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Reactivation of Lysozyme from Inactive HNB-Lysozyme

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The predominant reaction of lysozyme with 2-hydroxy-5-nitrobenzyl bromide, (HNB-Br), leads to the formation of an enzymatically inactive, labile product substituted at tryptophan 62. This species can revert to the native enzyme with the simultaneous loss of 2-hydroxy-5-nitrobenzyl alcohol (HNB-OH). The lability of the product in acidic or neutral solution depends upon three features of the HNB-lysozyme molecule: (1) the 2-hydroxy group of the HNB moiety, (2) a group which is readily reduced by borohydride, presumably an indolenine and (3) a particular structural conformation of the complex. A tentative mechanism for the hydrolysis reaction is presented.

The reaction between hen egg white lysozyme (EC 3.2.1.17) and 2-hydroxy-5-nitrobenzyl bromide (HNB-Br), in aqueous acidic solutions results in the formation of hydroxy-nitrobenzyl (HNB) derivatives on tryptophan residues of the enzyme (*1–6*). The products obtained range from nonlabeled to multiply-labeled lysozyme which can be separated by ion-exchange chromatography (*1*, *3*).

The monosubstituted lysozyme derivative was found to be unstable in acidic and neutral solutions at room temperature, decomposing to regenerate lysozyme and 2-hydroxy-5-nitrobenzyl alcohol [HNB-OH] (*1*, *2*, *7*).

In this paper we examine the nature of the regeneration process.

METHODS

Hen egg white lysozyme lots 9AA and 0CC, Trypsin-TPCK lot 0FA, and *Micrococcus lysodeikticus* lot 8JA were products of Worthington Biochemical Corp. The lysozyme had a specific activity of 10 500 units/mg using a modification of the turbidimetric method (*8*). Human lysozyme was a gift of Dr. E. F. Osserman of Columbia University. 2-Hydroxy-5-nitrobenzyl bromide (from Calbiochem) was recrystallized three times from hot benzene (mp 144.5–145.5°C), and iodoacetic acid was recrystallized from ethanol–petroleum ether, mp 81–82°C (uncorr.). 2-Methoxy-5-nitrobenzyl bromide was from Mann Research 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 (*9*). Buffer solutions had an ionic strength of 0.10 unless otherwise indicated.

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Lysozyme concentration was determined by the method of Folin–Ciocalteau (*10*) or by absorbance at 280 nm; $A^{1\%}_{280} = 2.51$, $\varepsilon = 35\,900$. HNB–lysozyme concentrations were determined by the same methods; however, in the ultraviolet method the absorbance of the HNB group at 280 nm was taken into account by the following equation:

Protein concentration =

$$\frac{OD_{280}\,(pH < 4) - 0.197 \times OD_{420}\,(pH > 11)}{35\,900}, \tag{1}$$

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.

The reaction of HNB-Br with lysozyme was accomplished by rapidly stirring a $CaCl_2$-dried acetone solution of HNB-Br into the lysozyme solution at 0°C. The concentrations of the HNB-Br solutions were such that the acetone never exceeded a final concentration greater than 10% v/v, in which the enzyme is stable. After the removal of nonprotein material from HNB–lysozyme by Sephadex G-10 gel filtration, the ratio of HNB to lysozyme was evaluated from the following formula which relates the concentration of the HNB group to the concentration of the protein:

Ratio of HNB to lysozyme =

$$\frac{OD_{420}\,(pH > 11)}{OD_{280}\,(1\%\ HAc) - 0.197\ OD_{420}\,(pH > 11)} \times 1.89, \tag{2}$$

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

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

Monosubstituted lysozyme, HNB–lysozyme, was prepared by reacting lysozyme, 2.00 g (0.139 mmoles) in 20 ml of 0.18 *M* acetic acid, pH 2.8, at 0°C with 80.7 mg (0.348 mmoles) of HNB-Br, dissolved in 2.0 ml of dry acetone. The mixture was then chromatographed on a jacketed column of Sephadex G-25 medium (5 × 36 cm) and maintained at 4°C by circulating water. The column was equilibrated and eluted with 0.18 *M* acetic acid, pH 2.8, and the entire solution containing the protein peak was immediately frozen and lyophylized. The reaction mixture products (1.98 g) were dissolved in 20.0 ml of equilibrating buffer at 4°C and applied to a 4 × 65-cm column of Bio-Rex 70, 100–200 mesh (Bio-Rad.), which had been equilibrated overnight at 4°C with 0.05 *M* borate–NaOH buffer, pH 9.80, at a flow rate of 100 ml per hour. The column was eluted with a two-step concentration gradient. Elution 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. After the elution of HNB-OH, which was characterized by its spectral properties (λ_{max} = 408 nm in basic solution), the proteins were 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 for enzyme activity. Those fractions containing inactive HNB-substituted lysozyme were pooled. Concentration was accomplished by diluting the pooled fractions with 3.5 vol 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. Protein was then eluted from the resin with 4 *M* NaCl, desalted with Sephadex G-10, and lyophylized.

A sample of the monosubstituted lysozyme fraction was rechromatographed on a longer column (0.8 × 100 cm) of Bio-Rex 70 (200–400 mesh) using the same buffer system for elution.

2-Methoxy-5-nitrobenzyl lysozyme (MNB-lysozyme) was prepared as follows: Identical aliquots of a lysozyme solution, 1.39×10^{-4} *M* in 0.18 *M* acetic acid, pH 2.80, were incubated at 30°C, and at timed intervals various amounts of 3.45×10^{-2} *M* 2-methoxy-5-nitrobenzyl bromide (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°C. All samples were subjected to gel filtration to remove unreacted MNB-Br, MNB-OH, and acetone, and the ratio of MNB to lysozyme was determined. The MNB-to-lysozyme ratio was evaluated from the following equation:

$$\frac{OD_{320}}{OD_{280} - 0.393\, OD_{320}} \times 3.78, \tag{3}$$

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

$$3.78 = \frac{\varepsilon_{280} \text{ lysozyme in } 1\,\% \text{ HAc}}{\varepsilon_{320} \text{ MNB group in } 1\,\% \text{ HAc}} = \frac{35\,900}{9500}.$$

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

The time course of the reactivation reaction was followed by assaying for enzyme activity and the HNB to lysozyme ratio. Rate constants for the pseudo first-order regeneration reaction, HNB-lysozyme (inactive) to lysozyme (active) plus HNB-OH, were determined from plots of log percent inhibition versus time. The percentage of inhibition at any time was calculated from the following equation:

$$\text{Percent inhibition} = 100 - \left(\frac{A_t - A_0}{A_\infty - A_0}\right)100, \tag{4}$$

where A_t = activity at any time; A_0 = activity at zero time; A_∞ = activity at completion of regeneration.

