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# The Nature of Amino Acid Side Chains Which Are Critical for the Activity of Lysozyme*

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ABSTRACT: Chemical modification by use of selective reagents has been applied to the side chains of lysine, histidine, tyrosine, and carboxyl residues in lysozyme. The properties of the lysozyme derivatives have been monitored by measuring (a) their ability to bind chitotriose and (b) their catalytic efficiency for hydrolysis of chitotriose or *Micrococcus lysodeikticus* cells. Thus, effects of chemical modification on binding properties or catalytic properties have been distinguished. In this manner it has been shown that neither lysine, histidine, nor tyrosine residues play a role in catalysis by lysozyme. Modification of carboxyl groups, however, by various chemical treatments abolishes catalytic activity. A new reagent for preferential esterification of protein carboxyl groups has been employed which has made it possible to isolate two singly esterified lysozyme derivatives. One of these derivatives has been shown to contain a labile carboxyl ester which is most likely at or close to the strong binding site for chitotriose. The other derivative has been shown to contain a single carboxyl ester and to be essentially inactive against both *M. lysodeikticus* and chitotriose as substrates, while retaining the ability to effect good binding of the trisaccharide.

From knowledge of the three-dimensional structure of lysozyme at 2-Å resolution (Blake *et al.*, 1965, 1967 a,b; Phillips, 1967) and knowledge of the structures of lysozyme–inhibitor complexes (Johnson and Phillips, 1965; Blake *et al.*, 1967a) now available, it appears that we should for the first time be close to understanding in detail the mechanism of action of an enzyme. Based on details of a nonproductive lysozyme–chitotriose complex in the crystalline state, it has been suggested as a result of further model building (Blake *et al.*, 1967b; Phillips, 1967) that the amino acid side chains most likely to play an active role in catalysis by the enzyme are the $\beta$- and $\gamma$-carboxyl groups of aspartic acid residue 52 and glutamic acid residue 35 in the amino acid sequence.

Numerous attempts have been made to identify by chemical means the amino acid side chains which affect catalysis by lysozyme. The approaches used have been reviewed (Jollès, 1964, 1967). In summary it has been claimed that oxidation (Fraenkel-Conrat, 1950) or reduction (Churchisch, 1962; Imai *et al.*, 1963, Jollès *et al.*, 1964) of disulfide bonds, photooxidation of histidine and aromatic amino acids (Weil *et al.*, 1952), oxidation (Horinishi *et al.*, 1964), iodination (Hartdegen and Rupley, 1964, 1967), or ozonization of tryptophan (Previero *et al.*, 1966, 1967) residues, acetylation of amino groups (Fraenkel-Conrat, 1950; Geschwind and Li, 1957), and esterification with methanol–HCl of carboxyl groups (Fraenkel-Conrat, 1950) all can cause inactivation of the enzyme.

In this communication we present the results of chemical modification, by various treatments, of the side chains of a number of amino acid residues in lysozyme. We have sought methods which to the best of our knowledge do not seriously disrupt the secondary or tertiary structures of the enzyme. An important feature of the investigation was the determination of whether inactivation caused by chemical modification of lysozyme resulted from an effect on the binding properties or on the catalytic properties of the enzyme derivatives.

## Experimental Section

*Materials.* Lysozyme (Lot No. 96B-8572) was purchased from Sigma Chemical Co. Acetic anhydride, iodoacetic acid, and hydroxylamine hydrochloride were purchased from Eastman Kodak Chemical Co. Iodoacetamide and acetylimidazole were purchased from Mann Research Laboratories. *O*-Methylisourea was obtained from the Aldrich Chemical Co. Ninhydrin was a product of the Pierce Chemical Co. *N*-Acetyl-D-glucosamine was obtained from the California Corp. for Biochemical Research. Chitotriose was prepared from a partial acid hydrolysate of chitin by a gel filtration procedure

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used in this laboratory (Raftery *et al.*, 1968c). *Micrococcus lysodeikticus* cells were purchased from Miles Laboratories Inc.

Triethyloxonium fluoroborate was synthesised from boron trifluoride etherate and epichlorohydrin (both from Matheson Coleman and Bell Co.) as previously described (Meerwein, 1937) and the product was stored under ether at 0° with desiccation.

The synthesis of $^{14}C$-labeled triethyloxonium fluoroborate was accomplished by incorporation of [$^{14}C$]diethyl ether into the oxonium salt by exchange. All preparatory manipulations for the exchange were carried out at −80° under a nitrogen atmosphere. A solution of the oxonium salt (0.78 g) in 7.0 ml of methylene chloride containing 1.0 mCi of [1-$^{14}C$]diethyl ether (74 mg, from New England Nuclear Corp.) was sealed by torch in a heavy-walled glass ampoule. The ampoule was incubated 22 hr in refluxing methylene chloride. The [$^{14}C$]-oxonium salt solution (1 ml) was added to 100 mg of 3,-5-dinitrobenzoic acid in 15 ml of methylene chloride. The mixture was refluxed for 4 hr. After evaporating the methylene chloride, washing the residue with 5% $NaHCO_3$, and recrystallizing the residue from EtOH–$H_2O$, 15 mg of ethyl 3,5-dinitrobenzoate (mp 82.9–83.7°) resulted with a specific activity of $5.0 \times 10^6$ dpm/mmole. The main bulk of the [$^{14}C$]oxonium salt was precipitated from methylene chloride solution as an oil by the addition of 20 ml of hexane. The supernatant was poured off and the oil was taken up into a syringe with 0.5 ml of dry acetonitrile. This solution containing about 0.5 g of [$^{14}C$]oxonium salt was treated with 188 mg of lysozyme in 12 ml of water at pH 4.5 in the manner described below.

Synthesis of 4-bromoacetamido-2-nitrophenol was accomplished by the following procedure. To a stirred, ice-cold solution of 20 g (0.13 mole) of 4-amino-2-nitrophenol (recrystallized from hot water) in 200 ml of a glacial acetic acid and saturated sodium acetate (1:1, v/v) mixture was added 20 ml (0.23 mole) of bromoacetyl bromide over a 5-min period. After the mixture was stirred for 1 hr at 0°, the resulting white solid was isolated by filtration, washed with 50% acetic acid, and air dried. Recrystallization from ethanol–water gave 6.1 g of product (17%), mp 130–131°. An analytical sample was recrystallized from benzene and had a melting point of 131–132°. *Anal.* Calcd for $C_8H_7BrN_2O_4$: C, 34.93; H, 2.57; Br, 29.05; N, 10.18. Found: C, 34.96; H, 2.63; Br, 29.30; N, 10.21.

*Analytical Methods.* Amino acid analyses were performed on a Beckman-Spinco Model 120B amino acid analyzer after prior hydrolysis of protein samples for 20 hr in constant-boiling HCl, under vacuum, at 105°. Color values of amino acids normally found in hydrolysates of lysozyme were calibrated by standard mixtures (Beckman Co., Spinco Division). Homoarginine was estimated using the standard arginine value. Carboxymethylhistidine was estimated using the standard histidine color value.

Amino-terminal groups were determined by reaction of protein samples with fluorodinitrobenzene (Sanger, 1945) followed by paper chromatography of dinitrophenylamino acids (Levy, 1954) after acid hydrolysis of the reacted proteins.

Quantitative ninhydrin analyses on lysozyme and some of its N-acetylated derivatives were performed according to a published method (Moore and Stein, 1954). Determination of *O*-acetyltyrosine in lysozyme was done by (a) estimating the difference in absorbance at 278 mμ between native and O-acetylated enzyme as described for other proteins (Riordan *et al.*, 1965); and (b) determination of esters as their hydroxamates (Hestrin, 1949) after reaction with hydroxylamine at pH 6.5 (Balls and Wood, 1956).

