THE REACTION OF 2-HYDROXY-5-NITROBENZYL BROMIDE WITH LYSOZYME
A STUDY OF INHIBITION AND REGENERATION

by

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List of Abbreviations

HNB-Br 2-hydroxy-5-nitrobenzyl bromide

HNB the 2-hydroxy-5-nitrobenzyl group

MNB-Br 2-methoxy-5-nitrobenzyl bromide

HNB:lysozyme lysozyme substituted with a non specified ratio of HNB to protein

HNB(1.05):lysozyme lysozyme substituted with 1.05 moles of HNB per mole of lysozyme

NAG N-acetyl glucosamine

NBS N-bromosuccinimide

RCM reduced and carboxymethylated

TPCK tosyl-L-phenylalanine chloromethyl ketone

BH_4 the borohydride anion

$BH_4(T_4)$ mixture of BH_4 and tritiated BH_4

PPO 2,5-diphenyloxazole

POPOP p-bis[2-(5-phenyloxazolyl)]-benzene

TEOFB triethyloxonium fluoroborate

NMR nuclear magnetic resonance

Abstract

The reaction of hen egg white lysozyme with 2-hydroxy-5-nitrobenzyl bromide (HNB-Br) has been studied. The major product of this reaction was shown to be an inactive, monosubstituted derivative formed by the reaction of HNB-Br with the tryptophan residue at position 62 in the enzyme. Studies of the HNB:lysozyme adduct indicated that a spontaneous hydrolysis occurs at acidic and neutral pH which is characterized by the liberation of 2-hydroxy-5-nitrobenzyl alcohol (HNB-OH) and the concomitant regeneration of enzymatic activity. The regeneration of activity and the release of HNB-OH proceed to 80 per cent completion, which corresponds to the extent of substitution of tryptophan 62 in the monosubstituted protein.

The factors which are concerned with the liberation of HNB-OH and the regeneration of enzymatic activity have been studied. Two groups within the HNB:tryptophan adduct have been implicated in the hydrolysis reaction. The 2-hydroxy group of HNB moiety is required since it has been shown that either acetylation of this group or the substitution of a methoxy group for the hydroxy group prevents hydrolysis. The indolenine $\rangle C{=}N-$ group which is presumed to be produced upon reaction of HNB-Br with tryptophan also appears to be necessary for hydrolysis since reduction of this group by borohydride prevents hydrolysis. In addition

to the groups indicated there is also a conformational requirement imposed upon the HNB:lysozyme adduct. Urea denaturation or reduction and carboxymethylation of the substituted protein prevents regeneration. The peptide carrying the label bound to tryptophan 62 is also stable with respect to liberation of HNB-OH. The effect of pH and the effect of deuterium incorporation on the rate of hydrolysis indicate a reaction which is subject to specific hydrogen ion catalysis involving a group with a pK_a of approximately 6.5 which participates in the hydrolysis process. A tentative identification of the group with $pK_a \sim 6.5$ as the indolenine $\rangle C{=}N-$ has been made.

The results of the studies concerned with the hydrolysis of HNB:lysozyme have been utilized to propose a tentative mechanism for this reaction.

I. Introduction

In 1922, Alexander Fleming discovered a substance in his own nasal mucous which was capable of dissolving certain bacteria (1). This discovery and the subsequent studies through the years have led to the point where this substance is perhaps the best understood of all complex biological materials. Fleming showed that the active material was a protein and he called this substance lysozyme because of its ability to lyse the bacterial cell wall.

Lysozymes (E.C. 3.2.1.17) (2) are widely distributed in nature, occurring in a great number of human organs and secretions as well as in other vertebrates, invertebrates, plants, bacteria and bacteriophages (3-9). With the exception of papaya lysozyme (10), molecular weight 25,000 and certain phage lysozymes (11), molecular weight about 18,000, all lysozymes are characterized as basic proteins, with molecular weights around 15,000, they all lyse certain bacterial.

The studies by Salton (12) in 1952, indicated that the substrate for lysozyme was the cell wall mucopolysaccharide of the bacteria Micrococcus Lysodeikticus. Berger and Weiser (13) subsequently showed that the hen egg white enzyme is capable of hydrolyzing chitin, a linear polymer of N-acetyl glucosamine which is joined by $\beta(1\rightarrow 4)$ linkages. Both types of substrate, either chitin or the cell wall

mucopolysaccharide, are polymers of $\beta(1 \rightarrow 4)$ linked N-acetyl glucosamine. However, in the cell wall mucopolysaccharide every alternate residue bears a 3-O-D-lactyl side chain, the lactyl ether of N-acetyl glucosamine being known as N-acetyl muramic acid. When the enzyme acts on chitin or other synthetic substrates of N-acetyl glucosamine polymers, the enzyme may be aptly termed an N-acetyl-glucosaminidase. However, when acting on cell wall saccharides, cleavage occurs only at the glycosidic bond of N-acetylmuramic acid and as such, the enzyme is termed an N-acetylmuramidase.

Hen egg white lysozyme is one of the most extensively studied enzymes and was the first enzyme for which the relation between structure and function has become clear. The primary structure of the enzyme was elucidated independently by Canfield (14,15) and Jolles (16,17). The protein consists of 129 amino acids which form a single polypeptide chain which is cross linked by four disulfide bonds. X-ray crystallographic studies, at $2A^{o}$ resolution, conducted by Phillips and his group (18,19), have been utilized to determine the three dimensional structure of the enzyme. The results of these studies indicate that the enzyme is roughly ellipsoidal in shape, with a deep cleft running up one side of the molecule. Further crystallographic studies on the structure of the enzyme have been conducted in the presence of various inhibitors (20-22). The results of the crystallographic studies with N-acetyl glucosamine and tri N-acetyl glucosamine have been discussed in great detail, along with

the results of many other studies, by Chipman and Sharon (23) with regard to the mechanism of action of the enzyme. The results of these studies can be described by referring to Figure 1.

The crystallographic study of the binding of tri N-acetyl glucosamine to lysozyme shows that the inhibitor binds to the protein within the cleft and fills approximately one half of it. The non reducing end of the inhibitor molecule is at the top of the cleft in a region designated subsite A, while the other two carbohydrate units are in regions designated as subsites B and C. A number of interactions between the inhibitor and the enzyme are observed, notably the formation of six hydrogen bonds of which four are interactions with the carbohydrate moiety in subsite C. These hydrogen bonds and additional interactions between the sugar residue in subsite C and the protein account for more than one half of the total free energy change in binding of a hexasaccharide to the enzyme (23). On the basis of models constructed with larger polysaccharides, three additional subsites D, E and F have been proposed. Examination of the model with six saccharide units leads to the following inferences: (i) Because of steric interactions between the enzyme and the CH_2OH group on carbon 5 of the pyranose residue at subsite D, the pyranose ring must be distorted from its normal conformation into a half chair conformation. (ii) If the cell wall oligosaccharide combines with the enzyme in the same way as the chitin oligosaccharides, the

muramic acid residues cannot fit into subsites A, C or E because of steric interactions between the lactyl side chain and the protein. The muramic acid residues can be bound, however, at subsites B, D and E because the lactyl side chain now projects outwards from the cleft. (iii) Since it is known that the position of cleavage of the cell wall polysaccharides occurs between muramic acid and glucosamine residues, the position of cleavage must be between residues B and C or D and E. (iv) Since tri N-acetyl glucosamine binds at subsites A through C and is a stable complex it is presumed that cleavage must occur between subsites D and E. Further examination of the model indicates that the most reactive groups on the enzyme in the region of subsites D and E are the carboxyl groups, of glutamic acid 35 and aspartic acid 52, which are found on either side of the $\beta(1\rightarrow 4)$ linkage which is the site of cleavage.

Both physical and chemical studies have been utilized to study the properties of the groups involved in the catalytic action of the enzyme. From the results of the interaction of tri N-acetyl glucosamine with lysozyme, many investigators have shown that two carboxyl groups with apparent pK values of about 6 and 4.2 are involved (24-30). Chemical modification studies by Koshland (31) and Parsons and Raftery (32-35) have both shown the importance of the carboxyl groups of aspartic acid 52 and glutamic acid 35 in the mechanism of action of the enzyme.

The implications of the model and the known involve-

ment of the carboxyl groups allows for the proposal of a mechanism of hydrolysis and its specificity with respect to the cell wall polysaccharide (20-22). Returning to Figure 1, the events leading to the rupture of the bacterial cell wall probably takes the following course: (i) The substrate is bound within the cleft and held stationary by hydrogen bonds and other forces. During this binding the fourth sugar residue, which binds at subsite D, is distorted and attains a half chair conformation. (ii) The glutamic acid residue at position 35, which has a high pK_a, protonates the glycosidic oxygen. (iii) Cleavage of the glycosidic bond yields a carbonium ion which is stabilized by the negative charge on aspartic acid 52, pK_a approximately 4. (iv) The disaccharide at subsites E and F diffuses away and the carbonium ion is attacked by water thus completing the hydrolysis.

The study of the mechanism of action of the enzyme has dealt primarily with the study of those amino acids which are directly responsible for the hydrolysis of the substrate. Although much is known about the actual mechanism of hydrolysis and the amino acids participating in this process, somewhat less information is available concerning the roles of other amino acids which are implicated in the action of the enzyme.

The importance of one or more tryptophan residues, in the action of the enzyme, has been implied by many investigators, utilizing methods ranging from X-ray

diffraction studies to chemical modification.

Of the six tryptophan residues in the molecule (14), three are on the surface of the cleft, these are residues 62, 63 and 108, and one additional tryptophan, residue 123, is exposed on the surface of the molecule. The exposure of the indole nucleus of the various tryptophans indicate that tryptophan 62 is the most exposed, with 60 per cent of the indole nucleus exposed, followed by residues 123, 63 and 128 in order of decreasing exposure (36). Additional X-ray crystallographic data in the presence of the inhibitor, tri N-acetyl glucosamine, implicate tryptophan residues 62 and 63 as being involved in the formation of hydrogen bonds with the inhibitor at subsite C (20-22). With respect to the binding of this inhibitor to lysozyme studied by X-ray methods, it is relevant to note that upon formation of the enzyme inhibitor complex the molecule moves, in such a way as to narrow the cleft with the indole side chain of tryptophan 62 moving some 0.75 A° (20-22). In addition to X-ray crystallographic studies, solvent perturbation studies have been performed and these results indicate the accessibility of four tryptophan residues to solvent (37, 38). Difference spectra studies have concluded that essentially one specific tryptophan residue is involved in the production of a difference spectra upon the binding of the substrate, chitin. This same tryptophan residue is preferentially oxidized by N-bromosuccinimide and it was shown that this residue is necessary for both activity and the difference spectrum since

the loss of this one residue led to an 80 per cent loss of both activity and the difference spectrum (39). Bello has indicated the availability of 3.5-4 exposed tryptophanyl residues in native lysozyme and 5.9-6.2 in the presence of 6M guanidine by thermal perturbation studies (40).

The results of many chemical modifications of hen egg white lysozyme also implicate tryptophan residues in the activity of the enzyme. Hayashi and co-workers (41) have demonstrated that N-bromosuccinimide oxidizes tryptophan 62 with the concomitant decrease of the enzymatic activity. Iodine, in equimolar amounts at pH 5.5, effects the selective oxidation of a different residue, namely tryptophan 108, and leads to the production of a completely inactive enzyme (42). In addition to the effect of iodine on tryptophan 108, Blake (43) has shown changes in the X-ray diffraction pattern of lysozyme crystals, iodinated at pH 4.7 with 5 moles of iodine per mole of protein. Under these conditions both tryptophan 108 and tryptophan 62 are oxidized. In contrast to the iodination studies, treatment of lysozyme with ozone in anhydrous formic acid led to the conversion of both tryptophan 108 and 111 to N-formylkynurenine residues without loss of enzyme activity (44). Photooxidation of lysozyme in the presence of methylene blue led possibly to the degradation of tryptophan 28 and most of the activity of the enzyme was lost (45). Treatment of the enzyme with hydrogen peroxide causes inactivation and it was concluded that the loss in activity occurred when 1.4 tryptophan

residues were modified (46). Veronese, Boccu and Fontana (47) have modified lysozyme by reaction with 2-nitro-4-carboxyphenylsulfenyl chloride. Analysis of the major substituted protein derived from reaction with one equivalent of the sulfenyl chloride showed the modification of tryptophan 108 with a 90 per cent loss in activity. In a second study, Shechter, Burnstein and Patchornik (48) have isolated the sulfenylated protein produced by reaction of lysozyme with 2-nitrophenylsulfenyl chloride. The major product was devoid of activity and was characterized as the sulfenylated derivative of tryptophan 62.

The different results of the chemical modification studies reflects either the lack of specificity of the reactions, or the experimental conditions imposed upon reaction. For example, the reaction of lysozyme with N-bromosuccinimide, as performed by Kronman, Robbins and Andreotti (49), demonstrated that in addition to tryptophan oxidation, residues of histidine and tyrosine are also modified. Also, the studies of Jori et al. (50) have shown that the loss in activity of lysozyme during methylene blue sensitized photooxidation resulted from the selective oxidation of methionine residues. The specificity of the sulfenylation reactions reported by Veronese et al. and Shecter et al. must be observed with respect to experimental conditions. The reaction which yields tryptophan 108 modified lysozyme was carried out in 25 per cent acetic acid with the addition of one equivalent of sulfenyl chloride,

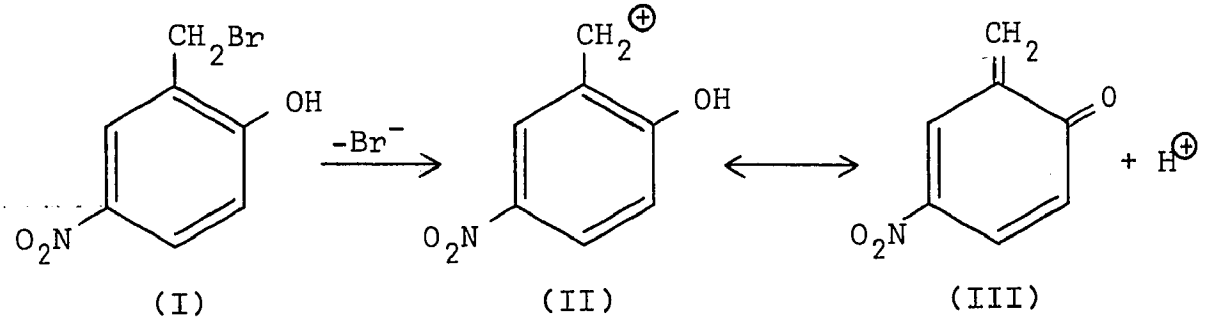
while modification of tryptophan 62 was performed in 0.1 M acetate buffer pH 3-4 with the addition of 40 equivalents of sulfenyl chloride.

A specific reagent is available which modifies only tryptophan using the appropriate conditions; it is 2-hydroxy-5-nitrobenzyl bromide, HNB-Br. It is a reagent which can be used under mild conditions. An examination of the specificity of the reagent towards modification of all amino acids shows that only tryptophan, cysteine and tyrosine are modified (51). In neutral or acidic solution, reaction with tryptophan is extremely rapid while cysteine is only one fifth as reactive as tryptophan and tyrosine does not react at all. In alkali, the reaction losses some specificity and both cysteine and tyrosine react to almost the same extent as tryptophan. Besides showing a high degree of selectivity in reaction with tryptophan, the reagent also has a high degree of reactivity. The determination of the rate of hydrolysis of the reagent in aqueous solution indicates a half life of less than thirty seconds and an approximation of the relative reactivities of tryptophan and water indicate that tryptophan is at least 10,000 times more reactive (51). The rapid hydrolysis of excess reagent and the mild conditions for reaction make this reagent suitable for modification studies since long incubation of protein and modifier are not necessary. In addition, the reaction of the reagent with proteins introduces a p-nitrophenol group which absorbs in the visible portion of the spectra in basic

solution and provides for the easy determination of the number of such groups incorporated.

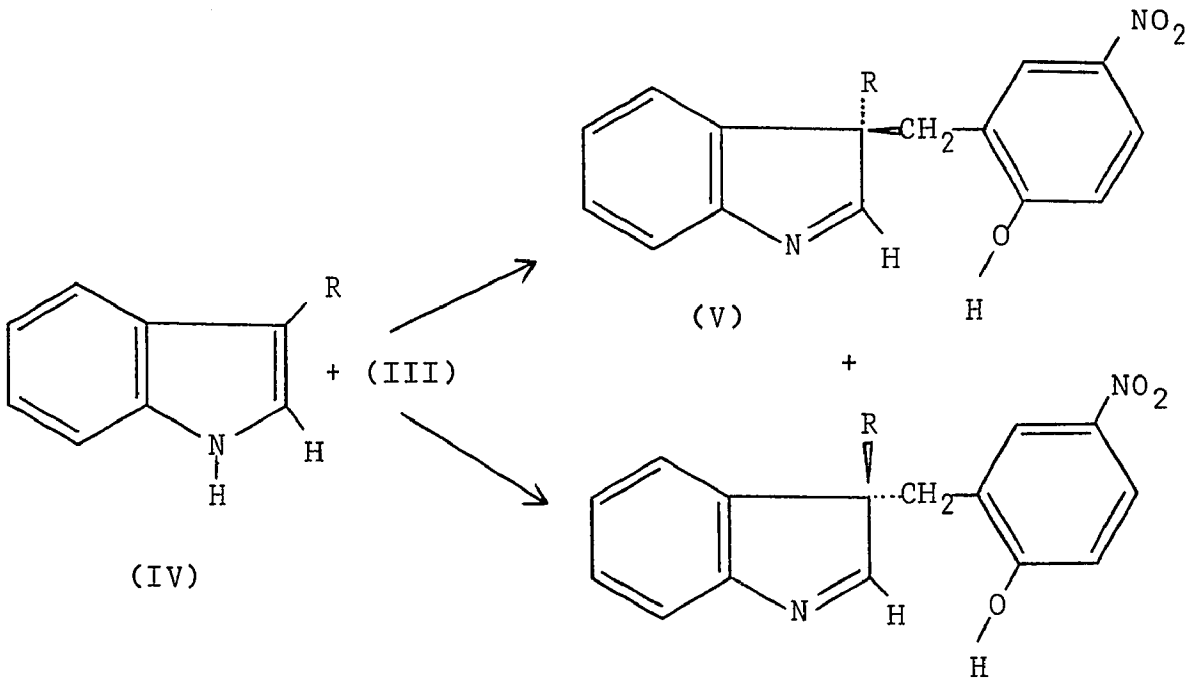
The high degree of selectivity, extreme reactivity under mild conditions and the relative ease of quantitation has led to a wide variety of uses for the reagent. In addition to the use of the reagent for the implication of tryptophan residues involved in the biological activity of proteins and enzymes (52-57), the reagent has been used to estimate the tryptophan content of proteins (40, 52,58-60) and to aid in the separation of tryptophan containing peptides (61).

The chemistry of the reaction of HNB-Br with tryptophan ethyl ester has been discussed in detail by Loudon and Koshland (62). The pertinent features of the reaction are shown in equations 1 through 3. The high reactivity of the HNB-Br reagent has been suggested to arise from the quinone methide species (III), shown in equation 1, which provides resonance stabilization for the 2-hydroxy-5-nitrobenzyl carbonium ion (II) (51). The quinone methide has been postulated to be the reactive species which is involved in the formation of a charge transfer complex with the 3 position of the indole nucleus (62). This charge transfer complex leads to reaction by a net transfer of a proton from the indole nitrogen to the phenolic oxygen, yielding the alkylated indole. Since the reaction of HNB-Br with the indole nucleus leads to the generation of a new asymmetric center, at position 3, two diasteromeric



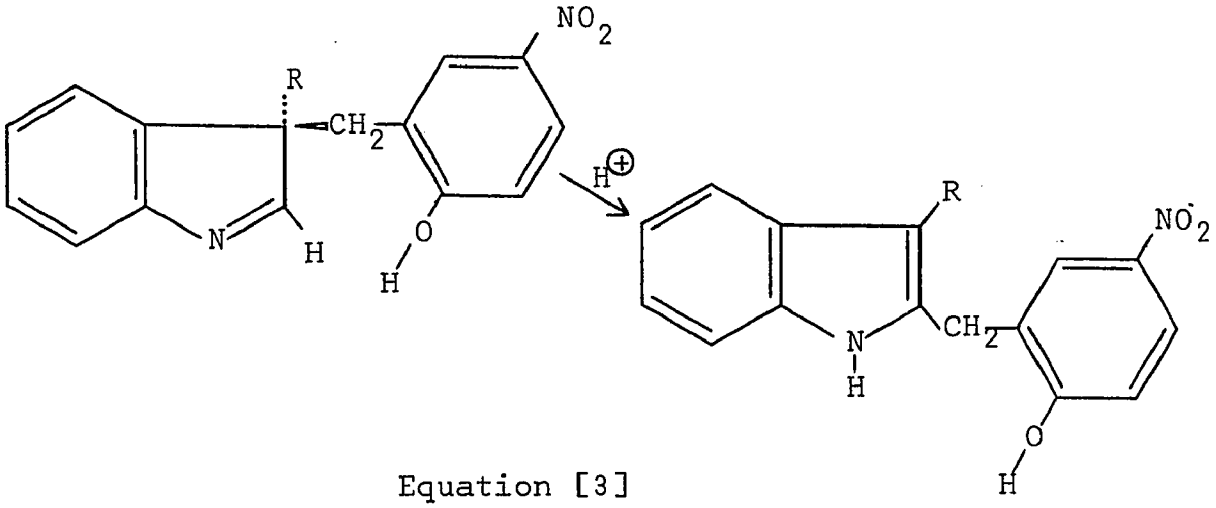
(I) (II) (III)

Equation [1]



(IV) (V)

Equation [2]



Equation [3]

products may be formed, equation 2. The 3,3 disubstituted indolenines formed, in equation 2, may undergo an acid catalyzed rearrangement to yield a 2,3 disubstituted indole, equation 3 (62).

A study of the involvement of tryptophan residues in the action of hen egg white lysozyme by modification with HNB-Br should be extremely specific due to the specificity and reactivity of this reagent and the lack of sulfhydryl groups in the enzyme which may interfere.

The reagent has been utilized in previous studies on lysozyme both to determine the number of tryptophans in the molecule (59), and to determine the number of such residues exposed (40). The effect of HNB-Br on the activity of lysozyme has been studied by Bewley and Li (52) who have shown the incorporation of two moles of HNB without any loss in activity. Additional studies concerned with the reaction of HNB-Br with lysozyme indicate the presence of one particularly reactive tryptophan residue which is necessary for activity and which appears to produce a labile HNB:lysozyme derivative (63-65). The lability of the HNB group in lysozyme, which leads to the regeneration of enzyme activity is analogous to the regeneration of inhibited acyl chymotrypsins studied by Kaiser (66-70). Both systems are characterized by the spontaneous reversion of the inhibited enzyme to the fully active enzyme with the concomitant release of the inhibitor moiety.

The present study is concerned with the characterization of the reaction of HNB-Br and hen egg white lysozyme with respect to the inhibition of the enzyme and the mechanism of the reaction which labilizes the HNB group.

II. Experimental

A. Reagents

Egg white lysozyme lots 9AA and OCC, Trypsin-TPCK lot OFA and Micrococcus Lysodeikticus lot 8JA were products of Worthington Biochemical Corp. Human lysozyme was a generous gift of Dr. E. F. Osserman. 2-hydroxy-5-nitrobenzyl bromide and N-acetyl glucosamine were obtained from Calbiochem Inc. Sephadex G-10 and G-25 were obtained from Pharmacia Fine Chemicals Corp. Bio-Rex 70 (100-200 mesh) and (200-400 mesh) were products of Bio-Rad Laboratories. Deuterated compounds were obtained from International Chemical and Nuclear Corp. Tritium labeled sodium borohydride was obtained from New England Nuclear Corp. Triethyloxonium fluoroborate was synthesized according to the method of Meerwein (71). All reagents obtained from commercial sources were of analytical grade and were used without any further purification with the exception of iodoacetic acid, which was recrystallized from ethanol-petroleum ether prior to use, M.P. 81-82° (uncorr.) and 2-hydroxy-5-nitrobenzyl bromide, which was recrystallized three times from hot benzene, M.P. 144.5-145.5° (uncorr.).

B. Buffers

Buffer solutions were all prepared at an ionic strength of 0.10 according to the methods outlined in the Biochemist's Handbook (72), unless otherwise specified.

C. Assay of lysozyme.

The activity of lysozyme was measured spectrophotometrically by following the decrease in absorbance at 450 nm, of 3.00 ml of an aged (at least three days old) suspension of Micrococcus Lysodeikticus at a concentration of 0.30 mg/ml in 0.10 M phosphate buffer pH 7.00 at 30°. Aliquots of enzyme contributing no more than a two per cent increase in volume were added to the substrate and the decrease in absorbance between the first and third minutes were taken as a measure of the activity. Changes in absorbance were recorded on a Leeds and Northrup Speedomax H-Azar recorder. The recorder, used in conjunction with a Bausch and Lomb Spectronic 70 spectrophotometer, a Philbrick logarithmic operator, model 4350, and model PR-30 regulated power supply and an Electronic Scientific Industries voltage divider, model DV 411, could be adjusted to give a full scale deflection for a change in absorbance as small as 0.050. One unit of activity is equal to a change in absorbance of 0.001 per minute, under the specified conditions. Specific activity is defined as activity per milligram of protein.

D. Determination of protein concentration.

Lysozyme concentration was determined by the method of Folin-Ciocalteau (73) or by absorbance at 280 nm; $A_{280}^{1\%} = 2.51$, $\epsilon = 35,900$. In the case of HNB:lysozyme, protein concentrations were determined by the same methods. In the ultraviolet method correction for the absorbance of the HNB group at 280 nm was determined by the following equation:

Protein concentration = Eq. [4]

$$\frac{O.D._{280}\ pH<4 - 0.197\ O.D._{420}\ pH>11}{35,900}$$

This is a corrected Beer's law equation where 0.197 is the ratio of the absorbance of the HNB chromophore at 280 nm in acid solution to its absorbance at 420 nm in basic solution.

E. Preparation of HNB:lysozyme or HNB:lysozyme derivatives.

Solutions of lysozyme, or the lysozyme derivatives in the appropriate solvents, were prepared and maintained at 0-2° in an ice bath. Solutions of HNB-Br were prepared in $CaCl_2$-dried acetone and were maintained in an ice bath. The reaction of HNB-Br with lysozyme was accomplished by rapidly adding, with stirring at 0-2°, the appropriate amount of HNB-Br solution to the lysozyme solution. In all reactions carried out the HNB-Br solution was prepared at a concentration such that the required molar excess of reagent could be added without bringing the acetone to a final concentration greater than 10 per cent v/v, such a concentration was shown to have no effect on the enzymatic activity. Within one

minute of the addition of HNB-Br to the protein, the solutions were assayed and either returned to the ice bath, frozen for future study, or chromatographed.