Deuterated HNB-lysozyme and deuterated lysozyme were prepared by first dissolving 11.0 mg of HNB-lysozyme or 15.0 mg of native lysozyme in 1.00 ml of D_2O and adding 6 *N* NaOD in D_2O until the pH was greater than 11. The resulting solution was left at room temperature for 3 hr. 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 × 25 cm) which was equilibrated and eluted with 0.18 *M* deuterated acetic acid in D_2O. The protein fractions isolated from the columns were lyophylized. Buffer solutions were prepared by adjusting 0.18 *M* acetic acid to pH 4.00 with NaOH and 0.18 *M* deuterated acetic acid 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. The activity of samples of lysozyme and deuterated lysozyme and the rates of regeneration of HNB lysozyme and deuterated HNB-lysozyme were determined.

Preparation, isolation, and characterization of HNB-peptide T_8–T_9 corresponding to residues 46–68 (*11*) (hereafter, HNB-peptide) to which the HNB group is attached was as follows. Native or HNB-lysozyme was reduced and carboxymethylated by the

procedure of Canfield and Anfinsen (*12*), except that the final precipitate was washed twice with 95% ethanol, suspended in water, and then lyophylized. The reduced, carboxymethylated (RCM) protein was suspended in 15 ml of water, adjusted to pH 10.0 with NaOH in order to wet the protein, and then the pH was adjusted to pH 8.0 with HCl. TPCK-treated trypsin, 8 mg, was added in small portions, over a 3-hr period, at room temperature. During digestion the solution was stirred and maintained at pH 8.0 by the addition of NaOH. After 3 hr the protein solution was centrifuged to remove any insoluble material. Addition of a buffer solution, consisting of 0.2 *M* pyridine–1.0 *M* acetic acid, pH 3.80, to the clear soluble tryptic digest of RCM-HNB-lysozyme in small aliquots with constant stirring was continued 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°C and the sample was centrifuged and the precipitate collected. The precipitate was washed twice with two 5.0-ml aliquots of pyridine–acetate buffer. The supernate and the subsequent wash solutions were analyzed for HNB absorbance and pooled. The washed precipitate was suspended in water, made slightly alkaline with dilute ammonium hydroxide, and lyophylized. The pooled supernate and wash solutions were frozen and the freeze–thaw extraction process was repeated. A sample of the isolated peptide was hydrolyzed for 20 hr at 110°C, with constant boiling 6 *N* HCl in vacuo. Amino acid analysis was accomplished on the long column of the Beckmann model 116 Amino Acid Analyzer.

The homogeneity of the native lysozyme and of HNB lysozyme preparations was determined by polyacrylamide disc gel electrophoresis. Seven percent acrylamide gels were prepared and run according to the method of Ornstein and Davis (*13*, *14*) 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 1 hr and were electrophoretically destained.

Reaction of HNB-lysozyme or of lysozyme with tritiated $NaBH_4$ ($NaBT_4$), was accomplished in protein solutions of 2.0 mg/ml in 0.50 *M* acetate-buffered solutions at pH 4.0 and 0°C. Because of the insolubility of the HNB-peptide at pH 4.0, its reduction was performed at pH 2.50 in a 1.0 *M* glycine–HCl buffer. Solutions of $NaBH_4$ or $NaBT_4$ were prepared in 10^{-4} *M* NaOH at concentrations between 0.20 and 0.40 *M* and stored at 0°C. Reactions of the protein, with either $NaBH_4$ or $NaBT_4$ were accomplished by their slow addition to the protein solutions while stirring rapidly at 0°C. The reaction mixture was immediately assayed for activity, HNB content, and for its ability to regenerate activity. The $NaBT_4$-reacted samples were counted in a Picker Nuclear Liquimat 220 scintillation counter after removing excess tritium by Sephadex G-10 gel filtration. 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% PPO, and 0.01% POPOP) and counted.

A large sample of tritium-labeled HNB-peptide was prepared from a solution of 25.0 mg of HNB-lysozyme in 5.0 ml of 0.50 *M* acetate buffer, pH 4.0, which was treated with 1.0 ml of a solution of 0.30 *M* $NaBT_4$ at 0°C. The solution was desalted by gel filtration and the protein fraction was recovered. It was then reduced, carboxymethylated, subjected to tryptic hydrolysis, and then pyridine acetate was added as previously described. During the course of purification aliquots were removed and analyzed for HNB concentration and for radioactivity.

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. (*15*). Estimation of the ester content was performed by the method of Hestrin (*16*). Samples of lysozyme or HNB-lysozyme were guanidinated by reaction with *O*-methylisourea according to the method of Kimmel (*17*). 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 (*18*).

Samples of lysozyme, HNB-lysozyme, and guanidinated lysozyme were acetylated at pH 7.50 with acetylimidazole as described by Riordan (*19*). The extent of acetylation was determined by following the loss in absorbance at 278 nm (*19*).

Tryptophan 62-oxidized lysozyme was prepared according to the method of Spande (*20*) utilizing *N*-bromosuccinimide. The extent of oxidation was determined from the equation derived by Spande and Witkop (*21*).

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-lysozyme 15% w/v in 0.18 *M* acetic acid, pH 2.80, were obtained at 18°C.

RESULTS

Preparation of HNB-Lysozyme

Although it was difficult to establish the stoichiometry of the HNB-Br lysozyme reaction because of the instability of the reagent in water, there appeared to be a maximum number of HNB groups that could be incorporated into the enzyme, under the conditions employed here. As shown in Fig. 1, 1.25 moles of HNB bound per mole of

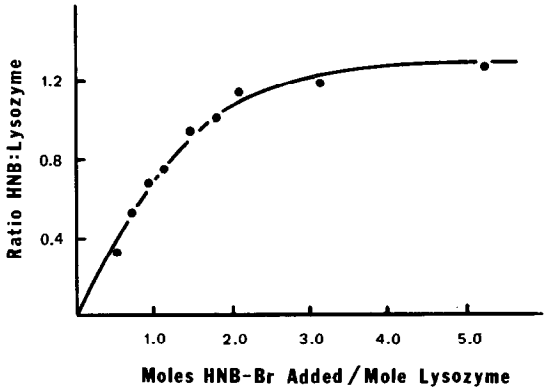


FIG. 1. Incorporation of HNB into lysozyme. Identical aliquots from a stock solution of lysozyme, 3.59×10^{-4} *M*, in 0.18 *M* acetic acid, pH 2.80, were reacted with varying volumes of 3.8×10^{-2} *M* HNB-Br in dry acetone at 0°C. After reaction all samples were passed through a column of Sephadex G-10 (0.9 × 25 cm) equilibrated, and eluted with 0.18 *M* acetic acid. Ratios of HNB to lysozyme were determined.

lysozyme resulted when a three-fold excess of HNB-Br was used. This led to complete inactivation of the enzyme, Fig. 2. By comparison, human lysozyme at a 20-fold molar excess of reagent incorporated 1.1 mole of HNB and retained at least 90% activity.