Carboxyl ethyl esters were determined (a) by converting them into hydroxamates at pH 7.0 or 9.0 (using 1 M $NH_2OH$) at 25° for 2 hr. This was followed by dialysis or gel filtration to remove excess hydroxylamine, lyophilization of the protein hydroxamates, and iodine oxidation to nitrite of the hydroxamates followed by determination as described elsewhere (Yasphe *et al.*, 1960); (b) as ethanol, liberated by mild basic hydrolysis in sealed ampoules, which was quantitatively determined by gas–liquid partition chromatography using 10% Carbowax 20M on a 60–80 mesh-column, 6 ft, at 90°. Flame ionization detection and *t*-butyl alcohol internal standards were used; (c) by basic saponification of the ester derivatives with standardized NaOH carried out in a pH-Stat at pH 10.0, at 37°, under nitrogen; or (d) by coupling of the enzyme ester to glycinamide with a water-soluble carbodiimide (Hoare and Koshland, 1967) using 5 M guanidinium hydrochloride as solvent. All protein samples were oxidized with performic acid (Hirs, 1956) before coupling. Comparison of the number of glycine residues (estimated by amino acid analysis) coupled to unmodified lysozyme under similar conditions gave, by difference, the number of carboxyl esters.

Column chromatographic separations were monitored by (a) absorbance of effluents at 280 mμ; (b) determination of protein by the Lowry procedure (Lowry *et al.*, 1951); (c) ninhydrin analysis using a Beckman-Spinco Model 120B amino acid analyser while employing a ninhydrin system previously described (Moore and Stein, 1954); or (d) manual ninhydrin analysis. Enzymic activity assays on lysozyme and its derivatives were performed under conditions (Perry *et al.*, 1965) where relative initial rates could be measured, using *M. lysodeikticus* cells as substrate. Activities of various enzyme preparations using chitotriose as substrate were performed in 0.1 M citrate buffer (pH 5.5) at 40° for 1 hr. Enzyme concentrations were $3 \times 10^{-3}$ M and chitotriose concentrations were $10^{-2}$ M. Quantitation of hydrolysis of chitotriose was effected by separation of aliquots of the hydrolysis mixtures on columns (1.0 × 100 cm) of Bio-Gel P-2 using 0.1 M NaCl as solvent. Concentrations of the mono-, di-, and trisaccharides were determined by the ferricyanide procedure (Park and Johnson, 1949).

Dissociation constants for the binding of chitotriose to lysozyme and lysozyme derivatives were determined by a method previously described (Dahlquist *et al.*, 1966). Dissociation constants for the binding of 2-acetamido-2-deoxy-D-glucopyranose to lysozyme and lysozyme derivatives were determined by proton magnetic resonance methods (Raftery *et al.*, 1968a,b).

Chromatography of lysozyme and its derivatives was performed on columns (0.6 × 45 cm) of the carboxylic cation exchanger Bio-Rex 70 (−400 mesh) utilizing two buffer systems. The first system was that previously described (Hirs, 1955) for purification of lysozyme, employing 0.2 M sodium phosphate (pH 7.18). The second buffer system utilized four chambers of a Varigrad (Technion Instruments). The first three chambers contained 0.16 M sodium phosphate (pH 7.2) and the fourth chamber contained 0.20 M sodium phosphate (pH 7.2) which was 0.5 M with respect to KCl. Each chamber contained 40 ml of buffer. Flow rates of 12 ml/hr were employed for such columns and the fraction size was 0.80 ml. All chromatography was performed in jacketed columns which were equilibrated and maintained at 7°.

To determine the specific activity of the $^{14}C$ derivatives, every odd fraction from an analytical chromatograph was suspended in 15 ml of Bray's scintillation solution (Bray, 1960) containing 4.5% thixotropic gel powder (Packard) and counted for 2 min. In order to compare directly the relative specific activities of the proteins and the benzoate standard, 0.65 mg of the $^{14}C$-labeled ethyl 3,5-dinitrobenzoate derivative was dissolved in 1.00 ml of dioxane. This solution (100 μl) was added to some of the previously counted vials throughout the chromatograph and these were recounted. This small amount of the 3,5-dinitrobenzoate was shown to give very little quenching. The counting efficiency at the beginning of the salt gradient was 69% and smoothly decreased to 63% at the end, as determined by a [$^{14}C$]-toluene standard. To calculate the ester content, the known extinction coefficient and molecular weight (Sophianopoulos *et al.*, 1962) were used for lysozyme.

*Preparative Methods.* A. GENERAL PROCEDURES. Guanidination was performed as previously described for reaction of lysozyme (Fraenkel-Conrat, 1950). Acetylation with acetic anhydride was carried out as described by the same author. Acetylation of tyrosine using acetylimidazole was employed using known procedures (Riordan *et al.*, 1965). Alkylation with iodoacetic acid, iodoacetamide, or 4-bromoacetamido-2-nitrophenol was performed by reaction at pH 5.5 (acetate, 0.1 M) of a 1% solution of lysozyme with an equal weight of alkylating agent. Reaction was allowed to proceed for periods of 18 hr, and was followed by dialysis against distilled water and lyophilization. Esterification in ethanol–HCl mixtures was done as described for a similar reaction in methanol–HCl (Fraenkel-Conrat, 1950).

B. ESTERIFICATION OF LYSOZYME WITH TRIETHYLOXONIUM FLUOROBORATE. A typical esterification was performed in the following manner. Lysozyme (188 mg) was dissolved in 15 ml of distilled water and the pH was adjusted to the desired value, pH 4.5, with dilute perchloric acid. Triethyloxonium fluoroborate (approximately 0.7 g) was dried under a stream of nitrogen and weighed. Approximately 0.2 g of dry acetonitrile was added to the salt and the resulting solution was weighed. A dry syringe was filled with the solution and the calculated weight containing 0.57 g of reagent was injected into the vigorously stirred lysozyme solution to give a final concentration of 0.2 M oxonium salt. The pH was maintained by a pH-Stat with addition of about 1 ml of 4 N NaOH. Reaction was over in about 20 min and a slightly cloudy solution resulted. The product was dialyzed against several changes of distilled water and lyophilized. A typical yield was 170 mg of esterified product. Derivatives were prepared in this manner at pH 4.0 and 4.5 with oxonium salt concentrations of 0.1 and 0.2 M. Large preparations involved reaction of 2.00 g of lysozyme in an identical manner on a larger scale.

C. ESTERIFICATION OF LYSOZYME IN THE PRESENCE OF CHITOTRIOSE. The esterification in the absence of substrate was carried out essentially as already described for reaction at pH 4.5 except that three additions of reagent were made at 20-min intervals. Esterification in the presence of substrate was similarly carried out on lysozyme which was preequilibrated for 2 min in $8 \times 10^{-3}$ M chitotriose solution before addition of the oxonium salt.

D. ESTERIFICATION OF LYSOZYME WITH MULTIPLE ADDITIONS OF ESTERIFICATION REAGENT. Lysozyme (500 mg) was dissolved in 40 ml of distilled water and the pH was adjusted to 4.5 with dilute perchloric acid. An acetonitrile solution of 1.52 g of triethyloxonium fluoroborate was added and the pH was maintained by addition of 8 N NaOH. The resulting slightly cloudy solution was dialyzed against distilled water for 2 hr after which a 1-ml aliquot was withdrawn for assay of enzymatic activity and the remainder was again subjected to the esterification reaction. The addition of reagent, followed by dialysis and activity assay, was carried out six times in all and yielded an esterified product which contained an average of 2.6 esters/molecule of enzyme and which had an enzymatic activity equal to 21% that of native lysozyme.

E. ESTERIFICATION OF LYSOZYME WITH HIGH CONCENTRATIONS OF ESTERIFICATION REAGENT. Lysozyme (500 mg) was dissolved in 40 ml of distilled water and the pH was adjusted to 4.5 with dilute perchloric acid. Triethyloxonium fluoroborate (7.6 g) was added in solid form to give a concentration of 1 M. The pH of the reaction mixture was maintained at 4.5 by addition of 8 M NaOH from a syringe until the pH no longer decreased (approximately 30 min). Some precipitation of protein occurred under these conditions of reaction. The reacted mixture was dialyzed against distilled water and lyophilized.

