I. PURIFICATION AND PARTIAL CHARACTERIZATION OF TESTICULAR HYALURONIDASE

II. MECHANISTIC STUDIES OF HUMAN LYSOZYME

Thesis by

Charles LaMonte Borders, Jr.

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To Cathy

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ABSTRACT

PART I

A new method is presented for the purification of testicular hyaluronidase involving ion exchange chromatography followed by gel filtration on Sephadex G-75. A highly purified hyaluronidase preparation has been obtained which contains 45,000 N. F. activity units per mg dry weight and which migrates as a single component on polyacrylamide gel electrophoresis at pH 4.3. The amino acid composition has been determined, and the enzyme has been shown to be a glycoprotein containing 5.00% mannose and 2.17% glucosamine (expressed as N-acetylglucosamine). The molecular weight of the purified hyaluronidase has been determined to be 61,000 by gel filtration methods. This value is in contrast to a molecular weight of 126,000 for the crude enzyme, as determined by similar techniques. A glycopeptide has been isolated from the purified hyaluronidase after total enzymatic digestion of the reduced carboxymethylated enzyme. This glycopeptide was shown to have a molecular weight of approximately 1,600, and to have a composition of $(\text{mannose})_7(\text{glucosamine})_2(\text{asp})_1$. The amino acid involved in the protein-carbohydrate linkage is thus aspartic acid.

PART II

It has been shown that when human lysozyme catalyzed glycosidic bond cleavage of chitobiose is carried out in the presence of methanol, transglycosylation occurs with production of methyl-N-acetyl-β-D-glucosaminide. Thus, retention of configuration occurs at the anomeric carbon of the disaccharide substrate during catalysis and eliminates the possibility of a single displacement mechanism for the enzyme. Use has been made of this transglycosylation reaction to enzymatically synthesize oligomeric p-nitrophenyl-β-D-glucosaminides from chitin oligosaccharides, p-nitrophenyl-N-acetyl-β-D-glucosaminide, and human lysozyme. Such saccharides serve as substrates for human lysozyme with release of p-nitrophenol. Similar synthesis of oligosaccharides from chitin oligomers, p-nitrophenyl-β-D-glucoside, and human lysozyme has been carried out. Since human lysozyme effects hydrolysis of the glucosidic bonds in such oligosaccharides, a mechanism involving anchimeric assistance of an N-acetyl group has been eliminated. Similar enzymatic synthesis and hydrolysis of oligosaccharide substrates from chitin oligomers and p-nitrophenyl-2-deoxy-β-D-glucoside has eliminated a mechanism involving acetamido or C-2 oxyanion participation by substrate due to general base catalysis by the enzyme.

Only two closely related possibilities remain to explain the mechanistic pathway of substrate during catalysis by human lysozyme.

The hydrolysis of chitobiose, chitotriose, and chitotetraose by human lysozyme under different sets of conditions has also been investigated. It was found that this enzyme could be distinguished from hen lysozyme by the product distributions obtained for hydrolysis by the two enzymes under identical reaction conditions.

The hydrolysis of p-nitrophenyl-β-D-chitobioside by human lysozyme was shown to follow Michaelis-Menton kinetics, with $K_M = 2.6 \times 10^{-2}$ M and $k_{cat} = 6.8 \times 10^{-5}$ mole·sec^{-1}·(mole enzyme)$^{-1}$.

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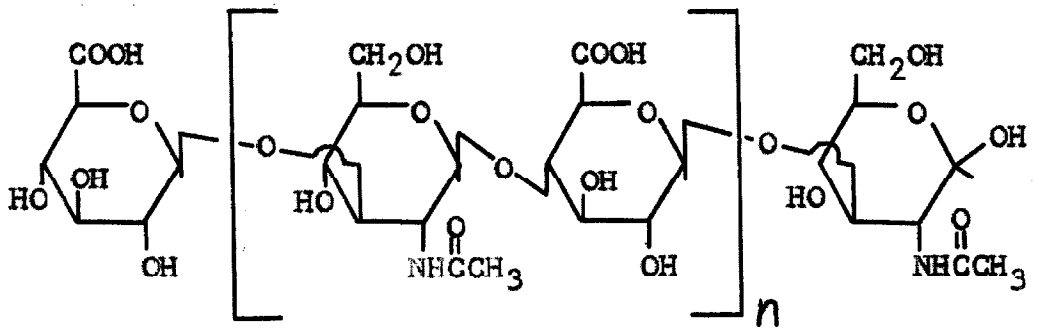
PART I

PURIFICATION AND PARTIAL CHARACTERIZATION OF TESTICULAR HYALURONIDASE

A. INTRODUCTION

1. History.

Hyaluronidase is the name given to a wide array of dissimilar enzymes from many different sources which have the common ability to cleave glycosidic bonds of hyaluronic acid (I). Although the first of such enzymes was obtained from microorganisms (1), the enzyme which has been the object of most investigations is bovine testicular hyaluronidase. This particular enzyme has been shown to be an endohexosaminidase (2), that is, it cleaves the glycosidic bonds of internal N-acetylhexosamine residues of hyaluronic acid. After exhaustive enzymatic digestion of the hyaluronic acid, the end products are 80 to 85% tetrasaccharide (I, n = 1) and 15 to 20% disaccharide (3). Testicular hyaluronidase has also been shown to possess transglycosylation activity (4,5), since since digestion of the hexasaccharide of hyaluronic acid (I, n = 2) gives



I

higher oligosaccharides as well as lower oligosaccharides.

These early studies were all done with heterogeneous enzyme preparations. In fact, despite many efforts directed towards the purification of testicular hyaluronidase, a homogeneous enzyme preparation had not actually been demonstrated up to the time this investigation was begun. Early efforts to purify the enzyme include those of Hahn (6), who used various salt fractionation procedures to obtain a somewhat purified preparation which he determined to have a sedimentation constant of 4.3 by using a separation cell during ultracentrifugation and determination of hyaluronidase activity in the upper and lower parts of the cell following centrifugation. Tint and Bogash (7) used salt fractionation, alcohol fractionation, and repeated salt fractionation to obtain a preparation which Malmgren (8) showed to have a sedimentation constant of 3.2 by measurement of hyaluronidase activity after centrifugation, and to contain 1.6% carbohydrate. Högberg (9) prepared a sample by salt fractionation, shaking with chloroform, and ion exchange chromatography on Amberlite XE-64 (a carboxylic acid cation exchanger). Malmgren (8) calculated this preparation to have a molecular weight of 11,000 by ultracentrifugation techniques, and he showed it to contain

2.0% carbohydrate.

Of the more recent attempts to purify and characterize testicular hyaluronidase, that reported by Brunish and Högberg (10) appears to be the best. Starting with material prepared by the method of Högberg (9), these investigators achieved extensive purification by ion exchange chromatography on IRC-50 (a carboxylic acid ion exchange resin) by using a salt gradient for elution of the enzyme. They reported their preparation to have an activity of about 40,000 i. u.[1,2] per mg, and that it was a glycoprotein with 5.2% carbohydrate and 5.0% hexosamine·HCl, but did not identify the types of neutral sugar and amino sugar residues involved. They determined the molecular weight to be 43,000 by the Archibald approach to sedimentation equilibrium method, but found the sample to be somewhat heterogeneous in the ultracentrifuge. They reported an amino acid analysis based on a single determination, and could not demonstrate the homogeneity of their preparation by any technique.

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[1]The abbreviations used are: i. u., international units; N. F., National Formulary; U. S. P., United States Pharmacopoeia; DEAE, diethylaminoethyl; BSA, bovine serum albumin; p. m. r., proton magnetic resonance; t. r. u., turbidity reducing units.

[2]An explanation of the various activity units used for hyaluronidase may be found on page 75.

A more recent attempt at the purification of testicular hyaluronidase has been reported by Soru and Ionescu-Stoian (11-13). These investigators used salt fractionation followed by a combined Sephadex - DEAE-Sephadex chromatography step. They reported a very high yield of the enzyme and claimed to have obtained a homogeneous preparation (11,12). However, their preparation possessed a very low specific activity compared to preparations obtained by other investigators, and, since homogeneity was investigated only by paper electrophoretic techniques (12), the validity of their findings is not obvious. They reported an amino acid composition for their preparation which was radically different from that of Brunish and Högberg, and were unable to detect any carbohydrates (13).

2. Reasons for the Present Investigation.

When the idea of investigating testicular hyaluronidase was first considered, there were several reasons for working on this enzyme. There have been extensive studies in this laboratory on hen egg white lysozyme directed towards the elucidation of its chemical and mechanistic properties, and since lysozyme and hyaluronidase are similar in a number of ways, it was thought that a comparative study of the two enzymes might be

enlightening.

Both lysozyme and hyaluronidase are β-glycosidases, and both have substrates which are chemically rather similar. Typical substrates for lysozyme include a cell wall polysaccharide containing the repeating dimeric structure N-acetylmuramic acid-N-acetylglucosamine with both glycosidic linkages in the β-(1→4) configuration, and chitin, a β-(1→4) linked polymer of N-acetylglucosamine. These polysaccharides are structurally quite similar to the substrate for hyaluronidase, hyaluronic acid (I). Both lysozyme and testicular hyaluronidase cleave the glycosidic bond of a N-acetylhexosamine of the β-configuration, and both yield low molecular weight oligosaccharides on exhaustive digestion of their respective substrates.

The interaction between lysozyme and low molecular weight oligosaccharides derived from chitin has been investigated in this laboratory by proton magnetic resonance spectroscopy by monitoring the methyl resonances of the N-acetyl groups of these oligosaccharides (14-19). Since hyaluronic acid also contains N-acetyl groups which should make similar studies possible with hyaluronidase, it was thought that p. m. r. studies of substrate-enzyme interactions could be extended to this system.

A decision was therefore reached to work on hyaluronidase with the idea of doing a comparative study between this enzyme and lysozyme for reasons listed above. Physicochemical studies of hyaluronidase were planned, with the possibility of mechanistic studies, in order to gain some insight into the similarities and differences between hyaluronidase and lysozyme. All these ideas were entertained with the understanding that a purified hyaluronidase preparation was commercially available, since a reputable supplier claimed to have available a "highly purified" material prepared by the method of Soru and Ionescu-Stoian (11,12).

Unfortunately, this commercial preparation was highly heterogeneous, as was shown later in this laboratory. The preparation of a homogeneous enzyme was attempted by repeating several different methods described in the literature, but to no avail. Thus, the problem of the purification of testicular hyaluronidase had to be overcome, and a new purification procedure had to be developed.

Since a thorough physicochemical characterization of hyaluronidase was not available (for the best preparation described in the literature to date (10) was characterized only in a superficial manner), it was decided to characterize the enzyme more completely.

It was hoped that a homogeneous preparation could be obtained and that the following characterizations could be achieved: positive demonstration of homogeneity; complete amino acid composition; identification of the kinds and amounts of neutral sugar and amino sugar residues involved; investigation into the composition of any glycopeptide components which could be prepared from the enzyme and determination of the mode of linkage of the carbohydrate portion to the protein backbone. Also, since there has been much disagreement in the literature on the molecular weight of hyaluronidase, it was hoped that a more reliable value of the molecular weight could be determined.

Bernfeld et al. (20) have found that, upon dilution, hyaluronidase is transformed into an enzymatically inactive form, recognizable by the progressive loss of specific activity. They attributed this phenomenon to a reversible dissociation of the active enzyme into inactive components. This finding, along with the different values for the molecular weight determined in different laboratories, indicated that testicular hyaluronidase might be composed of subunits, and it was hoped to investigate this idea more thoroughly as a part of the investigation to be described in this

dissertation.

B. RESULTS AND DISCUSSION

At the beginning of the present investigation, it was thought that a highly purified hyaluronidase was commercially available. Worthington Biochemical Corporation advertized a hyaluronidase preparation, isolated by the method of Soru and Ionescu-Stoian (11,12), which they quoted as having a specific activity of 3,400 U. S. P. units per mg dry weight. However, their claim that this was a "highly purified" preparation was not accurate, as will be shown in later sections. It was, in fact, highly heterogeneous. The preparation of a homogeneous enzyme was therefore attempted by first repeating several purification methods already described in the literature.

1. Preliminary Attempts at Purification of Testicular Hyaluronidase.

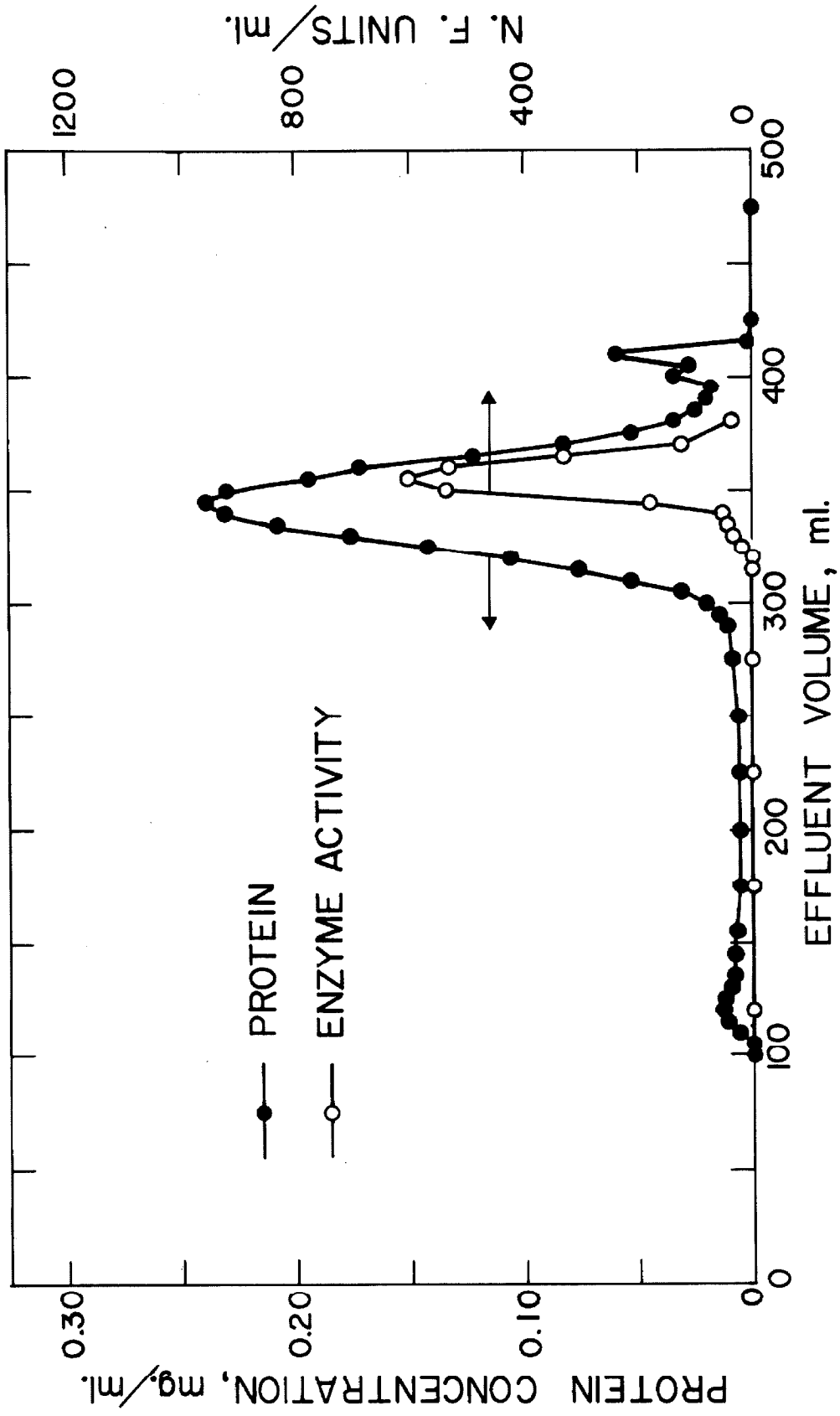
a. The Method of Soru and Ionescu-Stoian (11,12)-- When the DEAE-Sephadex chromatography step of Soru and Ionescu-Stoian was repeated in this laboratory, using as a starting material crude commercial hyaluronidase which had an activity of 360 N. F. units per mg, a somewhat purified protein material was isolated which had an activity of about 2,800 N. F. units per mg. This material had approximately the same specific activity

as the commercial preparation (labelled "highly purified") available from Worthington Biochemical Corporation, and since this latter material was shown to be highly heterogeneous, then it was assumed that the material prepared in this laboratory was also impure.

When the material purified by the method of Soru and Ionescu-Stoian as described above was dissolved in salt solution and subjected to gel filtration on Sephadex G-75 which had been equilibrated with distilled water at 4°C (a system which was subsequently used as the final step in the purification procedure developed in this laboratory), the hyaluronidase activity was held up on the column. It was eventually eluted in the back half of a broad protein peak which was eluted just ahead of the salt. The profile of this chromatogram (Figure 1) looked somewhat similar to that of the final step in the eventual purification scheme (Figure 8), but the specific activity of the hyaluronidase was much lower, only about 4,200 N. F. units per mg. Because of the low specific activity of the material obtained from the combined steps listed above, this approach was discontinued.

The material from the DEAE-Sephadex chromatography was subjected to ion exchange chromatography on Bio-Rex

Figure 1. Chromatography of DEAE-Sephadex-purified hyaluronidase on Sephadex G-75 in water.


PROTEIN CONCENTRATION, mg./ml.
0.30
0.20
0.10
0
N. F. UNITS/ml.
1200
800
400
0
PROTEIN
ENZYME ACTIVITY
100
200
300
400
500
EFFLUENT VOLUME, ml.

70 according to the method of Rasmussen (21). Hyaluronidase with a specific activity of about 8,000 N. F. units per mg was eluted from the column by the second buffer. The specific activity was still low compared to what was expected for pure hyaluronidase (as estimated by specific activities reported by other investigators, namely Brunish and Högberg (10)), and this approach was also discontinued.

b. The Method of Rasmussen (21)-- When crude hyaluronidase was subjected to ion exchange chromatography on Bio-Rex 70 using the two buffer system of Rasmussen (21), hyaluronidase with a specific activity of about 3,500 N. F. units per mg was obtained. This represented a ten-fold purification of the starting material. This method involved the application of the crude hyaluronidase in 0.1 M NaH_2PO_4, pH 6.0, to a column of Bio-Rex 70 which had been equilibrated with the same buffer. At this pH and ionic strength the hyaluronidase activity was tightly bound to the resin while most of the protein impurities passed directly through the column. The hyaluronidase was eluted from the resin by using a buffer of higher pH and ionic strength, in this case 0.3 M NaH_2PO_4, pH 7.7.

It was thought that further purification of the

material obtained by the method of Rasmussen as described above might be achieved by using a pH or ionic strength gradient on Bio-Rex 70. A pH-step gradient was first tried and it was found that all the hyaluronidase activity remained bound to the resin in 0.1 M NaH_2PO_4, pH 6.3, but was eluted by buffers of higher pH or ionic strength. Therefore, a salt gradient was set up using 0.1 M NaH_2PO_4, pH 6.31, with a gradient of zero to 1.0 M NaCl (Figure 2). As seen in Figure 2, the hyaluronidase activity was apparently eluted at a NaCl concentration of about 0.2 to 0.4 M, and had a maximum specific activity of about 14,000 N. F. units per mg.

Several attempts to find a single buffer system which would give a highly purified hyaluronidase on chromatography on Bio-Rex 70 led to a system of 0.10 M NaH_2PO_4, 0.075 M NaCl, pH 6.31. A typical chromatogram of hyaluronidase on Bio-Rex 70 using this buffer system is shown in Figure 3. The hyaluronidase activity peak was relatively symmetrical when compared to the protein peak, and the activity was constant at about 12,000 N. F. units per mg.

Although this system showed promise as a purification step, the column had a very low capacity for hyaluroni-

Figure 2. Chromatography of hyaluronidase purified by the method of Rasmussen (21) on Bio-Rex 70 using a NaCl gradient for elution.

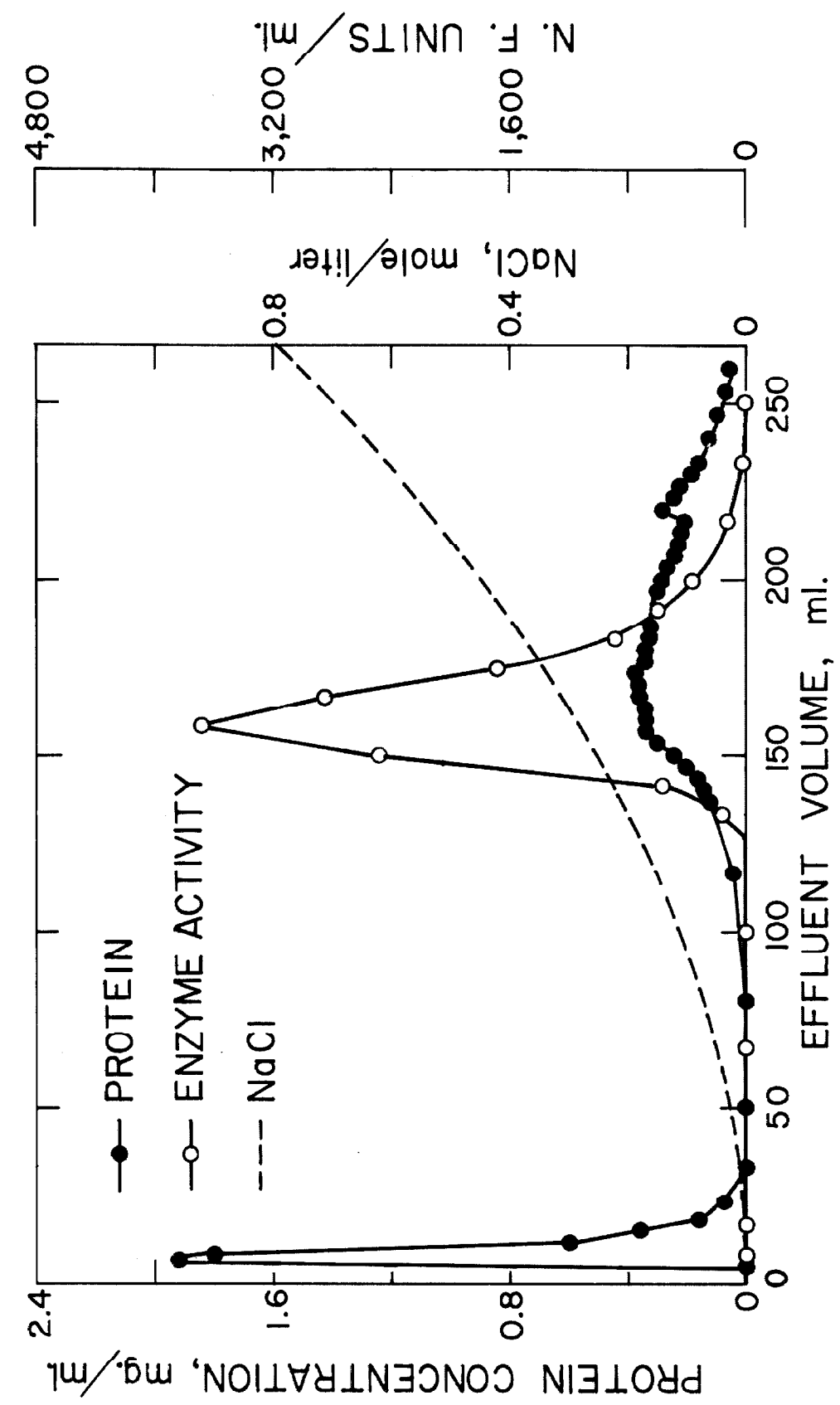
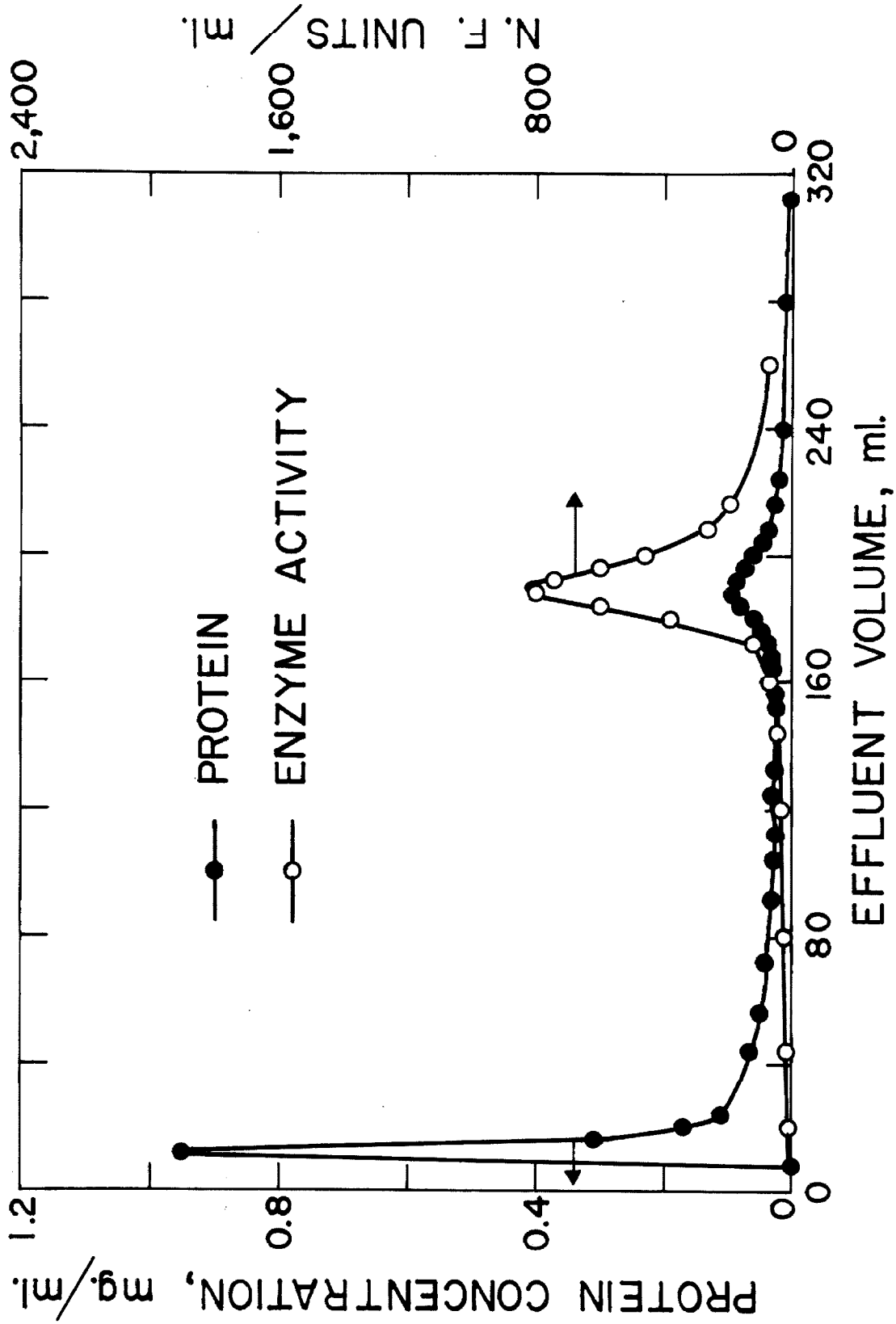

PROTEIN CONCENTRATION, mg./ml.
2.4
1.6
0.8
0
PROTEIN
ENZYME ACTIVITY
NaCl
0
50
100
150
200
250
EFFLUENT VOLUME, ml.
NaCl, mole/liter
0.8
0.4
0
N. F. UNITS/ml.
4,800
3,200
1,600
0

Figure 3. Chromatography of hyaluronidase purified by the method of Rasmussen (21) on Bio-Rex 70 in 0.10 M NaH_2PO_4, 0.075 M NaCl, pH 6.31.


PROTEIN CONCENTRATION, mg./ml.
1.2
0.8
0.4
0
N. F. UNITS / ml.
2,400
1,600
800
0
0
80
160
240
320
EFFLUENT VOLUME, ml.
PROTEIN
ENZYME ACTIVITY

dase. The highest protein concentration across the activity peak which could be obtained in several different runs using this procedure was less than 0.1 mg per ml. The system was abandoned as a purification method for this reason.

c. The Method of Högberg (9)-- Using chromatography on Amberlite XE-64 (an earlier term used for Bio-Rex 70) as the major purification step, Högberg reported to have prepared hyaluronidase of very high specific activity (9). The method consisted in adsorbing the enzyme on Amberlite XE-64, NH_4^+ form, washing the column successively with water, 0.1 M NH_4OAc, 0.1 M NH_4OH, and then eluting the hyaluronidase with a minimal amount of KCl-HCl, pH 1.5. Brunish and Högberg (10) used hyaluronidase prepared by this method as a starting material in their purification scheme. They claimed to have isolated hyaluronidase with a specific activity of about 20,000 i. u. per mg by this method of Högberg.

When it was attempted to repeat the procedure of Högberg in this laboratory, starting with crude commercial hyaluronidase which had an activity of 410 N. F. units per mg, the hyaluronidase which was isolated had an activity of only 3,100 N. F. units per mg. It was then noted that Högberg treated his crude hyaluroni-

dase solution by shaking it with 0.5 parts of chloroform before subjecting it to this procedure. It was thought that this might indeed be a critical step in the purification scheme. Another chromatogram was run using chloroform-treated hyaluronidase as the starting material, but the isolated product contained the same specific activity as the product which had not been treated with chloroform in a preliminary step.

It is not known why the material isolated by the method of Högberg (9) had a much lower specific activity than that reported by the original investigator, but the enzyme isolated in this laboratory was consistently lower in specific activity. For this reason, coupled with the fact that only 40% of the original activity was recovered by this procedure, this approach was discontinued.

d. Chromatography of Crude Testicular Hyaluronidase on Sephadex G-75 in 0.1 M NaCl Solution-- When it was subsequently shown in this laboratory that hyaluronidase which had been previously purified by the method of Rasmussen (21) could be highly purified by gel filtration chromatography on Sephadex G-75 in 0.1 M NaCl solution, it was thought that a gel filtration procedure might be a useful preliminary step in the puri-

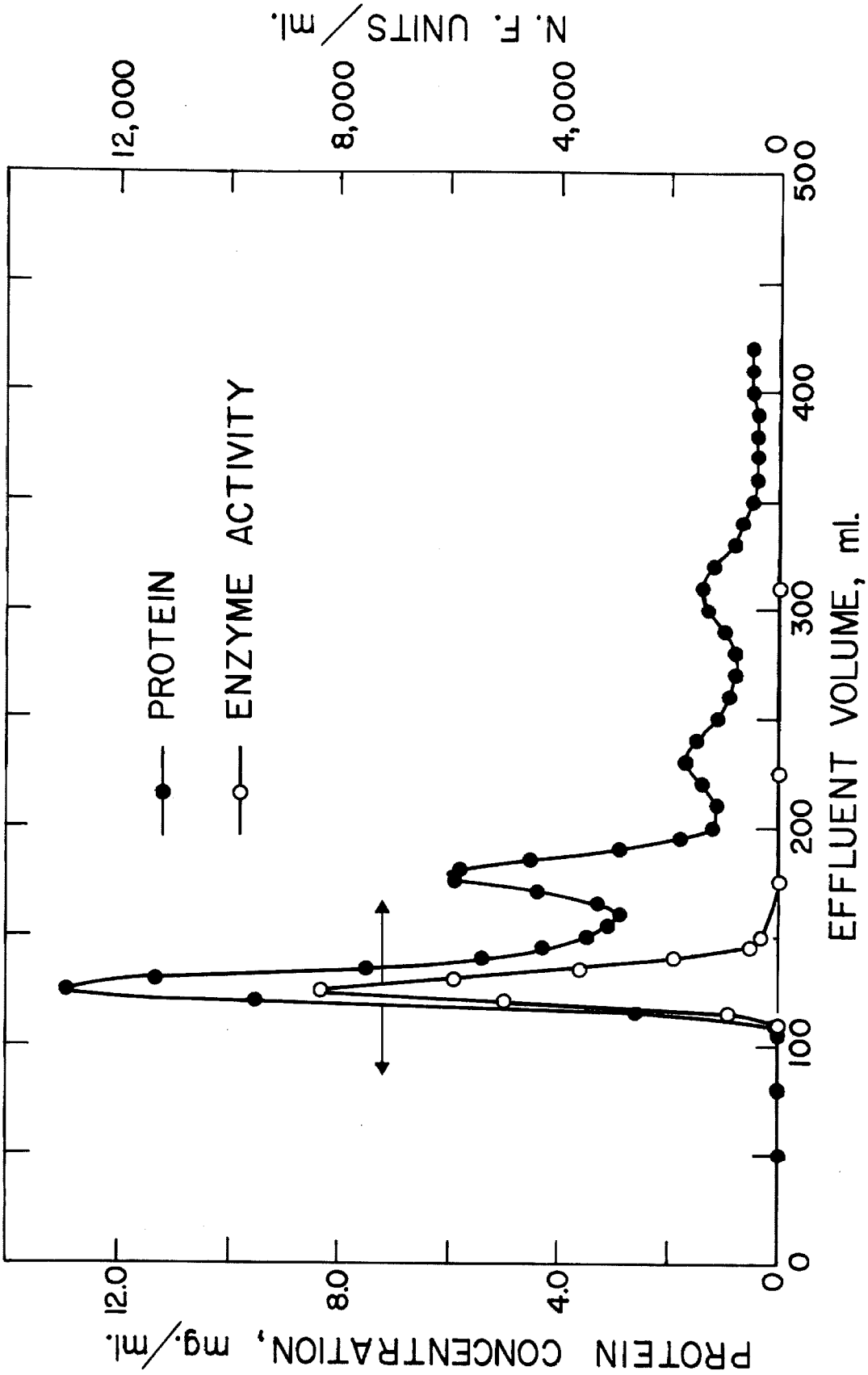
fication of crude hyaluronidase. However, when crude hyaluronidase with an activity of 410 N. F. units per mg was subjected to chromatography on Sephadex G-75 in 0.1 M NaCl, only a 1.5-fold purification resulted (Figure 4). This approach was therefore discontinued.

2. Final Scheme for the Extensive Purification of Testicular Hyaluronidase.

It was subsequently found that when hyaluronidase which had undergone preliminary purification by the method of Rasmussen (21) was subjected to gel filtration chromatography on Sephadex G-75 in 0.1 M NaCl solution, a substantial enrichment of specific activity took place. Thus when material with a specific activity of about 3,000 N. F. units per mg was used as the starting material, the resulting hyaluronidase had an activity of greater than 20,000 N. F. units per mg. The following purification scheme was based on this purification step.

a. Bio-Rex 70 Chromatography-- When crude commercial hyaluronidase (25.88 g) was applied to a column (6.0 x 43 cm) of Bio-Rex 70 which had been equilibrated with 0.1 M NaH_2PO_4, pH 6.0, most of the protein passed directly through the column along with a large amount of gold-colored impurity. The eluting

Figure 4. Chromatography of crude testicular hyaluronidase on Sephadex G-75 in 0.1 M NaCl solution.