Using a larger excess of reagent did not significantly increase the number of HNB moles bound (Fig. 1). The products which resulted when the enzyme inactivation was complete include mono- and disubstituted derivatives.

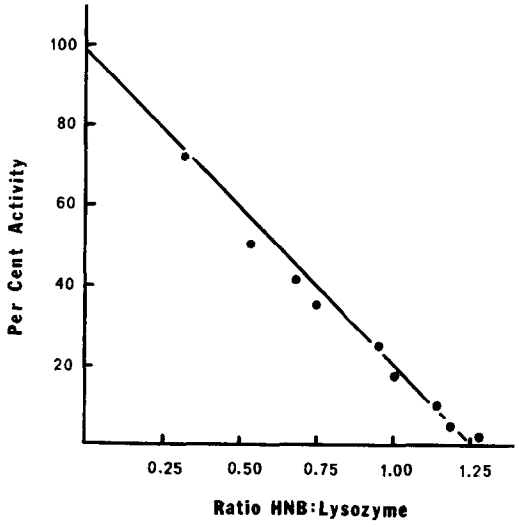


FIG. 2. Effect of HNB incorporation on the activity of lysozyme. Samples prepared as indicated in Fig. 1 were assayed immediately upon elution from Sephadex column prior to determination of HNB: protein ratios.

TABLE 1

PREPARATION AND ISOLATION OF HNB-LYSOZYME

Step	Protein (mg)	Ratio HNB: protein	Percent activity	Percent yield
Lysozyme	2000	—	100	100
HNB-lysozyme after G-25 gel filtration and lyophylization	1980	1.16	7.3	99
Bio-Rex 70 fractions 120–225	1720[a]	—	—	86
Pooled fractions after concentration, extraction, desalting, and lyophylization				
Fractions 120–155		1.05	5.1	
Fractions 156–185	1600[b]	1.03	5.1	80
Fractions 186–225		1.06	5.8	

[a] Determined from OD_{280} of the pooled fractions.
[b] Total protein derived from fractions 120–225.

Several fractions were obtained by Bio-Rex 70 column chromatography of the reaction mixture as given in Table 1. Fractions 120–225 were pooled and rechromatographed on a longer analytical column (0.8 × 100 cm) as shown in Fig. 3. Spectral examination of the material eluted in fractions 27–40 identified it as HNB-lysozyme. The minor fraction eluting after the main peak was identified as lysozyme by its activity and by the chromatographic elution pattern of pure lysozyme. Additional multiply labeled fractions were eluted from the preparative column with strong base and were

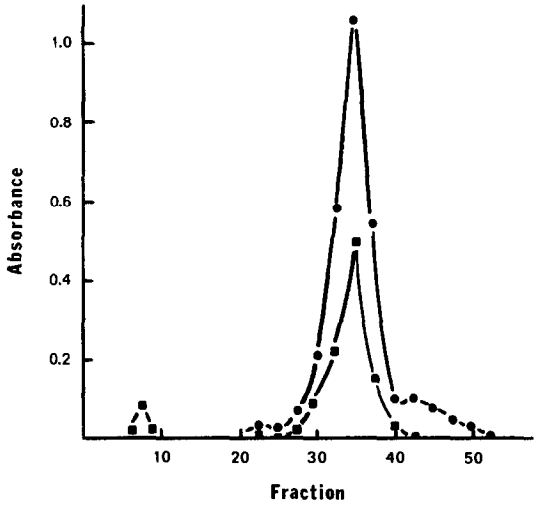


FIG. 3. Analytical chromatogram of purified HNB-lysozyme. A 10.0-mg sample of HNB-lysozyme was chromatographed on a column (0.8 × 100 cm) of Bio-Rex-70 (200–400 mesh). The column was equilibrated and eluted with 0.05 *M* borate–NaOH, pH 9.80. After elution of HNB-OH the buffer was changed to 0.23 M borate-NaOH, pH 9.80. The protein was eluted at room temperature at a flow rate of 27 ml/hr. Fractions of 5.3 ml were collected and absorbance at 280 nm (●–●–●) and 420 nm (■–■–■) was measured.

not examined further since they could not regenerate enzyme activity. During the course of the isolation of the substituted enzyme, regeneration occurred to the extent of about 5.5% under the conditions employed. HNB-OH as expected was obtained, peaking at fraction 9. The yield data for the isolation of HNB-lysozyme are given in Table 1.

Regeneration of Lysozyme Activity from HNB-Lysozyme

The analytical chromatogram of an HNB-lysozyme sample which was regenerated by incubating for 24 hr at pH 2.80 is given in Fig. 4. The decrease in the HNB-lysozyme peak at fraction 34 gave rise to a corresponding increase of the lysozyme activity and absorption peak at fraction 47 and the HNB-OH peak at fraction 9. Measurement of the areas under the peaks gave a ratio of HNB-lysozyme to lysozyme of about two to eight, which was consistent with the 80% loss of bound HNB and 70% gain of activity. The 20% residual HNB-lysozyme, stable to regeneraion, was also a monosubstituted derivative.

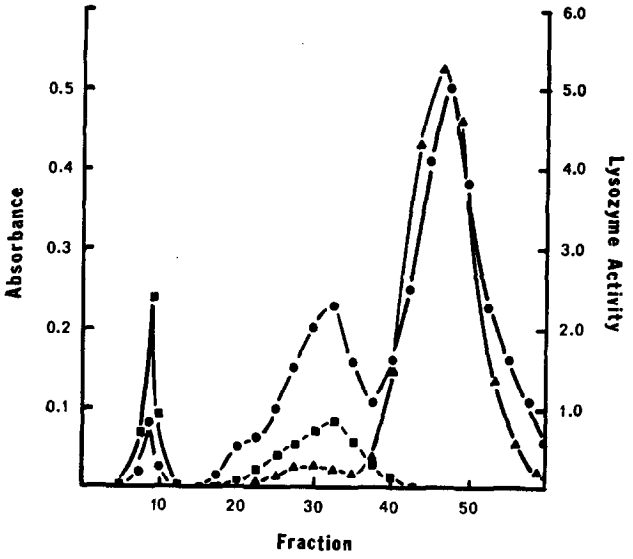


FIG. 4. Analytical chromatogram of regenerated HNB-lysozyme. A 20-mg sample of HNB-lysozyme was incubated at pH 2.8 and 37°C for 24 hr. After adjustment to pH 9.80 and centrifugation, the clear solution was chromatographed according to the procedure in the legend to Fig. 3, collecting fractions of 6.5 ml. Fractions were analysed for absorbance at 280 nm (●–●–●), 420 nm (■–■–■), and for enzymatic activity (▲–▲–▲).