F. ISOLATION OF ESTERIFIED DERIVATIVES OF LYSOZYME. Preparative chromatography was done on columns (5.0 × 70 cm) of Bio-Rex 70 (−400 mesh) at 7° using 0.2 M sodium phosphate (pH 7.18) as eluting buffer. Loads of 1.5–2.0 g of protein were applied to the columns. Fractions of 30–150 ml were collected using an Isco preparative fraction collector and effluents were monitored with an Isco Model UA-2 ultraviolet analyzer. For precise location of protein peaks fractions were analyzed by reading the absorbance at 280 mμ or by the Lowry method (Lowry *et al.*, 1951). After adjusting the pH to either 4 or 5 depending upon the component, rapid desalting of pooled fractions, which were sometimes of the order of 1000 ml, was effected by ultrafiltration at 7° to a small volume (about 40 ml) in an Amicon Ultrafiltration apparatus (Amicon Corporation) employing a UM-1 membrane followed by dialysis

against several changes of distilled water at 2–4°. The material was then lyophilized.

## Results

*Modification of Lysine Residues in Lysozyme.* A. GUANIDINATION. The reaction of lysozyme with *O*-methylisourea resulted in modification of all six lysine residues as previously described (Geschwind and Li, 1957). In the present instance this was judged from the amino acid composition of the product (Table I). Essentially, all lysine had disappeared and six residues of homoarginine (eluted after arginine on the basic column of a Beckman-Spinco amino acid analyzer) were found. The amino acid analysis results indicated that the composition of the guanidinated protein was in all other respects identical with that of native lysozyme. The enzymatic activity of guanidinated lysozyme was not different from native lysozyme at any pH value tested (Figure 1).

B. ACETYLATION. Acetylation of amino groups in lysozyme by a previously described method (Fraenkel-Conrat, 1950) led to products containing from four to six acetylated ε-amino groups of lysine as judged from both quantitative ninhydrin analysis and from amino acid analyses after reaction with *O*-methylisourea to convert residual free lysine into homoarginine (Table I). As found previously (Fraenkel-Conrat, 1950) the activity of the product when all six lysines were acetylated decreased to about 5–10% of that for native lysozyme. This activity measurement was made at pH 7 as were the previously described ones. When, however, fully ε-N-acetylated lysozyme was assayed over the pH range 2–10 the pH profile shown in Figure 1 was obtained. It was seen that in addition to a decrease in activity at all pH values the activity maximum had been shifted to lower pH. Also, the affinity of the acetylated lysozyme for chitotriose ($K_S = 4.7 \times 10^{-5}$ M) was decreased compared with that of native lysozyme ($K_S = 6.0 \times 10^{-6}$ M).

*Modification of the Single Histidine Residue in Lysozyme.* Two reports on alkylation of lysozyme at pH 5.5 by iodoacetic acid differ greatly in the results obtained. One of these (Jollès, 1964, 1967) claimed that a complex mixture of products was obtained during short periods of alkylation, none of which represented carboxymethylated histidine-modified lysozyme. The other report (Kravchenko *et al.*, 1964) presented evidence that under the conditions cited histidine was specifically alkylated since chromatographic separation of the products afforded two derivatives, representing the 1- and 3-carboxymethylhistidine lysozymes. We have repeated these experiments and our results agree with the latter report. Figure 2B shows the chromatographic pattern obtained. The major derivative (B-I) after acid hydrolysis was shown to contain 1-carboxymethylhistidine (Table I). Alkylation of lysozyme under the same conditions by iodoacetamide (Figure 2C, Table I) and by 4-bromoacetamido-2-nitrophenol (Figure 2D, Table I) yielded similar derivatives which were alkylated on N-1 of histidine as was shown by amino acid analysis.

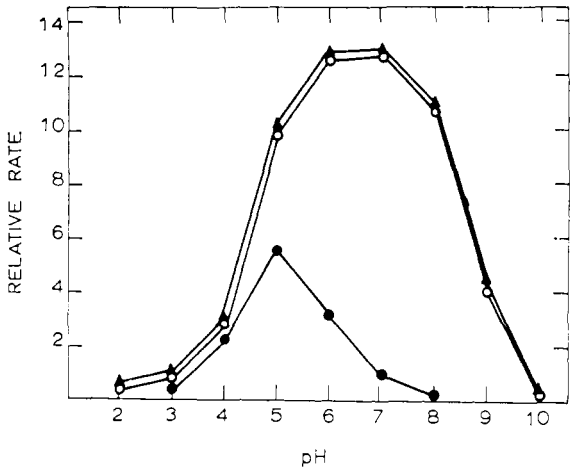


FIGURE 1: The relative activities of lysozyme (-▲-), guanidinated lysozyme (-O-), and acetylated lysozyme (-●-) as a function of pH.

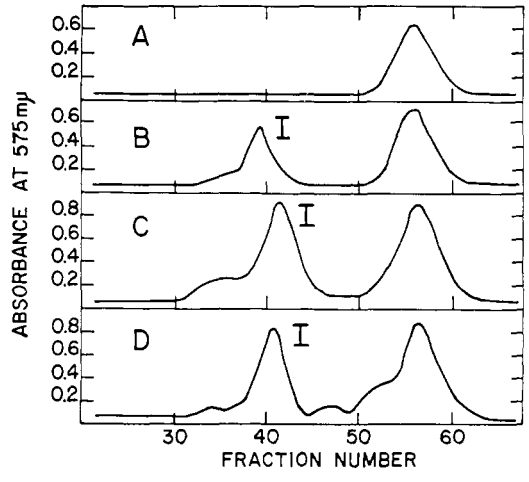


FIGURE 2: Chromatography of various histidine-alkylated derivatives of lysozyme on Bio-Rex 70 cation-exchange resin, at pH 7.18 in 0.2 M phosphate buffer. (A) Native enzyme. (B) Alkylation carried out with iodoacetic acid. (C) Alkylation carried out with iodoacetamide. (D) Alkylation carried out with 4-bromoacetamido-2-nitrophenol. Compounds labeled I are similar derivatives, each alkylated on N-1 of the histidine residue.

The enzymatic activities of all histidine-modified lysozymes were not different from that of untreated lysozyme despite the introduction of a negative charge, a carbamidomethyl group, or a bulky aromatic side chain, in addition to changing the $pK_a$ of histidine by alkylation of N-1.

*Modification of Tyrosine Residues in Lysozyme.* It has been shown (Simpson *et al.*, 1963; Riordan *et al.*, 1965) that treatment of proteins at neutral pH with acetylimidazole results in O acetylation of tyrosyl residues in contrast to N acetylation with acetic anhydride. Treatment of lysozyme with acetylimidazole resulted in two residues of *O*-acetyltyrosine being formed (Table II). The third tyrosyl residue of the enzyme did not react

TABLE I: Amino Acid Composition of Lysozyme Derivatives.