F. Gel filtration.

Gel filtration on columns of Sephadex G-10 (0.9x25 cm), equilibrated and eluted with 0.18 M acetic acid, pH 2.80, was routinely used for removal of any low molecular weight material for example, salts, HNB-OH, acetone, etc., from protein solutions prior to any determination, where such materials might interfere. The entire protein fraction which emerged prior to any small molecules was either studied immediately or frozen for subsequent studies.

G. Determination of HNB:lysozyme ratios.

Following the removal of non-protein material from HNB:lysozyme by gel filtration the ratio of HNB:lysozyme was evaluated from the following formula which compares the concentration of the HNB group to the concentration of the protein.

Ratio of HNB:lysozyme = Eq. [5]

$$\frac{O.D._{420}\ pH > 11}{O.D._{280}\ 1\%\ HAc - 0.197\ O.D._{420}\ pH > 11} \times 1.89$$

where 0.197 is the correction factor for the absorbance of the HNB group at 280 nm:

$$1.89 = \frac{\epsilon_{280}\ \text{lysozyme in 1\% HAc, (35,900)}}{\epsilon_{420}\ \text{HNB pH} > 11, \quad (19,000)}$$

H. Preparation and isolation of HNB (1.05):lysozyme.

Lysozyme, 2.00 gm (0.139 mmoles) in 20 ml of 0.18 M acetic acid pH 2.78, was reacted, at 0-2°, with 80.7 mg (0.348 mmoles) of HNB-Br, dissolved in 2.0 ml of $CaCl_2$-dried acetone. The entire reaction mixture was then chromatographed on a column of Sephadex G-25 medium (5 x 36 cm) which was jacketed and maintained at 3° by circulating water. The column was equilibrated and eluted with 0.18 M acetic acid pH 2.78 and the entire protein peak was immediately frozen and lyophyllized.

Bio-Rex 70 (100-200 mesh) was washed thoroughly and a column (4 x 65 cm) was prepared. The Bio-Rex 70 column was poured over a one cm layer of Sephadex G-25 coarse in order to assure good flow rates. The column was equilibrated overnight at 4° with 0.05 M Borate-NaOH buffer pH 9.80 at a flow rate of approximately 100 ml per hour.

HNB (1.16):lysozyme (1.98 gm) derived from the initial reaction of lysozyme with HNB-Br, was dissolved in 20.0 ml of equilibrating buffer at 4° and applied to the column. The column was eluted with a two step concentration gradient. Elution of the column was begun at a flow rate of 97 ml per hour with 0.05 M Borate-NaOH buffer pH 9.80 and 20 ml fractions were collected automatically. After the elution of HNB-OH, which was characterized by its spectral properties (λ_{max} = 408 nm in basic solution), the protein was eluted with 0.23 M Borate-NaOH buffer pH 9.80 at the same flow rate. Fractions were analyzed for absorbance at 280 nm and 420 nm

and also for enzymatic activity. Those fractions containing inactive HNB:lysozyme were pooled for concentration.

Concentration of the large volumes of solution was accomplished by diluting the pooled fractions with 3.5 volumes of water and adding small portions of dry Bio-Rex 70. The proteins become bound to the resin due to the decreased ionic strength of the medium. The solutions containing the resin were stirred in the cold for 15 minutes and the resin allowed to settle. The supernatant was analyzed for absorbance at 280 nm and the addition of resin was continued until the absorbance at 280 nm was less than five per cent of the original. The resin containing the bound protein was recovered by decanting and discarding the supernatant solution.

Protein was recovered from the resin by washing the resin on a coarse sintered glass funnel with 4 M NaCl. The washing procedure was continued until no further protein was extractable.

The solutions of HNB:lysozyme in 4 M NaCl were either frozen or immediately desalted on a column of Sephadex G-25 (5 x 36 cm) at 3°, eluted with 0.18 M acetic acid pH 2.80. The protein which emerges prior to NaCl was immediately frozen and lyophyllized.

I. Analytical chromatography of lysozyme and HNB:lysozyme on Bio-Rex 70.

Analytical column chromatography was performed on columns (0.8 x 100 cm) of Bio-Rex 70 (200-400 mesh). The elution of the column was accomplished with the same system used in the purification of HNB(1.05):lysozyme. Selected fractions were analyzed for absorbance at 280 nm and 420 nm and for enzyme activity.

J. Spectrophotometric titration of HNB-OH and HNB(1.05):lysozyme.

Solutions of HNB-OH and HNB(1.05):lysozyme were prepared at concentrations of approximately 5×10^{-5} M and their spectra in alkaline media (pH > 11) were recorded on a Cary Model 14 spectrophotometer. These samples were then adjusted to pH 2.40 and 4.00 respectively with HCl. The absorbance at 408 nm or 420 nm was measured with a Beckman DU monochromator equipped with a Gilford photometer. The samples were titrated by adding small aliquots of NaOH and the pH of the resulting solution was measured with a Radiometer pH meter type TTTlC. The repetitive process of adding NaOH, measuring the pH and measuring the absorbance, was continued until no further change in absorbance was observed. The pK_a for each sample was determined as the pH at the midpoint between maximum and minimum absorbance from a plot of absorbance versus pH.

K. Regeneration of HNB:lysozyme.

In all regeneration studies samples of HNB:lysozyme, HNB:lysozyme derivatives and native lysozyme, were prepared at concentrations of approximately 5.0 mg/ml in water at 0-2°. Various buffer solutions were utilized with or without the addition of appropriate materials and these were preincubated at the required temperatures. At zero time, an aliquot of the enzyme solution was added to the buffer to yield a final protein concentration of 0.50 mg/ml. After the addition of the protein, aliquots were removed at various time intervals and assayed for either enzyme activity or the ratio of HNB:lysozyme.

L. Determination of rate constants.

Rate constants for the pseudo first order regeneration reaction were determined from plots of log per cent inhibition versus time. The per cent inhibition at any time was calculated from the following equation:

$$\text{Per cent inhibition} = 100 - \left(\frac{A_t - A_o}{A_\infty - A_o}\right)100 \qquad \text{Eq. [6]}$$

where A_t = activity at any time, t.

A_o = activity at zero time.

A_∞ = activity at completion of regeneration.

Plots of log per cent inhibition versus time were linear and the half life was determined and used for the evaluation of the rate constant from the relationship:

$$k = \frac{0.693}{t_{1/2}} \qquad \text{Eq. [7]}$$

M. Difference spectra of HNB(1.05):lysozyme and native lysozyme with NAG.

The difference spectra with N-acetyl glucosamine were measured in matched quartz tandem cells of 0.875 cm path length on a Beckmann DU monochromator equipped with a Gilford photometer and a thermostated cell compartment maintained at 30°.

The difference spectrum for lysozyme and NAG was determined by the following procedure: 1.00 ml of lysozyme solution (2.0 mg/ml in 0.10 M phosphate buffer, pH 8.00) was placed in one compartment of each of two cells and 1.00 ml of 0.40 M NAG, in the same buffer, was added to the second compartment. The contents of one of the cells were mixed by inversion and the absorbance of both cells were measured versus buffer in the range of 350-260 nm. The difference spectrum was obtained by plotting the difference in absorbance of the mixed cell with respect to the unmixed sample as a function of wavelength.

The difference spectrum for HNB(1.05):lysozyme and NAG was determined in an identical procedure except that the concentration of NAG was 0.32 M and the spectrum was recorded in the region of 250-550 nm.

N. Determination of binding constants for HNB(1.05):lysozyme and native lysozyme with NAG.

The procedure used for the determination of the binding constants was essentially the same as that used for the generation of the difference spectra. A 1.00 ml aliquot of lysozyme, 7.14×10^{-5} M in 0.10 M phosphate buffer pH 8.00,

was placed in one compartment of each of two tandem cells. The second compartment of each cell received 1.00 ml of a solution of NAG in the same buffer at concentrations ranging from 0.005 to 0.400 M. The absorbance at 294 nm of the reference cell and the sample cell were measured. The contents of the sample cell were mixed and the absorbance of each cell was measured. The difference in absorbance of the sample cell relative to the reference cell was calculated taking into account changes observed in the reference cell.

The procedure for HNB(1.05):lysozyme and NAG was the same except that the concentration of HNB(1.05):lysozyme was 6.85×10^{-5} M and changes in absorbance were measured at 418 nm. Due to the instability of HNB:lysozyme the stock solution was maintained at 0-2° and was quickly warmed to 30° prior to observing the difference spectra.

The binding of NAG to lysozyme or HNB:(1.05):lysozyme may be described by:

$$\log \frac{[ES]}{1-[ES]} = n \log [NAG] - \log K \qquad \text{Eq. [8]}$$

where:

$[ES] = \dfrac{\text{difference spectra at [NAG]}}{\text{difference spectra at [NAG]}_{\rightarrow \infty}}$

$1-[ES] = E_o$ = free enzyme

n = number of binding sites for NAG

K = a single equilibrium constant related to n "conventional" equilibrium constants.

In deriving equation [8] the following assumptions are made:

1) $[NAG] \gg [E_o]$

2) the sites binding NAG are identical.

3) there are no interactions between the sites.

If the above assumptions are met, a plot of log [NAG] versus log $\frac{[ES]}{1-[ES]}$ should give a straight line whose slope is n and whose intercept is K. Difference spectra at [NAG] = ∞ were obtained from extrapolation of Eadie plots to infinite concentration.

O. Preparation of MNB:lysozyme.

Identical aliquots of a lysozyme solution, 1.39×10^{-4} M in 0.18 M acetic acid, pH 2.80, were incubated at 30° and at timed intervals various amounts of 3.45×10^{-2} M MNB-Br in dry acetone were added to give molar excesses of 0.25-10.0. The samples were assayed immediately and at various intervals during the incubation at 30°. All samples were subjected to gel filtration to remove unreacted MNB-Br, MNB-OH and acetone and the ratio of MNB:lysozyme was determined. The MNB:lysozyme ratio was evaluated from the following equation:

$$\frac{\text{O.D.(320 nm)}}{\text{O.D.(280 nm)} - 0.393\ \text{O.D.(320 nm)}} \times 3.78 \qquad \text{Eq. [9]}$$

where 0.393 is the ratio of the absorbance of the MNB group at 320 nm to its absorbance at 280 nm and;

$$3.78 = \frac{\epsilon(280)\ \text{lysozyme in 1\% HAc (35,900)}}{\epsilon(320)\ \text{MNB group in 1\% HAc (9,500)}}$$

P. Preparation of human HNB:lysozyme.

The procedure used for the preparation of human HNB:lysozyme was the same as that used for the hen egg lysozyme.

Q. Deuterium isotope effect on the regeneration of HNB(1.05):lysozyme.

Deuterated HNB(1.05):lysozyme and deuterated lysozyme were prepared by first dissolving 11.0 mg of HNB(1.05):lysozyme or 15.0 mg of native lysozyme in 1.00 ml of D_2O and adding 6N NaOD in D_2O until the pH was greater than eleven. The resulting solution was left at room temperature for three hours. After incubation the solution was cooled in an ice bath and brought to pH 3 with glacial deuterated acetic acid. The deuterated proteins were then subjected to gel filtration on a column of Sephadex G-10 (0.9 x 25 cm) which was equilibrated and eluted with 0.18 M DAc in D_2O. The protein fractions isolated from the columns were lyophyllized.

Buffer solutions for use in the regeneration system were prepared by adjusting 0.18 M HAc to pH 4.00 with NaOH and 0.18 M DAc in D_2O to pH 3.59 with NaOD so that both pH and pD equal 4.00. The pD was calculated from the relationship:

$$pD = pH + 0.41 \qquad \text{Eq. [10]}$$

Samples of deuterated and normal lysozyme and HNB:lysozyme were maintained under conditions which lead to regeneration by the usual method.

R. Preparation, isolation and characterization of HNB(0.96):peptide T_8-T_9.

Native or HNB(1.05):lysozyme was reduced and carboxymethylated by the procedure of Canfield and Anfinsen (74), except that the final precipitate was washed twice with 95 per cent ethanol, suspended in water and lyophyllized.

The RCM proteins were subjected to tryptic digestion. In a typical digestion 250 mg of RCM-protein was suspended in 15 ml of water. Thorough wetting of the protein was accomplished by adjusting to pH 10.5 with NaOH followed by reprecipitating the protein by adjusting to pH 8.0 with HCl. Digestion was performed by the addition of 8.0 mg of TPCK treated trypsin, in small portions, over a three hour period, at room temperature. During digestion the solution was stirred and maintained at pH 8.0 by the addition of NaOH. After three hours the protein solution was centrifuged to remove any insoluble material and the clear supernate was studied immediately or frozen for future use.

A buffer solution, consisting of 0.2 M pyridine - 1.0 M acetic acid, pH 3.80, was added to the soluble tryptic digest of RCM-HNB(1.05):lysozyme in small aliquots with constant stirring until the solution became turbid. A small excess of buffer was added and the resulting solution frozen. The frozen sample was allowed to thaw slowly at 4° and upon completion of the thawing process the sample was centrifuged and the precipitate collected. The precipitate was washed twice with two 5.0 ml aliquots of pyridine-acetate buffer. The initial supernate and the subsequent wash solutions were

analyzed for HNB absorption and pooled. The washed precipitate was suspended in water, made slightly alkaline with dilute ammonium hydroxide and finally lyophyllized. The pooled supernate and wash solutions were frozen and the freeze-thaw extraction process was repeated.

The same pyridine-acetate extraction procedure was applied to a sample of the tryptic digest of RCM-lysozyme.

A sample of the peptide isolated by pyridine-acetate precipitation was hydrolyzed, for 20 hours at 110°, with constant boiling 6N HCl in vacuo. Amino acid analysis was accomplished on the long column of the Beckmann model 116 Amino Acid Analyzer. The concentration of the sample was determined from the absorbance of the standard amino acid solution used to calibrate the column. An aliquot of the solution used for analysis was also used for a determination of the HNB concentration by spectral methods.

S. Polyacrylamide gel electrophoresis.

Samples of native lysozyme and HNB(1.05):lysozyme at zero time and at various times during the regeneration process were subjected to polyacrylamide disc gel electrophoresis. Seven per cent acrylamide gels were prepared and run according to the method of Orenstein and Davis (75, 76) at pH 8.6 and a current of 4 ma/gel, with the protein migrating toward the cathode. The gels were stained with amido black for one hour and were electrophoretically destained.

T. Reaction of HNB:derivatives or lysozyme with $NaBH_4(T_4)$.

Samples of HNB(1.05):lysozyme, native lysozyme or mixtures of both were prepared at a concentration of 2.0 mg/ml in 0.50 M acetate buffer, pH 4.0 and maintained at 0-2°. Solutions of $NaBH_4$ or $NaBH_4(T_4)$ were prepared in 10^{-4} M NaOH at concentrations between 0.20 and 0.40 M and also maintained at 0-2°. Reactions of protein with either BH_4 or $BH_4(T_4)$ were accomplished by the slow addition to the protein of various amounts of the appropriate BH_4 solutions while stirring rapidly and maintaining low temperature. Reaction products were immediately assayed for activity, HNB content and ability to regenerate activity. Those samples that were produced by reaction with $BH_4(T_4)$ were counted in a Picker Nuclear Liquimat 220 scintillation counter. Samples were prepared for counting by passage through a column of Sephadex G-10, eluted with 0.18 M acetic acid to remove excess tritium. Samples of up to 0.20 ml were mixed with 15.0 ml of a scintillation cocktail (850 ml toluene, 600 ml absolute ethanol, 0.6 per cent PPO and 0.01 per cent POPOP) and counted.

Reduction of HNB(0.96):peptide T_8-T_9 and HNB(1.05): lysozyme with $NaBH_4(T_4)$ was also performed. The same procedure was applied however due to the insolubility of the peptide at pH 4.0, the reduction was performed at pH 2.50 in a 1.0 M glycine-HCl buffer.

U. Preparation and isolation of $NaBH_4(T_4)$ reduced HNB:peptide.

A solution of 25.0 mg of HNB(1.05):lysozyme in 5.0 ml of 0.50 M acetate buffer, pH 4.0, was maintained at 0-2° and reacted with 1.0 ml of a solution of 0.30 M $NaBH_4(T_4)$. The products of the reaction were subjected to gel filtration and the protein fraction was recovered. The isolated protein was reduced and carboxymethylated, subjected to tryptic hydrolysis and pyridine acetate extraction, as previously described. During the course of purification aliquots were removed and analyzed for HNB concentration and radioactivity.

V. Esterification of lysozyme with TEOFB.

Lysozyme esters were prepared by esterification of the native enzyme with triethyloxonium fluoroborate. The procedure for the preparation of the esters and their isolation was identical with that of Parsons et al. (32). Estimation of the ester content was performed by the method of Hestrin (77).

W. Guanidination of lysozyme or HNB(1.05):lysozyme.

Samples of lysozyme or HNB(1.05):lysozyme were guanidinated by reaction with O-methylisourea according to the method of Kimmel (78). Estimation of the extent of reaction was made by determination of additional guanido groups incorporated into the proteins by a modification of the Sakaguchi reaction (79).

X. Acetylation of lysozyme or lysozyme derivatives.

Samples of lysozyme, HNB(1.05):lysozyme and guanidinated lysozyme were acetylated at pH 7.50 with acetyl imidazole as described by Riordan (80). The extent of acetylation was determined by following the loss in absorbance at 278 nm (80).

Y. Preparation of tryptophan 62 oxidized lysozyme.

Tryptophan 62 oxidized lysozyme was prepared according to the method of Spande (81) utilizing N-bromosuccinimide. The extent of oxidation was determined from the equation derived by Spande and Witkop (82).

Z. Nuclear magnetic resonance spectroscopy.

Nuclear magnetic resonance spectra were recorded on a Varian HR 220 equipped with a computer of average transients. The spectra of samples of native lysozyme and HNB(1.05):lysozyme 15 per cent w/v in 0.18 M acetic acid, pH 2.80, were recorded at 18°.

III. Results

A. The incorporation and subsequent regeneration of HNB:lysozyme species.

The products of the reaction of HNB-Br and lysozyme, produced at pH 2.80, at molar ratios of HNB-Br to lysozyme of 0.526 to 5.26 were analyzed for HNB content and enzymatic activity following gel filtration. Figure 2 shows that approximately 1.30 HNB groups are readily incorporated into lysozyme. The incorporation is closely paralled by a loss in enzymatic activity as seen in Figure 3. Extrapolation of the data in Figure 3 to zero activity yields a molar ratio of HNB to lysozyme of approximately 1.25. Following incubation, at pH 2.80 for 19 hours at 40°, the samples were again analyzed for HNB content and enzymatic activity following gel filtration. The results of the study are tabulated in Table I. The data indicate that all HNB:lysozyme species undergo regeneration of enzymatic activity and liberation of the HNB group, with an average of 81 per cent loss of the labile HNB group and 83 per cent return of activity. A correlation between the absolute number of HNB groups liberated as a function of the initial per cent inhibition is presented in Figure 4. The data indicates that a total of 1.0 moles of HNB per mole of protein are labile in the completely inhibited protein.

B. Effect of pH on the incorporation of HNB into lysozyme and regeneration of the products.

The effects of alteration of the pH in the reaction of HNB-Br with lysozyme are shown in Figures 5 and 6. The curves obtained for specific activity versus pH and ratio of HNB:lysozyme versus pH were both analyzed and inflection points, reflecting pK_a values of 4.05 and 4.15 respectively are obtained.

The data in Table II indicate that the reaction products formed at the various pH's are identical in terms of the lability of the HNB group, and the regeneration of enzymatic activity.

C. Preparation and isolation of HNB(1.05):lysozyme.

The results of the purification of HNB(1.05):lysozyme are tabulated in Table III. The final product obtained from fractions 120-225 in Figure 7 was isolated with an 80 per cent yield. The material with an HNB:lysozyme ratio of 1.16 which was applied to the Bio Rex 70 column must contain some disubstituted material by virtue of its composition. When elution of the Bio Rex 70 column was complete it was observed that there was some yellow material still firmly bound to the top of the column. This material could not be extracted by raising the ionic strength with 1.0 N NaCl. The material was successfully removed by heating the resin to 50° in the presence of 1.0 N NaOH. Spectral measurements indicated that this was multisubstituted HNB:lysozyme.

D. Chromatography of HNB:lysozyme on Bio Rex 70.

Figures 7 to 10 show the results obtained upon chromatography of HNB:lysozyme and mixtures of HNB:lysozyme and lysozyme on columns of Bio Rex 70 (100-200 mesh), with 0.23M Borate-NaOH buffer pH 9.80. The value of this chromatographic method can be clearly seen in Figures 8 and 9 which show the ease of separation of an artificial mixture of HNB-OH, HNB(1.05):lysozyme and native lysozyme; as well as the products formed during the regeneration of HNB(1.05): lysozyme. In addition to separation of HNB:lysozyme from native lysozyme, examination of Figure 7 reveals the separation of two minor components of HNB:lysozyme and a possible separation of what may be two major components. The non symetrical major peaks were pooled in three distinct fractions as indicated in Figure 7. Each of these fractions was isolated according to the methods previously described and characterized in terms of HNB content, per cent activity, extent of regeneration of activity and release of the HNB group. The data are given in Table IV.

The three fractions which were essentially identical were combined and constitute HNB(1.05):lysozyme. HNB(1.05): lysozyme was subjected to analytical chromatography on Bio Rex 70 and the results are shown in Figure 10 and also Table IV. The sample appears to be homogeneous with the exception of a shoulder prior to the main peak, and a minor fraction eluting after the major peak. This minor fraction, chromatographically distinct from the major protein, occupies

a position comparable to that of free lysozyme. It should be noted that during the course of isolation of the substituted protein, conditions which favor regeneration, i.e., maintenance at acidic pH, are encountered. The residual activity of approximately 5.5 per cent which is found in the purified samples is thus due to the formation of free lysozyme during purification. Examination of Figure 10 shows the presence of a small amount of HNB-OH which is consistent with the idea that the lysozyme and residual activity of the sample are due to hydrolysis of HNB:lysozyme.

The sample of HNB(1.05):lysozyme which was regenerated by incubation at pH 2.80 for 24 hours at 37° was chromatographed and the results of the chromatography are seen in Figure 9. An almost clear separation of residual HNB:lysozyme from native lysozyme can be seen. Approximate measurement of the areas under the two peaks give an approximate ratio of HNB:lysozyme to lysozyme of three to seven, which is consistent with a 70 per cent loss of HNB groups and 70 per cent gain of activity.

Characterization of the stable HNB:lysozyme species gives a ratio of HNB to lysozyme of approximately 1.0.

Additional chromatographic procedures were attempted in order to attain separation of the labile HNB:lysozyme and the stable HNB:lysozyme derivatives. Chromatography on Bio Rex 70 at pH 7.2 with 0.2M phosphate buffer, elution at pH 9.80 with Borate-NaOH buffers with a concentration gradient of 0.05M to 0.30M and elution at pH 9.80 with 0.05M

Borate-NaOH and a NaCl gradient of 0.00 to 0.50M were all unsuccessful.

E. Preparation and characterization of peptide T_8-T_9 from HNB(1.05):lysozyme.

The preparation of HNB:peptide T_8-T_9 was accomplished according to the methods previously described. The reduced carboxymethylated HNB(1.05):lysozyme was prepared in 80 per cent yield and the subsequent isolation of the peptide was accomplished with a yield of 74.4 per cent of the soluble products following tryptic digestion, as measured by O.D. at 420 nm. The yields at the various stages in the isolation of the peptide are given in Table V. An identical method was followed with native enzyme which gave an 86 per cent yield of reduced carboxymethylated protein but which failed to yield any precipitate with the pyridine extraction of the tryptic digestion products.

The peptide, isolated by pyridine acetate precipitation, which carries the HNB chromophore yielded an amino acid composition indicated in Table VI. The composition of the peptide coincides with the known composition of the peptides T_8 and T_9 of lysozyme (85). The sample used for amino acid composition was also analyzed for HNB concentration. With respect to the standard amino acid solution the concentration of the peptide is 1.33×10^{-5}M and the concentration of HNB determined spectrophotometrically is 1.27×10^{-5} which gives a HNB to peptide ratio of 0.96.

The HNB(0.96):peptide T_8-T_9, isolated from HNB(1.05):

lysozyme, corresponds to residues 46-68 in the native enzyme. This segment contains two tryptophan residues located at positions 62 and 63,one of which must therefore carry the label. The HNB:peptide is not a coprecipitate of peptide T_8 and T_9 since chromatography on Sephadex G-10 produces a single symetrical peak. The normal tryptic cleavage of the bond between arginine 61 and tryptophan 62 is thus apparently inhibited by the modification of one of the tryptophans, which permits the isolation of the combined peptides.

Samples of both RCM-HNB(1.05):lysozyme and HNB(0.96): peptide T_8-T_9 prepared in 0.18 M acetic acid pH 2.80 were subjected to incubation at 30° for 48 hours. Analysis of the HNB content following gel filtration indicated that both species are stable with respect to the loss of the HNB group.

F. Titration of HNB-OH and HNB(1.05):lysozyme.

Figure 11 shows the spectrophotometric titration curves for HNB-OH and HNB(1.05):lysozyme respectively. It is evident that the pK_a of the HNB phenolic group is increased from 6.91 to 7.70 when incorporated into the protein. In addition to the apparent change in pK_a for the HNB group, a distinct spectral shift from 408 nm to 421 nm is observed in basic solution of HNB-OH and HNB(1.05):lysozyme, Figure 12.

G. Difference spectra of lysozyme and HNB:lysozyme with NAG.

The results of the difference spectra of lysozyme and HNB(1.05):lysozyme with NAG are shown in Figures 13 and 14. The native enzyme produces a difference spectra in the

270-310 nm region with a maximum at 294.5 nm whereas the HNB(1.05):lysozyme produces a broad difference spectra in the visible region with a maximum at 418 nm in addition to a spectrum in the ultraviolet. The difference spectra for HNB (1.05):lysozyme must therefore be due to a perturbation of the environment of the HNB group.

The results in Figures 15 and 16, indicate a single binding site for NAG on lysozyme with a binding constant for the native enzyme of 27-28 mM, which is in good agreement with that of Dahlquist and Raftery (26). The HNB(1.05): lysozyme also binds NAG to a single site on the molecule but with a larger binding constant of 53-55 mM.