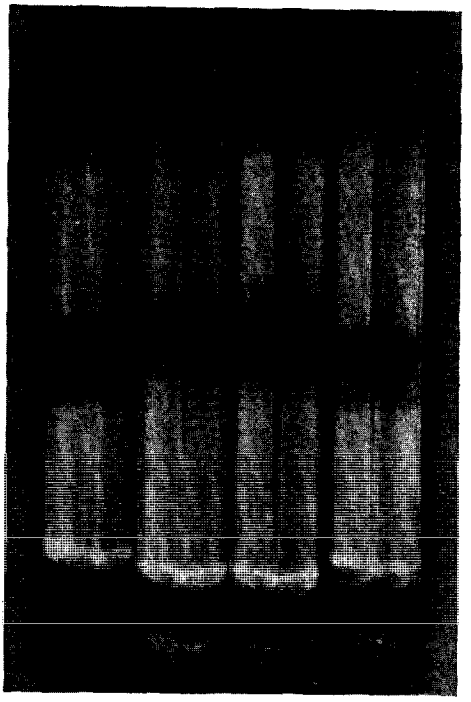


FIG. 5. Disc gel electrophoresis of lysozyme and HNB-lysozyme. Samples of native lysozyme (1) and HNB-lysozyme incubated for 0, 10, and 60 min, (2), (3), and (4), respectively, at pH 4.0 and 50°C were electrophoresed by the procedure of Ornstein and Davis (*13*, *14*) at a current of 4 MA/gel for 1 hr with reversed polarity. The origin is at the top of the gel and migration is toward the cathode.

These data and data presented below indicate the presence of two HNB-derivatives, both with an HNB-to-lysozyme ratio of one, but which were inseparable by all techniques applied. Chromatography on Bio-Rex 70 at pH 7.2 with 0.2 *M* phosphate buffer, elution at pH 9.80 with borate buffers with a concentration gradient of 0.05–0.30*M* and elution at pH 9.80 with 0.05 *M* borate and a NaCl gradient of 0.00–0.50 *M* failed to resolve HNB-lysozyme into separate species. Analytical gel electrophoresis of HNB-lysozyme gave only one band as shown in Fig. 5.

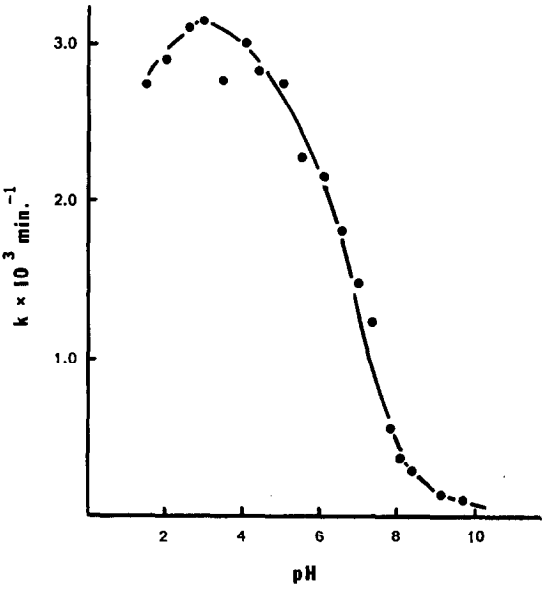


FIG. 6. Effect of pH on the rate of regeneration of HNB-lysozyme. Identical aliquots from a stock solution of HNB-lysozyme (5.0 mg/ml.) in water at 0°C were added to buffers at the indicated pH values preincubated at 30°C, to give a final protein concentration of 0.50 mg/ml. The samples were incubated at 30°C and aliquots were removed at 30-min intervals and assayed for regeneration of enzymatic activity. Rate constants were determined by the method described in the experimental section.

The rate of regeneration of HNB-lysozyme was optimal between pH 2 and 4 with a rapid decrease above pH 4 as shown in Fig. 6. The rate constant at each pH value was determined by measuring the rate of increase in lysozyme activity. The log of the rate of change was found to be linear with time over the complete reaction. Typical linearity data are shown in Fig. 7.

At the completion of the regeneration process all samples in the pH range of 2.5–8.4 showed an 80% recovery of enzyme activity. Samples of low pH, 2.05, 1.53, and 1.23 had activities of 60.5, 39.4, and 32.0%, respectively. The rate data at these low pH values were not linear throughout, indicating another reaction, possibly denaturation, was taking place.

The pH-regeneration rate curve in Fig. 6 has an inflection point at pH 6.50 indicating that a group with a p*K* of 6.5 may have been participating in the regeneration process.

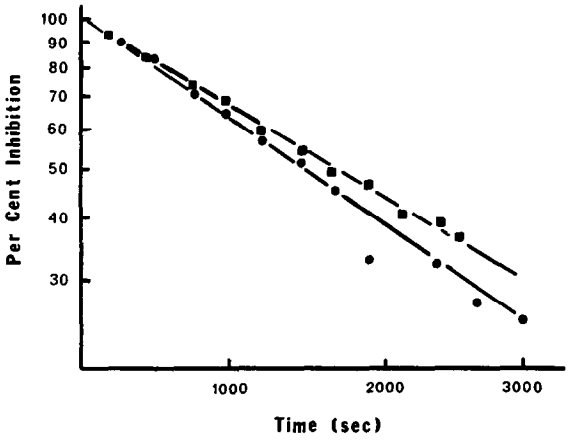


FIG. 7. Deuterium isotope effect on the regeneration of HNB-lysozyme. Samples of HNB-lysozyme (■–■) and deuterated HNB-lysozyme (●–●) both 0.50 mg/ml were incubated at 45°C in 0.18 *M* acetate buffer, pH 4.00, and 0.18 *M* deuterated acetate buffer, pD 4.09. Aliquots from each sample were assayed at various time intervals and precentage inhibition was calculated from Eq. (4).

Table 2 shows the effect of adding a nucleophilic ion, iodide, to the regeneration solution. A change in the iodide concentration from zero to 1.0 *M* resulted in a doubling of the rate of regeneration. Similar results were obtained with thiocyanate, but neither chloride nor bromide had an effect.

