# The Identification of Histidine-15 as Part of an Esteratic Site of Hen's Egg White Lysozyme*

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ABSTRACT: Lysozyme has been found to catalyze the hydrolyses of six carboxylic esters in an apparently bimolecular reaction. The observed second-order rates of lysozyme-catalyzed hydrolysis ($k_\psi = k_{cat}/K_m$) of all six esters were found to be dependent upon a basic group of $pK_{app} = 5.2 \pm 0.1$. The esteratic activity of lysozyme toward three of the esters, *o*-nitrophenyl acetate, *p*-nitrophenyl acetate, and sodium 3-nitro-4-octanoyloxybenzenesulfonate, is not inhibited by the competitive inhibitors of lysozyme's normal activity, *N*-acetylglucosamine and di-*N*-acetylchitobiose. The esteratic activity toward sodium 3-nitro-4-octanoyloxybenzenesulfonate dependent upon $pK_a = 5.2$ is eliminated by carboxymethylation of histidine-15. In addition the kinetically determined $pK_{app}$ for the hydrolysis of sodium 3-nitro-4-octanoyloxybenzenesulfonate is not altered, within experimental error, when determined in 30% dioxane–$H_2O$ (v/v). The esteratic site of lysozyme is, therefore, concluded to be centered around histidine-15 rather than the carboxyls of the glycosidic site. The mechanism of histidine-15 catalyzed ester hydrolysis is concluded to be nucleophilic since (a) the kinetic deuterium solvent isotope effect for sodium 3-nitro-4-octanoyloxybenzenesulfonate of $k_1(H_2O)/k_1(D_2O)$ is 1.19, and (b) the value of log $k_{cat}/K_m$ for *p*-nitrophenyl acetate fits a Brønsted plot for the nucleophilic reaction of various 4(5)-substituted imidazoles with this ester. The hydrogen bond between the histidine-15 imidazolyl group and the hydroxyl group of threonine-89 does not impart enhanced esteratic activity to lysozyme. Thus, hydrogen bonding between hydroxyl and imidazole is insufficient for the formation of a catalytically active site. Comparisons of lysozyme-catalyzed rates of ester hydrolysis with rates of reaction catalyzed by several nucleophiles indicates that lysozyme preferentially hydrolyzes esters having negatively charged substituents and long aliphatic chains. Examination of models of the substrate sodium 3-nitro-4-octanoyloxybenzenesulfonate and the lysozyme molecule around histidine-15 suggests several substrate orientations which would explain this preference on the basis of electrostatic and hydrophobic binding.

Lysozyme is the first enzyme to have its tertiary structure determined by X-ray crystallographic methods (Blake *et al.*, 1965, 1967; Phillips, 1967). The enzyme dissolves certain bacteria by catalytically hydrolyzing β-1,4-*N*-acetylmuramic acid–*N*-acetylglycosamine linkages in the carbohydrate polymer of bacterial cell walls (Sharon, 1967). Lysozyme has also been shown to hydrolyze β-1,4-linked oligomers of *N*-acetylglucosamine (Rupley and Gates, 1967; Rupley, 1967), β-aryl glycosides of di-*N*-acetylchitobiose (Osawa, 1966; Lowe, *et al.*, 1967) and tri-*N*-acetylchitotriose (Osawa and Nakazawa, 1966), and β-linked benzyl glycoside of di-*N*-acetylchitotriose (Zehavi and Jeanloz (1968)). In model studies, we (Piszkiewicz and Bruice, 1967, 1968) have shown that the acetamido group of 2-acetamido-2-deoxy-β-D-glucopyranosides anchimerically assists the hydrolysis of the glycoside bond in a stereospecific fashion. A recent study by Lowe and Sheppard (1968) has shown that lysozyme catalyzes the hydrolysis of *p*-nitrophenyl 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glycopyranoside with a value of $k_{cat}$ 20 times greater than *p*-nitrophenyl 4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranoside which substitutes a hydroxyl for an acetamido group vicinal to the β-glycoside bond. These results suggest that acetamido group participation may be involved in the glycosidic mechanism of lysozyme.

In the present study, our purpose was to determine if lysozyme could perform a catalytic function other than the hydrolysis of β-linked glycosides. The series of esters presented in Chart I were studied to determine if their hydrolyses could be catalyzed by lysozyme. It should be noted that these esters incorporate in their structures charged groups and aliphatic side chains which could facilitate electrostatic and hydrophobic binding to the enzyme.

Lysozyme has been shown to be inhibited by detergents such as sodium dodecyl sulfate (McLeod, 1941; Meyer *et al.*, 1947) and in general by alkyl sulfates, fatty acids, and aliphatic long-chain alcohols of 12 carbons or more (Smith and Stocker, 1949). In addition, 2.8% sodium dodecyl sulfate abolishes interaction between the substrate tri-*N*-acetylchitotriose and lysozyme (Rupley, 1967). This concentration has been shown (Glazer and Simmons, 1965) to produce no change in the helix

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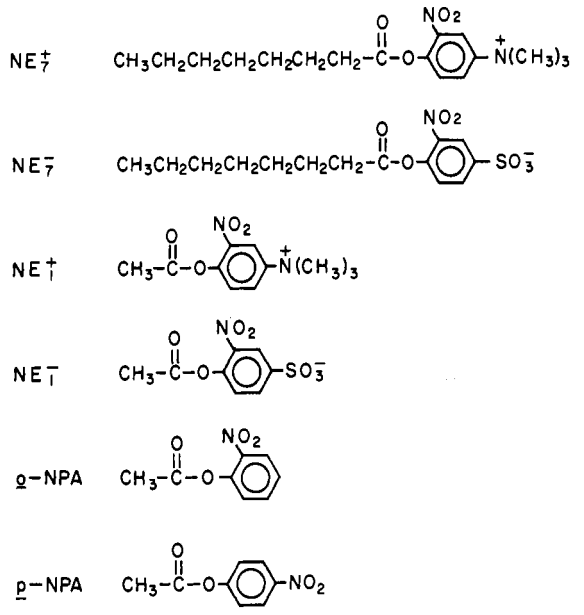
CHART I




content of the protein, but to alter the character of its tryptophan chromophores, presumably through specific interaction with them. Moreover, a tryptophan residue has been shown (Hayashi *et al.*, 1963) to be involved in the formation of the enzyme–substrate complex between lysozyme and poly-*N*-acetylglucosamine. Thus, it is tempting to conclude that the surface-active reagents inhibit lysozyme by binding to the enzyme at or near the active site. Most recently, Hayashi *et al.* (1968) have concluded on the basis of similar evidence that dimethylbenzylmyristylammonium chloride is a competitive inhibitor of lysozyme.

Phillips (1967) has suggested that the catalytically active functional groups of lysozyme are the glutamic acid-35 and aspartic acid-52 residues. Various mechanisms for catalysis of ester hydrolysis by carboxylates are well established (for a critical review, see Bruice and Benkovic, 1966); therefore, if a surface-active ester such as $NE_7^-$ [1] or $NE_7^+$ is bound to the active site, it might well be catalytically hydrolyzed.

The situation is complicated by the presence of a histidine residue in lysozyme. Imidazole catalysis of the hydrolysis of phenyl esters has been studied rather extensively (Bruice and Schmir, 1957, 1958a; Bruice and Benkovic, 1964; Bender and Turnquest, 1957; Kirsch and Jencks, 1964) and shown to be a very facile process, particularly in intramolecular reactions (Bruice and Schmir, 1958b; Bruice and Sturtevant, 1959; Pandit and Bruice, 1960). Moreover, the histidine residues of various proteins have been established as nucleophiles in ester hydrolyses, as in the hydrolysis of *p*-nitrophenyl acetate by metmyoglobin (Breslow and Gurd, 1962), and the hydrolysis of *o*-nitrophenyl hydrogen oxalate by ribonuclease A, in which Michaelis–Menton kinetics are observed (Bruice *et al.*, 1967).