PROTEIN CONCENTRATION, mg./ml.
12.0
8.0
4.0
0
N. F. UNITS / ml.
12,000
8,000
4,000
0
PROTEIN
ENZYME ACTIVITY
0
100
200
300
400
500
EFFLUENT VOLUME, ml.

buffer was changed to 0.3 M NaH_2PO_4, pH 7.7, and about 5 liters was passed through before the hyaluronidase was eluted as a broad peak (Figure 5). The fractions indicated by the arrow in Figure 5 were pooled and, after dialysis and lyophilization, yielded 2.13 g of protein material with an activity of 2,300 N. F. units per mg dry weight.

b. First Sephadex G-75 Chromatography in 0.1 M NaCl-- The protein material obtained from the Bio-Rex 70 chromatography above was passed through a column (5.0 x 76 cm) of Sephadex G-75 equilibrated with 0.1 M NaCl and the major part of the hyaluronidase activity was eluted in a trough between two major inactive components (Figure 6). The fractions indicated in Figure 6 were pooled and yielded hyaluronidase with an activity of 21,600 N. F. units per mg (representing a 77-fold purification of the crude starting material). These pooled fractions were used directly in the next step.

The preliminary chromatography on Bio-Rex 70 was essential in the purification procedure, as was evidenced by the fact that when crude commercial hyaluronidase was applied directly to a column of Sephadex G-75 equilibrated with 0.1 M NaCl, only a

Figure 5. Chromatography of crude hyaluronidase on Bio-Rex 70. The hyaluronidase was applied in 0.1 M NaH_2PO_4, pH 6.0, and eluted with 0.3 M NaH_2PO_4, pH 7.7.

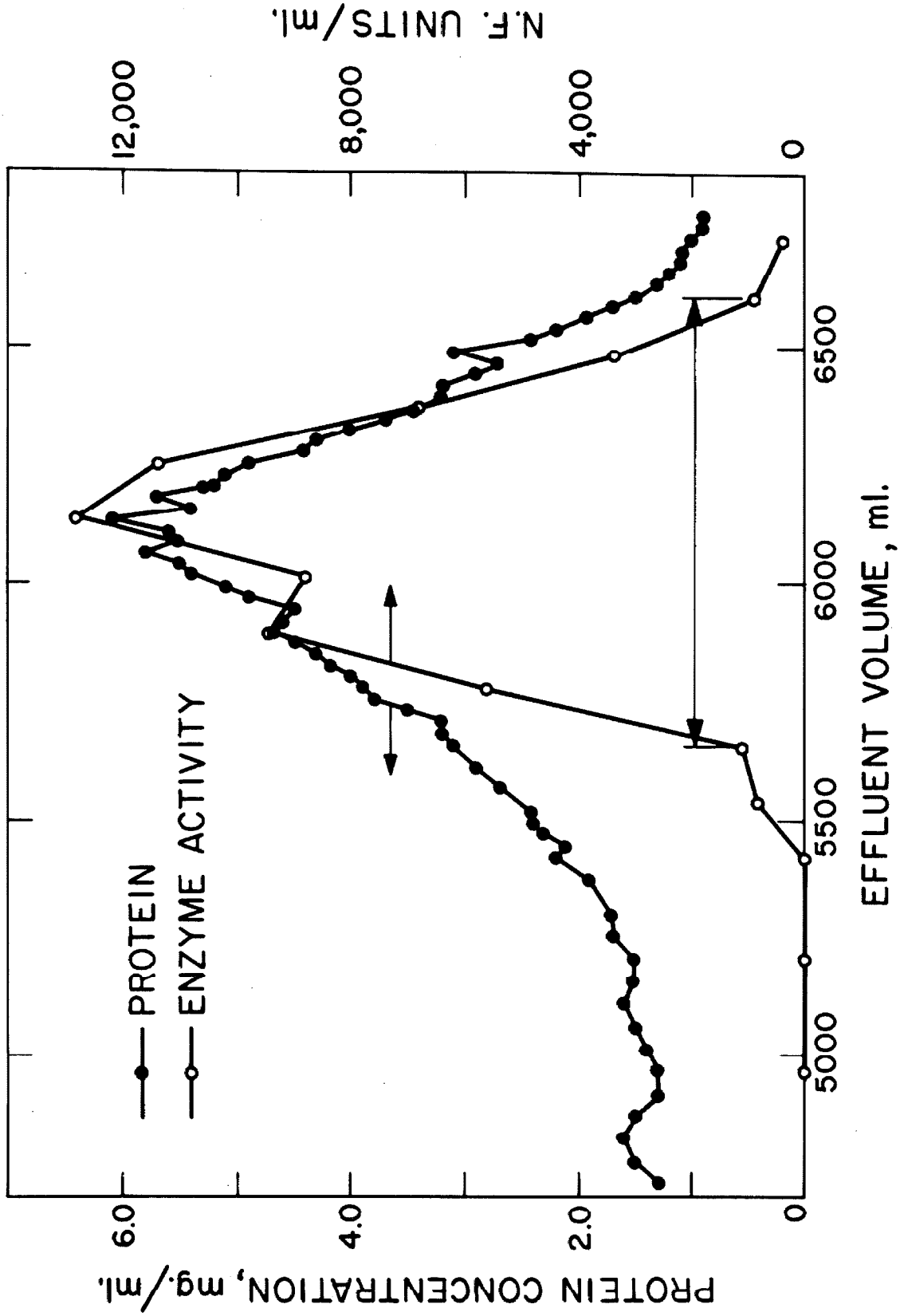
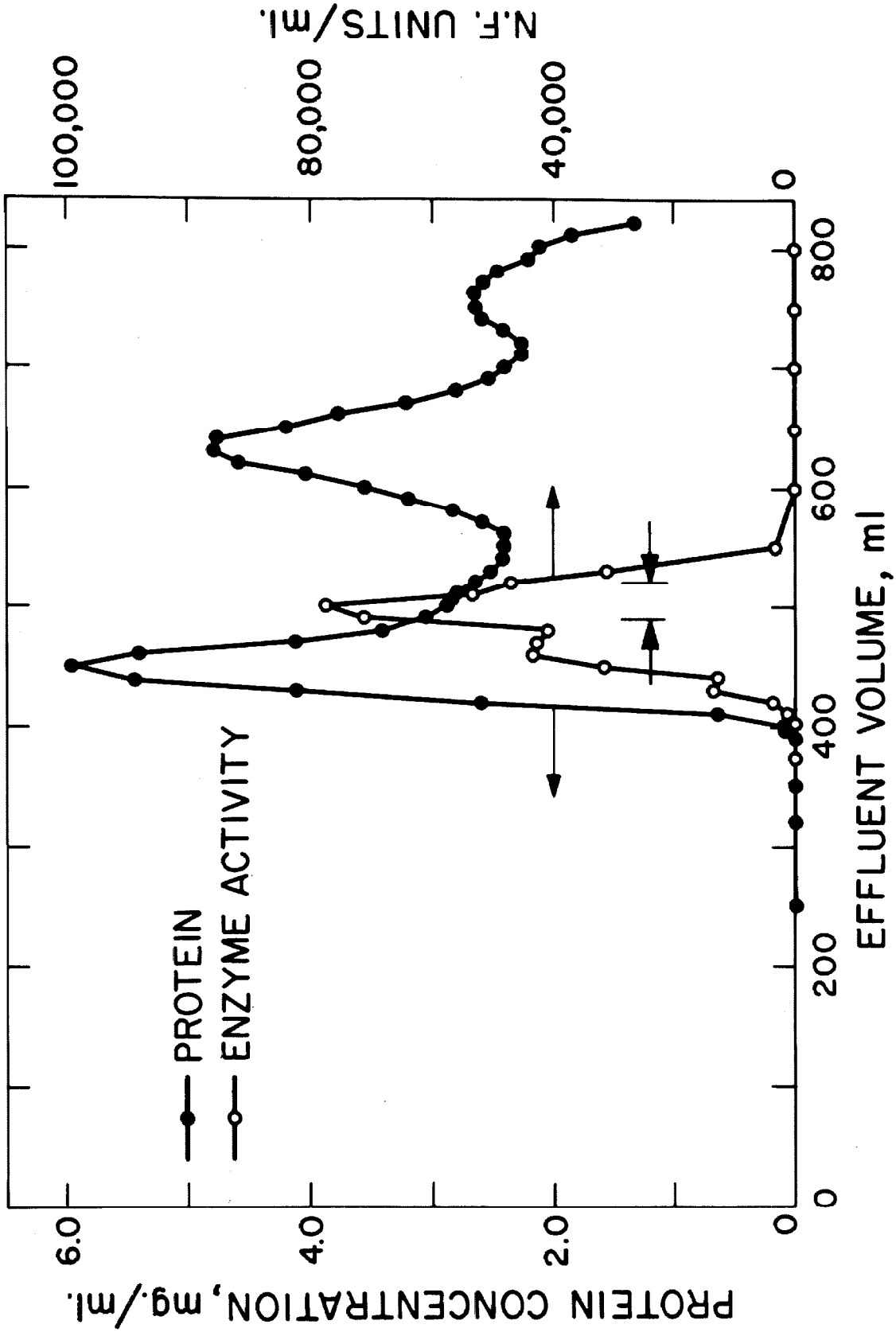

PROTEIN CONCENTRATION, mg./ml.
N.F. UNITS/ml.
EFFLUENT VOLUME, ml.
PROTEIN
ENZYME ACTIVITY
0
2.0
4.0
6.0
4,000
8,000
12,000
5000
5500
6000
6500

Figure 6. Chromatography of partially purified hyaluronidase on Sephadex G-75 in 0.1 M NaCl solution.


PROTEIN CONCENTRATION, mg./ml.
N.F. UNITS/ml.
EFFLUENT VOLUME, ml
PROTEIN
ENZYME ACTIVITY
0
2.0
4.0
6.0
40,000
80,000
100,000
200
400
600
800

1.5-fold purification resulted (Figure 4).

c. Second Sephadex G-75 Chromatography in 0.1 M NaCl-- The material obtained by Bio-Rex 70 chromatography and chromatography on Sephadex G-75 (in 0.1 M NaCl) was rechromatographed on Sephadex G-75 which had been equilibrated with 0.1 M NaCl. In this way additional purification was achieved (Figure 7). The fractions indicated in Figure 7 were pooled and yielded hyaluronidase with an activity of 31,000 N. F. units per mg. These pooled fractions were used directly in the next step.

d. Sephadex G-75 Chromatography in Water-- It is well known that many basic proteins are retained by Sephadex gels at very low ionic strength. Since previous experience with hyaluronidase in this laboratory had shown that it was indeed retained on Sephadex in the absence of salts, it was thought that a step such as this might be a valuable one in the purification scheme. That this was the case is demonstrated in Figure 8. A small amount of material devoid of hyaluronidase activity was eluted in the position at which hyaluronidase was normally eluted in the presence of 0.1 M NaCl. This was followed at a distance by a much larger protein peak, the front part of which was also devoid of hyaluronidase activity and the back part of which contained highly active hyaluronidase. The

Figure 7. Rechromatography of partially purified hyaluronidase on Sephadex G-75 in 0.1 M NaCl solution.

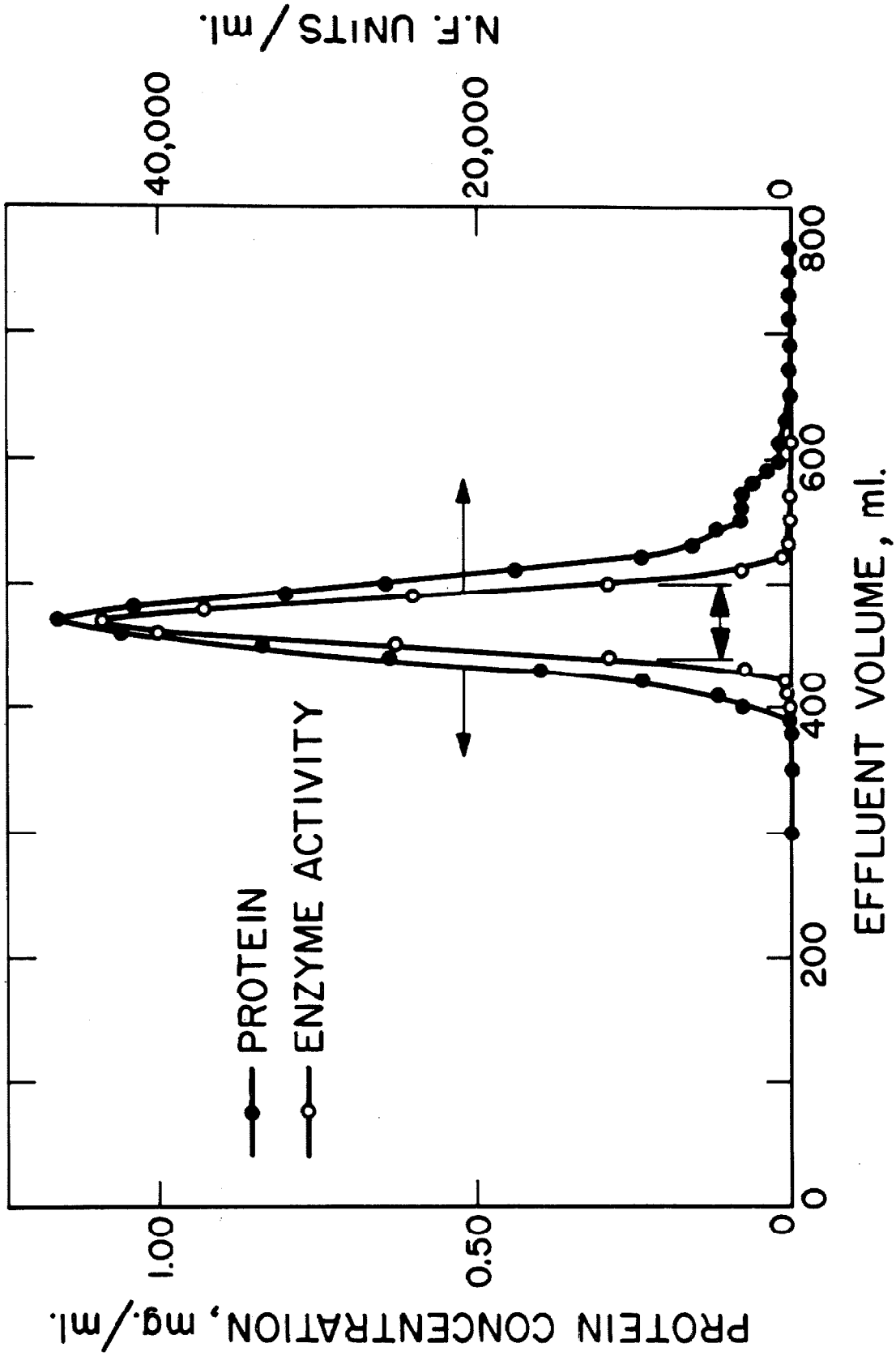
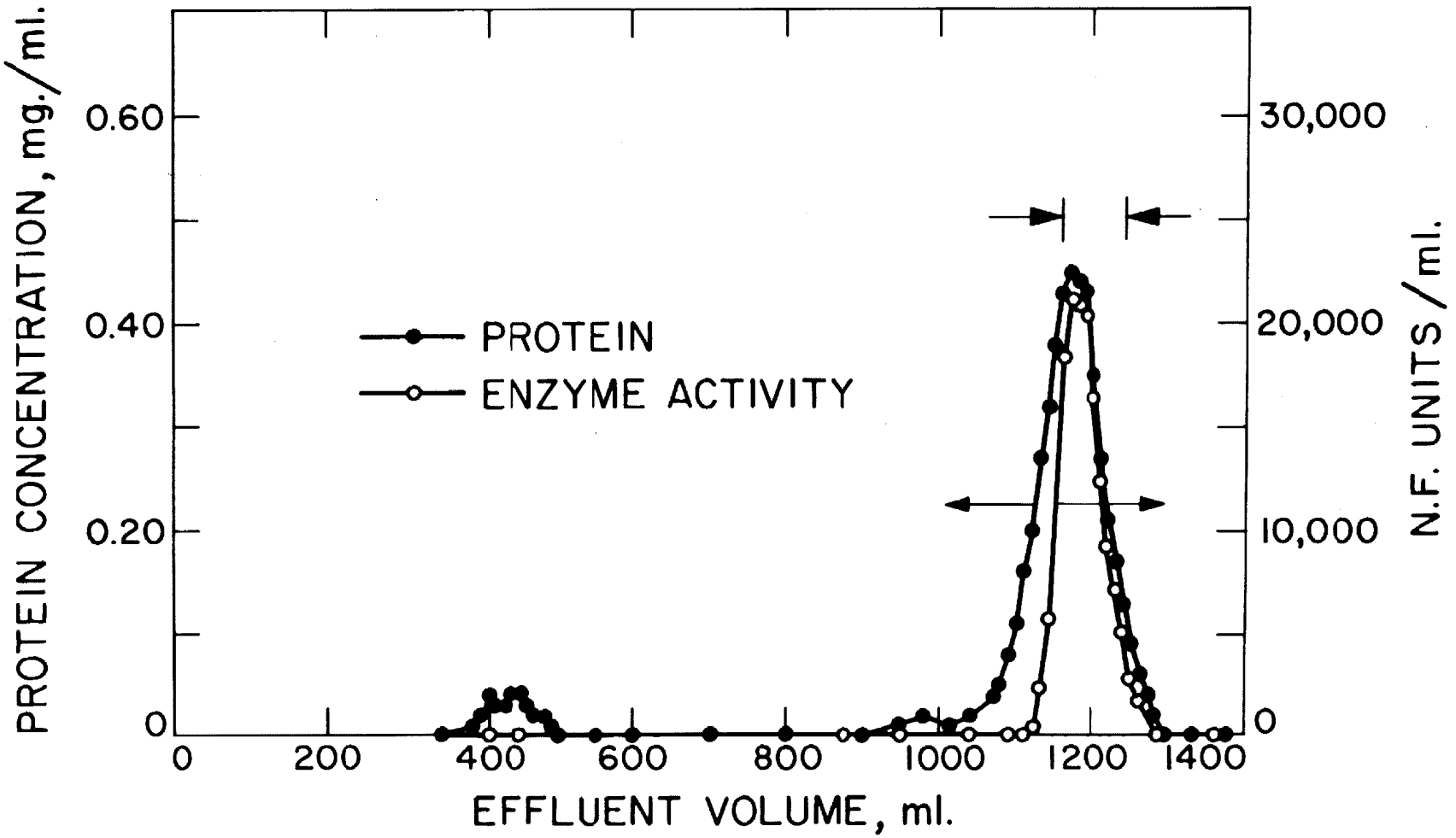

PROTEIN CONCENTRATION, mg./ml.
1.00
0.50
0
N.F. UNITS / ml.
40,000
20,000
0
PROTEIN
ENZYME ACTIVITY
0
200
400
600
800
EFFLUENT VOLUME, ml.

Figure 8. Gel filtration chromatography of partially purified hyaluronidase on Sephadex G-75 in water.


PROTEIN CONCENTRATION, mg./ml.
0.60
0.40
0.20
0
30,000
20,000
10,000
0
N.F. UNITS / ml.
PROTEIN
ENZYME ACTIVITY
0
200
400
600
800
1000
1200
1400
EFFLUENT VOLUME, ml.

fractions indicated by arrows had an essentially constant activity of 45,500 N. F. units per mg. When it was determined that the hyaluronidase had been eluted just ahead of the NaCl peak, the fractions of constant activity were pooled and lyophilized in a polyethylene container. The lyophilized material was stored in a dessicator below 0° C. It had an activity of 45,000 N. F. units per mg dry weight, and retained its activity even after storage as a frozen solution in distilled water for four months. A summary of the various steps in the purification procedure is given in Table I.

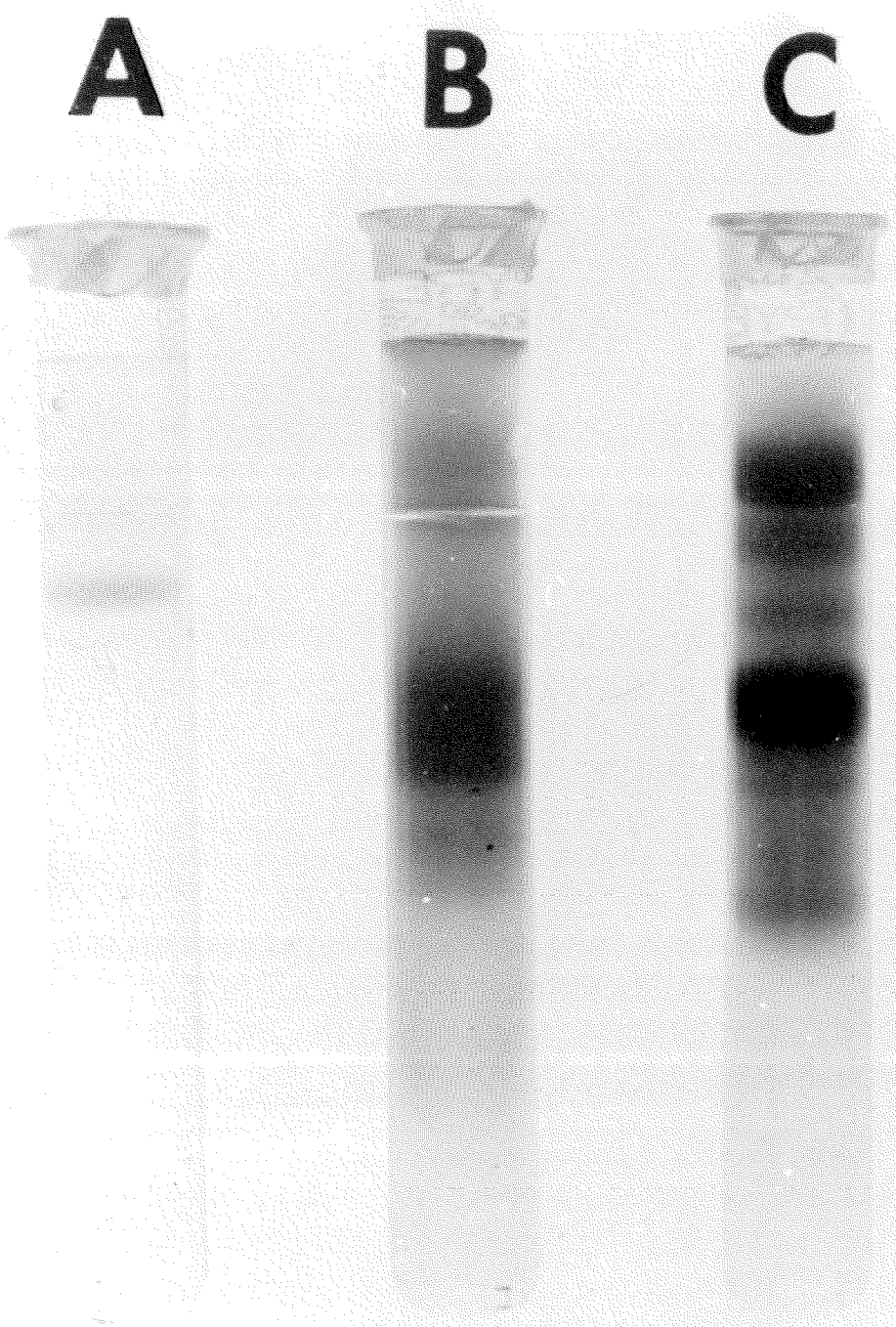
3. Disc Electrophoresis.

The homogeneity of the purified hyaluronidase was indicated by the presence of a single band in polyacrylamide gel electrophoresis at pH 4.3 (Figure 9) after the method of Reisfeld *et al.* (22). Hyaluronidase at various stages of purification is shown in Figure 9 and the degree of purification is evident. It is interesting to compare the hyaluronidase prepared as described in this dissertation with a preparation obtained by the method of Soru and Ionescu-Stoian (11,12) (purchased as "highly purified" hyaluronidase from Worthington), which was shown to contain four

Table 1. Summary of the Purification of Testicular Hyaluronidase.

Step	ml	mg protein/ ml	mg protein	N.F. units X 10^{-6}	N.F. units/ mg protein	Relative Activity	Yield (%)
Crude enzyme			25880	7.25	280	1.0	100
Bio-Rex 70			2130	4.90	2300	8.2	67.6
1st G-75 in 0.1 M NaCl	40	2.85	114	2.48	21600	77	34.2
2nd G-75 in 0.1 M NaCl	70	0.89	62	1.93	31100	111	26.6
3rd G-75 in water	90	0.32	28.8	1.31	45500	163	18.1

Figure 9. Disc electrophoresis patterns of hyaluronidase perparations at various stages of purification. A) 50 μg of highly purified hyaluronidase with an activity of 45,000 N. F. units per mg. B) 300 μg of hyaluronidase which had been purified by Bio-Rex 70 chromatography and which had an activity of 2,300 N. F. units per mg. C) 600 μg of crude commercial hyaluronidase with an activity of 280 N. F. units per mg.


A
B
C

major components and at least six minor components by disc electrophoresis (Figure 10).

Although polyacrylamide gel electrophoresis is not an absolute indication of homogeneity, it is one of the most diagnostic techniques which has been developed for this purpose. It is an extremely sensitive technique, and, since the preparation of hyaluronidase described in this work migrated as a single component under the conditions of electrophoresis, it was assumed to be a highly purified, if not homogeneous, preparation. Due to the small amount of material available, no further tests for homogeneity were made on this preparation.

4. Amino Acid and Carbohydrate Composition.

The amino acid and carbohydrate composition of hyaluronidase is given in Table II. The values are expressed as mmole residue per 100 g enzyme since only 76% by weight of the enzyme preparation could be accounted for by amino acids and carbohydrates. This is analogous to the preparation of Brunish and Högberg (10) in which only 75% by weight of their preparation could be accounted for as amino acids and carbohydrates. As a check against contamination by salt from the final Sephadex G-75 chromatography, a small amount of purified hyaluronidase was dialysed extensively against

Figure 10. Disc electrophoresis patterns obtained from A) 50 μg of highly purified hyaluronidase with an activity of 45,000 N. F. units per mg. B) 300 μg "highly purified" hyaluronidase prepared by the method of Soru and Ionescu-Stoian (11,12) with an activity of 3,450 N. F. units per mg.

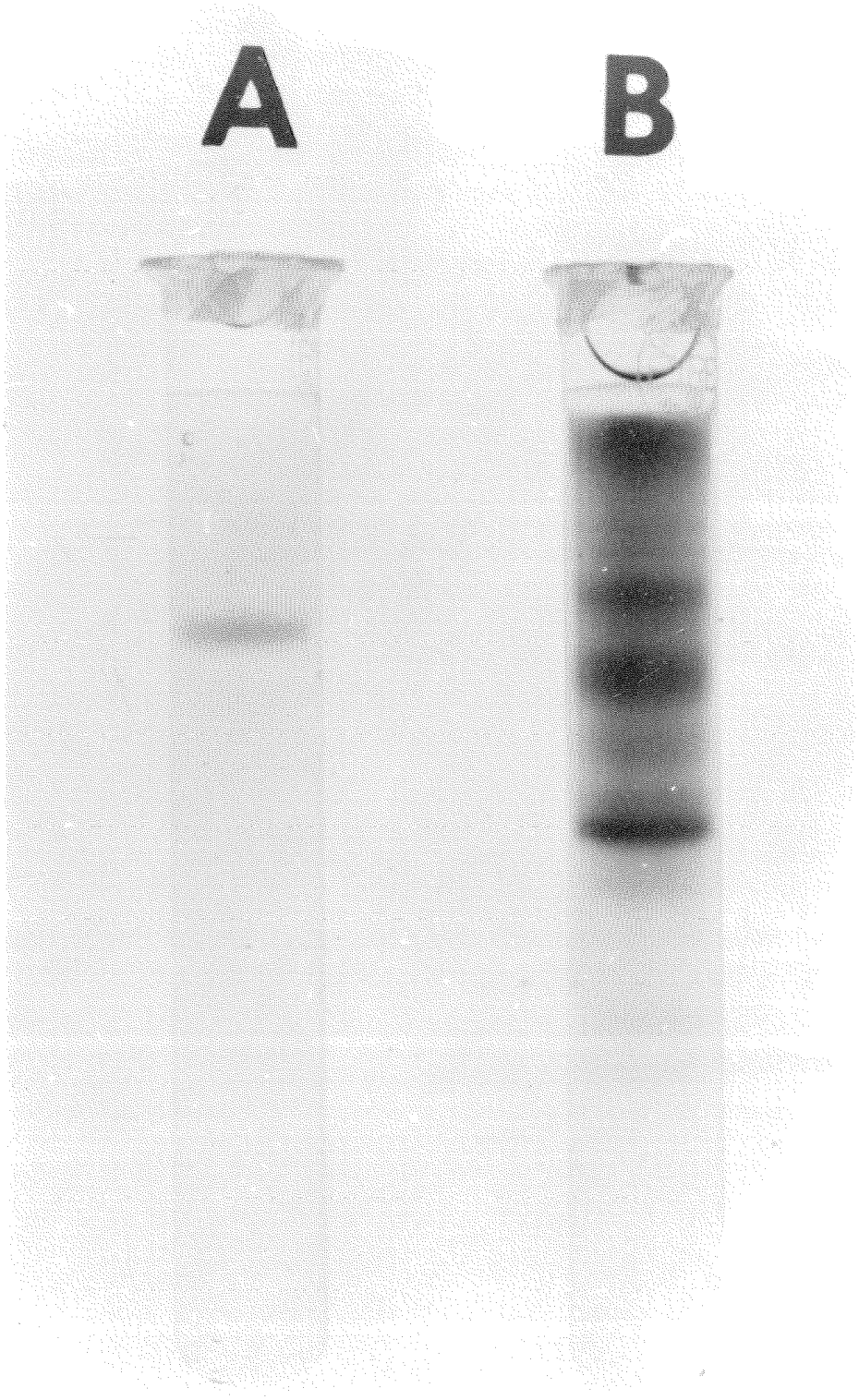

A
B

Table II. Amino Acid and Carbohydrate Composition of Testicular Hyaluronidase.

The 20- and 70-hour data are the average of 3 and 2 runs, respectively. Valine, isoleucine, leucine, serine, threonine, tryptophan, glucosamine, and mannose were determined as described in the text. Ammonia data was not corrected for contribution due to destruction of glucosamine.

Residue	Hydrolysis data (mμmole)		Average or extrapolated value	mmole residue/ 100 g enzyme	g residue/ 100 g enzyme	Brunish and Högberg(10)
	20 hour	70 hour				
Lys	25.9 ± 1.9	25.7 ± 0.4	25.8	37.2	4.77	4.21
His	9.3 ± 0.8	8.8	9.2	13.3	1.82	1.51
Arg	19.5 ± 1.6	19.3 ± 0.7	19.4	28.0	4.37	3.39
Asp	47.1 ± 2.5	47.3 ± 0.6	47.2	68.0	7.83	6.78
Thr	22.6 ± 0.6	22.0 ± 0.8	22.8	32.9	3.34	2.84
Ser	33.1 ± 1.3	28.7 ± 0.3	34.9	50.3	4.38	3.74
Glu	34.8 ± 0.8	35.9 ± 0.3	35.2	50.7	6.55	6.36
Pro	25.7 ± 1.9	25.4 ± 0.4	25.6	36.9	3.58	3.27
Gly	25.4 ± 1.1	26.6 ± 0.3	25.9	37.3	2.13	2.09
Ala	24.3 ± 0.6	24.3 ± 0.7	24.3	35.0	2.49	2.55
Cys/2	12.7 ± 2.3	13.0 ± 1.4	12.9	18.6	1.89	3.12
Val	29.2 ± 0.3	30.6 ± 0.2	30.6	44.1	4.37	4.20
Met	5.7 ± 1.0	5.1 ± 0.5	5.5	7.9	1.04	0.64
Ile	15.1 ± 0.7	17.6 ± 0.3	17.6	25.4	2.87	2.73
Leu	37.7 ± 0.4	37.5 ± 0.4	37.6	54.2	6.13	5.66
Tyr	17.1 ± 0.4	16.6 ± 0.3	16.9	24.4	3.98	3.22
Phe	17.1 ± 1.5	16.9 ± 1.0	17.0	24.5	3.61	3.29
NH_3	65.8 ± 4.0		65.8			
Try			(13.0)	(18.7)	3.48	2.43
Glucosamine (as N-acetylglucosamine)			(7.4)	(10.7)	2.17	
Mannose . . .			(21.4)	(30.8)	5.00	
Hexosamine·HCl						5.0
Neutral Sugars						5.2
					75.80 g	

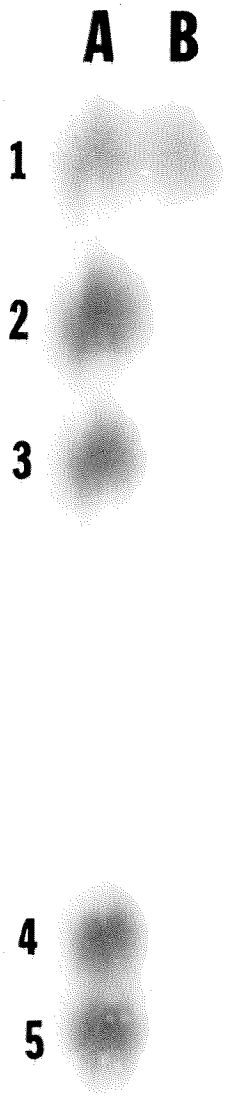
distilled water at 4° at a concentration of one mg per ml, without precipitation or loss of activity. Amino acid and sugar analysis of this dialysed enzyme again showed that only about 75% of its weight could be accounted for as amino acids and carbohydrates. It should be pointed out that the data were not corrected for moisture or ash (salts) in the material, or for the destruction of amino acid residues during hydrolysis under the influence of carbohydrates present in hydrolysis mixtures.

In comparing analyses obtained for the preparation described in this work with those of Brunish and Högberg, the largest discrepancies lie in the data for cystine/2 and glucosamine. These investigators found 3.12% cystine/2, while a value of 1.89% was found in the present study. To check this value, cystine plus cysteine were determined as carboxymethylcysteine after reduction and carboxymethylation by the method of Crestfield et al. (23). This gave a value for cystine plus cysteine which was within 2% of the value previously determined in the present study. It may be pointed out that the amino acid data of Brunish and Högberg were based on a single determination, and this may account for the observed difference.

Brunish and Högberg reported a value of 5.0% for the amino sugar content of hyaluronidase, as determined by wet chemical methods, while, in this work, glucosamine was found to be present in the amount of 2.17% (assuming it to be N-acetylglucosamine in the intact enzyme). This latter value was obtained by two different methods: (a) by determination after hydrolysis of the hyaluronidase in 1 N HCl; and (b) by extrapolation of the 6 N HCl hydrolyses data (20- and 70-hour hydrolysis times) to zero hydrolysis time. These different methods gave the same value within ± 5%. The absence of galactosamine and mannosamine was confirmed by the chromatographic analyses.

Brunish and Högberg reported a value of 5.2% for the neutral sugar content of their preparation. As shown in Figure 11, only mannose was detected in the preparation described in this work, and in the amount of 5.0%. The paper chromatogram depicted in Figure 11 indicates the absence of uronic acids, pentoses, and methylpentoses. For this reason, as well as the small amount of purified hyaluronidase available, no further tests for these sugars were performed.

Figure 11. Qualitative determination of the neutral sugars present in purified hyaluronidase by paper chromatography. A) Standard mixture of sugars: 1) mannose; 2) glucose; 3) galactose; 4) mannosamine; 5) glucosamine. B) Neutral sugars present in purified hyaluronidase; only mannose is observable.


A
B
1
2
3
4
5

5. Molecular Weight.

The elution pattern obtained from a mixture of 0.012 mg purified hyaluronidase plus 3 mg each of BSA, ovalbumin, and ribonuclease A, 1.5 mg chymotrypsinogen, and a trace of Blue Dextran when chromatographed on a Sephadex G-100 column (1.5 x 91.5 cm) is shown in Figure 12. The hyaluronidase was eluted immediately following the serum albumin, and when V_e *vs* log (molecular weight) was plotted for the standard proteins in the mixture (Figure 13), the molecular weight of the purified hyaluronidase was estimated as 61,000. In a separate determination using 0.24 mg purified hyaluronidase with the standard proteins described above, the molecular weight was again determined to be 61,000.

