PARTIAL PURIFICATION AND CHARACTERIZATION
OF DNASE I FROM THE INTESTINAL MUCOSA OF RAT

by

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ABSTRACT

DNase I activity has been found in the small intestine of the rat. This work involves a partial purification and characterization of this enzyme.

Crude enzyme extract was prepared by ultracentrifugation of the homogenate of washed mucosal scrapings. The DNase I activity of the crude enzyme preparation was not stable, it could be stabilized by divalent metal ions. The crude enzyme extract was chromatographed on DEAE cellulose in phosphate buffer and the adsorbed enzyme eluted with a phosphate gradient. The crude enzyme was shown to contain proteolytic enzymes.

The active material was freeze-dried, redissolved freed of phosphate and chromatographed on Sephadex G-100. The active material eluted from the Sephadex column was adsorbed on DEAE cellulose and eluted with a linear gradient of phosphate. This procedure gave a purification of 200-400 fold, relative to the crude enzyme extract. The product was not stable in the absence of Ca^{++} and contained proteolytic enzymes.

The molecular weight of the enzyme was estimated as $3.05 \times 10^{4}$ daltons by gel filtration on Sephadex G-100.

The properties of the rat enzyme were compared to those of the bovine enzyme. No significant difference
was found in molecular-weight, inhibition by Na⁺, K⁺, haemoglobin or iodoacetate, pH optimum, or metal ion requirements.

The interactions of Ca²⁺ with the DNA-DNase system were explored. In the presence of Mg²⁺, Ca²⁺ first activates and then inhibits DNase activity.
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INTRODUCTION

Until recently, the protein chemistry of DNase I was unknown because of the difficulties encountered in preparing highly purified samples of the enzyme. Thus, as late as 1967 molecular weight estimates varied between 60,000 and 33,000 daltons while the amino acid composition of the enzyme remained unknown. Germann and Okada (1) estimated a minimum molecular weight of 60,000 daltons by amino acid analysis, but the commercial DNase I preparation used was later shown (2) to be contaminated with 4 or 5 other proteins. The first accurate amino acid analysis was published by Lindberg (3) in 1967 and confirmed by Moore and Stein (4) in 1969. Liao et al (11) have sequenced the enzyme. Based on the amino acid and carbohydrate composition the molecular weight has been established as 30,072 daltons.

Moore, Stein and coworkers have made major contributions towards elucidating the physical and chemical properties of DNase I. Salnikow, Moore and Stein (5) chromatographed pancreatic DNase I on phosphocellulose in acetate buffer at pH 4.7 and separated DNase I into three fractions: A, B, and C. These fractions differed in number and type of sugar groups attached and in amino
acid content. DNase C contains one less histidine and one more proline than DNase A or B. The same workers have also detected a DNase D in small amounts.

Moore, Stein and coworkers (4) have demonstrated that the four half cystines in DNase I are present as S-S not S-H. They showed that the enzyme also contains sugar residues and is therefore a glycoprotein. The two disulphide bonds were found (6) to be extremely susceptible to reduction. A brief exposure to 0.05 M mercaptoethanal at room temperature caused complete reduction and inactivation of the enzyme. To achieve a similar state of reduction with most proteins, a denaturing agent such as 7 M urea is required. Once formed, the reduced enzyme is very stable; less than 5% of the reduced enzyme is reactivated when it is stored for 24 hours under aerobic conditions. Addition of calcium ion to the reduced enzyme preparation resulted in the complete recovery of activity within a few minutes, although only one S-S bond was reformed. However, only one S-S bond is necessary for activity, since the free S-H groups can be carboxymethylated without loss of enzyme activity.

It has been demonstrated that calcium ion has a strong stabilizing effect on the enzyme, protecting it from the action of proteolytic enzymes and high tempera-
tures (4). Moore and Stein have postulated that calcium may protect the DNase I found in pancreatic juice from the action of proteolytic enzymes which are present in large amounts.

Although calcium ion plays an important role in determining the three dimensional structure of the enzyme there is evidence that it is not strongly bound to the enzyme. Incubation of a sample of DNase I with $^{45}\text{Ca}$ followed by gel filtration at pH 7 removed all the $^{45}\text{Ca}$ from the enzyme protein.