Histidine-15 has been shown not to be involved in the normal enzyme–substrate interaction of hen's egg white lysozyme (Kravchenko *et al.*, 1963), and the X-ray crystallographic studies (Blake *et al.*, 1967; Phillips, 1967) confirm that this residue is not at the active site. Since the residue is at the surface, however, one might anticipate that the imidazolyl group would act as an effective catalyst if the ester could bind at this position on the surface. By inspection of the X-ray coordinates of Phillips[2] it is apparent that the hydroxyl of threonine-89 forms a hydrogen bond to the N-1 of the imidazolyl group of histidine-15. The conformations of these two groups resemble the conformation of histidine-57 and serine-195 of the active center of chymotrypsin (Matthews *et al.*, 1967). It would therefore be of interest to determine if the hydroxyl of threonine-89 of lysozyme could function in a manner similar to the serine hydroxyl of the serine esterases.

Hydrolysis of the esters of this study by either the carboxyls at the normal active site or the histidine at the surface would provide a valuable tool in the study of small molecule–protein interactions because the structures of both the enzyme and the substrate are known.

## Experimental Section

*Materials.* Three-times-crystallized lysozyme and Sephadex G-10 were obtained from Sigma Chemical Co. Iodoacetic acid was purchased from Aldrich Chemical Co. and recrystallized from benzene before use. Spectroquality Reagent *p*-dioxane was purchased from Matheson Coleman and Bell, and used without further purification. Bio-Rex 70 (100–200 mesh) and histidine methyl ester were obtained from Calbiochem. *N*-Acetylglucosamine was purchased from Nutritional Biochemicals Corp. Di-*N*-acetylchitobiose was prepared by Professor Rupley (Rupley, 1964), and generously supplied by him. All esters employed were synthesized for a previous study (Bruice *et al.*, 1968). The water used in preparing all solutions was deionized, then doubly distilled from an all-glass apparatus.

*Apparatus.* A Radiometer 22 pH meter equipped with a PHA 630 Pa scale expander and a type GK 2021C combined electrode was used to determine pH. The electrode was stored at the temperature of the kinetic measurements. All kinetic measurements were made using a Gilford 2000 recording spectrophotometer, a Ziess M4Q III monochromator equipped with a Gilford multiple-sample absorbance recorder, or a Zeiss M4Q III monochromator equipped with a Zieler multiple-sample absorbance recorder. Column chromatography was performed with the aid of a Gilson Model VL fractionator, and optical densities at 280 mμ of the individual fractions were determined with a Zeiss M4Q III monochromator. Amino acid analyses were generously per-

[1] Abbreviations used that are not listed in *Biochemistry* 5, 1445 (1966), are: $NE_7$, sodium 3-nitro-4-octanoyloxybenzenesulfonate; *p*-NPA, *p*-nitrophenyl acetate; *o*-NPA, *o*-nitrophenyl acetate.

[2] A Kendrew model constructed by Professor John A. Rupley was examined for this purpose and is shown in Figure 6.

formed for us by Professor John Riehm with a Beckman Model 120C amino acid analyzer.

*Kinetics.* All enzyme kinetic measurements were done at 30 ± 0.1° in aqueous buffers at $\mu = 0.1$. Buffers employed were potassium acetate (pH 3.80–6.25), potassium phosphate (pH 6.67–8.05), and Tris (pH 8.30–9.07). Ester hydrolysis was followed spectrophotometrically by observing the formation of substituted phenol at the isosbestic point of dissociated and undissociated forms. Thus, *p*-nitrophenol formation was observed at 348.6 mμ ($\epsilon$ 2.2 × $10^3$), 4-hydroxy-3-nitrobenzenesulfonate at 361.0 mμ ($\epsilon$ 2.04 × $10^3$), and 4-hydroxy-3-nitro-*N*-trimethylanilinium ion at 356.2 mμ ($\epsilon$ 2.00 × $10^3$). Preliminary kinetic experiments, using excess ester, then excess enzyme, indicated that the hydrolyses were first order in both. In no case where ester was in excess was an initial zero-order rate, characteristic of many enzyme-catalyzed reactions (Fruton and Simmonds, 1958), observed, nor was there observed in any case an initial burst followed by a slower, zero-order reaction as in the hydrolysis of *p*-NPA by α-chymotrypsin (Gutfreund and Sturtevant, 1956).

Since pseudo-first-order enzyme-catalyzed rates were often very slow, initial zero-order rates were determined. If it is assumed that the lysozyme-catalyzed hydrolyses of esters proceeds through an enzyme–substrate complex, the over-all reaction may be described by

$$\text{lysozyme} + \text{ester} \underset{}{\overset{K_m}{\rightleftharpoons}} \text{complex} \xrightarrow{k_{cat}} \text{product} \quad (1)$$

The Michaelis–Menton treatment (Fruton and Simmonds, 1958) may then be applied, so that

$$k_0 = \frac{k_{cat}[\text{lysozyme}][\text{ester}]}{K_m + [\text{ester}]} \quad (2)$$

where $k_0$ is the observed zero-order rate, $k_{cat}$ is the first-order rate constant for decomposition of enzyme–substrate complex to products, and $K_m$ is the dissociation constant for the intermediate complex. If, for a set of experiments at any constant pH, $k_0$ is plotted *vs.* [lysozyme][ester], the slope of the resulting line will be $k_{cat}/(K_m + [\text{ester}])$. If $K_m$ is much larger than [ester], the resulting line has a slope of $k_{cat}/K_m$ in units of a second-order rate constant ($k_\psi = k_{cat}/K_m$). Thus, if a zero-order kinetic treatment is applied, valid kinetic data are obtained for either ester complex formation or a simple bimolecular reaction.

In the present experiments, initial zero-order rates, $k_0$, were observed over approximately the first 10% of reaction. Second-order rate constants for the hydrolysis of esters by lysozyme ($k_\psi = k_{cat}/K_m$) were obtained as the slopes of plots of initial zero-order rate constants, $k_0$ (moles per minute as calculated from optical density per minute and the difference in $\epsilon$ of ester and substituted phenol), *vs.* the product of lysozyme and ester concentrations ([lysozyme][ester]). A minimum of five points was used for each such plot. Generally, ester concentration was held constant (5 × $10^{-5}$–2 × $10^{-4}$ M for various esters) while enzyme concentration was varied (up to 5 × $10^{-4}$ M).

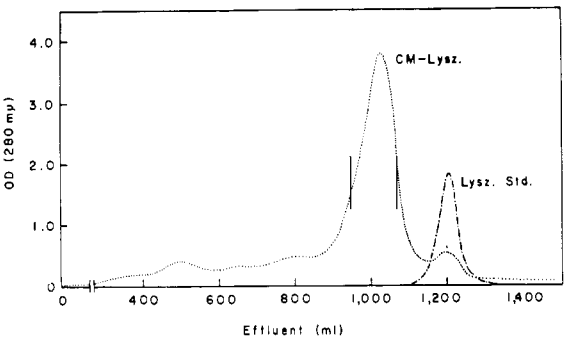



FIGURE 1: Chromatographic analyses of Bio-Rex 70. The dotted line shows the products of lysozyme treated with iodoacetate at pH 5.12 and 40° for 24 hr. Vertical bars indicate that portion of the effluent used.

