility that some of the effects noted with ketene, particularly after prolonged treatment, may be explained on the basis of acetylation of the aliphatic hydroxyl groups. For example, Miller and Stanley (235) found that about 70 per cent of the amino and 20 per cent of the phenol groups of tobacco mosaic virus could be acetylated with ketene without loss of activity. Further acetylation caused a decrease without marked increase in the amount of either amino or phenol groups reacted. Whereas it is possible that this may reflect the acetylation of a small number, perhaps even one sluggish essential amino or phenol group in the virus, the alternate possibility that essential aliphatic hydroxyl or other groups may have reacted deserves consideration. Schramm and Miller, who also studied this reaction intensively (286, 287), could not demonstrate alkaline reactivation of ketene-inactivated tobacco mosaic virus. This should have been possible if the inactivation had been due to acetylation of phenolic groups.

Of the polar groups in proteins not yet mentioned, the SH group is known to react (79a, 105). Those of egg albumin are acetylated by ketene at pH 5–6 more

[7] The phenyl acetate bond is hydrolyzed by long standing in neutral solution (232, 276). In attempts to determine the extent of acetylation of the phenol groups of proteins by hydrolyzing them with enzymes prior to determination of Folin color, the phenol acetate bonds were found to be split by the condition used for enzymatic digestion (114). The observation that ketene effected only a *temporary* detoxification of gonococcus and meningococcus (46) may be explained if it is assumed that the essential groups gave readily hydrolyzable acetates.

slowly than are the amino groups, possibly only at the rate at which they are unmasked through surface denaturation (79a). The thioacetate groups so formed are rapidly hydrolyzed by dilute alkali.

Several model substances were treated with ketene under rather more vigorous conditions than those usually used. Only slightly more acetyl groups were introduced into gliadin and protamine than could be accounted for on the basis of their known amino, phenol, hydroxyl, and SH contents. It may thus be concluded that the amide and guanidyl groups of proteins react with ketene very slowly, if at all (114). The indole groups of gramicidin appeared to be acetylated to a small extent by prolonged ketenization (108). Wood and Ross (342) observed that the chromogenic value of ketenized parathyroid hormone, after dilute alkaline hydrolysis, was 10–15 per cent lower (Folin color) than that of the untreated hormone, from which they inferred that additional chromogenic groups had reacted to form stable acetates. However, interpretations of Folin color changes are difficult (see footnote 5). Neuberger (247) mentioned the possibility that the imidazole groups of proteins might react with ketene in a Bamberger-fission type of reaction, but an intensively ketenized insulin was found to have an unchanged histidine content after hydrolysis (114). This does not exclude the possibility that the imidazole ring may have been acetylated.

It must thus be concluded that ketene falls short of being a good protein reagent in several respects: (*1*) Its action is not sufficiently specific for the amino groups. (*2*) It does not generally attack this type of grouping to completion. (*3*) As an unstable gas it needs special equipment for its preparation, is difficult to employ in accurately known amounts, and tends to surface-denature sensitive proteins. As an additional disadvantage, ketene appears to be extremely toxic (56, 343). It has been suggested that it might racemize some of the asymmetric carbon atoms of proteins if reaction is carried out in acid solution (180).

Acetic anhydride is a preferable reagent where acetylation of protein amino groups is desired.

2. Acetic anhydride

Preliminary observations indicate that acetic anhydride may be a valuable reagent for amino groups. The technique used by Hughes (178; *cf.* 166) involves causing a protein in cold sodium acetate-buffered solution to react with a limited amount of the reagent. With several proteins, the number of acetyl groups introduced has been found to be closely equivalent to the number of amino groups originally present (table 3) (114). Some proteins however—lysozyme, crotoxin—did not appear to react to completion. Phenolic groups were affected only slightly if at all. The technique appeared to cause no denaturation, since the acetyl derivative of egg albumin retained its masked SH group, and was heat-coagulable and soluble at its isoelectric point. As mentioned before, it cannot be concluded that acetic anhydride would react with all other proteins in a similar manner. Analytical controls are necessary.

Acetic and higher aliphatic anhydrides and chlorides have been used by many

investigators to modify proteins under more violent conditions than those described above. The reaction may then involve SH, phenol, aliphatic hydroxyl, indole, and possibly basic groups in addition to the amino groups (41, 42, 57, 110a, 135a, 136, 161).