TABLE 2

THE EFFECT OF NUCLEOPHILES ON THE RATE OF REGENERATION OF HNB-LYSOZYME[a]

Sample	Addition	pH	Temperature	$t_{1/2}$ (min)	k (min^{-1})
HNB-lysozyme	None	4.0	35°C	128	0.0054
HNB-lysozyme	0.03 *M* I^-	4.0	35°C	109	0.0064
HNB-lysozyme	0.10 *M* I^-	4.0	35°C	89	0.0078
HNB-lysozyme	0.30 *M* I^-	4.0	35°C	77	0.0090
HNB-lysozyme	1.00 *M* I^-	4.0	35°C	70	0.0099
HNB-lysozyme	None	4.0	40°C	48.5	0.0143
HNB-lysozyme	0.50 *M* I^-	4.0	40°C	32.5	0.0213
HNB-lysozyme	0.50 *M* SCN^-	4.0	40°C	31	0.0224

[a] Sodium acetate buffers, $\mu = 0.10$, containing the indicated addition, adjusted to pH 4.00, were preincubated at the desired temperatures. Initiation of this regeneration reaction was accomplished by the addition of aliquots from a stock solution of HNB-lysozyme, 10.0 mg/ml in water at 0°C, to the preincubated systems to yield a final protein concentration of 0.50 mg/ml. During the incubation aliquots were removed and assayed at 10-min intervals.

Only a minor isotope effect was observed for the rate of regeneration of deuterated HNB-lysozyme. At 45°C, 0.18 *M* acetate, pH 4.0, (p*D* 4.09), the *k* for HNB-lysozyme

was 2.43×10^{-2} min^{-1}, and the k for deuterated HNB-lysozyme was 2.88×10^{-2} min^{-1} giving a kH/kD of 0.84 ± 0.07 (Fig. 7).

The addition of 0.1 *M* *N*-acetylglucosamine (NAG) had little effect on the rate. The rate constant increased from 2.58×10^{-2} min^{-1} to 2.78×10^{-2} min^{-1} at 45°C and pH 4.00, 0.18 *M* acetate buffer.

Chemical modification of the lysine amino groups of the native enzyme or of the HNB-lysozyme to guanido groups using *O*-methylisourea had no effect on the regeneration rate. Similarly, acetylation of amino groups with acetylimidazole had no effect.

It was during the *O*-acetylation studies that it became apparent that the phenol group of HNB could be involved in the regeneration phase. Table 3 illustrates that mild

TABLE 3

REGENERATION OF ACETYLATED AND DEACETYLATED HNB-LYSOZYME

Sample	Treatment	Ratio HNB: lysozyme
acetyl-HNB-lysozyme[a]	Maintain at pH 2.80 2 hr at 0–2°C	0.92
acetyl-HNB-lysozyme[a]	Maintain at pH 2.80 2 hr at 50°C	0.67
acetyl-HNB-lysozyme[a]	Adjust to pH 11, after 5 min adjust to pH 3.0 Maintain at pH 3.0 2 hr at 50°C	0.26
HNB-lysozyme	Maintain at pH 2.80 2 hr at 50°C	0.28

[a] Prepared by reaction of HNB-lysozyme with a 545-fold excess of acetylimidazole at pH 7.50 according to the procedure of Riordan (*19*). After acetylation the protein was desalted by passage through Sephadex G-10 eluted with 0.18 *M* acetic acid, pH 2.80.

alkaline treatment which would be expected to deacetylate the HNB phenol ester resulted in an enzyme which could be regenerated. The two tyrosine residues were not involved since *O*-acetylation of lysozyme prior to the formation of the HNB-lysozyme had no effect on the regeneration process (Table 4).

The oxidation of lysozyme with *N*-bromosuccinimide (NBS) yielded a product with 20% of the original activity and a concomitant modification of 1.2 moles of tryptophan, including tryptophan 62. The data in Table 4 show that the incorporation of HNB was reduced from 0.96 moles to 0.36 moles per mole protein, while only 38% of the resulting HNB-lysozyme was labile.

The esterification of lysozyme with 0.2 *M* triethyloxonium fluoroborate at pH 4.50 produced products with an average of 1.13 ester groups/mole and an activity 50% that

TABLE 4

THE INCORPORATION OF HNB INTO VARIOUS LYSOZYME DERIVATIVES AND THE SUBSEQUENT REGENERATION OF THE HNB PROTEINS

Derivative[a]	Residues modified[b]	Ratio HNB-lysozyme		% Activity[d]			
		Initial	2 hr[c]	Initial	2 hr[c]	Δ Ratio	Δ% Act
Native lysozyme	—	0.955	0.208	14.5	86.1	0.747	71.6
Guanidinated lysozyme	Lysine (5.9/6.0)	0.932	0.204	13.7	72.0	0.728	58.7
Guanidinated and acetylated	Lysine (5.9/6.0) Tyrosine (2.1/3.0)	0.970	0.250	14.2	82.0	0.720	67.8
Acetylated	Tyrosine (2.1/3.0)	0.977	0.230	18.4	91.0	0.747	72.6
NBS oxidized	Tryptophan (1.2/6.0)	0.360	0.224	—	—	0.136	—
Glutamate 35 ethyl ester[e]	Glutamic 35 (1.0/1.0)	0.967	0.224	15.6	85.7	0.743	70.1
Aspartate 52 ethyl ester[e]	Aspartic 52 (1.0/1.0)	0.943	0.236	—	—	0.707	—
Guanidinated HNB-lysozyme[f]	Lysine (5.6/6.0)	0.917	0.292	21.6	78.5	0.625	56.9

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

[b] Residues modified/total residues present.

[c] Ratios and activity were determined after incubation for 2 hr at 55°C at pH 2.80.

[d] Percent activity was determined with respect to an identical sample not subjected to treatment with HNB-Br.

[e] The identity of the esters was made on the basis of specific activities and elution profile both of which were identical to those of Parsons et al. (*15*).

[f] Prepared by guanidination of HNB-lysozyme as previously described.

of the native enzyme. The ion-exchange chromatographic pattern was identical to that observed by Parsons et al. (*15*). The HNB lysozyme derivative of each of the four fractions were prepared. Their rates of regeneration were the same as that of unesterified HNB-lysozyme.