Ester hydrolyses by histidine methyl ester were performed at 30 ± 0.1° in aqueous buffers at $\mu = 0.1$ with KCl. Second-order rate constants for the hydrolyses of esters by histidine methyl ester, $k_\psi$, were obtained as the slopes of plots of the pseudo-first-order rate constants *vs.* histidine methyl ester concentration. Values of $k_{Nu}$ for histidine methyl ester hydrolysis of esters were then calculated as $k_{Nu} = k_\psi[K_a/(K_a + a_H)]$, where $K_a$ is the dissociation constant for the first proton and $a_H$ is the hydrogen ion activity as determined by the glass electrode.

*3-Carboxymethylhistidine-15 Lysozyme.* The histidine-15 residue of lysozyme was carboxymethylated in the 3 position by the method of Hartdegen and Rupley[3] (Hartdegen, 1967). Lysozyme (500 mg) was dissolved in 10 ml of 0.1 M acetate buffer at pH 5.1. To this was added a solution of 1.73 g of iodoacetic acid which had been brought to pH 5.1 by the addition of KOH. This reaction mixture (pH 5.12) was then heated at 40° for 24 hr. The product was desalted by passing it through a 2.5 × 40 cm column of Sephadex G-10 and eluting with a 0.05 M ammonium acetate buffer at pH 8.00. The Sephadex G-10 eluent showed two peaks at 280 mμ, the first with ultraviolet spectra similar to lysozyme and the second with ultraviolet spectra similar to iodoacetic acid. The fractions corresponding to the first peak were pooled and applied to a 2.2 × 23 cm column of Bio-Rex 70 which had been equilibrated with 0.05 M phosphate buffer at pH 7.18. The column was then eluted with a linear gradient formed by adding 0.2 M phosphate buffer (750 ml, pH 7.18) to 0.05 M phosphate buffer (750 ml, pH 7.18).

The elution pattern of protein from the Bio-Rex 70 column is shown in Figure 1. The major protein peak from the Bio-Rex 70 column, indicated by the vertical lines in Figure 1, was concentrated by lyophilization,

[3] The method employed is essentially that of Dr. Frank Hartdegen and Professor John A. Rupley, to whom all credit should be given, and is presented here with the permission of Professor Rupley. The procedure has been modified only insofar as to employ a shorter column and a greater elution gradient which has the advantage of decreasing the time necessary for purification.

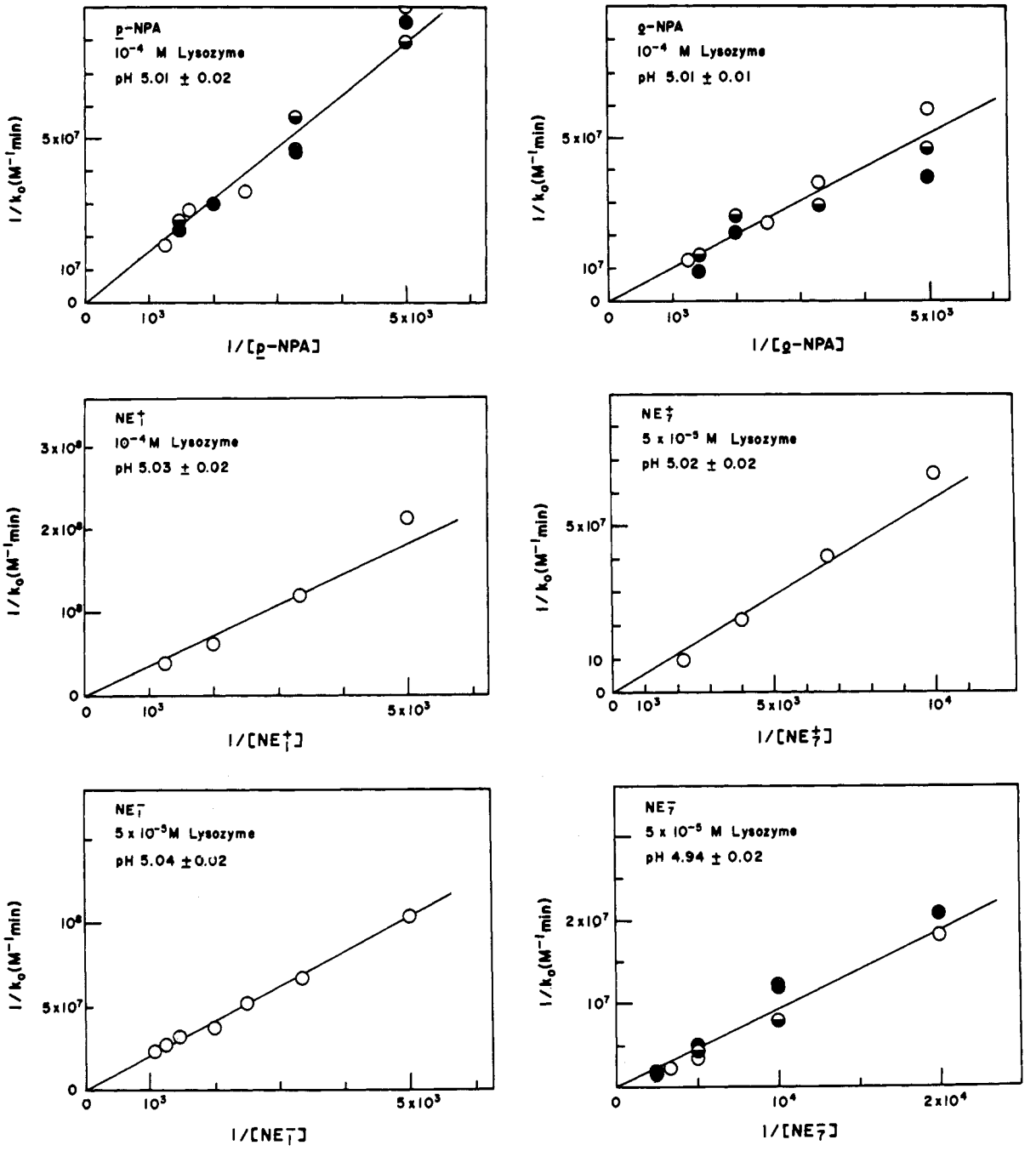



FIGURE 2: Lineweaver–Burk plots for the hydrolyses of esters by lysozyme. Open circles correspond to determinations with only ester and lysozyme present. Half-filled circles correspond to determination performed in the presence of $5 \times 10^{-2}$ M *N*-acetylglucosamine. Filled circles correspond to determinations performed in the presence of $2 \times 10^{-3}$ M di-*N*-acetylchitobiose.

dialyzed for 2 hr, then lyophilyzed to dryness. The amino acid analysis of the modified protein agreed well with the known amino acid composition (Canfield, 1963) but showed the elimination of the single histidine residue. Since no 1-carboxymethylhistidine was detected, the protein product was concluded to be 3-carboxymethylhistidine-15 lysozyme. This protein product was found to have an activity toward *Micrococcus lysodeikticus* 43 ± 2% of that of lysozyme (pH 7.00, 0.1 $\mu$ phosphate), in good agreement with the value of 40% determined previously (Hartdegen, 1967).