H. Gel electrophoresis.

The results of polyacrylamide gel electrophoresis are shown in Figure 17. Comparison of the rates of migration of the two proteins show that the native enzyme migrates faster than the HNB substituted protein. Lysozyme has an isoelectric pH of 10.5-11.0 (84) and under the conditions of electrophoresis at pH 8.6, it is positively charged. The introduction of the HNB group into the lysozyme molecule provides an additional ionizing species namely the phenolic hydroxyl group, pK_a = 7.70 which under the conditions of electrophoresis is negatively charged. The net effect of substitution of the protein with HNB-Br is thus to reduce the relative positive charge which reduces the mobility of the HNB substituted species.

Samples of HNB(1.05):lysozyme incubated at pH 4.00 and 50° were subjected to electrophoresis following incubation under the above conditions at the times indicated. The results of such incubations show the increased formation of a faster moving component, the native enzyme, with time and a parallel decrease in intensity of the slower moving component. These results are in accord with the observed increase in enzymatic activity of HNB(1.05):lysozyme under identical conditions, and are interpreted in terms of the cleavage of the HNB group which then yields the active native enzyme.

I. Effect of urea on regeneration of HNB(1.05):lysozyme.

The effect of 8M urea on HNB(1.05):lysozyme was examined with respect to the release of HNB-OH and the regeneration of enzymatic activity. Examination of the data in Table VII indicates that native lysozyme is unusually stable in urea solution at pH 9.80, requiring 44 hours to lose 99 per cent of its activity. This loss in activity is due partly to the conditions of the incubation i.e., 44 hours at 37° and pH 9.80 since it can be seen that the native enzyme incubated under identical conditions but in the absence of urea loses approximately 25-30 per cent of its activity. Samples of HNB(1.05):lysozyme incubated under identical conditions also show, to some extent, the loss of residual activity in the presence of urea and a small increase in activity in the absence of urea. The increase in

activity of HNB(1.05):lysozyme in the absence of urea can be explained by a slow spontaneous regeneration of HNB(1.05): lysozyme at 37° over a prolonged period of time. Removal of HNB-OH from HNB(1.05):lysozyme by gel filtration after 44 hours at 37° and pH 9.80 indicates a decrease in the ratio of HNB:lysozyme from 1.05 to 0.81 and an increase in specific activity from 600 to 1340. The activity observed for HNB: lysozyme after the incubation is smaller than would be expected on the basis of HNB-OH released, however, this effect can be explained if the loss in activity of the control lysozyme sample is taken into account.

Subsequent to the incubation of the samples at pH 9.80 and determination of activity and ratio data the samples were adjusted to pH 5.0 and incubated an additional 48 hours at 37°. Following the acidic incubation the samples were subjected to gel filtration and the relevant data can be found in Table VIII. HNB(1.05):lysozyme incubated in the absence of urea undergoes normal cleavage and regeneration of enzymatic activity. The correlation between HNB lost and activity gained was good when the final activity was corrected for the loss of activity in the control.

The samples of lysozyme and HNB(1.05):lysozyme which were both incubated in the presence of 8M urea are, at the termination of the experiment, devoid of any activity. This result is not surprising since urea, at high concentration, is an effective protein denaturant and leads to a loss of enzyme activity by destroying the tertiary structure of many

proteins. The stability of the HNB group in HNB(1.05): lysozyme to cleavage when incubated in urea solution must therefore reflect some kind of ordered structure which is necessary for release of this group.

J. Reaction of human lysozyme with HNB-Br.

Table IX indicates the results obtained from the reaction of HNB-Br with human lysozyme. Over a range of molar excesses of HNB-Br to lysozyme from 2.5 to 20 only a 10 per cent decrease in activity is observed. At a 20 fold molar excess of HNB-Br 1.10 moles of HNB are incorporated per mole of lysozyme. After incubation at pH 2.80 and 45° for 2.5 hours the ratio of HNB:lysozyme is essentially unchanged at 1.08. The small loss in activity of the enzyme derivative may be explained by a denaturation of the protein by acetone which attains a concentration of 20 per cent v/v. It is of importance to note that under identical reaction conditions hen egg lysozyme is completely inhibited at a five fold molar excess of HNB-Br.

K. Effect of pH on regeneration of HNB(1.05):lysozyme.

The rate of regeneration of HNB(1.05):lysozyme shows a marked dependence on pH as shown in Figure 18 and Table X. An apparent maximum in the rate of regeneration (Fig. 18) at pH 3.0 is, however, probably artifactual. In the determination of k, plots of per cent inhibition versus time normally yield a straight line indicating the pseudo first order nature of the reaction. Examination of the data for the regeneration at pH 1.53 shows that this relationship i.e.,

linearity is not observed. At the completion of the regeneration process all samples in the pH range of 2.5-8.4 show an approximately 80 per cent recovery of enzyme activity. Samples incubated at pH 2.03, 1.53, and 1.23 have recoveries of activity of 60.5, 39.4, and 32.0 per cent respectively. These results seem to indicate that the regeneration process is being blocked, either by denaturation of the protein or by some other mechanism.

In a separate experiment identical samples of HNB (1.05):lysozyme at pH 1.20 and pH 4.06 were allowed to regenerate at 35°. Aliquots removed at various time intervals were analyzed for HNB:protein ratios and activity. Samples incubated at pH 1.20 were shown to liberate 0.20 moles of HNB-OH per mole of protein and generate a 20 per cent gain in activity within two hours and no further liberation during an additional 5.5 hours at 35°. The sample incubated at pH 4.06 liberated 0.60 moles of HNB-OH per mole of protein and a 70 per cent gain of theoretical activity continuously over the 7.5 hours of incubation. After the incubation the pH of each sample was adjusted such that pH 1.20 became 4.00 and pH 4.06 became 1.20. Incubation was allowed to proceed for an additional 3.5 hours at 40° and the samples were reassayed. In either sample no additional activity could be generated.

The analysis of the dependence of rate with pH was made with respect to the ionization of a particular group within the protein inhibitor complex. When treated as a

titration curve, Figure 18 yields an apparent pK_a of 6.5 for a group participating in the regeneration process.

L. Effect of temperature on the regeneration of HNB(1.05):lysozyme.

The rate of regeneration of HNB(1.05):lysozyme at pH 4.06 was studied over a range of temperatures from 30-55°. An Arrhenius plot of the data (Fig. 19) shows that over the range of temperatures studied the energy of activation was constant with a value of 29.6 Kcal/mole.

M. Effects of salts on the regeneration of HNB(1.05):lysozyme.

The effect of various halides on the regeneration of HNB(1.05):lysozyme is seen in Figure 20. It is evident that at 0.1M, only iodide ion has any effect on the rate of regeneration of enzymatic activity. Table XI illustrates the effect of concentration of iodide on the regeneration rate and Figure 21 shows the relationship of iodide concentration on the rate of regeneration of HNB(1.05):lysozyme. In addition to iodide, which is a good nucleophile, the effects with thiocyanate were also studied at pH 4.0 and 7.0 and the results are shown in Table XI.

It has been observed that log $[I^-]$ is a linear function of log k (Fig. 21) and a change in iodide ion concentration from zero to 1.0 M yields a doubling of the rate of reaction (Table XI). These observations would seem to indicate that iodide ion is not participating directly in the hydrolysis reaction but may be acting catalytically.

N. Deuterium isotope effect on the regeneration of HNB(1.05):lysozyme.

Samples of HNB(1.05):lysozyme and deuterated HNB (1.05):lysozyme were subjected to conditions of regeneration at 45° in 0.18M acetate buffers at pH 4.00 and pD 4.09 respectively (Fig. 22). The rate constants determined for these reactions were 2.43×10^{-2} min.$^{-1}$ for HNB(1.05):lysozyme in normal acetate buffer and 2.88×10^{-2} min.$^{-1}$ for deuterated HNB(1.05):lysozyme in deuterated acetate buffer. Determination of the ratio of k_H/k_D yielded a value of 0.84±07. Control samples of normal lysozyme and deuterated lysozyme incubated under identical conditions showed no changes in activity over the same time interval.

O. Effect of NAG on HNB(1.05):lysozyme regeneration.

The modification of the environment of the HNB group observed in the difference spectra of NAG and HNB(1.05): lysozyme was further studied with respect to the effect of NAG on the regeneration of HNB(1.05):lysozyme. The consequence of the addition of 0.1M NAG to HNB(1.05):lysozyme was to increase the pseudo first order rate constant from 2.58×10^{-2} min.$^{-1}$ to 2.78×10^{-2} min.$^{-1}$ at 45° and pH 4.00.

P. Effect of HNB-Br on various modified lysozymes.

a) Guanidination.

The reaction of lysozyme or HNB(1.05):lysozyme with O-methyl isourea resulted in the appearance of 5.9 and 5.6 additional guanido groups respectively per mole of protein as determined by a modified Sakaguchi reaction. The

guanidinated lysozyme produced maintained full enzymatic activity in agreement with the findings of Parsons et al. (32), who also showed the appearance of six residues of homoarginine in the hydrolysis products of guanidinated lysozyme. The results in Table XII indicate that the derivatives formed by guanidination of HNB(1.05):lysozyme or the product of the reaction of HNB-Br with guanidinated lysozyme are both capable of undergoing cleavage of the HNB group and regeneration of enzymatic activity. The marked decrease in the change in ratio of HNB:protein, the decreased per cent of activity regenerated and the higher than normal per cent activity at T=0 in guanidinated HNB(1.05):lysozyme can be accounted for with respect to the method of its preparation. The starting material, HNB(1.05):lysozyme possessing approximately 6 per cent residual activity was subjected to dialysis at pH 6.0 for 22 hours following reaction with O-methyl isourea. Although dialysis proceeded at low temperature some regeneration of enzymatic activity and cleavage of the HNB group must have occurred. These results clearly indicate that modification of lysine residues by guanidination have virtually no effect on any of the parameters with regard to the reaction or subsequent regeneration of HNB:lysozyme.

b) Acetylation.

The effects of O-acetylation with N-acetyl imidazole on the regeneration of HNB:lysozyme can be approached from two different aspects, namely, effects due to O-acetylation

of residues of the protein and secondly effects due to O-acetylation of the HNB group.

The esterification of the phenolic hydroxyl of tyrosine by acetyl imidazole has been well documented (82). Two acetylated proteins were produced by the reaction of N-acetyl imidazole with native lysozyme or guanidinated lysozyme. The products produced by either reaction were analyzed for extent of O-acetylation and both products were shown to contain an average of 2.1 residues of O-acetylated tyrosine. The result of the acetylation of the native enzyme compared well with the results of Parsons et al. (32) who showed an average of 2.0 moles of O-acetyl tyrosine per mole of enzyme. The product of acetylation of the native enzyme yielded a preparation with 17 per cent residual activity whereas the product of the acetylation of guanidinated lysozyme possessed full enzymatic activity. N-acetyl imidazole is known to produce N-acetylated products by reaction with available free amino groups (80). The loss in activity of the native enzyme can therefore be explained by reaction of the free amino groups of lysine which are blocked in the guanidinated derivative. Both acetylated proteins were reacted with HNB-Br and the results of these reactions are given in Table XII. It was observed that neither derivative, whether O and N-acetylated or O-acetylated and guanidinated, participates in the reaction or regeneration of HNB:lysozyme.

The reaction of HNB(1.05):lysozyme with N-acetyl imidazole provides, in addition to the phenolic hydroxyl

groups of tyrosine, the phenolic hydroxyl group of the HNB moiety as a site for reaction. A quantitative determination of the extent of O-acetylation of the HNB group was unsuccessful. However, by analogy to the O-acetylation of N-acetyl tyrosine which occurs with a decrease in absorption at 275 nm (λ max) (82), a decrease in absorption at 320 nm (λ max for HNB-OH in acid solution) was observed upon acetylation. The experiments presented in Tables XIII and XIV indicate the results of the reaction of HNB(1.05):lysozyme with acetyl imidazole. The results must be examined with respect to the results presented in Table XII which indicate that neither O nor N acetylation of the native protein lead to any modification of the ability of HNB:lysozyme derivative to undergo regeneration. It is apparent from the data in Tables XIII and XIV that the acetylation of HNB(1.05): lysozyme leads to products with a reduced ability to release HNB. The reduced ability to release HNB, could be overcome by brief exposure to an alkaline environment. Phenolic esters are base labile and the correction of HNB released can be accounted for by the hydrolysis of the HNB ester to yield the phenol.

c) NBS Oxidation.

The oxidation of hen egg lysozyme by NBS yielded a product of approximately 20 per cent activity with the concomitant modification of 1.19 moles of tryptophan as determined by the method of Spande and Witkop (82). The product which contains predominantly oxidized tryptophan 62 compares

well with the product obtained by Spande and Witkop (81). The data presented in Table XII indicates that the incorporation of HNB into this derivative is significantly reduced. In addition to the reduced incorporation it was determined that, of the total HNB incorporated, only 38 per cent of this is labile; by comparison normal HNB:lysozyme contains approximately 75-80 per cent labile HNB groups. The residual activity found in the oxidized sample may be due to the presence of small amounts of native lysozyme and the small amount of normal HNB:lysozyme may thus account for the small amount of regeneration observed. It has been previously implied that the reaction of lysozyme with HNB-Br leads to the production of at least two HNB:lysozyme species, one of which is labile and the other stable. In the monosubstituted enzyme these species are produced in the ratio of approximately 80 to 20 respectively. In the tryptophan 62 oxidized enzyme this ratio is changed such that only 38 per cent of the incorporated HNB is labile while the remaining 62 per cent is stable. The change in ratio of labile to stable products and the decreased incorporation of HNB presumably reflect the modification of a critical residue.

d) Esterification.

The esterification of lysozyme with 0.2 M triethyloxonium fluoroborate at pH 4.50 produced a product with an average of 1.13 ester groups/mole and a specific activity of approximately 50 per cent that of the native enzyme. The esterified protein was subjected to ion exchange chromato-

graphy as described previously and the fractions indicated were pooled and their specific activities determined, Figure 23. Although it was not possible to determine the ester content of the various fractions due to the small quantities of protein isolated, the results of the specific activities and elution pattern was almost identical with that observed by Parsons et al. (32). It was assumed that the various fractions represented the native enzyme, the monosubstituted esters of aspartic acid residue 52 and glutamic acid residue 35, and a disubstituted ester of the two previously identified residues respectively. The native enzyme and each of the monosubstituted esters were reacted with HNB-Br and subjected to regeneration conditions. All samples incubated for 2 hours at 45° and pH 2.80 showed the release of approximately 80 per cent of the bound HNB. These results were taken as an indication that the esterification of either glutamic acid 35 or aspartic acid 52 has no effect on the ability of HNB:lysozyme to undergo regeneration.

Q. Reaction of MNB-Br with lysozyme.

The results of the reaction of lysozyme with MNB-Br at pH 2.80 are presented in Table XV and Figure 24. It can be seen from the table that the incorporation of the label is not instantaneous as is the comparable reaction of lysozyme and HNB-Br but requires a relatively long period. Figure 24 shows the parallel decrease in activity and increase in MNB:lysozyme ratio characteristic of the formation of an inactive lysozyme derivative. The most interesting

finding is included in Table XV. Samples which had less than 1 per cent activity at 2 hours were removed and subjected to gel filtration to remove excess unreacted MNB-Br, MNB-OH and acetone. These samples were returned to the bath at 30° and the incubation was continued from the three hour interval. At both 6 hours and at 19 hours no increase in activity was observed. It is apparent that 16 hours of incubation at 30° and pH 2.80 does not lead to the regeneration of enzymatic activity, compared with HNB(1.05):lysozyme which has a half life of regeneration of approximately 4 hours at 30° and pH 3.0. The MNB(1.0):lysozyme is therefore a stable inactive derivative.

R. Reduction of HNB(1.05):lysozyme with sodium borohydride.

The results of preliminary experiments dealing with the possible reaction of sodium borohydride with HNB(1.05):lysozyme, indicated that a reaction does take place and the product or products of this reaction are incapable of regeneration of activity or cleavage of the HNB moiety. These data are given in Table XVI. The decrease in labile HNB and the decreased extent of regeneration of enzymatic activity could be due to denaturation of the protein under the conditions of the reaction. In order to determine whether or not reaction conditions lead to denaturation, samples of HNB(1.05):lysozyme and native lysozyme were reacted together in the presence of BH_4. The results shown in Table XVII indicate that even under conditions where little or no HNB cleavage is observed, the activity of the native enzyme is unchanged.

It would appear that the observed loss in ability to cleave the HNB group and regenerate activity is not due to denaturation of the enzyme.

The results of the reaction of HNB(1.05):lysozyme with $BH_4(T_4)$ are presented in Table XVIII and Figure 25. In order to rule out any effects such as inhibition of the regeneration process by hydrolysis products of $BH_4(T_4)$ or non-specific tritium incorporation due to tritium-hydrogen exchange reactions, the following procedure was utilized. Duplicate samples of protein at each $BH_4(T_4)$ concentration were prepared by either adding the $BH_4(T_4)$ solution to a sample composed of 1.00 ml protein plus 1.00 ml buffer or adding the $BH_4(T_4)$ solution to a 1.00 ml sample of buffer and after a 10 minute period, to allow for hydrolysis of the $BH_4(T_4)$, adding 1.00 ml of the protein solution. The results in Table XVIII show that the hydrolysis products of $BH_4(T_4)$ have little or no effect on the enzyme since at all concentrations, the extent of regeneration of activity and the loss of the HNB group is constant. The data presented in Figure 25 suggests a direct relationship between the incorporation of tritium and the regeneration of enzyme activity.

The results shown in Table XVII have already been interpreted in terms of the stability of the native enzyme to denaturation under conditions leading to the cessation of HNB(1.05):lysozyme regeneration. The decreased extent of regeneration of HNB(1.05):lysozyme however may be a reflec-

tion of a decreased stability of this protein under the reaction conditions imposed. An assessment of the specificity of tritium incorporation, the extent of tritium incorporation, and the stability of the HNB(1.05):lysozyme species can however be made in a system where both protein and reducing agent concentrations are kept constant. Table XIX shows the results obtained in such a system where the $BH_4(T_4)$ concentration and protein concentration at different mole fractions of HNB(1.05):lysozyme to native enzyme are constant. Figure 26 provides evidence for the specificity of tritium incorporation since this incorporation is directly proportional to the mole fraction of HNB(1.05):lysozyme. The data also indicates that the decreased extent of regeneration is not due to the instability of the substituted protein, but must reflect the necessity of a reducible group in the protein for regeneration.

The use of radioactive isotopes in labelling studies provides an accurate method for the determination of the extent of such reactions. The specific radioactivity of the $BH_4(T_4)$ solution was determined from the known specific radioactivity of the undiluted BT_4 and the dilution factor and also from the concentration of the $BH_4(T_4)$ solution and the disintegration rate of appropriate aliquots. The specific radioactivity of the $BH_4(T_4)$ solution was determined to be 1.52±0.10 mc/mmole of $BH_4(T_4)$ or 0.380±0.025 mc/mmole of tritium. The data presented in Figure 25 and Table XIX allow a calculation of the specific radioactivity of the fully

tritiated protein. At a mole fraction of 1.00, HNB(1.05): lysozyme has a specific radioactivity of 0.348±0.07 mc/mmole when correction is made for the regeneration of twelve per cent which occurs. The ratio of the specific radioactivity of the reductant to that of the protein indicates the incorporation of 0.91±0.04 moles of tritium per mole of enzyme. The tritium incorporation observed is consistent with the mechanism of borohydride reductions which lead to reduction by the transfer of a single hydride ion from the reducing agent and a proton from the solvent to the bond being reduced.

The data presented in Figure 26 have already been discussed in terms of the specificity of tritium incorporation into HNB(1.05):lysozyme. In order to define these results more clearly and to gain further insight into the mechanism of the regeneration process, labelling experiments were performed with the intact protein as well as the peptide and isolation of the BH_4 reduced peptide from the BH_4 reduced protein was accomplished.

The incorporation of tritium into both HNB(1.05): lysozyme and HNB(0.96):peptide T_8-T_9 was accomplished and c.p.m./O.D.$_{420}$/ml values of 11,100 and 11,600 were obtained respectively. The results indicate an equivalency between the protein and peptide in terms of the reducibility of the bond being attacked by borohydride. The results presented in Table XX are consistent with the premise that reduction in the intact HNB(1.05):lysozyme takes place within a group in

the T_8-T_9 peptide.

S. Nuclear magnetic resonance spectra of lysozyme and HNB(1.05):lysozyme.

The N.M.R. spectra shown in Figure 27 indicate that in HNB(1.05):lysozyme a specific signal, at 2270 Hz., found in the native enzyme is absent. This signal, at 2270 Hz., has been identified by Glickson et al. (85) as the characteristic indole N-H proton of tryptophan 62. These results would appear to indicate the modification of tryptophan 62 in the HNB:lysozyme adduct.

IV. Discussion

The present study has dealt with essentially three major aspects of the reaction of HNB-Br with hen egg white lysozyme: the chemistry of the reaction; the isolation and characterization of the major substituted protein; and finally, those aspects of the regeneration of HNB:lysozyme which lead to a proposed mechanism to account for the lability of the HNB group.

A. The reaction of HNB-Br with lysozyme.

The reaction of HNB-Br with lysozyme results in a reaction which can be stoichiometrically defined. The adduct observed in this reaction has also been observed upon reaction of free tryptophan and other substituted indole compounds with HNB-Br (51,62,86). The two reactions, with protein and indole compounds, are also similar with respect to specificity and reactivity. Complete enzyme inhibition occurred within one minute and the reaction proceeded only at the indole nucleus of tryptophan.

The results presented here suggest that at least one residue within the protein is particularly reactive and that it is essential for enzyme activity. The high degree of reactivity of a single residue is not unexpected. Previous studies have shown that the six tryptophan residues of lysozyme (14) are not equally reactive towards various modifying agent (41-48), which is due in part to their unequal

availability for reaction (36-40). A concomitant decrease in enzyme activity with increasing HNB incorporation occurred until 1.25 moles of HNB were bound per mole of protein, whereby the enzyme was fully inhibited.

The results of the incorporation of HNB into the protein and the effect of such incorporation on the activity of the enzyme give some clues towards an understanding of the reactions which lead to the incorporation of the label. The observation has been made throughout this study that the extent of protein inhibition is only about 80 per cent of that which would be expected on the basis of HNB incorporation. This lack of correspondence may be attributed to one of two factors; either, the incorporation of the HNB group at a particular residue has no effect on the activity of the enzyme, or, the additional HNB group incorporated is being added to an already substituted protein molecule. The results of the subsequent studies on the regeneration of the HNB:lysozyme products favors the second alternative.

It has been observed that the fully inhibited protein carries 1.27 moles of bound HNB per mole of protein. Following the release of 1.00 moles of HNB, the product obtained was a species which still contained 0.27 moles of bound HNB and was 25 per cent inhibited. These results indicated that the HNB:protein which remained, following regeneration of the labile derivative, was an inactive derivative. This observation thus rules out the possibility that the incorporation of HNB at some other residue has no effect on enzyme activity.

The reaction of the enzyme with HNB-Br would appear to be explained in terms of two reactions. The initial and major reaction of the protein and modifier leads to the production of the inactive labile derivative. This reaction is then followed by the further substitution of the primary product by an additional HNB group which is incorporated at a slower rate and produces the stable derivative. Since it has been shown that both the labile and stable derivatives are inactive, such a sequence of events would explain the non coincidence of HNB incorporation with respect to inhibition of the protein. This sequence of events was further substantiated by the finding that there was a direct correlation between the extent of inhibition and the extent of lability of the HNB group (Fig. 4). It has been observed that all species produced, in the reaction of lysozyme with HNB-Br, regenerated 80 per cent of their activity and released 80 per cent of their bound HNB groups. Thus, the necessity for the incorporation of approximately 1.25 moles of HNB per mole of protein for full inhibition can be explained with respect to the reactions indicated.

The inactive protein produced by reaction with HNB-Br and carrying 1.27 moles of bound HNB must, by virtue of its composition, be a mixture of monosubstituted and disubstituted proteins. The disubstituted protein may be two types: a product formed by the attack of HNB-Br on two different tryptophan residues; or a product formed on a single tryptophan residue which bears two moles of the label.

A single disubstituted tryptophan residue appears not to be favored. The reaction of indoles or substituted indoles with alkyl halides first leads to products by alkylation at carbon 3 of the indole nucleus (86-90), which then yield 3,3 disubstituted indoles. The 3,3 disubstituted indoles are known to undergo rearrangements in acid solution with the migration of one of the groups at C-3 to C-2 to form 2,3 disubstituted indoles (62,86,87,91). Thus, the sequence of events leading to the formation of a disubstituted HNB-tryptophan would require the initial alkylation at C-3, followed by the migration of one of the groups at C-3 to C-2, followed by the attack at C-3 of a second HNB group. Such a sequence of events seems unlikely since it would require that the migration be more rapid than HNB-Br hydrolysis in order to allow for the reaction of a second molecule of HNB-Br at C-3. In addition, the reaction of HNB-Br with the protein was performed at pH 2.80, in 0.18 M acetic acid and at low temperature, which are conditions that do not favor indolenine rearrangements. Such reactions occur only under more drastic conditions, for example, the rearrangement of HNB-tryptophan ethyl ester required refluxing in concentrated HCl-absolute ethanol, 1:3 v/v (62). However, there are some indications that at very low pH, HNB(1.05):lysozyme does undergo a possible indolenine rearrangement. In studies of the effect of pH on the rate of regeneration of enzyme activity, samples incubated at pH 1.2, 1.5 and 2.0 regenerated 32, 40 and 60 per cent activity respectively. Additional

samples in the pH range of 2.5-8.4 all regenerated 80 per cent activity. Since the native enzyme was stable under these acidic conditions it is possible that the reduced extent of regeneration was due to an indolenine rearrangement.

The previous discussion would appear to rule out the possibility of disubstitution on a single tryptophan residue and thus require that the additional label be found on one or more different tryptophans. Barman, has shown (92), that an HNB(1.2):lysozyme species has the label distributed as follows; 1.02 moles HNB bound to tryptophan 62 or 63 or both, 0.07 moles bound to tryptophan 28, 0.04 moles bound to tryptophan 108 or 111 or both and 0.03 moles bound to tryptophan 123.

The pH dependence of the incorporation of HNB into lysozyme further supports the conclusions of the previous discussion. Studies of the reaction of HNB-Br with tryptophan indicate a pH independent reaction (51). Thus, the observed pH dependence of the reaction of HNB-Br with lysozyme is a function of the protein. The analysis of the effect of pH on the incorporation of HNB into lysozyme implicated a group with a pK_a of 4.1, which is the pK_a of a carboxyl group. The studies of Donovan et al. (93) and Ikeda (30) have indicated changes in the structure of lysozyme with changes in pH. The pattern of incorporation of HNB, may therefore simply reflect the modification of the protein structure leading to a decreased extent of HNB incorporation.

