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The Reversible Reaction of Protein Amino Groups with *exo-cis*-3,6-Endoxo-Δ^4-tetrahydrophthalic anhydride

THE REACTION WITH LYSOZYME

BY M. RILEY AND R. N. PERHAM
Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, U.K.

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1. The reaction of *exo-cis*-3,6-endoxo-Δ^4-tetrahydrophthalic anhydride with amino groups of model compounds and lysozyme is described. 2. Reaction with the ϵ-amino group of N^{α}-acetyl-L-lysine amide gives rise to two diastereoisomeric products; at acid pH the free amino group is liberated with anchimeric assistance by the neighbouring protonated carboxyl group with a half-time of 4–5h at pH 3.0 and 25°C. 3. The amino groups of lysozyme can be completely blocked, with total loss of enzymic activity. Dialysis at pH 3.0 results in complete recovery of the native primary and tertiary structure of lysozyme and complete return of catalytic activity. 4. The specificity of reaction of this and other anhydrides with amino groups in proteins is discussed.

It has been recognized for some time that amide linkages situated in close proximity to a carboxyl group can be hydrolysed by a mechanism that involves intramolecular catalysis by that carboxyl group (Leach & Lindley, 1953). The most favourable situation for such catalysis is that in which the amide and carboxyl functions are eclipsed and incapable of free rotation (for review see Kirby & Lancaster, 1970), and in recent years this principle has been utilized as a means of reversibly blocking protein amino groups. Thus maleic anhydride (Butler, Harris, Hartley & Leberman, 1967, 1969), 2-methylmaleic (citraconic) anhydride and dimethylmaleic anhydride (Dixon & Perham, 1968) have been shown to react with protein amino groups to give the corresponding dicarboxylic acid monoamides in which the protonated species of the favourably situated carboxyl group anchimerically assists the hydrolysis of the amide bond. Hence the maleyl, citroconyl and dimethylmaleyl groups, though stable at neutral and alkaline pH, may be removed at acid pH.

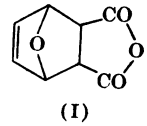
These anhydrides have a great many potential uses, e.g. in sequence work, as a method of inhibiting tryptic cleavage at lysine residues, as a means of purifying lysine-containing peptides by diagonal electrophoretic techniques (Perham & Jones, 1967; Perham, 1967) and in effecting the disaggregation of multimeric proteins (Gibbons & Perham, 1970). To accommodate these adequately it would be desirable to have an extension to this range of anhydrides, since the conditions necessary for the removal of maleyl groups may be too severe for some purposes, whereas dimethylmaleyl (and possibly citraconyl) groups may be too labile for others. Further, these three reagents possess activated olefinic bonds and the irreversible nucleophilic addition of protein thiol groups is a distinct possibility, especially in proteins that possess reactive cysteine residues. For these reasons we looked for an anhydride that would give derivatives more labile than maleyl groups but less labile than citraconyl groups, and preferably an anhydride with no activated olefinic bonds.

It is known that mono-*p*-methoxyphenyl esters of ETPC* are approximately five times as labile as those of maleic acid (Bruice & Pandit, 1960*a*,*b*) and it was therefore expected that ETPA (I) would possess the properties we sought. The present paper describes the reaction of this reagent with model compounds and with lysozyme (EC 3.2.1.17).

MATERIALS AND METHODS

Reagents and enzymes. Maleic anhydride was purchased from Emanuel Ltd., Alperton, Middx., U.K., and purified

* Abbreviations: ETPA, *exo-cis*-3,6-endoxo-Δ^4-tetrahydrophthalic anhydride: ETPC, *exo-cis*-3,6-endoxo-Δ^4-tetrahydrophthalic acid; ETP, *exo-cis*-3,6-endoxo-Δ^4-tetrahydrophthaloyl.