Poulas and Price (7) have shown that the addition of calcium ion changes the optical rotatory dispersion spectrum. They examined the optical rotatory dispersion and circular dichroism spectra of DNase I in the presence and absence of calcium ions and concluded that calcium ion binding causes significant perturbations of tryptophan and tyrosine residues. As the calcium ion binds, the environment of these residues becomes increasingly non polar. This change has been interpreted as indicating that the enzyme is more rigid in the presence of calcium ion.

Price (8) has shown that DNase IA at pH 7.5 has two sites which bind calcium ion strongly and three weak binding sites. One of the strong binding sites is
calcium specific, since a 1000 fold excess of manganese or magnesium ions does not affect calcium binding to this site. However manganese and magnesium can compete with calcium for the second site. It seems likely, therefore, that the binding of calcium to the first site is responsible for a change in conformation of the DNase I molecule which buries the tyrosine and tryptophan within the molecule and thereby confers stability on the molecule in the presence of proteolytic enzymes.

Histidine has been implicated as part of the active site. Price, Moore and Stein (9) found that DNase I could only be inactivated by iodoacetate at pH 7.2 if divalent ions were present. Although there are 6 histidine residues in DNase I, alkylation with $^{14}$C iodoacetate gave only one labelled histidine. The inactivated protein was digested and found to contain one residue of 3 carboxymethyl histidine per molecule.

Hugle and Stein (10) selectively nitrated a single tyrosine residue and inactivated DNase I. The nitrated enzyme could not be stabilized against chymotryptic digestion by calcium. Addition of calcium ion no longer induced change in the O.R.D. spectrum. Although calcium ion did not stabilize the
nitrated enzyme against mercaptoethanal reduction, removal of excess mercaptoethanal followed by addition of calcium resulted in the rapid reformation of the S-S bonds. This suggests the presence of two calcium ion binding sites, only one of which is affected by nitration.

The complete amino acid sequence of DNase IA published by Moore and Stein (11) is shown in figure 1. The disulphide bond necessary for enzymic activity links residues 170 and 206, while the non essential disulphide bond forms a small loop between residues 98 and 101. The histidine carboxymethylated by Price, Moore and Stein in the presence of divalent metal ions was found to be histidine 131. The selectively nitrated tyrosine described by Hugle and Stein was found to be tyrosine 62. The sequence shows no repeating sections and there is no evidence to suggest the existence of two active sites.
Figure 1. The above sequence and the pairing of the half-cystine residues has been derived for bovine pancreatic DNase I A by Moore, Stein and coworkers.
KINETICS OF DNase I

The mechanism of degradation of DNA by DNase and the role of metal ions in the reaction are poorly understood. Erkama and Suutarinen (12) proposed that divalent metal ions combine with DNA to form a metalo-substrate. They used Mg-DNA as a substrate and found that added Mg$^{+2}$ was required for maximal activity, suggesting that a metalo-enzyme was also necessary. Other investigators have reached similar conclusions but the details of the mechanism remain unresolved (2).

Without speculating as to its significance Lee (13) and Melgar and Goldthwait (14) demonstrated non linear behavior of DNase I using the Lineweaver-Burk plot (31). Perlguitz and Hernondez (15), using purified Mg-DNA as a substrate, observed a biphasic curve for the activation of the enzyme by magnesium ion. Replotting the two portions of this curve as Lineweaver-Burk plots they obtained upward curved plots. The magnesium ion activation curve was sigmoidal in form and was therefore analyzed by Perlguitz and Hernondez using the equation first developed by Hill to investigate the uptake of oxygen by haemoglobin. They used the form:

$$\frac{v}{V_m - v} = \frac{Mg^{++}}{K'}$$
and found \( n = 2 \) for both sections of the curve. This suggests that two metal ions combine sequentially at each site. However, the experiments performed by Perlgut and Hernandez were done at pH 5.0 and recent work (8) has established that DNase 1 has a different number of metal ion binding sites at pH 5.0 than at pH 6.8.