It has been observed that the substituted proteins

produced at the various pH's are identical with respect to their ability to regenerate 79 per cent of their activity and release 76 per cent of their HNB. This data would seem to corroborate the theory that the reaction of HNB-Br with lysozyme first involves the formation of the labile derivative which is then additionally substituted. If such a situation was not obtained it would be reasonsable to expect a change in the distribution of the products. In addition to supporting the theory pertaining to the incorporation of the label, the results of this study suggests that the stable derivative is not formed by an indolenine rearrangement. Such rearrangements have been shown to require an acidic media and even the product formed at pH 7 showed the presence of about 25 per cent of HNB:lysozyme which failed to regenerate.

B. The isolation and characterization of HNB(1.05): lysozyme.

The isolation of HNB(1.05):lysozyme by chromatography on Bio-Rex 70 indicated the presence of two minor and one major non-symmetrical HNB:lysozyme components. The separation of the two minor fractions and the major non-symmetrical fractions may be attributed to the separation of various HNB: lysozyme derivatives labelled at different tryptophan residues or to the separation of a single HNB:lysozyme species with different amide contents. Tallan and Stein (94) have shown such heterogeneity of commercial lysozyme preparations. Following the chromatographic separation various fractions

from the major peak were pooled and characterized. The characterization of these fractions indicated that they all had the same properties, i.e., residual activity, HNB content and extent of both, regeneration of activity and release of the HNB group. The combined fractions constituted HNB(1.05):lysozyme. Rechromatography of HNB(1.05):lysozyme on an analytical column of Bio-Rex 70 (200-400 mesh) as well as polyacrylamide gel electrophoresis showed that the material was homogeneous. HNB(1.05):lysozyme is thus, essentially a monosubstituted derivative devoid of activity. Both the regeneration of enzyme activity and the release of HNB from HNB(1.05):lysozyme went to 80 per cent completion.

Evidence that the tryptophan residue which is labelled with the labile HNB group is tryptophan 62 is as follows: 1) Tryptophan 62 is the most exposed tryptophan in the molecule (36); 2) it is the residue which is preferentially sulfenylated (48), and 3) oxidized by N-bromosuccinimide (41). 4) The results presented here showed that the reaction of HNB-Br and lysozyme, oxidized at tryptophan 62, was not favorable. The oxidized enzyme possessed approximately 20 per cent of the activity of the native enzyme. If it is assumed that the activity of the oxidized enzyme was due to residual native enzyme, then the decreased incorporation of HNB points towards tryptophan 62 as the site of primary alkylation and the source of the labile HNB group.

5) The isolation of the monosubstituted HNB-peptide,

T_8-T_9 implicates either tryptophan 62 or 63 as the site of labelling. The peptide was isolated after tryptic digestion. It contains the labelled tryptophan and it is the predominant product of the modification reaction since it was obtained in 80 per cent yield. This value for the yield of the peptide also corresponds to the extent of lability of the HNB group in HNB(1.05):lysozyme. Knowledge of the peptide sequence indicates the site of the label. The T_8-T_9 peptide, which can only be isolated from the substituted protein, has an arginine residue at position 61 which in the native enzyme is susceptible to tryptic digestion. However, in the HNB(1.05):lysozyme, no such cleavage occurred. The residue adjacent to arginine 61 is tryptophan 62, thus in the native enzyme this bond is cleaved, whereas in the HNB:enzyme this bond remains intact. It would appear that the modification of the protein leads to the inhibition of the tryptic hydrolysis, and the stability of this bond may be due to the steric inhibition produced by the incorporation of the bulky HNB moiety on tryptophan 62.

6) Finally, the results of the NMR studies indicated substitution at tryptophan 62. Consistent with the assignment of the indole NH proton of tryptophan 62 at 2270 Hz., by Glickson et al. (85), the present studies showed the lack of this band at 2270 Hz. for HNB(1.05):lysozyme and this was taken as an indication of the specific modification of tryptophan 62 in the HNB derivative.

These data and the inhibition data substantiate the

findings of other studies (41,48,64), which have shown that the loss of enzyme activity occurs concomitantly with the modification of tryptophan 62. Examination of the three dimensional structure of the protein would appear to support the conclusion that the inhibition of the enzyme is caused by the introduction of the bulky HNB group, which interferes with the binding of the substrate. The HNB group projects outward into the cleft and thus provides the necessary steric inhibition to the binding of the substrate.

Although the data are in agreement with the studies cited, they are in contrast with those of Bewley and Li (52), who have shown the incorporation of two moles of HNB per mole of protein with the retention of 90 per cent of enzyme activity. These results are different, perhaps, because their reactions were run in the presence of 2-chloroethanol which may have led to the exposure of other tryptophan residues, not essential for activity.

The purified HNB(1.05):lysozyme was further characterized by studying the effects of the introduction of the HNB group on the binding of the inhibitor, N-acetyl glucosamine.

The binding of NAG to HNB(1.05):lysozyme showed an increase in the binding constant, from 27-28 mM in the native enzyme, to 53-55 mM in the HNB derivative. The anomeric forms of the NAG molecule bind differently to the lysozyme molecule. Blake *et al.* have shown (21), that the binding of NAG to lysozyme requires the formation of hydrogen bonds. They are formed between the N-H and carbonyl oxygen of the

N-acetyl side chain in the carbohydrate with the main chain C=O and N-H groups of amino acids 107 and 59 respectively. These interactions are constant for NAG molecules in either the α or β forms but the remaining interactions are different. β -NAG molecules form hydrogen bonds between their O(6) and O(3) atoms and the indole NH groups of tryptophan 62 and 63.

The α -NAG molecules, on the other hand, appear to form a hydrogen bond between O(1) and the main chain NH of residue 109. Dahlquist and Raftery have shown a difference in the binding constants for these anomeric NAG molecules (95). The determination of the binding constant for a mixture of anomeric NAG molecules with the native enzyme is therefore a composite of the binding constants for the individual anomers. The increased binding constant of NAG with HNB(1.05):lysozyme may therefore reflect the elimination of one of these modes of binding, presumably the one which involves the β anomer binding with tryptophan 62.

It has been observed that certain physical properties of the HNB group are modified when this group is bound to the protein. With respect to the hydrolysis product of HNB-Br, which is HNB-OH, these changes were characterized as; an apparent increase in the pK_a, from 6.9 to 7.7 of the phenolic hydroxyl group of the HNB moiety in HNB(1.05):lysozyme, and a shift in wavelength of maximal abosorption from 408 nm to 421 nm in the absorption spectrum of HNB(1.05):lysozyme determined in alkaline solution. These changes in the

properties of the HNB group, when bound to the protein, may therefore be a reflection of the modification of the environment surrounding this group.

In summary, the main properties of the substituted lysozyme are:

1) HNB(1.05):lysozyme is a monosubstituted inactive derivative capable of regenerating 80 per cent of enzyme activity and releasing 80 per cent of its HNB groups.

2) Tryptophan 62 is the major substituted residue and the source of the labile HNB group.

C. Factors concerned with the lability of the HNB group.

The characterization of HNB(1.05):lysozyme as composed of 80 per cent of a labile component and 20 per cent of a stable component allows the use of this mixed derivative in lability studies. Since the total amount of the labile derivative can be determined such an impure system could be utilized to study the factors leading to this lability.

The lability of the HNB group appears to require a particular conformation within the protein. Experiments with urea denatured HNB(1.05):lysozyme, the reduced and carboxymethylated HNB(1.05):lysozyme and the HNB(0.96):peptide T_8-T_9, reveal that the HNB group is stable with respect to its liberation. It would appear that the inability of these products to release their HNB groups must reflect a change in the orientation of the amino acids present in these species, relative to HNB(1.05):lysozyme. Thus, the unique

conformation found in HNB(1.05):lysozyme would appear to be necessary for the HNB group lability.

A comparison of the primary structure of hen egg white lysozyme, human leukemia lysozyme and bovine α-lactalbumin suggest a common ancestral gene since there are many homologous regions in the proteins (96). In addition to sequential homologies, X-ray diffraction studies have indicated structural homologies between the human and the egg enzymes (97). It would appear from the X-ray diffraction studies that the three dimensional structures of the two enzymes are similar and the active site is essentially unchanged.

The most striking difference between the two enzymes is the change in the amino acids which comprise subsite C. The replacement of the tryptophan residue at position 62 in the egg enzyme by a tyrosine which occurs at position 63 in the human enzyme (the equivalent of position 62 in the egg enzyme) (98), probably leads to a hydrogen bonding deficiency between the O(6) atom of the sugar residue at subsite C and tyrosine. The substitution of the tryptophan by tyrosine would displace the phenolic hydroxyl group (the equivalent of the indole NH) by about 2A° (97).

Although the two enzymes appear to be structurally and sequentially homologous it has been observed that they are different with respect to the activity and lability of the HNB derivatives. Thus, the egg enzyme incorporated one

mole of HNB and produced an inactive, labile derivative, whereas the human enzyme also incorporated one mole of HNB but the product was stable and active. The modification of the structurally similar protein, bovine α-lactalbumin, with HNB-Br also provides evidence for a unique conformation necessary for the lability of the HNB group in the egg enzyme. The studies of Barman (99) indicate the incorporation of HNB into bovine α-lactalbumin and the stability of the product formed.

The unique structural and conformational requirement may be a reflection of the juxtaposition of various residues within the protein which may either participate directly in the hydrolysis, or which may be necessary to maintain the HNB adduct in an orientation which is favorable for the cleavage of the HNB group. These two possibilities may be discussed in terms of the hydrolysis, as either facilitated by groups on the enzyme or by a specific ion or ions from the solution. Protein modification studies including esterification, guanidination and acetylation have indicated that the groups modified by these reactions play no role in the regeneration process, however, other groups may be implicated.

The energy of activation of the cleavage process was 30 Kcal per mole, which is high compared to the activation energies of enzymatic processes which fall within the range of 1-25 Kcal per mole (100). It appears that the hydrolysis of HNB:lysozyme is not an enzymatic reaction as a catalytic process but rather a chemical reaction which occurs

because of a structural requirement which is provided by the enzyme.

The participation of two specific groups within the tryptophan-HNB adduct are necessary for the hydrolytic process. They are the 2-hydroxy group of the HNB moiety and the indolenine >C=N- group.

The implication of the phenolic hydroxyl group of the HNB moiety comes from the results of two studies. The compound 2-methoxy-5-nitrobenzyl bromide was capable of alkylating the protein and the product of this alkylation was a monosubstituted protein which was without activity. Furthermore, the MNB:lysozyme thus produced was stable with respect to the regeneration of enzyme activity or loss of the MNB group. An additional indication of the requirement of the 2-hydroxy group comes from the results of the reaction of HNB(1.05):lysozyme with N-acetyl imidazole. The data demonstrated that the extent of regeneration could be blocked by acetylation of the 2-hydroxy group in the HNB moiety. Moreover, following treatment of the acetylated derivative under conditions which lead to the deacetylation of acetylated phenols, there was a restitution of the ability of the protein to release all of its HNB groups. This further supports the importance of the 2-hydroxy group.

The evidence for a group such as the indolenine >C=N- group as a necessary functional group came from the results of the borohydride experiments. Previous studies (101-103) have shown that indolenines are susceptible to

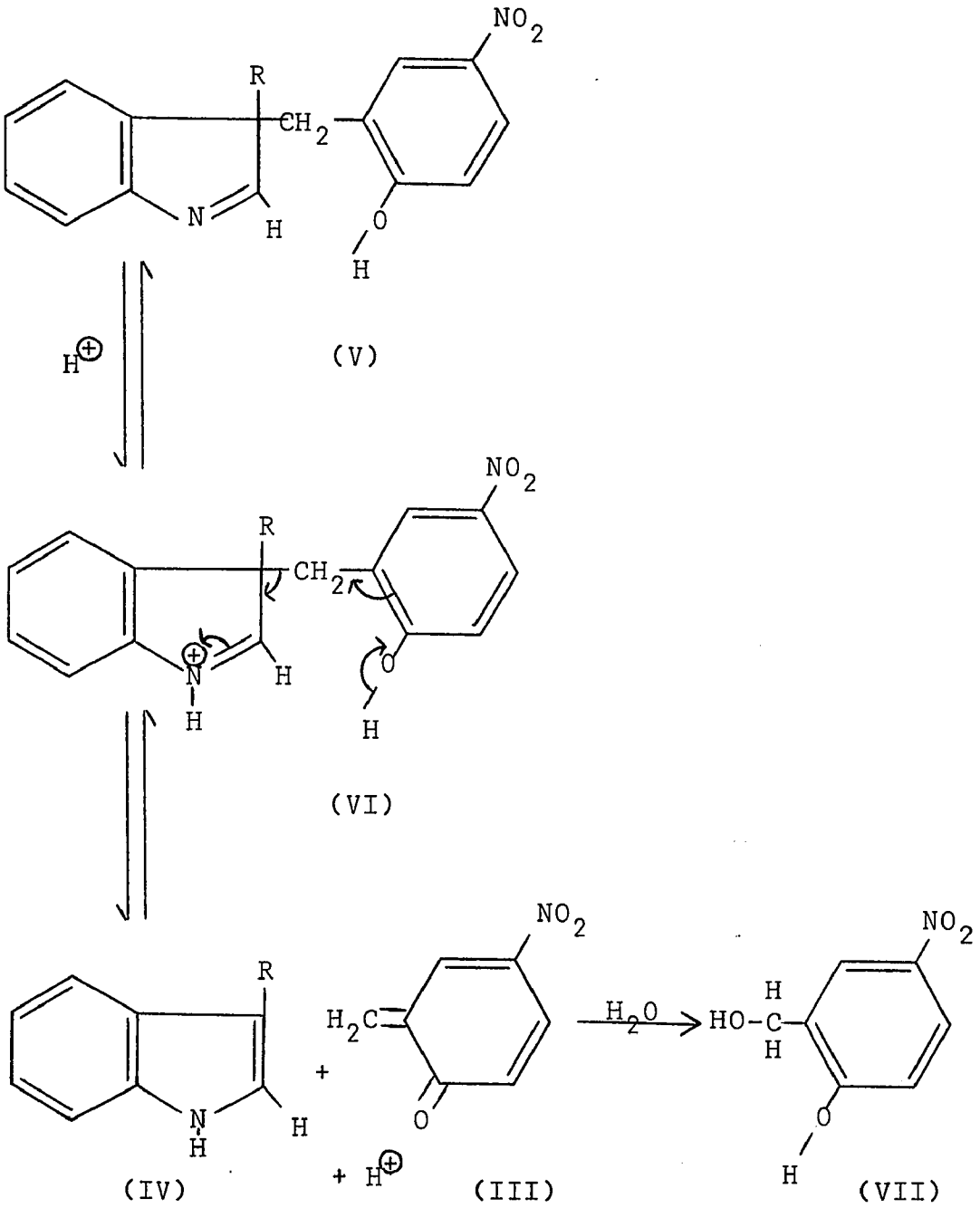
reduction with borohydride. The results reported here indicate that the reduction of HNB(1.05):lysozyme with tritium labelled borohydride leads to the production of a species which is incapable of regeneration and has incorporated one mole of tritium per mole of protein. Thus, the HNB:lysozyme may have an indolenine structure.

An examination of the various factors which determine the rate of hydrolysis of HNB(1.05):lysozyme and the knowledge of the groups participating in this process leads to the proposal of a possible mechanism for this reaction. This mechanism is presented in Scheme 1.

D. Proposed mechanism.

The reaction mechanism proposed in Scheme 1 contains three steps: 1) The initial protonation of the indolenine (V). 2) The rearrangement of the electrons in the protonated indolenine (VI), to yield tryptophan (IV) and the quinone methide (III). 3) The solvation of the quinone methide to yield HNB-OH (VII). These steps are, in part, substantiated by evidence already presented and, further supported by the following results.

The argument has already been made that the regeneration process appears to be dependent upon some type of specific orientation of the groups within the protein. The observation that NAG and HNB(1.05):lysozyme gives rise to a difference spectrum, characteristic of the HNB group, implies that the binding of NAG to the protein leads to a perturbation of the environment of this group. Such a perturbation


NO2
R
CH2
N
H
O
H
H⊕
(V)
NO2
R
CH2
N⊕
H
H
O
H
(VI)
R
N
H
H2C
O
NO2
+
+ H⊕
(IV)
(III)
H2O
HO-C
H
NO2
O
H
(VII)

Scheme 1

may thus be reflected in the rate of hydrolysis of HNB(1.05): lysozyme in the presence of NAG. The results presented here would seem to indicate an increase in the first order rate constant from 2.58×10^{-2} min^{-1} to 2.78×10^{-2} min^{-1} upon the addition of NAG. Although these results are not very dramatic nor very definitive they may be taken as an indication of the necessity of a particular orientation.

The results of deuterium isotope studies on the rates of chemical reactions can often be utilized in a definition of the reaction mechanism. As an example, in the case of reactions which are subject to specific hydrogen ion catalysis, it is found that the rates of such reactions proceed faster in D_2O than in H_2O by factors generally ranging from 25 to 100 per cent (104). The results presented in this study indicated a 19 per cent increase in the rate of regeneration of HNB(1.05):lysozyme in a deuterated as opposed to a non deuterated system. This observation would thus appear to indicate that the hydrolysis reaction is subject to specific hydrogen ion catalysis. Such reactions require a prior equilibrium between the substrate and a proton followed by the rate determining step (105). These steps are borne out by the proposed mechanism which requires the initial protonation of the indolenine (V) prior to the cleavage of the labile HNB group.

In conjunction with the deuterium isotope effect indicating the need for the protonation in a prior equilibrium between (V) and a proton, the results of the pH-rate profile

of the regeneration, apparently also reflect the need for protonation of the HNB(1.05):lysozyme species. The analysis of the pH-rate profile points to a group with an apparent pK_a of 6.5, as the group involved in the protonation. An examination of the groups within the protein which have pK_a values in this region indicate several possibilities including: the phenolic hydroxyl group of HNB, pK_a = 7.7; glutamic acid residue 35, pK_a about 6 (24-30); and histidine residue 15, pK_a = 5-5.5 (106). The participation of any of these groups seems unlikely because of; 1) the large difference in pK_a (compare 7.7 for HNB:lysozyme with 6.5), 2) the results of chemical modification which excludes glutamic acid 35 since the ester:HNB complex is labile, 3) proximity, histidine 15 is far removed from tryptophan 62 (18,19). One remaining possibility is that the HNB reaction leads to an indolenine $\rangle C{=}N-$.

Indolenines are fairly well characterized bases (107, 108), and may be protonated in the pH region studied. A thorough search of the literature provided no specific information about the pK_a values for indolenines. However, examination of other heterocyclic compounds which contain the $\rangle C{=}N-$ group provide some evidence that indolenines could have a pK_a in the region observed. Phenanthridine and quinoline are both polynuclear heterocyclic compounds containing the $\rangle C{=}N-$ group and have pK_a values of 4.5 and 4.9 respectively (109). The pK_a's of various substituted Δ^1 pyrrollines are a closer approximation to the presumed

indolenine. These compounds are closely analogous to indolenines since they are the pyrrole ring in the indole nucleus with a single double bond between nitrogen and carbon and have pK_a's ranging from 6.8 to 8.0 (110, 111). The assignment of the indolenine group as a participant in the hydrolysis reaction, can thus be tentatively made.

Finally it has been shown that the addition of nucleophiles increased the rate of regeneration of HNB(1.05): lysozyme. The effect of iodide ion on the rate was not due to a non specific salt effect since chloride or bromide ions did not effect the rate of regeneration. It is possible that the iodide or thiocyanate ion effect is concerned either with the stabilization and subsequent removal of the reaction product (III) or with the solvation or conformation of the protein.

The implication of the various factors concerned with the lability of the HNB group may now be summarized with respect to the mechanism proposed in Scheme 1.

The implication of the 2-hydroxy group in the HNB moiety comes from an examination of structures (III) and (VI) in the reaction sequence. It is apparent that neither the 2-methoxy nor the acetylated hydroxy compounds can undergo the reshuffling of electrons necessary to generate the quinone methide (III). Just as apparent is the necessity of the indolenine $\rangle C{=}N-$ group which must accept a proton and subsequently donate a pair of electrons to the positively charged nitrogen. Both the deuterium isotope effect and the

pH-rate dependence also indicate the requirement of the protonation of (V) for the hydrolysis of the adduct. The conformational requirements can be explained in terms of the orientation of the HNB group and the indole nucleus. In the redistribution of the electrons shown in (VI) one would expect a particular orientation which would allow for a smooth redistribution of the electrons between the two ring systems. The reaction sequence shown in Scheme 1 indicates that the products (III) and (IV) can recombine to form the indolenine (VI). It has been postulated that such a reaction is obtained upon the reaction of HNB-Br with tryptophan (51,62). If the quinone methide reacts with the indole nucleus, no regeneration will be observed. It is only when the quinone methide reacts with the solvent and produces HNB-OH (VII), that regeneration of enzyme activity occurs. The increase in the rate of regeneration in the presence of nucleophiles, like iodide or thiocyanate, may be a reflection of a competition between these nucleophiles and the indole nucleus for the quinone methide.

This brings us to a final question. Why does this particular HNB:protein adduct undergo regeneration? It is evident from the previous discussion that the requirement of a unique conformation is essential for the regeneration process. It is tempting to assume that the uniqueness of this conformation is a characteristic of only this one protein. Indeed, there are indications that this statement may be true. Studies of the reaction of HNB-Br with various indole

derivatives have shown that following the attack of the reagent to form these indolenines, the indolenines undergo cyclization by the attack of the 2-hydroxy group or a free amino group on carbon 2 of the indolenine (58,62,112,113). These reactions are apparently very facile and the cyclic products are apparently very stable. Since such structures result in the loss of the indolenine nucleus they would be stable with respect to the proposed mechanism. The ability of the HNB:lysozyme to incorporate tritium by borohydride reduction seems to indicate the presence of an indolenine and the unique lability of the HNB group on tryptophan 62 in this enzyme may therefore be due to some type of inhibition of this cyclization reaction. It must be pointed out, however, that the HNB:peptide also incorporates tritium, which implies the presence of an indolenine, but the peptide is stable towards release of the HNB group. This observation may appear to contradict the previous statement but it may be explainable in terms of a different conformation for the protein and peptide which prevent cyclization but does not allow for the release of the HNB group from the peptide.

The results of the present investigation may be compared with the results of Kaiser et al., who have studied the reactions of chymotrypsin with a number of cyclic esters (109-113). Kaiser's studies have indicated that the reaction of chymotrypsin and specific cyclic esters of sulfur, carbon and phosphorous are extremely rapid and produce an acyl

enzyme in which the acyl group is presumably bound to the active site serine residue at position 195. The acyl enzymes thus produced are inactive but they spontaneously revert to the active enzyme at pH 7.6 and 25°, $k = 6.7 \times 10^{-4}$ sec^{-1} (for desulfonylation) (66). This reversion to the active enzyme is characterized by the release of the acyl substituent. The spontaneous reversion to the active enzyme has been examined with respect to the group participating in this reaction and it has been shown that the group participating in the desulfonylation reaction is a 2-hydroxyl group (67), which is generated upon cleavage of the cyclic ester. Thus the labilization of the acyl substituent in the acyl chymotrypsin and the labilization of the HNB group in HNB:lysozyme appear to involve the participation of a group or groups within the modifier-enzyme complex which are responsible for the observed lability and the regeneration of enzyme activity.

V. Conclusions and Significance

The conclusions and significance of the present study bear in general upon the subject of protein modification and the use of specific protein modifications to gain an insight into the mechanism of action of enzymes. The observation that hen egg white lysozyme is inhibited by a reaction leading to the incorporation of an easily determinable label on tryptophan 62 supports other studies which have previously implicated this residue in the mechanism of action of this enzyme. The further observation that this HNB label is labile, however, has a great deal of significance with respect to the use of HNB-Br or any other group specific label. The results presented here, in conjunction with the observation of Kaiser, should serve to alert investigators probing the mechanism of action of enzymes, that the use of specific inhibitors may lead to false conclusions if such inhibitors lead to labile products which may go undetected. With respect to the lability of the HNB group in lysozyme, the present study has led to a definition of a possible mechanism to account for the lability and a method has also been provided to stabilize the labile group. The stabilization, by borohydride reduction, of the labile HNB group can now permit the use of HNB-Br as a means of introducing a specific reporter group on tryptophan 62 in order to probe the cleft region in the hen egg white lysozyme molecule.

Table I

Incorporation of HNB into lysozyme and the subsequent regeneration of HNB:lysozyme.

Molar ratio HNB/protein[a]	HNB:protein[b] T=0	HNB:protein[b] T+19	% Inhibition[c] T=0	% Inhibition[c] T+19	% HNB released	% Activity regenerated	Moles HNB released
0.526	0.319	0.056	28.2	4.4	82.4	84.2	0.236
0.737	0.534	0.095	51.1	9.2	82.2	82.0	0.439
0.947	0.678	0.122	58.6	8.5	82.0	85.4	0.566
1.16	0.758	0.128	65.1	9.7	83.1	85.0	0.630
1.47	0.945	0.177	74.9	8.8	81.3	88.2	0.768
1.79	1.017	0.175	82.7	12.2	82.6	85.3	0.842
2.10	1.140	0.308	90.5	25.0	70.5	72.4	0.830
3.16	1.180	0.273	95.0	19.4	76.8	79.6	0.910
5.26	1.270	0.270	98.0	25.3	78.7	74.2	1.000

(a) 1.00 ml aliquots of a stock solution of lysozyme, 3.59×10^{-4} M, in 0.18 M acetic acid, pH 2.80 were reacted with varying volumes of 3.78×10^{-2} M HNB-Br in dry acetone to yield the appropriate molar excess of reagent.

(b) The ratio of HNB:lysozyme was determined, following gel filtration, at zero time and following 19 hours of incubation at pH 2.80 and 40°.

(c) Activity was determined immediately after reaction, T=0, and following 19 hours of incubation at pH 2.80 and 40°.

Table II

Effect of pH on incorporation and characterization of HNB:lysozyme.

pH	HNB:lysozyme T=0(a)	HNB:lysozyme T+2(b)	Specific Activity T=0(a)	Specific Activity T+2(b)	% HNB Lost	% Activity Regenerated
1.09	1.001	.345	2073	11193	65.5	73.5
2.00	1.049	.271	1350	11390	74.2	76.6
2.50	1.016	.243	1463	12036	76.0	81.0
2.97	.956	.235	1969	12183	75.4	81.6
3.51	.909	.247	2451	12331	77.8	82.1
4.02	.690	.138	4843	12686	80.0	81.2
4.47	.585	.140	6771	13265	76.4	85.9
5.02	.377	.099	8847	13380	73.8	80.3
6.07	.325	.077	9893	13270	76.3	73.4
7.07	.323	.075	10110	13360	76.7	74.1

(a) Lysozyme solutions were prepared at concentrations of 1.39×10^{-4}M in 0.10 M buffers at the pH values indicated. Each sample was reacted with HNB-Br 7.0×10^{-2}M. Following reaction the proteins were assayed and subjected to gel filtration prior to ratio determinations.