(I)

further by recrystallization from chloroform. Furan was obtained from BDH (Chemicals) Ltd., Poole, Dorset, U.K., and was purified by distillation immediately before use. 2,4,6-Trinitrobenzenesulphonic acid was from Eastman Kodak Ltd., Kirkby, Lancs., U.K.; solutions, made up in glass-distilled water, were stored in the dark at 4°C and were discarded after 24h. Guanidine hydrochloride was prepared from guanidine carbonate as described by Nozaki & Tanford (1967).

N^{α}-Acetyl-L-lysine amide acetate (lot S-1614) was purchased from Cyclo Chemical Corp., Los Angeles, Calif., U.S.A. Paper electrophoresis at pH6.5 gave a major spot with mobility +0.86 relative to aspartic acid (Offord, 1966) corresponding to the desired compound, together with a trace impurity neutral at pH6.5. It was used without further purification and was determined by hydrolysis and amino acid analysis.

ETPA was synthesized from maleic anhydride and furan as described by Diels & Alder (1929) and recrystallized once from chloroform, m.p. (uncorr.) 115–116°C (decomp.). Its structure and purity were confirmed by i.r. and n.m.r. spectroscopy. The product of this Diels–Alder reaction is now known to be the *exo-cis* isomer rather than the *endo-cis* isomer (Woodward & Baer, 1948).

Lysozyme (hen's-egg white, thrice recrystallized, lot no. 582) was purchased from Seravac Laboratories Ltd., Maidenhead, Berks., U. K. Trypsin (twice recrystallized) was from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Cellulose acetate electrophoresis. Electrophoresis of proteins was carried out on Gelman Sepraphore III strips in 40mM-sodium barbitone buffer, pH8.5, at 12V/cm for 75min. Protein was stained with 0.25% (w/v) Ponceau S in 3% (w/v) trichloroacetic acid–3% (w/v) sulphosalicylic acid.

High-voltage paper electrophoresis. Paper electrophoresis was carried out as described by Perham (1967). Amino acids and their ETP derivatives were detected on paper by staining with ninhydrin–cadmium acetate (Heilmann, Barrollier & Watzke, 1957) or by the chlorine test of Rydon & Smith (1952). ETP derivatives could also be detected by spraying the paper with 1% (w/v) $KMnO_4$, when they appeared as yellow spots on a pink background. Arginine and its derivatives were specifically stained by means of the Sakaguchi reaction (Jepson & Smith, 1953).

The electrophoretic mobilities of amino acids and their derivatives were measured relative to aspartic acid (defined as −1.0) (Offord, 1966). At pH6.5 the position of neutral amino acids was taken as the zero; at pH8.9 mobilities were measured from the origin.

Amino acid analysis. Samples of protein were hydrolysed with 6M-HCl in sealed evacuated tubes at 105°C for 24h and the HCl was removed *in vacuo* over NaOH pellets. Amino acid analysis was carried out on a Beckman model 120C automatic analyser. The values for serine and threonine were corrected by 6 and 3% respectively for losses during hydrolysis (Perham, 1967). No corrections were made for incomplete release of valine and isoleucine.

Assay of protein amino groups. Free amino groups in lysozyme and its derivatives were assayed with 2,4,6-trinitrobenzenesulphonic acid by the method of Habeeb (1966). Before the assays the protein solutions were dialysed against 0.02M-sodium borate buffer, pH8.6, to remove NH_4HCO_3, which interferes with this assay. Assuming complete reaction with the amino groups of unmodified lysozyme, a value of 1.24×10^4 for the molar extinction coefficient of the trinitrophenylamino group at 340nm was determined in trial experiments and used throughout.

Reduction and carboxymethylation of proteins. A 15mg portion of protein was dissolved in 1ml of 0.2M-tris–HCl buffer, pH8.4, containing 7M-guanidine hydrochloride. Then 0.1ml of dithiothreitol (65mg/ml) was added, and the tube was flushed with N_2 and left at room temperature (20°C) for 2h. Then 0.1ml of sodium iodoacetate (235mg/ml, neutralized with M-NaOH) was added, and the tube was flushed again with N_2 and left in the dark for 30min at 20°C. The alkylation was stopped by adding 0.5ml of 2-mercaptoethanol and the protein was dialysed against several changes of 0.5% (w/v) NH_4HCO_3.