| Amino Acid | Lysozyme (residues/molecule) Theory | Control | Guanidinated Lysozyme | Acetylated Lysozyme[b] | Acetylated, Guanidinated Lysozyme | 1-Carbamidomethylhistidine Lysozyme | 1-Carbamidomethylhistidine Lysozyme | *N*-(3-Nitro-4-hydroxyphenyl)-carbamidomethyl Lysozyme |
|---|---|---|---|---|---|---|---|---|
| Asp | 21 | 20.9 | 21.3 | 21.5 | 21.0 | 21.2 | 20.9 | 20.7 |
| Thr | 7 | 6.6 | 6.8 | 6.7 | 6.9 | 6.9 | 6.8 | 6.8 |
| Ser | 10 | 9.0 | 8.7 | 8.5 | 8.7 | 8.7 | 8.6 | 8.5 |
| Glu | 5 | 5.2 | 5.2 | 5.0 | 5.1 | 5.0 | 4.9 | 4.8 |
| Pro | 2 | 2.0 | 2.2 | 2.1 | 2.10 | 1.90 | 2.1 | 2.0 |
| Gly | 12 | 11.9 | 12.6 | 12.2 | 12.6 | 12.2 | 12.1 | 12.1 |
| Ala | 12 | 12.0[a] | 12.0[a] | 12.0[a] | 12.0[a] | 12.0[a] | 12.0[a] | 12.0[a] |
| Cys(1/2) | 8 | | 4.2 | 4.7 | 4.7 | 4.6 | 4.0 | 4.7 |
| Val | 6 | 5.2 | 5.6 | 5.7 | 5.6 | 5.7 | 5.6 | 5.5 |
| Met | 2 | | 1.9 | 1.8 | 1.9 | 1.8 | 1.7 | 1.9 |
| Ile | 6 | 5.2 | 5.7 | 5.6 | 5.6 | 5.3 | 5.3 | 5.4 |
| Leu | 8 | 7.5 | 7.6 | 7.7 | 7.7 | 7.5 | 7.4 | 7.7 |
| Tyr | 3 | 2.9 | 3.0 | 2.9 | 2.9 | 2.7 | 2.9 | 2.8 |
| Phe | 3 | 3.0 | 2.9 | 2.9 | 2.9 | 2.8 | 2.8 | 2.8 |
| Trp | 6 | | | | | | | |
| Lys | 6 | 5.8 | 0.05 | 5.8 | 0.05 | 5.9 | 5.9 | 5.8 |
| His | 1 | 1.1 | 1.05 | 1.0 | 1.0 | 0.02 | 0.01 | 0.03 |
| Homoarginine | | | 5.9 | 10.6 | 5.9 | | | |
| 1-Carboxymethylhistidine | | | | 0.2 | | 0.93 | 0.95 | 0.92 |
| 3-Carboxymethylhistidine | | | | | | | | |
| Cys-$SO_3H$ | 8 | 7.80 | | | | | | |
| Met-$SO_2$ | 2 | 1.85 | | | | | | |

[a] All values are expressed as molar ratios normalized to a value of 12.00 for alanine. [b] This material was treated with *O*-methylisourea to convert all nonacetylated lysine residues to homoarginine.

even after prolonged treatment with several additions of reagent. The estimations of extent of modification were made using both (a) the decrease in absorbance at 278 mμ associated with *O*-acetyltyrosine relative to tyrosine (Simpson *et al.*, 1963) and (b) estimation of acetohydroxamate following treatment with hydroxylamine at pH 6.5 (Balls and Wood, 1956). The enzymatic activity of the bis-O-acetylated lysozyme derivative was decreased to 30% that of native lysozyme at pH 7.0 (Table III). It was found, however, that the acetylation was not specific for tyrosine, approximately three lysine residues being ε-N-acetylated during the shortest reaction time used (Table II). This was estimated by quantitative ninhydrin analysis (Moore and Stein, 1954). To overcome this nonspecific reaction the O acetylation was performed on fully guanidinated lysozyme since we had shown that this material possessed full enzymatic activity. Reaction of guanidinated lysozyme with acetylimidazole resulted in acetylation of all three tyrosines in the molecule (Table II). This result was surprising in view of the previous difficulty of fully acetylating the tyrosines of native lysozyme. The simplest interpretation is that guanidination caused a structural change in th protein which made the third tyrosine residue accessible for acetylation. This structural change did not, however, have any ill effects on the enzymatic activity of the modified enzyme since guanidinated and O-acetylated lysozyme was 100% active when compared with native lysozyme (Table III).

*Modification of Carboxyl Groups in Lysozyme.* We have prepared an ethyl ester derivative of lysozyme by treatment with dry ethanol–HCl, followed by dialysis and lyophilization. Table IV shows that as determined by three methods the average ethyl ester content was found to be 5.3 moles/mole of enzyme. Our figure of 5.3 ethyl esters can probably be raised to 6.3 since, as is subsequently shown in later sections of this communication, lysozyme can form one ethyl ester which is labile and it is likely that this ester was hydrolyzed during these particular ester determinations.

The activity of our ethyl ester derivative when assayed for its ability to lyse *M. lysodeicticus* cells was between 3 and 5% that of native lysozyme. We also tested the ability of this material to bind the inhibitor *N*-

TABLE II: Acetylation of Lysozyme and Guanidinated Lysozyme with Acetylimidazole.

| Protein Reacted | Addn of Reagent | Addnl Treatment | *O*-Acetyltyrosine Residues/Molecule Method | | Amino Groups Molecule (Ninhydrin)[b] |
|---|---|---|---|---|---|
| | | | $\Delta A_{280\,m\mu}$ | Hydroxamate[a] | |
| Lysozyme | 0 | None | 0 | 0.2 | 7 |
| Lysozyme | 1 | None | 1.8 | 2.1 | 4 |
| Lysozyme | 3 | None | 1.9 | 2.0 | 3 |
| Lysozyme | 4 | 8 M urea | 2.8 | 2.9 | 1.5 |
| Guanidinated lysozyme | 2 | None | 3.1 | 2.9 | 1.2 |

[a] Values listed in this column are not corrected for the amount (0.2 residue/molecule) obtained in the lysozyme blank. [b] The ninhydrin color value obtained from unmodified lysozyme was taken as being equal to seven amino groups (*i.e.*, six ε-lysine amino groups and one α-amino group) from the known composition of the enzyme (Table I).

TABLE III

| Derivative | Residue Modified | Total Such Residues in Enzyme | Derivative Formed (No.) | $K_S$ (M)[a] | % Act. at pH 7[b] |
|---|---|---|---|---|---|
| 1 | Lysine | 6 | Homoarginine (6) | $7.0 \times 10^{-6}$ | 100 |
| 2 | Lysine | 6 | ε-*N*-Acetyllysine (6) | $4.7 \times 10^{-5}$ | 15[c] |
| 3 | Histidine | 1 | 1-Carboxymethylhistidine (1) | $6.5 \times 10^{-6}$ | 100 |
| 4 | Histidine | 1 | 1-Carbamidomethylhistidine (1) | $6.3 \times 10^{-6}$ | 100 |
| 5 | Histidine | 1 | 1-*N*-(3-Nitro-4-hydroxyphenyl)-carbamidomethylhistidine (1) | | 100 |
| 6 | Tyrosine | 3 | *O*-Acetyltyrosine (3) | $6.3 \times 10^{-6}$ | 100 |
| | Lysine | 6 | Homoarginine (6) | | |

[a] Dissociation constant for binding of chitotriose as determined by a spectrophotometric method (Dahlquist *et al.*, 1966). The dissociation constant for native lysozyme–chitotriose was $6.6 \times 10^{-6}$ M. [b] Activity assay using *M. lysodeikticus* as substrate. Activity of native lysozyme was taken as 100. [c] The pH optimum of enzymatic activity has been shifted to lower pH values in this derivative as is shown in Figure 1.

acetyl-D-gluocosamine. We have previously shown (Raftery *et al.*, 1968a,b) that association of 2-acetamido-2-deoxy-D-glucopyranose and lysozyme can be studied by proton magnetic resonance spectroscopy. It has been shown (Dahlquist and Raftery, 1968) that the α and β anomers of 2-acetamido-2-deoxy-D-glucopyranose while competing for the same binding site on lysozyme do not bind in precisely the same way to that site. The chemical shift undergone by the acetamido methyl group of 2-acetamido-2-deoxy-D-glucopyranose in the presence of lysozyme ethyl ester indicates that 2-acetamido-2-deoxy-D-glucopyranose binds to this modified enzyme. However, in the present instance the resonances of the α and β anomers were not separated. The chemical shift data are plotted in Figure 3 according to a method previously described (Raftery *et al.*, 1968a,b). The dissociation constant obtained

TABLE IV: Ethyl Ester Content of Ethanol–HCl Esterified Lysozyme.

| Method of Analysis | Moles of Ester/Mole of Enzyme |
|---|---|
| 1. Ethoxyl content[a] | 5.0 |
| 2. Hydroxamate[b] | 4.9 |
| 3. Glycinamide incorporation[c] | 5.3 |

[a] This analysis was performed by the Microanalytical Laboratory, University of California, Berkeley, Calif. [b] Performed according to Yasphe *et al.* (1960). [c] Performed by the method of Hoare and Koshland (1966, 1967) using 5 M guanidinine hydrochloride as solvent for the coupling reaction on the performic acid oxidized protein.