In contrast to the elution pattern of the purified hyaluronidase, when crude commercial hyaluronidase was run on the same column and located by activity measurements, its molecular weight was determined to be 126,000. This difference in molecular weight may reflect the presence of a "carrier" protein to which hyaluronidase has been reported to be attached (8). The observed difference could reflect the existence of hyaluronidase as a dimer in the crude testicular

Figure 12. Gel filtration pattern of A) Blue Dextran, B) BSA, C) Ovalbumin, D) Chymotrypsinogen, E) Ribonuclease, and purified hyaluronidase on Sephadex G-100.

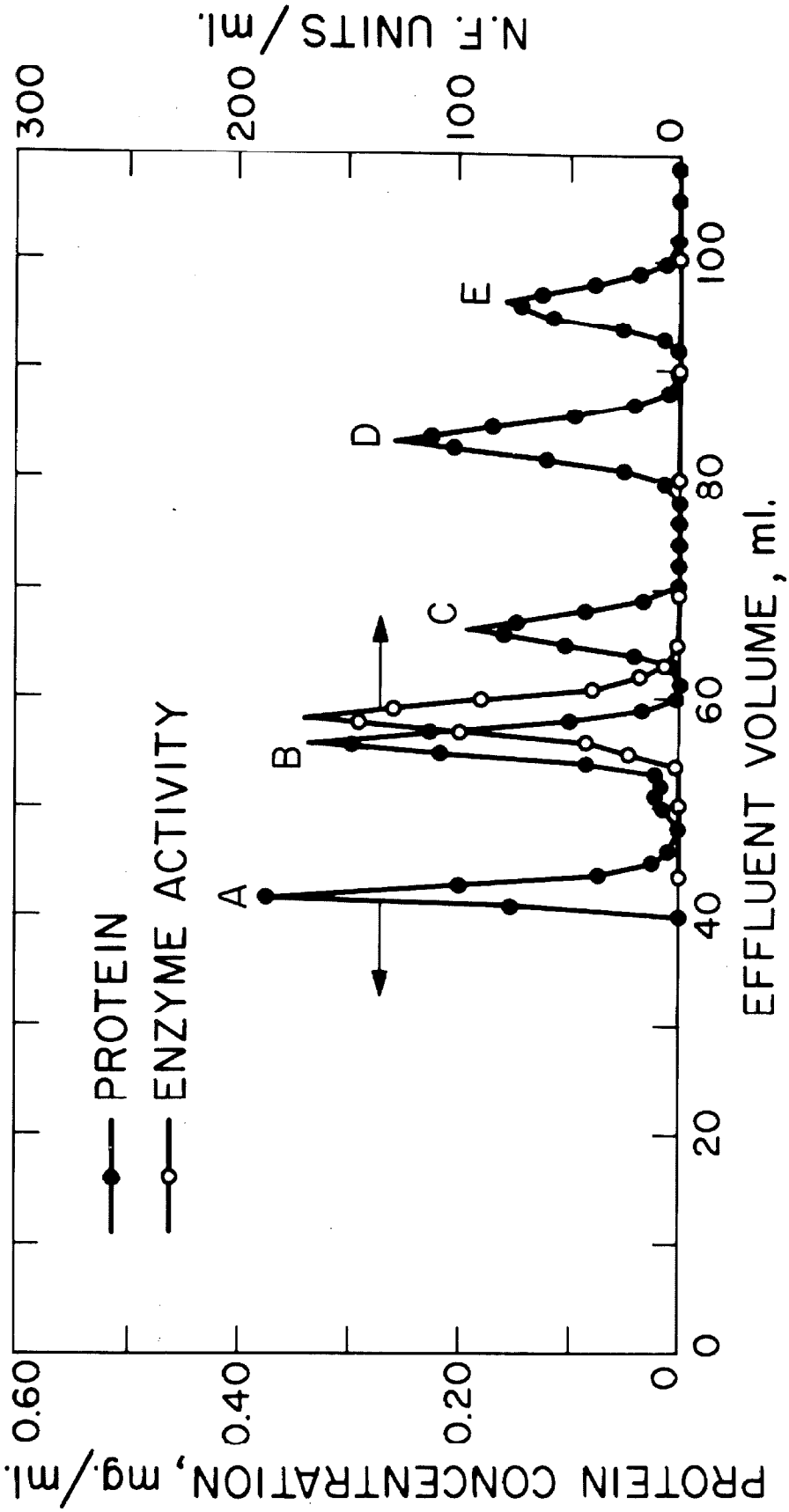
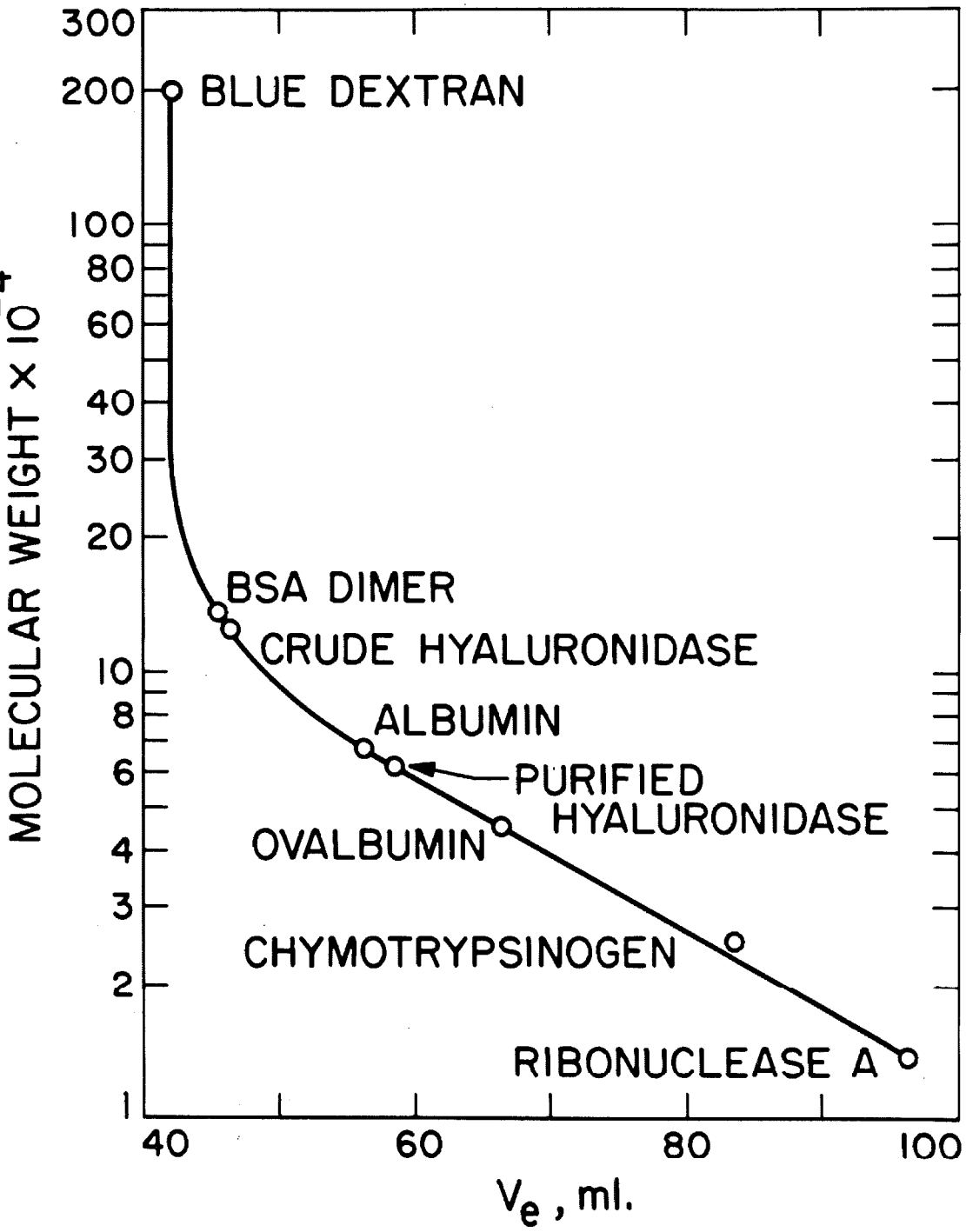

PROTEIN CONCENTRATION, mg./ml.
0.60
0.40
0.20
0
N.F. UNITS / ml.
300
200
100
0
EFFLUENT VOLUME, ml.
0
20
40
60
80
100
PROTEIN
ENZYME ACTIVITY
A
B
C
D
E

Figure 13. Molecular weight determination of crude hyaluronidase and of highly purified hyaluronidase by gel filtration on Sephadex G-100 columns.


300
200
100
80
60
40
30
20
10
8
6
4
3
2
1
MOLECULAR WEIGHT X 10^{-4}
BLUE DEXTRAN
BSA DIMER
CRUDE HYALURONIDASE
ALBUMIN
PURIFIED
HYALURONIDASE
OVALBUMIN
CHYMOTRYPSINOGEN
RIBONUCLEASE A
40
60
80
100
V_e, ml.

extract. It is most likely that the Bio-Rex 70 chromatography step in the purification procedure was responsible for the lower molecular weight observed for the purified enzyme, since hyaluronidase which had been partially purified by Bio-Rex 70 chromatography and the first Sephadex G-75 chromatography was shown to yield a peak of enzymatic activity on the Sephadex G-100 column which corresponded to a molecular weight of 61,000. The Bio-Rex 70 step could lower the molecular weight of the hyaluronidase by removing a "carrier" protein, or it could do so by removal of a cofactor, such as a divalent cation, which might be necessary for the dimerization.

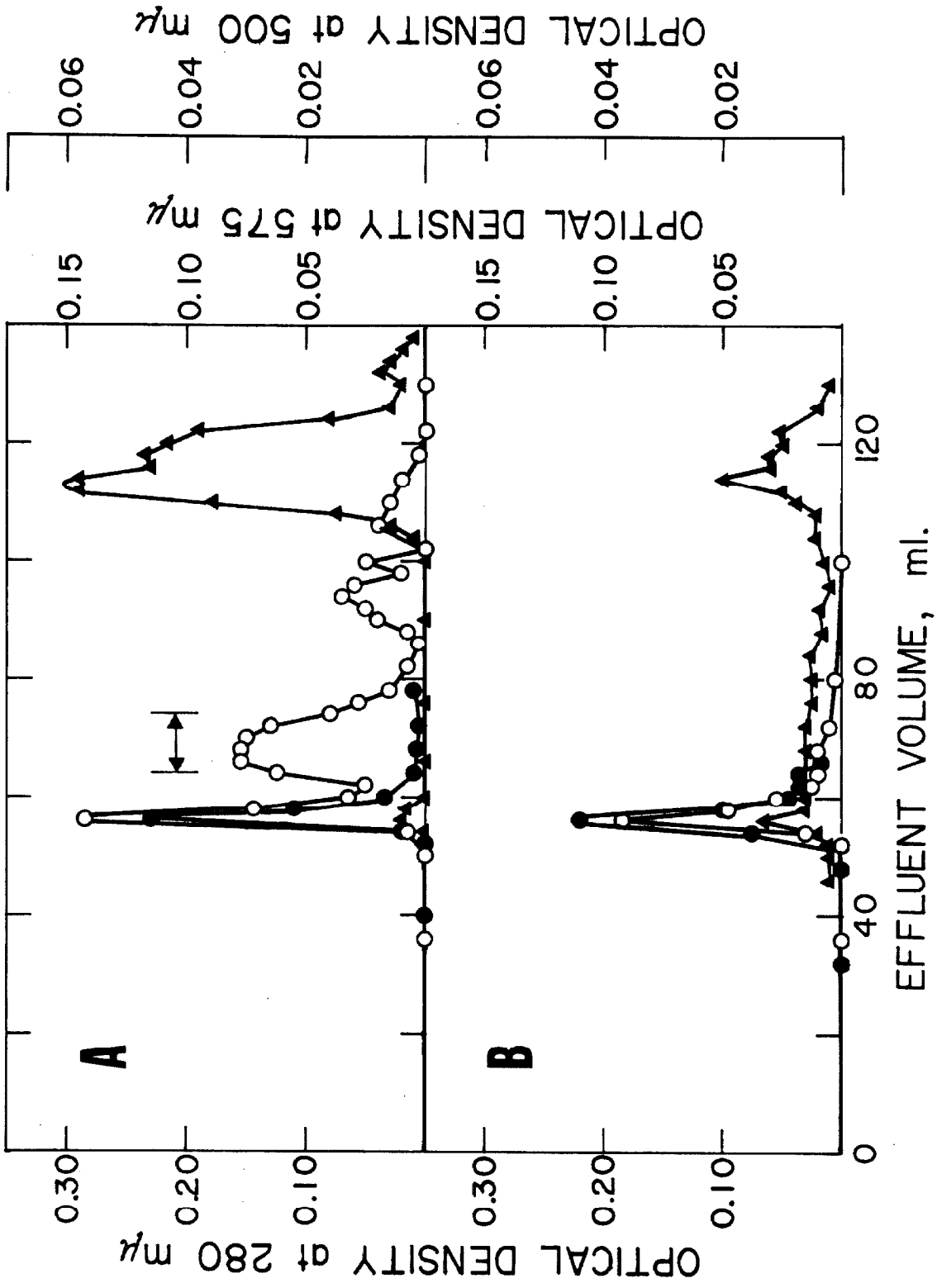
6. Studies on the Glycopeptide From Purified Testicular Hyaluronidase.

Since hyaluronidase was shown to be a glycoprotein in this work, it was thought that an investigation into the make-up of the carbohydrate moiety and the determination of the amino acid residue involved in the protein-carbohydrate linkage would be enlightening. It was also hoped to determine the size of the carbohydrate moiety and, if possible, the number of such components attached to the protein. The best method of degrading the polypeptide portion of hyaluronidase while keeping the carbohydrate portion intact

seemed to be the use of some combination of proteolytic enzymes for the degradation. This would enable the peptide bonds of the protein backbone of hyaluronidase to be cleaved while leaving the protein-carbohydrate linkage intact, as well as the carbohydrate portion.

In order to degrade the polypeptide portion of hyaluronidase, the enzyme was first reduced and carboxymethylated by the method of Crestfield et al. (23). The reduced carboxymethylated enzyme was then subjected to total enzymatic hydrolysis by consecutive degradation by the protease, subtilisin, and then by the peptidase, aminopeptidase M. The enzymatically degraded hyaluronidase was then chromatographed on Sephadex G-25 which had been equilibrated with 0.1 M NaCl, and the elution diagram was as shown in Figure 14a. When a blank of the proteolytic enzymes used for hydrolysis, less the hyaluronidase, was chromatographed on the same column, the elution diagram was as shown in Figure 14b. From a comparison of Figure 14a with Figure 14b, it can be seen that a carbohydrate component was present in the hyaluronidase digest which was eluted after the proteolytic enzymes in this chromatographic system. It can be seen that the carbohydrate material was relatively free of ninhydrin-positive and UV-adsorbing

Figure 14. A) Chromatography of enzymatically degraded hyaluronidase on Sephadex G-25 in 0.1 M NaCl solution. The fractions were checked for protein (—●—) by determining the optidensity at 280 mμ, for carbohydrate (—○—) by the orcinol method (OD_{500}), and for amino acids (—▲—) by the ninhydrin method (OD_{575}).
B) Chromatography of blank containing proteolytic enzymes, less the hyaluronidase, on Sephadex G-25 in 0.1 M NaCl solution.


OPTICAL DENSITY at 280 mμ
OPTICAL DENSITY at 575 mμ
OPTICAL DENSITY at 500 mμ
A
B
EFFLUENT VOLUME, ml.
0
40
80
120
0.10
0.20
0.30
0.05
0.10
0.15
0.02
0.04
0.06

species, and is thus most likely the glycopeptide from purified hyaluronidase.

The Sephadex G-25 column was standardized for molecular weight determinations by using the disaccharide, tetrasaccharide, and hexasaccharide of hyaluronic acid. On comparing the elution volume of the glycopeptide with the standard saccharides (Figure 15), it was determined that the glycopeptide had a molecular weight of approximately 1,600.

When the glycopeptide was analysed for amino acid and carbohydrate composition, the results shown in Table III were obtained. From these data, along with the value of 1,600 for the molecular weight as determined above, the composition of the glycopeptide can be formulated as $(\text{mannose})_7(\text{glucosamine})_2(\text{asp})_1$. The molecular weight of such a glycopeptide, assuming the glucosamine to be present as N-acetylglucosamine, would be 1,673 - a value which is in good agreement with the experimentally determined value of 1,600.

It was attempted to further purify the glycopeptide by rechromatography on Sephadex G-25 which had been equilibrated with 2% (v/v) acetic acid at 4° C. The fractions were checked only for carbohydrate, and again one major component was present. After standard-

Figure 15. Molecular Weight determination of the glycopeptide of purified hyaluronidase by gel filtration on Sephadex G-25.

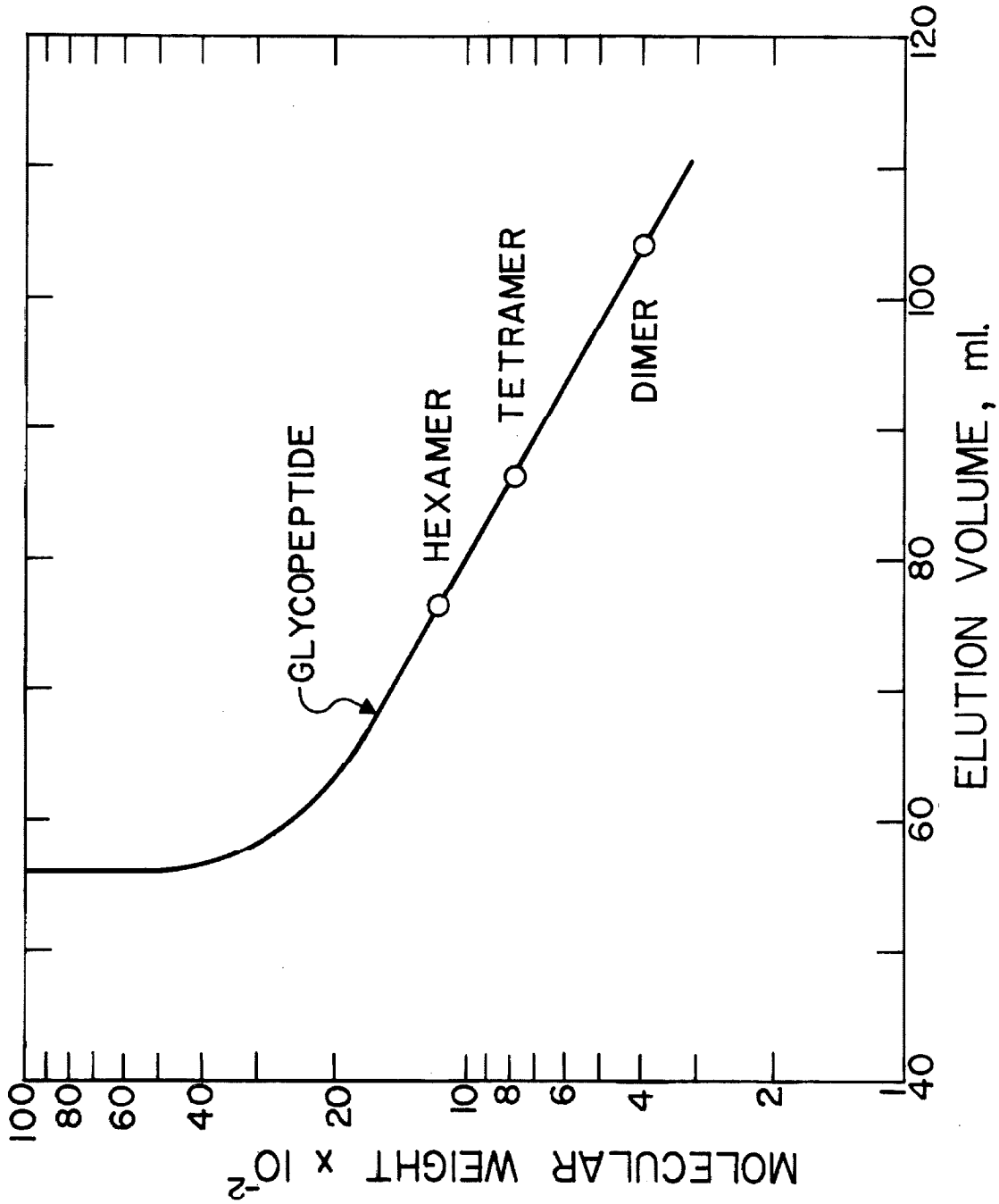

MOLECULAR WEIGHT x 10^{-2}
100
80
60
40
20
10
8
6
4
2
1
40
60
80
100
120
ELUTION VOLUME, ml.
GLYCOPEPTIDE
HEXAMER
TETRAMER
DIMER

Table III. Composition of the Glycopeptide from Purified Testicular Hyaluronidase.

The amino sugar and amino acid values were determined on the amino acid analyzer after hydrolysis with 1 N HCl as described in the text. Mannose was determined by the orcinol procedure as described in the text. Concentrations of each residue are expressed in m mole per ml.

Residue	Glyco-peptide	Blank	Corrected Glyco-peptide	Relative Ratio*
Mannose	82.1	29.4	52.7	7.4
Glucosamine	14.3	0.0	14.3	2.0
Asp	8.3	2.0	6.3	0.9
Thr	3.0	0.5	2.5	0.3
Ser	3.4	1.0	2.4	0.3
Gly	2.3	1.6	0.7	0.1
Ala	1.6	0.8	0.8	0.1
Val	3.8	1.3	2.5	0.3
Leu	1.9	0.0	1.9	0.2

*Based on a value of 2.0 for glucosamine.

izing the column for molecular weight determination, using the oligosaccharides of hyaluronic acid as outlined above, it was found that the carbohydrate component again had a molecular weight of approximately 1,600 - in good agreement with the value determined previously. When this repurified glycopeptide was again analysed for amino acid and amino sugar composition after acid hydrolysis, results very similar to those in Table III were obtained. The ratio of glucosamine to aspartic acid in this case was 2.0/1.1, and there were lesser amounts (about 1/3 or less of the aspartic acid value) of threonine, serine, and valine, and trace amounts of other amino acids as shown in Table III.

To date only three amino acid residues have been implicated in protein-carbohydrate linkages in different glycoproteins (24). Aspartic acid was found to form the linkage of protein to carbohydrate in egg albumin and α_1-acid glycoprotein, and threonine and serine were found to be involved in such linkages in submaxillary gland glycoproteins. It has been shown that in the case of egg albumin, an N-acetylglucosamine residue at the reducing end of a heterosaccharide is linked through its reducing group to the amide

nitrogen of an asparaginyl residue (25). This is also the type of linkage which is most likely present in α_1-acid glycoprotein (26). Carubelli *et al.* (27) have shown that in ovine submaxillary gland glycoprotein the predominant type of linkage between the carbohydrate group and the polypeptide backbone is an O-glycosidic linkage involving the reducing group of an N-acetyl-galactosamine and the hydroxyl group of serine and threonine residues.

From the analytical data on the glycopeptide from testicular hyaluronidase, it is reasonable to infer that the amino acid involved in the protein-carbohydrate linkage in this enzyme is an aspartic acid residue. Because of the small amount of material available, further studies on the glycopeptide were not carried out. But, if there is any analogy between this study and previous studies on glycoproteins (24-26), it is most likely that the linkage involves a glycosidic-type bond between the amide nitrogen of an asparaginyl residue and the anomeric carbon of a carbohydrate residue at the reducing end of a heterosaccharide - possibly a glucosamine residue, since all previous studies in this area have indicated an amino sugar residue in such a linkage.

The presence of a significant amount of threonine and serine in the amino acid analyses of the glycopeptide is of some concern. However, in a comparison of the amino acid composition of acid-hydrolyzed hyaluronidase with that arrived at after total enzymatic hydrolysis of reduced, carboxymethylated hyaluronidase, the threonine values are the same, on a relative basis, in both cases. A comparison of the values obtained for serine by these two different hydrolysis methods is complicated by the fact that, in the chromatographic system used, serine was eluted along with asparagine and glutamine. But at least these results tend to further rule out threonine as a linking residue.

In order to determine the number of heterosaccharide groups per enzyme molecule, the fact that only 76% of the weight of the enzyme could be accounted for by amino acids and sugars must be resolved. In the case where 76% of the weight of hyaluronidase could be accounted for, the amount of mannose plus N-acetylglucosamine = 7.17%. If the enzyme is considered to contain three heterosaccharide groups with the composition $(\text{mannose})_7(\text{N-acetylglucosamine})_2$, then the following carbohydrate composition could be expected:

$$\frac{3 \times 1,560}{61,000} \times 100 = 7.67\%$$

This value would be in line with the fact that three heterosaccharide groups were present per molecule of molecular weight 61,000.

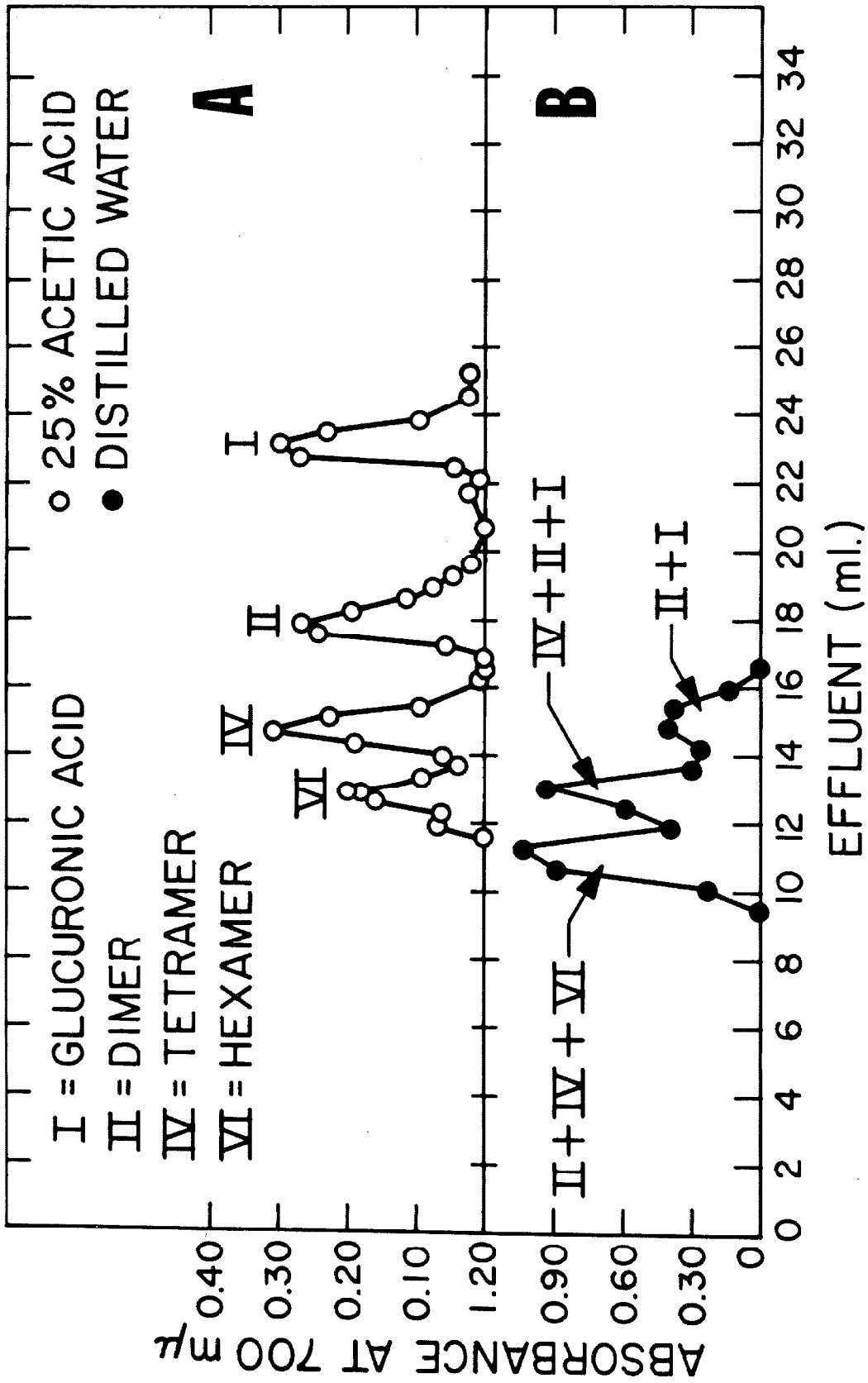
However, if the value of 76% by weight of amino acids and sugars is considered to be an artifact, and if, in fact, the analyses determined in this laboratory represented 100% of the weight of the enzyme, then a value of four heterosaccharide groups per molecule of molecular weight 61,000 would be consistent.

7. Chromatography of Hyaluronic Acid Oligosaccharides on Bio-Gel P-2.

For several years, Bio-Gel P-2 has been used in this laboratory to separate the oligosaccharides of chitin on a preparative scale (28). Since this technique separates these molecules on the basis of size, it was thought that such a technique might prove useful in the preparation of the oligosaccharides of hyaluronic acid. These oligosaccharides had previously been separated by ion exchange chromatography on Dowex 1 (29).

The behavior of the disaccharide, tetrasaccharide, and hexasaccharide of hyaluronic acid on chromatography on Bio-Gel P-2 was dependent on the eluant used (Figure 16a & b). When distilled water was used as

Figure 16. Chromatography of glucuronic acid and the disaccharide, tetrasaccharide, and hexasaccharide of hyaluronic acid on Bio-Gel P-2 in A) 25% acetic acid, and B) distilled water.


I = GLUCURONIC ACID
II = DIMER
IV = TETRAMER
VI = HEXAMER
o 25% ACETIC ACID
● DISTILLED WATER
A
B
I
II
IV
VI
IV+II+I
II+I
II+IV+VI
ABSORBANCE AT 700 mμ
0.40
0.30
0.20
0.10
1.20
0.90
0.60
0.30
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
EFFLUENT (ml.)

eluant, there was very little separation observed (Figure 16b). The sugars were apparently prevented from entering the gel pores and were eluted near the void volume of the column. However, when 25% acetic acid was used as eluant, there was very good separation of the oligosaccharides (Figure 16a).

These results can be explained if it is assumed that the repulsion of the oligosaccharides by the gel in distilled water was due to repulsion of the carboxyl groups of the glucuronic acid moieties of the oligosaccharides by any free carboxyl groups present in the gel. In the presence of 25% acetic acid this repulsion was minimized and penetration of the oligosaccharides into the gel pores was observed, with the result that the oligosaccharides were separated from one another.

8. Discussion.

One of the main advantages of the purification procedure for hyaluronidase described in this work is that the handling of the purified protein at various stages was kept at a minimum. It is known that dialysis of highly purified hyaluronidase against distilled water at low concentrations of enzyme causes considerable denaturation (10). In the procedure described in this work, dialysis of highly purified hyaluronidase

was avoided, and thus losses by denaturation were minimized.

Although the specific activity, amino acid composition and carbohydrate composition of the purified hyaluronidase are not grossly inconsistent with those reported by Brunish and Högberg for their preparation (10), they are in disagreement with those reported by Soru and Ionescu-Stoian for their preparation (11-13). Since the preparation described here had a much higher specific activity than that of Soru and Ionescu-Stoian and migrated as a single component in polyacrylamide gel electrophoresis while such electrophoresis of a sample prepared by the method of Soru and Ionescu-Stoian showed heterogeneity, it is obvious that the preparation described in this dissertation is more representative of the native enzyme in highly purified form.

Attempts were made to use the method of Högberg (9) as the preliminary step of the purification procedure. However, hyaluronidase with a specific activity of only 3,000 N. F. units per mg was obtained. Brunish and Högberg reported a preparation obtained by this method to have a specific activity of about 20,000 i. u. per mg (10). The reason for this discrepancy between

their findings and those reported here are not known. Since only 40% of the hyaluronidase activity was recovered by the method of Högberg (9) in this laboratory, while 65% recovery was attained using the method of Rasmussen (21), the latter was employed as the preliminary step. The material obtained by the method of Högberg (9) was not tested by Sephadex G-75 chromatography.

There has been disagreement in the literature regarding the molecular weight of hyaluronidase. In studying a sample prepared by the method of Högberg, Malmgren (8) reported a molecular weight of 11,000, based upon sedimentation velocity and diffusion data, and a value of 14,000 using the Archibald principle of approach to sedimentation equilibrium. Brunish and Högberg, on the other hand, reported a molecular weight of 43,200 for their preparation as determined by the Archibald method and extrapolation to zero centrifugation time (10). They noted, however, that the apparent molecular weight decreased with increased time of centrifugation, and attributed this phenomenon to a heterogeneous hyaluronidase sample.

When the molecular weight of the purified hyaluronidase described in this dissertation was determined

by gel filtration techniques, a value of 61,000 was obtained. This value was independent of concentration over a 20-fold range, indicating that the concentration dependence of the molecular weight was negligible. The value for the molecular weight of the purified enzyme is in sharp contrast to the value of 126,000 obtained for the crude hyaluronidase preparation as determined by the same method. One plausible explanation of this difference between the crude and purified hyaluronidases is that in the crude extract the enzyme is associated with a carrier protein as postulated by Malmgren (8). Another explanation of this difference is that the enzyme exists as a dimer in the crude extract and as a monomer in the purified form, and that the Bio-Rex 70 chromatography removed a cofactor, possibly a divalent cation, which is necessary for the dimerization.

An unresolved aspect of this investigation is that only 76% of the weight of the enzyme could be accounted for by constituent amino acids and carbohydrates. This result does not seem artifactual since a similar result was obtained on analysis after prolonged dialysis of the purified hyaluronidase against distilled water. Brunish and Högberg were able to account for

only 75% of the weight of their preparation as amino acids and carbohydrates, although their data were based on a single determination (10). The values obtained in the present investigation were not corrected for moisture, but it seems unlikely that this preparation of hyaluronidase contained up to 25% moisture.

It is believed that the data reported in this dissertation on the glycopeptide of testicular hyaluronidase are the first,in several respects, for an enzyme molecule. There have been studies on serum glycoproteins, such as α_1-acid glycoprotein, on the albumin of hen egg, and on submaxillary gland glycoproteins (see reference 24 for a review). This is, however, the first of such studies to report the molecular weight and composition of a heterosaccharide derived from a glycoprotein enzyme, and the first to report the amino acid involved in the protein-carbohydrate linkage of such an enzyme.

The technique of determining the amino acid involved in such a linkage which was used in this work - that is, digestion with subtilisin and then with aminopeptidase M, and then chromatography on Sephadex G-25 - also represents a new approach. Previously, other workers have degraded their glycoproteins for such an analysis by using a series of proteolytic enzymes and

purifying the glycopeptide by various techniques. In most cases, the peptide moiety was heterogeneous and this made subsequent analysis and interpretation of results more difficult. The use of subtilisin and aminopeptidase M in this study was apparently sufficient to degrade hyaluronidase almost entirely to its heterosaccharide plus linking amino acid, and this technique should prove useful in other investigations.

In conclusion, the results presented in this dissertation show that testicular hyaluronidase can be isolated from crude testicular extracts as a highly purified preparation by use of a simple ion exchange step, followed by gel filtration. The resulting preparation has been shown to possess a higher specific activity than previously reported preparations. It has also been shown to have a molecular weight of 61,000, in contrast to previous conflicting reports. It has been further shown to be a glycoprotein containing three of four heterosaccharide components of composition $(\text{mannose})_7(\text{glucosamine})_2$ per molecule, and that the amino acid involved in the protein-carbohydrate linkage is aspartic acid.