Preparation of peptide 'maps'. Reduced and carboxymethylated proteins were digested with 1% (w/v) trypsin in 0.5% (w/v) NH_4HCO_3, pH8.0, at 37°C for 2h. The buffer was then removed by freeze-drying. Peptide 'maps' were prepared as described by Gibbons & Perham (1970) and stained with ninhydrin–cadmium acetate (Heilmann *et al.* 1957).

Reaction of ETPA with amino acids. Solutions of ϵ-DNP-L-lysine hydrochloride, L-arginine hydrochloride, glycine, and N^{α}-acetyl-L-lysine amide acetate in water (10–20mM) were adjusted to pH9.0 and allowed to react with ETPA at 20°C, with stirring, the pH being maintained at 8–9 by addition of 0.2M-NaOH. The anhydride was added in several portions; 1–2equiv. was generally required for complete reaction as judged by paper electrophoresis at pH6.5 and pH8.9.

Kinetics of hydrolysis of ETP-acetyl-lysine amide. A solution of N^{α}-acetyl-lysine amide (12mM) in water was allowed to react quantitatively with ETPA as described above and was then diluted with water to a final concentration of 5.6mM. Hydrolysis was initiated by mixing 1.5ml of this solution with 3.5ml of the appropriate buffer solution and incubating at 25°C. The buffers used were: sodium oxalate, 0.15M with respect to oxalate (pH2.0); sodium citrate, 0.15M with respect to citrate (pH3.0, pH4.0 and pH5.0); sodium phosphate, 0.15M with respect to phosphate (pH6.0). After various time-intervals 0.3ml samples of the hydrolysis mixture were removed and hydrolysis was terminated by addition of 4.7ml of 0.15M-sodium borate buffer, pH8.6. Free amino groups were then assayed by adding 1.0ml of 2,4,6-trinitrobenzenesulphonic acid (1.5mg/ml in glass-distilled water), incubating at 37°C for 75min and reading the extinction at 335nm against a blank made up with 0.3ml of water. At pH2.0, pH3.0 and pH4.0, the extinction at 'infinite time' was obtained by direct observation, but at pH5.0 and pH6.0 where the rate of hydrolysis is very low it was obtained by extrapolation (Freedman & Radda, 1968) by using the relation:

$$\Delta E^{\infty} = \frac{\Delta E_b^2 - \Delta E_a \cdot \Delta E_c}{2\Delta E_b - (\Delta E_a + \Delta E_c)}$$

where ΔE_a, ΔE_b and ΔE_c are the extinctions at times t_a, t_b and t_c respectively and $t_b - t_a = t_c - t_b$. The results were plotted in accordance with the first-order rate law: $k_{obs.}\cdot t = 2.303[\log(\Delta E^{\infty}_{335} - \Delta E^{0}_{335}) - \log(\Delta E^{\infty}_{335} - E^{t}_{335})]$.

Assay of lysozyme and its derivatives. Solutions of

lysozyme or its derivatives in 0.5% (w/v) NH_4HCO_3 were diluted just before assay with 0.1M-sodium phosphate buffer, pH6.24, and their enzymic activities were determined by the *Micrococcus lysodeikticus* assay method of Shugar (1952).

Reaction of ETPA with lysozyme. Lysozyme dissolved in 0.2M-sodium borate buffer, pH8.8, at 12mg/ml was allowed to react at 2°C with a 12-fold molar excess of ETPA over protein amino groups. The ETPA was added in several lots with continuous stirring and the pH was maintained at 8.5–9 with M-NaOH. The reagent dissolved slowly during the reaction. Lysozyme is precipitated on the first addition of ETPA but redissolves within 5min. When the reaction was complete the modified protein was purified by dialysis against 0.5% (w/v) NH_4HCO_3, pH8, at 4°C.

For unblocking, ETP-lysozyme was dialysed against two changes of 0.1M-sodium citrate buffer, pH3.0, at 25°C for 30h. The protein was precipitated within 15min but gradually redissolved and was all back in solution after 23h. The sodium citrate was finally removed by dialysis against 0.5% (w/v) NH_4HCO_3 at 2°C.

