N-Acetylimidazole: A Reagent for Determination of "Free" Tyrosyl Residues of Proteins*

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ABSTRACT: The reactivity of *N*-acetylimidazole with functional groups of amino acid residues of copolymers and proteins has been studied. The ε-amino groups of all copolymers and proteins examined interact with acetylimidazole to a much lesser extent than with acetic anhydride. The ε-amino groups of carboxypeptidase do not react. In the limited number of instances examined both reagents acetylate sulfhydryl groups to a similar extent. The data obtained thus far did not reveal significant interactions with aliphatic hydroxyl groups. Acetylimidazole acetylates all of the tyrosyl residues of copolymers and denatured proteins. However, the reagent acetylates only the "free" tyrosyl residues in native proteins. The spectral changes due to *O*-acetylation of tyrosine are pronounced and allow a convenient and accurate measure of the number of tyrosines reacted. The results of colorimetric determinations of hydroxamates, subsequent to deacetylation of *O*-acetyltyrosine with hydroxylamine, are in quantitative agreement with those from spectral changes on acetylation. The present data in regard to the numbers of "free" and "buried" tyrosyl residues correlate well with those on several proteins studied previously by the method of J. L. Crammer and A. Neuberger (1943, *Biochem. J. 37*, 302). The relative specificity of acetylimidazole, the mildness of the reaction conditions, and the simplicity of the analytical procedure render it a suitable reagent both for the determination of "free" tyrosyl residues in proteins and of their potential contribution to function.

Monocarboxylic acid anhydrides, but particularly acetic anhydride, have long been employed for chemical modification of amino and thiol groups of proteins and peptides (Fraenkel-Conrat, 1959). Recently we have demonstrated the usefulness of these reagents in acylating the phenolic hydroxyl group of tyrosine as well (Riordan and Vallee, 1963). Dicarboxylic acid anhydrides also modify this group, but the products are intrinsically unstable and hydrolyze via intramolecular nucleophilic catalysis (Riordan and Vallee, 1964). Acylation of tyrosine is accompanied by distinctive spectral changes, thereby allowing ready detection (Simpson *et al.*, 1963; Riordan and Vallee, 1963).

In the course of studies on carboxypeptidase, *N*-acetylimidazole was found to be a useful reagent for the acetylation of tyrosyl residues. Moreover, the reagent seemed milder than acid anhydrides, as indicated by physicochemical evidence (Bethune *et al.*, 1964). Such findings prompted the present investigation to determine the reaction specificity of acetylimidazole both toward "free" and "buried" tyrosyl residues and toward other reactive groups of polypeptides and proteins. A preliminary account has been presented (Wacker *et al.*, 1964).

Materials

Acetylimidazole was prepared by the method of Boyer (1952) or purchased from K & K Laboratories. Since the reagent is quite hygroscopic it was stored *in vacuo* over phosphorus pentoxide. At the first signs of moisture it was dissolved in benzene, dried with sodium sulfate, and recrystallized. Acetic anhydride (Eastman) was redistilled prior to use. Carboxypeptidase A, muscle aldolase, chymotrypsinogen, α-chymotrypsin, ovalbumin, ovomucoid, ribonuclease, and trypsin (Worthington), horse liver alcohol dehydrogenase (Boehringer), bovine insulin (Lilly), bovine serum albumin (Ortho Pharmaceutical), conalbumin (Sigma), bovine hemoglobin (Pentex), sperm whale myoglobin (Mann), poly-L-lysine, and thiolated gelatin-highbloom, G-10 (Schwartz), were all used without further purification. Seryl–glutamic acid copolymer was a gift of Dr. E. R. Blout, the glutamic-lysyl-tyrosine tricopolymer of Dr. T. J. Gill, III, the tyrosyl–glutamic acid and lysyl-tyrosine copolymers of Dr. H. Sober, and the random tyrosyl-glutamic and block copolymer of tyrosyl–glutamic acid of Dr. G. Fasman.