## Results

*Hydrolysis of Esters by Lysozyme.* Lysozyme was found to catalyze the hydrolysis of the six carboxylic acid esters of Chart I. The kinetic data for all six esters, when subjected to a Lineweaver–Burk plotting treatment (Lineweaver and Burk, 1934), could be fit best by a line intersecting the origin (Figure 2). Thus, for all six esters the mechanism of lysozyme-catalyzed hydrolysis is apparently bimolecular. The spectrophotometrically determined pH–log rate profiles for the lysozyme-cata-

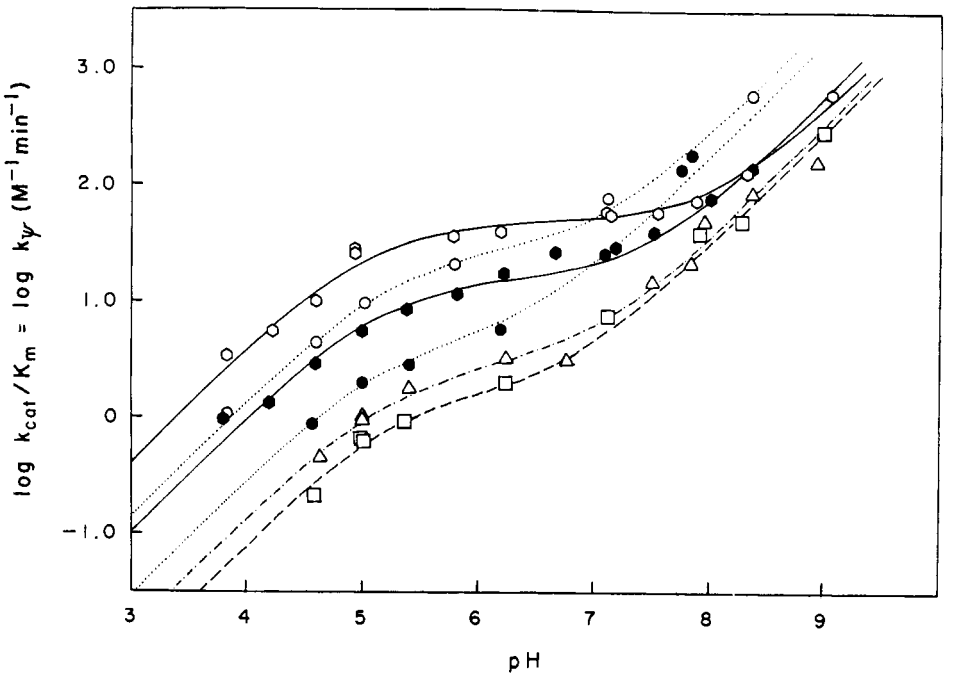



FIGURE 3: Spectrophotometrically determined pH–log ($k_{cat}/K_m$) profiles for the hydrolyses of esters by lysozyme. Points are experimental, and the curves are calculated from the over-all rate expression of eq 3 and the derived constants of Table I. (○—○) $NE_7^-$, (○- - -○) $NE_1^-$, (●—●) $NE_7^+$, (●----●) $NE_1^+$, (△·—·△) *o*-NPA, and (□—□) *p*-NPA.

lyzed hydrolyses of these esters are presented in Figure 3. The second-order rate constants ($k_{cat}/K_m = k_\psi$) for the hydrolyses of all esters by lysozyme follow the over-all rate expression of eq 3. Here $k_1$ is a second-order rate

$$k_\psi = k_{cat}/K_m = \frac{k_1 K_{a_1}}{K_{a_1} + a_H} + \frac{k_2 K_w}{a_H} \qquad (3)$$

constant for catalysis dependent upon the mole fraction of the free-base form of a functional group of the enzyme of $pK_{app} = pK_{a_1} = 5.2 \pm 0.1$; $k_2$ may be interpreted either as an apparent third-order rate constant for catalysis by $OH^-$ and enzyme, or as a second-order rate constant for catalysis dependent upon the mole fraction of the free base form of a functional group of the enzyme of $pK_{app} \geq 9$. Equations 3 and 4 are kinetically equivalent in the pH range employed (*i.e.*, $k_3 = k_2 K_w / K_{a_2}$). In Fig-

$$k_\psi = \frac{k_1 K_{a_1}}{K_{a_1} + a_H} + \frac{k_3 K_{a_2}}{K_{a_2} + a_H} \qquad (4)$$

ure 3 the points are experimental and the curves constructed from the most satisfactory solution of eq 3. A tabulation of the derived values of $K_{a_1}$, $k_1$, and $k_2 K_w$ used in constructing the lines in Figure 3 are presented in Table I.

The catalytic activity of lysozyme in hydrolyzing glycosidic linkages shows a pH optimum between approximately pH 4 and 6 (Rupley, 1967; Osawa and Nakazawa, 1966; Morgan and Riehm, 1968). The approximate bell-shaped pH-dependency curve has been suggested (Phillips, 1966) to result from the necessity for the terminal carboxyl group of Asp-52 to be dissociated and that of Glu-35 ($pK_a$ near 6.5, Rupley, 1967) to be associated. Inspection of Figure 3 reveals no like dependence of the esteratic activity of lysozyme upon pH.

The glycosidic activity of lysozyme has been shown to be inhibited by *N*-acetylglucosamine (Wenzel *et al.*, 1962) and di-*N*-acetylchitobiose (Rupley, 1964), presumably by competition with the substrate for the active site. In addition, the binding constants of these inhibitors to lysozyme have been determined (Dahlquist *et al.*, 1966). Therefore, the esterolyses were run in the presence of these compounds. However, when *N*-acetylglucosamine ($5 \times 10^{-2}$ M) was added to reaction mixtures of lysozyme and *p*-NPA, *o*-NPA, and $NE_7^-$ at a concentration in the range of its binding constant (Dahlquist *et al.*, 1966; Rupley, 1967) no inhibition of ester hydrolysis was observed (Figure 2). Similarly when di-*N*-acetylchitobiose ($2 \times 10^{-3}$ M) was added at a concentration approximately an order of magnitude greater than its binding constant (Dahlquist *et al.*, 1966; Rupley, 1967) no inhibition of the hydrolyses of *p*-NPA, *o*-NPA, and $NE_7^-$ was observed (Figure 2). Thus, it is

TABLE I: Derived Constants for the Hydrolyses of Esters by Lysozyme.

| Ester | $pK_{a_1}$ | $k_1$ ($M^{-1}$ $min^{-1}$) | $k_2K_w$ ($min^{-1}$) |
|---|---|---|---|
| *p*-NPA | 5.3 | 1.58 | $3.10 \times 10^{-7}$ |
| *o*-NPA | 5.3 | 2.75 | $3.63 \times 10^{-7}$ |
| $NE_1^+$ | 5.2 | 4.37 | $1.82 \times 10^{-6}$ |
| $NE_7^+$ | 5.2 | $1.58 \times 10^1$ | $6.03 \times 10^{-7}$ |
| $NE_1^-$ | 5.3 | $2.75 \times 10^1$ | $2.76 \times 10^{-6}$ |
| $NE_7^-$ | 5.1 | $5.00 \times 10^1$ | $4.37 \times 10^{-7}$ |

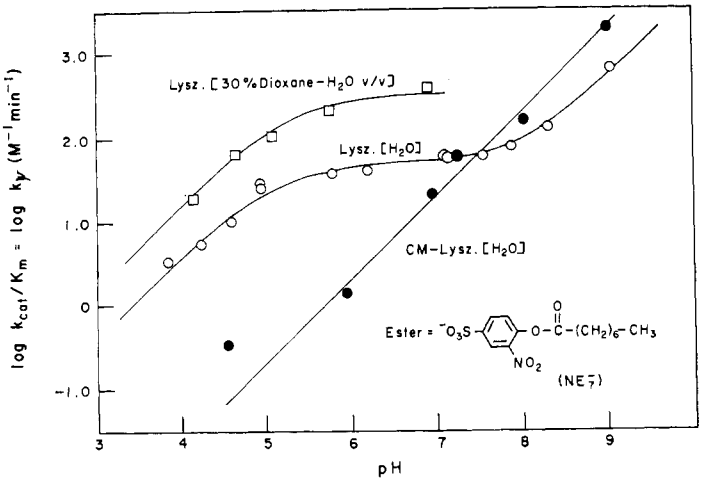



FIGURE 4: Spectrophotometrically determined pH–log ($k_{cat}/K_m$) profiles for the hydrolysis of $NE_7^-$ in water, in 30% aqueous dioxane, and by 3-carboxymethylhistidine-15 lysozyme. Points are experimental and lines are calculated from the appropriate equation, 3 or 5 and constants given in the text.

not likely that *N*-acetylglucosamine and di-*N*-acetylchitobiose bind to lysozyme at the same site involved in the catalysis of ester hydrolysis.