Reaction of MNB-Br with Lysozyme

The results of the reaction of lysozyme with MNB-Br at pH 2.80, in Table 5, shows that the inactivation of the enzyme was slower than the reaction with HNB-Br. Figure 8 shows that the decrease in activity resulted in an increase in the MNB-lysozyme ratio, which led to an inactive lysozyme derivative. The findings included in Table 5 demonstrated that MNB-lysozyme was stable to regeneration. Those samples which had less than 1 % activity at 2 hr were removed and subjected to gel filtration to remove excess unreacted MNB-Br, MNB-OH, and acetone, and the ratio of MNB to lysozyme was determined. These samples were reincubated at pH 2.8 and at 30°C. At both 3 hr and at 16 hr no increase in activity was observed and there was no loss of the label, (the MNB-

TABLE 5

THE INACTIVATION OF LYSOZYME BY 2-METHOXY-5-NITROBENZYL BROMIDE

Molar excess[a] MNB-Br	Percent activity[b] 1 min	0.5 hr	1.0	2.0	6.0	19.0	% Inhibition	Ratio MNB: lysozyme
—	100	100	100	100	100	100	0.00	0.00
0.25	94	80	75	76	87	78	22	0.254
0.50	90	71	68	56	60	61	39	0.460
0.75	92	63	55	45	40	36	64	0.610
1.0	98	53	41	30	25	22	78	0.763
1.5	95	35	25	9.3	4	3.7	86	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 0.18 *M* 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] Percent activity was determined at the indicated times during incubation of MNB-Br with lysozyme at pH 2.80 and 30°C.

[c] These samples were removed at 2 hr and subjected to gel filtration, After the removal of MNB-Br, MNB-OH, and acetone, the protein fraction was returned to the bath and incubation allowed to proceed from the 3-hr interval to test for reactivation or change in MNB content.

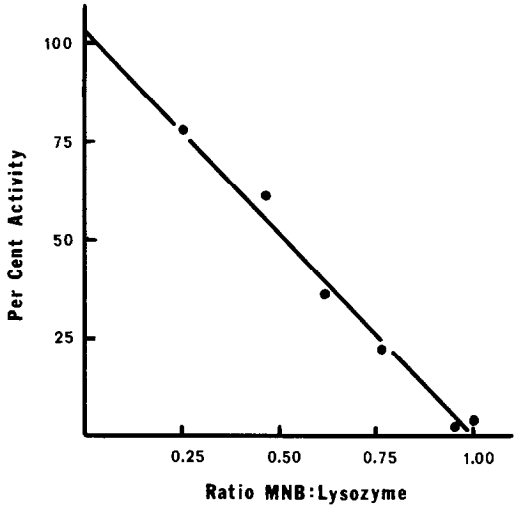


FIG. 8. The inactivation of lysozyme by MNB-Br. Identical aliquots from a stock solution of lysozyme, 1.39×10^{-4} *M*, in 0.18 *M* acetic acid, pH 2.80, were reacted with varying volumes of a stock solution of MNB-Br, 3.45×10^{-2} *M* in dry acetone. Upon completion of reaction, as judged from either total inhibition or constant activity, the samples were passed through Sephadex G-10 to remove hydrolysis products and unreacted MNB-Br and the ratio of MNB to protein was determined as well as enzymatic activity.

lysozyme ratio did not change), during the 16-hr period. This observation may be compared with HNB-lysozyme which at this pH and temperature had a half-life of reactivation of about 4 hr.

Reduction of HNB-Lysozyme and HNB-Peptide (T_8-T_9)

A reaction between sodium borohydride and HNB-lysozyme, led to product(s) which were stable to regeneration of activity and to cleavage of the HNB moiety. These data are given in Table 6. To determine if these results were caused by denaturation, samples of HNB-lysozyme and native lysozyme were reacted together in the presence of $NaBH_4$. Denaturation apparently did not occur since the activity of the native enzyme was unchanged.

TABLE 6

EFFECT OF BOROHYDRIDE ON THE REGENERATION OF HNB-LYSOZYME

Sample	$[BH_4]$	% Activity[b]		Ratio HNB: lysozyme[c]
		Initial	After 2 hr[c]	
HNB-lysozyme[a]	—	9.0	79	0.218
HNB-lysozyme[a]	0.0004	8.0	68	0.258
HNB-lysozyme[a]	0.0020	7.9	58	0.416
HNB-lysozyme[a]	0.0095	6.7	21.5	0.784
HNB-lysozyme[a]	0.040	6.1	12.5	0.823

[a] One milliliter HNB-lysozyme 6.9×10^{-5} *M* in 0.5 *M* acetate buffer was reacted with 0, 2.0, 10.0, 50.0, and 250.0 μl 0.20 *M* $NaBH_4$ at pH 4.0 and 0°C.

[b] Relative to native lysozyme.

[c] After 2-hr incubation at pH 4.0 and 55°C.

The results of the reaction of HNB-lysozyme with $NaBT_4$ are presented in Table 7 and Fig. 9. The hydrolysis products of borohydride had little or no effect on the enzyme, since at all concentrations the extent of regeneration of activity and the loss of the HNB group were constant. The data presented in Fig. 9 indicate an inverse relationship between the incorporation of tritium and the regeneration of enzymatic activity.

The stability of the native enzyme to denaturation under conditions leading to the cessation of HNB-lysozyme regeneration has already been indicated. The decreased extent of regeneration of HNB-lysozyme however may have been a reflection 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-lysozyme species could, however, be made in a system where both protein and reducing agent concentrations were kept constant. Figure 10 shows the results obtained in such a system where the $NaBT_4$ concentration and protein concentration at different mole fractions of HNB-lysozyme to native enzyme were constant, and it provides evidence for the specificity of tritium incorporation, since this incorporation was directly proportional to the mole fraction of HNB-lysozyme. The data indicated

TABLE 7

THE INHIBITION OF THE REGENERATION OF ENZYMATIC ACTIVITY AND THE INCORPORATION OF TRITIUM IN HNB-LYSOZYME BY $NaBT_4$

Sample no.[a]	$[NaBT_4]$	Specific activity		Δ Specific activity	cpm/mg[c]	cpm/mg[d]
		Initial	After 2 hr[b]			
1	None	790	10 050	9260	0	—
2	0.000455	840	9640	8800	3690	1910
3	0.000455	760	10 230	9470	1780	—
4	0.00224	780	8790	8010	7700	4890
5	0.00224	630	10 080	9450	2810	—
6	0.0108	770	5900	5130	21 000	18 640
7	0.0108	660	9600	8940	2360	—
8	0.0454	790	2450	1660	41 400	35 700
9	0.0454	740	9690	8950	5700	—

[a] Odd-numbered samples prepared by the addition of the enzyme to hydrolyzed samples of $NaBT_4$. HNB-lysozyme 6.95×10^{-5} *M*.

[b] After 2 hr at pH 4.00 and 55°C.

[c] Corrected for sample one, no $NaBT_4$ equal 0 cpm.

[d] Corrected for nonspecific tritium incorporation.