3. Aromatic isocyanates

Hopkins and Wormall (175, 176) found a fairly close correlation between the amount of bromine introduced into proteins by reaction with *p*-bromophenyl isocyanate and the decrease in amino nitrogen, and concluded that the reagent reacted *only* with the amino groups. In contrast, Miller and Stanley (235) reported that phenyl isocyanate reacted not only with the amino groups but also with 30 to 40 per cent of the phenolic groups of tobacco mosaic virus under the conditions used. Miller (231) later showed that these values were erroneously high. In view of the difficulties involved in interpreting Folin color values

TABLE 3

Effect of acetic anhydride[a] on reactive groups of proteins

PROTEIN	ORIGINAL AMINO-GROUPS[c]	LOSS IN AMINO-GROUPS[c]	ACETYL GROUPS INTRODUCED
	gram-moles/10⁴ g.	*gram-moles/10⁴ g.*	*gram-moles/10⁴ g.*
Bovine serum albumin	8.5	7.9	8.5
Egg albumin	4.7	4.0	4.7
Insulin	5.0	4.5	4.5

[a] 1.2 parts acetic anhydride was added dropwise to a stirred solution of 1 part of the protein (5 per cent) in half-saturated sodium acetate solution held at 0–15°C.

[b] The SH groups of egg albumin were not affected by the reagent, but it is possible that available SH groups, such as those of myosin, would be acetylated. The phenolic groups, as estimated from the change in Folin color from that of the original protein, were in no case decreased by more than 10 per cent. The colors obtained at pH 8 were 81 to 95 per cent of those obtained at pH 11 (162) (see footnote 5).

[c] Van Slyke manometric procedure (327a); 15 min. reaction time.

(see footnote 5), it now appears that participation of phenol groups in the reaction may not have been definitely proven. It is of interest that inactivation by phenyl isocyanate of tobacco mosaic virus was not demonstrated (235, 287). Fraenkel-Conrat *et al.* (109) found no evidence for the reaction of the phenol groups of egg albumin with phenyl isocyanate at pH 8.0, but recent experiments indicate that even under the mildest conditions insulin combines with more chlorophenyl isocyanate than can be accounted for by its amino groups alone (114).

However, as with acid anhydrides and chlorides, the specificity of phenyl isocyanate may be dependent upon the amount of reagent used. Thus, Sizer (300) found that reaction of one part phenyl isocyanate to two parts chymotrypsin did not destroy the enzyme activity, whereas a reagent–protein ratio of 2.5 to 1 resulted in immediate destruction. Since other experiments indicated that the phenol but not the amino groups of chymotrypsin were essential, this

observation suggests that the phenol groups had reacted with the excess phenyl isocyanate. The need for analytical controls is evident.

Phenyl isocyanate reacts with the SH groups of low-molecular-weight compounds (127) and proteins (105). The SH groups of native egg albumin react with *m*-chlorophenyl isocyanate even at 0°C., and more rapidly than do the amino groups (114). Some evidence indicates that the guanidyl groups of proteins may react to a slight extent with phenyl isocyanate in aqueous solution at pH 8 (114, 127, 144).

The isocyanate reaction has been used to introduce several types of aromatic and heterocyclic groups into proteins (76, 272). Where analyses have been made (76), part of the amino groups appeared to have reacted. Jensen and Evans (185) used phenyl isocyanate to show that phenylalanine occurred at the amino end of some of the peptide chains in insulin. Roche *et al.* (270) have recently extended this technique to several other proteins.

The usefulness of aromatic isocyanates as specific agents for the amino groups of proteins is limited by the possibility of reaction with the phenol groups and also by the following additional disadvantages. The reagents and their reaction products with water are water-insoluble, and the protein derivatives also may be quite insoluble in aqueous solution (109). This insolubility makes difficult both the interpretation of biological assays and also group analyses by colorimetric analytical methods. Attempts to determine the degree of involvement of phenol groups have been unsatisfactory; the derivatives resist enzyme digestion (114), and mild alkaline treatment yields aniline, which contributes to high Folin color values (232).

4. Carbon suboxide

The reaction of carbon suboxide with proteins has been studied in some detail (322). This compound is a gas resembling ketene in many of its properties. It reacts at similar rates with the amino and phenolic groups of serum albumin and, presumably, other proteins. Both types of groups can be almost completely substituted. The derivatives are half-amides and half-esters of malonic acid. The phenolic ester is readily hydrolyzed, even at neutrality. Tracy and Ross (323) found that carbon suboxide inactivated pepsin, and that part of the activity was regained after gentle hydrolysis, in agreement with Herriott's findings (162, 163) concerning the essential rôle of the phenolic groups for peptic activity.

Carbon suboxide (at pH 5–6) attacked the SH groups of native egg albumin more rapidly than it did either the amino or the phenolic groups (105). Model experiments with amino acids suggest that imidazole, guanidyl, and aliphatic hydroxyl groups do not react (274). The lability of the phenolic ester makes possible the use of carbon suboxide as a specific reagent for amino groups, since derivatives can be freed of all but amino-bound malonyl groups by mild alkaline hydrolysis. The derivatives are of interest in that the substitution of a free carboxyl group for each amino group results in proteins with isoelectric points considerably lower than those of the original proteins. However, most of the disadvantages listed for ketene are equally valid for carbon suboxide.