(b) Following two hour incubation at pH 2.80 at 53°.

Table III

Preparation and isolation of HNB(1.05):lysozyme.

Fraction	Protein(mg)	HNB: protein	Per cent Activity	Per cent Yield
Lysozyme	2,000	-	100	100
HNB:lysozyme after G-25 gel filtration	1,980	1.16	7.3	99
Bio-Rex 70 fractions 120-225	1,720[(a)]	-	-	86
Pooled fractions following concentration, extraction, desalting and lyophyllization				
fractions 120-155		1.05	5.1	
fractions 156-185	1,600[(b)]	1.03	5.1	80
fractions 186-225		1.06	5.8	

(a) Determined from $O.D._{280}$ of the pooled fractions.

(b) Total protein derived from fractions 120-225.

Table IV

Characterization of HNB:lysozyme fractions from Bio Rex 70 columns.

Fraction	Ratio	%Act.	Ratio[a]	% Act.[a]	ΔRatio	Δ% Act.
120-155	1.05	5.1	.29	78.5	.76	73.4
156-185	1.03	5.1	.29	79.0	.74	73.9
186-225	1.06	5.8	.27	82.5	.79	76.7
Frac. 35 Fig. 10[b]	1.06	6.8	.25	88.5	.81	81.7

(a) After incubation for 3 hours at 50° and pH 2.80.

(b) The sample applied was a portion of the final product derived from fractions 120-225.

Table V

Isolation of HNB(0.96):peptide T_8-T_9.

Fraction	Protein (mg)	Total O.D.$_{420}$	Yield
HNB(1.05):lysozyme	250	330	100
RCM-HNB(1.05):lysozyme	197	254	78.8
Supernate of tryptic digestion[a]	-	225	100
Supernate of pyridine-acetate precipitate (I)	-	42	-
First pyridine acetate wash (II)	-	14	-
Second pyridine acetate wash (III)	-	2	-
Redissolved pyridine acetate precipitate	26	168	74.4[b]
Combined I, II, III, freeze thaw supernate	-	51	-

(a) 190 mg of RCM-HNB(1.05):lysozyme subjected to tryptic digestion.

(b) Based on soluble tryptic digestion products.

Table VI

Amino acid composition of peptide T_8-T_9.

Amino Acid	Ratio Sample/Std[a]	Residues[c] Actual	Amino Acid Residues Corrected	
			Sample	Peptide T_8-T_9[d]
CM-Cys.	.133[b]	1.00	1	1
Asp.	.832	6.25	6	6
Thr.	.232	1.74	2	2
Ser.	.211	1.59	2	2
Glu.	.133	1.00[c]	1	1
Pro.	-	-	-	-
Gly.	.399	3.00	3	3
Ala.	.028	0.21	0	0
Cys.	-	-	-	-
Val.	-	-	-	-
Met.	-	-	-	-
Ile.	.236	1.78	2	2
Leu.	.116	0.87	1	1
Tyr.	.105	0.80	1	1
Phe.	-	-	-	-

(a) Standard amino acid calibration solution 0.10 mmoles/ml.

(b) No CM cysteine was available as a standard so that the ratio is determined by assuming absorption of CM-cysteine is equal to the average of all the other amino acids.

(c) Relative to glutamic acid which is defined as 1.00 residues.

(d) Reference (83).

Table VII

The denaturation of lysozyme and HNB(1.05):lysozyme by urea.

Sample[(a)]	[Urea]	Specific Activity at time (hrs.) 0	2.5	20	44
Lysozyme	-	10,900	10,000	8,370	8,430
Lysozyme	8M	10,300	10,000	2,880	70
HNB(1.05): lysozyme	-	600	575	725	825
HNB(1.05): lysozyme	8M	550	425	100	0

(a) The samples were prepared at a concentration of 2.78×10^{-4}M in 0.23M Borate-NaOH buffer, pH 9.80 and incubated in the presence or absence of 8M urea at 37° for the indicated times.

Table VIII

The regeneration of HNB(1.05):lysozyme in 8M urea.

Sample	Specific Activity T=0[a]	Specific Activity T+48[b]	Ratio HNB:Protein T=0[a]	Ratio HNB:Protein T+48[b]
Lysozyme	8,620	8,800	-	-
Lysozyme + 8M urea	140	188	-	-
HNB(1.05): lysozyme	1,340	7,330	0.81	0.30
HNB(1.05): lysozyme + 8M urea	0	0	1.05	1.06

(a) Determined after 44 hours incubation at 37°, pH 9.80.

(b) Samples at 44 hours adjusted to pH 5.0 and incubated an additional 48 hours at 37°.

Table IX

Effect of HNB-Br on human lysozyme.

Sample	Molar Excess HNB-Br[a]	Per cent Activity	Ratio[b] HNB: lysozyme	Ratio[c] HNB: lysozyme
Human lysozyme	-	100	-	-
" "	2.5	103	-	-
" "	5.0	96	-	-
" "	7.5	97	-	-
" "	10.0	90	-	-
" "	15.0	89	-	-
" "	20.0	90	1.10	1.08

(a) Duplicate 1.00 ml samples of human lysozyme, 6.95×10^{-5}M in 0.18M acetic acid pH 2.80, were reacted with varying volumes of 7.00×10^{-3}M HNB-Br in dry acetone. Following the addition of an aliquot of HNB-Br the sample was immediately assayed and an additional aliquot of HNB-Br was added. This process of HNB-Br was continued until a 20 fold molar excess of HNB-Br was attained.

(b) Ratio of HNB:lysozyme determined following gel filtration.

(c) Ratio determined after 2.5 hours of incubation at 45° in 0.18M acetic acid pH 2.80.

Table X

Effect of pH on the first order rate constant for the regeneration of HNB(1.05):lysozyme.

pH	$t_{1/2}$ (min)	k x10^3 min^{-1}
1.23	-	-
1.53	253(a)	2.74
2.03	242	2.86
2.59	223	3.10
2.96	220	3.14
3.45	250	2.77
4.04	230	3.01
4.41	245	2.83
5.02	252	2.75
5.51	305	2.27
6.05	320	2.16
6.57	382	1.81
6.95	471	1.47
7.37	567	1.22
7.82	1240	0.558
8.10	1920	0.361
8.39	2480	0.279
9.12	5840	0.119
9.70	9300	0.075

(a) Determined by taking the initial slope.

Table XI

The effect of various nucleophiles on the rate of regeneration of HNB(1.05):lysozyme.

Sample	Addition	pH	Temperature	$t_{1/2}$(min)	k min^{-1}
HNB(1.05): lysozyme	none	4.0	35°	128	.0054
" "	0.03M I^-	"	"	109	.0064
" "	0.10M I^-	"	"	89	.0078
" "	0.30M I^-	"	"	77	.0090
" "	1.00M I^-	"	"	70	.0099
" "	none	"	40°	48.5	.0143
" "	0.50M I^-	"	"	32.5	.0213
" "	0.50M SCN^-	"	"	31	.0224
" "	none	7.0	"	107	.0065
" "	0.5M I^-	"	"	54	.0128
" "	0.5M SCN^-	"	"	54	.0128

Table XII

The incorporation of HNB into various lysozyme derivatives and the subsequent regeneration of the HNB:proteins.

Derivative[a]	Residues Modified[c]	Ratio HNB:protein T=0	Ratio HNB:protein T+2 hrs[d]	% Activity[e] T=0	% Activity[e] T+2[d]	ΔRatio	Δ% Act.
Native lysozyme	-	.955	.208	14.5	86.1	.747	71.6
guanidinated lysozyme	lysine (5.9/6.0)	.932	.204	13.7	72.0	.728	58.7
guanidinated and acetylated	lysine (5.9/6.0) tyrosine (2.1/3.0)	.970	.250	14.2	82.0	.720	67.8
acetylated	tyrosine (2.1/3.0)	.977	.230	18.4	91.0	.747	72.6
NBS oxidized	tryptophan (1.2/6.0)	.360	.224	-	-	.136	-
guanidinated HNB(1.05): lysozyme[b]	lysine (5.6/6.0)	.917	.292	21.6	78.5	.625	56.9

(a) The HNB:derivatives were produced by reaction of 1.00 ml protein, 6.9×10^{-5}M in 0.18M acetic acid pH 2.80 with 0.0050 ml HNB-Br, 6.9×10^{-2}M.

(b) Prepared by guanidination of HNB(1.05):lysozyme as previously described.

(c) Residues modified/total residues present.

(d) Ratios and activity were determined following incubation for 2 hours at 55°.

(e) Per cent activity was determined with respect to an identical sample not subjected to treatment with HNB-Br.

Table XIII

Acetylation of HNB(1.05):lysozyme.

Sample	Ratio HNB:lysozyme T=0	T+3hours[b]	T+5hours[c]
HNB(1.05): lysozyme	1.04	0.29	0.28
acetyl-HNB(1.05): lysozyme[a]	1.06	0.49	0.28

(a) Prepared by reaction of HNB(1.05):lysozyme with a 435 fold excess of acetyl imidazole.

(b) Determined after three hour incubation at 45° and pH 2.80.

(c) Following three hour incubation both samples adjusted to pH > 11, after 10 minutes adjusted to pH 3.0 and incubated an additional 2 hours at 45°.

Table XIV

Regeneration of acetylated and deacetylated HNB(1.05):lysozyme.

Sample	Treatment	Ratio HNB: lysozyme
acetyl-HNB(1.05): lysozyme[a]	Maintain at pH 2.80 two hours at 0-2°	0.92
" "	Maintain at pH 2.80 two hours at 50°	0.67
" "	Adjust to pH 11, after 5 minutes adjust to pH 3.0. Maintain at pH 3.0 two hours at 50°	0.26
HNB(1.05):lysozyme	Maintain at pH 2.80 3 hours at 50°	0.28

(a) Prepared by reaction of HNB(1.05):lysozyme with a 545 fold excess of acetyl imidazole.

Table XV

The inhibition of lysozyme by 2-methoxy-5-nitrobenzyl bromide.

Molar Excess[a] MNB-Br	Per Cent Activity[b] 1 min	0.5 hrs	1.0	2.0	6.0	19.0	% Inhibition	HNB: lysozyme
-	100	100	100	100	100	100	0.00	0.00
0.25	94	80	75	76	87	78	22	.254
0.50	90	71	68	56	60	61	39	.460
0.75	92	63	55	45	40	36	64	.610
1.0	98	53	41	30	25	22	78	.763
1.5	95	35	25	9.3	4	3.7	96	1.00
2.0	90	29	13	4.9	1	1.4	99	0.95
4.0	84	15	3.4	1	<1	<1	100	1.00
7.0	82	14	4.2	<1[c]	<1	<1	100	0.90
10.0	79	15	3.8	<1[c]	<1	<1	100	1.10

(a) Ten identical 1.00 ml aliquots of a stock solution of lysozyme 1.39×10^{-4}M in 0.18M acetic acid pH 2.80 were reacted with varying volumes (1.0-40.0 μl) of a stock solution of MNB-Br, 3.45×10^{-2}M in dry acetone.

(b) Per cent activity was determined at the indicated times during incubation of MNB-Br with lysozyme at pH 2.80 and 30°.

(c) These samples were removed at 2 hours and subjected to gel filtration, following the removal of MNB-Br, MNB-OH and acetone, the protein fraction was returned to the bath and incubation allowed to proceed from the three hour interval.

Table XVI

Effect of borohydride on the regeneration of HNB(1.05):lysozyme.

Sample	$[BH_4]$	% Activity T=0	% Activity T+2[b]	Ratio HNB:lysozyme[b]
HNB(1.05):lysozyme[a]	-	9.0	79	.218
" "	.0004	8.0	68	.258
" "	.0020	7.9	58	.416
" "	.0095	6.7	21.5	.784
" "	.040	6.1	12.5	.823

(a) 1.00 ml HNB(1.05):lysozyme 6.9×10^{-5}M was reacted with 0, 2.0, 10.0, 50.0 and 250.0 μl 0.20M $NaBH_4$ at pH 4.0.

(b) Following two hours incubation at pH 4.0 and 55°.

Table XVII

The effect of sodium borohydride on the regeneration of HNB(1.05):lysozyme.

Sample[a]	$[BH_4]$	Activity/μl[b]	HNB:protein	Activity/μl[c]	HNB:protein[c]
HNB(1.05): lysozyme	0	64	0.73	167	0.18
+ native lysozyme at 0.74 mole fraction HNB:lys.	0.0065	63	"	113	0.35
	0.025	64	"	71.5	0.64
	0.084	62.3	"	61.5	0.67

(a) A sample of lysozyme plus HNB(1.05):lysozyme was prepared at a concentration of 1.39×10^{-4}M in 0.50M acetate buffer pH 4.00 and was reacted with 0.40M $NaBH_4$ to give the appropriate final concentrations indicated.

(b) Activity given in terms of O.D./min/μl $\times 10^3$.

(c) Following incubation at pH 4.00 for 2 hours at 55°.

Table XVIII

The inhibition of the regeneration of enzymatic activity and the incorporation of tritium in HNB(1.05):lysozyme by $NaBH_4(T_4)$.

Sample[a] #	$[BH_4(T_4)]$	Specific Activity T=0	Specific Activity T+2 hrs[b]	Δ Specific Activity	cpm/mg[c]	cpm/mg[d]
1	none	790	10,050	9260	0	-
2	0.00455	840	9,640	8800	3,690	1,910
3	0.00455	760	10,230	9470	1,780	-
4	0.00224	780	8,790	8010	7,700	4,890
5	0.00224	630	10,080	9450	2,810	-
6	0.0108	770	5,900	5130	21,000	18,640
7	0.0108	660	9,600	8940	2,360	-
8	0.0454	790	2,450	1660	41,400	35,700
9	0.0454	740	9,690	8950	5,700	-

(a) Odd numbered samples prepared by the addition of the enzyme to hydrolyzed samples of $BH_4(T_4)$. HNB(1.05):lysozyme 1.39×10^{-4}M.

(b) After two hours at pH 4.00 and 55°.

(c) Corrected for sample one, no $BH_4(T_4)$ equal 0 cpm.

(d) Corrected for non specific tritium incorporation.

Table XIX

Incorporation of tritium as a function of mole fraction HNB(1.05):lysozyme.

Mole fraction[a] HNB(1.05):lysozyme	cpm/mg[c] protein	Per cent[d] regeneration
1.000	48200	11.3
0.801	35200	12.0
0.598	30200	11.8
0.389	21500	12.1
0.175	9825	12.1
0.000	0	-
1.000[b]	0	100.0

(a) Two stock solutions of HNB(1.05):lysozyme and native lysozyme both at a concentration of 1.39×10^{-4}M were mixed in the appropriate proportions to yield various mole fractions. All mole fractions determined from the HNB:protein ratios.

(b) This sample was regenerated by usual procedures and serves as a reference for the regeneration of samples reacted with $BH_4(T_4)$.

(c) Determined after reactions of 1.00 ml protein solution 1.39×10^{-4}M with 0.250 ml $NaBH_4(T_4)$ 0.329M in 0.50M acetate buffer pH 4.00. Following incubation for 2 hours at 55° aliquots of each fraction were counted. Counts per minute are corrected for non specific hydrogen-tritium exchange.

(d) Relative to the sample incubated in the absence of BH_4.

Table XX

Purification of tritium labeled HNB:peptide T_8-T_9 from tritiated HNB(1.05):lysozyme.

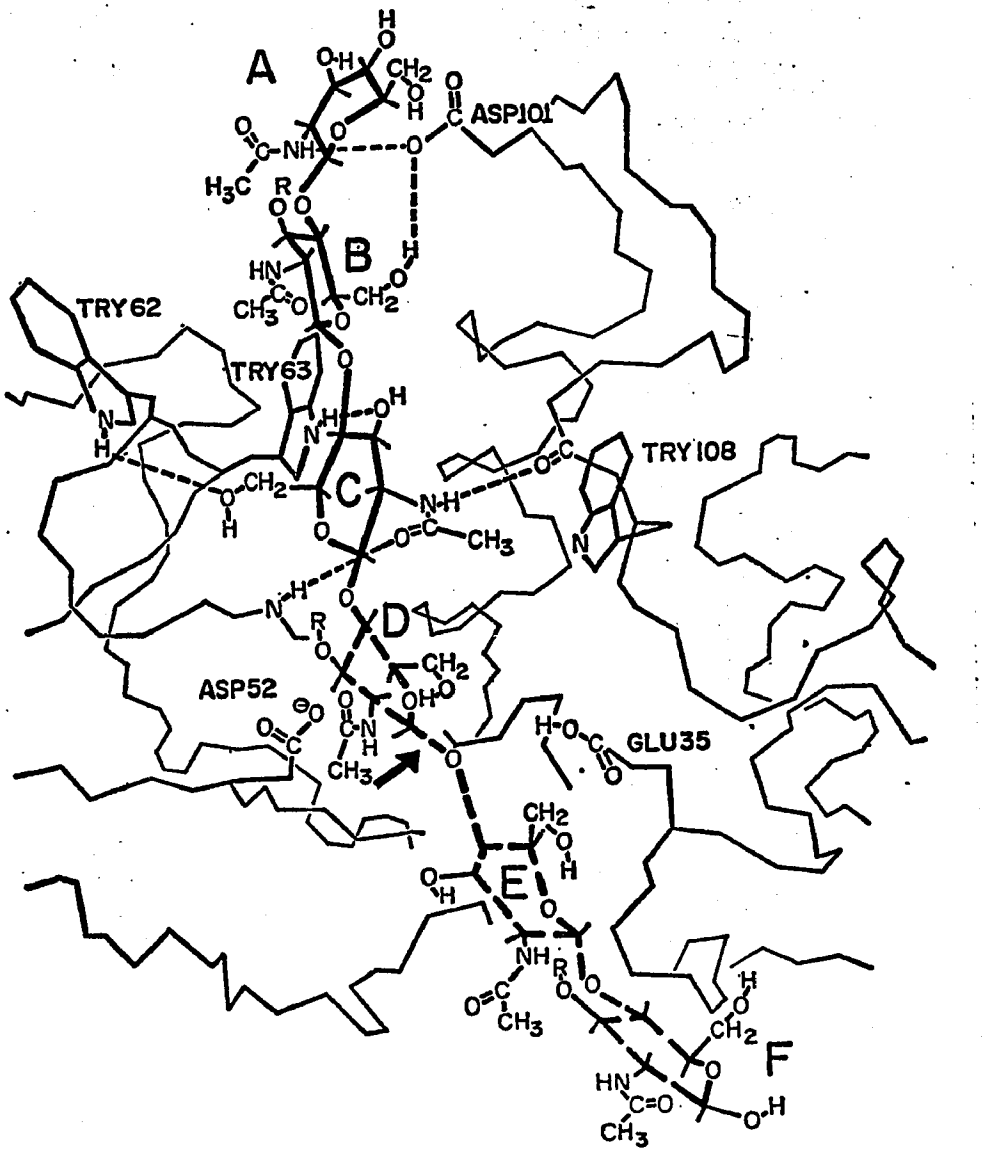
Sample	Volume of solution(ml)	Total[(a)] $O.D._{420}$	Total[(b)] cpm x 10^{-5}	Specific Activity x 10^{-4}
Tritiated HNB(1.05):lys.	9.0	33.4	4.21	1.26
RCM tritiated HNB(1.05):lys.	5.0	28.6	3.58	1.25
Soluble tryptic digestion products	5.1	26.8	3.62	1.35
Pyridine acetate supernate and wash solutions	10.1	5.95	0.77	1.29
Pyridine acetate precipitate HNB:peptide T_8-T_9	5.0	19.3	2.68	1.39

(a) Determined by dilution of 0.10 ml aliquots with 0.90 ml 1N NaOH.

(b) Aliquots of 0.10 ml were counted and corrected by the use of the appropriate blank.

Figure 1

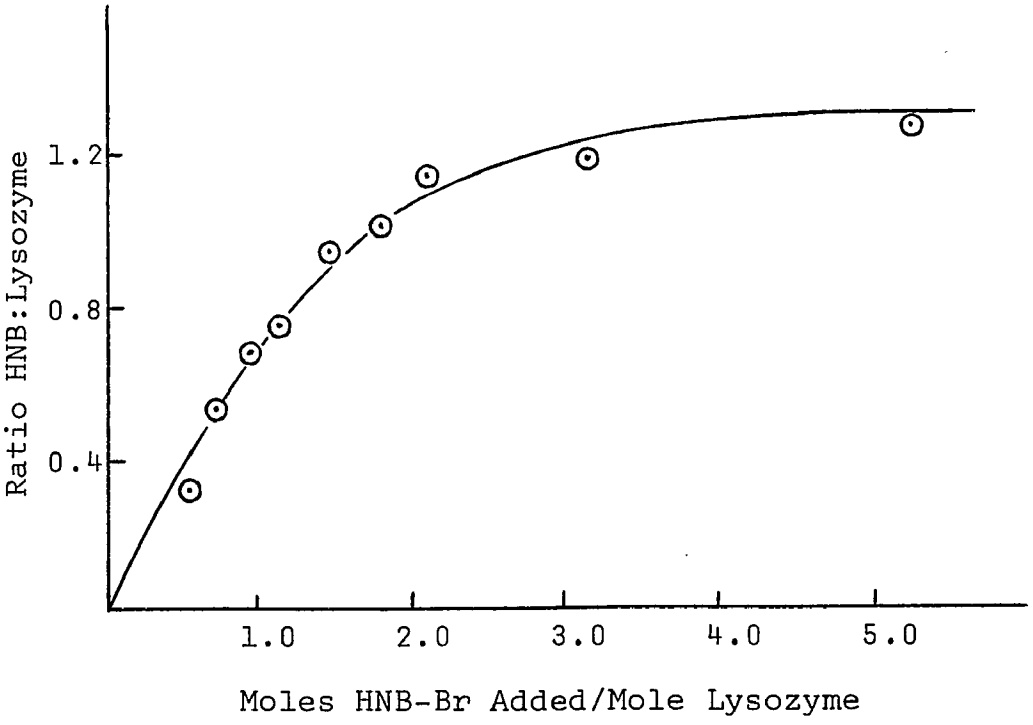
The three-dimensional model of the area around the active site of the lysozyme-substrate complex.



The pyranose rings of the substrate are shown in heavy lines, with the monosaccharide units of the lysozyme-tri N-acetyl glucosamine complex (A-C) solid and those placed by model building (D-F) dashed. Except for some groups of particular interest, only the peptide backbone of the protein is shown. The light dashed lines are the six hydrogen bonds between tri N-acetyl glucosamine and the enzyme. The groups R are hydrogens in the case of chitin substrates, and lactyl groups in cell wall saccharides. The arrow indicates the bond cleaved. Taken from Chipman and Sharon (23).

Figure 2

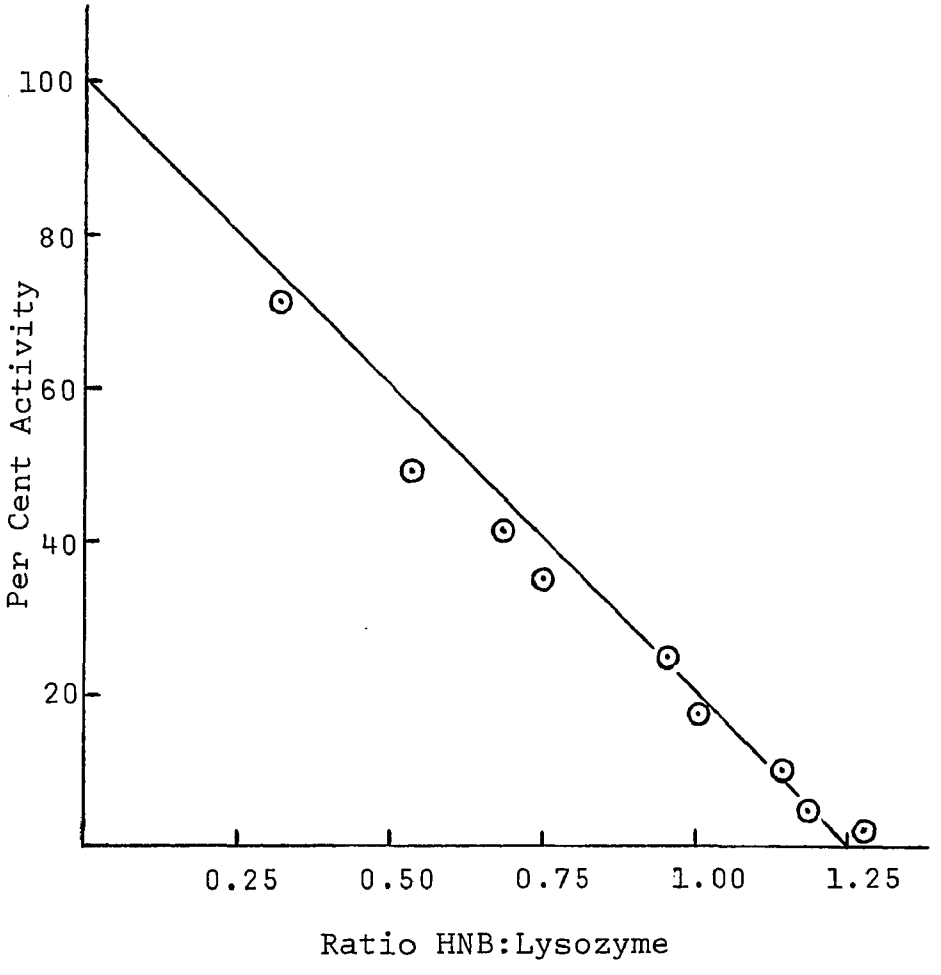
The stoichiometry of HNB-Br incorporation into lysozyme at pH 2.80.



The reaction of lysozyme with HNB-Br was performed under the conditions given in Table I. Following gel filtration the extent of HNB incorporation was determined.