RESULTS

Reaction of ETPA with amino acids. When ETPA was allowed to react with the two optically active amino acids, ε-DNP-L-lysine and L-arginine, and samples of the resulting reaction mixture were subjected to electrophoresis at pH6.5 and pH8.9, it was observed that each amino acid gave rise to two reaction products, which separated at both pH values, migrating towards the anode (Table 1). In each case the slower-moving component was present in slightly greater amount than the faster-moving one. However, on reaction of ETPA with glycine or with N^{α}-acetyl-L-lysine amide only a single product could be detected by paper electrophoresis at pH6.5 and 8.9. The mobilities of the products produced by reaction of ETPA with these model compounds support the assumption that ETPA acylated the free amino groups to give the corresponding dicarboxylic acid monoamides. The two products arising from reaction with ε-DNP-lysine and with arginine are presumably the two diastereoisomers that would be expected to result from reaction of ETPA with an optically active amino acid. Glycine, being optically inactive, can give rise to but a single product, and with N^{α}-acetyl-lysine amide it would appear that the two diastereoisomeric products are sufficiently similar to be unresolvable by paper electrophoresis.

Kinetics of hydrolysis of ETP-N^{α}-acetyl-L-lysine amide. As a model for the ε-amino groups of proteins we chose N^{α}-acetyl-L-lysine amide. In a preliminary experiment a solution of this compound that had quantitatively reacted with ETPA was adjusted to pH2 with M-hydrochloric acid. The pH was maintained at 2–3 by periodically adding M-hydrochloric acid, and samples were removed at various times and applied to paper for electrophoresis at pH6.5. Hydrolysis of the derivative was essentially complete after approx. 30h at pH2–3 and 20°C, indicating the half-life under these conditions to be about 4–6h. Finally, after about 48h at pH2–3 a sample corresponding to about 1μmol of unblocked N^{α}-acetyl-lysine amide was applied to paper as a 1cm band and subjected to electrophoresis at pH6.5. The only spot that could be detected was that corresponding to free N^{α}-acetyl-lysine amide, indicating that exposure of this derivative to acid pH results simply in intramolecular hydrolysis of the amide bond with complete regeneration of the free amino group.

Table 1. *Electrophoretic mobilities at* pH6.5 *of the products of reaction of ETPA with amino acids*

The yields of the products from ε-DNP-L-lysine were determined by elution with 0.1M-potassium phosphate buffer, pH6.0, and by reading the extinction at 360nm against a paper blank. The relative yields of those from L-arginine were judged (++ and +) by visual inspection.

Amino acid	No. of products	Mobility (pH6.5)	Yield (%)
ε-DNP-L-lysine	2	−0.50	57.6
		−0.73	42.4
L-Arginine	2	−0.27	++
		−0.54	+
Glycine	1	−1.20	
N^{α}-Acetyl-L-lysine	1	−0.50	

The accurate determination of the rate of hydrolysis of ETP-N^{α}-acetyl-lysine amide as a function of pH was performed at 25°C as described above. At all pH values plots of log $(\Delta E^{\infty}_{335} - \Delta E^{t}_{335})$ versus time were linear, indicating that the kinetics of hydrolysis were first-order. The experimentally determined half-times ($t_{\frac{1}{2}}$) of hydrolysis are: pH2.0, 283min; pH3.0, 284min; pH4.0, 364min; pH5.0, 2350min; pH6, 21200min. A plot of log $k_{obs.}$ versus pH (Fig. 1) shows that the rate constants increase, below pH6.0, with a slope of −1.0, reaching a steady maximum at approx. pH4.0, indicating hydrolysis via intramolecular participation by the protonated species of the neighbouring carboxyl group. The kinetic pK (p$K_{app.}$) value determined from this plot is 4.13, which means that at pH3.0 and below the rate of intramolecular hydrolysis will be essentially maximal.