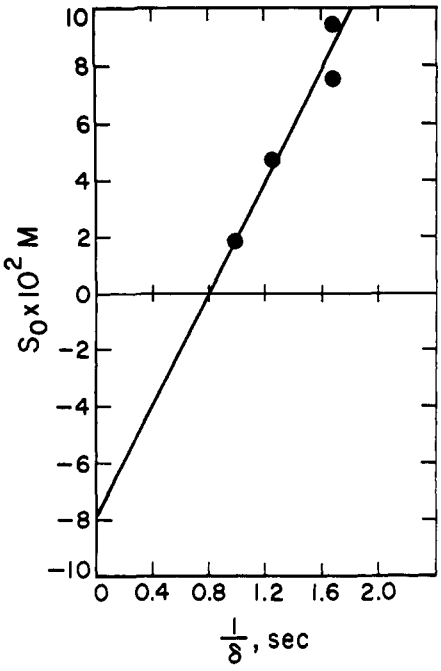


FIGURE 3: Plot of the inverse of the chemical shift of the acetamido methyl resonance of 2-acetamido-2-deoxy-D-glucopyranose (at mutarotational equilibrium) in association with the ethyl ester derivative of lysozyme *vs.* the total concentration of 2-acetamido-2-deoxy-D-glucopyranose. Measurements were made in 0.1 M citrate buffer (pH 5.5) at 40° and 60 MHz, lysozyme concentration $2.8 \times 10^{-3}$ M.

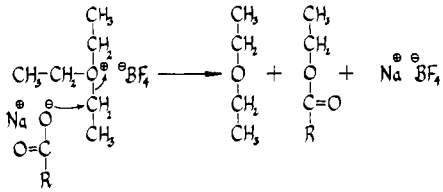

FIGURE 4: The pathway for the reaction of triethyloxonium fluoroborate with carboxyl groups.

from this plot is $K_S = 8 \times 10^{-2}$ M. The $\Delta$ value, which is discussed later, is 0.58 ppm. Thus association of 2-acetamido-2-deoxy-D-glucopyranose is weaker with the lysozyme ester than with lysozyme where $K_{\alpha\text{-2-acetamido-2-deoxy-D-glucopyranose}} = 1.8 \times 10^{-2}$ M and $K_{\beta\text{-2-acetamido-2-deoxy-D-glucopyranose}} = 3.5 \times 10^{-2}$ M (Dahlquist and Raftery, 1968). It was also shown that the ethyl ester derivative of lysozyme had a decreased affinity for chitotriose as determined by an ultraviolet difference spectral technique (Dahlquist *et al.*, 1966) with $K_S = 1.0 \times 10^{-4}$ M, compared with $K_S = 6 \times 10^{-6}$ M for unmodified lysozyme.

For reasons discussed later, we sought a method which would selectively esterify carboxyl groups in the enzyme, which would do so with no irreversible structural alterations in the protein, and which would possibly discriminate among the many carboxyl groups present. Accordingly, triethyloxonium fluoroborate, a powerful ethylating reagent, was tested. Its reaction with carboxyl groups proceeds according to the pathway in Figure 4. Most of the reagent is hydrolyzed in aqueous solution with the uptake of base.

*Specificity of the Esterification of Lysozyme with Triethyloxonium Fluoroborate.* The amino acid composition of lysozyme esterified in 1.0 M oxonium salt at pH 4.5 and containing 5.5 esters as estimated by saponification did not differ from that of unmodified lysozyme (Table V), indicating that ethylation of histidine, methionine, cystine, lysine, arginine, serine, threonine, or tyrosine had not occurred. In addition, no new ninhydrin-positive components were visible on the traces obtained from the amino acid analyses columns. Gas–liquid partition chromatography, saponification, and hydroxamate formation all indicated the formation of protein esters. One further check of the specificity of the reaction was performed. In case random O alkylation of amides had occurred, albeit unlikely in aqueous solution, end-group analysis by the Sanger (1945) method was carried out on the product containing 5.5 esters to identify any new amino-terminal residues which would have resulted from the spontaneous hydrolysis of *O*-ethylamides in the primary sequence. By comparison with a simultaneous end-group determination on the parent lysozyme no new amino-terminal residues were detected. Thus it was concluded that the alkylation reaction was specific for esterification of carboxyl groups.

Although complete inactivation of lysozyme by many moderate additions of oxonium salt (Figure 5) was not possible (the lowest activity found equalled 21% that of native lysozyme), a strong indication that carboxyl groups are important in catalysis was obtained by performing the esterification in the presence of chitotriose, a known substrate (Wenzel *et al.*, 1962) for the enzyme. Esterification at pH 4.5 with three additions of reagent caused a decrease in activity from 100 to 24%; in the presence of the trisaccharide inactivation by similar treatments was limited since the product retained 55% of its initial activity. This suggests that partial protection of carboxyl groups which are susceptible to esterification is effected by the binding to lysozyme of chitotriose.

Although 5.5 moles of ethyl ester could be introduced per mole of lysozyme and the relative enzymatic activity could be reduced to 6%, such a procedure was not followed in subsequent work since it was obvious (Figure 5) that the enzymatic activity of lysozyme decreased to 50% of its original value when a limited number of esters (0.8) had been introduced. It seemed, therefore, that some selectivity was operating in the esterification, due perhaps to special reactivity of critical carboxyl groups.

*Esterification of Lysozyme at pH 4.0.* Analytical chromatography of the product formed by reaction of lysozyme with 0.1 M triethyloxonium fluoroborate at pH 4 revealed that in addition to unmodified lysozyme, only one main chromatographic component was found. Figure 6B shows a typical chromatographic pattern for such a preparation. Cochromatography of peak I with lysozyme in the same system confirmed their identity.

In addition, the enzymatic activity of peak I was found to be 100% that of the parent lysozyme. The activity of the material in peak III was found to be constant across the peak and to be equal to 57% that of the parent lysozyme, if determined immediately after emerging from the column, when assayed for its ability to lyse suspensions of *M. lysodeikticus*.

## Isolation of Component III

Reaction of 2 g of lysozyme in 160 ml of water with triethyloxonium fluoroborate (0.1 M) at pH 4.0 gave 1 g of component III when isolated as described in the Experimental Section at pH 4.0 (except during chromatography). Analytical chromatography of the isolated material showed that it was pure component III.

Determination of the binding constant for association of chitotriose with component III, utilizing a spectrophotometric method (Dahlquist *et al.*, 1966), yielded a dissociation constant, $K_S$, of $3 \times 10^{-4}$ M which showed that relative to unmodified lysozyme ($K_S = 6 \times 10^{-6}$ M) the binding strength was decreased. It seems reasonable to infer that a carboxyl group in or near the three contiguous binding sites on the enzyme surface (Dahlquist *et al.*, 1966) was esterified in component III. The amino acid composition of component III is given in Table V. The ester content of this derivative was shown to be 0.7 ester group/molecule by conversion into the hydroxamate and estimation by the method of Yasphe *et al.* (1960).

An interesting property of component III is that it reverts to lysozyme on standing in phosphate buffer (pH 7.2) at room temperature over a 48-hr period. Thus the ester formed is labile, possibly due to the properties of the parent carboxylate. Full enzymatic activity was regenerated, relative to lysozyme, as a result of reversion to the parent enzyme. Figure 6C shows the chromatographic position of the reverted product. It occupies a position in the elution diagram identical with that occupied by native enzyme. This is evidence that the ester formed in component III came from a carboxylate group, not an asparagine or a glutamine. This also indicates that component III is homogeneous.