Figure 3

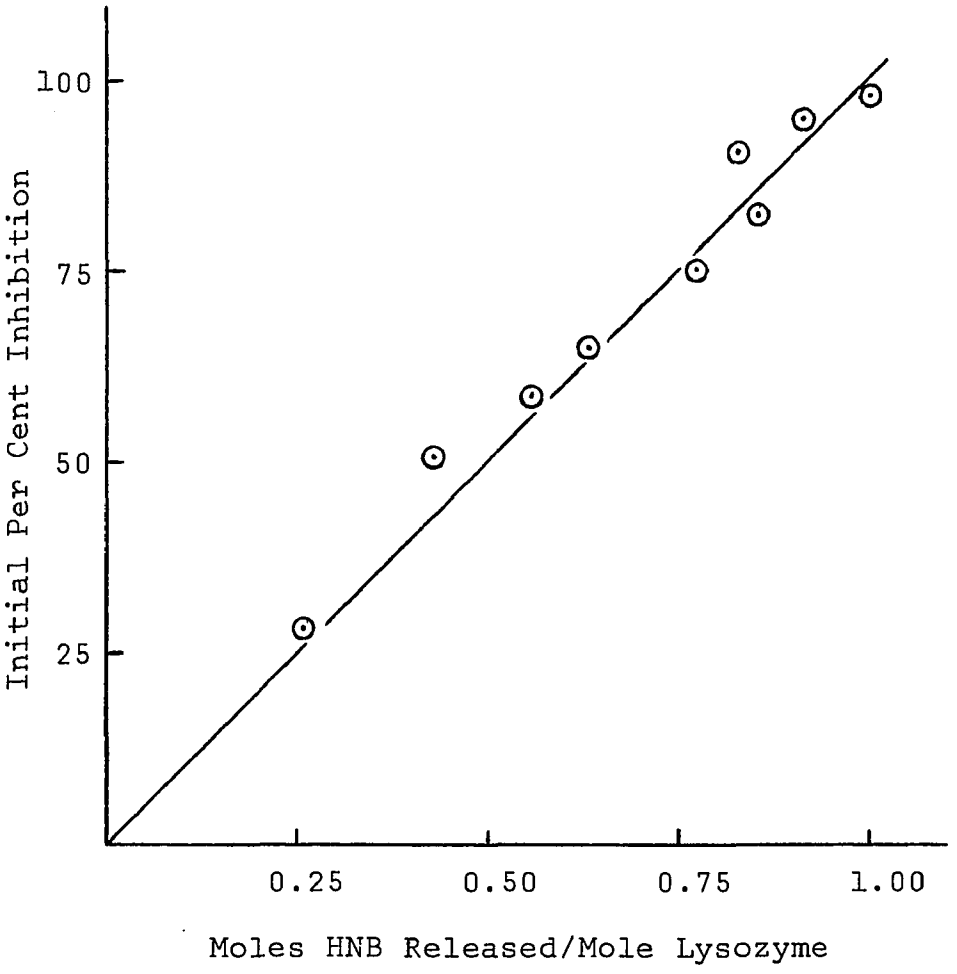
The inhibition of lysozyme by HNB-Br.



Samples of HNB:lysozymes produced under reaction conditions given in Table I were assayed for activity. Per cent activity was determined with respect to a sample of native enzyme treated under identical conditions in the absence of HNB-Br.

Figure 4

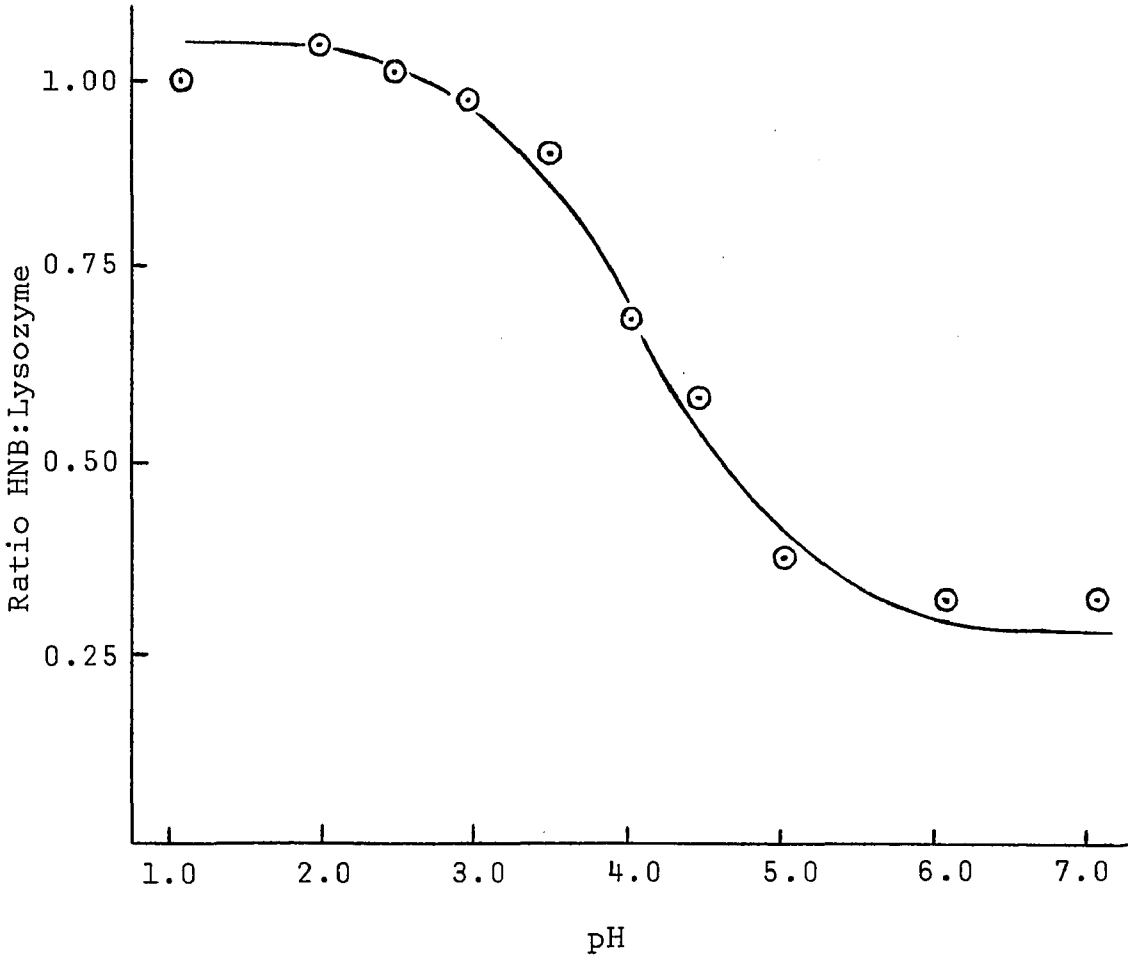
The relationship of labile HNB groups and enzyme inhibition.



Samples of HNB:lysozyme (Table I) were regenerated at pH 2.80 and 40° for 19 hours. The ratio of HNB:lysozyme after incubation minus the ratio of HNB:lysozyme prior to incubation was taken as a measure of HNB groups which are labile.

Figure 5

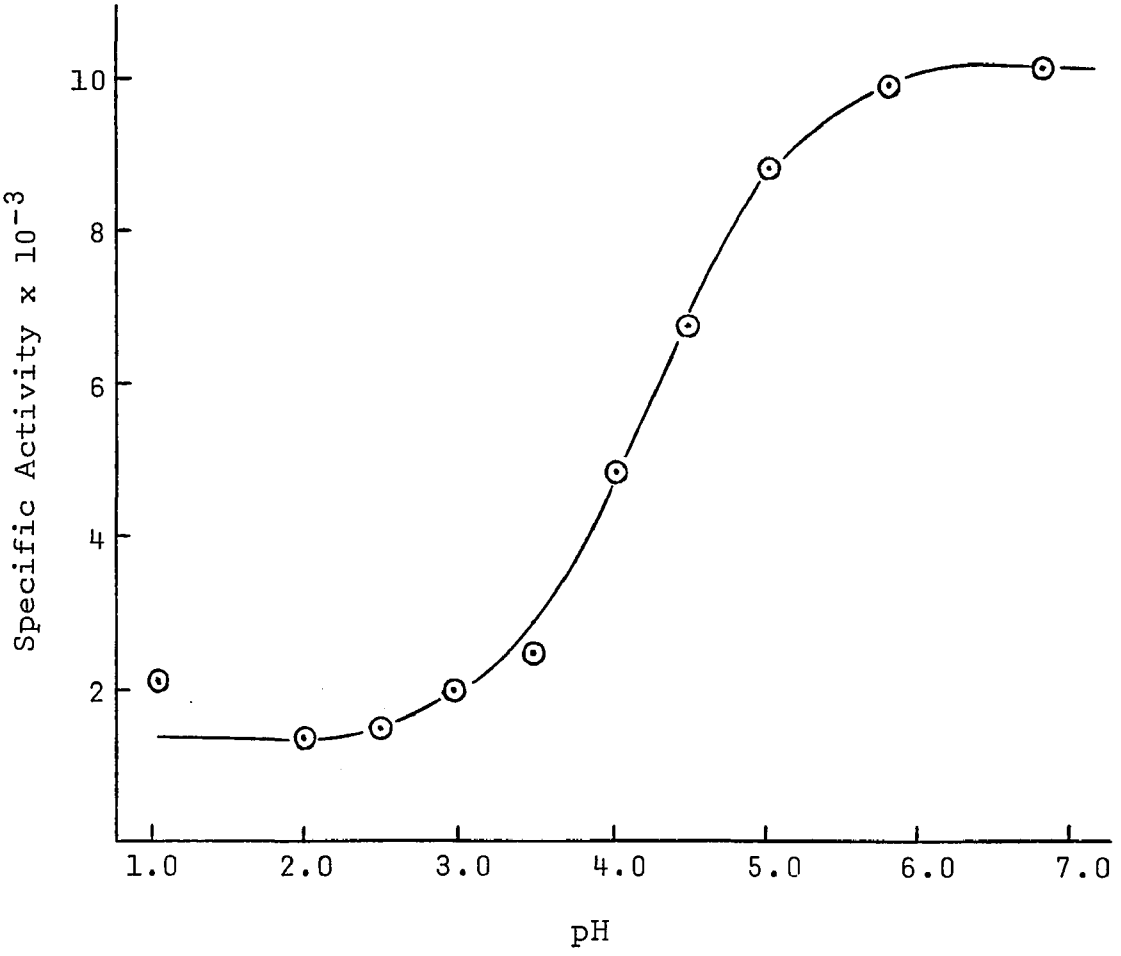
The effect of pH on the incorporation of HNB groups into lysozyme.



Conditions as given in Table II.

Figure 6

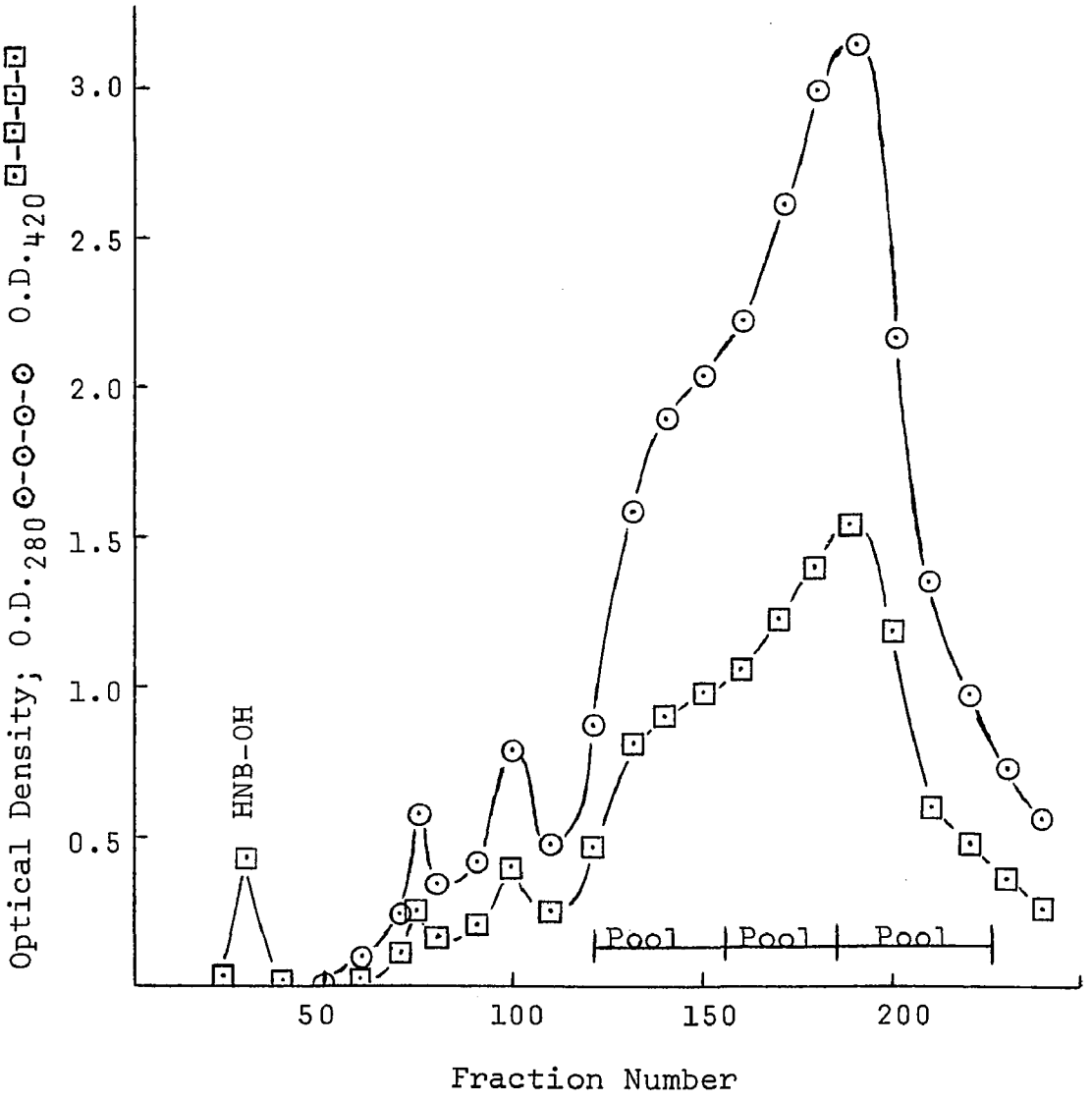
Specific activity of HNB:lysozyme produced at different pH's.



Conditions as given in Table II.

Figure 7

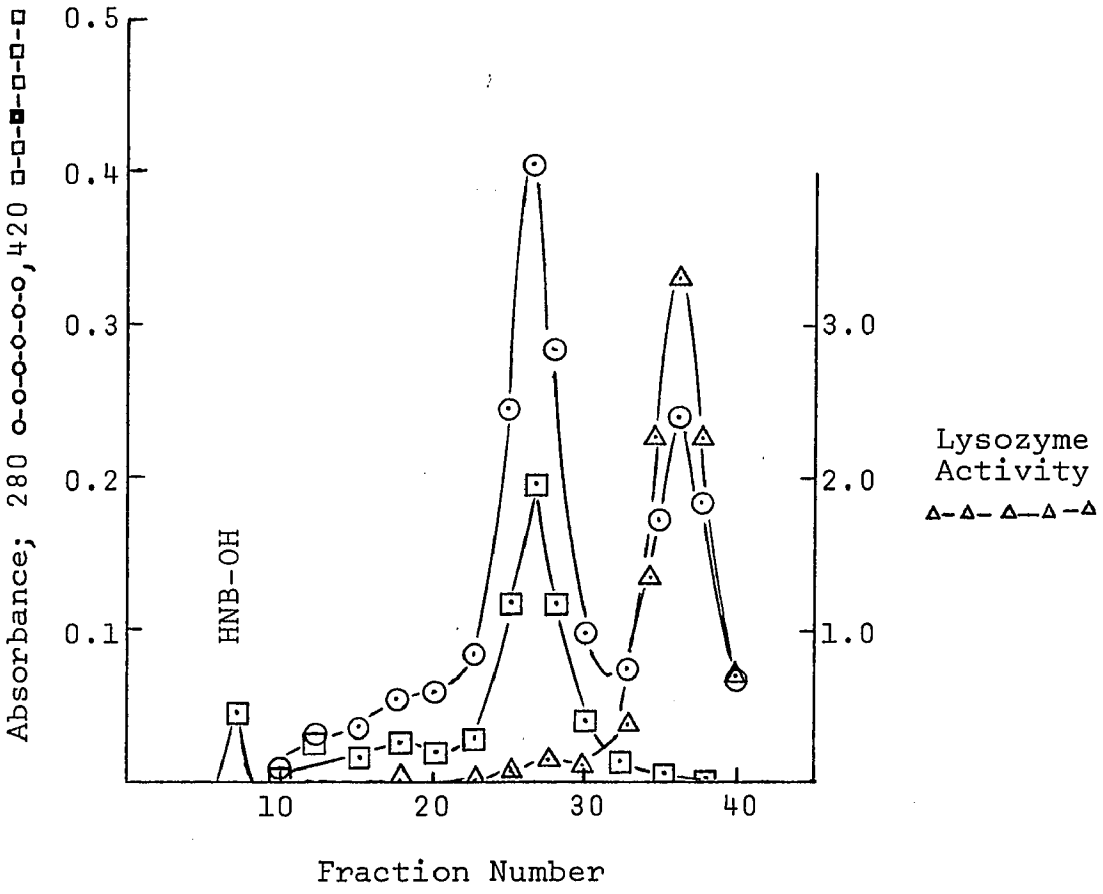
Chromatography of HNB(1.16):lysozyme on Bio-Rex 70.



1.98 gm (HNB(1.16):lysozyme was dissolved in 20 ml, 0.05 M Borate-NaOH buffer, pH 9.80. HNB-OH was eluted with 800 ml buffer at a flow rate of 97 ml/hr, at 4°. Elution continued with 0.23 M borate-NaOH buffer, pH 9.80 at the same flow rate. Fractions of 20 ml were collected. The pooled fractions indicated were isolated as described in Experimental section.

Figure 8

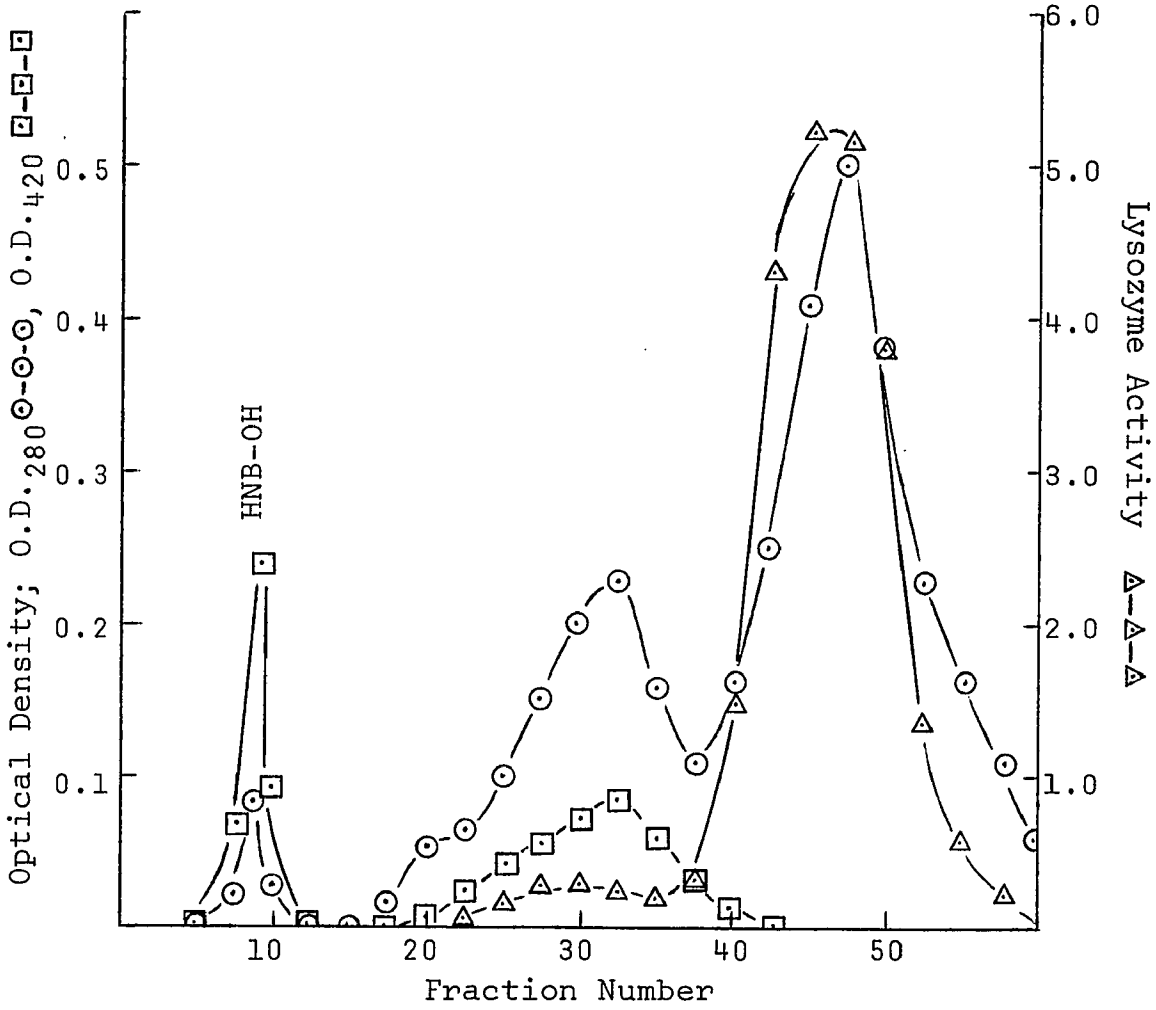
Analytical chromatography of a mixture of lysozyme and HNB(1.05):lysozyme.



A sample composed of 4 mg HNB(1.05):lysozyme and 2 mg native lysozyme was chromatographed according to the method outlined in Experimental section. Elution proceeded at a flow rate of 27 ml/hr and fractions of 5.0 ml were collected.

Figure 9

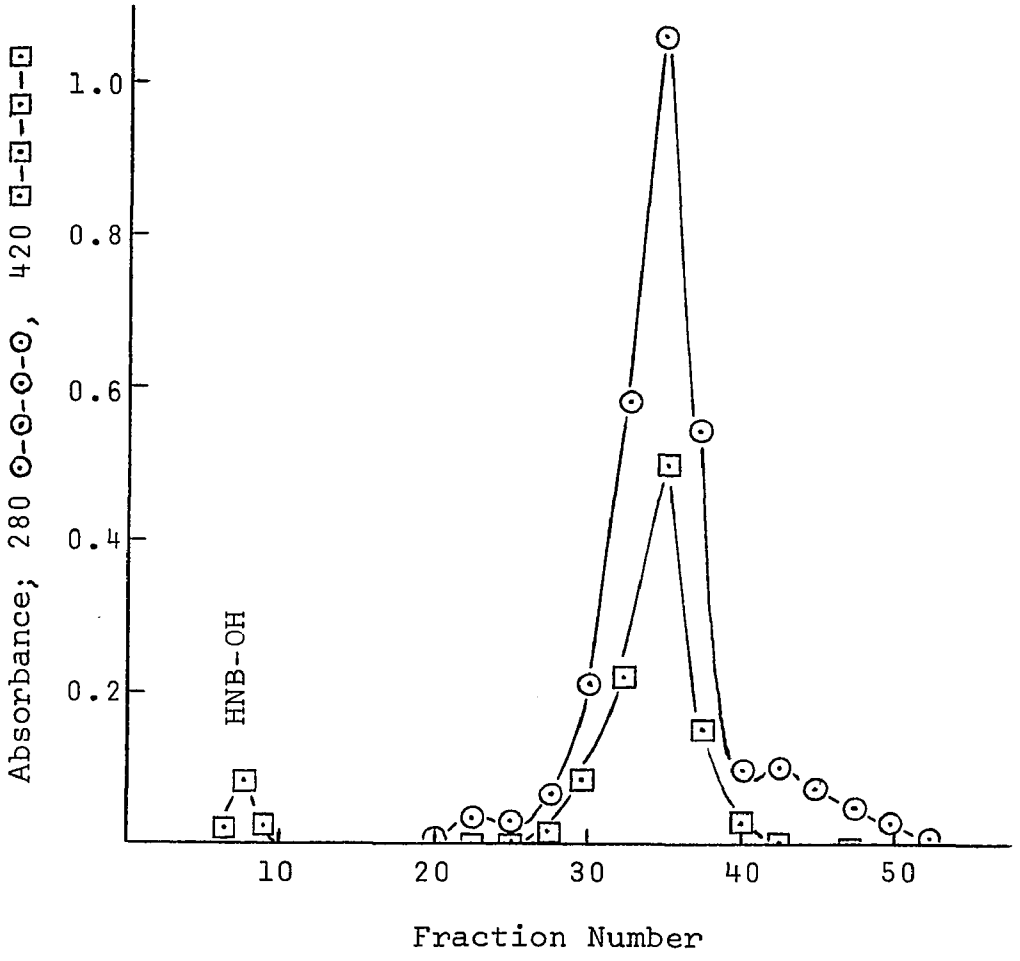
Analytical chromatography of the products formed in the regeneration of HNB(1.05):lysozyme.



A 20 mg sample of HNB(1.05):lysozyme was incubated for 24 hrs. at pH 2.80 and 37°. The products were adjusted to pH 9.80 and centrifuged. The clear supernatant solution was chromatographed according to methods outlined in Experimental section. Elution proceeded at a flow rate of 27 ml/hr and fractions of 6.5 ml were collected.