The results presented above had indicated that the two diastereoiosmeric products obtained from reaction of ETPA with N^{α}-acetyl-L-lysine amide were sufficiently similar as to be unresolvable by paper electrophoresis. The first-order kinetics of hydrolysis observed here indicate that the two

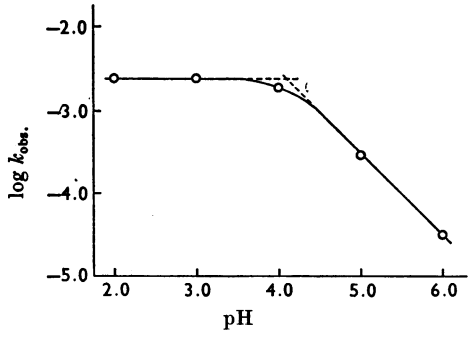


Fig. 1. First-order rate constants ($k_{obs.}$) for the hydrolysis of ETP-N$^{\alpha}$-acetyl-L-lysine amide at various pH values.

diastereoisomers are also kinetically indistinguishable.

Reaction of lysozyme with ETPA and unblocking. To test whether ETPA could be used for reversibly blocking protein amino groups, we chose lysozyme as a model. The reasons for this choice were based on several considerations: it is a reasonably small monomeric protein, the primary (Canfield, 1963; Canfield & Liu, 1965; Jollès, Jauregui-Adell, Bernier & Jollès, 1963) and tertiary (Blake, Mair, North, Phillips & Sarma, 1967) structures of which have been well studied; the six ϵ-amino and the single α-amino groups are well-exposed and react rapidly and quantitatively with 2,4,6-trinitrobenzenesulphonic acid (Freedman & Radda, 1968) and should therefore also react smoothly with ETPA; it is a very stable protein that will regenerate its native conformation even after complete reduction and denaturation (Isemura, Takagi, Maeda & Imai, 1961), and so should be well able to tolerate the conditions used for removing the blocking groups.

In a preliminary experiment it was found that a fivefold excess of ETPA over protein amino groups was sufficient to block more than 90% of the amino groups, as judged by assay with 2,4,6-trinitrobenzenesulphonic acid. In all subsequent experiments a 12-fold excess of reagent was used to bring about complete blocking. In the early stages of reaction with ETPA the protein is precipitated, presumably as the isoelectric point is lowered by the introduction of negatively charged groups, but it redissolves as reaction proceeds. To remove the ETP groups, the fully blocked protein was dialysed at 25°C against two changes of 0.1M-sodium citrate buffer, pH 3.0, for 30h. The protein was precipitated during the first 30min of dialysis at pH 3.0, but was all back in solution again after about 23h. Finally it was dialysed against 0.5% (w/v) ammonium hydrogen carbonate, pH 8.0, at 4°C. Dialysis at pH 3.0 for 30h was sufficient to regenerate more than 95% of the amino groups, as judged by assay with 2,4,6-trinitrobenzenesulphonic acid, consistent with the half-time of hydrolysis of 4–5h found for model compounds.

Properties of blocked, unblocked and native lysozyme. To check whether any irreversible reactions had taken place either during reaction with ETPA or during the unblocking procedure, samples of fully blocked, unblocked and native lysozyme were reduced and carboxymethylated and then subjected to amino acid analysis after hydrolysis with 6M-hydrochloric acid. Under these conditions, any amide or ester bonds of ETPC would be completely hydrolysed. Therefore, in the absence of any peculiar side-reactions, the amino acid compositions of all three samples should be identical. This, indeed, was found to be the case within experimental error (±3%). A more sensitive criterion for regeneration of primary structure is a comparison of tryptic peptide 'maps'. Samples of native and unblocked lysozyme were reduced and carboxymethylated, and the tryptic peptide 'maps' were compared. No differences could be seen between the 'maps' of native and unblocked samples, indicating that, within the limits of this system, the primary structure had been completely regenerated.