*Esterification of Lysozyme at pH 4.5.* Analytical chromatography (0.20 M phosphate system) of the product formed by reaction of lysozyme with 0.2 M triethyloxonium fluoroborate at pH 4.5 gave the pattern shown in Figure 7A. It was seen that in addition to a small amount of lysozyme (I), three chromatographic components (II, III, and IV) were observed. Component III

TABLE V: Amino Acid Composition of Lysozyme Ester Derivatives.[a]

| Amino Acid | Theory | Lysozyme Control | Lysozyme/Ester[c] | Triethyloxonium Fluoroborate Treated Lysozyme[d] | Component II[e] | Component III[f] |
|---|---|---|---|---|---|---|
| Asp | 21 | 20.8 | 20.6 | 20.8 | 20.5 | 21.0 |
| Thr | 7 | 6.5 | 6.2 | 6.5 | 6.5 | 7.1 |
| Ser | 10 | 9.0 | 8.7 | 9.0 | 9.3 | 9.7 |
| Glu | 5 | 5.2 | 4.9 | 4.9 | 5.1 | 4.9 |
| Pro | 2 | 2.0 | 2.0 | 1.9 | 2.0 | 2.0 |
| Gly | 12 | 11.9 | 12.0 | 12.0 | 12.2 | 12.3 |
| Ala | 12 | 12.0[b] | 12.0 | 12.0 | 12.0 | 12.0 |
| Cys(1/2) | 8 | | | 5.8 | 5.9 | 5.8 |
| Val | 6 | 5.2 | 5.1 | 5.2 | 5.3 | 5.2 |
| Met | 2 | | | 1.8 | 1.8 | 1.7 |
| Ile | 6 | 5.3 | 5.2 | 5.4 | 5.6 | 5.7 |
| Leu | 8 | 7.5 | 7.7 | 7.7 | 7.8 | 7.9 |
| Tyr | 3 | 2.8 | 2.7 | 3.0 | 3.0 | 2.9 |
| Phe | 3 | 3.0 | 2.8 | 2.9 | 3.0 | 2.8 |
| Trp | 6 | | | | | |
| Lys | 6 | 5.8 | 5.6 | 5.5 | 5.5 | 5.6 |
| His | 1 | 1.1 | 0.9 | 0.9 | 0.9 | 0.9 |
| Arg | 11 | 10.7 | 10.3 | 10.2 | 10.4 | 11.0 |
| Cys-$SO_3H$ | | 7.8 | 7.5 | | | |
| Met-$SO_2$ | | 1.8 | 1.8 | | | |

[a] Values are expressed in residues per molecule. [b] All molar ratios were calculated relative to a value of 12.00 for alanine. [c] This material was obtained by esterification of lysozyme in ethanol–HCl. [d] This material had been treated with 1.0 M triethyloxonium fluoroborate at pH 4.5 and was shown to contain an average of 5.5 ester groups/molecule of enzyme by base uptake. [e] This material was isolated chromatographically and was shown to be essentially devoid of enzymatic activity. It contained one ethyl ester per molecule. [f] This material was also isolated chromatographically and was shown to contain one labile ester. See text for description of its isolation and properties.

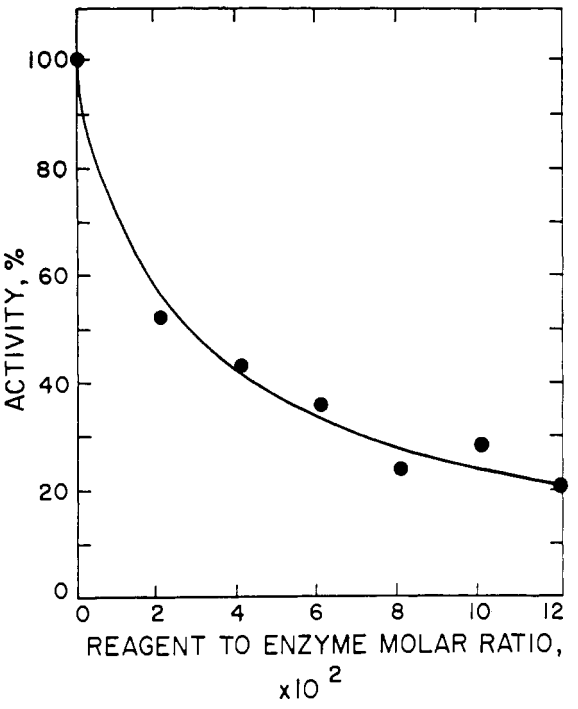


FIGURE 5: A plot of the per cent activity remaining after successive treatments of lysozyme with 0.2 M oxonium salt. The ethylations were carried out at pH 4.5 at a lysozyme concentration of $8.5 \times 10^{-4}$ M.

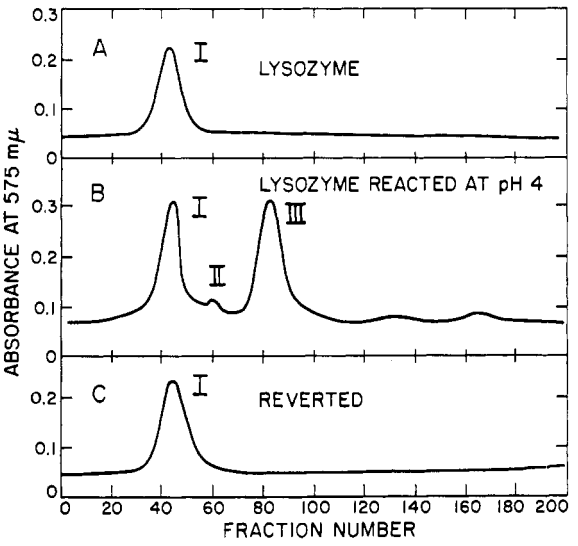


FIGURE 6: Chromatography of ethylated derivatives of lysozyme on Bio-Rex 70 cation-exchange resin. (A) Native lysozyme. (B) Lysozyme treated with 0.1 M triethyloxonium fluoroborate at pH 4.0. Component I is the unreacted lysozyme. Component II was not obtained in sufficient quantity to identify. Component III is a monoethyl ester of lysozyme. (C) Component III incubated at pH 7.2 for 48 hr at room temperature.

was eluted at a position corresponding to that of the major derivative obtained from esterification at pH 4.0. Assay of the peaks I, II, III, and IV showed that component I had a specific enzymatic activity equal to that

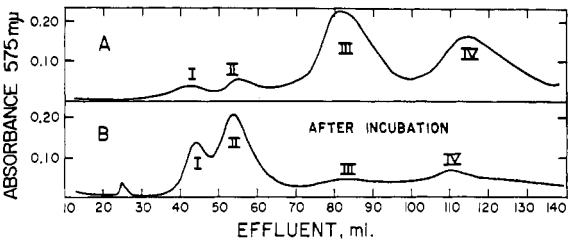


FIGURE 7: Chromatography on Bio-Rex 70 of ethylated derivatives of lysozyme formed at pH 4.5. (A) Lysozyme with 0.2 M triethyloxonium fluoroborate. Component IV is a diethyl ester containing the esters of components II and III. (B) The above mixture was incubated at 7.2 for 20 hr at room temperature.

of lysozyme, while both components II and IV appeared to be inactive. Component III had a specific activity equal to 55% that of native lysozyme, which corresponded to that of the major derivative obtained upon esterification at pH 4.0. The most interesting material in the present instance was component II, since it chromatographed just behind lysozyme and was therefore considered to have a low number of carboxyl ester groups. Its yield in the total mixture was, however, disappointingly low. It was found that if a sample of the reaction mixture containing components I, II, III, and IV was left at room temperature in 0.2 M phosphate, pH 7.18, for 20 hr and then chromatographed, components III and IV reverted to lysozyme and component II, respectively. Figure 7B shows the pattern obtained. Thus the yield of component II could be increased. It seemed reasonable that the most reactive carboxyl at pH 4.0 was esterified in high yield at pH 4.5, but that, in addition, a second carboxyl residue was esterified, both in lysozyme and in the ester derivative labeled III, giving rise to components II and IV, respectively. It was found that component II contained an ester of expected stability unlike component III.