5. Miscellaneous

The Schotten–Baumann technique has been used with benzoyl chloride and benzoic anhydride to introduce benzoyl groups into proteins. Selectivity can be achieved by the use of minimum quantities of the reagent. Thus Mellon *et al.* (226) treated casein with benzoyl chloride and found that, until more than 50 per cent of the amino groups had been acylated, only negligible amounts of extra benzoyl were introduced. With more vigorous reaction conditions, benzoyl groups can be introduced also on many other polar groups. Goldschmidt and Schön (134) used benzoic anhydride. Goldschmidt and Kinsky (133) carefully acylated egg albumin with *m*-chlorobenzoyl chloride. They showed that part of the acyl groups introduced were attached to the amino groups and that these were quite resistant to hydrolysis, while the rest were of varying lability to alkali at low temperatures. Other studies are in agreement (2, 3). Benzoyl chloride also reacts with the imidazole and guanidyl groups of protamines (81, 98). The possibility that the imidazole groups of proteins may undergo a Bamberger fission with benzoyl chloride has been suggested (309).

Carbobenzoxy chloride has been used with insulin (128) and tobacco mosaic virus (236). It appeared to attack amino groups more specifically than ketene, phenyl isocyanate, *p*-chlorobenzoyl chloride, or benzenesulfonyl chloride since, for the same number of amino groups reacted, there were fewer phenolic groups involved. Benzenesulfonyl chloride was used by Gurin and Clarke (143) with gelatin. About 30 per cent more sulfur was introduced than could be accounted for by the decrease in amino groups. In pyridine solution, toluenesulfonyl chloride attacked the phenol groups of tyrocidine more rapidly than it did the amino groups (66), contrary to the usual order of reaction of acylating agents in aqueous solution.

Azides react with proteins in cold aqueous alkaline dioxane (54, 68, 69, 70, 179, 229). Clutton *et al.* (68) suggested that reaction occurred only with the α-amino nitrogen groups, but more of the reagent (*O*-glucosido-*N*-carbobenzoxytyrosyl azide) was introduced than could be accounted for on this basis (69), and the titration curves also did not conform to this hypothesis (68). The serum globulin derivative was heat-coagulable (69).

Phosphorylation of proteins has been accomplished by reaction with phosphorus oxychloride in dilute alkaline solution (156, 224, 267). Phenol, amino, aliphatic hydroxyl, and other groups probably are involved (224). In contrast, treatment with phosphorus pentoxide dissolved in phosphoric acid appears to achieve phosphorylation of the hydroxyl groups without involving many of the other polar groups (101). The biological activity of insulin is largely retained after such treatment (117).

E. NITROUS ACID

The reaction of nitrous acid with the amino groups of proteins is the basis for the Van Slyke methods for their determination (327). In addition, phenol, SH, and other groups are involved. According to Philpot and Small (256), in

the presence of excess nitrite, the reaction with the amino group is "pseudobimolecular," and that with the phenol groups is "unimolecular." Investigators have used rate studies to interpret the results of the reaction of nitrous acid on biological proteins. Usually, if inactivation is rapid, it is concluded that the amino groups are essential; if slow, the phenol groups are considered to be necessary, particularly if the rate of inactivation conforms to a first-order reaction (137, 211, 300, 335).

Philpot and Small (256) studied the action of nitrous acid on pepsin. Under the conditions used (pH 4.0, 1 *M* sodium nitrite, 0°C.), the reaction with the amino group appeared to be essentially complete in 30 min., while reaction with the phenolic group had scarcely begun. Weill and Caldwell (335) identified the first reaction of nitrous acid on β-amylase as an oxidation, presumably of SH groups, since the inactivation could be completely reversed by reducing agents. The slower, irreversible inactivation was considered to be due to reaction with the phenolic groups. It has recently been found that, with the mild conditions used by Philpot and Small (256), most of the SH groups of egg albumin were oxidized, but the amino and phenolic groups were only slightly affected; serum albumin showed a partial loss (about 40 per cent) of the latter two types of groups (114). Philpot and Small (256) showed that the rate of reaction of the phenol groups of pepsin was the same as that of tyrosine (as measured by color development). When the reaction slowed, about 50 per cent of the phenol groups had reacted and 50 per cent of the peptic activity had been destroyed. Since tryptophan reacted much more rapidly than tyrosine with nitrous acid, these authors concluded that the indole groups in pepsin could not be involved in the inactivation by nitrous acid. However, the rate of reaction of the indole groups in pepsin may well differ from that of the amino acid. The difficulty in relying on rates of reaction for information concerning reactive groups is illustrated in a recent paper in which Li (200) showed that the rate of iodination of the phenol groups of serum albumin was accelerated in the presence of urea, to a rate even more rapid than that of tyrosine itself. A study of the effect of denaturants on the extent and rate of reaction of nitrous acid on pepsin might yield interesting information.