Due to the small amount of purified hyaluronidase obtained from the crude testicular extracts in this study (~0.1% by weight) the studies which could be

carried out on the purified enzyme were limited. Thus, neither mechanistic studies, chemical modification studies, nor p. m. r. studies, all of which were originally planned when this work was initiated, could be carried out.

C. EXPERIMENTAL

1. Materials.

The crude testicular hyaluronidase which was used as the starting material for all studies was obtained from Sigma Chemical Company. It ranged in specific activity from 280 to 412 N. F. units per mg, with the material used in the final purification procedure (Type I, Lot #27B-1200) having an activity of 280 N. F. units per mg. Hyaluronic acid (Type III, Lot #36B-2460-1), bee venom (Grade 1, Lot #45B-1610), bovine serum albumin (Fraction V), ribonuclease (Type 1-A, bovine pancreas, 5x crystallized), Blue Dextran (B-2000), and Sephadex G-25, G-75, and G-100, all in bead form (10-40μ) were also obtained from Sigma Chemical Company. "Highly purified" hyaluronidase (Lot #HSEP 6LB, 3450 U. S. P. units per mg) and chymotrypsinogen (crystallized, salt free) were obtained from Worthington Biochemical Corporation. Bio-Rex 70 (minus 400 mesh), Bio-Gel P-2 (200-400 mesh), Dowex 1-X8, and Dowex 50 were obtained from the California Corporation for Biochemical Research. DEAE-Sephadex A-50 (medium) was obtained from Pharmacia Fine Chemicals. Egg albumin (crystallized) was purchased from Armour Chemical Company. Subtilisin (Carlsberg) was a generous gift of Prof. E. L. Smith.

Aminopeptidase M was acquired from Henley and Company, New York. National Formulary Standard hyaluronidase was obtained from the National Formulary Reference Standards section of the American Pharmaceutical Association. Oligosaccharides of hyaluronic acid were initially prepared by the method of Barker et al. (29). All other chemicals, reagent grade or the best grade available, were purchased from commercial sources.

To minimize inactivation of the purified hyaluronidase due to contact with glass (30), polyethylene test tubes were used in the preparative work and in all the assays of enzymatic activity.

2. Assay.

Hyaluronidase activity was determined turbidimetrically by a modification of the method described in the Worthington Enzymes catalogue (31). The substrate solution was made up to contain 0.40 mg hyaluronic acid per ml in 0.1 M NaH_2PO_4, 0.15 M NaCl, pH 5.3. To 0.50 ml of the substrate solution was added 0.45 ml of the pH 5.3 buffer and the mixture was preincubated at 37° C for at least 0.5 hours. Then an appropriate amount of enzyme material (0.05 - 2.0 μg) in 0.05 ml buffer was added and the mixture was incubated for 10.0 minutes at 37° C. The reaction was stopped by dilution with 9.0 ml of acid albumin solution (made up by dissolving 2.5 g

BSA in 250 ml of 0.5 M NaOAc, pH 4.2, adjusting the pH to 3.0-3.1 with conc. HCl, heating for 30 minutes at 90-95° C, allowing to cool, and then adding 0.5 M NaOAc, pH 4.2, to a total volume of 1 liter). After 10 minutes, the optical density at 420 mμ was read and the activity was expressed in N. F. units by comparison of the results with a standard curve made up by using known amounts of N. F. standard hyaluronidase.

In the early stages of this work, hyaluronidase activity was expressed in turbidity reducing units by using the formula given in the Worthington Enzymes catalogue. Subsequently, activity was expressed in N. F. units by comparison with a simultaneously run standard hyaluronidase preparation. Other common ways of expressing hyaluronidase activity are in international units (i. u.), after a method developed by the World Health Organization (32), and in U. S. P. units after a standard preparation of the United States Pharamcopoeia (33). For lack of any reason to assume otherwise, the following relationship was assumed to hold:

1 U. S. P. unit = 1 i. u. = 1 N. F. unit

It was determined in this laboratory that 1 N. F. unit $\cong$ 0.7 t. r. u. by determining the activity of a single sample by the two different methods and determining the ratio of the two activity values. All activities in

this dissertation are expressed in N. F. units. Where the activity of a sample was originally determined in t. r. u., the relationship 1 N. F. unit = 0.7 t. r. u. was used to convert the values to N. F. units.

3. Chromatography of Crude Testicular Hyaluronidase on DEAE-Sephadex.

The procedure described here is similar to that of Soru and Ionescu-Stoian (11,12). Crude hyaluronidase (2.0 g, 360 N. F. units per mg) was dissolved in 30 ml of 0.02 M NaH_2PO_4, 0.056 M NaCl, pH 6.0, and applied to a column (3.4 x 30 cm) of DEAE-Sephadex A-50 which had been equilibrated with the same buffer at 4° C. The flow rate was adjusted to 50 ml per hour and 10 ml fractions were collected. Protein was determined by the method of Lowry et al. (34), using as a standard hyaluronidase which had been purified by chromatography on Bio-Rex 70 after the method of Rasmussen (21). Hyaluronidase activity of random fractions was determined. It was found that most of the enzyme activity was eluted with a large amount of protein material in the void volume of the column. The first few fractions with the highest specific activity were pooled, dialysed against several changes of distilled water at 4° C, and lyophilized.

4. Chromatography of DEAE-Sephadex-Purified Hyaluronidase on Sephadex G-75 in Water.

Hyaluronidase (15 mg) which had been purified as described in the previous section was dissolved in 10 ml of 0.1 M NaCl solution and applied to a column (2.4 x 70 cm) of Sephadex G-75 which had been equilibrated with distilled water at 4° C. The flow rate was adjusted to 15 ml per hour and 5 ml fractions were collected. Protein concentration and hyaluronidase activity of the fractions were determined as described previously.

5. Bio-Rex 70 Chromatography of DEAE-Sephadex-Purified Hyaluronidase.

DEAE-Sephadex-purified hyaluronidase (20 mg) was chromatographed on a column (0.9 x 40 cm) of Bio-Rex 70, H^+ form, at 4° C after the method of Rasmussen (21). The resin was first equilibrated with 0.1 M NaH_2PO_4, pH 6.0, and the hyaluronidase was applied in 5 ml of the same buffer. The flow rate was adjusted to 2 ml per hour and the column was washed with 20 ml of the first buffer (0.1 M NaH_2PO_4, pH 6.0), and then with 0.3 M NaH_2PO_4, pH 7.7, until the hyaluronidase activity was eluted. Two ml fractions were collected, and the protein concentration and hyaluronidase activity were located as described previously.

6. Chromatography of Hyaluronidase Purified by the Method of Rasmussen on Bio-Rex 70 Using a Salt Gradient for Elution.

Hyaluronidase (50 mg) which had been purified by the method of Rasmussen (21), and which had a specific activity of about 3,000 N. F. units per mg, was dissolved in a minimal amount of 0.10 M NaH_2PO_4 buffer, pH 6.31, and applied to a column (0.9 x 20 cm) of Bio-Rex 70, H^+ form, which had been equilibrated with the same buffer at 4° C. A salt gradient was set up by using three chambers of a Varigrad apparatus, the first two chambers containing 100 ml each of 0.10 M NaH_2PO_4, pH 6.31, and the third containing 100 ml of 0.10 M NaH_2PO_4, 1.0 M NaCl, pH 6.31. The flow rate was adjusted to 28 ml per hour and 1.7 ml fractions were collected. Protein concentration and hyaluronidase activity were located as previously described.

7. Chromatography of Hyaluronidase Purified by the Method of Rasmussen on Bio-Rex 70 in 0.10 M NaH_2PO_4, 0.075 M NaCl, pH 6.31.

Hyaluronidase (21.5 mg) which had been purified by the method of Rasmussen (21), and which had a specific activity of 3,000 N. F. units per mg, was dissolved in 0.5 ml of 0.10 M NaH_2PO_4, 0.075 M NaCl, pH 6.31, and

applied to a column (0.9 x 20 cm) of Bio-Rex 70, H^+ form, which had been equilibrated with the same buffer at 4° C. The flow rate was adjusted to 30 ml per hour and 4 ml fractions were collected. Protein concentration and hyaluronidase activity were determined as previously described.

8. Chromatography of Crude Testicular Hyaluronidase on Sephadex G-75 in 0.1 M NaCl Solution.

Crude hyaluronidase (310 mg) in 10 ml of 0.1 M NaCl solution was applied to a column (2.5 x 64 cm) of Sephadex G-75 which had been equilibrated with 0.1 M NaCl at 4° C. The flow rate was adjusted to 18 ml per hour and 5 ml fractions were collected. Protein concentration and hyaluronidase activity were determined as described in previous sections.

9. Chromatography of Crude Testicular Hyaluronidase on Bio-Rex 70 by the Method of Högberg (9).

Crude hyaluronidase (2.0 g), with a specific activity of 412 N. F. units per mg, was dissolved in 60 ml of distilled water and applied to a column (0.9 x 14 cm) of Bio-Rex 70, NH_4^+ form. After the protein had been applied, the column was washed successively with distilled water (210 ml), 0.1 M NH_4OAc (150 ml), 0.1 M NH_4CH (25 ml), and then with enough KCl-HCl, pH 1.5, to elute the hyaluronidase. Ten ml fractions

were collected, and protein concentration and hyaluronidase activity were determined as described in previous sections.

10. Final Scheme for the Purification of Testicular Hyaluronidase.

The first step in the purification procedure was a modification of the method of Rasmussen (21). Crude hyaluronidase (25.88 g, 280 N. F. units per mg) was dissolved in 750 ml of 0.1 M NaH_2PO_4, pH 6.0. The pH was adjusted to 5.9 with 50% NaOH solution, and this hyaluronidase solution was applied to a column (6.0 x 43 cm) of Bio-Rex 70 (minus 400 mesh), H^+ form, which had been equilibrated with 0.1 M NaH_2PO_4, pH 6.0. The column was set up in a constant temperature enclosure at 14° C. After all the protein solution had been applied, the column was washed with 4 liters of 0.1 M NaH_2PO_4, pH 6.0, at a rate of 150-200 ml per hour. The eluting buffer was then changed to 0.3 M NaH_2PO_4, pH 7.7, and 12 ml fractions were collected. The protein concentration of each fraction, and the hyaluronidase activity of random fractions, was determined as previously described. The tubes containing hyaluronidase activity were pooled, dialysed against 6 changes of distilled water at 4° for a total of 30 hours, centri-

fuged to remove a small amount of insoluble material, and lyophilized.

The purified hyaluronidase obtained above (2.10 g) was dissolved in 50 ml of 0.1 M NaCl solution and applied to a Sephadex G-75 column (5.0 x 76 cm) which had been equilibrated with 0.1 M NaCl at 4° C. The flow rate was adjusted to 50 ml per hour and 10 ml fractions were collected. Protein concentration and hyaluronidase activity were determined and the appropriate fractions were pooled and reapplied directly to a second column (5.0 x 72 cm) of Sephadex G-75 which had also been equilibrated with 0.1 M NaCl at 4° C. Protein concentration and hyaluronidase activity were again determined and the appropriate fractions were pooled and applied to a column (5.0 x 70 cm) of Sephadex G-75 which had been equilibrated with distilled water at 4° C. The flow rate was adjusted to 30 ml per hour and 10 ml fractions were collected. The protein concentration and hyaluronidase activity were determined as previously described, and it was determined that the major protein peak was eluted just ahead of the NaCl by locating the latter with $AgNO_3$. The fractions of constant hyaluronidase activity were pooled and lyophilized in a polyethylene container to give a highly purified preparation of hyaluronidase.

11. Disc Electrophoresis.

Disc electrophoresis at pH 4.3 was carried out by the method of Reisfield et al. (22), using a 15% polyacrylamide gel, omitting all but the small-pore gel, and applying the samples in 10% sucrose solution. Electrophoresis was carried out at 4 ma per tube for 90 minutes. The gels were stained with 1% Amidoschwarz in 7% acetic acid and destained by washing with 7% acetic acid.

12. Amino Acid and Carbohydrate Analysis.

Acid hydrolysis of hyaluronidase prior to amino acid analysis was carried out with 6 N HCl for 20 and 70 hours at 108° C in evacuated sealed tubes. The amino acids were determined on an automatic amino acid analyser (Beckman Spinco Model 120B) on a 0.9 x 50 cm column. Amino sugars were determined on the same column after hydrolysis for 20 hours with 1 N HCl at 108° C in evacuated sealed tubes. In the chromatographic system used, they were eluted after phenylalanine. As a check, amino sugars were also determined by linear extrapolation of the data obtained after hydrolysis in 6 N HCl (20- and 70-hour hydrolysis times) to zero hydrolysis time. Threonine and serine were determined by plotting the data obtained after hydrolysis for 20 and 70 hours in 6 N HCl in a linear fashion and ex-

trapolating to zero hydrolysis time. Data obtained from the 70-hour hydrolyses were used to obtain values for valine, leucine, and isoleucine. The tyrosine-tryptophan ratio was determined in 0.1 M NaOH by the method of Bencze and Schmid (35). Cystine plus cysteine was also determined as carboxymethylcysteine after reduction and carboxymethylation according to the method of Crestfield et al. (23).

Neutral sugars were quantitatively determined using the orcinol test described by Hartley and Jevons (36), with mannose as standard. Neutral sugars were qualitatively identified by descending chromatography on Whatman No. 1 paper for 24 hours using ethyl acetate: pyridine:water (12:5:4) as solvent (37). They were identified by comparison with simultaneously run standards. The sugars were detected by the $AgNO_3$-NaOH method (38), with destaining and preserving by treatment with $Na_2S_2O_3$. For analysis of carbohydrates, one mg of the purified enzyme was dissolved in 1 ml of 1 N HCl and hydrolysed at 108° for 3 hours in an evacuated sealed tube. After drying, the hydrolysate was taken up in 1 ml water and applied to a column (0.9 x 10 cm) of Dowex 50-X8, 200-400 mesh, H^+ form, eluted with water, and the first 30 ml were evaporated to dryness

on a rotary evaporator with a bath temperature of 35°. The dried neutral sugars were taken up in 2.0 ml water and a 1.0 ml aliquot was used for quantitative analysis while the remainder was used for qualitative tests.

13. Molecular Weight Determination.

Molecular weight determinations were made on Sephadex G-100 (bead form) by the method of Andrews (39). A column (1.5 x 91.5 cm) of Sephadex G-100 was equilibrated at 20° C with 0.10 M NaH_2PO_4, pH 5.3, containing 0.15 M NaCl and saturated with chloroform. This buffer was chosen to approximate the conditions of the assay used to determine hyaluronidase activity. The column was standardized with Blue Dextran, BSA, BSA dimer, ovalbumin, chymotrypsinogen, and ribonuclease A. Purified hyaluronidase was run simultaneously with the standard proteins. Fractions of 1.0 ml were collected, the standard proteins were located by reading optical densities at 280 mμ, while the hyaluronidase was located by activity measurements. Duplicate runs of different hyaluronidase concentrations were made. Crude hyaluronidase was run with the Blue Dextran marker and was located by activity measurements.

14. Studies on the Glycopeptide From Purified Testicular Hyaluronidase.

Purified hyaluronidase (5.8 mg) was reduced and

carboxymethylated by the method of Crestfield et al. (23). After washing and air-drying the reduced carboxymethylated enzyme, a total of 7.3 mg of alkylated material was isolated. About 0.4 mg of this material was hydrolyzed in 6 N HCl for 20 hours at 108° C in an evacuated sealed tube in order to compare the data with the previously determined amino acid composition of hyaluronidase.

The remaining amount of reduced carboxymethylated hyaluronidase (6.9 mg) was subjected to total enzymatic hydrolysis in the following manner. The material was added to 1.0 ml of 0.1 M NaH_2PO_4, pH 7.0, and subtilisin (0.30 mg) in 0.10 ml of the same buffer was added. A drop of toluene was added to prevent microbial growth and the mixture was incubated at 37° C until all the hyaluronidase had dissolved, and then for an additional four hours. After this time, aminopeptidase M (2.0 mg) was added as a solid and the mixture was incubated for another 20 hours. A blank was also run without the hyaluronidase being included.

At the end of the alloted time, 0.05 ml of the reaction mixture, and, separately, an equivalent amount of the blank solution, was chromatographed on the amino acid analyser. The remaining amount of the sample

solution was applied to a column (1.5 x 92 cm) of Sephadex G-25 which had been equilibrated with 0.1 M NaCl solution at 4° C. The flow rate was adjusted to 10 ml per hour and 2.0 ml fractions were collected. The fractions were checked for protein by determining the optical density at 280 mμ, for amino acids by the ninhydrin reaction (40), and for carbohydrate by the orcinol method (36). The blank was chromatographed on the same column and checked in a similar manner.

To get an estimate of the molecular size of the glycopeptide eluted from the G-25 column, the column was standardized for molecular weight determination by chromatography of the disaccharide, tetrasaccharide, and hexasaccharide of hyaluronic acid under similar conditions, locating the oligosaccharides by the ferricyanide method (41). The log (molecular weight) vs elution volume for this standard mixture was plotted, and the size of the glycopeptide from hyaluronidase was estimated using this data.

The fractions containing carbohydrate, and free of protein and most extraneous amino acids, were pooled and stored at -10° C until needed. The same fractions from the chromatogram of the blank solution were also pooled and stored at -10°. Aliquots from the sample solution and the blank solution were analysed quantita-

tively for carbohydrate by the orcinol method (36), using mannose as a standard. Aliquots of sample solution and blank solution were also evaporated to dryness and each was subjected to acid hydrolysis at 108° C in evacuated sealed tubes under two different sets of conditions; in 1 N HCl for 4 hours, and in 6 N HCl for 20 hours. Amino acid and amino sugar analyses of each sample were made on the amino acid analyser.

In an attempt to purify the glycopeptide further, the sample obtained from the G-25 column as described above was evaporated to a small volume and applied to a column (1.5 x 90 cm) of Sephadex G-25 which had been equilibrated with 2% (v/v) acetic acid at 4° C. The conditions of chromatography and the methods of analysis of the resulting chromatogram were the same as those listed above. The fractions containing the glycopeptide were pooled and lyophilized. The small amount of material which was obtained was taken up in a small aliquot of distilled water and used for amino acid analysis, as well as for amino sugar analysis, after hydrolysis in 1 N HCl for 23 hours at 108° C in an evacuated sealed tube.

15. Chromatography of Hyaluronic Acid Oligosaccharides on Bio-Gel P-2.

A mixture of glucuronic acid (70 μg), and the disaccharide (110 μg), tetrasaccharide (130 μg), and hexasaccharide (160 μg) of hyaluronic acid in 0.05 ml of distilled water was applied to a column (0.5 x 93 cm) of Bio-Gel P-2 which had been equilibrated with distilled water at 25° C. The flow rate was set at 5 ml per hour and fractions of 0.59 ml were collected. Each fraction was checked for reducing sugars by the ferricyanide test (41).

When chromatography was repeated on Bio-Gel P-2 which had been equilibrated with 25% (v/v) acetic acid, the conditions were the same as above except that 0.34 ml fractions were collected.

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PART II

MECHANISTIC STUDIES OF HUMAN LYSOZYME

A. INTRODUCTION

1. History.

In 1922, Alexander Flemming (1) reported to the Royal Society of London the discovery of a "remarkable bacteriolytic element found in tissues and secretions." The substance was called "lysozyme" and the strain of gram-positive cocci found to be particularly susceptible to its lytic action was designated Micrococcus lysodeikticus. In this first paper (1), it was observed that lysozyme was widely distributed and that it was highly concentrated in hen egg white, and in human tears, saliva, sputum, and nasal secretions. Significant activity was also found in blood serum and leukocytes, and in abnormal urine "containing much albumin and pus," but not in normal urine. The apparent similarity or identity of the lysozymes of different human tissues and body fluids and their similarity to the lysozyme of egg white was indicated by the observation that organisms grown in the presence of, and resistant to, egg white lysozyme were found to be comparably resistant to the lysozyme activity of human tissues and secretions (1).

Following these initial observations, no significant progress was made in further characterizing lysozyme until Meyer and his co-workers (2) developed

a procedure for purifying the enzyme from acetone-dried egg white by precipitation with flavianic acid. They characterized the enzyme as "a basic polypeptide, having a nitrogen content of 15.3% and giving a number of protein reactions." Subsequently, Alderton et al. (3) isolated egg white lysozyme by using bentonite as an adsorbent and eluting the lysozyme with 5% pyridine adjusted to pH 5.0 with sulfuric acid. They isolated the enzyme in 85 to 90% yield by this procedure, and characterized it as a basic protein (isoelectric point between 10.5 and 11.0) of low molecular weight (about 17,000). They further demonstrated that the lysozyme could be crystallized at pH values ranging from 3.5 to 10.8. Wetter and Deutsch (4), using the method of Alderton et al. (3), prepared 7 times crystallized egg white lysozyme which was found to have an isoelectric point of 11.35 at 0.1 ionic strength, an electrophoretic mobility of $+2.90 \times 10^{-5}$ $cm^2 \cdot v^{-1} \cdot sec^{-1}$ at pH 8.6 in diethyl barbiturate buffer, and a sedimentation constant of 2.11. From the sedimentation and diffusion constants and the apparent specific volume, a molecular weight of 17,200 was calculated.

More recently, detailed studies of the amino acid composition, tryptic and chymotryptic peptides, and amino acid sequence of egg white lysozyme have been

carried out by Jollès (5-10) and by Canfield (11-14) and their associates. From these studies it is apparent that hen egg white lysozyme is a single polypeptide chain of 129 amino acid residues, with lysine at the amino-terminal position and leucine at the carboxyl-terminal position. The molecular weight of hen egg white lysozyme, based on sequence determinations, is 14,307. There are eight half-cystines (four disulside bridges) and six tryptophan residues.

The three dimensional structure of egg white lysozyme has been elucidated to 2 Å resolution by the use of X-ray crystallographic techniques (15,16). Parallel studies have been carried out in the same laboratory on the interactions between the enzyme and various inhibitors (17-19).

Because of the availability of large quantities of hen egg white lysozyme, this protein has been the most extensively studied lysozyme to date. However, there have been some studies on lysozymes isolated from other sources, including plants (20,21), rabbit, rat, and dog spleen and kidney (22,10,7), human kidney (23), placenta (24), saliva (10,25) and normal and leukemic leukocytes (10,26). These studies have been reviewed by Jollès (27). They have established certain chemical and structural similarities among different lysozymes,

including acid and heat stability, basicity with isoelectric points in the range of pH 10 to 11, and comparable substrate specificities. In addition, all lysozymes of animal origin seem to have a similar molecular weight in the range of 14,000 - 15,000.

2. The Present Study.

Recently, Osserman and Lawlor (28) have found that patients suffering from monocytic and monomyelocytic leukemia excrete a large amount of lysozyme (0.6 to 2.6 g per day) in the urine. They have isolated and purified this lysozyme and have characterized it physicochemically and immunochemically. It has a molecular weight of 14,000-15,000, as determined by ultracentrifugation techniques, and its amino acid composition agreed very closely with those determined for lysozymes from human placenta (24), saliva (25), and leukocytes (10,26). Because of these data, along with the immunochemical evidence of the identity of the leukemic urine lysozyme with the lysozyme of human tears, Osserman and Lawlor (28) stated that the same enzyme was probably present in all human tissues and secretions.

The human lysozyme is different from that of hen egg white in several respects. The amino acid compositions are clearly different, as shown in Table I.

Table I. Amino Acid Compositions of Human Lysozyme and Hen Egg White Lysozyme.

Residue	Human Lysozyme (28)	Hen Egg White Lysozyme (13)
Lys	5	6
His	1	1
Arg	12	11
Asp	17	21
Thr	5	7
Ser	6	10
Glu	10	5
Pro	3	2
Gly	11	12
Ala	13	12
Cys/2	6	8
Val	8	6
Met	2	2
Ile	5	6
Leu	8	8
Tyr	6	3
Phe	2	3
Try	(5)	6
Total	125	129

Also, it was reported (28) that the rabbit antiserum to human lysozyme failed to inhibit the activity of the hen egg white lysozyme. It was also reported (28) that human lysozyme had from 3 to 12 times the lytic activity against M. lysodeikticus when compared with the hen lysozyme.

It has been reported (29) that leukemic urine lysozyme can be crystallized in the pH range from 4.5 to 10.5. D. C. Phillips at Oxford is presently investigating the three dimensional structure of human lysozyme (30), and R. E. Canfield at the Columbia College of Physicians and Surgeons is determining its amino acid sequence (31). Thus, it is conceivable that in the near future the three dimensional structure of human lysozyme will be known on a level comparable with that of hen egg white lysozyme.

Raftery and his co-workers (32,33) have recently investigated the mechanistic pathway traversed by substrate during catalysis by hen egg white lysozyme. They have also studied the activity of hen lysozyme against selected low molecular weight substrates, among them chitotriose and chitotetraose (34), and p-nitrophenyl-β-D-chitobioside (35). Since human lysozyme could now be obtained by the method of Osserman and Lawlor (28) in larger quantities than previously

available, it was thought that a comparative study of human lysozyme and hen lysozyme might be both feasible and enlightening.

The investigation described in this dissertation was thus initiated in order to gain some insight into the similarities and differences between the catalytic mechanisms and substrate specificities of hen egg white lysozyme and human lysozyme.

B. RESULTS

1. The Anomeric Form of the Product of Human Lysozyme Hydrolysis.

Two recent reports (32,36) have indicated that hen egg white lysozyme can cleave chitobiose slowly to yield N-acetyl-D-glucosamine (and higher oligosaccharides as a result of transglycosylation). When this reaction was carried out in the presence of methanol, the product of the enzymatic cleavage was observed to partition between methanol and water (32,33), with the result that a significant amount of methyl glucosaminide was formed. Raftery and Rand-Meir (33) were able to detect only methyl-N-acetyl-β-D-glucosaminide by paper chromatography of the products of the reaction. Dahlquist and Raftery (32) studied the transglycosylation reaction in a quantitative manner by using ^{14}C labelled chitobiose as substrate, and showed β-methyl-NAG* to be present to an extent of at least 99.7% in comparison to the α-product. Since the glycosidic

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*The abbreviations used are: β-methyl-NAG, methyl-N-acetyl-β-D-glucosaminide; α-methyl-NAG, methyl-N-acetyl-α-D-glucosaminide; p-nitrophenyl-β-NAG, p-nitrophenyl-N-acetyl-β-D-glucosaminide; NAG, N-acetyl-D-glucosamine; NAGal, N-acetyl-D-galactosamine; NAM, N-acetylmuramic acid; dpm, decompositions per minute.

bond of chitobiose is known to be of the β-configuration, this indicated that the hydrolysis proceeded with retention of configuration.

In order to explore the mechanistic properties of human lysozyme and compare them with hen lysozyme, similar experiments were run using human lysozyme. Figure 1 shows a chromatogram (on a column of Bio-Gel P-2) of products of the reaction between human lysozyme and chitobiose. It can be seen that there was hydrolysis of chitobiose by human lysozyme to an extent of about 75% under the conditions of the experiment. There also seemed to be a small amount of chitotriose formed, indicating that transglycosylation also took place.

The next step was to run the reaction in a buffer containing methanol to see if partitioning between methanol and water could be observed. Figure 2 depicts a paper chromatogram of the reaction mixture after being worked up as described in the experimental section. All of the glycoside formed corresponded to β-methyl-NAG. No trace of the corresponding α-glycoside was observed.

The partitioning reaction was studied in a more quantitative manner by using ^{14}C labelled chitobiose

Figure 1. Chromatogram on a column (1.0 x 100 cm) of Bio-Gel P-2 of the products obtained from the hydrolysis of chitobiose (10 mg) by human lysozyme (10 mg) in 200 μl 0.1 M citrate, pH 5.5, for 20 hours at 40° C. Each fraction was checked for the presence of reducing sugars by the ferricyanide method (67) (OD_{700}). E = enzyme; I = NAG; II = chitobiose.

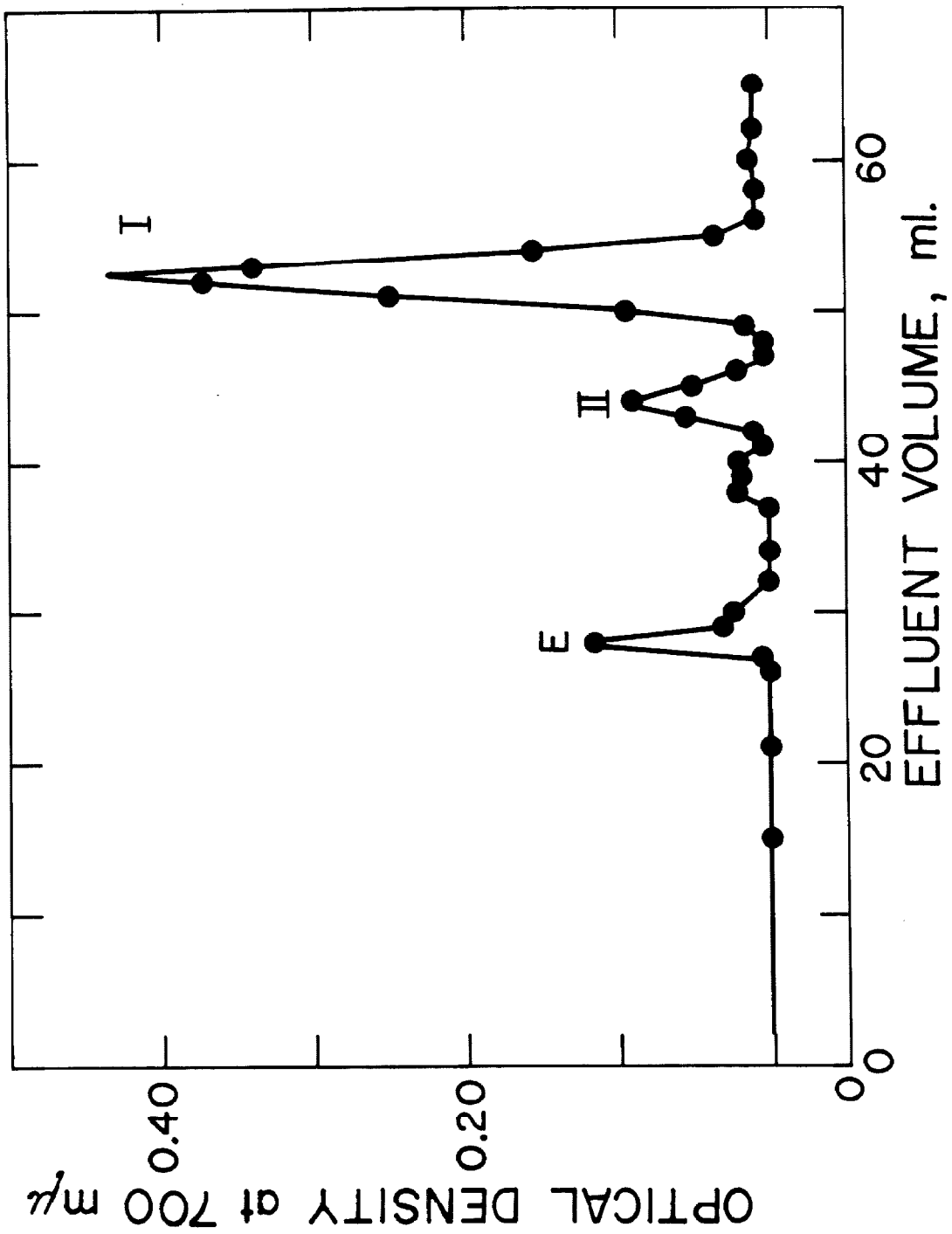
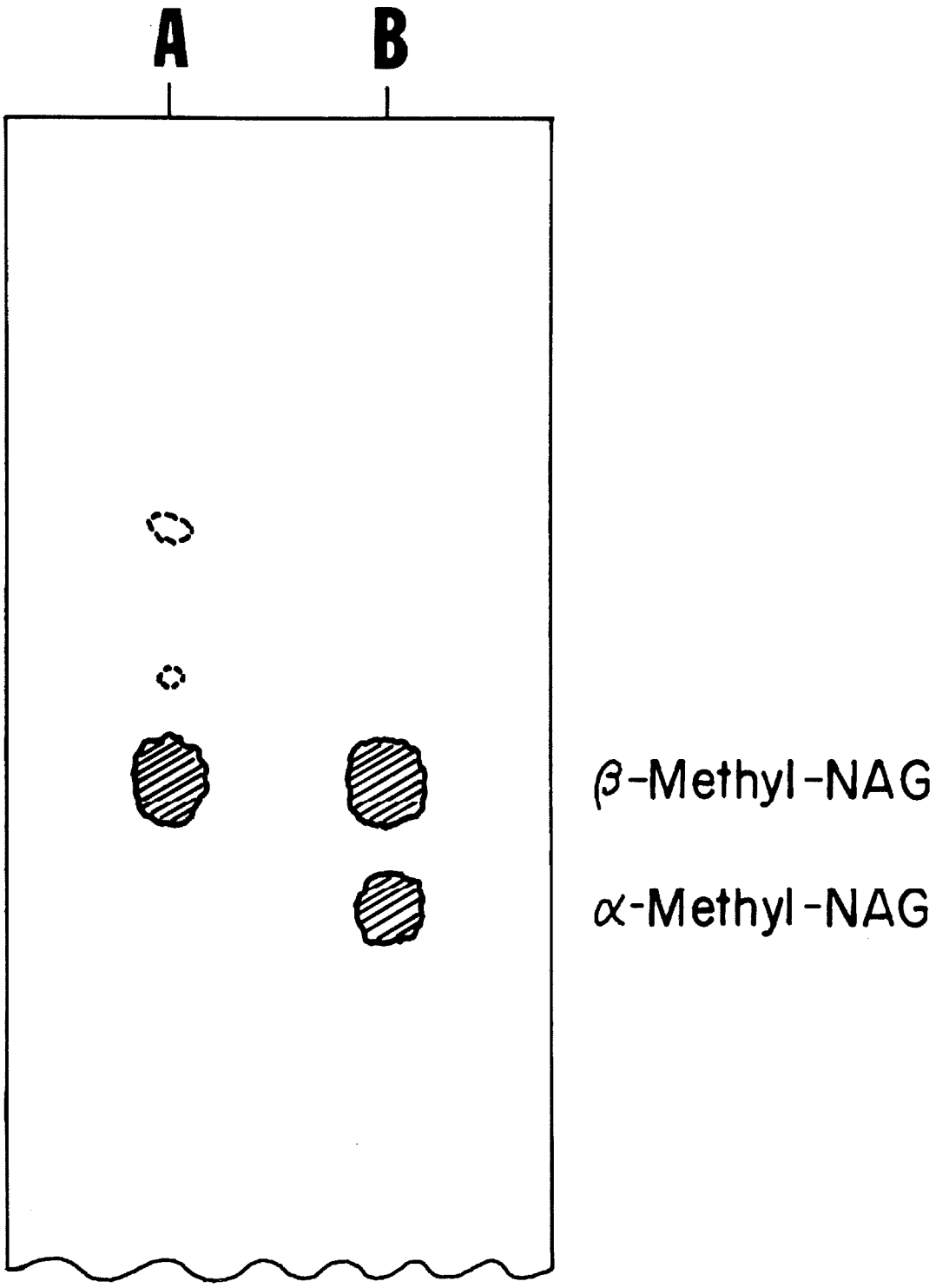

OPTICAL DENSITY at 700 mμ
0.40
0.20
0
0
20
40
60
EFFLUENT VOLUME, ml.
I
II
E

Figure 2. Tracing of paper chromatographic separation of products obtained from human lysozyme catalyzed cleavage of chitobiose in the presence of methanol. A) products of the transfer reaction; B) standard mixture of β-methyl-NAG and α-methyl-NAG.