Where $^{14}$C-labeled oxonium salt was employed for reaction at pH 4.5, analytical chromatography with the gradient system of the $^{14}$C-labeled esters gave the pattern shown in Figure 8. Components I, II, III, and IV were identified by their relative positions and by their enzymatic activities. The low yields of components II and IV were due to the fact that because of the difficulties in handling it, the [$^{14}$C]oxonium salt in the lysozyme reaction turned out to be only about 0.15 M. A fifth enzymatically active component, not eluted with 0.20 M sodium phosphate, also appeared but was not investigated. Essentially all of the protein and radioactivity applied to the column was eluted. The radioactivity peaks followed the protein peaks well and it was readily seen that components IV and V contained about twice the counts relative to $OD_{281}$ that components II and III did. Only background levels were found under the lysozyme peak, component I. The calculated specific activities confirmed that component II (inactive) contains 0.96 ester group/molecule, that component III (labile) contains 1.0 ester, and that component IV (inactive and labile) contains 1.9 esters.

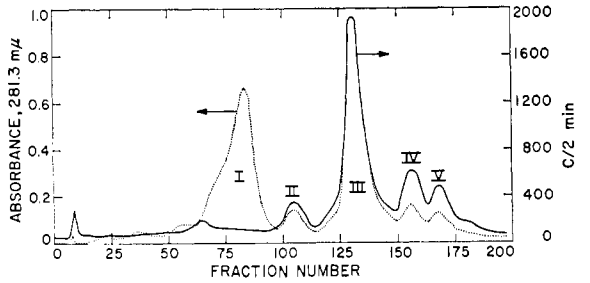


FIGURE 8: Chromatography with gradient system on Bio-Rex 70 of $^{14}$C-labeled derivatives of lysozyme formed at at pH 4.5 with about 0.15 M oxonium salt.

## Isolation of Component II

Reaction of 2.0 g of lysozyme with 0.2 M triethyloxonium fluoroborate at pH 4.5 under the conditions outlined in the Experimental Section yielded 1.9 g of total derivative after dialysis and lyophilization. This was dissolved in 200 ml of 0.2 M phosphate (pH 7.18) and left at room temperature for 20 hr. Chromatography on a preparative column partially separated lysozyme and component II. Material (220 mg) which was predominately component II was isolated at pH 5.0 as described above. The enzymatic activity of isolated component II was shown to be 10–12% that of native lysozyme.

It was of interest to investigate the properties of this preparation using chitin oligosaccharides as substrates and inhibitors. Determination of the dissociation constant of the complex formed between component II and chitotriose gave a value for $K_S$ of $7 \times 10^{-5}$ M compared with $K_S = 6 \times 10^{-6}$ M for lysozyme–chitotriose (Dahlquist *et al.*, 1966). This indicated that a carboxyl group at or close to the binding site for chitotriose had been esterified in component II.

Since chitotriose also serves as a substrate for lysozyme the capability of component II to hydrolyze the trisaccharide was also tested. It was shown that treatment of the trisaccharide ($10^{-2}$ M) with component II ($3.0 \times 10^{-3}$ M) at 40° for 1 hr resulted in formation of chitobiose and *N*-acetyl-D-glucosamine to the extent of only 4% that formed by native enzyme under similar conditions.

Rechromatography of component II on a similar preparative column gave the pattern shown in Figure 9. Assay of the enzymatic activity of each tube showed that the activity was eluted from the column ahead of component II, and was therefore due to contaminating lysozyme. The yield of the rechromatographed component II was 200 mg. The enzymatic activity of rechromatographed component II varied from preparation to preparation and was of the order of 0–1.9% the specific activity of native lysozyme when assayed using *M. lysodeicticus* as substrate. The relative specific activity of one of the better preparations was shown to be less than 0.5% throughout the pH range 2–10. The occasional small amount of activity was probably due to other contaminating single esters which were formed from labile double esters, such as component V in Figure 8, during the incubation at pH 7.2. When the incubation was not carried out and the activity of component II in the effluent of an analytical column was determined, essentially zero activity was found. The amino acid composition of component II is given in Table V.

In addition to the use of $^{14}$C-labeled reagent to estimate the ethyl ester content of components II, III, and IV as already described, the ethyl ester content of the isolated component II was determined by reaction of the unesterified carboxyl groups with glycinamide in 5 M guanidinium hydrochloride according to Hoare and Koshland (1967), and determination of the number of glycine residues incorporated by amino acid analysis. Comparison with unmodified lysozyme which had been similarly coupled with glycinamide gave by difference the number of carboxyl groups which had been modified by esterification. Table VI shows the results obtained, and these confirm that component II contains a single ethyl ester which is derived from a carboxyl group.

Another interesting property of component II was that on standing at room temperature at pH 2.0 enzymatic activity was slowly regenerated to a maximum of 60% that of native lysozyme after 20 hr. Higher values were not obtained due to denaturation at the acid pH. The specific enzymatic activity fell from 60% to lower values on further standing.

TABLE VI: Estimation of Free Carboxyl Groups in Lysozyme and in Derivative II by Coupling with Glycinamide.[a,b]

| Amino Acid | Theory | Control Lysozyme | Lysozyme Coupled | Derivative II Coupled |
|---|---|---|---|---|
| Asp | 21 | 20.85 | 21.0 | 20.37 |
| Thr | 7 | 6.59 | 6.91 | 6.41 |
| Ser | 10 | 9.00 | 9.65 | 8.72 |
| Glu | 5 | 5.20 | 5.32 | 4.93 |
| Pro | 3 | 2.80 | 2.82 | 2.71 |
| Gly | 12 | 11.90 | 23.8 | 22.77 |
| Ala | 12 | 12.00[c] | 12.00 | 12.00 |
| Cys(1/2) | 8 | | | |
| Val | 6 | 5.20 | 5.70 | 5.31 |
| Met | 2 | | | |
| Ile | 6 | 5.30 | 5.70 | 5.12 |
| Leu | 8 | 7.50 | 7.40 | 7.37 |
| Tyr | 3 | 2.85 | 2.80 | 2.79 |
| Phe | 3 | 3.00 | 2.91 | 2.83 |
| Trp | 6 | | | |
| Lys | 6 | 5.82 | 5.75 | 5.61 |
| His | 1 | 1.10 | 1.01 | 0.97 |
| Arg | 12 | 11.70 | 11.62 | 11.47 |
| Cys-$SO_3H$ | 8 | 7.80 | 7.70 | 7.39 |
| Met-$SO_2$ | 2 | 1.85 | 1.85 | 1.79 |

[a] The method used was that of Hoare and Koshland (1966, 1967) as described in the Experimental Section. [b] Values are expressed as residues per molecule. [c] All molar ratios were calculated by normalizing to a value of 12.00 for alanine.

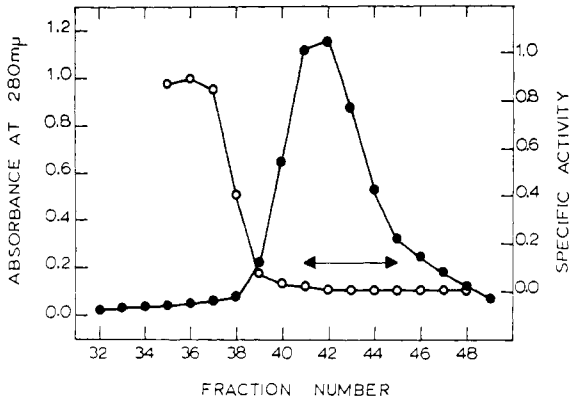


FIGURE 9: A plot of the rechromatography of component II (-●-) and the enzymatic activity of each fraction (-○-).

## Discussion

Virtually all the type of side chains in lysozyme which are susceptible to chemical modification have been implicated in reference to its activity. The apparent nonspecificity in the destruction of the lytic ability has doubtless been due partly to the unavailability of a well-defined substrate with which to distinctly separate binding function from catalytic action. In the present instance this difficulty has been overcome by use of chitotriose, a small molecule which serves as a lysozyme substrate, albeit a poor one. Chitotriose has been shown to bind to the enzyme by X-ray analysis methods (Blake *et al.*, 1967a), by difference spectral techniques (Dahlquist *et al.*, 1966; Rupley *et al.*, 1967), by fluorescence techniques (Lehrer and Fasman, 1966, 1967), and by proton magnetic resonance methods (Raftery *et al.*, 1968a, b; Dahlquist and Raftery, 1968, 1969). The binding of chitotriose has been shown to involve three contiguous strong binding sites by these methods and the stability of the complex formed suggests that it is a nonproductive one. Thus, this association affords a means of studying the strong binding region of the enzyme, while cleavage of chitotriose to chitobiose and 2-acetamido-2-deoxy-D-glucopyranose, presumably at another site on the enzyme, allows estimation of catalytic activity.