*Hydrolysis of $NE_7^-$ by Lysozyme in 30% Aqueous Dioxane.* The imidazolium group of histidine residues of proteins generally has a p$K_a$ of 5.6–7.0 (Edsall, 1943). In the pH range 4–6 the contribution from the first term of eq 3 and 4, which shows a dependence upon a group of the enzyme with p$K_{a_1}$ = 5.2 ± 0.1, is dominant. In assessing the significance of the carboxyl and imidazolyl groups in the hydrolyses of esters by lysozyme, additional studies concentrated on this pH range. Hydrolyses of the ester with the greatest $k_1$ value, $NE_7^-$, were studied in aqueous dioxane. In going from water to 30% aqueous *p*-dioxane, the p$K_a$ of acetic acid increases from 4.76 to 5.65 (interpolated from values given by Bell and Robinson, 1961). Thus, if a carboxyl group were responsible for the catalysis through $k_1$, a similar increase in p$K_{a_1}$ would be expected in going from an aqueous system to 30% aqueous *p*-dioxane. In going from water to 30% aqueous *p*-dioxane, the p$K_a$ of histidine methyl ester decreases slightly from 5.23 to 5.07 (determined by half-neutralization). Thus, if histidine-15 were responsible for $k_1$, no significant shift in p$K_{a_1}$ would be expected in going from aqueous solution to 30% aqueous *p*-dioxane. The pH–log rate profile for the hydrolysis of $NE_7^-$ by lysozyme in 30% aqueous *p*-dioxane solvent is presented in Figure 4. The points are experimental and the line is calculated from the most satisfactory solution of eq 3, using the values $k_1 = 3.47 \times 10^2$ and $K_{a_1} = 5.00 \times 10^{-6}$. The value of p$K_{a_1}$ = 5.3 determined in 30% aqueous *p*-dioxane is, within experimental uncertainty, unchanged from the value determined in water. Thus, an interpretation of catalysis of ester hydrolysis by histidine-15 rather than a carboxyl group is in agreement with the experimental evidence.

*Hydrolysis of $NE_7^-$ by 3-Carboxymethylhistidine-15 Lysozyme.* As a definitive experiment, the hydrolysis of $NE^-_7$ by 3-carboxymethylhistidine-15 lysozyme was studied. The pH–log rate profile for the hydrolysis $NE_7^-$ by 3-carboxymethylhistidine-15 lysozyme is included in Figure 4. The hydrolysis of $NE_7^-$ was found to follow the rate equation

$$k_{cat}/K_m = k_\psi = \frac{k_2 K_w}{a_H} \qquad (5)$$

where $k_2K_w = 2.0 \times 10^{-6}$ M$^{-1}$ min$^{-1}$. Since the alteration of the histidine-15 residue by carboxymethylation brings about the elimination of the kinetic term $k_1[K_{a_1}/(K_{a_1} + a_H)]$ of eq 3 and 4, catalysis by histidine-15 must be responsible for this term. In addition, it is of interest to note that the carboxymethylation of histidine-15 brings about an increase in $k_2K_W$ for $NE_7^-$ of 4.6-fold.

*Kinetic Deuterium Solvent Isotope Effect.* The lysozyme-catalyzed hydrolysis of $NE_7^-$ was also carried out in $D_2O$. Imidazole shows a shift in p$K_a$ of +0.47 in going from $H_2O$ to $D_2O$ (p$K_a$ = 7.10 in $H_2O$, and p$K_a$ = 7.57 in $D_2O$) (Bruno and Bruice, 1961). If the imidazolyl group of histidine-15 is assumed to undergo a similar, positive shift in p$K_a$ in going from $H_2O$ to $D_2O$, the determined second-order rate constant $k_{cat}/K_m = k_\psi$ in $D_2O$ would approximately equal $k_1$ at values of pH = pD > 6.7. The lysozyme-catalyzed hydrolysis of $NE_7^-$ was carried out in $D_2O$ at pH = pD = 6.84, with the value of pD being determined using the glass electrode correction factor of Fife and Bruice (1961). A value of $k_{cat}/K_m = k_\psi = k_1$ of 4.18 × 10 M$^{-1}$ min$^{-1}$ was determined, and $k_1(H_2O)/k_1(D_2O)$ = 1.19. This result suggests that proton transfer is not involved in the rate-determining step of the $k_1$ term. Histidine-15 therefore would appear to be acting as a nucleophile rather than as a general base.

*Hydrolyses of Esters by Selected Nucleophiles.* In the lysozyme-catalyzed hydrolyses of the six esters examined, no enzyme–substrate complex formation was observed, as would be demonstrated by the determination of a Michaelis–Menton constant, $K_m$. To determine if any lysozyme–ester binding did take place before reaction, the second-order rate constants for enzyme-catalyzed hydrolysis ($k_1$ of eq 3 and 4) were compared with the second-order rate constants, $k_{Nu}$, for hydrolyses of these esters by several nucleophiles.

For example, histidine methyl ester with a p$K_a$ of 5.23 (Bruice and Schmir, 1958a) is structurally and chemically similar to histidine-15 of lysozyme which has a p$K_{app}$ of 5.2 ± 0.1 (Table I). If no enzyme–substrate binding was involved in the lysozyme-catalyzed hydrolyses of the six esters, the rate of lysozyme-catalyzed hydrolysis, $k_1$, should be similar, and barring steric effects the susceptibility to nucleophilic attack by histidine methyl ester and lysozyme should be linearly related. Thus, one would expect *a priori* that a plot of $k_1$ *vs.* $k_{Nu}$ would be linear with approximate slope +1 if no binding were involved in $k_1$. Determined values of $k_{Nu}$ for histidine methyl ester are as follows: *p*-NPA, $8.46 \times 10^{-1}$ M$^{-1}$ min$^{-1}$; *o*-NPA, $2.50 \times 10^{-1}$ M$^{-1}$ min$^{-1}$; $NE_1^+$, $1.21 \times 10^1$ M$^{-1}$ min$^{-1}$; $NE_7^+$, $1.21 \times 10^1$ M$^{-1}$ min$^{-1}$; $NE_1^-$, 9.75 M$^{-1}$ min$^{-1}$; and $NE_7^-$, 6.59 M$^{-1}$ min$^{-1}$. The plot of $k_1$ *vs.* $k_{Nu}$ for the six esters is presented in Fig-