A
B
β-Methyl-NAG
α-Methyl-NAG

and working up the reaction mixture as described in the experimental section. Figure 3 shows the results obtained. There was a small amount of a ^{14}C-containing compound which chromatographed with a R_f value almost equal to that of α-methyl-NAG. This compound was not α-methyl-NAG, however, since its peak fractions 70 and 71 did not coincide with the tritiated α-methyl-NAG carrier peak which had peak fractions 67, 68, and 69. It is possible, however, that this apparent impurity could mask a low level of α-methyl-NAG. Dahlquist and Raftery also observed a similar fast-running radioactive impurity in their study of the reaction catalyzed by hen lysozyme (32). Table II shows the $^{3}H/^{14}C$ ratios of the fractions corresponding to the β-methyl-NAG and α-methyl-NAG peaks. These figures allow a comparison of both the constancy of the ratio across each peak and also of the relative amounts of β-methyl-NAG to α-methyl-NAG formed during the transglycosylation. By comparing the $^{3}H/^{14}C$ ratio of the α-methyl-NAG to that of the β-methyl-NAG, it was found that the ^{14}C content of the α-methyl-NAG area was only 0.1% of that of the β-methyl-NAG peak. At least some, if not all, of this ^{14}C was due to the faster-running impurity. These results indicate that

Figure 3. Nomogram of radioactivity measurements of fractions eluted from the paper chromatogram employed to separate the products of human lysozyme action on chitobiose in the presence of methanol. Solid lines (———) denote ^{14}C disintegrations of residual chitobiose-N,N'-^{14}C and of products of hydrolysis and transglycosylation catalyzed by human lysozyme. Dashed lines (- - -) (enclosing shaded area) denote ^{3}H disintegrations of added β-methyl-NAG and α-methyl-NAG.

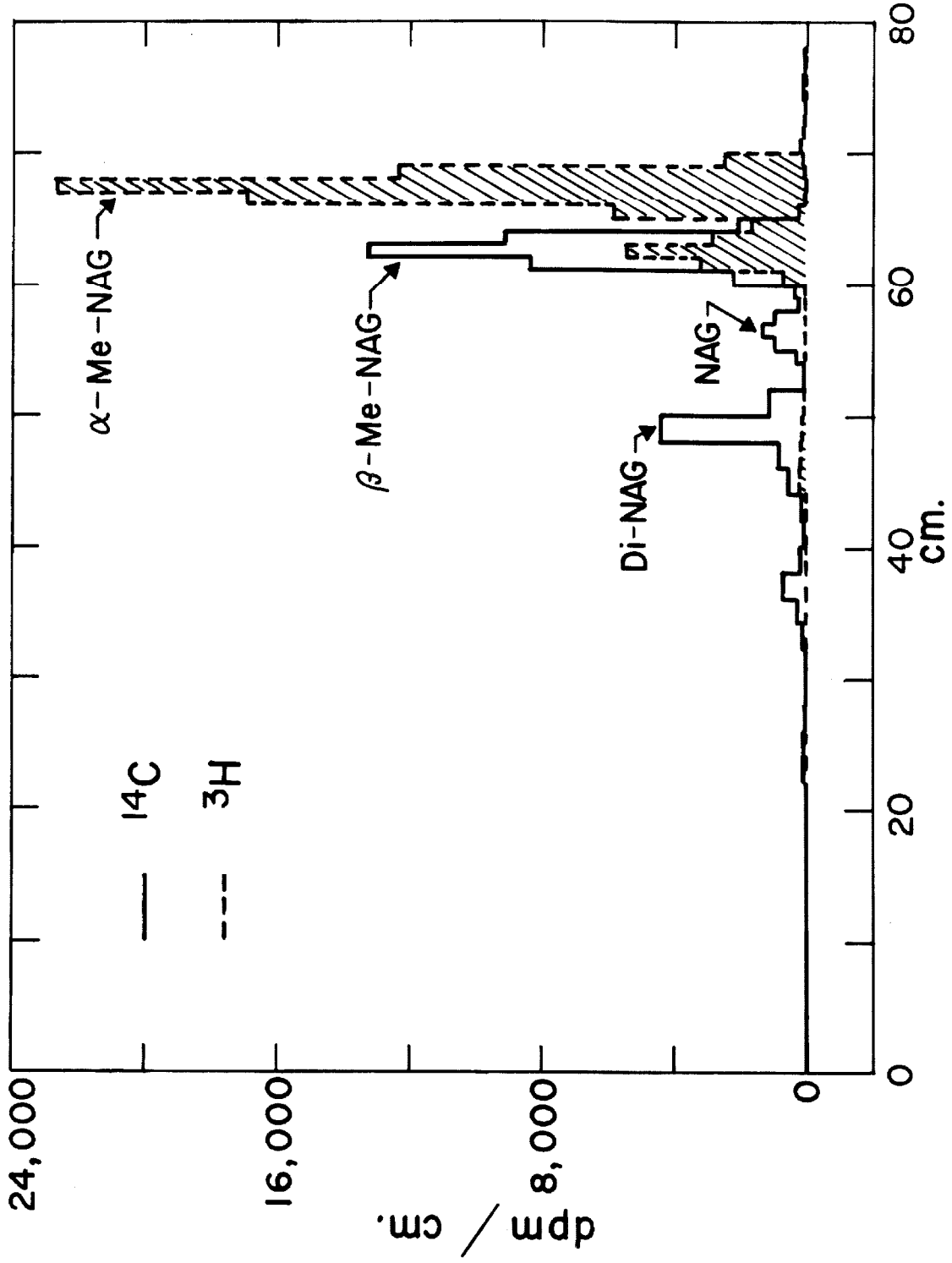

24,000
16,000
8,000
0
dpm / cm.
0
20
40
60
80
cm.
α-Me-NAG
β-Me-NAG
NAG
Di-NAG
14C
3H

Table II. Ratio of $^{3}H/^{14}C$ for the Fractions Corresponding to β-Methyl-NAG and α-Methyl-NAG.

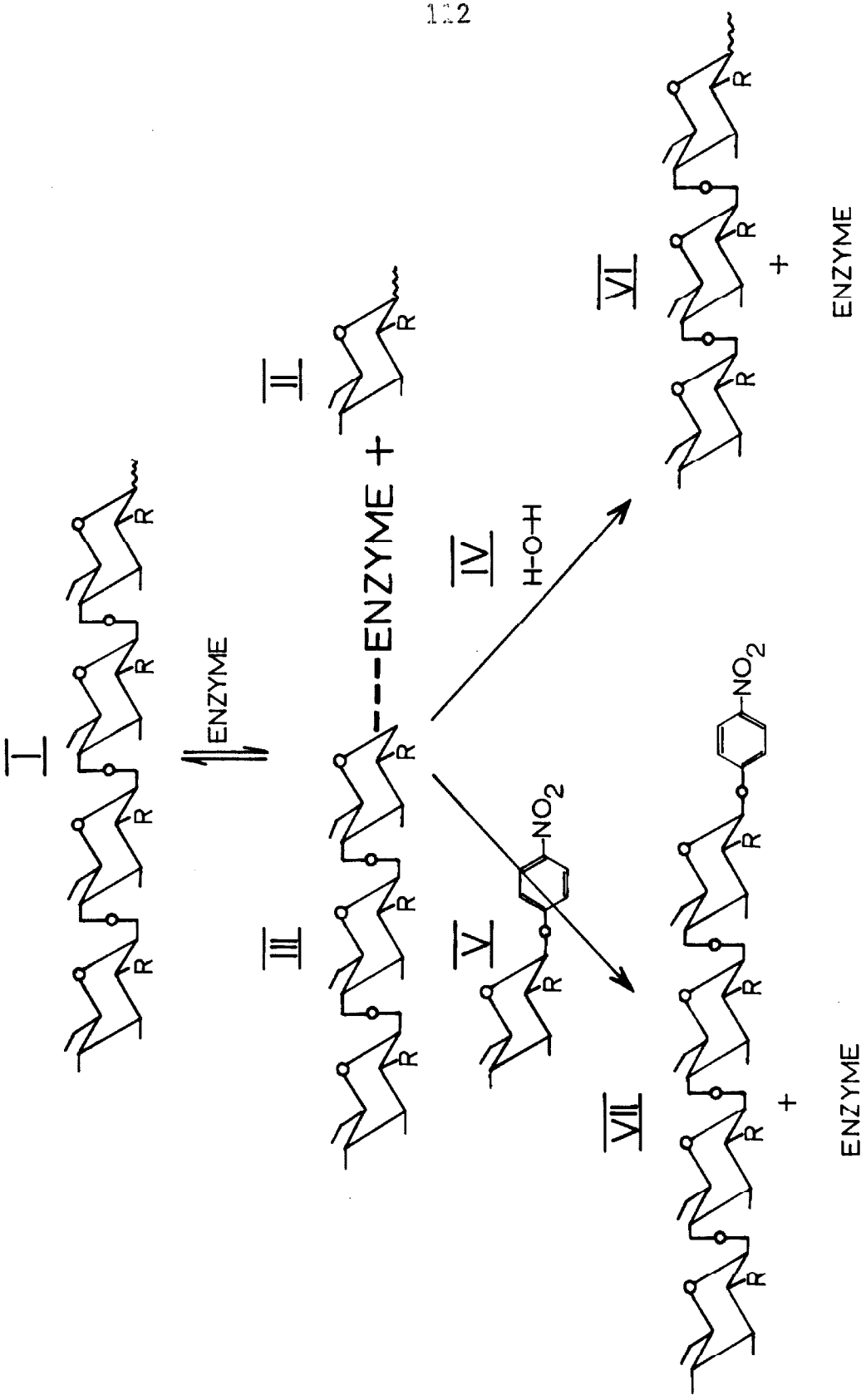
Compound	Fract. No.	$^{3}H/^{14}C$
β-Methyl-NAG	61	0.471
	62	0.498
	63	0.501
	64	0.437
α-Methyl-NAG	67	490
	68	914
	69	273
	70	25.7

the transfer reaction catalyzed by human lysozyme formed the β-product with a very high level of stereospecificity (at least 99.9%). Since the glycosidic linkage in chitobiose is β-(1→4), the reaction involved retention of configuration.

2. Human Lysozyme Catalyzed Hydrolysis of p-Nitrophenyl Glycosides in the Presence of Chitin Oligosaccharides.

It has recently been shown by Raftery and Rand-Meir (33) that when p-nitrophenyl glycosides were incubated with hen lysozyme and chitotetraose, transglycosylation occurred and oligosaccharides containing a p-nitrophenyl group were obtained. Thus it appeared that the scheme outlined in Figure 4 was serving to generate p-nitrophenyl glycosides. Whether these oligosaccharides served as substrates which released p-nitrophenol or not depended on the geometry of the sugar residue involved in the p-nitrophenyl glycoside. Since it was conceivable, from the methanol partitioning experiment, that human lysozyme is similar to hen lysozyme in its mechanistic properties, it was decided to see if this similarity extended to the transfer of p-nitrophenyl glycosides and hydrolysis of the resulting oligosaccharides to release p-nitrophenol. The results obtained when different p-nitrophenyl glycosides were

Figure 4. Scheme for the synthesis of oligomeric p-nitrophenyl glycosides from chitotetraose and p-nitrophenyl-N-acetyl-β-D-glucosaminide by human lysozyme mediated glycosyl transfer. The scheme suggests that chitotetraose (I) cleavage by human lysozyme results in formation of free NAG (II) and a chitotriose-enzyme complex (III), which can, on reaction with water (IV), give enzyme and free chitotriose (VI) or, with another acceptor such as p-nitrophenyl-N-acetyl-β-D-glucosaminide (V) give enzyme and p-nitrophenyl-β-chitotetraoside (VII). Other related products are also possible, depending on which bond in the tetrasaccharide (I) is cleaved by the enzyme.


I
ENZYME
II
---ENZYME +
III
IV
H-O-H
V
NO_2
VI
+
ENZYME
VII
NO_2
+
ENZYME

incubated with human lysozyme in the presence of chitin oligosaccharides are shown in Figure 5. It can be seen that p-nitrophenyl-β-D-glucoside, p-nitrophenyl-2-deoxy-β-D-glucoside, and p-nitrophenyl-β-NAG all yielded products which released p-nitrophenol, and it is apparent that the scheme outlined in Figure 4 was serving to generate p-nitrophenyl glycosides. However, no release of p-nitrophenol was observed when p-nitrophenyl-α-D-glucoside, p-nitrophenyl-β-D-xyloside, and p-nitrophenyl-β-NAGal were used. The relative rates of release of p-nitrophenol for the different p-nitrophenyl glycosides are given in Table III.

The results of this experiment were very similar to the results obtained with hen lysozyme (33). It can be seen that the relative rate of release of p-nitrophenol was greatest when p-nitrophenyl-2-deoxy-β-D-glucoside was used. The rate using this compound was 4 times that obtained when p-nitrophenyl-β-NAG was used, and approximately 16 times greater than that obtained using p-nitrophenyl-β-D-glucoside. These relative values compared favorably with those (8 and 16 times greater, respectively) obtained from similar studies using hen lysozyme (33).

The effect of pH on the release of p-nitrophenol from p-nitrophenyl-β-D-glucoside in the presence of

Figure 5. Time study of the human lysozyme mediated release of p-nitrophenol from mixtures of the enzyme (3.5×10^{-4} M) and chitin oligosaccharides (5×10^{-3} M) with: p-nitrophenyl-2-deoxy-β-D-glucoside (2.5×10^{-2} M), —○—; p-nitrophenyl-N-acetyl-β-D-glucosaminide (2.5×10^{-2} M), —□—; p-nitrophenyl-β-D-glucoside (2.5×10^{-2} M), —▲—; p-nitrophenyl-α-D-glucoside (2.5×10^{-2} M), —●—; p-nitrophenyl-N-acetyl-β-D-galactosaminide (2.5×10^{-2} M), —●—; and p-nitrophenyl-β-D-xyloside (2.5×10^{-2} M), —●—.

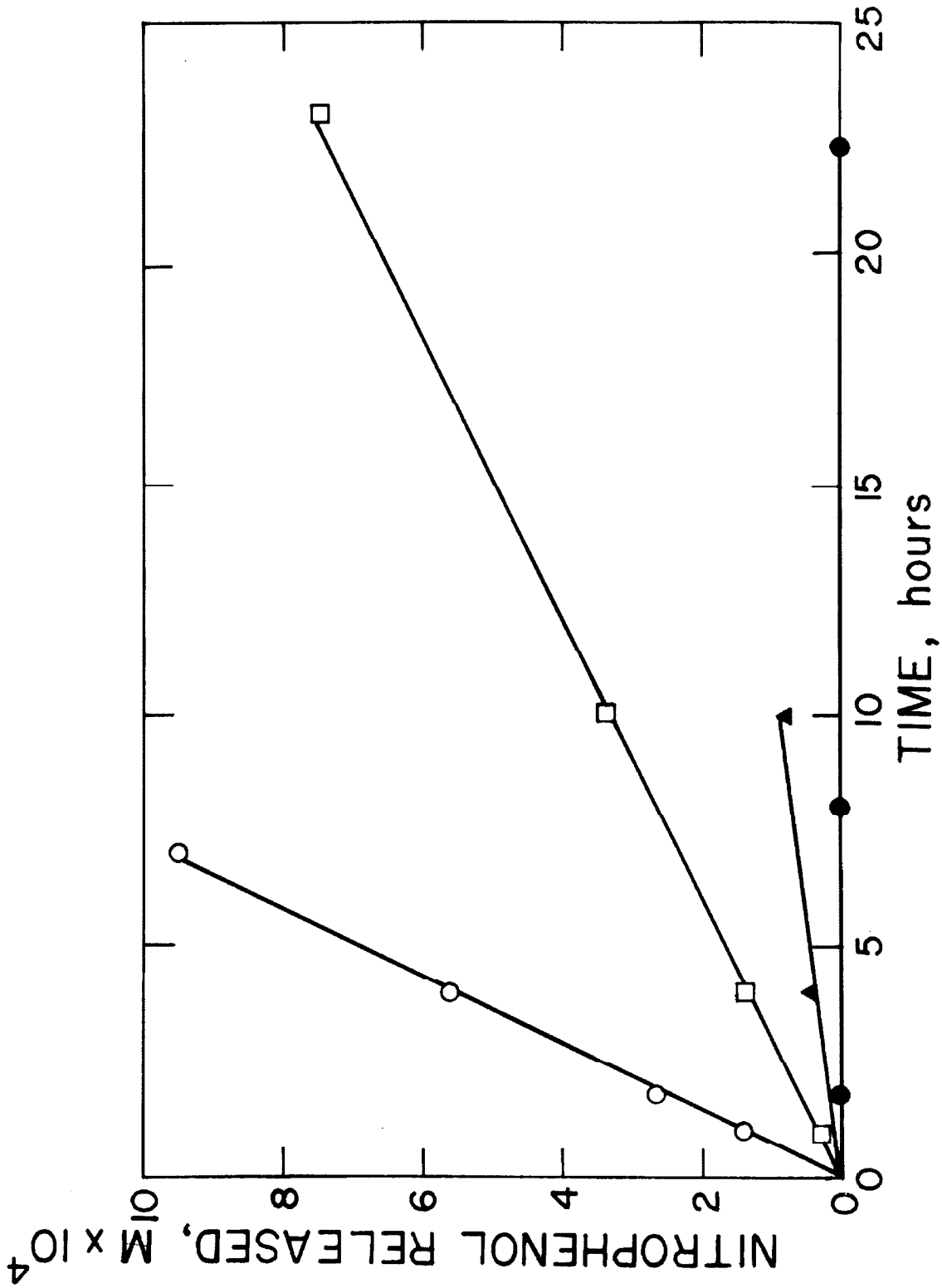

NITROPHENOL RELEASED, M x 10⁴
0
2
4
6
8
10
0
5
10
15
20
25
TIME, hours

Table III. Relative Rate of Release of p-Nitrophenol from Several p-Nitrophenyl Glycosides in the Presence of Human Lysozyme and Chitin Oligosaccharides.

p-Nitrophenyl Glycoside	Relative Rate*
β-2-deoxyglucoside	4.2
β-NAG	1.0
β-glucoside	0.26
α-glucoside	0.00
β-NAGal	0.00
β-xyloside	0.00

*Based on a value of 1.0 for p-nitrophenyl-N-acetyl-β-D-glucosaminide.

human lysozyme and chitin oligosaccharides was also measured and the results are shown in Figure 6. The reaction showed a pH maximum of pH 4.7-4.8. While it is difficult, because of the complexity of the reaction involved, to relate this curve directly to dissociations of side-chains of the enzyme which could be involved in catalysis, the results tend to implicate a basic group with pK_a ~3.5 and an acidic group with pK_a ~6.5 as being important in the reaction being observed. The pH dependence of the reaction is strikingly similar to that of hen lysozyme for the same reaction (33). This is another indication that human lysozyme and hen lysozyme are very similar to one another in their mechanisms.

3. Discussion on the Mechanism of Action of Human Lysozyme.

a. Possible Mechanisms for Glycoside Hydrolysis by Human Lysozyme-- Raftery and Rand-Meir (33) have determined, by analogy with model studies on the hydrolysis of simple glycosides, that several mechanistic pathways are possible for the enzymatic hydrolysis of glycosides, and their observations are applicable to human lysozyme. On the basis of studies on model compounds by different investigators, the mechanisms depicted in Figure 7 are considered possible for the

Figure 6. The pH dependence of human lysozyme (3.5×10^{-4} M) catalyzed release of p-nitrophenol from p-nitrophenyl-β-D-glucoside (1.0×10^{-1} M) in the presence of chitin oligosaccharides (5×10^{-3} M). In the pH range 2.2-5.5, 0.1 M citrate buffers were used, and in the pH range 6.0-8.0, 0.1 M citrate-phosphate buffers were used (70).

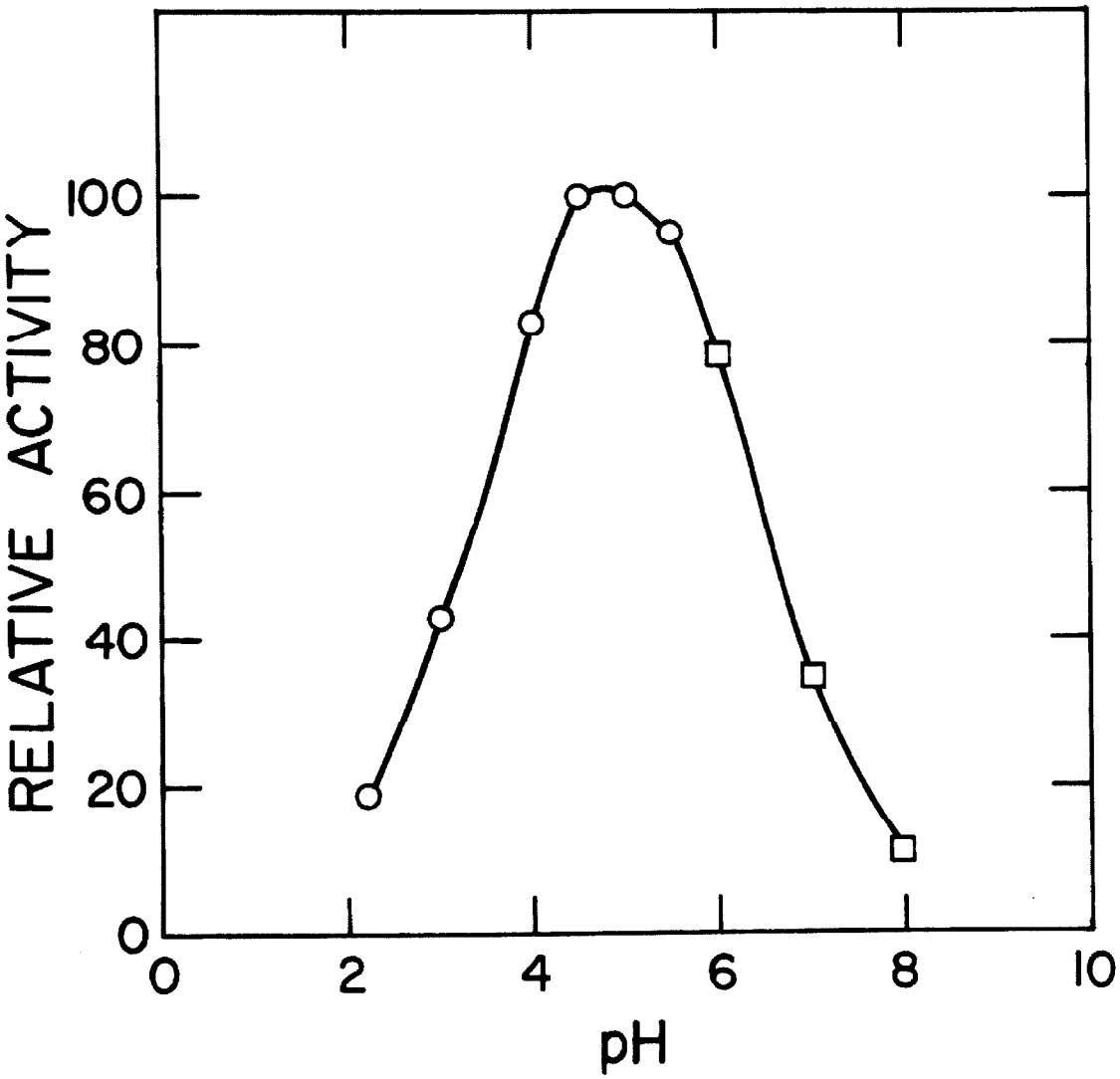
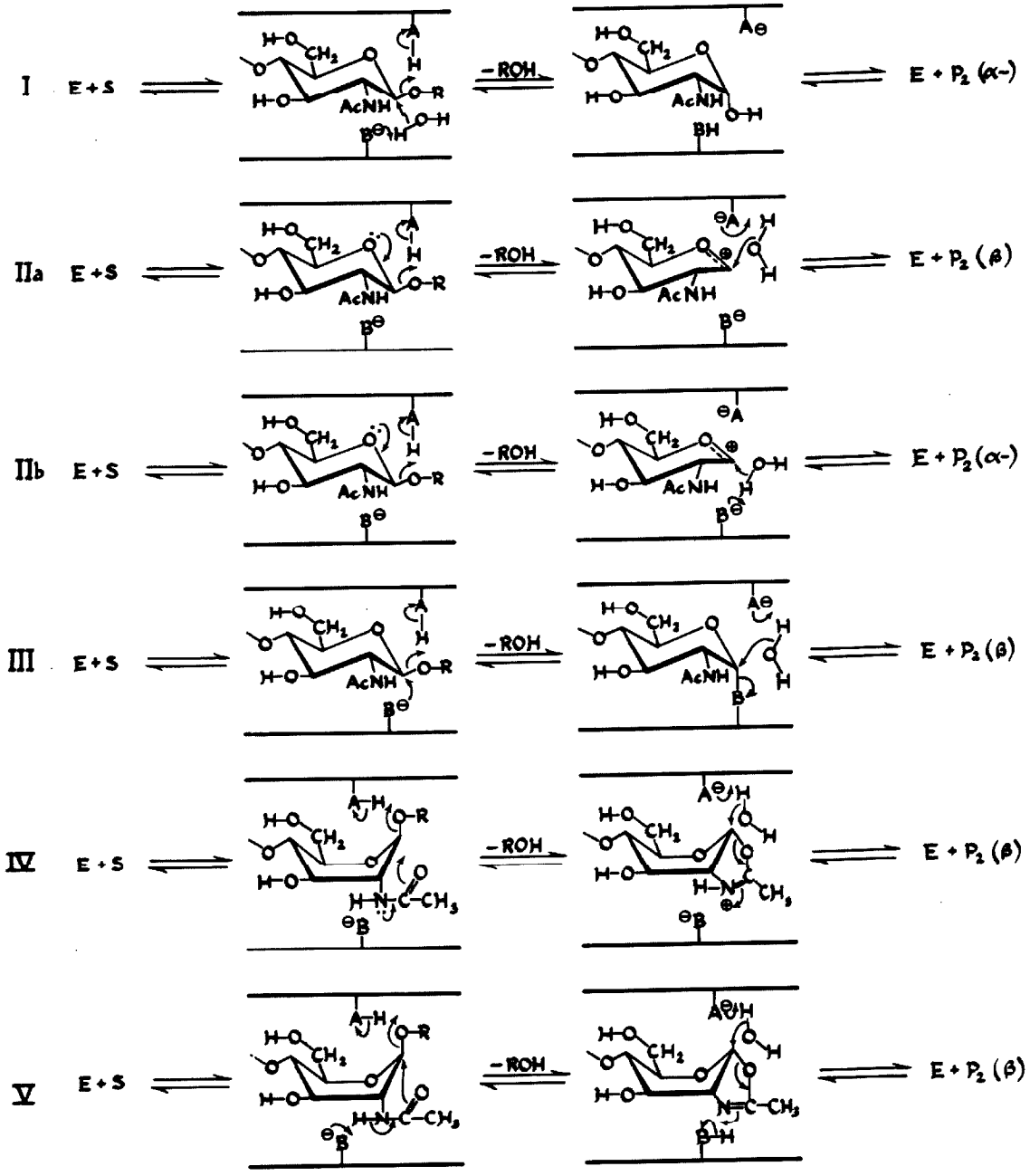

RELATIVE ACTIVITY
100
80
60
40
20
0
0
2
4
6
8
10
pH

Figure 7. Depiction of mechanistic pathways possible for substrate during human lysozyme catalyzed cleavage of glycosidic bonds. AH and $B^{\ominus}$ denote an acid and a base, respectively, which are situated at the catalytic site of the enzyme. Further explanations are given in the text.


I E+S
-ROH
E + P2 (α-)
IIa E+S
-ROH
E + P2 (β)
IIb E+S
-ROH
E + P2 (α-)
III E+S
-ROH
E + P2 (β)
IV E+S
-ROH
E + P2 (β)
V E+S
-ROH
E + P2 (β)

human lysozyme catalyzed hydrolysis of glycosidic bonds. All mechanistic pathways are depicted to occur on the surface of an enzyme molecule with a propinquous acidic and basic group (HA and $B^{\ominus}$, respectively) being involved. There are five different pathways represented and each is depicted as involving a general acid catalyzed step. Mechanisms I, III, IV, and V can be written without this step, protonation occurring after the leaving group (RO^{-}) has departed.

Mechanism I is a single-step reaction which involves displacement of the aglycone by an incoming water molecule to form the reducing sugar product. The consequence of this mechanism is that the configuration at C_1 is inverted in the product. There are no known analogies for this mechanism in studies of the non-enzymatic hydrolysis of model glycosides. In enzymatic reactions, however, it is conceivable that an enzyme-bound water molecule could function in the suggested manner.

Mechanism IIa and IIb depict the formation of a carbonium ion from the conjugate acid of the substrate. Such a step, that is one involving general acid catalysis, has been claimed in model studies of the acid catalyzed hydrolysis of o-carboxylphenyl-β-D-

glucoside (37), in another simple phenolic acetal (38), as well as in the hydrolysis of poly- and oligouronides (39). The carbonium ion resulting from the human lysozyme catalyzed reaction could react immediately with water or could be stabilized as an ion pair (40,41) by a propinquous base on the enzyme. Attack by a water molecule could give rise to a product with its configuration at C_1 retained (pathway IIa), inverted (pathway IIb), or mixed. The configuration actually observed would most likely be a result of steric factors imposed by the enzyme.

Carbonium ion intermediates have been demonstrated in solvolytic reactions of 2,3,4,6-tetra-O-methyl-α-D-glucopyranosyl chloride (42) and in the reactions of methoxymethyl chloride (43). Acid catalyzed hydrolysis of glycosides (44,45) and acid catalyzed anomerization of methyl glycosides (46) are also considered to follow this mechanistic pathway. In all of these examples, the conjugate acid of the glycoside was formed by specific acid catalysis. On the basis of these and similar model studies it has been suggested that glycosidases in general could utilize a carbonium ion mechanism (47), and, more recently, similar speculation about the lysozyme catalyzed reaction has been attempted (48).

Mechanism III involves general acid and nucleophilic catalysis, yielding an enzyme-substrate intermediate which is covalently bonded. This type of mechanism has been previously put forward as a scheme to account for retention of configuration (due to two displacements at C_1) in enzymatic glycoside hydrolysis (49). There are no studies on model compounds which have been shown to involve such a scheme.

In mechanism IV, general acid catalysis by the enzyme is again suggested to form the conjugate acid of the glycoside. Nucleophilic attack by the neighboring acetamido group could result in an oxazoline structure which on reaction with water would give a product with the β-configuration. The existence of such intermediates has been demonstrated in some reactions undergone by β-chloro-peracetylated-D-glucosamine and related compounds (50), but not in any enzyme-catalyzed reactions. Such an anchimeric effect has been suggested, however, for lysozyme catalyzed hydrolysis of glycosidic bonds (51,52). A recent report (53) has demonstrated an anchimeric assistance by the acetamido group in the spontaneous hydrolysis of o- and p-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside.

Mechanism V is closely related to IV in that anchimeric assistance again results in formation of an oxazoline intermediate. However, in mechanism V, the enzyme is shown to act as a general base by abstracting a proton from the 2-acetamido nitrogen with simultaneous displacement of the glycosidic leaving group.

Although it has been clearly established that acid-catalyzed hydrolysis of glycosidic bonds proceeds by an S_N1 mechanism (48,54), and that base-catalyzed hydrolysis of certain aryl glycosides proceeds by S_N2 mechanisms (55,56), in no case has the mechanism of an enzyme-catalyzed hydrolysis of glycosidic bonds been elucidated. Obviously, the studies on various model compounds may serve as excellent guides to approach an understanding of such enzymatic reactions.

b. Distinguishing Between the Possible Mechanisms--The results of the described investigation allow distinction between several of the mechanisms outlined in Figure 7. Since it was shown that human lysozyme-catalyzed transglycosylation of NAG from chitobiose to methanol resulted in formation of β-methyl-NAG without detectable amounts of the α-product, it is clear that mechanism I, which involves a single displacement reaction mediated by the enzyme, does not describe the

mechanistic pathway of substrate during catalysis. The same result also eliminates mechanism IIb, i. e., a carbonium ion giving rise to a product of inverted configuration. It does not, however, eliminate mechanism IIa, a carbonium ion intermediate giving rise to a product of retained configuration. It was hoped to obtain positive evidence for mechanism IIa by finding a small percentage of α-methyl-NAG in the experiment using ^{14}C labelled chitobiose in the presence of methanol. However, the results obtained did not lead to such a conclusion since β-methyl-NAG was obtained to at least 99.9%, and the presence of small amounts of impurities did not allow quantitation beyond this value.

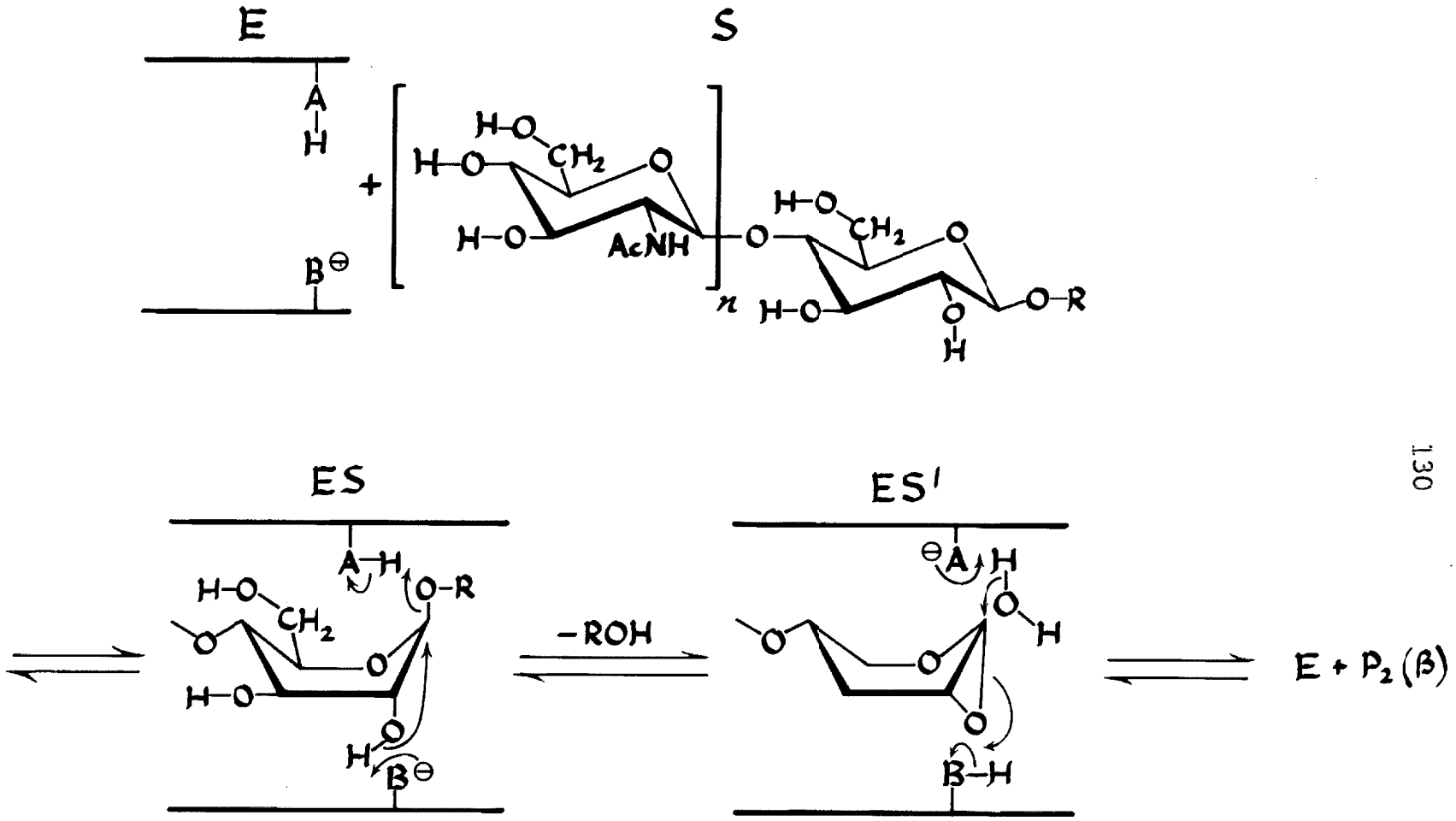
The transglycosylation reaction catalyzed by human lysozyme has allowed the synthesis of a variety of new saccharides whose behavior in the presence of the enzyme have an important bearing on the mechanism. As expected, p-nitrophenyl oligosaccharides of NAG made in this way served as substrates which released p-nitrophenol. The finding that p-nitrophenyl-β-D-glucosyl-$(NAG)_n$ also served as substrates for human lysozyme, with p-nitrophenol being released through hydrolysis of the glucosidic bond, is of considerable

interest. This indicated that human lysozyme could effect hydrolysis of glycosides other than those of oligosaccharides containing only NAG (β-(1→4) linked) or NAG-β-(1→4)-NAM units. The acetamido groups of the natural substrates are therefore not necessary for catalysis to occur, but most likely are involved in binding, i. e., formation of the enzyme-substrate complex. These findings are very similar to those of Raftery and Rand-Meir (33) in their investigation of hen lysozyme. These data eliminate the possibility of anchimeric assistance by the acetamido side chain in catalysis of the glycosidic bond, and thus mechanism IV is eliminated as being the pathway traversed by substrate during catalysis by human lysozyme.