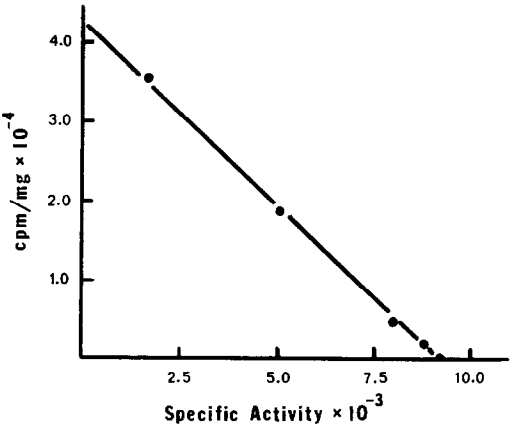


FIG. 9. Effect of tritiated sodium borohydride reduction on the regeneration of HNB-lysozyme. Aliquots of a stock solution of HNB-lysozyme (2.00 mg/ml) in water at 0°C were diluted to a final concentration of 1.00 mg/ml with 0.5 *M* acetate buffer, pH 4.0, at 0°C. The aliquots were reacted with; 0-, 2-, 10-, 50-, or 250-μl aliquots of 0.227 *M* tritiated sodium borohydride at 0°C. Controls for nonspecific tritium incorporation were prepared by adding $NaBT_4$ to acetate buffer prior to the addition of the enzyme. After incubation for 2 hr at pH 4.0 and 55°C all samples were passed through Sephadex G-10 to remove unbound tritium. Aliquots of each sample were counted and their specific activities determined.

that the decreased extent of regeneration was not due to the instability of the substituted protein, but must reflect the necessity of a reducible group in the protein for regeneration.

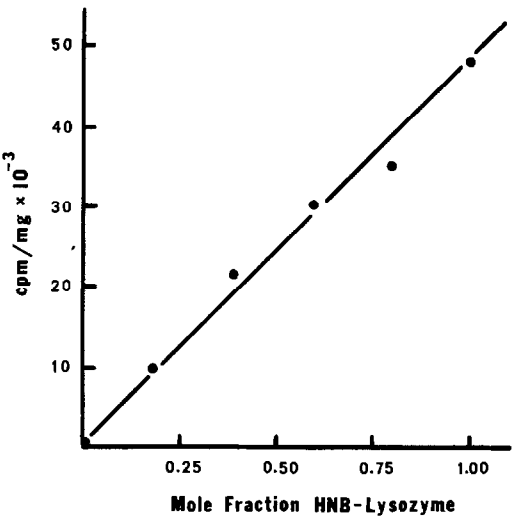


FIG. 10. Dependence of tritium incorporation on the mole fraction of HNB-lysozyme. Stock solutions of HNB-lysozyme and native lysozyme both 1.39×10^{-4} *M* in 0.5 *M* acetate buffer, pH 4.0 and 0°C, were appropriately mixed to give samples of the indicated mole fractions. Each sample (1.00 ml) was reacted with 0.250 ml of tritiated sodium borohydride, 3.29×10^{-1} *M*, and incubated at pH 4.0 and 55°C for 2 hr. After incubation all samples were passed through Sephadex G-10 and aliquots were counted and assayed.

The use of radioactive isotopes in labeling studies provided an accurate method for the determination of the extent of such reactions. The specific radioactivity of the $NaBT_4$ solution was determined to be 1.52 ± 0.10 mCi/mmole of $NaBT_4$ or 0.380 ± 0.025 mCi/mmole of tritium. The data presented in Fig. 9 allowed a calculation of the specific radioactivity of the fully inhibited protein. HNB-lysozyme had a specific radioactivity of 0.348 ± 0.07 mCi/mmole when correction was made for the regeneration of 12% which occurred. The ratio of the specific radioactivity of the reductant to that of the protein indicated the incorporation of 0.91 ± 0.04 moles of tritium per mole of enzyme.

The data presented in Fig. 10 have already been discussed in terms of the specificity of tritium incorporation into HNB-lysozyme. In order to define these results more clearly and to gain further insight into the mechanism of the regeneration process, labeling experiments were performed with the intact protein as well as the peptide, and isolation of the $NaBT_4$ reduced peptide from the $NaBT_4$ reduced protein was accomplished.

The incorporation of tritium into both HNB-lysozyme and HNB-peptide was accomplished and cpm/OD_{420}/ml values of 11 100 and 11 600 were obtained, respectively. The results indicated an equivalency between the protein and peptide in terms of the reducibility of the bond being attacked by borohydride. The results presented in Table 8 are consistent with the premise that reduction in the intact HNB-lysozyme took place within a group in the T_8-T_9 peptide.

Nuclear Magnetic Resonance Spectra of Lysozyme and HNB-Lysozyme

The NMR spectra shown in Fig. 11 indicated that in HNB-lysozyme, a specific signal, at 2270 Hz, found in the native enzyme was absent. This signal, at 2270 Hz, has

TABLE 8

PURIFICATION OF TRITIUM-LABELED HNB-PEPTIDE T_8-T_9 FROM TRITIATED HNB-LYSOZYME

Sample	Volume of solution (ml)	Total[a] OD_{420}	Total[b] cpm × 10^{-5}	Specific activity × 10^{-4}
Tritiated HNB-lysozyme[c]	9.0	33.4	4.21	1.26
RCM tritiated HNB-lysozyme	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 1 *N* NaOH.

[b] Aliquots of 0.10 ml were counted and corrected by the use of the appropriate blank.

[c] Prepared by the addition of 300 μmoles $NaBT_4$ to 1.8 μmoles (25 mg) of HNB-lysozyme at pH 4.0 and 0°C. Hydrolysis products of $NaBT_4$ were removed by passage of labeled protein through Sephadex G-10 eluted with 0.18 *M* acetic acid.

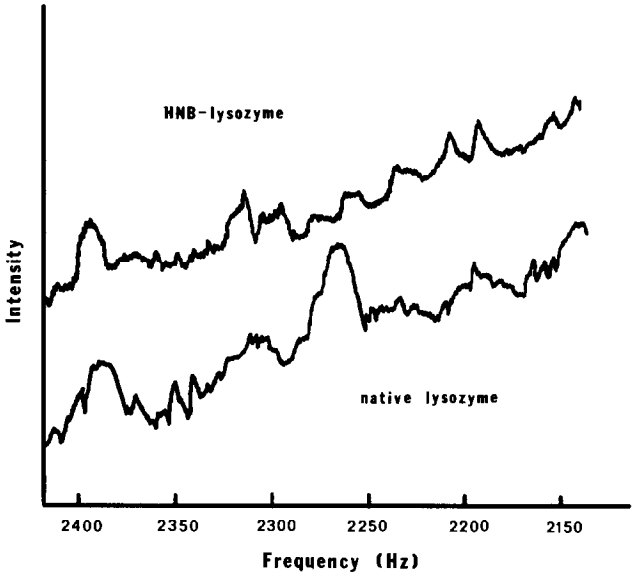


FIG. 11. Nuclear magnetic resonance spectra of HNB-lysozyme and native lysozyme. Samples, 15% w/v in 0.18 *M* acetic acid, pH 2.8, were analysed at 18°C. The spectra are the results of 33 and 35 computer average transient scans for lysozyme and HNB-lysozyme, respectively.

been identified by Glickson et al. (22) as the characteristic indole N–H proton of tryptophan 62. This result indicated the modification of tryptophan 62 in the HNB-lysozyme adduct.