Methods

Except when otherwise indicated, acetylation with acetylimidazole was performed at room temperature by

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incubating the protein or polymer (4 mg/ml) with the reagent in 3 ml of 0.05 M sodium borate buffer, 0.02 M Veronal, or 0.01 M Tris at *p*H 7.5.[1] This *p*H was adopted since it is the maximum for the stability of acetylimidazole. In general, a 60-fold molar excess of acetylimidazole was employed and the reaction was allowed to proceed for 1 hour. Because of the high absorptivity of acetylimidazole at 245 mμ (Stadtman, 1954) the reaction mixture had to be separated on a 1- × 30-cm column of Sephadex G-25, simultaneously terminating the acetylation; in some instances excess acetylimidazole was removed by dialysis. Controls were treated in the same manner but without addition of acetylimidazole. An alternative procedure would be to allow the residual acetylimidazole to undergo spontaneous decomposition. The eluate fraction from gel filtration containing protein or tyrosyl polymer was diluted to an extent that the absorbance of the control sample at 280 mμ was 0.8–0.9. Spectra were measured with a Cary Model 15 spectrophotometer.

Acetylation with acetic anhydride was carried out between 0 and 4°, the *p*H being maintained at 7.5 by means of a *p*H-stat (Radiometer, Copenhagen). Otherwise the procedure was essentially as described for acetylimidazole.

The degree of amino-group substitution was measured by the ninhydrin procedure of Moore and Stein (1954) using phenylalanine as a standard. Acetylation of tyrosine was determined by decrease in absorbance at 278 mμ (Simpson *et al.*, 1963). The alkaline hydroxamate procedure of Hestrin (1949) was employed to measure *O*-acetylation. Sulfhydryl groups were measured by argentometric titration (Benesch *et al.*, 1955).

TABLE I: Acetylation of Amino Groups of Polymers and Proteins with Acetylimidazole and Acetic Anhydride.[a]

Polymer or Protein	Amino Groups Acetylated: Acetylimidazole (%)	Amino Groups Acetylated: Acetic Anhydride (%)
Poly-L-lysine	34	100
Poly-L-lysyl-L-tyrosine (5:1)	31	100
Poly-L-glutamyl-L-lysyl-L-tyrosine (52:32:16)	20	81
Carboxypeptidase A in 2 M NaCl	0	68
Carboxypeptidase A in 0.05 M β-phenylpropionate	0	66
Hemoglobin	3	57
Chymotrypsinogen	10	65
Ovomucoid	11	90
Ovalbumin	20	58
Bovine serum albumin	23	62
Myoglobin	29	61
Iron conalbumin	36	70
Ribonuclease	35	95

[a] Acetylation with acetylimidazole was carried out at 20° with a 60-fold molar excess of reagent. The protein or polymer (4 mg/ml) was dissolved in 0.05 M borate or 0.01 M Tris buffer, *p*H 7.5. After 60 minutes an aliquot of the reaction mixture was removed for amino group analysis by ninhydrin (Moore and Stein, 1954). Acetylation with acetic anhydride was carried out at 0° on the *p*H-stat but otherwise as with acetylimidazole.

Results

Acetylation of Amino Groups. Amino groups of proteins are readily acetylated with acetic anhydride, the reagent most commonly employed for this purpose. The procedure is generally carried out in the presence of half-saturated sodium acetate (Fraenkel-Conrat, 1957). While intended to act both as a buffer and as a catalyst, sodium acetate, in fact, directs the specificity of the anhydride since any *O*-acetyltyrosyl residues which might be formed would be hydrolyzed under these conditions (Bender and Neveu, 1958). In these studies, however, sodium acetate was omitted and acetylation with acetic anhydride was performed on the *p*H-stat. Table I compares the percentage of amino groups acetylated by acetylimidazole and by acetic anhydride as determined with ninhydrin. Acetic anhydride completely acetylates the amino groups of poly-L-lysine and of poly-L-lysyl-L-tyrosine (5:1), while acetylimidazole modifies these groups only 34 and 31%, respectively. Acetic anhydride modifies 81% of the amino groups of tricopolymer, glutamyl-L-lysyl-L-tyrosine (52:32:16), but with acetylimidazole only 20% substitution is found.

The amino groups of proteins also react to different degrees with these acetylating agents. The anhydride blocks 68% of the amino groups of carboxypeptidase A. However, acetylimidazole fails to acetylate these groups.

Carboxypeptidase is normally soluble only in 1–2 M NaCl. It was thought that the high salt concentration might interfere with the reactivity of amino groups. Hence, acetylation with acetylimidazole was carried out in 0.02 M Veronal and 0.05 M β-phenylpropionate, since the enzyme is readily soluble without salt in low concentrations of this agent. However, neither the elimination of salt nor the presence of β-phenylpropionate increased the reactivity of amino groups to acetylation with acetylimidazole (Table I, line 5).