Since the disposition of the 2-hydroxyl and p-nitrophenyl glycosidic groups in the nitrophenyl-β-glucosides is the same stereochemically as the 2-acetamido and p-nitrophenyl glycosidic groups in β-NAG-glycosides, the possibility of general base catalysis by the enzyme causing an anchimeric effect in the substrate (Figure 7, V) was not eliminated by the finding that p-nitrophenyl glucosides served as substrates. It has recently been shown that in the spontaneous hydrolysis of o- and p-nitropheny-β-NAG, the acetamido side chain participates anchimerically

through formation of an oxazoline intermediate (53). In the same study, the 2-hydroxyl group of o- and p-nitrophenyl-β-D-glucoside was shown to provide anchimeric assistance in the spontaneous hydrolysis of the compounds. In these studies it was shown that participation by the acetamido side chain was more effective by a factor of 10^3 than participation by the 2-hydroxyl group. In the event, however, of the enzyme providing a general base for abstraction of a proton from the acetamido side chain as shown in mechanism V (Figure 7), the hydrolysis of p-nitrophenyl glucosidic bonds could be a result of general base catalysis by the enzyme through abstraction of a proton from the 2-hydroxyl residue, thus allowing C-2 oxyanion participation as depicted in Figure 8. Thus mechanism V could operate with substrates containing 2-hydroxyl groups as well as those with 2-acetamido groups. To test this possibility, substances of the general formula p-nitrophenyl-2-deoxy-β-D-glucosyl-$(NAG)_n$ were tested for human lysozyme catalyzed release of p-nitrophenol. As shown in Figure 5, human lysozyme catalyzed the release of p-nitrophenol from such compounds. Thus, mechanism V can be eliminated on this basis. The greater rate of release of p-nitrophenol exhibited by the 2-deoxy-glucoside substrates when compared with analogous

Figure 8. Possible mechanistic pathway for human lysozyme catalyzed hydrolysis of p-nitrophenyl-β-D-glucopyranosides, involving general acid and general base catalysis by the enzyme and C-2 oxyanion participation by the substrate.


E
S
A
H
B⊖
AcNH
n
ES
ES'
-ROH
E + P2 (β)

glucose compounds (the observed relative rates were 16:1, as shown in Table III) is of interest since it has been shown that acid catalyzed hydrolysis of methyl-2-deoxy-β-D-glucoside proceeds at a rate which is approximately 10^3 times that of methyl-β-D-glucoside (57). It is not plausible, however, due to the complexity of the enzyme catalyzed reaction, to interpret the present results as indicating a carbonium ion mechanism.

The results obtained in this study allow definition of the specificity of human lysozyme in very precise terms. It has been shown that acetamido groups are necessary for catalysis to occur, but that such side chains are necessary only for the binding of the substrate to human lysozyme and are not involved in the actual hydrolytic reaction. The requirement for β-(1→4) glycosidic bonds between pyranose rings was made apparent, since p-nitrophenyl-2-acetamido-β-D-galactosyl-$(NAG)_n$ compounds did not serve as substrates which released p-nitrophenol. The requirement for the β-configuration in the glycosidic bond undergoing hydrolysis has been shown to be a strict requirement since no hydrolysis to p-nitrophenol was observed when p-nitrophenyl-α-D-glucosyl-$(NAG)_n$ was used as substrate. There also seemed to be a dependence on C-6 of the

pyranose ring containing the glycosidic bond to be cleaved, since p-nitrophenyl-β-D-xylosyl-$(NAG)_n$ did not serve as a substrate which released p-nitrophenol. These findings were all in contrast to the variability which was allowable at C-2.

In conclusion, it may be stated that, of those reasonable mechanistic pathways (Figure 7) by means of which human lysozyme could cleave glycosidic bonds, the present studies have eliminated: (a) a single displacement mechanism (I); (b) a carbonium ion mechanism which gives rise to α-anomeric products (IIb); (c) a mechanism involving participation of the acetamido side chain of NAG-containing substrates (IV); and (d) a mechanism involving anchimeric assistance by the substrate due to general base catalysis by the enzyme (V). The remaining possibilities are: (a) a carbonium ion mechanism which gives rise to β-anomeric products (IIb); or (b) a double displacement mechanism involving an enzyme-bound intermediate and which also gives rise to β-products. If the carbonium ion intermediate exists, it is possible that it is stabilized by a nearby basic group since the human lysozyme-intermediate complex is long lived, as evidenced by the transglycosylation observed with numerous acceptors. It is possible that the base involved in the stabilization of

a carbonium ion (as an ion pair) or in a covalently bonded glycosyl-enzyme intermediate is a carboxylate anion. The pH profile of the human lysozyme catalyzed hydrolysis of p-nitrophenyl-β-D-glucosyl oligosaccharides (Figure 6) implicated a group of pK_a ~3.5 which is involved in catalysis, and this is likely to be a carboxyl group.

These studies on the mechanistic pathway traversed by substrate during human lysozyme catalyzed hydrolysis indicate that human lysozyme is very similar to hen egg white lysozyme (32,33) in its mechanistic properties. The mechanisms of both enzymes can be described as either: (a) a carbonium ion mechanism giving rise to β-products; of (b) a double displacement mechanism involving an enzyme-bound intermediate which also gives rise to β-products. The pH profile for the release of p-nitrophenol from p-nitrophenyl-β-D-glucoside in the presence of chitin oligomers is also very similar for the two enzymes. Human lysozyme was shown to have a pH optimum of 4.7-4.8 for this reaction, while hen lysozyme has been shown previously to have a pH optimum of about 5.0 (33). These results tend to indicate that both enzymes operate by very similar, if not the same, mechanisms.

4. Hydrolysis of Chitotriose and Chitotetraose by Human Lysozyme.

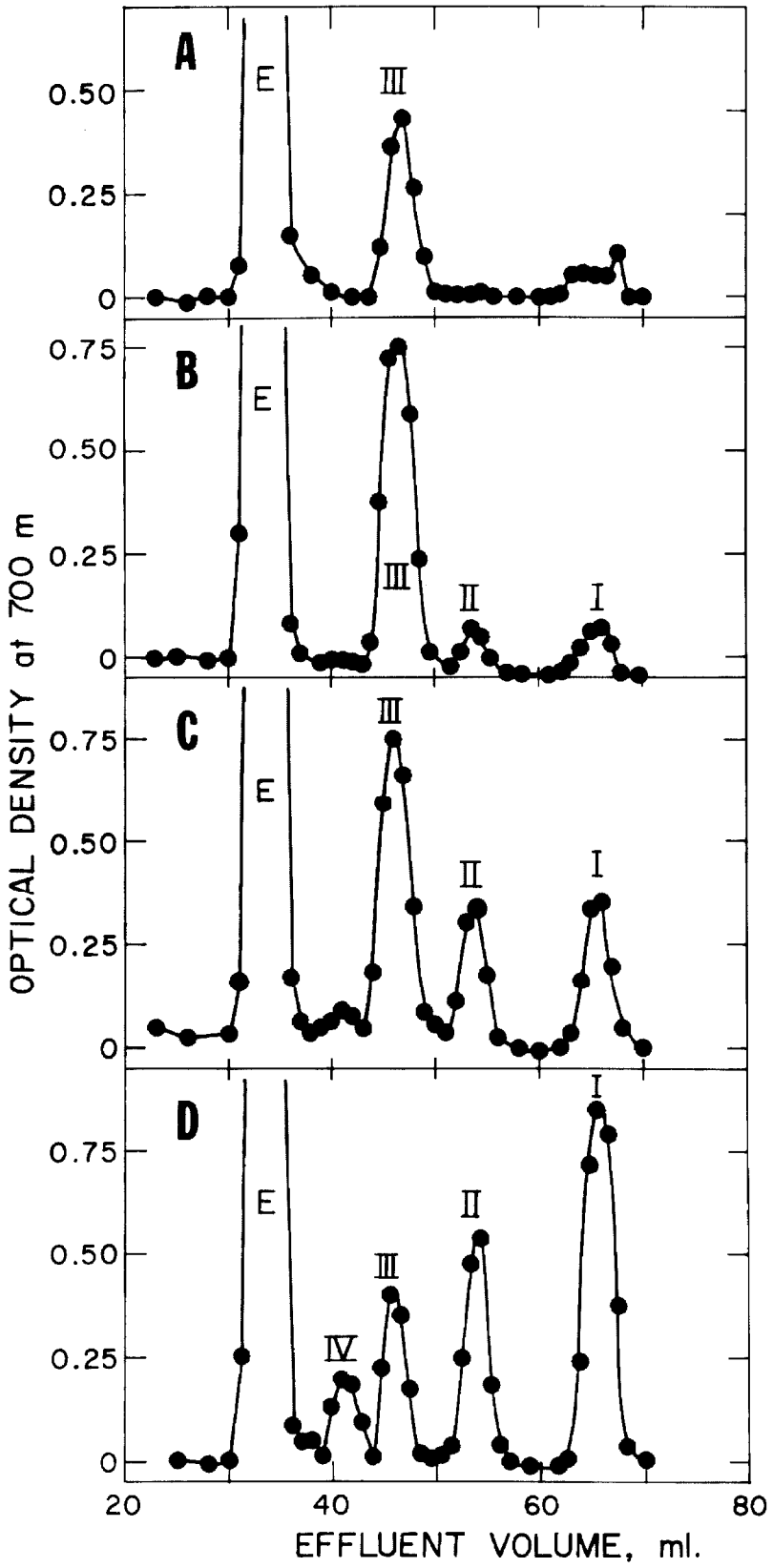
It has been shown that hen egg white lysozyme hydrolyzes the β-(1→4) glycosidic bonds of oligosaccharides obtained from chitin (58,59). A small substrate of this type for which hydrolysis has been observed is chitotriose. Lysozyme can hydrolyze either of the two glycosidic bonds in chitotriose (60,36), with its preference varying with the conditions used (34). Another chitin oligosaccharide which serves as a substrate for hen lysozyme is chitotetraose. Under certain conditions, the enzyme has been shown to hydrolyze all three glycosidic bonds in the tetrasaccharide, with the bond nearest the reducing end and the one nearest the non-reducing end being hydrolyzed more readily (36). Under other conditions, selective hydrolysis of the glycosidic bond nearest the reducing end was observed (34).

Since this investigation had shown human lysozyme to be very similar to hen lysozyme in many ways, it was decided to compare the two enzymes in their activities toward these low molecular weight substrates. When chitotriose was incubated with human lysozyme at different concentrations of substrate, and the reaction products were analyzed by gel filtration chromatography,

the results shown in Figure 9 were obtained. As can be seen, the distribution of reaction products depended on the relative concentrations of human lysozyme and chitotriose used. When $[\text{chitotriose}]/[E] = 0.7$ was used (Figure 9a), there was no significant hydrolysis even after 20 hours reaction time. There did appear to be a small amount of reducing material present in the area where NAG was normally eluted. However, this was considered to be an artifact, possibly due to a slight contamination of the chitotriose used for the study by chitotetraose, since no chitobiose was detected among the reaction products.

When $[\text{chitotriose}]/[E] = 1.2$ was used (Figure 9b), small amounts of monomer and dimer were formed in approximately equimolar amounts, indicating that the specific cleavage of trimer was being observed with no detectable transglycosylation. This conclusion was reinforced by the results obtained when a value for $[\text{chitotriose}]/[E]$ of 1.7 was used (Figure 9c). In this instance, the chromatogram indicated that there was approximately the same amount of chitotriose present as in the experiment where $[\text{chitotriose}]/[E] = 1.2$ was used, but that there were larger amounts of monomer and dimer formed, again in approximately equimolar amounts. Finally, when a ratio of

Figure 9. Gel filtration on a column (1.0 x 100 cm) of Bio-Gel P-2 of mixtures obtained from the hydrolysis of chitotriose by human lysozyme (3×10^{-3} M) in 0.1 M citrate buffer, pH 5.5, at 40° C: A) 2×10^{-3} M chitotriose for 20 hours; B) 3.6×10^{-3} M chitotriose for 2.7 hours; C) 5.0×10^{-3} M chitotriose for 2.0 hours; and D) 1.0×10^{-2} M chitotriose for 1.5 hours. Aliquots from each fraction were checked for the presence of reducing sugars by the ferricyanide method (67) (OD_{700}). E = enzyme; I = NAG; II = chitobiose; III = chitotriose; IV = chitotetraose.


A
B
C
D
E
I
II
III
IV
0.50
0.25
0
0.75
20
40
60
80
OPTICAL DENSITY at 700 m
EFFLUENT VOLUME, ml.

$[\text{chitotriose}]/[E]$ = 3.3 was used (Figure 9d), the amount of monomer formed was significantly greater than that of dimer, and there was a considerable amount of chitotetraose detected, indicating that the hydrolysis pattern was being complicated by transglycosylation.

The cleavage of chitotetraose by human lysozyme was next investigated under varying conditions (Figure 10). When a ratio of $[\text{chitotetraose}]/[E]$ = 0.7 was used (Figure 10a), approximately equimolar amounts of monomer and trimer were formed, and very little dimer was detected. This indicated that specific cleavage of chitotetraose was being observed, with cleavage occurring at either the glycosidic bond nearest the non-reducing end or the one nearest the reducing end. These results were in sharp contrast to those obtained when $[\text{chitotetraose}]/[E]$ = 33 was used (Figure 10b). In this case, the principal product was chitobiose, and a detectable amount of chitopentaose was observed, indicating that the hydrolysis pattern was being complicated by transglycosylation.

When chitotetraose which had been labelled with ^3H at the reducing end was incubated with human lysozyme at a ratio of $[\text{chitotetraose-}^3H]/[E]$ = 0.7, the results shown in Figure 11 were obtained. Specific cleavage was again observed (there were approximately

Figure 10. Gel filtration on a column (1.0 x 100 cm) of Bio-Gel P-2 of mixtures obtained from the hydrolysis of chitotetraose by human lysozyme in 0.1 M citrate, pH 5.5, at 40° C: A) human lysozyme (3×10^{-3} M) and chitotetraose (2×10^{-3} M) for 0.6 hours; and B) human lysozyme (3×10^{-4} M) and chitotetraose (1.0×10^{-2} M) for 1.0 hour. Aliquots from each fraction were analyzed for the presence of reducing sugars by the ferricyanide method (67) (OD_{700}). E = enzyme; I = NAG; II = chitobiose; III = chitotriose; IV = chitotetraose; V = chitopentaose.

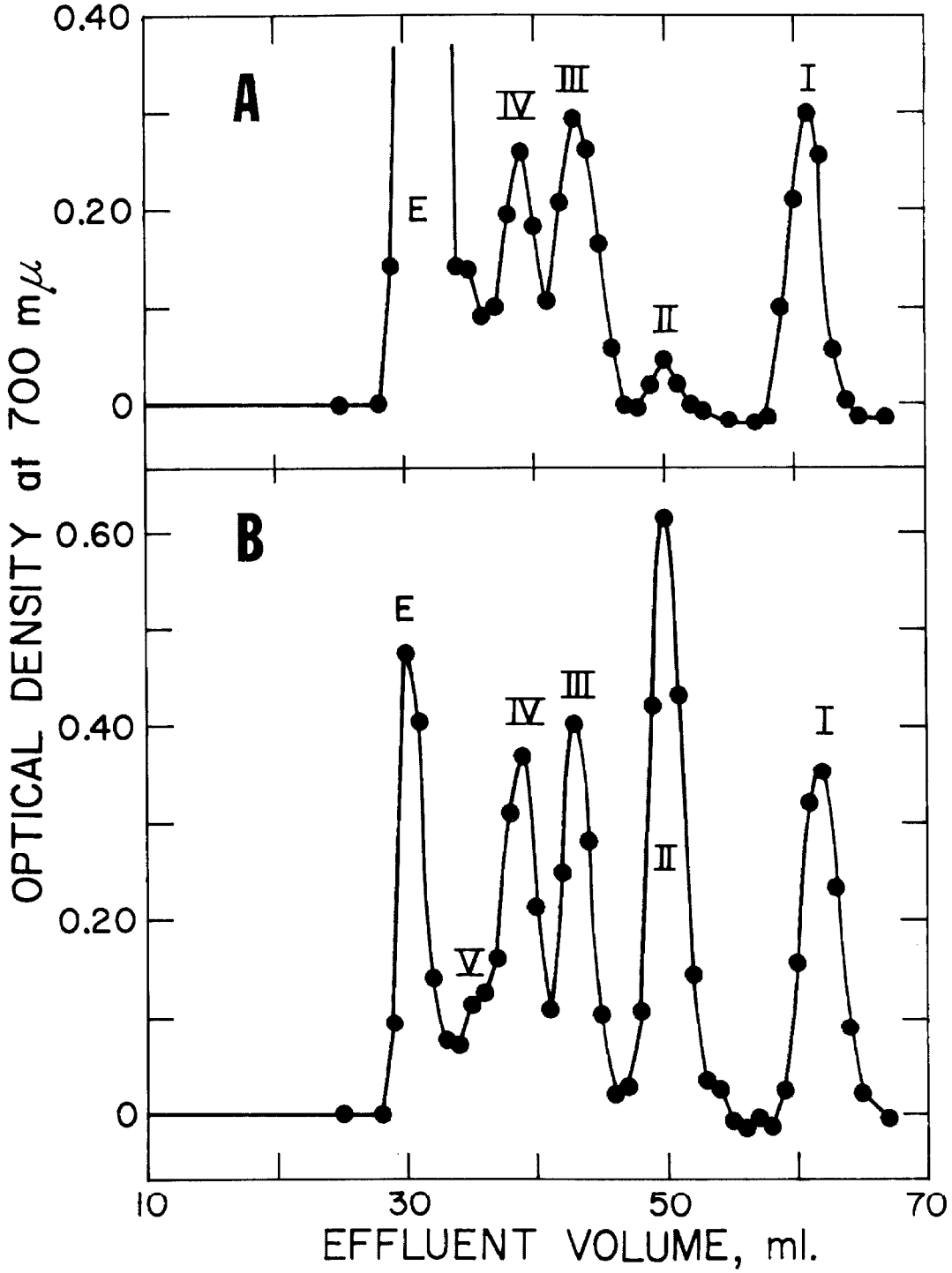
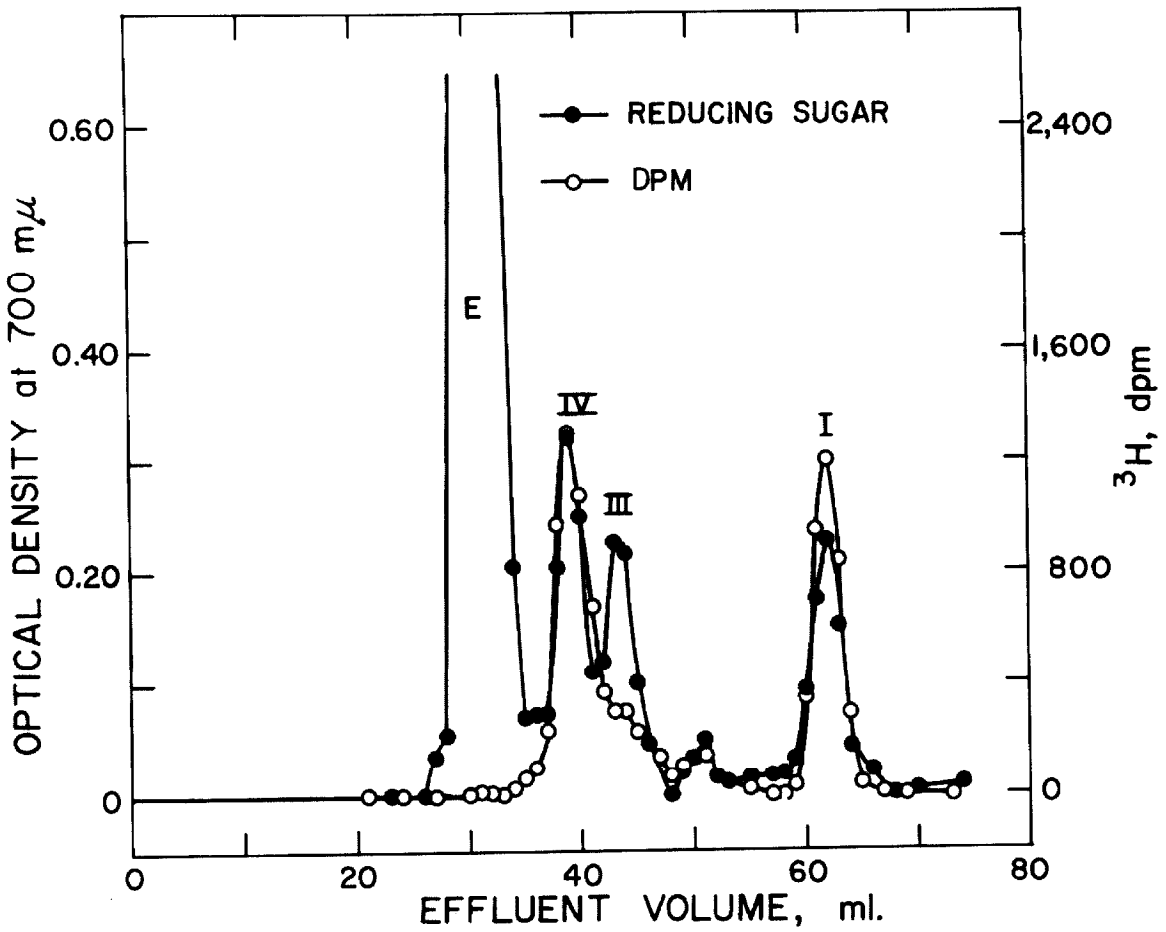

A
B
E
IV
III
II
I
V
0.40
0.20
0
0.60
0.40
0.20
0
10
30
50
70
OPTICAL DENSITY at 700 mμ
EFFLUENT VOLUME, ml.

Figure 11. Gel filtration on a column (1.0 x 100 cm) of Bio-Gel P-2 of the mixture obtained from the hydrolysis of chitotetraose-^{3}H (labelled at the reducing end) (2×10^{-3} M) by human lysozyme (3×10^{-3} M) in 0.1 M citrate, pH 5.5, for 0.5 hour at 40° C. Separate aliquots from each fraction were checked for the presence of reducing sugars by the ferricyanide method (67) (OD_{700}), and for ^{3}H content by liquid scintillation methods. E = enzyme; I = NAG; III = chitotriose; IV = chitotetraose.


REDUCING SUGAR
DPM
E
IV
III
I
0.60
0.40
0.20
0
2,400
1,600
800
0
OPTICAL DENSITY at 700 mμ
^{3}H, dpm
0
20
40
60
80
EFFLUENT VOLUME, ml.

equimolar amounts of monomer and trimer , but very little dimer, formed). When the fractions were checked for ^{3}H content by liquid scintillation methods, it was found that the specific activity of the NAG peak was about the same as that of the chitotetraose-^{3}H peak, while the chitotriose peak contained very little radioactivity. This indicated that under conditions where [E]>[chitotetraose], specific cleavage of the tetrasaccharide at the glycosidic bond nearest the reducing end was being observed.

5. Hydrolysis of p-Nitrophenyl-β-D-chitobioside by Human Lysozyme.

Several investigators have studied the hydrolysis of p-nitrophenyl-β-D-chitobioside by hen lysozyme (61,35). These studies indicated that the release of p-nitrophenol from this compound upon digestion with hen lysozyme was very slow. However, by working at high enzyme and substrate concentrations, Rand-Meir et al. (35) were able to demonstrate that the hydrolysis of this compound could be described by Michaelis-Menton kinetics (62), and they were able to determine the K_M and k_{cat} of this compound for hydrolysis by hen lysozyme.

Since human lysozyme had been shown in the present investigation to be very similar to hen lysozyme, the

study of the hydrolysis of p-nitrophenyl-β-D-chitobioside by human lysozyme was undertaken in order to see if the similarity extended to this system. Figure 12 shows a Lineweaver-Burk plot (63) of the data from the hydrolysis of p-nitrophenyl-β-D-chitobioside by human lysozyme. From these data it was determined that K_M = 2.6×10^{-2} M and $k_{cat} = 6.8 \times 10^{-5}$ mole·sec^{-1}·(mole enzyme)$^{-1}$. These values compare favorably with values of $K_M = 1.1 \times 10^{-2}$ M and $k_{cat} = 5.0 \times 10^{-5}$ mole·sec^{-1}·(mole enzyme)$^{-1}$ for the hydrolysis of p-nitrophenyl-β-D-chitobioside by hen lysozyme (35).

6. Discussion.

Human lysozyme and hen lysozyme are very similar to one another in their mechanisms of glycosidic bond hydrolysis (see II.B.3). They are also qualitatively very similar to one another in their hydrolysis of chitin oligosaccharides, although from a quantitative viewpoint they appear to be somewhat different. Thus, human lysozyme and hen lysozyme both hydrolyze chitobiose, but to different degrees under the same conditions. Figure 13 compares the extent of hydrolysis of chitobiose by human lysozyme, as determined in this investigation, with that by hen lysozyme (34) under the same conditions of hydrolysis. It can be seen that human lysozyme cleaved chitobiose to NAG to an extent of

Figure 12. Lineweaver-Burk plot for the hydrolysis of p-nitrophenyl-β-D-chitobioside by human lysozyme in 0.1 M citrate buffer, pH 5.5, at 40° C. From these data, it was determined that $K_M = 2.6 \times 10^{-2}$ M, and $k_{cat} = 6.8 \times 10^{-5}$ mole·sec^{-1}·(mole enzyme)$^{-1}$.

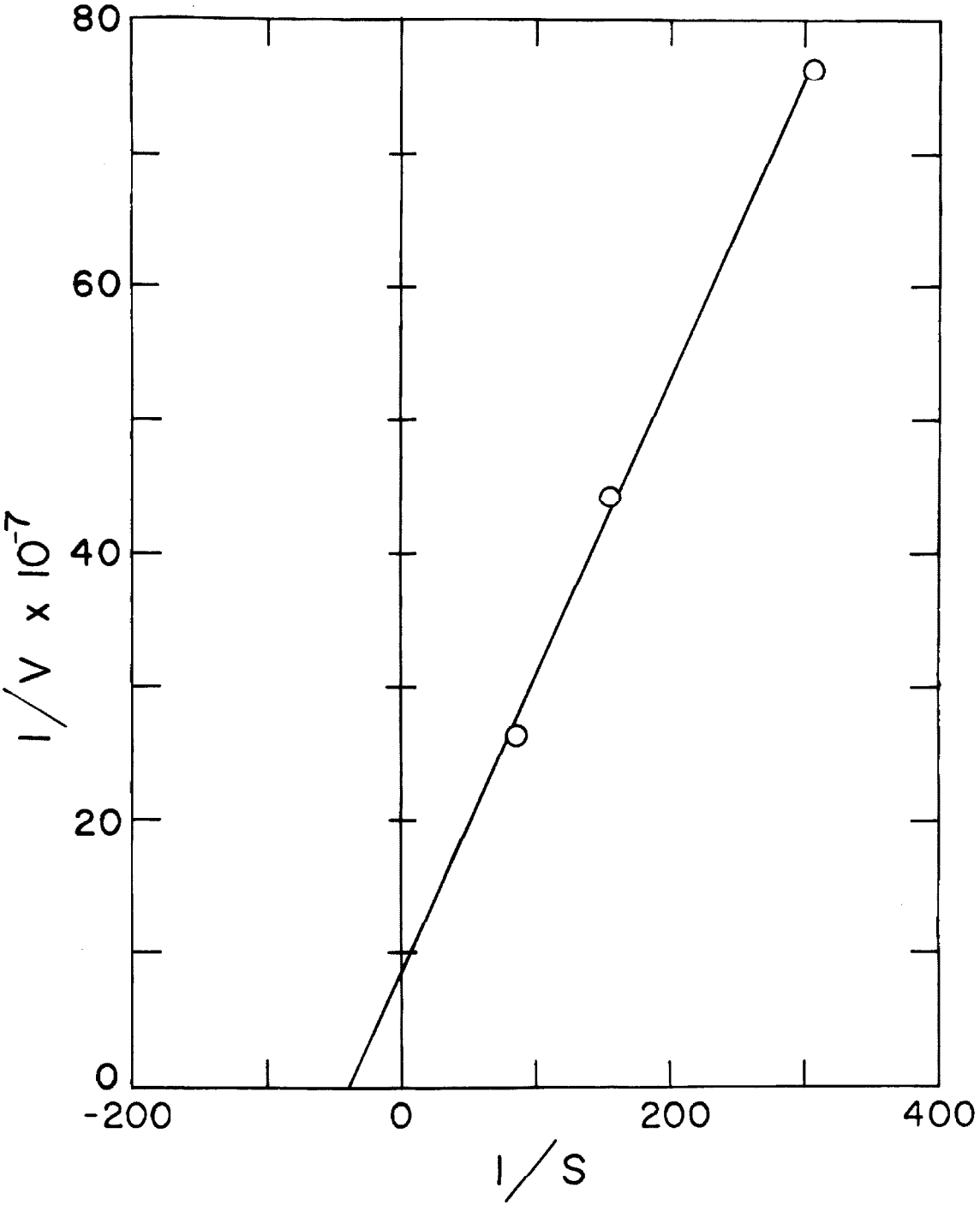
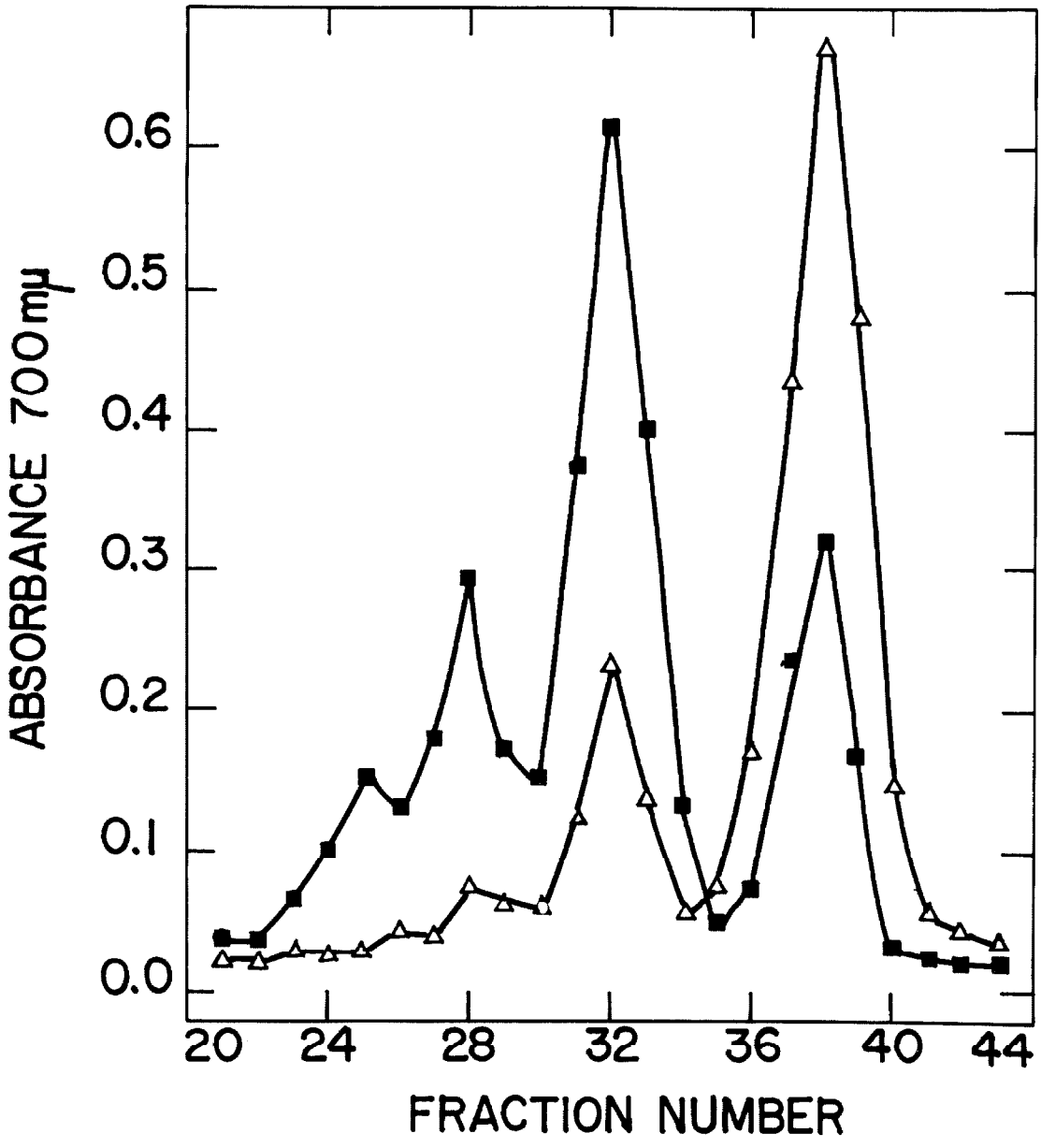

80
60
40
20
0
1/V x 10^-7
-200
0
200
400
1/S

Figure 13. Comparison of the hydrolysis of chitobiose by human lysozyme (—Δ—) and hen lysozyme (—■—) under identical conditions. The reaction conditions were: chitobiose (50 mg/ml) and enzyme (50 mg/ml) in 0.1 M citrate buffer, pH 5.5, for 20 hours at 40° C. The reaction mixtures were chromatographed on a column (1.0 x 100 cm) of Bio-Gel P-2, and each fraction was checked for the presence of reducing sugars by the ferricyanide method (67). I = NAG; II = chitobiose; III = chitotriose; IV = chitotetraose.