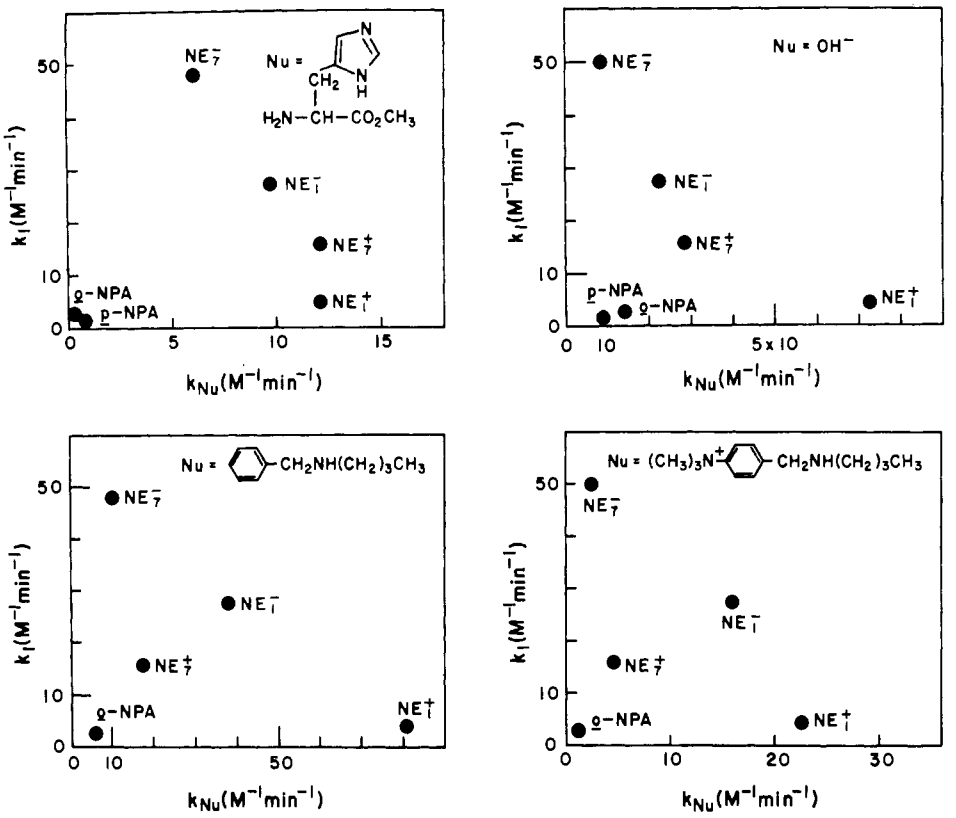



FIGURE 5: Plots of $k_1$ for lysozyme-catalyzed hydrolyses of esters *vs.* $k_{Nu}$ for the hydrolyses of esters by several nucleophiles. Values of $k_1$ are from Table I. Values of $k_{Nu}$ are given in text. Values of $k_{Nu}$ for hydroxide ion catalyzed hydrolyses of *p*-NPA and *o*-NPA are from B. Holmquist and T. C. Bruice (manuscript in preparation). All other values of $k_{Nu}$ are from Bruice *et al.* (1968).

ure 5, and is obviously nonlinear. By inspection of the values of $k_1$ and Figures 5 a general trend may be observed: lysozyme, in contrast to histidine methyl ester, preferentially hydrolyzes esters with negative charges ($NE_1^-$ *vs.* $NE_1^+$ and $NE_7^-$ *vs.* $NE_7^+$) and long aliphatic chains ($NE_7^-$ and $NE_7^+$). The same general trend in reactivities may be observed in the plots of $k_1$ *vs.* $k_{Nu}$ ($M^{-1}$ $min^{-1}$) for the nucleophiles hydroxide ion, benzylbutylamine, and *p*-trimethylaminobenzylbutylamine presented in Figure 5. Therefore, lysozyme does not act simply as a nucleophile toward the six esters in a bimolecular reaction. Some degree of substrate binding is indicated, and is exhibited by the fact that esters with negative charge and long aliphatic chains are preferentially hydrolyzed.

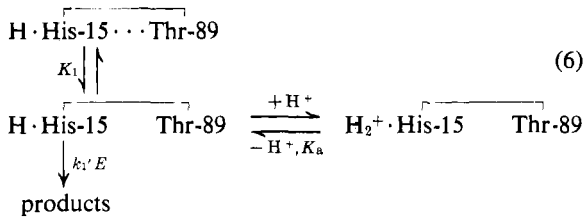
## Discussion

The results of this study demonstrate that hen's egg white lysozyme has the ability to hydrolyze esters. The pH–log ($k_{cat}/K_m$) profiles for the hydrolysis of the six esters studied (Chart I) demonstrate that in the lower pH region, the rate of hydrolysis ($k_{cat}/K_m = k_\psi$) is dependent upon a basic group of $pK_{a_1} = 5.2 \pm 0.1$. Catalysis by histidine-15 rather than catalysis by glutamic acid-35 or aspartic acid-52, the two carboxyl residues at the active site (Blake *et al.*, 1967; Phillips, 1967), is indicated for the following reasons: (a) *N*-acetylglucosamine and di-*N*-acetylchitobiose, known inhibitors of lysozyme (Wenzel *et al.*, 1962; Rupley, 1964) do not inhibit the hydrolyses of *p*-NPA, *o*-NPA, and $NE_7^-$ by lysozyme; (b) the $pK_{app}$ determined in 30% aqueous dioxane is, within experimental uncertainty, unchanged from that determined in water, a result consistent with imidazolyl group catalysis but not consistent with carboxyl group catalysis; and (c) the modification of histidine-15 by carboxymethylation results in the elimination of the term $k_1$, dependent upon a group of $pK_{a_1}$.

The mechanism of histidine-15 catalysis in the hydrolyses of the esters tested is probably nucleophilic for the following reasons. First, the kinetic deuterium solvent effect in the hydrolysis of $NE_7^-$ of $k_1(H_2O)/k_1(D_2O) = 1.19$ suggests that proton transfer is not involved in the rate-determining transition state. Thus, nucleophilic attack by a imidazolyl nitrogen of histidine-15 rather than general base catalysis is suggested. Second, the value of $k_1$ with a $pK_{a_1}$ of 5.2 for *p*-NPA fits the line of a Brønsted plot (not shown) for the nucleophilic-catalyzed hydrolysis of *p*-NPA by various 4(5)-substituted imidazoles (Bruice and Lapinski, 1958). Since *p*-NPA would not be anticipated to be complexed to the enzyme surface through either strong lyophobic or electrostatic attraction, the reaction of this substrate with the enzyme can be considered to be a true second-order reaction. Since lysozyme as a nucleophile fits a Brønsted plot for the reaction of 4(5)-substituted imidazoles with *p*-NPA (Bruice and Lapinski, 1958), there is no reason to postulate any nucleophilic role for threonine-89. Lastly,

since no stable enzyme–substrate intermediate was detected kinetically, no attempt was made to isolate such a species.

The $pK_{app}$ of the imidazolyl group of histidine-15 is unusually low (Edsall, 1943) and in the range where it could be mistaken for a carboxylic acid. The reason for this low value of $pK_{app}$ is not immediately evident. Though arginine-14 is juxtaposed to histidine-15 in the crystal structure (Figure 6) as a surface side chain it is free to move away from the histidine to alleviate electrostatic interaction. Hydrogen bonding of the imidazolyl nitrogen of histidine-15 to the hydroxyl group[2] of threonine-89 appears to offer a logical reason for the low $pK_{app}$ value. From eq 6 the kinetics of eq 7 may be derived.




(6)

$$k_\psi = \frac{k_1' K_1}{[K_1 + 1]} \left[ \frac{K_a[K_1 + 1]/K_1}{K_a[K_1 + 1]/K_1 + a_H} \right] \quad (7)$$

Inspection of eq 7 reveals the following relationships

$$pK_{app} = -\log \frac{K_a[K_1 + 1]}{K_1}$$
$$k_1 = \frac{k_1' K_1}{[K_1 + 1]} \quad (8)$$

If the hydrogen bond between histidine-15 and threonine-89 is sufficiently strong to bring about an apparent decrease in $pK_a$ then $K_1 > 1$. If it is assumed that $pK_a$ is normal for histidine in peptide linkage ($\approx 6.5$) then it follows that $K_1 \approx 5 \times 10^{-2}$ and the hydrogen bond is associated with a free energy of *ca.* $-1.8$ kcal mole$^{-1}$. On the basis of the known value of $K_a$ and anticipated value of $K_1$, $k_1'$ (the true rate constant for reaction of histidine-15 with ester) would be $20k_1$. For *p*-NPA a value of $k_1' = 3.2 \times 10^1$ could then be calculated. This value of $k_1'$ for an imidazole of $pK_a = 6.5$ shows a positive deviation of less than an order of magnitude from the Brønsted plot for the reaction of 4(5)-substituted imidazoles with *p*-NPA (Bruice and Lapinski, 1958). It is reasonable to conclude that the lowering of the $pK_a$ of histidine-15 is a result of hydrogen bonding by the hydroxyl of threonine-89. This result, therefore, gives kinetic evidence that a hydrogen bond (specifically of the threonine hydroxyl to the histidine imidazole groups) with an energy of $\Delta F \approx -1.8$ kcal/mole may exist on the surface of a protein.