The reactivity of the amino groups of other proteins toward acetylimidazole was also studied. In hemoglobin, chymotrypsinogen, and ovomucoid 11% or fewer of these groups reacted with acetylimidazole, while acetic anhydride modified at least 57%. Acetylation of the other proteins listed in Table I was somewhat more extensive, 20–36% with acetylimidazole, and 58–95% with acetic anhydride.

The time course of the reaction of amino groups with acetylimidazole was examined with poly-L-lysine,

[1] The effect of higher molarities of Tris is discussed later.

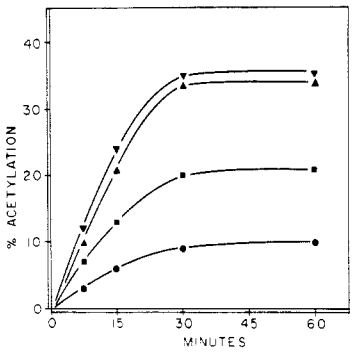

FIGURE 1: Acetylation of ε-amino groups with acetylimidazole. Acetylation of poly-L-lysine (▼), ribonuclease (▲), ovalbumin (■) and chymotrypsinogen (●) was carried out as in Table I. The degree of modification was determined with ninhydrin (Moore and Stein, 1954).

ribonuclease, ovalbumin, and chymotrypsinogen (Figure 1). Although the extent of acetylation is different for each, maximal reaction with a 60-fold molar excess of acetylimidazole occurred within 30 minutes.

Acetylation of Aliphatic Hydroxyl Residues. On incubation of poly-L-seryl-L-glutamate (1:9) with acetylimidazole formation of *O*-acetylserine did not occur, as indicated by the absence of acethydroxamate formation after treatment with hydroxylamine at *p*H 12.2 (Table II, lines 1 and 2). With acetic anhydride only about 0.2 mole of *O*-acetylserine per mole of polymer was formed (lines 1 and 3). Significant *O*-acetylation of the aliphatic hydroxyl groups of proteins was not found under the conditions employed. Thus, in acetyl-bovine serum albumin, a total of 2.3 acetyl groups was found labile at alkaline *p*H and 2.0 moles was accounted for by the deacetylation of *O*-acetyltyrosine (Table II, line 4) (*vide infra*). Similarly, a particular sample of acetylribonuclease containing 0.9 *O*-acetyltyrosyl residue yielded 1.4 moles of hydroxamate at *p*H 12 per mole of enzyme (Table II, line 5).

Acetylation of Thiol Residues. Acetylimidazole and acetic anhydride acetylate thiol groups both of model compounds and of proteins. While both completely acetylate the single —SH group of glutathione and the 21 —SH groups of thiolated gelatin (Table III), the results with proteins were more variable. Thus, acetylimidazole did not react with the 22.5 thiol groups of this aldolase preparation, while acetic anhydride acetylates three to four of them. None of the 28 titratable thiol residues of horse liver alcohol dehydrogenase react with either reagent at *p*H 7.5 in 0.1 M sodium phosphate buffer, but on denaturation of alcohol dehydrogenase with sodium dodecyl sulfate 18 thiol groups were acetylated with acetylimidazole and 12 with acetic anhydride.

Acetylation of Tyrosyl Residues. Acetylation of *N*-acetyltyrosine or of tyrosyl residues produces a characteristic decrease in absorption between 250 and 300 mμ (Braude, 1949; Schlögl *et al.*, 1953; Simpson *et al.*, 1963; Riordan and Vallee, 1963). The essential features of the spectral changes are here indicated as a matter of convenience. The absorption maximum of

TABLE II: Acetylation[a] of Aliphatic Hydroxyl Residues with Acetylimidazole (Ac_I)[b] and Acetic Anhydride (Ac_A).

	Hydroxamates (mole/mole)	*O*-Acetyltyrosyls (mole/mole)
Poly-L-seryl-L-glutamate (1:9)	0.0	
Ac_I poly-L-seryl-L-glutamate (1:9)	0.0	
Ac_A poly-L-seryl-L-glutamate (1:9)	0.2	
Ac_I bovine serum albumin	2.3	2.0
Ac_I ribonuclease	1.4	0.9[c]

[a] Acetylations were carried out as in Table I. Hydroxamates were determined at *p*H 12.2 by the method of Hestrin (1949). *O*-Acetyltyrosyl residues were determined spectrophotometrically. [b] The prefixes Ac_I and Ac_A refer to protein or polymer acetylated with acetylimidazole and acetic anhydride, respectively. [c] The sample of Ac_I ribonuclease used in this analysis was acetylated with a 60-fold molar excess of acetylimidazole.