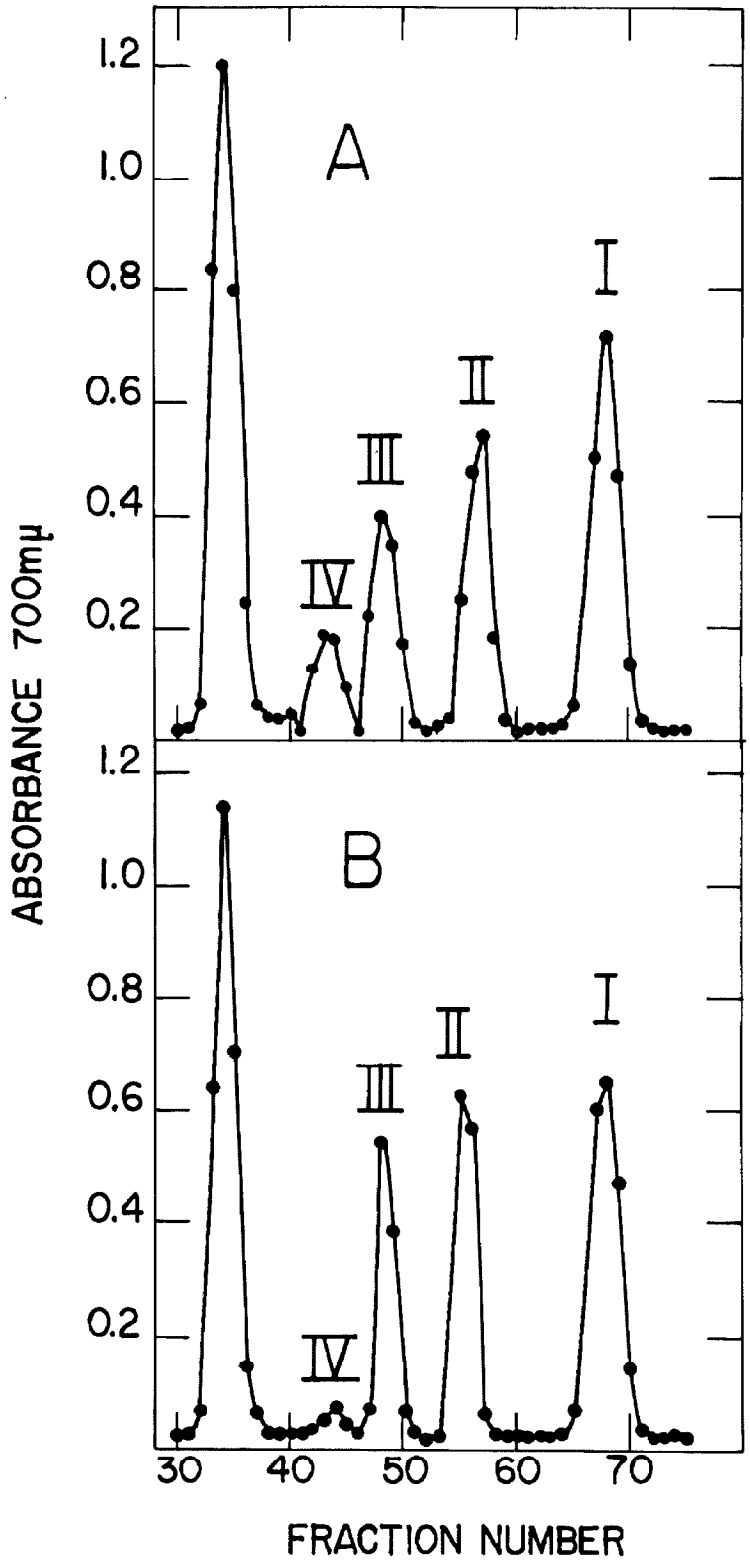

ABSORBANCE 700mμ
0.6
0.5
0.4
0.3
0.2
0.1
0.0
20
24
28
32
36
40
44
FRACTION NUMBER

about 75% hydrolysis, with only a trace of trimer being observed. Analysis of the products of the reaction catalyzed by hen lysozyme, however, shows that NAG was formed in an amount corresponding to only about 20% hydrolysis, and much larger amounts of chitotriose and chitotetraose were observed.

This quantitative difference between hen and human lysozymes is again demonstrated by the extent of hydrolysis of chitotriose by both enzymes under identical conditions (Figure 14). It can be seen (Figure 14a) that the human lysozyme catalyzed hydrolysis resulted in NAG being formed to a greater degree than chitobiose, and that a significant amount of chitotetraose was formed. In the hen lysozyme catalyzed reaction, however, it was observed (34) that monomer and dimer were formed in approximately equimolar amounts, and that very little chitotetraose was present (Figure 14b).

Dahlquist and Raftery (34) have shown that when chitotriose (2×10^{-3} M) was incubated with hen lysozyme (3×10^{-3} M), specific hydrolysis of the glycosidic bond nearest the reducing end of the trisaccharide was obtained. However, when chitotriose was incubated with human lysozyme under the same conditions, as described in this dissertation, no detectable

Figure 14. Comparison of the hydrolysis of chitotriose by A) human lysozyme, and B) hen lysozyme, under identical conditions. The reaction conditions were: 1.0×10^{-2} M chitotriose and 3×10^{-3} M enzyme in 0.1 M citrate buffer, pH 5.5, for 1.5 hours at 40° C. The reaction mixtures were chromatographed on a column (1.0 x 100 cm) of Bio-Gel P-2, and each fraction was checked for the presence of reducing sugars by the ferricyanide method. I = NAG; II = chitobiose; III = chitotriose; IV = chitotetraose.


A
B
I
II
III
IV
1.2
1.0
0.8
0.6
0.4
0.2
ABSORBANCE 700mμ
30
40
50
60
70
FRACTION NUMBER

hydrolysis occurred (Figure 9a). When conditions where $1 < \frac{[\text{chitotriose}]}{[\text{enzyme}]} < 2$ were used (Figures 9b and 9c), however, equimolar amounts of monomer and dimer were formed and very little tetramer was observed. These results indicated that when enzyme is present in excess of trimer, a 1:1 chitotriose:enzyme complex is formed which is non-productive, i. e., the bound trimer molecule does not overlap the catalytic site. However, when the concentration of trimer is increased so that it is present in slight excess of enzyme, it is possible for a 2:1 chitotriose:enzyme complex to be formed. In this 2:1 complex the second molecule of bound trimer encompasses the catalytic site and is hydrolyzed to chitobiose and NAG. By controlling the concentrations of chitotriose and human lysozyme so that only a slight excess of trimer is present at any time, it appeared that transglycosylation could be minimized and that specific cleavage of chitotriose could be obtained.

It was hoped that this specific cleavage could be positively demonstrated by using as substrate chitotriose which had been labelled with ^{14}C in the reducing end. This attempt was unsuccessful, however, due to a radioactive contaminant in the chitotriose-^{14}C which complicated the experiment to such a degree that no

significance could be placed on the results.

Dahlquist and Raftery (34) have also shown that when chitotetraose (2×10^{-3} M) was incubated with hen lysozyme (3×10^{-3} M), specific cleavage of the glycosidic bond nearest the reducing end of the tetrasaccharide was obtained. Figure 11 shows the results of a similar experiment using human lysozyme. Chitotetraose which had been labelled with ^{3}H in the reducing end was used as substrate, and it was observed that the NAG which was formed had approximately the same specific activity as the chitotetraose-^{3}H, while the chitotriose had a very low specific activity. It was concluded that human lysozyme also cleaved chitotetraose specifically at the glycosidic bond nearest the reducing end under conditions where [E] > [chitotetraose].

It was shown in this investigation that p-nitrophenyl-β-D-chitobioside was hydrolyzed by human lysozyme under certain conditions, and that the hydrolysis reaction could be described by Michaelis-Menton kinetics. The K_M and k_{cat} for p-nitrophenyl-β-D-chitobioside were calculated from these data, and it was found that $K_M = 2.6 \times 10^{-2}$ M and $k_{cat} = 6.8 \times 10^{-5}$ mole·sec^{-1}·(mole enzyme)$^{-1}$ for the human lysozyme catalyzed hydrolysis. These values compare with

values of $K_M = 1.1 \times 10^{-2}$ M and $k_{cat} = 5.0 \times 10^{-5}$ mole·sec^{-1}·(mole enzyme)$^{-1}$ for the hen lysozyme catalyzed hydrolysis of this compound. This indicates that the two enzymes are similar to one another in their hydrolysis of p-nitrophenyl-β-D-chitobioside. However, this substrate binds to hen lysozyme more strongly by a factor of 2.5, but is hydrolyzed by this enzyme at a rate which is only 0.7 times that of the human lysozyme catalyzed hydrolysis.

In summation, it has been demonstrated that the mechanism of glycosidic bond hydrolysis by human lysozyme is very similar to that of hen lysozyme (32, 33). The mechanistic possibilities for human lysozyme have been narrowed down to either: (a) a carbonium ion mechanism which gives rise stereospecifically to β-anomeric products; or (b) a double displacement mechanism which also results in retention of configuration, i. e., β-anomeric products are formed. It has also been demonstrated that human lysozyme is qualitatively very similar to hen lysozyme in its hydrolytic action towards chitin oligosaccharides and towards p-nitrophenyl-β-D-chitobioside. However, human lysozyme is distinguishable from hen lysozyme on the basis of the product distributions observed after hydrolysis of the chitin oligosaccharides, and on the basis of the

K_M and k_{cat} values obtained for the hydrolysis of p-nitrophenyl-β-D-chitobioside.

C. EXPERIMENTAL

1. Materials.

Human lysozyme was a generous gift of Dr. R. E. Canfield. Chitin oligosaccharides obtained from acid hydrolysis of the polysaccharide (59) were fractionated as described elsewhere (64). Methyl-2-acetamido-2-deoxy-β-D-glucopyranoside was synthesized by previously published procedures (65). Methyl-2-acetamido-2-deoxy-α-D-glucopyranoside was purchased from Pierce Chemical Company. It was freed from a 10% contamination by the corresponding β-glycoside by chromatography on charcoal-celite columns (65). p-Nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside, p-nitrophenyl-β-D-glucoside, and p-nitrophenyl-2-acetamido-2-deoxy-β-D-galacto-pyranoside were purchased from Cyclo Chemical Company. Pierce Chemical Company was the source of p-nitrophenyl-β-D-xyloside and p-nitrophenyl-α-D-glucoside. p-Nitro-phenyl-2-deoxy-β-D-glucoside was synthesized from 2-deoxy-D-glucose (66). p-Nitrophenyl-β-D-chitobioside was synthesized from chitobiose (35). N,N'-diacetyl-^{14}C-chitobiose was synthesized as described elsewhere (32). The mixture of tritiated α- and β-methyl-N-acetyl-D-glucosaminides was the same one that has been described elsewhere (32). The specific activity of the glycoside mixture was 1 mc per mmole. The relative

concentration of α- and β-methyl-NAG in this mixture was determined by integration of the methoxyl resonances obtained in a 60 MHz proton magnetic resonance spectrum of the sample. The ratio of α- to β-methyl-NAG was found to be 1.9:1.0. Chitotetraose which was labelled with ^{3}H in the reducing end was synthesized by F. W. Dahlquist (34). Amberlite MB-1 was obtained from Mallinckrodt Chemical Works. Bio-Gel P-2 (200-400 mesh) was obtained from Bio-Rad Laboratories, Richmond, California. All other chemicals, reagent grade or the best available, were purchased from commercial sources.

2. Human Lysozyme Catalyzed Hydrolysis of Chitobiose.

Human lysozyme (10 mg, 7×10^{-7} mole) and chitobiose (10 mg, 2.4×10^{-5} mole) were dissolved in 200 μl of 0.1 M sodium citrate buffer, pH 5.5, a drop of toluene was added, and the mixture was incubated for 20 hours at 40° C. At the end of this time, a small amount of precipitate was removed, after dilution of the mixture to 1.0 ml with water, by filtration through a Millipore filter (40 μ pore size). The filtered solution was then applied to a column (1.0 x 100 cm) of Bio-Gel P-2 which had been equilibrated with 0.1 M NaCl at 25° C. The flow rate was adjusted to 20 ml per hour and 1.0 ml fractions were collected. Ten μl

from each fraction was assayed for reducing sugars by the ferricyanide method (67).

3. Transglycosylation by Methanol During Human Lysozyme Catalyzed Hydrolysis of Chitobiose.

Human lysozyme (10 mg) and chitobiose (10 mg) were dissolved in 200 μl of 0.1 M sodium citrate buffer, pH 5.5, which was 8 M in methanol. A drop of toluene was added and the stoppered mixture was incubated at 40° C for 15 hours. At the end of this time, a small amount of precipitate was removed, after dilution of the mixture to 15 ml with water, by filtration through a Millipore filter (40 μ pore size). The human lysozyme was removed from the filtrate by ultrafiltration through a Diaflo membrane (UM-1) using a Diaflo ultrafiltration apparatus (Amicon Corporation). The citrate buffer was removed from the ultrafiltrate by treatment with Amberlite MB-1 resin for one hour. This treatment also removed most of the free sugars, i. e., those with potential reducing groups. After the resin was removed by filtration, the mixture was lyophilized.

Descending paper chromatography of the lyophilized material was performed on Whatman No. 3 paper, using pyridine/ethyl acetate/water (1/2/2) (upper phase) as the developing solvent. A standard containing α- and β-methyl-NAG was run alongside the reaction products.

The solvent was allowed to drip off the paper during the development of 24 hours. After thorough drying of of the paper, the separated saccharides were located using a modification (W. J. Dreyer, personal communication) of the chlorine peptide-bond spray (68).

When N,N'-diacetyl-^{14}C-chitobiose was used in the partitioning reaction, the conditions were the same as described in the above section except that all quantities were twice as great. After the allotted reaction time, 0.4 mg each of α- and β-methyl-NAG were added to the reaction mixture along with 2.4×10^5 dpm of the mixture of α- and β-methyl-NAG-^{3}H. The resulting mixture was subjected to filtration, ultrafiltration, deionization, and lyophilization as described in the above section. Paper chromatography was performed as described above for a total of 30 hours. A standard mixture of α- and β-methyl-NAG was also run alongside the reaction products. After development and thorough drying of the paper, it was cut in half lengthwise. The half containing the standard mixture was subjected to the peptide bond spray described above to locate the α- and β-methyl-NAG. The other half of the chromatogram was cut into 2 cm strips, except for that part containing the radioactive α- and β-methyl-NAG which was cut into 1 cm strips, and these were eluted

with water. An aliquot from each fraction was removed and, after addition to 15.0 ml of Bray's solution (69), the ratio of ^{3}H to ^{14}C was determined by liquid scintillation methods in a Packard Model 3324 TriCarb scintillation spectrophotometer.

4. Transglycosylation of p-Nitrophenyl-glycosides in the Human Lysozyme Catalyzed Hydrolysis of Chitin Oligosaccharides and Hydrolysis of the Resulting p-Nitrophenyl-oligosaccharides.

Stock solutions of human lysozyme (20 mg per ml, 1.4×10^{-3} M) and p-nitrophenyl glycosides (5.0×10^{-2} M) in 0.1 M citrate buffer, pH 5.5, which contained 10% (v/v) p-dioxane, were made up freshly prior to use. A stock solution of a mixture of chitotetraose, chitopentaose, and chitohexaose (in approximately equimolar amounts) (20 mg per ml, $\sim 2 \times 10^{-2}$ M) was made up in the same buffer. This same preparation of chitin oligomers was used throughout these experiments. Aliquots of the stock solutions were mixed to give final concentrations of enzyme equal to 3.5×10^{-4} M, p-nitrophenyl glycoside equal to 2.5×10^{-2} M, and chitin oligomers equal to $\sim 5 \times 10^{-3}$ M. Blanks for the cleavage of the individual p-nitrophenyl glycosides by human lysozyme were made up by using the same mixture mentioned above, less the chitin oligomers. Each

mixture, to which a drop of toluene was added to prevent microbial growth, was incubated at 40° C in a stoppered tube. Estimation of the release of p-nitrophenol was performed by adding aliquots (100 µl) of the incubation mixtures to 3.0 ml of 0.2 M potassium tetraborate, pH 9.5, and, following filtration to remove any insoluble material, reading the adsorbance of the resulting solution at 400 mµ in a Beckman DB spectrophotometer. A molar extinction coefficient of 18,000 was used for the quantitative estimation of p-nitrophenol.

5. <u>The Effect of pH on the Release of p-Nitrophenol from p-Nitrophenyl-β-D-glucoside in the Presence of Human Lysozyme and Chitin Oligosaccharides</u>.

Stock solutions of human lysozyme (1.4×10^{-3} M), p-nitrophenyl-β-D-glucoside (2.0×10^{-1} M), and the same mixture of chitotetraose, chitopentaose, and chitohexaose ($\sim 2 \times 10^{-2}$ M) used above were made up in buffers of the appropriate pH containing 10% (v/v) p-dioxane. In the pH range 2.2-5.5, 0.1 M citrate buffers were used, and in the pH range 6.0-8.0, 0.1 M citrate-phosphate buffers (70) were used. Aliquots from the stock solutions were mixed to give final concentrations of enzyme equal to 3.5×10^{-4} M, p-nitrophenyl-β-D-glucoside equal to 1.0×10^{-1} M, and chitin oligomers equal to $\sim 5 \times 10^{-3}$ M. The same method

as outlined for the p-nitrophenyl glycosides was used to estimate the p-nitrophenol released at each pH value after incubation of each reaction mixture at 40° C for 16 hours.

6. Human Lysozyme Catalyzed Hydrolysis of Chitotriose.

A stock solution of chitotriose (1.0×10^{-2} M) in 0.1 M citrate buffer, pH 5.5, was made up freshly before each experiment. Reaction mixtures were made up by weighing out 10.9 mg samples of human lysozyme into a test tube and adding aliquots of the stock chitotriose solution, and aliquots of buffer where necessary, so that a total of 250 μl of solution was added. The resulting mixtures were stoppered and incubated at 40° C for an appropriate period of time, and then applied to a column (1.0 x 100 cm) of Bio-Gel P-2 which had been equilibrated with 25% acetic acid (v/v) at room temperature. The column was eluted with the same solvent, the flow rate was set at 15 ml per hour, and 1.0 ml fractions were collected. Aliquots from each fraction were analyzed for reducing sugars by the ferricyanide method (67).

7. Human Lysozyme Catalyzed Hydrolysis of Chitotetraose.

A stock solution of chitotetraose (1.0×10^{-2} M) was made up in 0.1 M citrate buffer, pH 5.5, and

stored under toluene until use. In the case where [E] > [chitotetraose] , 11.0 mg human lysozyme was weighed into a test tube and 50 μl of the stock chitotetraose solution and 200 μl of buffer were added to give final concentrations of 3×10^{-3} M enzyme and 2×10^{-3} M chitotetraose. This mixture was incubated at 40° for 0.6 hour and then applied to a column (1.0 x 100 cm) of Bio-Gel P-2, which had been equilibrated with 25% (v/v) acetic acid, and was eluted with the same solvent. The flow rate was 15 ml per hour and 1.0 ml fractions were collected. An aliquot from each fraction (500 μl) was checked for the presence of reducing sugars by the ferricyanide method (67).

In the case where [chitotetraose] >> [E], 3.3 mg of human lysozyme was dissolved in 750 μl of the stock chitotetraose solution to give final concentrations of 3×10^{-4} M enzyme and 1.0×10^{-2} M chitotetraose. The mixture was incubated at 40° C for 1.0 hour, and then applied to the P-2 column and worked up as described in the above section. In this case, 100 μl aliquots were checked for the presence of reducing sugars.

8. Specificity of Chitotetraose Cleavage by Human Lysozyme.

When chitotetraose which had been labelled with ^{3}H at the reducing end was used as substrate, an enzyme

concentration of 3×10^{-3} M and chitotetraose-^{3}H concentration of 2×10^{-3} M were used and the mixture was incubated at 40° C for 0.5 hour. After application of the reaction mixture to the P-2 column and work-up as described previously, 500 µl aliquots from each fraction were removed and, after addition to 15.0 ml of Bray's solution (69), were analyzed for ^{3}H by liquid scintillation methods as described in previous sections. An additional aliquot from each fraction was analyzed for reducing sugars by the ferricyanide method (67).

9. Determination of K_M and k_{cat} of the Human Lysozyme Catalyzed Hydrolysis of p-Nitrophenyl-β-D-chitobioside.

Stock solutions of human lysozyme (1.7×10^{-3} M) and p-nitrophenyl-β-D-chitobioside (1.3×10^{-2} M) in 0.1 M citrate buffer, pH 5.5, containing 10% (v/v) p-dioxane, were made up freshly prior to use. Aliquots of the stock solutions were taken and, after dilution to 1.0 ml with buffer, gave three mixtures which were 1.7×10^{-4} M in enzyme and 1.17×10^{-2} M, 6.50×10^{-3} M, 3.25×10^{-3} M, respectively, in p-nitrophenyl-β-D-chitobioside. The reaction mixtures were incubated at 40° C in stoppered tubes. Estimation of the release of p-nitrophenol in each mixture was made by adding

aliquots (200 μl) of each mixture to 3.0 ml of 0.2 M potassium tetraborate, pH 9.5, and, following filtration to remove any insoluble material, reading the adsorbance of the resulting solutions at 400 mμ. A molar extinction coefficient of 18,000 was used for the quantitative estimation of p-nitrophenol. The values of K_M and k_{cat} for p-nitrophenyl-β-D-chitobioside were obtained by a Lineweaver-Burk plot (63) of the resulting data.

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Abstracts of Propositions

Proposition I

Several compounds suitable for active-site-directed irreversible inhibitors of creatine kinase are proposed.

Proposition II

It is proposed that adenyl (3'→5') adenosine-2,8-d_2 be synthesized to provide a direct assignment of the adenine protons for use in proton magnetic resonance studies of ApA.

Proposition III

It is proposed that the mechanism of a nucleophilic aromatic substitution reaction be investigated by proton magnetic resonance spectroscopy.

Proposition IV

Several compounds are proposed as bifunctional alkylating reagents for the cross-linking of protein molecules.

Proposition V

A study of the effect of different groups at the 6-position of different saccharides on the hydrolysis of these saccharides by lysozyme is proposed.

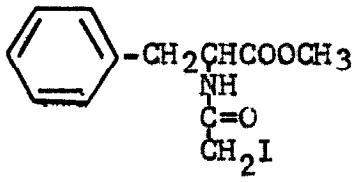
Proposition I

In the past few years several "active-site-directed irreversible inhibitors" have been used to identify amino acid residues at the active sites of several enzymes (1-10). The idea behind this technique is to design a bifunctional reagent for the enzyme under consideration so that it closely resembles the substrate of the enzyme and binds reversibly to the active site, and so that it contains a functional group capable of forming a covalent bond to some group at the active site and thus inhibit the enzyme irreversibly.

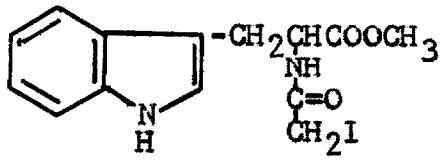
Baker has used such substrate analogues to study several enzymes, among them succinoadenylate kinosynthetase, thymidylate synthetase, thymidine kinase, folic reductase, and guanine deaminase (1). Gundlach and Turba used L- and D-N-iodoacetylphenylalanine methyl ester (I) and D,L-N-iodoacetyltryptophan methyl ester (II) to alkylate a methionine residue (met-192) at the active site of chymotrypsin (2). They found that Ib and II were more effective and that the corresponding chloro-derivatives did not react. Hartley showed that phenoxymethyloxirane (III) reacts with chymotrypsin to alkylate methionine-192 (3).

In a more elegant experiment, Schoellmann and Shaw showed that 1-chloro-3-tosylamido-4-phenylbutan-2-one

(TPCK) (IV), the chloromethyl ketone analogue of phenylalanine, also alkylates chymotrypsin at the active site, but instead of attacking a methionine residue it attacks a histidine residue (his-57) (4). Similarly, Shaw showed that 1-chloro-3-tosylamido-7-aminoheptan-2-one (TLCK) (V), the chloromethyl ketone analogue of lysine, irreversibly inhibits trypsin by alkylating a histidine residue (his-46) (5,6). Smillie studied the alkylating ability of phenoxymethyl chloromethyl ketone (PMCK) (VI) on chymotrypsin and found it to irreversibly inhibit the enzyme by alkylation of the same histidine (his-57) as TPCK (7).



Ia, D-isomer
b, L-isomer



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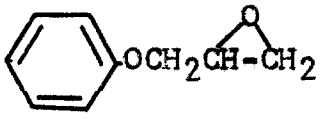

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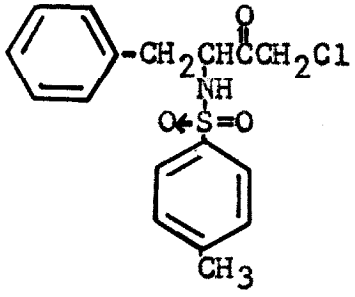

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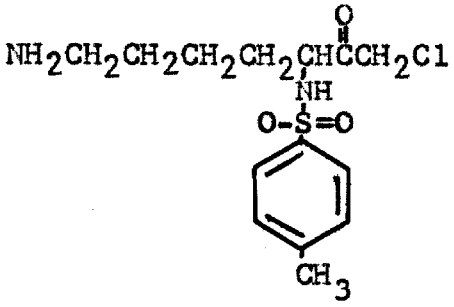

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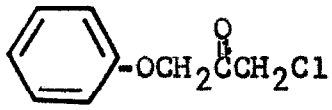
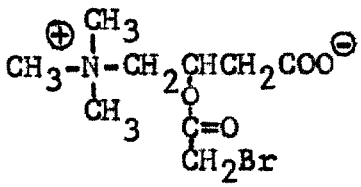
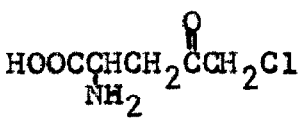


VI

More recently, Chase and Tubbs have shown that bromoacetylcarnitine (VII) and bromoacetyl $\overline{\text{CoA}}$ irreversibly inactivate carnitine acetyltransferase (8). In the same article these authors also state that bromoacetyl $\overline{\text{CoA}}$ inactivates other acetyltransferases, such as choline acetylase. Meister has shown that 2-amino-4-oxo-5-chloropentanoic acid (VIII) irreversibly blocks the glutamine binding site of E. Coli carbamyl synthetase (9). And Meloche has shown that bromopyruvate irreversibly alkylates the active site of 2-keto-3-deoxy-6-phosphogluconic aldolase (10).

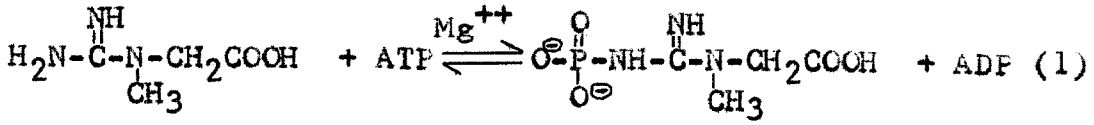


VII



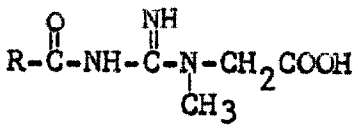
VIII

It is proposed that an active-site-directed irreversible inhibitor be designed and investigated for the enzyme creatine kinase. This enzyme catalyzes the following reaction:

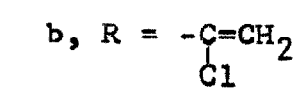


Creatine (IX) Phosphocreatine (X)

A possible candidate for such an alkylating inhibitor would be chloroacetylcreatine (XIa). This could be synthesized by an adaption of the method of Traube (11) for the chloroacetylation of guanidine.



XIa, R = $-CH_2Cl$



Chloroacetylcreatine should bind reversibly to the enzyme at the active site, at the position which accomodates creatine (IX) or phosphocreatine (X). Once bound to the active site, XIa could alkylate an amino acid residue in the immediate vicinity and irreversibly inactivate the enzyme. Degradation of the enzyme and identification of the alkylated residue would give some

insight as to a particular amino acid present at the active site.

Creatine kinase contains two unusually reactive cysteine residues. It has been shown that the enzyme is a dimer, and that the amino acid sequence around each of these "reactive" sulfhydryl groups is the same (12), thus it is probable that there is one reactive cysteine per active site. These "reactive" cysteine residues can be alkylated with iodoacetic acid or iodoacetamide (13), or with 2,4-dinitrofluorobenzene (DNFB) (14), with parallel loss of enzymatic activity. These "reactive" cysteines are so reactive that at high enzyme concentrations they can be alkylated by a stoichimetric amount of alkylating reagent (14). In view of the high reactivity of these cysteine residues towards alkylating reagents, care would have to be taken in order to avoid exclusive alkylation of these "reactive" cysteine residues by the "active-site-directed irreversible inhibitor" of creatine kinase.

Although it is known that alkylation of the essential cysteine is parallelled by loss of enzymatic activity, it is unlikely that this residue is involved in substrate binding because it is not protected from alkylation by the presence of either substrate, or by the presence of both substrates, but only by the presence

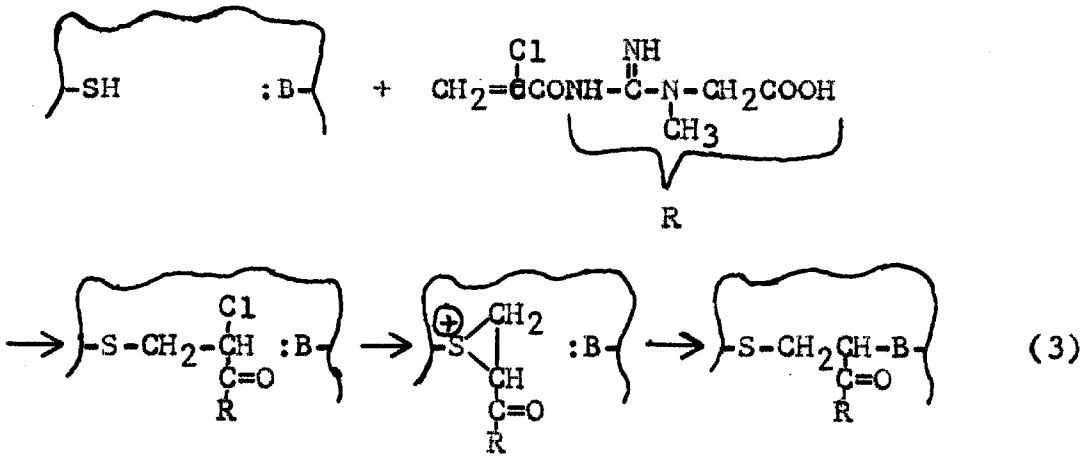
of both substrates plus the activating metal ion (15). From this evidence it would seem that the alkylation of the "reactive" cysteine by iodoacetic acid or DNFB is a diffusion controlled process. If this is so, then by working at very low enzyme and chloroacetylcreatine concentrations, random alkylation should be minimized and alkylation could be limited to those residues in the immediate vicinity of the alkylating inhibitor bound to the active site.

In the event that the "reactive" cysteine is alkylated exclusively even under the above conditions, there are several other methods which might enable one to bypass this difficulty. First, since it is very likely that the "reactive" cysteine is not directly involved in substrate binding (15), then it might be possible to protect this residue and still have an enzyme which binds the substrates efficiently. It could be protected irreversibly as the carboxymethyl- or carboxamidomethyl-derivative by first treating the enzyme with iodoacetic acid or iodoacetamide before incubating it with XIa. Or it could be protected reversibly with thiosulfate before incubation with XIa. Studies would have to be made on the ability of the protected enzyme to bind substrates and substrate analogues before this type of study could be carried out.

In the event that neither of the above methods proved successful, another alternative is available. By using an alkylating inhibitor such as 2-chloroacrylylcreatine (XIb), any problems encountered in the above methods might be avoided. It is well known that acrylic acid derivatives react with free sulfhydryl groups to give thioethers in the following manner:

$$\text{Enzyme-SH} + CH_2{=}CHCOOH \longrightarrow \text{Enzyme-S-}CH_2CH_2COOH \qquad (2)$$

By using the 2-chloroacryic acid derivative of creatine, it is conceivable that a covalently bound alkylating inhibitor could be generated near the active site of the enzyme (scheme 3). This enzyme-bound reagent could covalently attach to another residue in the immediate vicinity through a sulphur mustard intermediate.



This would lead to a cross-linked enzyme in which one of the residues is the reactive cysteine and the other would be a residue in the immediate vicinity of this cysteine. Degradation of the enzyme and identification of the alkylated residues would give useful information regarding residues involved at the active site.

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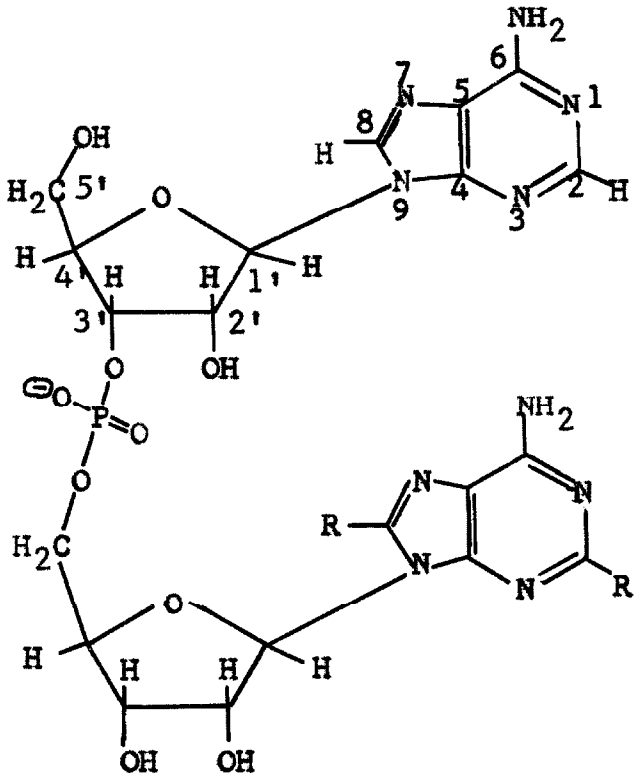
Proposition II

Chan and co-workers have recently engaged in proton magnetic resonance studies of dinucleoside monophosphates in order to accumulate information on the intramolecular base-stacking interactions of these biologically important molecules (1,2). These studies are complementary to recent findings in many laboratories which show that the base-stacking interactions of adjacent nitrogenous bases along the polynucleotide chains make a definite contribution to the stability and conformational properties of nucleic acids (3-15).