The reaction of nitrous acid with tyrosine or the phenol groups of proteins has been shown to involve (*a*) introduction of a nitroso group ortho to the hydroxyl, (*b*) reduction of the nitroso group to an amine, and (*c*) diazotization of the amino group (241, 256). The observations of Eagle (85) indicate that similar reactions may occur with tryptophan and the indole groups of proteins. Extensively "deaminated" proteins have decreased histidine (313, 338), arginine (313), and cystine (170) contents. However, the effect of short-time nitrous acid treatment on the indole, guanidyl, and imidazole groups and on the disulfide linkages of proteins has not been investigated. Studies with the amino acids have shown that tryptophan, arginine, and cystine are attacked slowly (282).

The many reactions that may occur when nitrous acid reacts with proteins would appear to limit its usefulness as a specific reagent. Certainly it should not

be used to show the essentiality of SH, amino, or phenol groups without thorough analytical control and separate confirming experiments with different reagents.

F. IODINE

Iodine has been used both as an oxidizing and as a substituting reagent. In dilute acid solutions, particularly of high iodide concentration, oxidation of SH groups appears to be fairly specific. The SH groups of native egg albumin could be completely oxidized at pH 3–5 by stoichiometric amounts of iodine if the oxidation was carried out at 0°C. and in 1 *N* potassium iodide (10, 11). The SH group was oxidized beyond the disulfide stage unless the reaction mixture was kept cold (8). On the other hand, the SH groups of tobacco mosaic virus reacted with iodine at neutrality only in solutions of *low* iodide concentration and in the presence of excess iodine (2.5 times the stoichiometric amount) (11). The virus remained active under these conditions, but with increasing amounts of iodine and at elevated temperature, substitution of the phenol groups and inactivation occurred. Iodine has often been used as an oxidizing agent in the study of SH enzymes (*cf.* 31, 157, 158, 223, 336).

In neutral and alkaline solution, iodine has found utility as a reagent for the phenol groups. Li (198) and Berger and Shaffer (26) studied the kinetics of the reaction of iodine with tyrosine and concluded that they were explainable on the assumption that hypoiodous acid is the primary iodinating reagent. The rate of reaction was inversely proportional to the hydrogen-ion concentration and the square of the iodide concentration and directly proportional to the buffer concentration (199a). These conclusions are in accord with the observations that an acid medium and high concentrations of iodide are preferable when oxidation of SH groups is the desired objective.

Harington and Neuberger (148) iodinated insulin in aqueous or alcoholic ammonia solution. The product had an iodine content of 15.4 per cent, corresponding to the formation of diiodotyrosine quantitatively from the tyrosine residues. The Millon test was negative, and the activity was reduced to 10–15 per cent of that of insulin itself. Seventy per cent of the iodine could be removed by catalytic hydrogenation, and 30–50 per cent of the original activity was thereby regained.

Neuberger (246) obtained an iodinated zein of iodine content conforming to its tyrosine content, and Li *et al.* (204) obtained similarly good agreement with the iodine content of iodinated lactogenic hormone and its original tyrosine content. Herriott (163) studied the iodination of pepsin and identified at least 82 per cent of the iodine as diiodotyrosine in an hydrolysate. In a further study, Herriott (165) added limited amounts of iodine to pepsin, then accounted for 65 per cent of the introduced iodine as monoiodotyrosine. Only 2 per cent of the total phenol groups were involved. The identity of this compound has been questioned (149) and defended (166). Ludwig and von Mutzenbecher (213) accounted for 39 per cent of the iodine in an iodinated casein preparation (total iodine content, 3.5 per cent) as *dl*-monoiodotyrosine. However, kinetic studies on the iodination of tyrosine (198) indicated that the introduction of the first

iodine atom is the rate-controlling step, the reaction of the second atom being so rapid that there is very little monoiodotyrosine in the reaction mixture at any particular time. Experiments with lactogenic hormone appeared to be in accord with the concept that iodination of protein phenol groups follows a similar course (204). The mechanism of iodination of the tyrosine groups in proteins may require reinvestigation.

Li (200) found that the rate of fixation of iodine by serum albumin and pepsin was a function of the degree of denaturation. Whereas with the native proteins the rate was slower than with tyrosine, the addition of iodine in urea solution was more rapid, and in heated urea solution the reaction rate exceeded that observed with the free amino acid.[8] These data are interpreted as revealing an increased availability of the tyrosine groups with increasing denaturation. Analytical results indicated that the amount of iodine introduced was equivalent to the amount of tyrosine reacted as determined by the Lugg procedure (215); hence other groups could not have been affected. It may be mentioned that monoiodotyrosine, if present, would give a color by this method (149, 165, 269a).