TABLE III: Acetylation of Thiol Groups by Acetylimidazole and Acetic Anhydride.[a]

	Moles Ag^+/ Mole		
	Control	Acetylimidazole	Acetic Anhydride
Glutathione	1.0	0.0	0.0
Thiolated gelatin	21.0	0.0	0.0
Aldolase	22.5	22.5	19.0
Liver alcohol dehydrogenase	28.2	28.2	28.2
Liver alcohol dehydrogenase + sodium dodecyl sulfate	29.0	9.9	16.0

[a] Acetylations were carried out as in Table I except for liver alcohol dehydrogenase, which was acetylated in 0.1 M phosphate, *p*H 7.5, ±0.01 M sodium dodecyl sulfate. Thiol groups were measured by argentometric titration (Benesch *et al.*, 1955).

N-acetyltyrosine at 275 mμ is shifted to 263 mμ on *O*-acetylation with a decrease in the molar absorptivity at 275 mμ, ϵ_{275}, from 1360 to 200, a decrease of 1160. The spectrum of *N,O*-diacetyltyrosine resembles that of phenylalanine. Exposure of *N,O*-diacetyltyrosine to hydroxylamine restores the original spectrum by deacylation; the chemical basis of this change is verified by measurement of the resultant hydroxamate (Simpson *et al.*, 1963; Riordan and Vallee, 1963).

Acetylimidazole was first employed as a protein-acetylating agent on carboxypeptidase A (Simpson *et al.*, 1963). The changes in the spectrum of carboxypeptidase are analogous to those produced by *O*-acetylation of tyrosine. Therefore, the number of tyrosyl groups modified can be calculated on the basis of the molar absorptivities of *N,O*-diacetyl- and *N*-acetyltyrosine. The results are in agreement with those obtained independently using ^{14}C-labeled acetylimidazole (Vallee, 1964).

Acetylimidazole modifies only six to seven of the nineteen tyrosyl residues of carboxypeptidase (Simpson *et al.*, 1963). Since seven tyrosine groups have a *pK* of 9.5, these findings suggest that the secondary and tertiary structure of the protein, or the microscopic environment of the groups, or a combination of these factors suppresses the ionization of the remaining twelve tyrosines and prevents them from reacting with acetylimidazole (*vide infra*).

A number of tyrosine copolymers and both native and denatured proteins, all of known tyrosine content, were acetylated with acetylimidazole. A 60-fold molar excess of reagent was generally required to produce maximal acetylation, though for ribonuclease and α-chymotrypsin a *180*-fold molar excess was needed.

All of the random copolymers studied, varying in their proportion of tyrosine and glutamic acid and/or lysine, were acetylated readily and completely (Table IV). A copolymer of glutamic acid and tyrosine in which all of the tyrosyls occurred as a "block" of the C-terminal portion of the molecule was also acetylated. In this instance only 18% of the tyrosyl groups were modified.

TABLE IV: Acetylimidazole-reactive Tyrosyl Residues of Copolymers.[a]

Copolymer	Tyrosyl Acetylation (%)
L-Glutamyl-L-tyrosine (4:1)	100
L-Glutamyl-L-tyrosine (1:1)	100
L-Lysyl-L-tyrosine (5:1)	100
L-Glutamyl-L-lysyl-L-tyrosine (52:32:16)	100
L-Glutamyl-L-tyrosine (95:5) ("block" copolymer)	18

[a] Acetylations were carried out as in Table I. The change in absorptivity on acetylation of these polymers is exactly analogous to that for *O*-acetylation of *N*-acetyltyrosine. The degree of acetylation is expressed as a percentage since exact molecular weights of the polymers were not known.

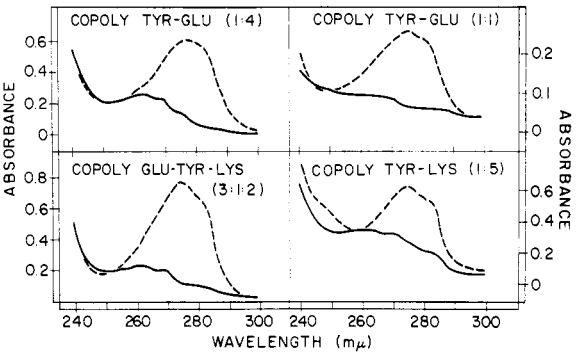

FIGURE 2: Acetylation of tyrosyl residues in copolymers with acetylimidazole. Acetylations were carried out as in Table I. Spectra of the unmodified (---------) and acetylated (———) polymers were determined after removal of excess acetylimidazole by gel filtration.