DISCUSSION

The formation of HNB-lysozyme resulted in enzyme inactivation within 1 min by the modification of one tryptophan residue which was particularly reactive and which was essential for enzyme activity. The residue which carried the labile HNB group, after reaction of lysozyme with HNB-Br, has been identified as tryptophan 62. Other investigations have also shown that tryptophan 62 is the most reactive tryptophan in the protein. It is preferentially sulfenylated by 2-nitrophenyl-sulfenyl chloride (*23*) and oxidized by *N*-bromosuccinimide (*24*). Barman (*3*) has shown 1 mole of HNB, in HNB-lysozyme containing a total of 1.2 moles of HNB bound per mole of enzyme, was associated with peptide T_8-T_9 which includes both tryptophan 62 and 63. Our observation that the specific modification of tryptophan 62 by *N*-bromosuccinimide oxidation leads to a substantial reduction in incorporation of the HNB group and the observed loss in signal of the indole N–H proton of tryptophan 62 by NMR studies provide direct evidence that substitution occurred at tryptophan 62.

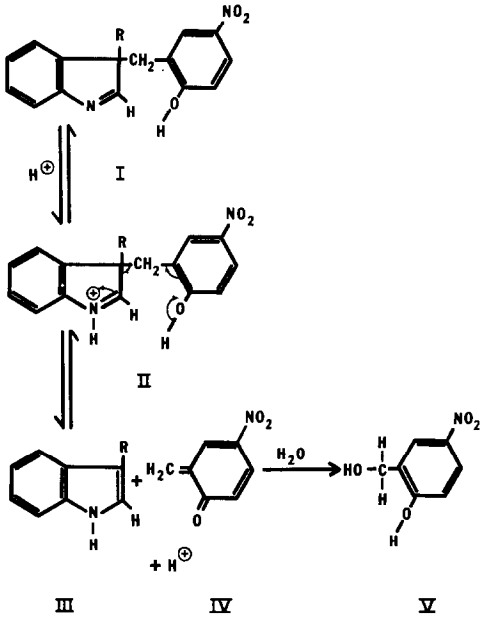
The regeneration of lysozyme activity and the formation of HNB-OH from inactive HNB-lysozyme appears to require the participation of at least two functional groups within the complex.

The first, the 2-hydroxy group of the HNB moiety, is based on the findings that blocking this group by acetylation with acetylimidazole resulted in a derivative from which no lysozyme activity could be regenerated. Treatment of lysozyme with an HNB-Br analogue, one with a methylated 2-hydroxy group, MNB-Br, resulted in an enzyme derivative which could not be regenerated. Although the enzyme residue which reacted with MNB-Br was not identified, the fact that the reaction specificity of HNB-Br and MNB-Br are the same with tryptophan, leads to the assumption that the two reagents reacted the same way with the enzyme.

The second, an easily reducible double bond, was also indicated since reduction with tritiated sodium borohydride resulted in the formation of a stable tritiated HNB-lysozyme. The amount of tritium incorporated into native lysozyme and into HNB-lysozyme under the same experimental conditions indicated the specific incorporation of 1 mole of tritium per mole of the substituted protein. One atom of tritium per molecule of HNB-peptide was also incorporated. The data suggest the same structural feature in both the HNB-lysozyme and the HNB-peptide. The structure which may be formed by alkylation of the indole nucleus of tryptophan by HNB-Br is that of an indolenine (*25*). The reduction of a >C=N- bond by borohydride would result in the uptake of 1 tritium atom.

The implication of the 2-hydroxy group of HNB and the indolenine, as well as the results of other studies, appear to support the postulated mechanism indicated in Scheme I.

The proposed mechanism consists of three parts: [1] Protonation of the indolenine–HNB complex (I). [2] Cleavage of the protonated complex (II) to yield tryptophan (III)



Scheme 1

and a quinone methide (IV). [3] Solvation of the quinone methide to yield HNB-OH (V).

The necessity of the 2-hydroxy group is apparent since neither the 2-methoxy derivative nor the *O*-acetylated derivative can generate the quinone methide (IV). The indolenine >C=N- group participates by donating a pair of electrons to the positively charged nitrogen.

The mechanism was substantiated by the following observations: [1] The pH-rate profile indicated a reaction which was acid catalyzed and the deuterium isotope effect indicated that the reaction was subject to specific hydrogen ion catalysis (*26*). [2] The hydrolysis products of the protonated HNB-indolenine complex could form via a quinone methide intermediate. Horton and Koshland have postulated that the quinone methide is the reactive intermediate in the formation of an HNB–tryptophan adduct (*27*). Application of the principle of microscopic reversibility with respect to formation of the adduct, therefore, lends credence to the hydrolysis reaction. Since the products of the hydrolysis reaction can recombine to yield the HNB–indolenine adduct, regeneration is only observed when the quinone methide is solvated. The observed increase in the rate of regeneration in the presence of nucleophiles like iodide or thiocyanate may thus have been a reflection of a competition between these nucleophiles and the indole nucleus for the quinone methide. [3] An additional component of regeneration which does not appear in the above reaction pertains to protein conformation. This was deduced from the observations that: (1) HNB-OH did not split off from the HNB-peptide and (2) release of HNB-OH did not occur in HNB-lysozyme denatured in 8 *M* urea or in reduced and carboxymethylated HNB-lysozyme.

With respect to the contribution of protein structure to the regeneration reaction, it has been shown that lysine ε amino groups, phenolic hydroxyl groups of tyrosine, and

the carboxyl groups of aspartic acid 52 and glutamic acid 35 play no role in the reactivation process.

The results of the present investigation may be compared with the results of Kaiser et al., who have studied the reaction of chymotrypsin with a number of cyclic esters (*28–33*). These studies have indicated that the reaction of chymotrypsin and specific cyclic esters of sulfur, carbon, and phosphorus are extremely rapid and produce an acyl enzyme in which the acyl group is bound to the active site serine residue. The acyl enzymes are inactive but they spontaneously revert to the active enzyme, $k = 6.7 \times 10^{-4}$ sec^{-1} at pH 7.6 and 25°C for desulfonylation (*28*). An analogy can be drawn between the regeneration of HNB-lysozyme and of acyl chymotrypsin in that inactive enzymes are formed at the active site by covalent inhibition which spontaneously revert to active enzymes with the concomitant release of the inhibitor moiety.

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