Melgar and Goldthwait found that the mechanism of action of DNase 1 on DNA may be switched from a single hit mode to a double hit mode. The single hit mode is so named since a single event breaks both strands of the substrate DNA at the same locus; the double hit mode involves random hits with each hit breaking only a single strand. Double strand breaks thus occur as a result of accumulating random single strand breaks. Perlgut and Hernandez, using labelled DNA trapped in an acrylamide gel as an assay system, showed that there was an initial lag in the rate of release of labelled DNA fragments from the gel when magnesium ion alone was used as the activator. This initial lag was eliminated when manganese ion, cobalt ion, calcium ion, or a mixture of calcium ion and magnesium ion was used as an activator. The addition of sodium ion or potassium ion reestablished the lag. Melgar and Goldthwait (14) postulated that the presence of the lag indicated a double hit mechanism.
while its absence indicated a single hit mechanism. This interpretation has been confirmed by viscometric experiments analyzed by the method of Bernardi and Sadron (16) and by ultracentrifugation analysis (17).

Three possible mechanisms for single hit kinetics have been considered by Melgar and Goldthwait:

1) The enzyme may associate to form a dimer which then attacks both strands simultaneously. This is very unlikely as no dimeric form of DNase I has been reported despite intensive investigation.

2) The enzyme may have two active sites. This possibility has been eliminated by the recent work of Liao et al (11) who elucidated the entire amino acid sequence of the enzyme.

3) Binding of one DNase I molecule to DNA may facilitate the binding of a second enzyme molecule at the same site. No experimental evidence for this hypothesis exists; indeed no experiments have been reported which attempt to test this suggestion.
THE LINKAGE SPECIFICITY OF DNase I

The linkage specificity of DNase I is of great interest to those who wish to sequence DNA. If an unequivocal preference could be demonstrated the enzyme could be used as an analytical tool. Many groups have investigated this problem and contradictory data have accumulated (18).

The early experiments involved the digestion of DNA until the reaction reached equilibrium and subsequent analysis of the products. Such experimental design assumes a constant linkage specificity throughout the reaction. This assumption remains unproven. Later work (19) has shown that the DNase I reaction exhibits autoretardation. The discrepancies between different workers' results were further explained by Bollum (20) who discovered that the nature of the activating ion affects the specificity of reaction. He digested an artificial homopolymer dI:dC with DNase I in the presence of magnesium ion and found that the dC chain was completely resistant to attack. Addition of a trace of calcium ion or changing the divalent ion to manganese resulted in complete digestion of both strands.
It has been suggested (22) that the first few scissions made by DNase I are highly specific and that specificity decreases during the reaction. Sicard et al (22) showed by analytical ultracentrifugation that the scissions made in DNA by the enzyme tend to cluster; DNase I behaved as a region specific enzyme in these experiments. Unfortunately the early specificity exhibited by DNase I cannot be verified until some means of sequencing the terminal bases of long oligonucleotides is developed. Since the reason for determining the base specificity of DNase I was to use it in sequencing, it seems that data on the specificity of DNase I must await the development of some new technique for sequencing DNA.

**PROTEIN INHIBITOR**

In 1945 Laskowski (23) first reported a protein inhibitor of DNase I. It was purified from the hypertrophic epithelium of the pigeon crop gland. Protein inhibitors have subsequently been found in other tissues (24). Lindberg (25) purified two DNase I inhibitors from bovine spleen. He determined the molecular weight and amino acid composition of one which he designated "spleen inhibitor II".
Spleen inhibitor II was shown by gel filtration to form a 1:1 molecular complex with DNase I. A sample of DNase I was chromatographed on Sephadex G-100 and eluted at the expected position. Addition of excess inhibitor to DNase I abolished the peak of enzyme activity which was replaced by a peak eluting at the position expected for 1:1 enzyme-inhibitor complex. This complex could be dissociated in 3 M urea to give DNase I and an irreversibly denatured inhibitor. The inhibitor was specific for DNase I. It did not inhibit DNase II, endonuclease I from E. Coli or RNase.