When, as in these copolymers, tyrosine is the only chromophore in the range from 260 to 310 mμ, the spectral changes on *O*-acetylation can be observed directly and easily (Figure 2). In proteins, however, the change on *O*-acetylation of tyrosine must be differentiated from the contribution to the total absorbance of variable numbers of tryptophanyl, methionyl, and those tyrosyl residues which remain unmodified. Thus, in proteins, the practical limit of detection of *O*-acetyltyrosine is determined by the ratio of the absorbance due to this modification to the total absorbance due to all these other species.

Hence, a number of proteins differing in the number and type of these constituent chromophores were *O*-acetylated with acetylimidazole. As expected, the changes in molar absorptivity of the proteins at 278 mμ vary both as a function of the total number of tryptophanyl residues and of the tyrosyl residues which remain unmodified (Figure 3). Thus, the spectrum of native insulin, e.g., which has few or no residues other than tyrosine absorbing near 280 mμ, closely resembles that of tyrosine alone, and, hence, the changes on acetylation are the most conspicuous among the proteins studied. On the other hand, for proteins containing high percentages of tryptophan such as carboxypeptidase or bovine serum albumin the spectral alterations are less obvious.

The decrease at 278 mμ, $\Delta\epsilon_{278}$ = 1160 per mole, on *O*-acetylation of *N*-acetyltyrosine has been used to determine the number of tyrosyl residues modified in each protein (Table V). All of the tyrosyl residues of insulin and ovomucoid are acetylated. However, in most proteins only some of the tyrosyl residues known to be present by amino acid analysis (Table V, column 3) react with acetylimidazole. In most instances the

TABLE V: Acetylimidazole-reactive Tyrosyl Residues Compared with "Free" and Total Tyrosyl Residues of Proteins.[a]

	O-Acetyltyrosyl Residues (mole/mole)	Tyrosyl Residues (mole/mole)		
		"Free"	Total	Ref.
Myoglobin	0.8	1	3	[b]
Insulin	4.1	4	4	[c]
α-Chymotrypsin	2.0	2	4	[d]
Ovomucoid	5.3		5	[e]
Ribonuclease	3.0	3	6	[f]
Ovalbumin	1.5	2?	9–10	[g]
Trypsin	6.7	6	10	[a]
Liver alcohol dehydrogenase	6.1		10	[h]
Hemoglobin	7.6	8	12	[i]
Iron conalbumin	5.0	5	18	[i]
Carboxypeptidase A	5.9	7	19	[j]
Bovine serum albumin	4.0		20	[k]
Rabbit muscle aldolase	10.0	11–13	40–42	[l]

[a] The sources of the proteins are given under Methods and Materials. Acetylations were carried out as in Table I except for α-chymotrypsin and ribonuclease, where a 180-fold molar excess of acetylimidazole was employed. Tyrosyl acetylation was determined from the decrease in molar absorptivity using a factor of 1160 per tyrosyl residue per mole, as described in the text. [b] Hermans (1962). [c] Crammer and Neuberger (1943). [d] Havsteen and Hess (1962). [e] Stevens and Feeney (1963). [f] Shugar (1952); Tanford *et al.* (1955a). [g] Inada *et al.* (1964). [h] T. K. Li, unpublished data. [i] Wishnia *et al.* (1961). [j] Simpson *et al.* (1963). [k] Tanford *et al.* (1955b). [l] Hass and Lewis (1963).

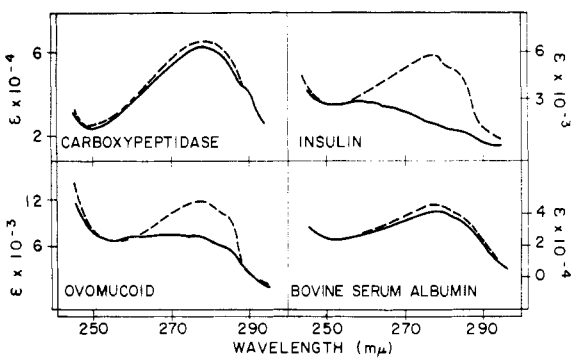

FIGURE 3: Acetylation of tyrosyl residues in proteins with acetylimidazole. Acetylations were carried out as in Table I. Spectra of the native (---------) and acetylated (———) proteins were determined after removal of excess acetylimidazole by gel filtration.