As a part of these studies, Chan and Nelson investigated adenyl (3'→5') adenosine (ApA) (Ia) by proton magnetic resonance spectroscopy (2). After studying the p. m. r. spectrum as a function of concentration, temperature, solution pH, and concentration of added purine, they determined that the stacking interaction between the two adenine rings in ApA is relatively strong, and that the adenine rings are stacked with each of the bases preferentially oriented in the <u>anti</u> conformation as in a similar dApdA (dA = deoxyadenosine) segment in helical DNA (16). They base this conclusion on the assignment of the adenine proton resonances in ApA as, in order of increasing field, $H_8(5')$, $H_8(3')$, $H_2(5')$, $H_2(3')$. The H_2 protons can be

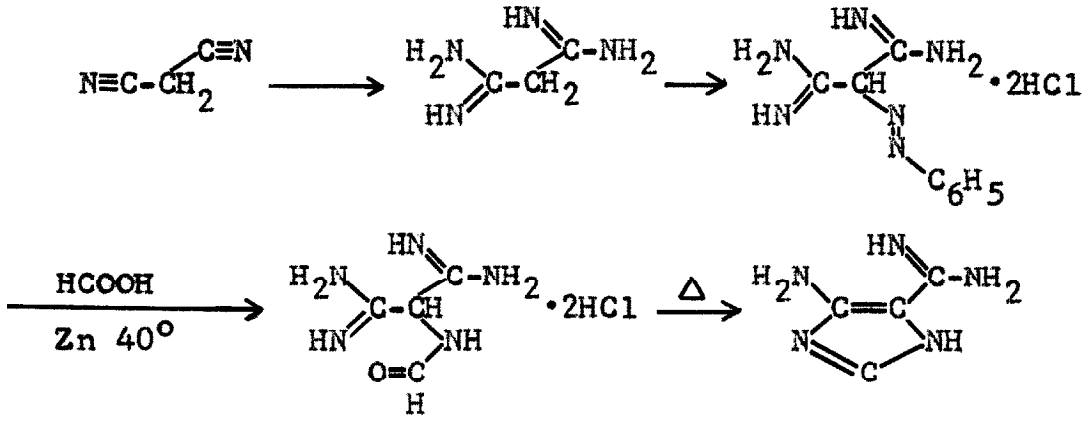
distinguished from the H_8 protons since the latter are readily exchanged in D_2O at elevated temperature (17). However, the 3'-protons are distinguished from the 5'-protons only by indirect spectral evidence.



Ia, R = H
b, R = D

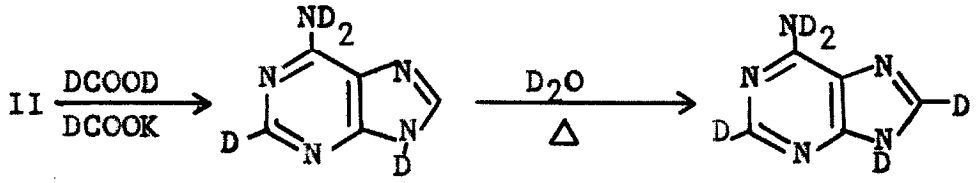
Considering the importance of the correct assignment of the 3' and 5'-proton resonances to the conclusions regarding the base-stacking interactions, it is proposed that ApA labled with $D_8(5')$ and $D_2(5')$ (Ib) be synthesized to provide the basis for a direct assignment of the

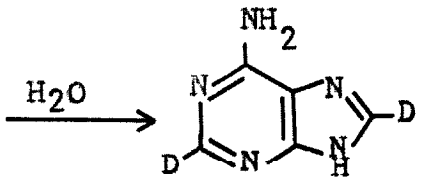
adenine protons for use in the proton magnetic resonance studies of ApA. A synthetic scheme, based on the work of Shaw (18), is given below:



II

Compound II could then be converted to adenine-2-d_1, then to adenine-2,8-d_2 (III), by the following reactions:

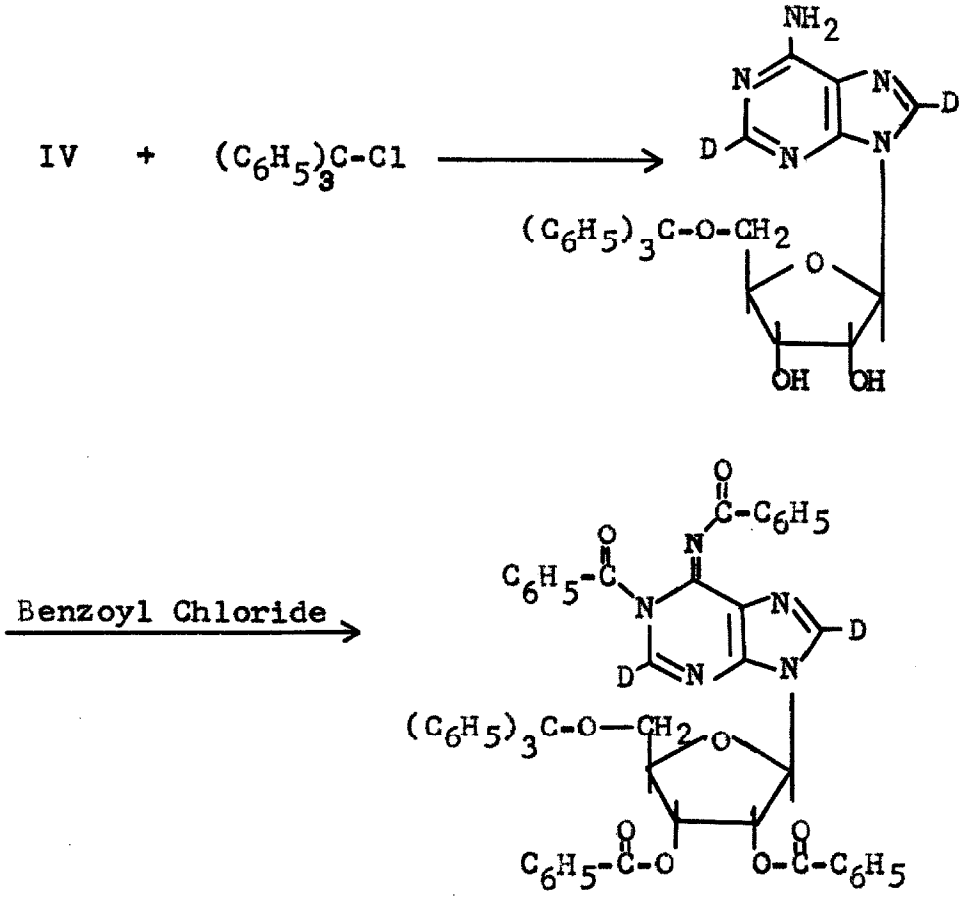


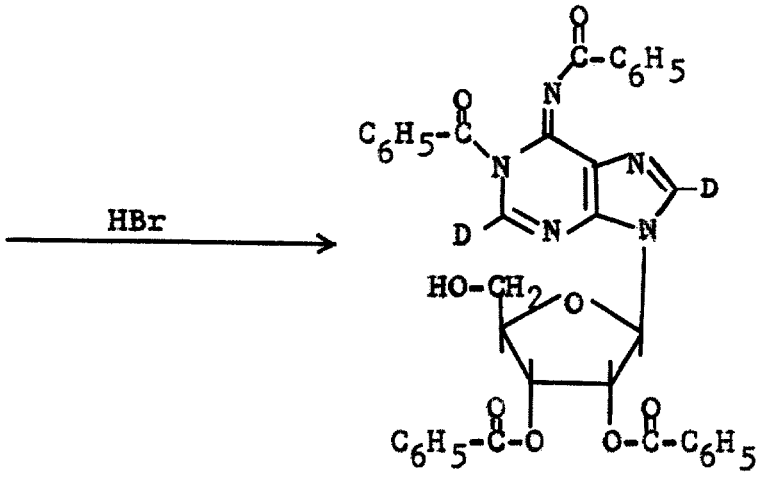


III

Then III could be converted to adenosine-2,8-d_2 (IV) by either synthetic means or by microorganisms (19). The latter method would probably be a better one as it goes in much higher yield.

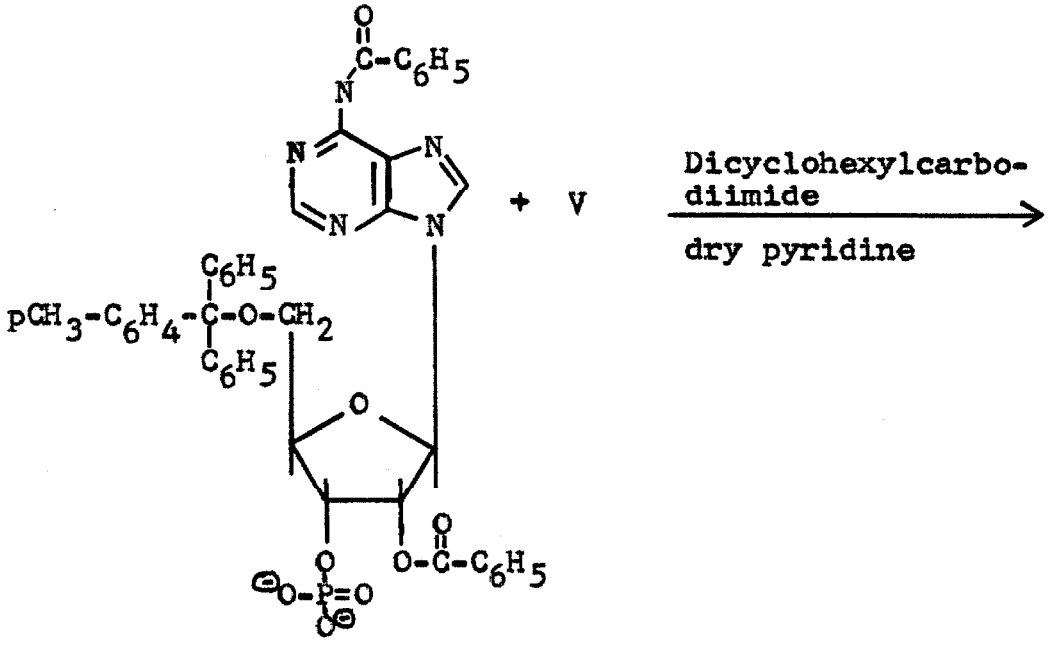
Using the elegant synthetic methods of Khorana and co-workers (20,21), IV could be converted to adenyl (3'→5') adenosine-2,8-d_2 (Ib). First, IV could be converted to N^1,N^6, 2', 3'-O-tetrabenzoyl-adenosine-2,8-d_2 (V) in greater than 60% yield by the method of Smith, et al. (20):

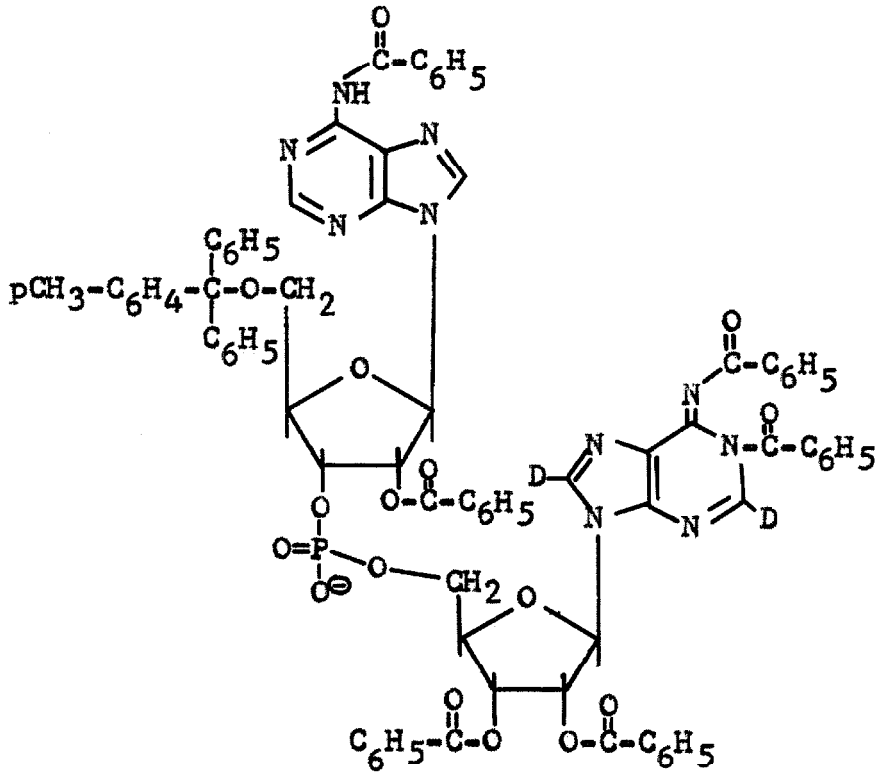




V

Then V could be coupled with unlabled adenosine-3'-monophosphate containing the appropriate blocking groups to give the fully blocked and appropriately labled ApA (VI) (21):





VI

Then the blocking groups could be removed from VI to give adenyl (3' → 5') adenosine-2,8-d_2 (Ib).

All that would remain would be to take the p. m. r. spectrum of Ib and to make the correct assignments.

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Proposition III

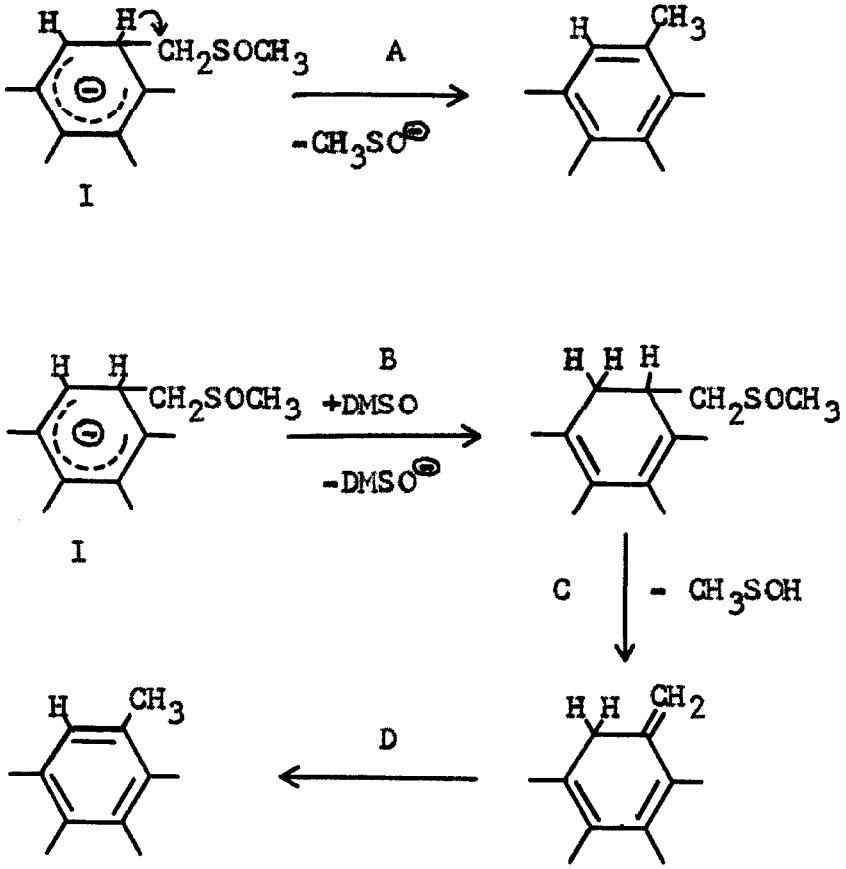
Within the past few years it has been shown that the sodium and potassium salts of dimethyl sulfoxide (dimsylsodium and dimsylpotassium) in dimethyl sulfoxide solution are efficient methylating agents for many condensed polycyclic aromatic and heteroaromatic compounds (1-3). Some of the compounds which have been studied are listed in Table 1.

Table 1. Methylation of Condensed Polycyclic Aromatics with $DMSO^{\ominus}$ at 70°C (2).

Compound	Base/Compound (mole ratio)	Reaction Time (hr)	Position of Attack (% yield)
Anthracene	5	2	9-(67)
Phenanthrene	10	4	9-(93)
Acridine	10	4	9-(98)
Isoquinoline	5.5	4	1-(98)
Quinoline	5.5	4	4-(96)

The aromatic substitution must proceed via nucleophilic attack of dimsyl anion. The products are in agreement with the calculated charge densities of the heteroaromatic compounds (4). The reaction can

be envisioned as proceeding through the negatively charged σ-complex (I). There are several mechanisms by which this complex could decay to products; it could involve a 1,2-hydride shift with liberation of methylsulfenate anion (A), or it could decay by protonation of the σ-complex by solvent (B), followed by elimination of the elements of sulfenic acid (C), and subsequent fast aromatization (D).



This type of system deserves further study. It is proposed that the mechanism of this type of reaction be investigated by running the reaction in DMSO-d_6, using either NaH or t-BuOK as base, and that the products be analyzed indirectly for the extent of deuterium incorporation by monitoring the reaction by proton magnetic resonance spectroscopy. An appropriate polycyclic aromatic hydrocarbon would be phenanthrene. The 9, 10-hydrogen atoms of phenanthrene show a strong, uncoupled resonance at 373.1 cps (5), and this could serve as one point of observation. The other point of observation would be in the area where the methyl protons of 9-methylphenanthrene (the normal reaction product) would fall. By comparing the relative areas of each peak as a function of reaction time, one could get some idea as to the mechanism of the reaction going on. If the product contained one methyl hydrogen and one hydrogen at the 10 position, then mechanism A would be consistent. However, if the product contained only one hydrogen atom distributed between the methyl carbon and the 10 position, then mechanism BCD would be consistent, with D going through an intramolecular collapse. Of course,there are several variations of these mechanisms which might be involved, and other mechanisms which have not been considered, which could

be implicated by the experimental results.

Since a serious problem which might be encountered in the above experiment would be isotopic exchange of starting material prior to reaction, it would be necessary to monitor the deuterium content of the starting material remaining at different intervals during the reaction. If this proved difficult in DMSO-d_6 because of the rapid rate of methylation, a solvent system of DMSO-d_6 - t-BuOD - t-BuOK might be used to advantage. This system serves to slow down the rate of methylation considerably in other methylation reactions (1,6). An indication that the exchange might not be too rapid is that the methylation of 9-deuterophenanthrene resulted in only about 50% reduction of the deuterium content in the monomethylation product (3).

Another serious problem involved in an investigation such as the one proposed here would be the possible isotopic exchange of products. Thus a control experiment whereby the normal methylated product is subjected to reaction conditions, and the extent of deuterium incorporation is determined, would be in order. Since base is effectively used up as the methylation reaction proceeds, any exchange of products might be minimized by using equivalent amounts of base and starting material.

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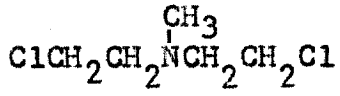
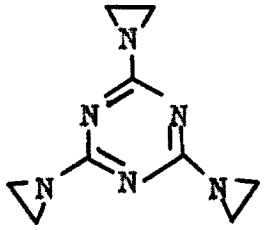
Proposition IV

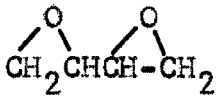
Nearly all the studies reported in the literature on the use of bifunctional alkylating inhibitors have been conducted along the lines of cancer chemotherapy. There are several publications which outline the use of such reagents as anti-tumor agents (1,2). In general, the types of bifunctional inhibitors which are normally used in such studies can be divided into four areas (1): a) nitrogen mustards, such as nitrogen mustard itself (I); b) ethylenimines, such as 2,4,6-tris (1-aziridinyl)-s-triazine (II); c) diepoxides, such as diepoxybutane (III); and d) disulfonic acid esters such as Myleran (IV). These polyfunctional reagents derive their anticancer activity from the fact that they are able to covalently cross-link various macromolecules which are necessary for tumor growth and thus inhibit their function or their reproductive process (in the case of DNA's).



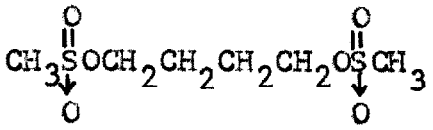
I



II



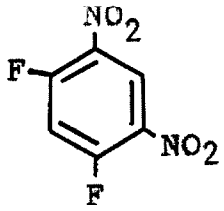
III



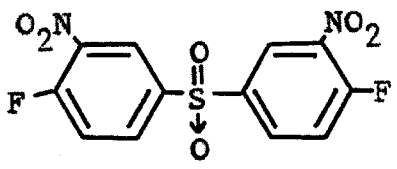
IV

There have been a few cases where bifunctional reagents have been used on purified enzymes or proteins (3-7). Zahn used 1,5-difluoro-2,4-dinitrobenzene (FFD) (V) to form covalent cross-links in insulin (3), and was able to draw several conclusions concerning the proximity of several residues in the three dimensional configuration of this protein. Wold used p,p'-difluoro-m,m'-dinitrodiphenylsulfone (VI) in similar studies on serum albumin (4).

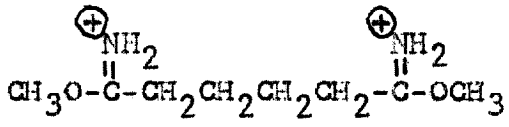
More recently, Marfey has used V in studies on ribonuclease (5,6), and has shown that lys-7 and lys-41 are in close proximity in the native conformation of this enzyme. Wold (7) has used the diimido ester, dimethyladipimidate (VII), to cross-link lys-7 to lys-37, and lys-31 to lys-37, in ribonuclease.



V



VI

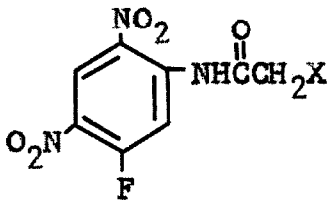


VII

The use of such bifunctional reagents on a purified enzyme should provide useful information concerning the enzyme. In the case of an enzyme whose amino acid sequence is known, such as ribonuclease, the relative proximity of certain amino acid residues could be determined, and some insight might be gained into the tertiary structure of the enzyme. By varying the distance between the alkylating functions of such reagents, it is possible that different residues could be cross-linked and the average intramolecular distances between a greater number of residues could be obtained. By varying the reactivity of the functional group, it is possible that a greater range of selectivity could be achieved.

One disadvantage of nearly all of the bifunctional alkylating inhibitors presently available is that they are usually symmetrical reagents and contain functional groups of equal selectivity and reactivity. There are instances where a bifunctional reagent containing groups of widely different reactivity could be used to advantage in the study of certain enzymes. It is thus

proposed that the bifunctional alkylating reagent, 2,4-dinitro-5-chloroacetamido-1-fluorobenzene (VIIIa) be synthesized and used in such studies. This compound could be synthesized from 2,4-dinitro-5-amino-1-fluorobenzene, which is commercially available, by chloroacetylation.



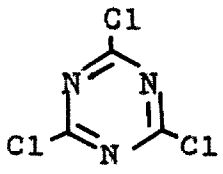
VIII a, X = Cl
b, X = Br
c, X = I

It is conceivable that by the right choice of conditions, lysine-histidine, lysine-methionine, cysteine-histidine, and cysteine-methionine cross-links could be introduced into an enzyme or protein using this reagent. These combinations of cross-links have not been obtained in the past by other investigators. By changing the chloroacetyl functional group to bromoacetyl and iodoacetyl in the proposed bifunctional reagent, a range of reactivities and selectivities could be accomplished.

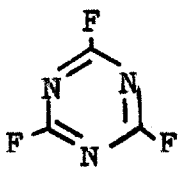
One instance where VIIIa might be useful in the determination of the three dimensional relationship between certain residues in an enzyme molecule would be in studies on the enzyme myokinase. This enzyme contains an unusually reactive cysteine residue which can be titrated stoichimetrically with 2,4-dinitrofluorobenzene (DFB) to give a completely inactive enzyme (8). By using VIIIa instead of DFB, it should be possible to generate an alkylating group at a specific site in the enzyme, and it is conceivable that a second residue in the immediate vicinity could be alkylated specifically and a cross-linked enzyme would result.

Another type of reagent which is as yet unexplored in this area, but which might prove very useful in cross-linking studies, is trichloro-s-triazine (IX). This is an activated heteroaromatic system and the chlorine atoms are susceptible to nucleophilic displacement. But, although all three chlorines are equally reactive in the parent molecule, replacement of the first chlorine atom by a more electronegative group such as a substituted amine or an alkoxide group has a deactivating effect on the other positions. Thurston, et al. (9) investigated the preparation of

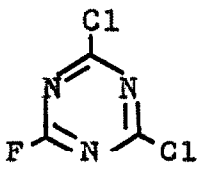
aminochloro-s-triazines from IX and ammonia in aqueous media and found that at 0-5° they obtained the monoamino compound, and that the diamino compound was obtained only at 40-45° C. Bitter and Zollinger (10) likewise reported that the first chlorine in IX is displaced at 0-10°, the second at 20-30°, and the third at 50-70°.



IX



X



XI

There have been no reports in the literature on the reaction of IX with purified proteins, although the trifluoro compound (X) has been used to determine the state of tyrosine residues in certain proteins (11-16). Trifluoro-s-triazine reacts with several amino acid side chains, among them ionized tyrosine, histidine, cysteine, and possibly tryptophan (11), but no cross-linking activity has been reported for this reagent.

It is conceivable that by an appropriate choice of reaction conditions, trichloro-s-triazine might react rather selectively with certain residues of a

protein molecule at low temperatures, and that a cross-linked protein might be obtained on raising the temperature of the reaction mixture. If difficulties are encountered in reacting the reagent with a protein at low temperatures, good use might be made of an unsymmetrical reagent such as 2,4-dichloro-6-fluoro-s-triazine (XI) (17).

This type of investigation might prove very useful in studies of the three dimensional relationships between different residues in certain proteins.

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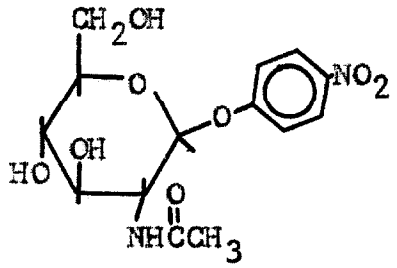
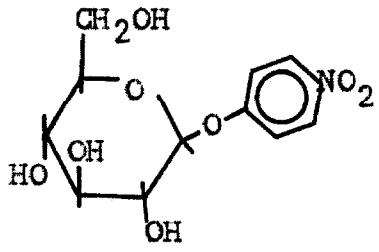
Proposition V

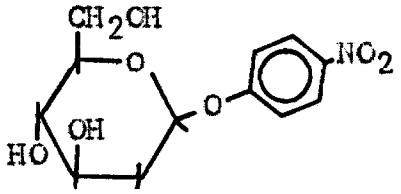
It has recently been shown that when lysozyme-catalyzed glycosidic bond cleavage of chitotetraose is carried out in the presence of p-nitrophenyl-N-acetyl-β-D-glucosaminide (I), transglycosylation occurs with the result that oligomeric p-nitrophenyl-N-acetyl-β-D-glucosaminides are enzymatically synthesized (1,2). Such oligosaccharides serve as substrates for lysozyme which release p-nitrophenol. Similar enzymatic synthesis of oligosaccharides from chitotetraose, lysozyme, and either p-nitrophenyl-β-D-glucoside (II) or p-nitrophenyl-2-deoxy-β-D-glucoside (III) was carried out, and in each case p-nitrophenol was released.



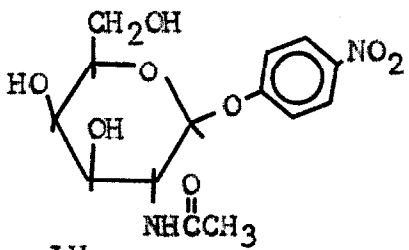
I



II



III



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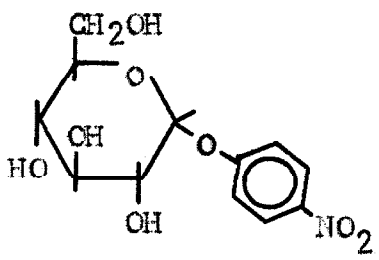

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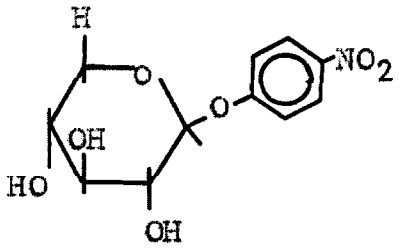
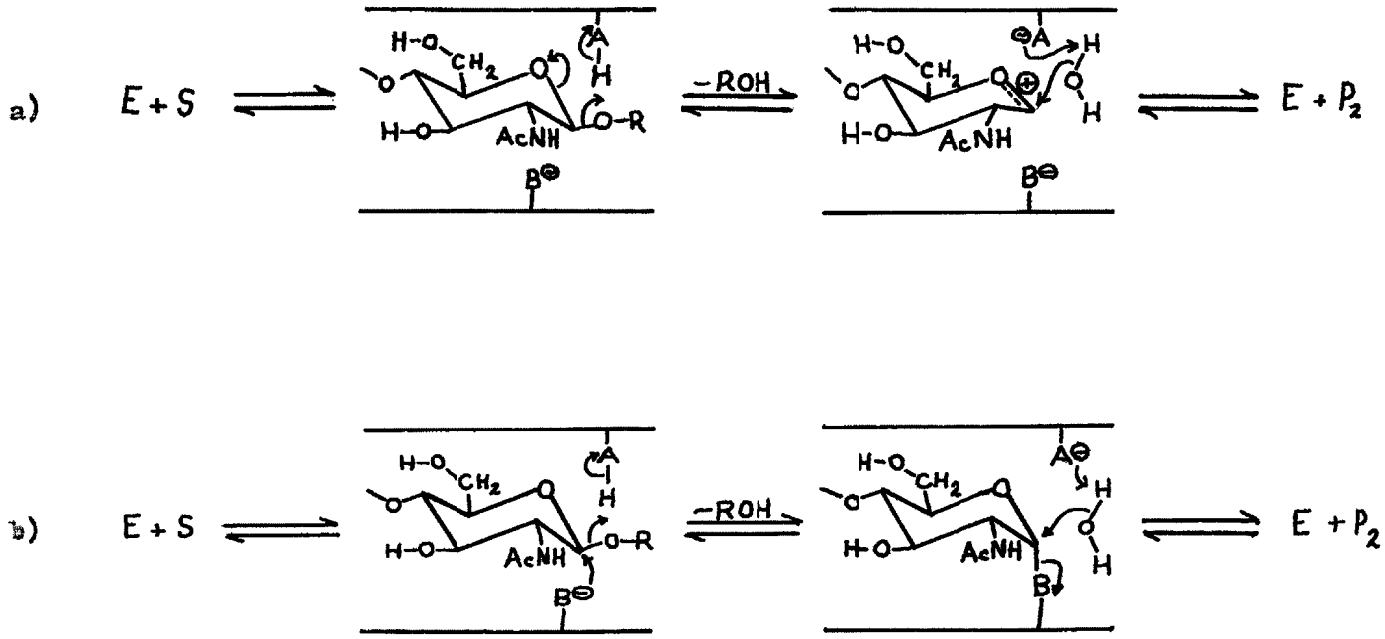


VI

The above-mentioned experiments are significant because they eliminate a mechanism involving anchimeric assistance of an N-acetyl group or C-2 oxyanion participation by substrate due to base catalysis by the enzyme. Since it has been shown that the lysozyme-catalyzed hydrolysis of glycosidic bonds proceeds with retention of configuration at carbon atom 1, that is, hydrolysis products of the β-configuration are obtained (3), this leaves only two closely related possibilities to explain the mechanistic pathway of substrate during catalysis by lysozyme. Either the hydrolysis proceeds by way of a carbonium ion which adds water stereo-specifically to give β-products (Figure 1a), or by a double displacement mechanism involving acid and nucleophilic catalysis and proceeding through a covalently bound enzyme-substrate intermediate (Figure 1b).

When transfer experiments are carried out using

Figure 1. Possible mechanistic pathways for substrate during lysozyme-catalyzed cleavage of glycosidic bonds. AH and $B^{\ominus}$ denote an acid and a base respectively which are situated at the catalytic site of the enzyme. a) "Cationic" mechanism. b) "Double displacement" mechanism.



p-nitrophenyl-N-acetyl-β-D-galactosaminide (IV), p-nitrophenyl-α-D-glucoside (V), or p-nitrophenyl-β-D-xyloside (VI), products containing the p-nitrophenyl group bonded to an oligosaccharide of at least four sugar residues are formed, but no p-nitrophenol is released (1,2). The results are summarized in Table 1.

An interesting result shown in Table 1 is the formation of intermediate molecular weight products containing the p-nitrophenyl group in the reaction using VI. Although IV, V, and VI all form products containing the p-nitrophenyl group connected to an oligosaccharide of at least four sugar residues, only in the case of p-nitrophenyl-β-D-xyloside (VI) are intermediate-sized products formed. An explanation of this result is that IV and V are transferred in a transglycosylation process, but that the p-nitrophenyl oligosaccharides thus formed do not bind effectively to the active site of the enzyme and thus no cleavage to smaller p-nitrophenyl-containing oligosaccharides or release of p-nitrophenol is observed. The p-nitrophenyl-β-D-xyloside, on the other hand, is also transferred, but the resulting oligosaccharide binds effectively and the internal glycosidic bonds are cleaved, although there is no release of p-nitrophenol.

Table 1. Reaction products from the reaction of lysozyme, chitotetraose, and p-nitrophenyl glycosides in 0.1 M citrate, pH 5.0, 10 % (v/v) dioxane, at 40° C.

p-Nitrophenyl Glycoside	High M. Wt. product containing p-nitrophenyl	Intermediate M. Wt. product containing p-nitrophenyl	Release of p-nitrophenol
β-NAG*	+	+	+
β-Glucose	+	+	+
β-2-Deoxyglucose	+	+	+
β-NAGal**	+	−	−
α-Glucose	+	−	−
β-Xylose	+	+	−

*N-Acetylglucosamine

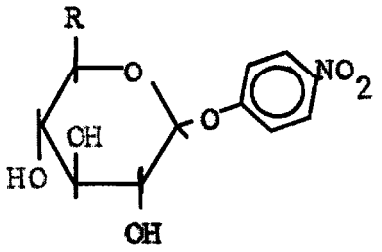
**N-Acetylgalactosamine

In comparing the structure of p-nitrophenyl-β-D-glucoside (II) with that of VI, it is seen that the only difference between the two is that the $-CH_2OH$ attached to carbon atom 5 of II is replaced by -H in VI. It has been shown that a β-(1→4) linkage results when II is used in transfer experiments (4), and it is proposed that the nature of the bond resulting from the transfer of VI be determined in a similar manner. If it could be demonstrated that a (1→4) bond resulted in each case, then the only explanation for the observed results would be that C-6 of the pyranose ring containing the glycosidic bond to be cleaved is necessary for hydrolysis.

Phillips constructed a lysozyme-substrate model from crystallographic data (5), and from the results of his model building he decided that carbon atom 6 and its adjacent oxygen atom in the sugar residue containing the glycosidic bond to be cleaved make "uncomfortably close" contacts with atoms in the enzyme molecule unless this sugar residue is distorted a little out of its most stable "chair" conformation into a conformation in which carbon atoms 1,2, and 5 and oxygen atom 5 all lie in a plane. In other words, he says that C-6 is important for steric reasons in that

it forces its sugar residue into a conformation which facilitates hydrolysis.

It is proposed that a study be made of the effect of different substituents at the 6 position of p-nitrophenyl-β-D-glucosides on the release of p-nitrophenol in the reaction system described above. Some of the compounds which could be studied are VIIa-g.



VIIa, R = $-CH_2OH$ (II)
b, R = $-CH_2SH$
c, R = $-COCH$
d, R = $-H$ (VI)
e, R = $-CH_3$
f, R = $-CH_2OCH_3$
g, R = $-COOCH_3$

From the results of such an investigation, it should be possible to draw some conclusion as to the role played by carbon atom 6 in the lysozyme catalyzed hydrolysis of glycosidic bonds. The substituent in the 6 position could be important because of stereochemical interactions which result in the enzyme-substrate complex, as proposed by Phillips (5), or possibly

for other reasons which could be revealed by the results of the proposed investigation.

References

1) M. A. Raftery and T. Rand-Meir, in press(1968).

2) C. L. Borders, Jr. and M. A. Raftery, in press(1968).

3) F. W. Dahlquist and M. A. Raftery, in press(1968).

4) T. Rand-Meir and M. A. Raftery, in press(1968).

5) D. C. Phillips, Scientific American 215, 78 (November, 1966).