**BIOLOGICAL ROLE OF DNase**

The physiological role of DNase I remains uncertain although there is much indirect evidence which suggest that DNases are involved in DNA synthesis. Allfrey and Mirsky (26) found a positive correlation between the DNase content of a variety of animal tissues and the rate of tissue proliferation or regeneration. Other investigators have extended these observations to plants (27) and bacteria (28).
Although there is much circumstantial evidence, no experimental data exist which indicate precisely how DNase is involved in DNA synthesis. It is known that some bacterial cells possess restriction enzymes which destroy DNA not labelled with the cells "tag" - a sugar group on the 5-hydroxymethylcytosine (29). DNases could serve a similar function in the mammalian cell, however, "tagged" mammalian DNA has never been observed. Alternatively, the enzyme could serve as a repair enzyme by removing mismatched bases and unordered loops of single stranded DNA caused by ultraviolet radiation damage. However, this is an unlikely function of the DNase I since it prefers native to single stranded DNA. DNases with the opposite preference have been reported (59).

Richardson, Schildkraut and Kornberg (30) showed that small amounts of endonuclease I added to a DNA polymerase system greatly increased the rate of synthesis by "nicking" the B. Subtiles DNA primer-template and producing additional 3' OH groups. It is attractive to speculate that DNase I functions by "nicking" the DNA primer-template, especially as it has been suggested that the first few breaks made by the enzyme are highly specific.
The presence of DNases in the cell throughout the entire cell cycle implies that the cell possesses a mechanism for the control of these powerful nucleolytic agents. Protein inhibitors of DNase I are well characterized but their biological function is not well delineated. It has not been possible to dissociate the inhibitor enzyme complex under physiological conditions. While it seem likely that DNases are involved in synthesis, the mechanism of their involvement remains obscure.
A wide variety of methods have been employed to assay DNase I activity. These include loss of viscosity by DNA solutions (31), release of acid soluble oligonucleotides (32), release of bound DNA from a polyacrylamide matrix (33), hydrolysis of DNA in a DNA gel (34), the loss of biological activity of circular phage DNA (35), and immunological methods (36). Three assays have been used in the present work. First, the estimation of the hyperchromicity of a DNA solution, second the measurement of the amount of acid soluble oligonucleotides released, and third the determination of the amount of DNA hydrolysed in a DNA agar gel by measuring the zone of clearing.

**KUNITZ ASSAY**

The Kunitz assay measures the increase in absorbance at 260 nm of a DNA solution after the addition of enzyme. In the present work, the assay was modified by replacing the acetate buffer, pH 5.0, by M.E.S. buffer, pH 6.8. Cuvettes containing 0.15 mg of DNA in 2.9 ml of
0.1 M M.E.S. buffer, pH 6.8, 10 mM in manganese ion were prepared. The reaction was started by the addition of enzyme solution. The mixture was stirred quickly and a continuous record of the change in absorbance at 260 nm with time was obtained using a Cary 15 spectrophotometer. One unit of enzyme activity was defined as the amount producing an increase in A_{260} of 0.001 per minute under the assay conditions used. This assay is very time consuming and, in the presence of large amounts of protein, precipitation can give false results.

**ACID SOLUBLE Oligonucleotide assay**

The acid soluble oligonucleotide assay used here is a modification of the method described by Bernardi (37). Exactly the same solutions were used as described for the Kunitz assay. The assay mixtures were incubated in Sorvall centrifuge tubes at 37°C and 0.1 ml of enzyme solution was added to each mixture to start the reaction. After 15 minutes each reaction was stopped by the addition of 0.5 ml of 12% perchloric acid to give a final volume of 3.5 ml. The tubes were cooled in an
ice water bath for 10 minutes, and then centrifuged at 10,000 x g for 10 minutes. The absorbance at 260 nm of the supernatants was measured using a Cary 15 spectrophotometer. One unit of enzyme activity was defined as the amount producing an absorbance change at 260 nm of 1.0 in 15 minutes under the assay conditions. This assay is much less time consuming than the Kunitz assay since a large number of assays can be performed simultaneously. In addition, interfering proteins are removed by precipitation with perchloric acid.