TABLE VI: Effect of Denaturation on the Reactivity of Tyrosyl Residues of Proteins with Acetylimidazole.[a]

Protein	*O*-Acetyltyrosyl Residues (mole/mole)	Total Tyrosyl Residues (mole/mole)
Insulin in 8 M urea	4	4
α-Chymotrypsin in 8 M urea	4	4
Carboxypeptidase A in 8 M urea	19	19
Reduced and alkylated ribonuclease	6	6

[a] Conditions for acetylation were as in Table I, except for the presence of 8 M urea, as indicated. Tyrosyl acetylation was determined as in Table V.

number of acetylimidazole-reactive tyrosines (column 1), when compared with the number of "free" tyrosines measured spectrophotometrically by *p*H titration (column 2), coincides closely.

In order to explore the effect of secondary and tertiary structure on acetylation of tyrosyl residues insulin, as a control, α-chymotrypsin, and carboxypeptidase were acetylated in 8 M urea, and ribonuclease was reduced, alkylated, and then acetylated. Denaturation renders *all* of the phenoxy groups of these proteins susceptible to acetylation (Table VI).

The chemical characteristics and concentration of the buffer ion can be a significant variable in the acetylation reaction with acetylimidazole. Thus, the presence of 0.05 M Tris buffer significantly reduces the number of tyrosyl residues of carboxypeptidase which are acetylated when compared with the results observed in 0.01 M Tris, borate, or Veronal (Table VII). In the presence of 0.05 M Tris only 2.0 tyrosyl residues are acetylated, while in 0.01 M Tris and the other buffers the number of *O*-acetyltyrosyl residues is close to 6. In 0.05 M Tris,

TABLE VII: Effect of Different Buffer Ions on the Acetylation of Tyrosyl Residues of Carboxypeptidase A with Acetylimidazole.[a]

Buffer	Molarity	*O*-Acetyltyrosyl Residues (mole/mole)
Sodium barbital	0.02	5.7
Sodium borate	0.05	6.2
Tris chloride	0.01	6.1
Tris chloride	0.05	2.0

[a] Except for the buffers, acetylations were carried out as in Table I. Tyrosyl acetylation was determined as in Table V.

peptidase activity may be as high as 50% of the native enzyme, indicating incomplete acetylation of the active-center tyrosyl residues, since with the other buffers cited in Table VII it decreases to less than 5% (see also Bender *et al.*, 1965). Moreover, the decreased peptidase and increased esterase of fully acetylated carboxypeptidase return toward those of the native enzyme on storage or dialysis of the acetyl enzyme in 0.05 M Tris buffer. Such data show that Tris deacylates *O*-acetyltyrosine residues as a function of time. This process may also account for the incomplete acetylation when performed in 0.05 M Tris (Table VII), though Tris ions may additionally have a protective effect against acetylation. Clearly, high concentrations of nucleophilic buffer ions in the ambient environment employed for acetylation should be avoided (see also Dayan and Wilson, 1964; Caplow and Jencks, 1962).

Discussion

These studies on a number of amino acid polymers and proteins were undertaken to investigate the specificity of acetylimidazole toward reactive groups of proteins. The reaction is milder and more selective than that with the widely employed acid anhydrides. Thus, acetic anhydride, e.g., has been shown to denature carboxypeptidase partially (Bethune *et al.*, 1964) and also other proteins (Lee *et al.*, 1963). Further, while acetic anhydride acetylates a substantial fraction of the NH_2 groups of carboxypeptidase, no *N*-acetylation by acetylimidazole was detected at all under the conditions employed.[2] This difference in the specificity of these two agents in regard to the *N*-acetylation of carboxypeptidase appears to represent an extreme. Both in synthetic polypeptides and proteins, acetylimidazole acetylates the ε-amino groups of lysine to a far smaller extent than does acetic anhydride, but the difference is not qualitative (Table I).