The present results show that whereas modification of various amino acid side chains resulted in decreased specific activity of the enzyme it has been possible to determine that in most cases this reduction resulted from lowering the affinity of the enzyme for substrate. The results obtained from acetylation of lysine residues illustrate this point. In the pH range 6–7 (the normal pH for assay) the activity of $\epsilon$-N-acetylated lysozyme was only a fraction (15–20%) of the activity of the native enzyme. This was due, however, to a decreased affinity for substrate (Table III) as well as to a shift in the pH profile of catalysis (Figure 1). Thus, although modification of all six lysines in the enzyme caused some changes in its catalytic properties inactivation was not achieved. Similar results have been recently reported by Yamasaki *et al.* (1968a,b) on the properties of acetylated lysozyme. The shifting of the pH profile to lower values could be indicative of two active sites on the enzyme, one being inactivated by the acetylation reaction and presumably requiring lysine residues. Such a possibility was deemed unlikely from the results obtained in the present study by conversion of all six lysine residues into homoarginines on reaction with *O*-methylisourea. The accompanying change in $pK_a$ of lysine(s) essential for catalysis would be expected to extend the alkaline limb of the pH profile of catalysis to higher pH values. As shown in Figure 1, such an effect was not observed.

The nonessentiality of the single histidine residue in the enzyme was confirmed in the present study since alkylation by three different reagents (iodoacetic acid, iodoacetamide, and 4-bromoacetamido-2-nitrophenol) did not have any effect on either the binding properties of the enzyme or its catalytic efficiency. These results confirm the similar alkylation studies of Kravchenko (1964) employing iodoacetic acid. It is felt that the results obtained by Kravchenko and by us eliminate the necessity of histidine for the catalytic function of lysozyme. This result is in accord with the X-ray analysis studies of Blake *et al.* (1965, 1967) who have shown that the single histidine residue of the enzyme occurs on the surface of the molecule remote from the binding cleft.

The acetylation of tyrosine residues in lysozyme has also been shown to have no effect on the catalytic properties of the enzyme. It was necessary to use guanidinated lysozyme to prove this point conclusively, however, since the acetylating agent, acetylimidazole, was not specific for O acetylation, causing some concomitant N acetylation of lysines. When such N acetylation was prevented it was possible to show that O-acetylated lysozyme had not lost any binding capability or catalytic efficiency when compared with native enzyme.

When lysozyme was treated with reagents which result in the modification of many of the carboxyl groups most of the enzymatic activity was abolished (Fraenkel-Conrat, 1950; Donovan *et al.*, 1960). Thus, modification by the method of Hoare and Koshland (1966, 1967) with resultant incorporation of seven glycinamide residues or esterification in ethanol–HCl with formation of 5.3 ethyl esters gave products which indicated that carboxyl groups might be critical for the activity of lysozyme. However, such a conclusion, although agreeing with the suggestions of Blake *et al.* (1967a), is tenuous when based only on the modification of all "available" carboxyls. The inactivation might be due to effects not directly related to modification of the one or two catalytically important (and perhaps not "available") carboxyls.

That the lysozyme derivative containing 5.3 ethyl esters, prepared in ethanol–HCl, has essentially the native conformation is indicated by the binding studies. Both 2-acetamido-2-deoxy-D-glucopyranose, and chitotriose bind to the derivative. The somewhat larger dissociation constants (for $\beta$-2-acetamido-2-deoxy-D-glucopyranose, only twice as large with the derivative as with native enzyme) could be due to direct steric interactions between the ester groups and the inhibitors. In any case, the retention of substantial inhibitor binding indicates that the tertiary structure of the enzyme in the region of the binding sites was not seriously disrupted by esterification. This is further supported by the fact

that 2-acetamido-2-deoxy-D-glucopyranose binds to a site in this derivative that gives rise to an upfield chemical shift (0.58 ppm) in the proton magnetic resonance signal of the acetamido methyl groups similar to that given by β-2-acetamido-2-deoxy-D-glucopyranose, methyl-β-2-acetamido-2-deoxy-D-glucopyranose, and methyl-α-2-acetamido-2-deoxy-D-glucopyranose in association with the native enzyme (0.54 ppm) (Raftery *et al.*, 1968b). It seems, then, that a lysozyme derivative containing a single ester group most likely would be in the native conformation.

We sought a method which would selectively esterify carboxyl groups in aqueous solution under mild conditions. To this end, we tested triethyloxonium fluoroborate [$(C_2H_5)_3O^+BF_4^-$]. The capability of this compound to effect ethylation of various nucleophiles is well documented (Booth and Martin, 1949). The reagent discriminates between carboxyl side chains probably on the basis of their $pK_a$ values (as well as, of course, on the basis of steric requirements) since the oxonium ion is known to attack anions much more rapidly than the corresponding neutral species.

In addition to amino acid analyses and the amino-terminal group determination, many different types of ester analyses were utilized to prove that this powerful alkylator attacks only carboxyl groups. Agreement was found between methods that in effect detected that carboxyl groups had disappeared and other methods that indicated ester groups present. Treatment of lysozyme with the reagent resulted in a decrease of enzymatic activity. When the trisaccharide, chitotriose, was included in the reaction mixture, some protection against the inactivation was afforded. Thus it seemed that this esterification reagent was ideally suited to investigation of the role played by particular carboxyl groups in lysozyme. It was possible to isolate two esterified derivatives of the enzyme and to determine some of their properties.

A derivative which was formed in a highly preferential esterification at pH 4.0 was shown to contain a single labile ester, to bind chitotriose much less efficiently than native lysozyme (by a factor of 20), but to retain 57% of the specific activity of the enzyme when assayed against *M. lysodeikticus*. Thus, it is probable that the carboxyl group esterified is one which is at or close to the strong binding site (nonproductive complex) for chitotriose rather than at the catalytic site. The lability of this derivative which allows it to revert to lysozyme on standing in solution at pH 7 most likely results from whatever environment makes it the most reactive carboxyl in the enzyme.

The second reactive carboxyl group in lysozyme was esterified in fair yield by reaction with triethyloxonium fluoroborate at pH 4.5. Higher yields of this derivative were isolated by allowing hydrolysis of a labile ester in derivative IV to occur. Thus a doubly esterified enzyme was converted into a singly esterified species which displayed the stability expected of a normal ethyl ester. This derivative was shown to bind chitotriose less efficiently than native lysozyme (by a factor of 10) but to possess only 4% of the specific activity of lysozyme toward hydrolysis of chitotriose and 10–12% toward hydrolysis of *M. lysodeikticus*. Rechromatography of this preparation resulted in its separation from contaminating lysozyme with the result that its specific activity, using *M. lysodeikticus* as substrate, was found to be, in the best preparations, 0–0.5% that of native enzyme. Because of the nature of the substrate it was difficult to be more quantitative than this. In addition, assay of activity using chitotriose as a substrate was complicated by the conditions used, 40° for 1 hr at pH 5.5. It was not possible to eliminate the possibility of limited ester hydrolysis. However, we feel that this derivative is essentially devoid of enzymatic activity. Therefore, it seems reasonable to suggest that the integrity of a single carboxyl residue is essential for lysozyme catalysis. This result is in agreement with the suggestions of Blake *et al.* (1967a) based on model building of saccharide molecules and fitting these into the cleft in the surface of their lysozyme model, as extensions of the known lysozyme–chitotriose nonproductive complex. It is also in agreement with the pH profile for lysozyme catalysis of various synthetic substrates (Raftery and Rand-Meir, 1968). It would seem therefore that a specific carboxyl side chain in lysozyme is critical for catalysis by the enzyme although it is not possible from the present studies to specify the exact role played by this group. Further work is being directed toward the identification of this particular residue regarding its position in the amino acid sequence of the enzyme.

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