It is of interest to note that the calculated value of $k_1'$ is still approximately 5000-fold lower than $k_{cat}/K_m$ for the serine esterase chymotrypsin (Faller and Sturtevant, 1966). Thus, hydrogen bonding between hydroxyl and imidazole is insufficient for the formation of a catalytically active site.

In the experiments described, no enzyme–substrate complex formation was observed, as would be demonstrated by the determination of a Michaelis–Menton constant, $K_m$. The failure to observe an enzyme saturation effect with any of the six esters examined does not, however, eliminate the possibility of the formation of an enzyme–substrate complex. It is reasonable to infer that the limited solubility of the various esters precludes bringing substrate concentration to a value approaching that of $K_m$. Similar inabilities to determine $K_m$ experimentally have been observed in the hydrolysis of *p*-NPA by chymotrypsin (Faller and Sturtevant, 1966) and in the hydrolysis of *p*-nitrophenyl trimethylacetate by elastase (Bender and Marshall, 1968).

Comparison of the second-order rate constants for enzymic, $k_1$, and nucleophilic, $k_{Nu}$, catalysis of ester hydrolysis virtually assures binding of esters to lysozyme. For all esters investigated, the order of $k_{Nu}$ is as anticipated on the basis of electronic and steric considerations (*i.e.*, rate constants decrease in the order $NE_1^+ \approx NE_7^+ > NE_1^- > NE_7^- \gg$ *p*-NPA $>$ *o*-NPA). In the case of ester hydrolysis by lysozyme, however, the order of rate constants is not as anticipated for a simple bimolecular reaction ($NE_7^- > NE_1^- > NE_7^+ > NE_1^+ >$ *o*-NPA $>$ *p*-NPA). Clearly a negative charge on the ester facilitates esteratic activity ($NE_1^-$ *vs.* $NE_1^+$ and $NE_7^-$ *vs.* $NE_7^+$) as does an aliphatic side chain ($NE_7^-$ *vs.* $NE_1^-$ and $NE_7^+$ *vs.* $NE_1^+$).

If binding of ester to enzyme occurs as in eq 9, then

$$\text{ester} + \text{lyszome} \underset{}{\overset{K}{\rightleftharpoons}} \text{ES} \xrightarrow{k_r} \text{product} \quad (9)$$

the determined value of $k_1$ equals $k_rK$. Since the $pK_a'$ of the imidazolyl group of histidine methyl ester is, within experimental error, identical with the $pK_{app}$ of the imidazolyl group of histidine-15 we may assume the difference in the values of $k_{Nu}$ and $k_1$ to correspond to an increase in the free energy of the ground state in the case of the enzymic reaction due to the juxtaposing of imidazolyl and ester bond brought about by the binding of substrate. It follows, therefore, that the difference in the calculated values of $\Delta F_{Nu}^{\ddagger}$ for histidine methyl ester and $\Delta F_1^{\ddagger}$ for lysozyme can be related to the free energy of binding, $\Delta F_K$, of substrate (Table II). Comparison of $\Delta F_K$ for *o*-NPA and *p*-NPA reveals that a substituent

TABLE II: Calculated Free Energies of Binding of Substrates to Lysozyme.

| Ester | $\Delta F_K$ (kcal mole$^{-1}$) |
|---|---|
| *p*-NPA | −0.4 |
| *o*-NPA | −1.4 |
| $NE_1^+$ | +0.6 |
| $NE_7^+$ | −0.2 |
| $NE_1^-$ | −0.4 |
| $NE_7^-$ | −1.4 |

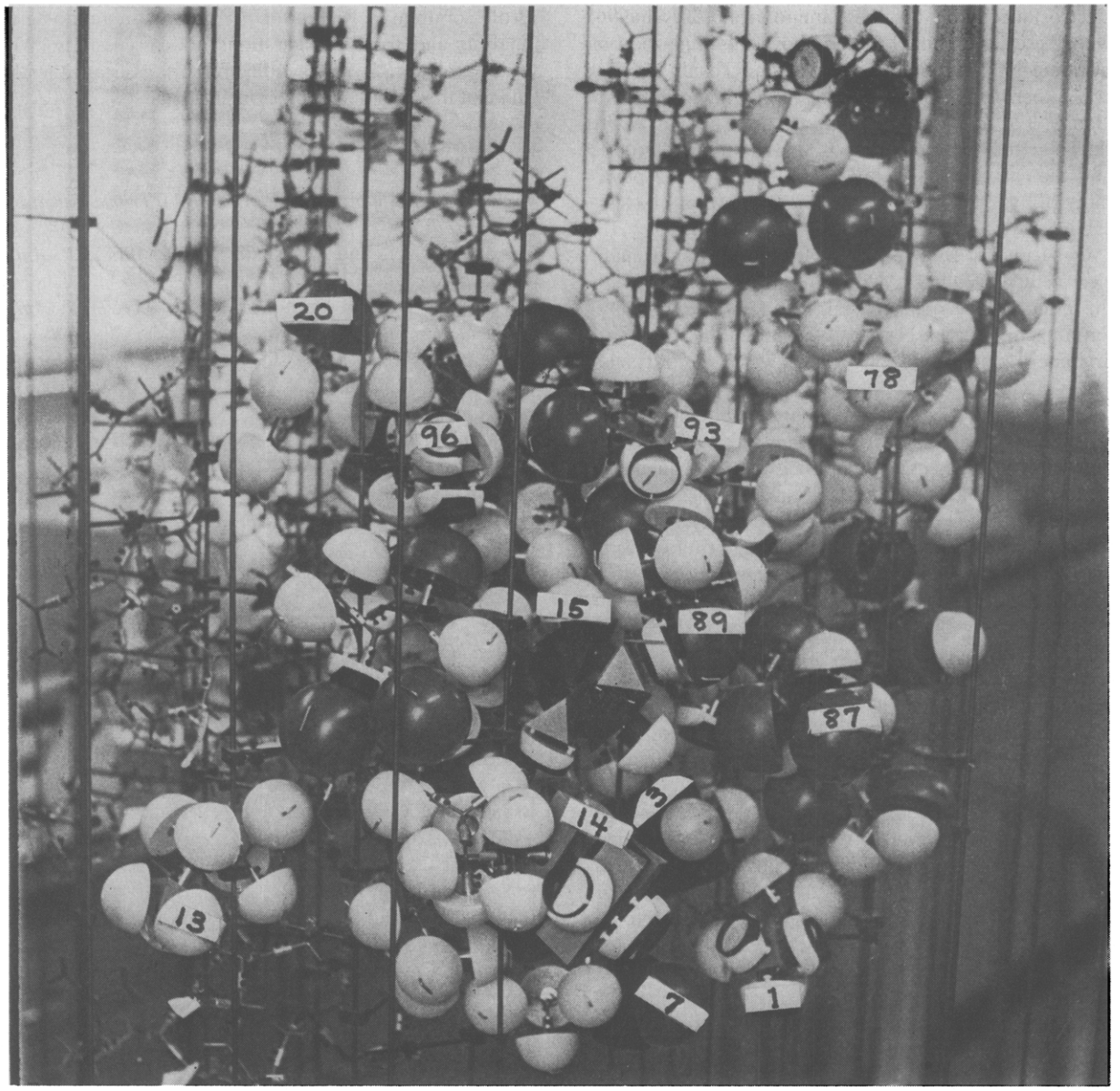



FIGURE 6: Model of the lysozyme molecule showing the esteratic site. Kendrew model components form the backbone and Courtauld space-filling models define the enzyme surface. The glycosidic site is on the reverse side of the molecule and cannot be seen. In this picture, the hydroxyl of threonine-89 can be seen hydrogen bonded in the 1-nitrogen of histidine-15. The model was constructed from coordinates supplied to Professor John A. Rupley by Dr. D. C. Phillips.