Since our studies were limited to carboxypeptidase, which does not contain free sulfhydryl groups, the reactivity of thiol groups with acetylimidazole was not previously studied (Vallee, 1964). Both acetylimidazole and acetic anhydride acetylate the thiol group of glutathione and those of thiolated gelatin and proteins, though not all of the thiol groups of proteins necessarily react. In fact, these reagents do not acetylate any of the thiol groups of native horse liver alcohol dehydrogenase though these are acetylated partially after denaturation of the protein with a detergent (Table III). "Sluggish" reactivity of "unavailable" or "masked" protein thiol groups has long been recognized and has been attributed to steric hindrance by the secondary or tertiary structure of the protein (Mirsky and Pauling, 1936; Neurath *et al.*, 1944; Boyer, 1959), though other explanations also have been offered (Boyer, 1959).

No significant acetylation of the aliphatic hydroxyl groups of either synthetic polypeptides or proteins was found either with acetylimidazole or acetic anhydride at neutral *p*H (Table II). Earlier studies have demonstrated that acetic anhydride acetylates about 10% of the aliphatic hydroxyl groups of trypsin and serum albumin, but the molar ratios of anhydride to protein in those studies were much higher than those employed here (Uraki *et al.*, 1957). Thus, while acetylimidazole completely acetylates the tyrosine residues of synthetic polymers, its reactivity is not limited to its interaction with these groups. Rather, the reactivity of acetylimidazole is typical of high-energy acyl compounds as is readily apparent from studies with model compounds (Jencks and Carriuolo, 1958). As with other reagents, the degree of acetylation of the various reactive groups of proteins is determined by the reaction specificity of acetylimidazole, by the chemical environment of the side chains, and, further, by the composition of the reaction mixture, as exemplified by the effect of Tris ions (Table VII).

Nor are such considerations limited to the proper use of acetylimidazole; they also apply to the mode of action of acetic anhydride. Lack of appreciation of these circumstances has already led to complications in the interpretation of data obtained on acetylation of carboxypeptidase with acetic anhydride in the presence of half-saturated sodium acetate. Under these conditions, tyrosyl residues, including those involved in activity, are *not* acetylated, accounting for the failure of Ando and Fujioka (1962) to notice enzymatic changes on acetylation of the enzyme. The effect of sodium acetate in the acetylating mixture is quite analogous to that of Tris. Acetate, in high concentrations, can catalyze the hydrolysis of phenolic esters (Bender and Neveu, 1958) and, under the appropriate conditions, this fortuitous circumstance permits the utilization of acetic anhydride as a reagent specific for amino groups (Fraenkel-Conrat, 1957).

[2] By increasing the concentrations of acetylimidazole substantially, some acetylation of amino groups of carboxypeptidase can be brought about without, however, further affecting the *O*-acetylation of tyrosyl residues.

Dependence of the reactivity of tyrosyl residues on protein conformation is clearly apparent from the present studies. While all tyrosyl residues were acetylated readily in random synthetic copolymers (Table IV), their reactivity in proteins and the single "block" copolymer here examined appears to be limited to those which are "free" and therefore readily accessible to the reagent (Tables IV and V). Since the initial observations by Crammer and Neuberger (1943), spectrophotometric titrations have revealed at least two classes of tyrosyl residues in many proteins (Beaven and Holiday, 1952; Wetlaufer, 1962). Those with pK values between 9.5 and 10.5 have been termed "free," and are thought to be exposed at the surface of the protein molecule in direct contact with the ambient medium and therefore readily accessible both to OH^- and (as shown here) to acetylation. Those tyrosyls ionizing above pH 10.5 have been called "buried," and are considered to be either within the interior of the protein, or interacting with other residues in some manner and therefore relatively inaccessible to OH^- (Edsall, 1963), hence unreactive to acetylation. Following the destruction of protein conformation by denaturation, all of the tyrosines titrate with the same pK. Operationally similar "buried" and "free" tyrosines have also been identified in tyrosine-containing block copolymers (Pesce *et al.*, 1964).

The present studies with acetylimidazole at pH 7.5 indicate a very close correlation between the number of readily acetylated tyrosine groups of a given protein, as measured by the characteristic change in the spectrum, to those which dissociate with a pK near 9.5. The acetylation reaction is carried out under conditions where most proteins are stable, is reversible by hydroxylamine, and is quite simple and less time consuming than spectrophotometric titrations, while giving comparable results. The reaction apparently constitutes a suitable procedure to differentiate "free" from "buried" tyrosyl groups in proteins. The disrupting effect of acetic anhydride on protein conformation obviated analogous studies with this reagent (Bethune *et al.*, 1964).