A possible side-reaction of anhydrides (see the Discussion section) is acylation of protein hydroxyl groups, in particular those of serine and threonine residues. The identity of the tryptic peptide 'maps' indicates that if such reactions had occurred they were completely reversed under the conditions used for unblocking. However, this still leaves unanswered the question whether in the modified protein reaction had been confined entirely to protein amino groups. Acylation of protein hydroxyl groups would give rise to minor components of increased acidity that should be detectable by electrophoresis on cellulose acetate strips. Samples of fully blocked, unblocked and native lysozyme were therefore submitted to electrophoresis on strips of cellulose acetate in 40mM-sodium barbitone buffer, pH 8.5, for 75min and the strips were then stained with Ponceau S. Native and unblocked lysozyme both gave single bands migrating identically towards the anode; the blocked derivative gave a somewhat smeared band migrating towards the cathode. This experiment confirmed very nicely that all the ETP groups introduced during the conditions used for quantitative blocking of amino groups were removed by dialysis at pH 3.0 and 25°C for 30h. However, there remained the possibility that the smeared band given by the blocked derivative was due to the presence of minor components arising from acylation of protein hydroxyl groups. Accordingly, samples of the three proteins were treated with M-hydroxylamine in 0.2M-*N*-ethylmorpholine–acetic acid buffer, pH 8.2, at 25°C for 2h, and the proteins were purified by exhaustive dialysis

against 0.5% (w/v) ammonium hydrogen carbonate, pH 8.0, and freeze-dried. Treatment with hydroxylamine under these conditions is sufficient to destroy the comparable maleyl ester linkages in tobacco-mosaic-virus protein (L. King & R. N. Perham, unpublished work), while leaving maleylamino bonds intact. However, electrophoresis of the various lysozymes after hydroxylamine treatment gave exactly the same pattern as before, with the blocked derivative still producing a somewhat smeared band migrating towards the cathode. On this basis it would appear unlikely that the smearing of the blocked protein is due to the presence of ETP ester components, although the possibility cannot be rigorously excluded. What emerges very clearly from these experiments is that if such side reactions occur they are completely reversible under the deblocking conditions.

Samples of the lysozyme solutions that had been dialysed against 0.5% (w/v) ammonium hydrogen carbonate, pH 8.0, were diluted with 0.1M-sodium phosphate buffer, pH 6.24, and assayed immediately by the method of Shugar (1952). Since at pH 6.24 the half-time of hydrolysis of ETP-amino bands is in excess of 14 days it can be safely assumed that, during the time required for dilution and assay, unblocking did not occur to any significant extent. The relative specific activities of the three proteins were: 0.0 (blocked); 1.07 (unblocked); 1.0 (native).

DISCUSSION

The acylation of an amino group by ETPA, with resulting fission of the anhydride ring, is accompanied by the generation of a new asymmetric centre. If the molecule containing the amino group itself contains a centre of asymmetry one expects to obtain two diastereoisomeric products. Since the products are diastereoisomeric, the transition states leading to them will also be diastereoisomeric and therefore different in free energy, and hence they should be differentiated kinetically. In general, the difference in free energy between diastereoisomeric transition states, and the resulting preferential synthesis of one diastereoisomeric product over the other is greatest when the two asymmetric centres are close together (for review see Eliel, 1962). The two products obtained in unequal yield from reaction of ETPA with the α-amino group of ϵ-DNP-L-lysine and L-arginine are therefore presumably diastereoisomers. An entirely analogous situation is seen in the reaction of β-phenylglutaric anhydride with L-α-phenethylamine to give two diastereoisomeric monoamides in yields of 60% and 40% (Schwartz & Carter, 1954). In support of this conclusion it is found that reaction of ETPA with the α-amino group of glycine, a molecule with no centre of asymmetry, gives only a single product.