Figure 10

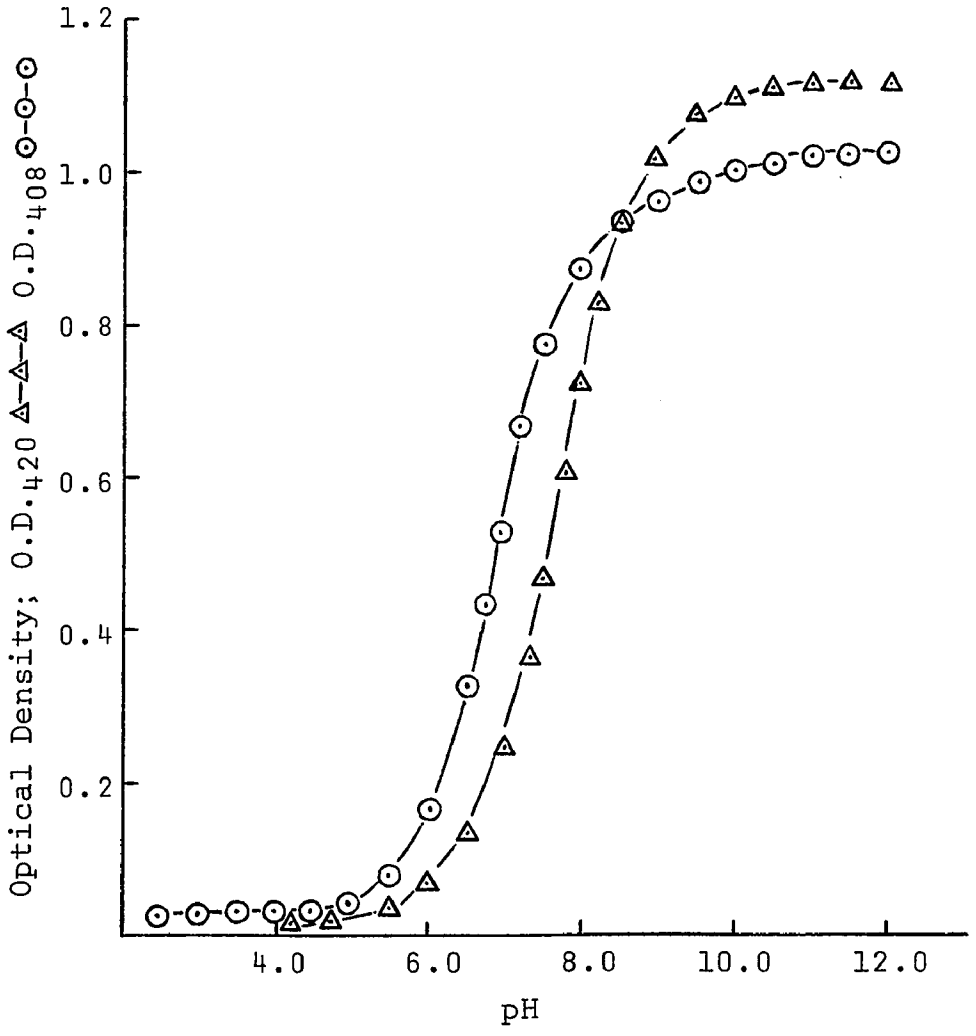
Analytical chromatography of HNB(1.05):lysozyme.



A 10 mg sample of HNB(1.05):lysozyme chromatographed according to method outlined in Experimental section. Elution proceeded at a flow rate of 27 ml/hr and fractions of 5.3 ml were collected.

Figure 11

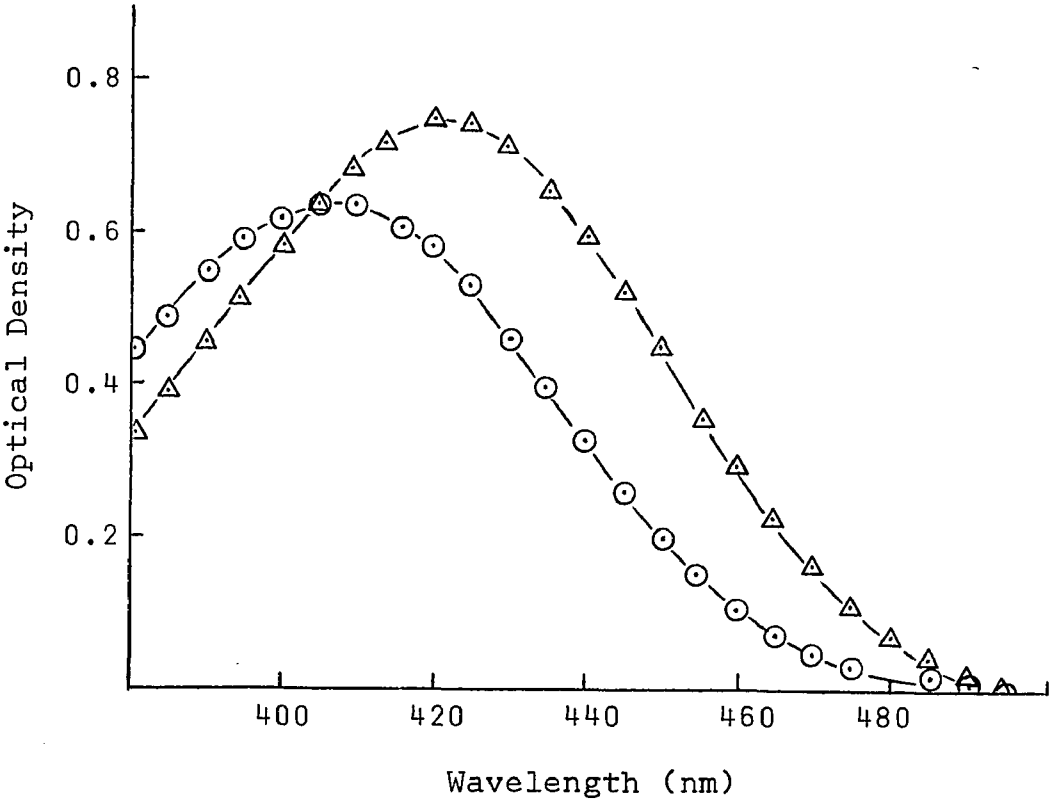
Spectrophotometric titration of HNB-OH and HNB(1.05):lysozyme.



Samples of HNB-OH ⊙-⊙-⊙-⊙ and HNB(1.05):lysozyme Δ–Δ–Δ at approximately 5×10^{-5} M were adjusted to various pH's with NaOH and optical densities were measured at either 408 nm (HNB-OH) or 420 nm (HNB(1.05):lysozyme).

Figure 12

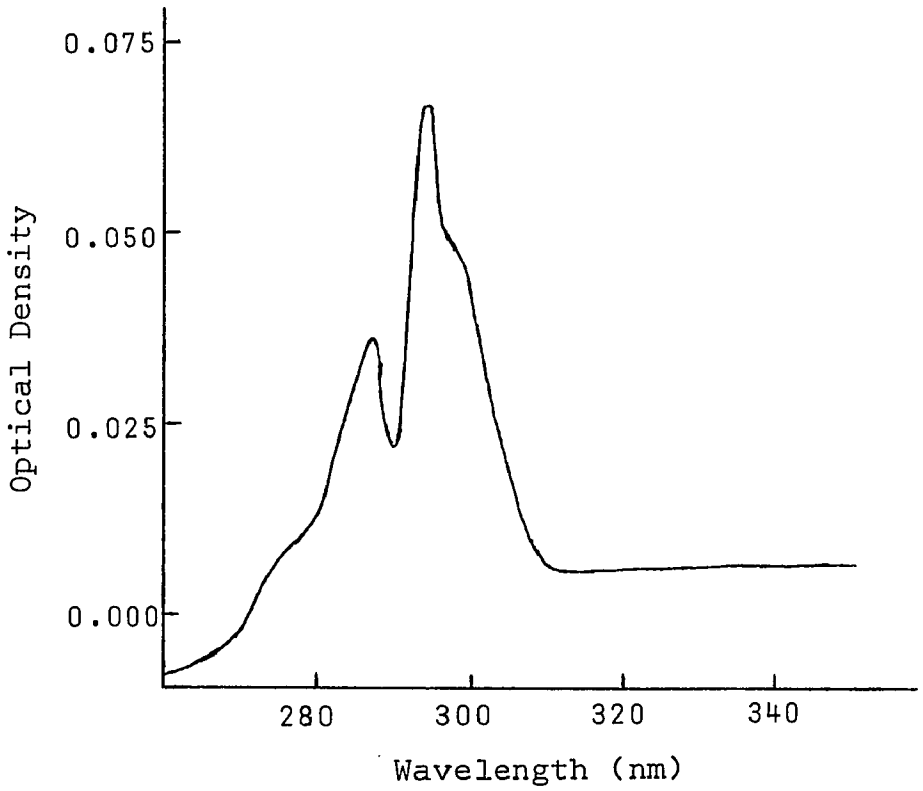
Visible spectra of HNB-OH and HNB(1.05): lysozyme in alkaline solution.



The visible spectra of HNB-OH ⊙-⊙-⊙-⊙ approximately 3.3×10^{-5} M and HNB(1.05):lysozyme △-△-△-△ approximately 4×10^{-5} M both in 0.9 M NaOH were recorded on a Cary model 14 spectrophotometer.

Figure 13

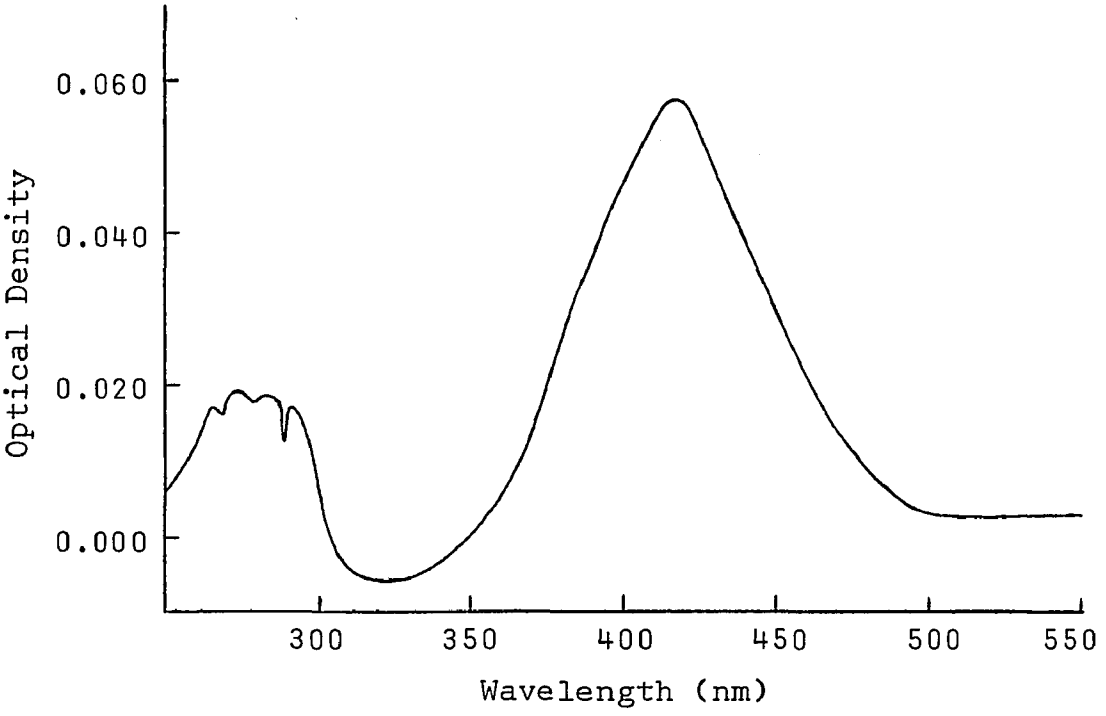
Difference spectrum of N-acetyl glucosamine and lysozyme.



The difference spectrum of lysozyme, 6.9×10^{-5} M and NAG, 0.20 M both in 0.10 M phosphate buffer pH 8.0 was produced at 30°.

Figure 14

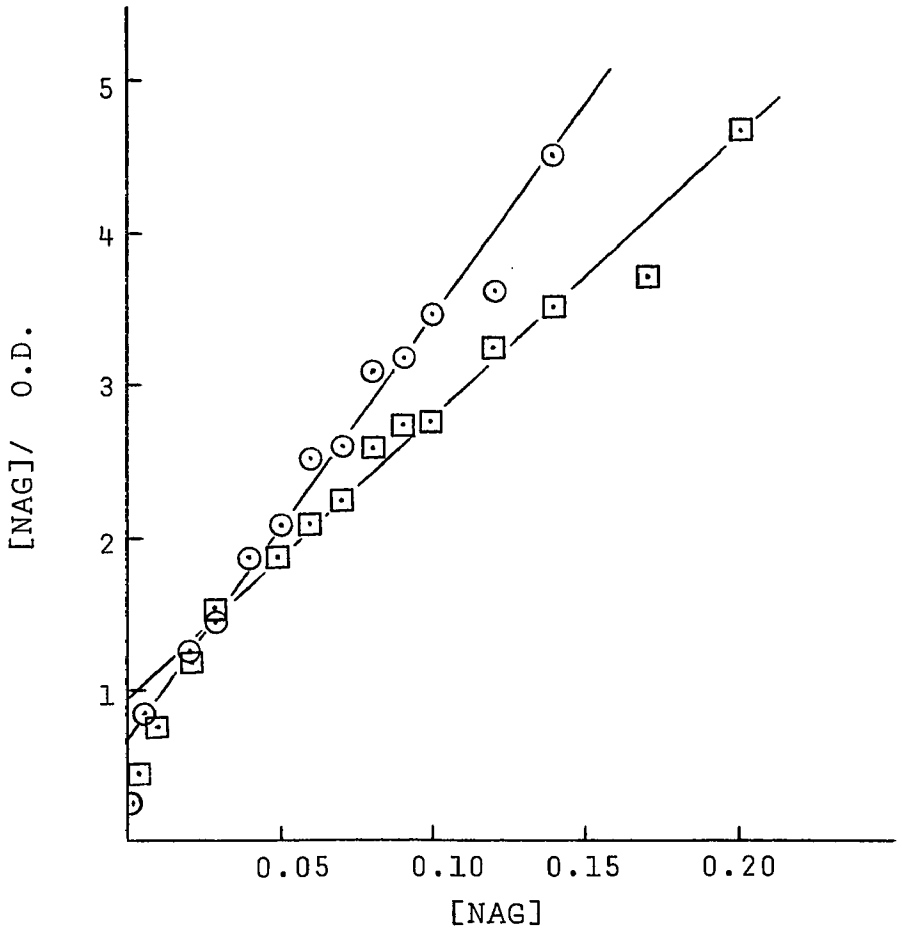
Difference spectrum of N-acetyl glucosamine and HNB(1.05):lysozyme.



Conditions as in Figure 13 except concentration of NAG, 0.16 M.

Figure 15

Eadie plots for the binding of N-acetyl glucosamine to lysozyme and HNB(1.05):lysozyme.

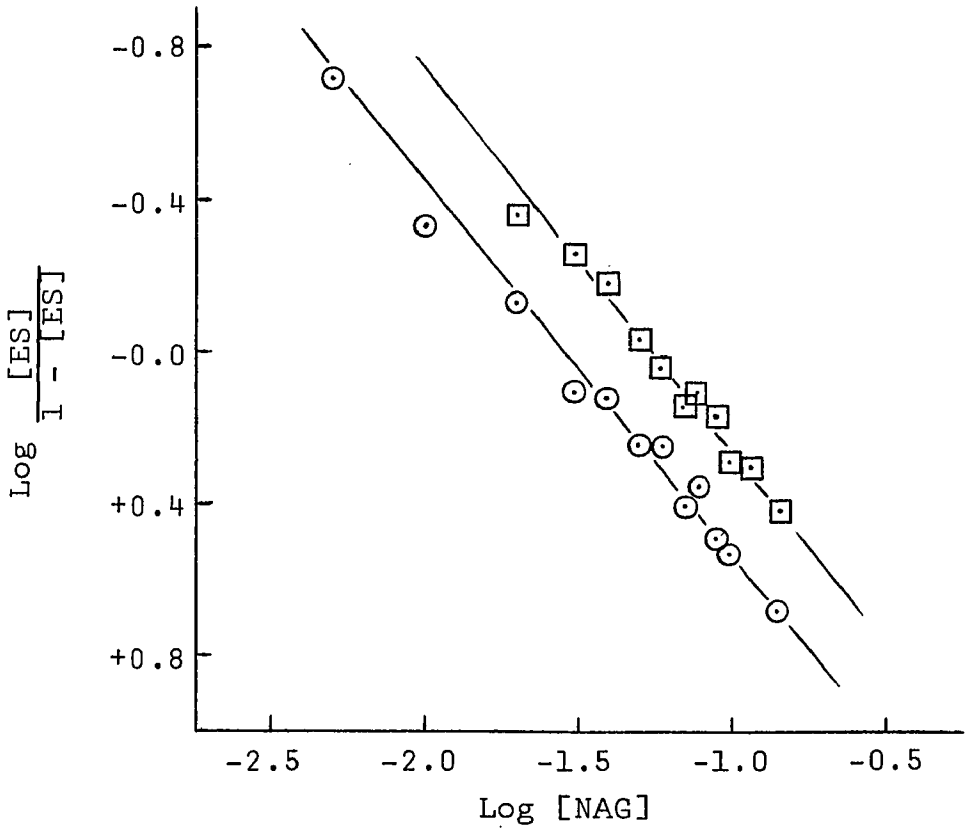


Lysozyme ⊙-⊙-⊙-⊙-⊙

HNB(1.05):lysozyme ⊡-⊡-⊡-⊡-⊡

Figure 16

Scatchard plots for the binding of N-acetyl glucosamine to lysozyme and HNB(1.05):lysozyme.

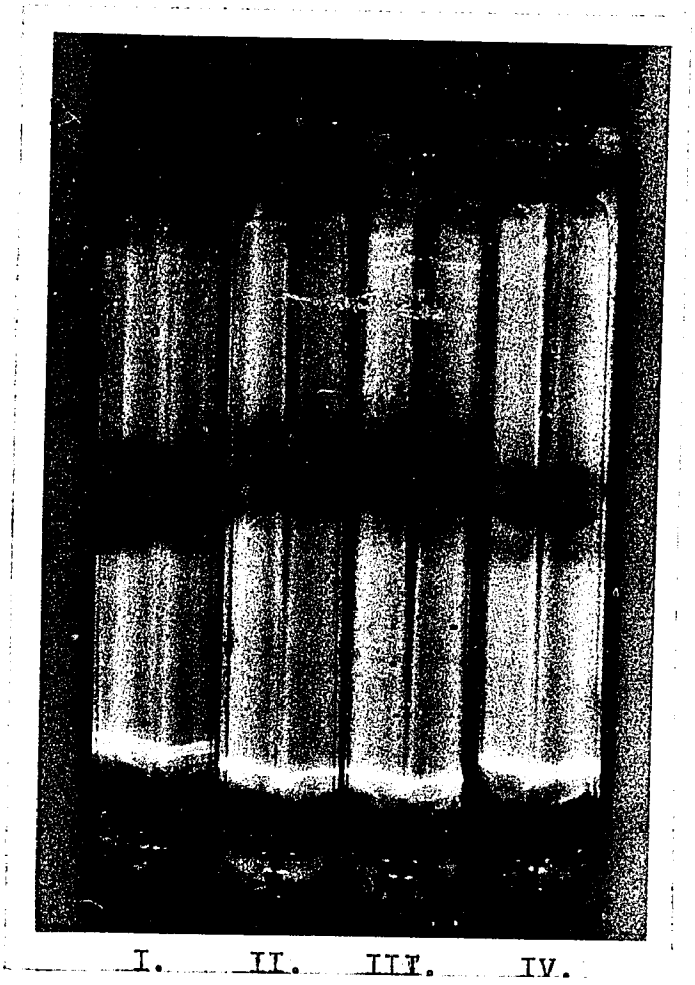


Lysozyme ⊙-⊙-⊙-⊙-⊙

HNB(1.05):lysozyme ⊡-⊡-⊡-⊡-⊡

Figure 17

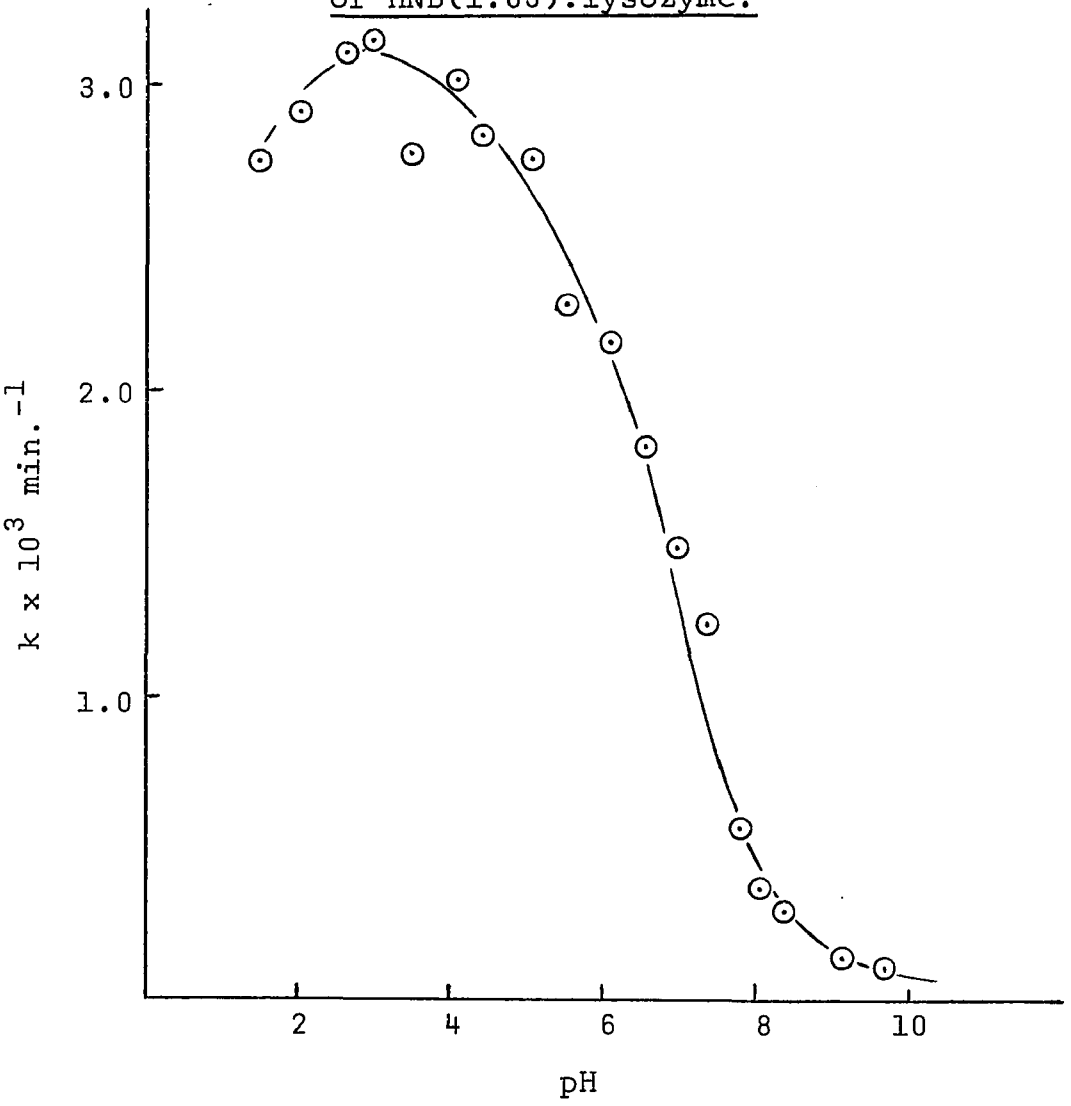
Polyacrylamide gel electrophoresis of lysozyme and HNB(1.05):lysozyme.



Gel I, native lysozyme; gels II, III and IV (HNB(1.05): lysozyme incubated for 0, 10 and 60 minutes at pH 4.00 and 50°. The origin is at the top of the gel and migration is towards the cathode.

Figure 18

The effect of pH on the rate of regeneration of HNB(1.05):lysozyme.



HNB(1.05):lysozyme, 0.50 mg/ml, in buffers of various pH's was incubated at 30° and rate constants for the regeneration of enzyme activity were determined.

Figure 19

Arrhenius plot for the regeneration of HNB(1.05):lysozyme at pH 4.06.