There are minor discrepancies between the values obtained by acetylation and spectrophotometric titration. Thus, six tyrosyl groups of carboxypeptidase react with acetylimidazole while seven titrate "normally." Similarly, the values observed for trypsin and aldolase with acetylimidazole do not coincide with the literature data obtained by titration. Perhaps certain tyrosyl residues are only partially buried in "crevices" or "cavities" near the protein surface. Considerations pertinent to the assignment of tyrosines to intermediate categories have already been discussed (Herskovits and Laskowski, 1962).

Clearly, the differentiation of tyrosyl residues into "normal" and "abnormal" on the basis of pH titration is operational and their classification as "free" and "buried" constitutes an interpretation, albeit supported strongly by the effects of denaturing agents which abolish the differences both in ionization of tyrosyls and of their reactivity toward acetylimidazole (Table VI). To what extent either titration data or reactivity toward acetylimidazole constitute indices of the degree of exposure of tyrosyl residues must ultimately be determined by a direct approach. The present studies help to discern, however, how closely the structure of the protein in solution is related to its crystalline structure as defined by X-ray diffraction.

The data on myoglobin are most revealing in this regard. Titration demonstrates pK values of 10.3, 11.5, and >12.8 for each of its three tyrosyl residues, respectively, the first thought to be "free," the last one "buried" (Hermans, 1962). Employing the terminology of Herskovits and Laskowski (1962), the group with an intermediate pK might be "partially buried." X-Ray data reveal that one tyrosyl residue is buried within the molecule, hydrogen bonded to a main-chain carbonyl residue (Kendrew, 1962). Two tyrosyl residues are exposed at the surface of the molecule; but one of these has a possible, weak interaction with the hydroxyl group of threonine, in all probability accounting for the group titrating with the intermediate pK of 11.5. By acetylation two residues are "buried" and one is "free." Hence, the precise designation and its structural meaning for each tyrosyl residue derives from and requires information obtained by all three analytical techniques, titration, chemical modification, and X-ray diffraction.

The arrangement of most residues other than tyrosyls in relation to the conformation of the molecule is gauged largely on differences in the rates of chemical modification of their functional groups. Sufficient data for comparison between pH titration and chemical modifications analogous to those here reported are therefore not available for these other groups.

It is pertinent to point out that two tyrosyl residues are involved in the mechanism of action of carboxypeptidase since an increase in esterase and abolition of peptidase activity correlate with the *O*-acetylation of two tyrosyl residues (Simpson *et al.*, 1963; Riordan and Vallee, 1963, 1964). In another proteolytic enzyme, pepsin, tyrosyl residues also have been thought to be functional (Herriott, 1935), though this protein was not examined here because of its sensitivity to the experimental conditions employed. Two other proteolytic enzymes, trypsin and chymotrypsin, were investigated, however. In both, several tyrosyl residues were acetylated at pH 7.5 (Table V), though changes in enzymatic activity were not detected. These experiments suggest that the "free" tyrosines of these enzymes are not required for activity, and under these conditions stable *O*-acetylserine is not formed at the active sites of these enzymes. However, acetylation of trypsin in 8 M urea with acetylimidazole indicates the participation of *buried* tyrosyls in the maintenance of the active site (J. F. Riordan, W. E. C. Wacker, and B. L. Vallee, in preparation).

The availability of site-specific, selective reagents has resulted in the discovery that the reactive group of specific amino acid residues is involved in the mechanism of enzyme action. Thus the abolition or modification of enzymatic function, subsequent to specific

chemical modification of the aliphatic hydroxyl group of serine, the sulfhydryl group of cysteine, the imidazolyl group of histidine, and the ε-amino group of lysine, has been cardinal in the identification of the catalytic role of these functional groups in various classes of enzymes. The existence of an appropriate reagent, selective for a specific group, is prerequisite for the recognition of the functional importance of that residue, of course. The relative specificity of acetylimidazole for exposed tyrosyl groups, the mildness of the reaction conditions, and the simplicity of the analytical procedure all constitute attributes demanded of a site-specific, selective reagent.

Both the reactivity of tyrosyl residues, dependent upon protein conformation and structure, and the identification of their participation in the mechanism of carboxypeptidase action are good indices that the specificity of acetylimidazole should permit an evaluation of the hypothesis that a class of "tyrosyl enzymes" is yet to be identified.

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