The papers so far cited appear to show that iodine in nearly neutral or alcoholic or aqueous ammoniacal solution reacts solely with the tyrosine groups of proteins (containing no SH groups). However, Pauly (253) found sturine to bind an amount of iodine which corresponded to its histidine content, and Bauer and Strauss (20, 21, 22, 23) found that globin bound iodine in excess of that required for its tyrosine content and showed that iodination of the imidazole groups had occurred. In this connection it is interesting to note that globin iodinated with limited amounts of iodine did not show the expected change of dissociation constant in alkaline solutions (72), characteristic of proteins containing iodized tyrosine (246). Li (199) found that the rate of iodination of tyrosine was 30 to 100 times faster than that of histidine and pointed out that the imidazole groups of proteins may react with iodine if the reagent is present in excess and the reaction time is prolonged. But Brunings (53a) has shown that the iodination of histidine requires more vigorous conditions than that of histidine anhydride; hence imidazole groups in proteins may react at rates considerably different from those observed with histidine itself.

The possibility of the reaction of the indole groups of proteins with iodine has not been properly assessed. Chymotrypsinogen (6 per cent tryptophan) did not add iodine in dilute acid solution (7), but, at pH 7, acetyltryptophan and gramicidin (40 per cent tryptophan content) decolorize iodine rapidly (114), suggestive evidence that indole groups in proteins may react similarly. It is necessary to postulate that imidazole and indole, and even other groups, can add iodine in order to account for the very large amounts introduced into some proteins by recent workers (45, 155, 245, 294; *cf.* 42a).

Evidence is presented by Philpot and Small (257) that pepsin may be inactivated by small amounts of iodine without participation of the phenol groups in the reaction, but interpretation of the work is rendered difficult by their reliance

[8] From the results of unpublished experiments, Anson (9, 10) suggests that the oxidation of tyrosine itself may be accelerated in the presence of urea.

on the Folin color reaction (see footnote 5) to detect changes in the phenol groups.

Thus, with iodine, as with the other reagents previously discussed, additional analytical work appears to be required before its usefulness as a specific reagent for the phenol groups can be assured.

Under more extreme conditions, iodine converts the tyrosine of proteins into thyroxine (146, 213, 264). Discussion of this interesting reaction is outside the scope of this review.

The effects of bromine and chlorine on proteins under mild conditions have been studied in much less detail. Wormall (344) reacted serum proteins with bromine water and found the product to contain 4.7 per cent bromine. Blumenthal and Clarke (44) showed that bromine water oxidized some protein disulfide bonds to sulfate. Although considerable amounts of chlorine reacted with serum proteins, none was introduced (344), the reaction appeared to be exclusively oxidative. Consden *et al.* (73) demonstrated the presence of cysteic acid in chlorinated wool. Stumpf and Green (316a) found dilute chlorine solutions to inactivate SH enzymes but not to affect enzymes not requiring this group. The strong oxidative properties of these halogens make them of doubtful utility as specific reagents.

G. FORMALDEHYDE

The reaction of formaldehyde with proteins is complicated and its usefulness as a specific reagent is, therefore, limited. An excellent review of the published work until 1945 is available (118). Additional important papers have since appeared, but unless evidence of reaction with specific groups was presented, they have not been quoted in this brief summary.

In neutral solution, the immediate reaction with proteins is combination with the free amino groups (basis for the "formol titration"). However, this is an equilibrium type reaction and is partly or completely reversible by dilution, dialysis, in the presence of the formaldehyde precipitant, dimedon, or under the conditions of the Van Slyke amino nitrogen analysis. With longer time of reaction, the formaldehyde slowly becomes more firmly bound and the amino nitrogen content is decreased (212, 331). Even the latter reaction is partially reversible by dialysis against dilute acid (114, 275). Thus it may be expected that the results obtained with formaldehyde-treated, biologically active proteins would depend to a large extent not only upon the reaction conditions used, but also upon the techniques applied to the protein from the time the reaction was completed until the derived protein was assayed.[9]

Instances of reactivation of formaldehyde-inactivated proteins have been observed for bacteriophage (290), Type I pneumococcus antibody (65), parathyroid hormone (326), and tobacco mosaic virus[9] (275). In some cases where the immediate reaction with formaldehyde has been used to demonstrate the essen-

[9] Different techniques in the handling of their preparations may explain the inability of Kassanis and Kleczkowski (188) to repeat the reversible inactivation of tobacco mosaic virus by formaldehyde demonstrated by Ross and Stanley (275).

tiality of amino groups, the assay has been performed on the solution containing formaldehyde (207, 210). It is reasonable to suppose that activity might have been regained if the formaldehyde had been removed as, for example, by dialysis. However, when the reaction is permitted to proceed for longer periods of time, the changes that occur are progressively less reversible by dialysis (114).