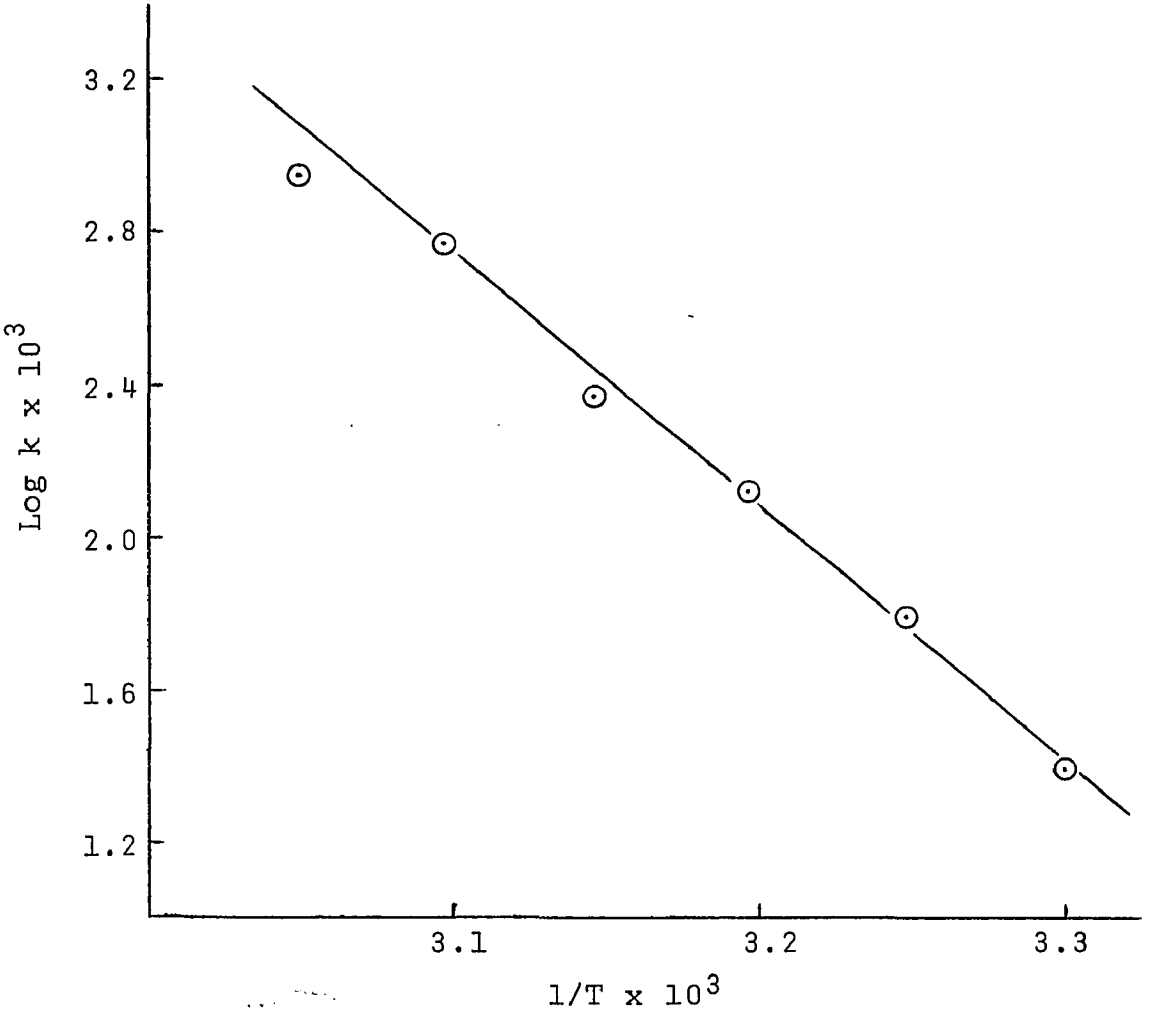
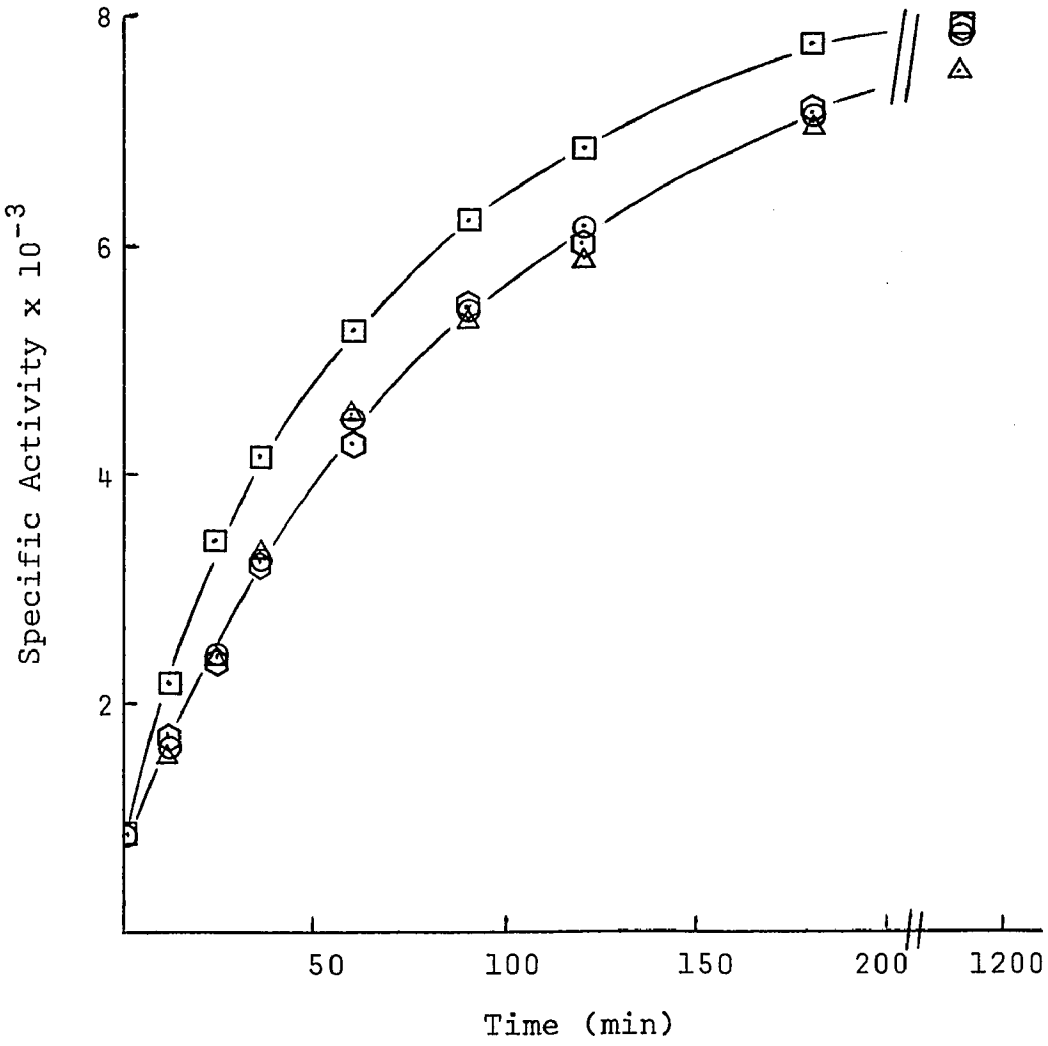


Figure 20

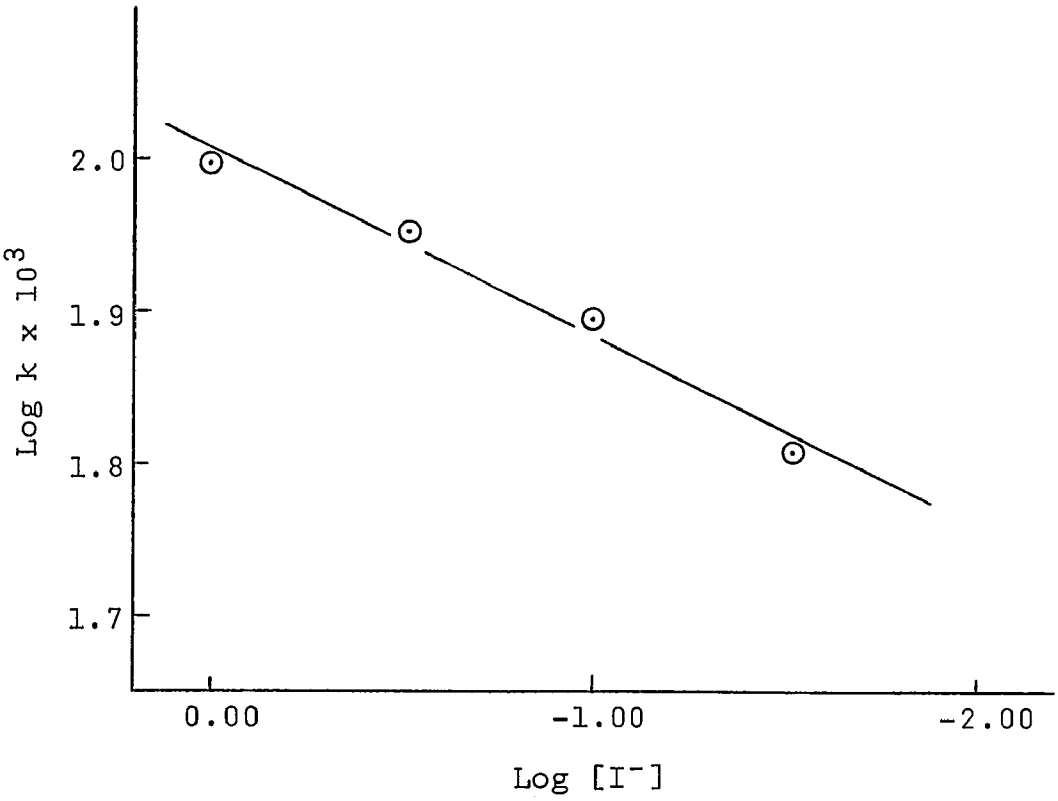
The effect of halides on the regeneration of HNB(1.05):lysozyme.



Samples of HNB(1.05):lysozyme, 0.50 mg/ml in 0.10 M acetate buffer, pH 4.0 in the presence of; ⊙-⊙-⊙-⊙ no halide, △-△-△-△ 0.10 M Cl^-, ⬡-⬡-⬡-⬡ 0.10 M Br^- and ⊡-⊡-⊡-⊡ 0.10 M I^-, were incubated at 40°.

Figure 21

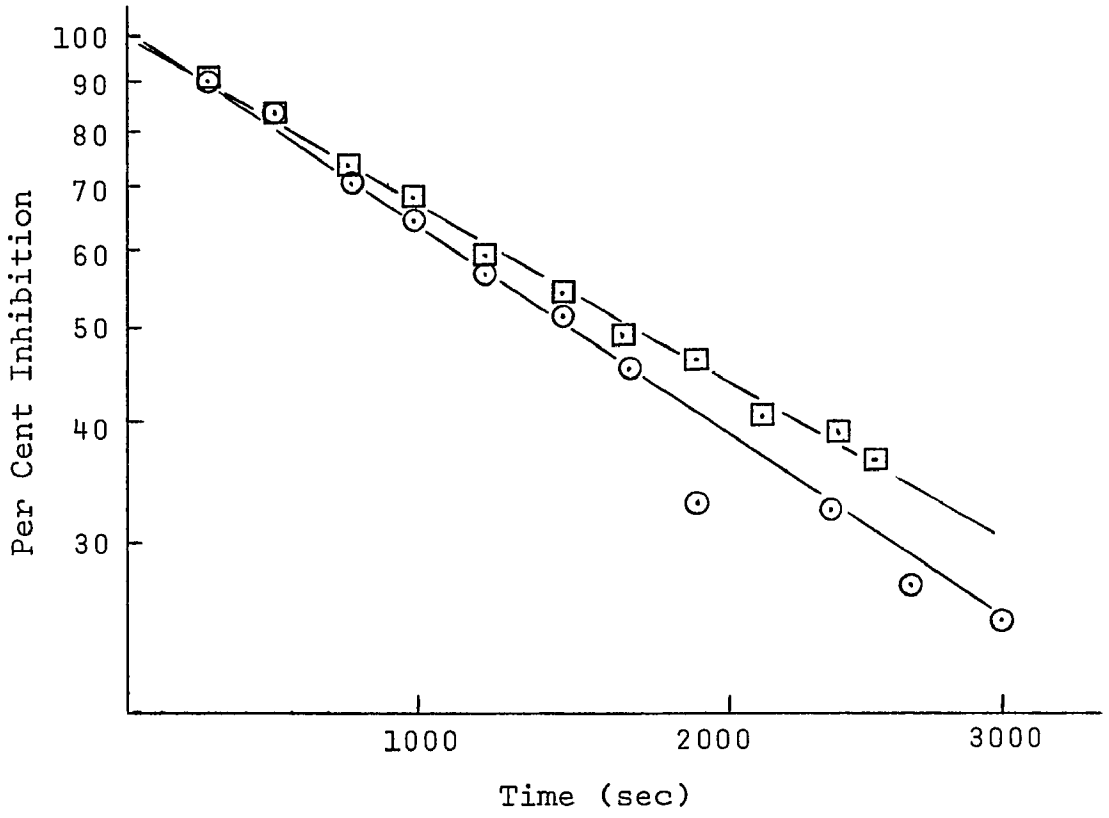
The effect of iodide ion concentration on the rate constant of HNB(1.05):lysozyme regeneration.



HNB(1.05):lysozyme, 0.50 mg/ml in 0.10 M acetate buffer, pH 4.0 in the presence of the indicated concentrations of NaI was incubated at 35° and the rate constants were determined as described in Experimental section.

Figure 22

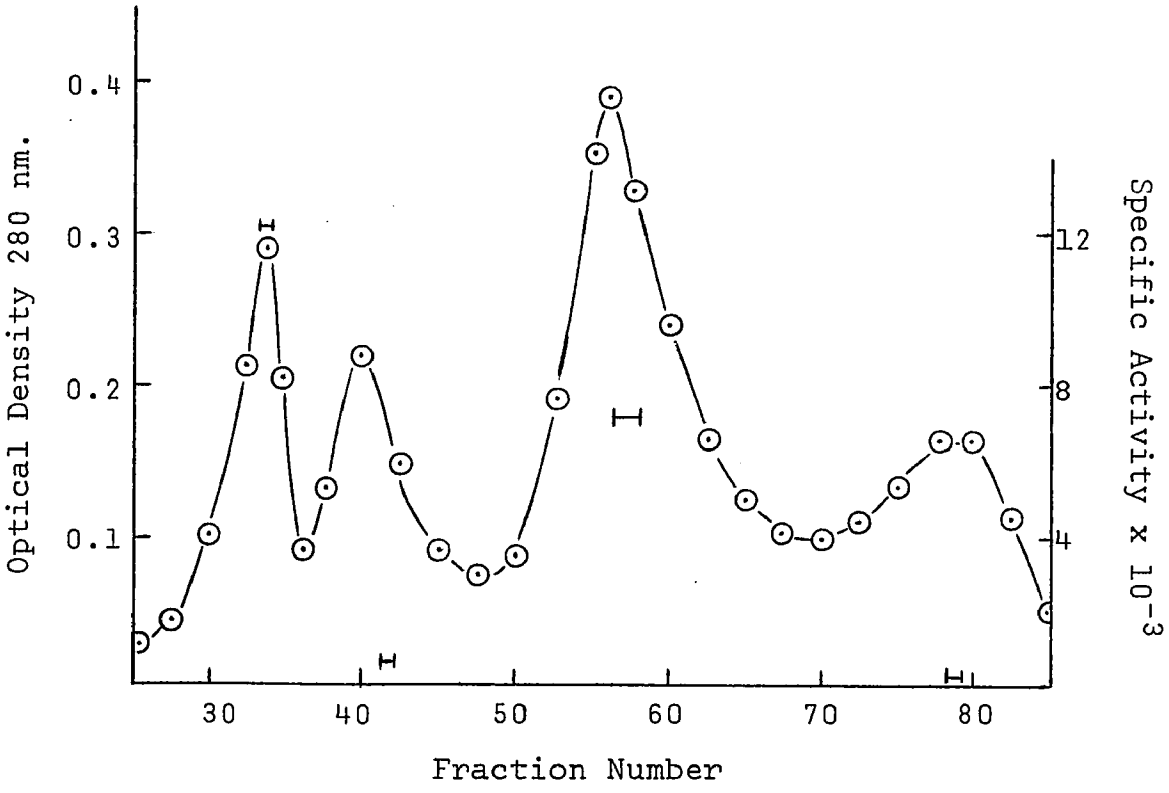
Deuterium isotope effect on the regeneration of HNB(1.05):lysozyme.



Samples of HNB(1.05):lysozyme ⊡-⊡-⊡-⊡ and deuterated HNB (1.05):lysozyme ⊙-⊙-⊙-⊙ in 0.18 M acetate buffer, pH 4.00 and 0.18 M deuterated acetate buffer, pD 4.09 respectively were regenerated at 45°.

Figure 23

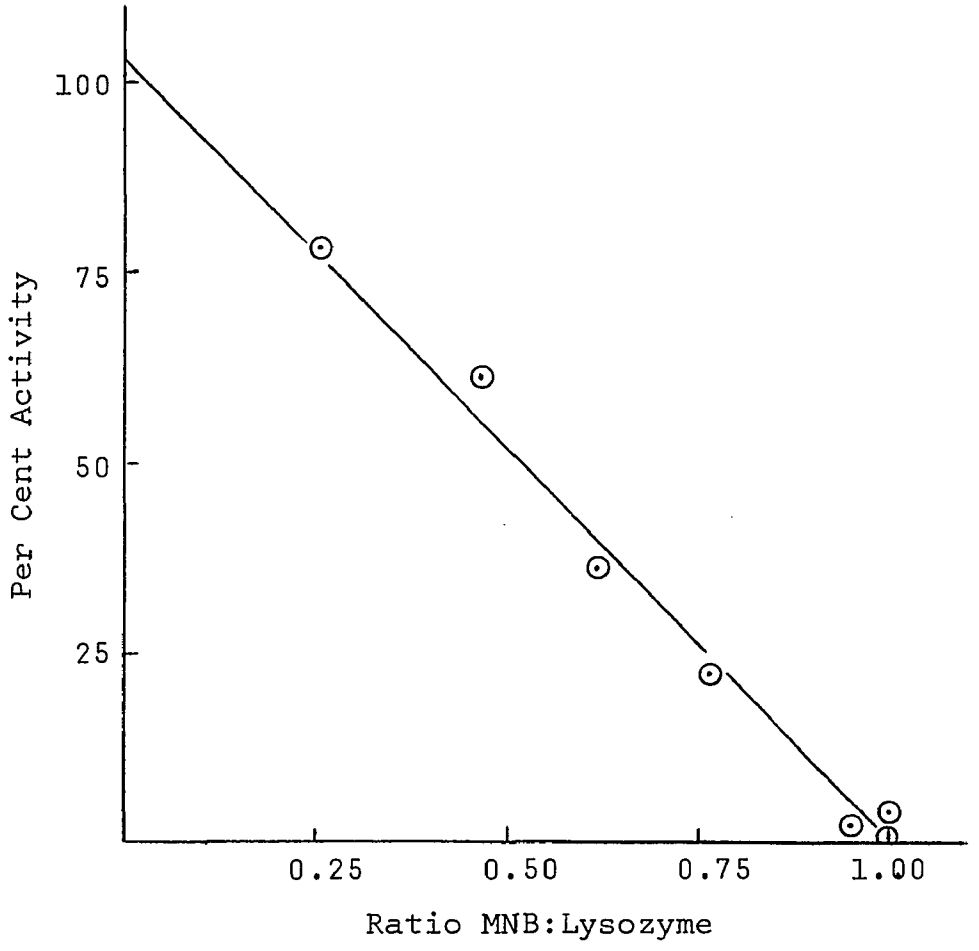
Chromatography of lysozyme ester.



Lysozyme ester, 10 mg in 0.03 M NaCl-0.20 M phosphate buffer, pH 7.18 was applied to a column (0.9 x 46 cm) of Bio-Rex 70 (200-400 mesh) which was equilibrated and eluted with this same buffer. Fractions of 1.6 ml were collected at a flow rate of 37 ml/hr. The ⊢⊣ fractions were pooled and specific activity determined.

Figure 24

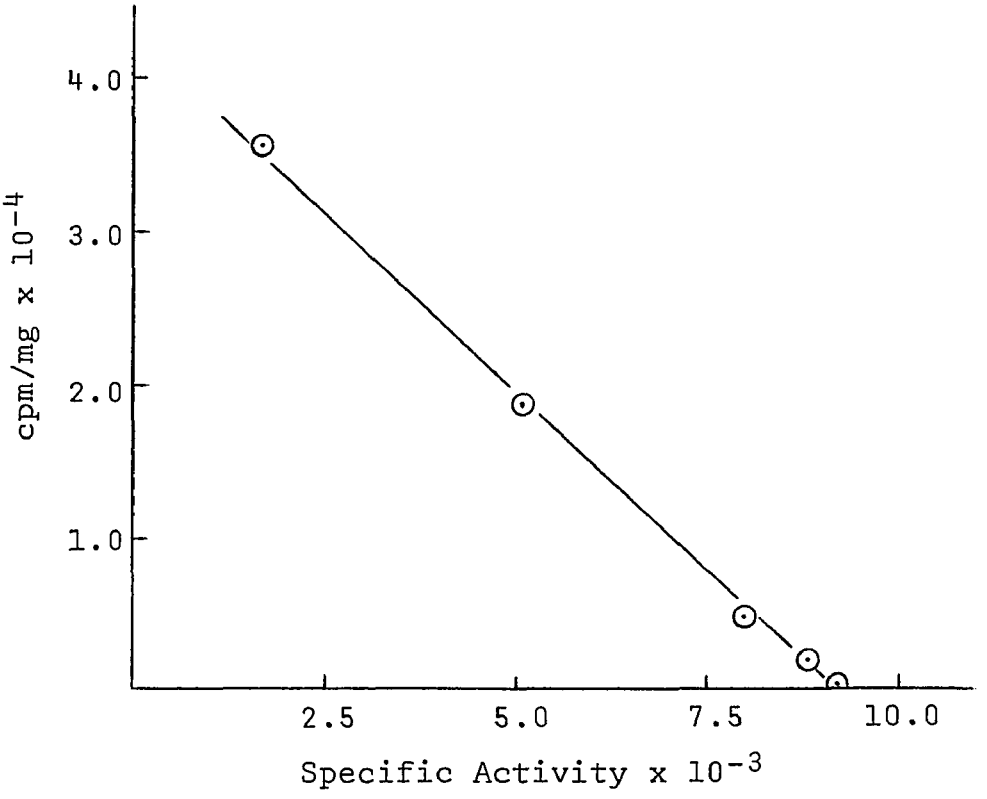
The inhibition of lysozyme by MNB-Br.



Samples of MNB:lysozyme were prepared by reactions indicated in Table XV.

Figure 25

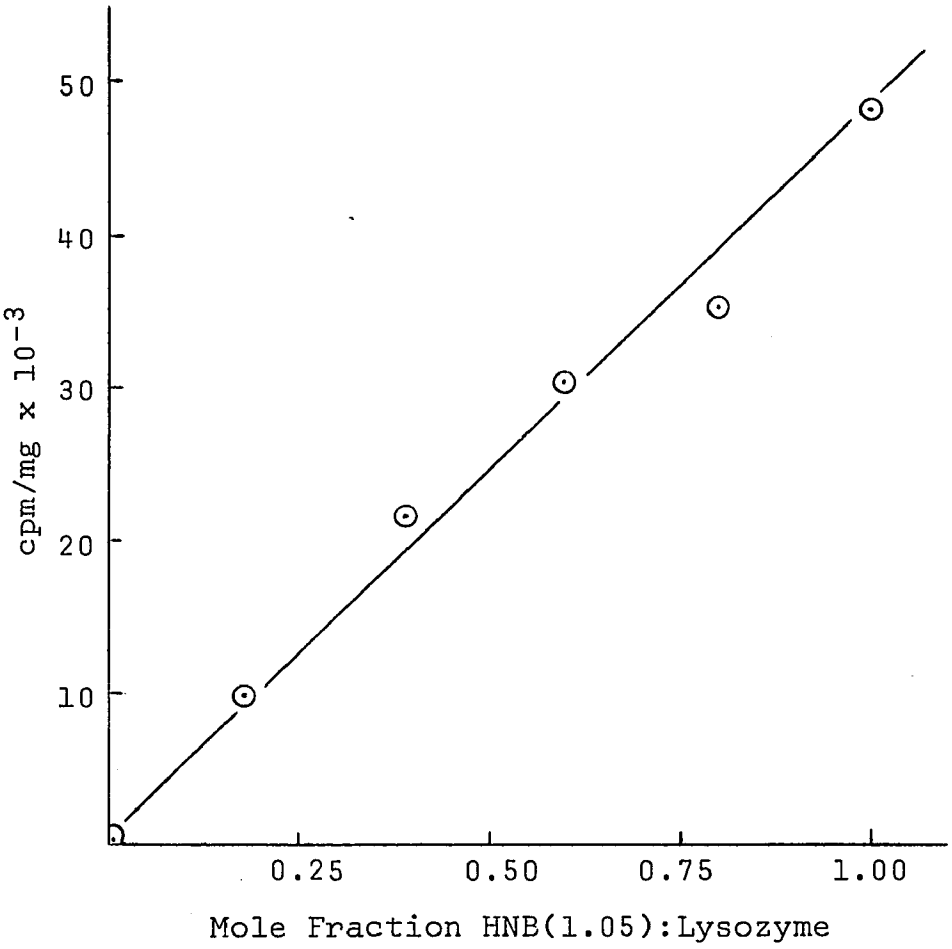
Extent of HNB(1.05):lysozyme regeneration as a function of tritium incorporation.



Conditions as in Table XVIII.

Figure 26

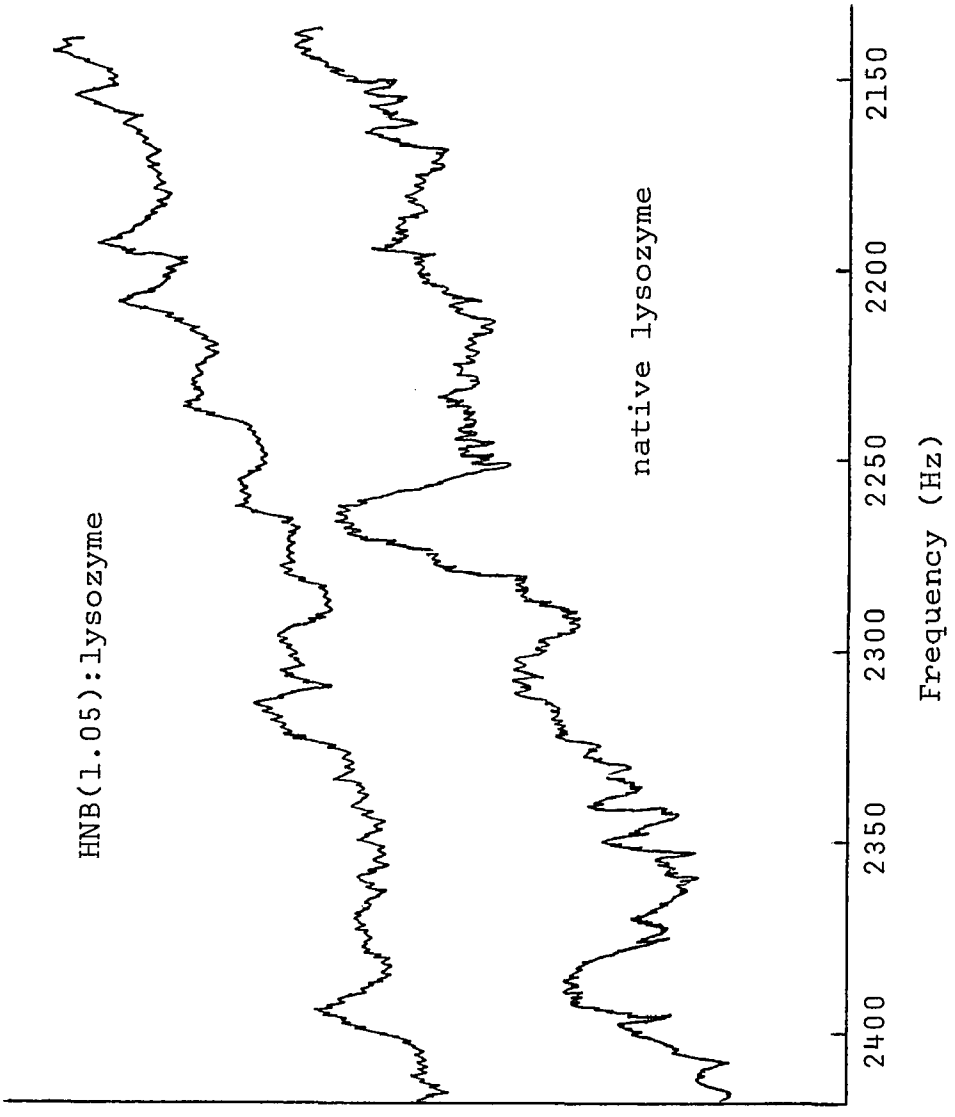
Dependence of tritium incorporation on the mole fraction of HNB(1.05):lysozyme.



Conditions as in Table XIX.

Figure 27

Nuclear magnetic resonance spectra of lysozyme and HNB(1.05):lysozyme.



Spectra of lysozyme and HNB(1.05):lysozyme, 15 per cent w/v in 0.20 M acetic acid, adjusted to pH 3 were recorded at 18°. The spectra are the results of 33 and 35 computer of average transients scans for lysozyme and HNB(1.05): lysozyme respectively.

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