in the *para* position of the aromatic ring is unfavorable to binding. Comparison of $\Delta F_K$ for $NE_1^+$ and $NE_1^-$ with the value for *o*-NPA reveals that a positively charged group in the *para* position provides a much larger repulsive force. This repulsion suggests interaction with a positively charged group of the enzyme near histidine-15 at the esteratic site of the enzyme. This suggestion is in accord with the greater binding of $NE_1^-$ compared with $NE_1^+$ ($\Delta\Delta F_K = -1.0$ kcal mole$^{-1}$) and of $NE_7^-$ compared with $NE_7^+$ ($\Delta\Delta F_K = 1.6$ kcal mole$^{-1}$). If $\Delta F_K$ for $NE_1^+$ is compared with that for $NE_7^+$ it is seen that extension of the alkyl group by six methylenes decreases $\Delta F_K$ by $\approx 0.8$ kcal mole$^{-1}$ while a like comparison for $NE_1^-$ and $NE_7^-$ shows a decrease in $\Delta F_K$ of 1.0 kcal mole$^{-1}$. The contribution toward the free energy of hydrophobic binding *via* a minimum contact by a single methylene group has been suggested to contribute *ca.* $-0.3$ kcal mole$^{-1}$ to the free energy of binding (Némethy and Scheraga, 1962). Employing this value it may be estimated that for $NE_7^+$, 2.7 of the six extra methylene groups are in contact with a lyophobic group on the enzyme surface, while for $NE_7^-$, 3.3 methylene groups are in contact with the lyophobic region.

Possible binding orientations of $NE_7^-$ to the surface of lysozyme at its esteratic site may be determined by examining the region around histidine-15 for positively charged groups and lyophobic regions. A photograph of the side of the enzyme possessing the esteratic site is presented in Figure 6. Four possible orientations of $NE_7^-$ at the esteratic site have been examined by placing a model of the substrate on the model of the enzyme.

ORIENTATION A. The *p*-sulfonate group of $NE_7^-$ may

electrostatically bind to the $\epsilon$-amino group of lysine-96, with the ester carbonyl falling above the imidazolyl of histidine-15. The terminus of the octanoyl chain of $NE_7^-$ could then fall into the nonpolar hole having phenylalanine-3 at its base. This nonpolar hole is flanked by the polar guanido of arginine-14, the $\epsilon$-amino of lysine-1, and the carboxyl of aspartic acid-87. Thus, the possibility of hydrophobic binding at this site would be lessened.

ORIENTATION B. The *p*-sulfonate group might again electrostatically bind to the $\epsilon$-amino of lysine-96, with the ester linkage placed over histidine-15. The alkyl terminus might then bind to the exposed methylenes of arginine-14 and extend toward the methylenes of lysine-13.

ORIENTATION C. The *p*-sulfonate may bind to the guanido of arginine-14 with the ester linkage over histidine-15. The alkyl chain would then fall over the methylenes of threonine-89 and extend toward isoleucine-78.

ORIENTATION D. The *p*-sulfonate of $NE_7^-$ may bind at the $\epsilon$-amino of lysine-1 with the phenolic ring crossing the lyophobic pocket and again placing the ester carbonyl near histidine-15. The alkyl terminus might then hydrophobically bind to the methylenes of threonine-89. In this orientation, however, the $\epsilon$-amino of lysine-1 is too far from the imidazolium of histidine-15 to allow optimum electrostatic interaction.

Consequently, of the four possible enzyme–substrate orientations examined, those most likely to explain ester binding are orientations B and C.

This study has demonstrated that the single imidazolyl group of hen's egg white lysozyme (histidine-15), though hydrogen bonded to threonine-89, has the nucleophilicity toward *p*-NPA of a 4(5)-substituted imidazole of similar $pK_a$. This histidine residue has been shown to be the only effective nucleophilic site at neutral pH toward the six esters studied. Furthermore, negatively charged substituents and long aliphatic chains of the esters enhance the substrate reactivity presumably through electrostatic attraction and lyophobic binding to positively charged functional groups and lyophobic regions in the vicinity of histidine-15. With a knowledge of those characteristics of structure which enhance substrate reactivity and the knowledge of the tertiary structure of lysozyme, it should be possible to design synthetic substrates which will exhibit a greater degree of binding to the esteratic site than seen in this study. If such binding were of sufficient magnitude, a kinetically observable enzyme–ester complex formation, characteristic of most enzymatic reactions, would result. We are currently examining this possibility. Thus, in the study of model enzymes, it may prove easier to synthesize a substrate to fit a known nucleophilic site on a protein than to produce a synthetic enzyme to act upon a known substrate.

## Acknowledgments

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# The Effects of pH on the Kinetics of Human Liver Ornithine–Carbamyl Phosphate Transferase*

Philip J. Snodgrass

ABSTRACT: The effects of pH on the kinetics of human liver ornithine–carbamyl phosphate transferase have been examined. As the pH of the assay increases, the concentration of ornithine which gives maximum activity decreases, and inhibition becomes apparent at each pH if an optimal ornithine concentration is exceeded. The $K_m$ for ornithine decreases as the pH is raised from 6 to 8, but the $K_m$ for the zwitterion form ($pK'_2 = 8.69$) remains constant over this range, indicating that the zwitterion is the actual substrate of human ornithine–carbamyl phosphate transferase.

Further, pH does not affect the $K_m$ for carbamyl phosphate. Hence, when either carbamyl phosphate or the zwitterion of ornithine bind, apparently no groups at the active center of the enzyme or of the enzyme–substrate complex ionize. However, the effect of pH on $V_{max}$ implies that an ionizing group in the enzyme–substrate complex with an apparent $pK$ of 6.6 affects the rate of the breakdown of the complex into free enzyme and products.

Carbamyl phosphate–L-ornithine carbamyl transferase (EC 2.1.3.3) from rat or beef liver catalyzes the formation of citrulline by a single displacement reaction in which ornithine and carbamyl phosphate are bound simultaneously to separate sites on the enzyme (Reichard, 1957; Joseph *et al.*, 1963). Although the latter kinetic study indicates that carbamyl phosphate is bound in part through its phosphate moiety, the presence of groups on the enzyme which might play a role in substrate binding or in the catalytic process have not been sought by standard kinetic methods. As a part of a study on enzymes which participate in the urea cycle of human liver, we have therefore examined ornithine carbamyl transferase (OCT)[1] for the effects of pH on $K_m$ and $V_{max}$ of both substrates.

* From the Department of Medicine, Harvard Medical School, and the Peter Bent Brigham Hospital, Gastrointestinal Division, Boston, Massachusetts. *Received May 13, 1968.* This investigation was supported by U. S. Public Health Service Research Grant AM-07848 from the National Institutes of Health of the Department of Health, Education, and Welfare. A preliminary account has been presented (Snodgrass, 1967).

[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: OCT, ornithine–carbamyl phosphate transferase.