The guanidyl group also reacts slowly with formaldehyde at neutrality and room temperature (107, 112, 316b). Eaton (88) found the Sakaguchi test for guanidyl groups to be decreased in diphtheria toxin treated with formaldehyde, but Ross and Stanley (275) found no change in this test with formalized tobacco mosaic virus.[10]

In alkaline solution, not only the amino, but also the indole (108), amide (107), and guanidyl groups add formaldehyde rapidly (107, 316b). The amino compound, like that obtained at neutrality, is reversibly dissociable. The other combinations are more stable, thus making it possible to obtain protein derivatives in which the amide, indole, and guanidyl, but not the amino groups, are combined.

The reaction of protein SH groups with formaldehyde has not been studied in detail. Anson (8) indicated that those of egg albumin react only slowly with formaldehyde. The SH groups of native egg albumin seem to be largely unaffected by formaldehyde at room temperature and neutrality, although a readily dissociable compound may be formed (114; *cf.* 262, 288).

A number of investigators have reported variable or slow inactivation of biological proteins with formaldehyde. Where analytical data were not obtained, the results have not been included in table 2. The following references include a partial list (24, 35, 38, 43, 60, 275, 326).

Exact interpretations of the reaction of formaldehyde with proteins are complicated by cross-linking reactions. Cross-linking of guanidyl groups has been demonstrated with protamine (112). Of more general significance may be the condensation reaction of aminomethylol groups ($RNHCH_2OH$) with amide, imidazole, indole, phenol, and perhaps still other groups to give stable methylene linkages. These reactions can occur under conditions where neither the amino nor the other polar groups by themselves give stable formaldehyde addition products, or where they do so only slowly (114). Many toxoid preparations require long periods of incubation, e.g., 2 weeks at 37°C., conditions which are favorable for cross-linking. It is thus possible that groups that do not by themselves combine with formaldehyde, may be involved in toxoid formation through the secondary cross-linking reactions.

H. MERCAPTIDE-FORMING AGENTS

The advantages of organic mercury and trivalent arsenic compounds as specific agents for SH groups have been discussed in Section II,A. Of these, *p*-chloromercuribenzoate, first proposed by Hellermann (158), has been the most useful. Phenylmercuric nitrate depresses the activity of non-SH enzymes (74, 75). Var-

[10] Ross and Stanley (275) observed a decrease in the Folin color value, but this has been recognized as an illustration of the pitfalls of the colorimetric technique (108, 188; footnote 5).

ious trivalent arsenicals (19, 297) as well as other mercury compounds (19, 158, 159, 336) act similarly, although their effectiveness appears to vary (19, 223). Results with arsenious oxide derivatives should be interpreted with caution, since they may reduce disulfide linkages. Bersin (30) reported that *p*-aminophenylarsenoxide may partially activate papain (*cf.* 71).

Stocken and Thompson (314) treated a reduced keratin (hair) preparation with lewisite (chlorovinyldichloroarsine). The amount of arsenic bound and its stability suggested that one arsenic molecule combines with two SH groups.

I. MISCELLANEOUS

1. 1,2-Epoxides

Low-molecular-weight, water-soluble epoxides such as ethylene oxide and 1,2-propylene oxide react readily with proteins under mild conditions (106). The carboxyl, amino, phenolic, and SH groups react to form esters, substituted amines, aromatic ethers, and thioethers, respectively. Reaction with amino groups occurs much more readily in alkaline than in acid solution. The products, like other esterified proteins, show isoelectric points raised by 2 to 3 pH units. Attempts to make this a specific technique of esterification have not been successful, since the phenolic groups appeared to react under all conditions used. This conclusion was drawn from Folin analyses and may need substantiation (114).

2. War gases: mustard gas and nitrogen mustards

Some of the extensive work accomplished during the war on the reaction of various war gases on proteins has now been published in detail. The data so far available indicate that many protein groups may participate, but it appears probable that reaction with SH groups may be the most rapid and also that responsible for the intense physiological reactions (17, 102, 138, 153, 235a; *cf.* 166). According to Kinsey and Grant (190) mustard gas [bis(2-chloroethyl) sulfide or H] reacts with protein imidazole, amino, thioether, and hydroxyl groups of threonine at pH 9.3. At pH 7.4, the hydroxyl group does not react. Microbiological assays of H-treated casein hydrolysates indicated that both thioether and amino groups reacted more completely at the higher pH. Herriott *et al.* (167) and Banks *et al.* (17) found the amino content of proteins unchanged after H-treatment at pH 5–6 (*cf.* 240). Carboxyl groups reacted over a wide range of pH values, and this reaction appeared to be fairly specific at pH 5.5–6.0 (with the possible exception of SH groups), since the amount of H introduced into several proteins was roughly equivalent to the number of carboxyl groups esterified (78, 167). However, the extent of involvement of phenol groups is not known (see Section II,I), and the rôle of methionine was not studied.