The observation that the reaction of ETPA with the ϵ-amino group of N^{α}-acetyl-L-lysine amide gives rise apparently to only one product, as judged by paper electrophoresis, indicates that in this case the two diastereoisomeric products are physically very similar if not identical. Further, the simple linear plots obtained by assuming first-order kinetics of hydrolysis show that the two diastereoisomeric products are also kinetically indistinguishable. This is entirely as expected since the two asymmetric centres are well separated and little or no asymmetric induction should occur. From the point of view of protein chemistry, therefore, the complication of ETPA reacting with ϵ-amino groups to give two products that hydrolyse at different rates does not arise. One possible minor complication is that the two products produced by reaction with the α-amino group of a protein may have half-lives of hydrolysis different from those arising from reaction with ϵ-amino groups. However, one would expect this difference to be slight and not a major drawback.

The pH-dependence of the hydrolysis of the ETP derivative of N^{α}-acetyl-lysine amide clearly indicates that the protonated form of the carboxyl group is the active species in catalysis. The kinetically determined pK is 4.13 and the rate of hydrolysis should therefore be maximal below pH 3.0. The observation that essentially all the amino groups of lysozyme are regenerated after approx. 30h dialysis at pH 3.0 and 25°C is consistent with the assumption that the ETP groups are released under these conditions with a half-life of 4–5h, in agreement with the data for model compounds, indicating that the insolubility of the modified protein does not significantly affect the rate of hydrolysis.

ETP-amino groups are, then, a good deal more labile than maleylamino groups, which have a half-life of 11h at pH 3.5 and 37°C (Butler *et al.* 1969), conditions that might conceivably cause hydrolysis of some labile bonds in proteins. On the other hand, they are significantly more stable than citraconyl groups. Thus the two isomeric products obtained by reaction of citraconic anhydride with the α-amino group of arginine hydrolyse at pH 2.0 and 20°C with half-lives of 1–2h (Dixon & Perham, 1968). Of more relevance, however, is the situation that obtains with ϵ-amino groups, and it seems that in this case citraconyl groups are somewhat more labile. For example, it has been shown (I. Gibbons, unpublished work) that the isomeric products resulting from the reaction of citraconic anhydride with the ϵ-amino group of N^{α}-acetyl-L-lysine amide both hydrolyse with a half-life of approx. 20min at pH 2.0 and 20°C. Above this pH the two isomers diverge considerably in their rates of hydrolysis. Presumably at pH 2.0 both forms are completely protonated and the rate constants become identical. Thus the stability of the ETP-amino group falls

conveniently between those of the maleylamino and citraconylamino groups, which may have some usefulness in the chemical modification of peptides and proteins.

The occurrence of reaction at residues other than lysine in the use of these various anhydrides calls for some discussion. One possible side-reaction is acylation of the hydroxyl groups of serine, threonine and tyrosine residues. Substantial acylation of serine and threonine residues has been observed during maleylation of tobacco-mosaic-virus protein (L. King & R. N. Perham, unpublished work) and of immunoglobulins (Freedman, Grossberg & Pressman, 1968). Thiol esters could also be produced, but would presumably break down spontaneously at the pH of the reaction. The work of Thanassi & Bruice (1966) on the hydrolysis of monohydrogen phthalate esters has shown that the mechanism of hydrolysis and the values of the associated rate constants are dependent on the pK_a of the conjugate acid of the leaving group. If this pK_a is greater than approx. 13.5 the protonated carboxyl group is the active catalytic species; if it is less than approx. 13.5 the unprotonated carboxyl group is involved in catalysis. Although the mechanisms of hydrolysis of monoesters of ETPC, maleic acid and citraconic acid have not been investigated as fully, the available evidence (Bruice & Pandit, 1960*a*,*b*; Pekkarinen, 1954) indicates that for these reagents also the same general phenomenon is true. Now the pK_a of the hydroxyl group of *N*-acetylserine amide is 13.6 (Bruice, Fife, Bruno & Brandon, 1962) and it is found that the rate constant for the hydrolysis of *O*-phthaloyl-*N*-acetylserine amide is independent of pH between pH 2.0 and pH 4.3, indicating the expected participation by both the protonated and the unprotonated carboxyl groups (Thanassi & Bruice, 1966). Finally, it should be noted that the first-order rate constant for the hydrolysis of phenyl hydrogen phthalate is 10^4–10^6 times that for the hydrolysis of methyl hydrogen phthalate but the rate constant for the hydrolysis of phthalamic acid is about the same as that for methyl hydrogen phthalate hydrolysis (Bender, Chow & Chloupek, 1958; Thanassi & Bruice, 1966). The implications of this with respect to the specificity of reaction of these various anhydrides with protein amino groups are therefore as follows. Any tyrosine esters that formed would be unstable and would break down rapidly at neutral and alkaline pH. Any serine or threonine esters that were produced would certainly be labile at acid pH and would be completely cleaved under the conditions used to liberate the free amino groups, as found in the experiments reported above. They would also possibly be labile at alkaline pH, although the rate of hydrolysis would be very much lower (several thousand-fold) than the corresponding tyrosine esters. Therefore to ensure that reaction is confined entirely to amino groups the minimum quantity of anhydride that is required to block quantitatively the amino groups should be used and the pH maintained during reaction between 8 and 9, when the amino group is a very much better nucleophile than is the hydroxyl group; once reaction is complete the modified protein should be dialysed extensively at alkaline pH to destroy any ester bonds that may have formed. With these conditions for the reaction of ETPA with lysozyme it is found that reaction is confined almost entirely, if not exclusively, to the amino groups. Finally, it may be noted that a short treatment with hydroxylamine at alkaline pH can also be used selectively to cleave labile ester bonds as a convenient alternative to prolonged dialysis at alkaline pH.