The sensitivity of the Kunitz assay and the acid soluble oligonucleotide assay were compared. A series of dilutions of crude enzyme extract were prepared and assayed by both methods. Figure 2 illustrates that the acid soluble oligonucleotide assay is insensitive to low enzyme concentrations. Insensitivity to low concentrations of enzyme in the early stage of the reaction are characteristics of all assays which depend on the liberation of acid soluble oligonucleotides. This is because the first breaks in the DNA strand caused by DNase I are unlikely to produce oligonucleotides small
Fig. 2. A comparison of the sensitivities of the Kunitz assay and the acid soluble oligonucleotide assay. Dotted line represents the Kunitz assay, the solid line represents the acid soluble oligonucleotide assay. All assays were done in 0.1 M Tris buffer, pH 6.8, containing 10 mM Mn ++.
enough to be acid soluble. Larger acid insoluble oligonucleotides are not detected by this assay.

GEL ASSAY

The DNA gel assay developed by Jarvis and Lawrence (34) was also used. Agar was dissolved in a mixture of 5 ml of water and 10 ml of 0.1 M Tris-HCl buffer, pH 7.8, with heating a boiling water bath. The hot solution was allowed to cool to approximately 60° C and 20 mg of DNA dissolved in 5 ml of 0.01 M NaCl were added to give a final concentration of 1 mg per ml of DNA. The solution was made 0.01 M with respect to manganese ion. The solution was pipetted with a warm pipet onto glass slides outlined with plastic tape. A hole 3 mm in diameter was punched in the centre of the agar coated slide. The slides were incubated at 37° C for 20 hours and developed by immersion in 0.5 M HCl for 1 minute. Enzyme activity was indicated by the appearance of a clear circle surrounding the centre well. The area of this circle was related to the level of DNase I activity.

The gel assay was several fold more sensitive than either of the two assays described previously. Figure 3 shows a comparison of the gel and acid
Fig. 3. A comparison of the sensitivities of the gel assay and the acid soluble oligonucleotide assay. Samples were assayed in duplicate by both assays and the results plotted.
soluble oligonucleotide assay. Since the gel assay system was saturated at relatively low levels of DNase I activity, it cannot be used for quantitative analysis. However, it is ideal for qualitative studies and was frequently used to detect enzyme activity eluting from columns as it was the only assay used here which was not inhibited by phosphate ion (Figure 4). It was particularly valuable for the detection of enzyme activity which eluted from columns with phosphate gradients.

**PREPARATION OF THE CRUDE ENZYME EXTRACT**

Five male Wistar rats, each weighing 200-250 grams, were stunned by a blow on the head and then decapitated. The small intestine was removed immediately and flushed with cold 0.02 M phosphate buffer containing 10 mM Mg^{++}. The intestine was placed on a cold surface and split lengthwise. The intestinal mucosa cells were then scraped off with a glass slide.

The mucosal tissue was homogenized at 700 rpm in a Sorvall Omnimix for 5 minutes. The resulting foamy homogenate was centrifuged at 10,000 x g for 5 minutes. The supernatant was recentrifuged
Fig. 4. Inhibition of the gel and acid soluble oligonucleotide assay by phosphate. Dotted line represents activity by the gel assay, solid line represents activity by the acid soluble oligonucleotide assay.
at 100,000 x g for 1 hour in a Beckman model L ultracentrifuge. The pellet was discarded and the supernatant solution was designated the crude enzyme preparation. In general, 500-700 units of activity (acid soluble oligonucleotide assay) in a total volume of 100-125 ml were obtained.

Purification of DNase I on DEAE Cellulose

DEAE cellulose, DE-32, was suspended in 0.5 M HCl for 1 hour and then washed with distilled water on a Buchner funnel until the filtrate reached a pH of 4. The cake of cellulose was suspended in 0.5 M NaOH for 1 hour. It was then filtered on a Buchner funnel and resuspended in 0.5 M NaOH for a further hour. The cellulose was washed with distilled water until the pH of the filtrate was between 7 and 8.