Model experiments indicate that H may react *in acid solution* primarily with the thioether linkages of proteins to form sulfonium-type addition compounds (310). It has been reported that the imidazole groups react with H at pH 7.6 (78, 190, 243, 329).

Other war gases and related compounds have been studied in somewhat less detail. Fruton *et al.* (125) concluded from model experiments with amino acids

and amino acid derivatives that the nitrogen mustards (e.g., bis(2-chloroethyl)-methylamine, etc.) would react with the (*1*) imidazole groups, (*2*) thioether groups, (*3*) amino groups (less rapidly with the ϵ than with the α), and (*4*) the free carboxyl groups of proteins.

Mustard sulfone, mustard sulfoxide, divinyl sulfone, and other derivatives also react with proteins but fewer details are yet available (17, 25, 307). Mustard sulfone (HO_2) reacts primarily with the SH and amino groups of proteins (17). Reaction with the latter differentiates this reagent from H (25). The sulfoxide (HO) does not react with proteins (17). Divinyl sulfone reacts with protein SH groups (138) and possibly also with the amino and phenol groups (307). Mustard and other war gases have been found to cause mutations in *Drosophila*. The results of investigations so far described indicate that chemical reactions are responsible (11a).

3. Aldehyde and ketone reagents

The activities of a few enzymes are markedly inhibited by low concentrations of hydrazine, hydroxylamine, and semicarbazide (e.g., 123, 126, 220, 318). The conclusion is drawn that the enzymes possess a keto or aldehyde group essential for their activity. However, such results should be ascribed to the presence of prosthetic groups or coenzymes containing ketone or aldehyde groups (such as pyridoxal). No protein group is known to react with these reagents in the low concentrations used. The inhibiting effect of phenylhydrazine on a papain preparation (27) has not been adequately explained.

IV. Discussion

Table 4 contains a compilation of the effects of protein reagents on the various groups, assigned somewhat arbitrarily on the basis of information in the literature and experience gained in this Laboratory. The practice of using several reagents for determining the essentiality of any one group for biological activity is sound, and thorough analytical control of each reaction is strongly recommended.

Table 2 contains much of the data in the literature on the essential groups of biologically active proteins. Knowledge in this field is still fragmentary, but may be expected to expand rapidly with the development of new specific reagents and the accumulation of more information concerning those that have already been found useful. Reagents tagged with radioactive or other isotopic elements should prove extremely helpful in determining the amounts introduced into proteins.

The concept has been tacitly accepted that biologically active proteins have essential groups and that, when these are "covered" or "substituted", the protein can no longer function. This oversimplification is necessary because of the limited information yet available. However, it might be pertinent to mention some of the factors that may complicate the interpretation of the effects of modifying reagents. For example, esterification of the carboxyl groups of insulin results in inactivation (table 2). Is this due to the essentiality of carboxyl

TABLE 4

*Effects of reagents on protein groups under conditions most favorable for specificity**

REAGENTS	AMINO	GUANIDYL	IMIDAZOLE	INDOLE	ALIPHATIC HYDROXYL	AMIDE	THIOETHER	DISULFIDE	SULFHYDRYL	PHENOL	CARBOXYL
Oxidizing agents:											
Iodosobenzoate, porphyrindin, ferricyanide, iodine[a]	−	−	−	−	−	−	−	−	3+	−	−
Hydrogen peroxide[b]					−	−	2+		3+		−
Reducing agents:											
Cysteine, thioglycolic acid, thioglycol, cyanide, sulfite[c]	−	−	−	−	−	−	−	3+	−	−	−
Alkylating agents:											
Iodoacetate, iodoacetamide[d]	±					−		−	2+	±	−
Dinitrofluorobenzene[e]	3+	−	+			−		−	3+	3+	−
Acylating agents:											
Ketene[f]	3+	−		±	±	−	−	−	+	2+	−
Acetic anhydride[g]	3+	−	−	−	−	−	−	−	−	±	−
Phenyl isocyanate[h]	3+	±			−	−	−	−	3+	?	−
Carbon suboxide[f]	2+	−	−		−	−	−	−	3+	2+	−
Azides, benzoyl, carbobenzoxy, benzenesulfonyl chlorides, etc.[i]	3+				−	−	−	−	+	±	−
Concentrated sulfuric acid[j]	−	−	−	−	3+	−	−	−	3+	+	−
Nitrous acid[k]	3+	−		+?	−	−		−	3+	2+	−
Iodine[l]	−	−	±	+?	−	−			3+	3+	−
Formaldehyde (pH 7-8)[m]	(3+)[m]	+		−	−	−	−	−	±	−	−
Formaldehyde (pH 11)[m]	(3+)[m]	3+	−	3+	−	3+			+	−	−
Epoxides[n]	+				−	−		−	2+	2+	3+
Mustard gas[o]	−	−	+		−	−	+	−	3+	?	2+
Acid-alcohol[p]	−	−	−	−	−	−	−	−	−	−	3+
p-Chloromercuribenzoate[q]	−	−	−	−	−	−	−	−	3+	−	−
Diazonium compounds[r]		−	3+	+	−	−	−	−		2+	−