Maleic anhydride, citraconic anhydride and dimethylmaleic anhydride (and the corresponding acids) all possess activated olefinic bonds, and nucleophilic addition to protein thiol groups could conceivably occur when using these reagents. It is known that maleic acid can alkylate simple thiol compounds, e.g. cysteine and GSH, under mild conditions (Morgan & Friedmann, 1938), and it has been found (Gibbons & Perham, 1970) that reaction of rabbit muscle aldolase with an amount of citraconic anhydride sufficient to block quantitatively the amino groups gives rise to significant loss of the thiol groups in this protein. For the purposes of 'diagonal' electrophoretic techniques these difficulties can be circumvented by prior carboxymethylation of the protein thiol residues. For enzymological studies, however, this cannot be resorted to and the use of these anhydrides might be restricted. In the experiments reported in this paper, the question of alkylation of thiol residues did not arise since lysozyme contains only four disulphide bridges. However, the double bond in ETPA is not activated by conjugation with carbonyl groups and addition to thiol residues is very unlikely. Further, with the corresponding saturated derivative of ETPA, *exo-cis*-3,6-endoxo-hexahydrophthalic anhydride, the possibility of alkylation of thiol groups does not exist, and preliminary experiments (M. Riley, unpublished work) indicate that the reaction of this compound with amino groups and the kinetics of hydrolysis of the resulting amides are indistinguishable from the results with ETPA.

Although modification of the amino groups of lysozyme with ETPA causes complete loss of activity, exposure of the blocked lysozyme to the unblocking conditions is sufficient to regenerate full enzymic activity, so that not only the primary structure but also the tertiary structure is fully regained after reaction and unblocking. It is known that modification of the amino groups of lysozyme with *O*-methylisourea (Parsons *et al.* 1969) and with

methyl picolinimidate (Benisek & Richards, 1968) does not result in total loss of activity and that, in contrast with earlier findings (Fraenkel-Conrat, 1950), acetylation of lysozyme amino groups causes loss of only 60% of the activity with a shift in the pH optimum of the enzyme to a lower pH (Yamasaki Hayashi & Funatsu, 1968). Further, fully acetylated lysozyme still retains full activity towards glycol-chitin as a substrate (Yamasaki *et al.* 1968). The complete loss of activity (at pH 6.24) in lysozyme that has fully reacted with ETPA may therefore be due to one of several causes: perhaps the enzyme is unfolded owing to repulsion between the introduced negative charges or perhaps only the pH optimum of the reaction catalysed is shifted. In any event one should be aware of the danger in being too dogmatic in interpreting the effect of chemical modification of particular residues on enzymic activity.

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