* The symbols used have the following significance: 3+, 2+, and + indicate the relative rapidity or extent of reaction, with 3+ denoting the most rapid reaction. ± indicates reactions that may or may not occur under the conditions suggested. − indicates those reactions either that have been shown not to occur or that appear improbable from organic chemical considerations. ? indicates those reactions for which more information is required. Spaces have been left blank where there is a possibility of reaction but no evidence is available. Allocation of the proper symbols for each reaction is difficult because of the differences between proteins. The choices are arbitrary.

The conditions of the reaction given in the following footnotes are those suggested by users of these reagents. For references to the original literature, see table 2 and the text.

[a] pH 7, 0.001–0.01 *M*, 0–25°C., 5–30 min. The specificity of *iodine* as an oxidizing agent requires a high concentration of iodide ions, pH 1–7.

[b] pH 6.6, 0.005 *M*, 25°C., 0.5–40 hr.

[c] pH 7–8, 0.001–0.1 *M*, 25°C., 0.5–4 hr.

[d] pH 7–8, 0.05–0.1 *M*, 0–25°C., 0.5–2 hr.

[e] pH 7–8, 0.17 *M*, 25°C., 2 hr.

[f] pH 5–8, 0–25°C., 5–30 min.

[g] pH 7–8, 0°C., 30 min. (see table 3).

[h] pH 7–8, 0–25°C., 0.5–2 hr., reagent-protein ratio, 0.5–2.5:1.

TABLE 4—*Continued*

[i] pH 7–9, 0–25°C., 0.5–2 hr., limited amounts of reagent.'
[j] −18° to 0°C., 10–30 min.
[k] pH 4, 1 *M*, 0°C., 30 min.
[l] pH 5–11, −5° to 25°C., 0.5–3 hr., limited amount of iodine, low iodide concentration. See also footnote a.
[m] 25°C., 1–2 *M*, at pH 7–8, 1 hr.; at pH 11, 10 min. Amino groups react rapidly but reversibly. After isolation by dialysis, amino groups are essentially free.
[n] pH 5–6, 1–2 *M*, 25°C., 1–4 days.
[o] pH 5–6, 25°C., 0.5–4 hr.
[p] 0.01–0.1 *M* mineral acid in absolute alcohol, 0–25°C., 1–2 days.
[q] pH 7, 10^{-5} to 10^{-2} *M*, 25°C., 5–30 min.
[r] pH 7–9, limited amounts of reagent, 25°C., 30 min.

groups, or to a change in the charge distribution in the protein? The latter interpretation is favored by the observations of Reiner and Lang (265), who noted that insulin derivatives with azo compounds containing basic groups were inactive while those containing acidic groups were active, although, presumably, the same protein groups were involved. Similarly, the introduction of strongly charged acid sulfate groups on the hydroxyl groups did not destroy insulin activity (130); possibly the introduction of basic substituents *at the same place* would be detrimental. In all cases, including those just mentioned, the possibility must be considered that the formation of a derivative of a "non-essential" group might influence unfavorably the activity of an adjacent but unreacted "essential" group.

Possibly of greater importance than the determination of the essentiality of various groups of biologically active proteins, will be studies of the properties of those derivatives that retain activity. For example, the transformation of toxins to toxoids has so far been accomplished only by empirical methods. In these cases one biological activity (toxicity) is changed, while another (antigenicity) is retained. Knowledge of the groups involved should lead to better control of these processes (*cf.* 251). Similar selective changes in the activities of enzymes and hormones may be possible, but this field has hardly been touched (*cf.* 323). Tobacco mosaic virus can withstand considerable chemical modification without loss of activity (11, 235, 236, 286). Some partially inactivated derivatives were found to have differing specific activities when assayed on different plants (236). Although the derivatives so far obtained all have propagated as the original virus, possible means of producing "chemical mutants" have not yet been exhausted.

It is to be hoped that the application of specific reagents to these various types of proteins will help to enlighten the mystery concerning their mode of function in the life process.

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