The recycled cellulose was stored at room temperature as a suspension in 0.5 M potassium phosphate buffer until used. The DEAE cellulose slurry was poured in a column (2.5 x 20 cm) and allowed to settle under gravity. The column was washed with equilibrating buffer until the conductivities of eluant and effluent were identical.
The crude enzyme preparation (approximately 1500 mg protein) was applied to the equilibrated column and eluted with the equilibrating buffer until the absorbance at 280 nm of the washings dropped below 0.2. A linear gradient of 0.02 M to 0.25 M phosphate was applied and 10 ml fractions collected. The fractions were assayed by the acid soluble oligonucleotide assay and those containing DNase I activity were pooled and freeze dried immediately after elution.

The used columns were washed with 0.5 M potassium phosphate buffer, pH 6.8, to remove as much protein as possible and the top 2-3 cm of DEAE 32 cellulose were discarded. The DEAE cellulose was recycled each time; it could be reequilibrated and used directly but the yield and purification of the enzyme decreased.

**REMOVAL OF PHOSPHATE**

The freeze dried enzyme preparation obtained from the DEAE cellulose chromatography step contained large amounts of phosphate. It was necessary to remove this phosphate before chromatography
on Sephadex G-100 to avoid the formation of calcium phosphate.

The freeze dried material was dissolved in a minimum volume of distilled water and 1 M calcium chloride solution was added dropwise to precipitate most of the phosphate present. The white precipitate was removed by centrifugation. This precipitate could not be redisolved in distilled water and possessed no DNase I activity. It dissolved in dilute acid and was probably $\text{Ca}_3(\text{PO}_4)_2$.

It was essential to add the calcium chloride solution carefully to avoid precipitating all the phosphate and thereby removing the buffer. In some experiments the freeze dried powder was dissolved in Tris buffer instead of water so that an excess of calcium ion could be added to remove all the phosphate buffer.

**CHROMATOGRAPHY ON SEPHADEX G-100**

A column 2.5 x 90 cm was filled with Sephadex G-100 and allowed to settle overnight at 4\(^\circ\) C. It was washed with equilibrating buffer (0.025 M Tris-HCl, pH 7.5 containing 5 mM $\text{Ca}^{++}$) until the $A_{280}$ of the effluent was zero. The enzyme
preparation, which had been treated to remove phosphate, was loaded onto the column and subsequently eluted with equilibrating buffer.

The elution positions of the proteins were determined by measuring the absorption of each fraction at 280 nm and enzyme activity was detected by the gel assay and by the acid soluble oligonucleotide assay.

RECHROMATOGRAPHY ON DEAE

A DEAE column (1 x 10 cm) was packed and equilibrated as described for the 2.5 x 20 cm column. The partly purified DNase I preparation obtained from the Sephadeox chromatography step was applied directly to the column. The column was eluted with a gradient of 0.02 M to 0.1 M phosphate buffer, pH 6.8. Loss of DNase I activity was prevented by collecting 2 ml fractions in tubes which contained 1.0 ml of 0.1 M Tris-HCl buffer, pH 7.5, 0.25 M in CaCl₂. This removed all the phosphate and left the enzyme in a buffer containing calcium ion.

MOLECULAR WEIGHT DETERMINATION ON SEPHADEX G-100

A 2.5 cm x 1 m Pharmacia column, equipped with flow adaptors, was filled with swollen Sephadex
G-100 beads. The outlet was maintained no more than 20 cm below the liquid surface until the column was packed. The final height of the column was 90 cm. A continuous flow of equilibrating buffer (0.1 M Tris-HCl, pH 7.5, 5 mM in CaCl₂) was maintained through the column at all times. Samples were dissolved in no more than 5 ml of the equilibrating buffer, which was also used for elution. Five ml fractions were collected.

The marker proteins used were RNase A, Ovalbumin, and chymotrypsinogen A. RNase was detected by its enzyme activity (38) while chymotrypsinogen and ovalbumin were detected by measuring their absorbance at 280 nm using a Cary 15 spectrophotometer.

Samples of partly purified DNase I were desalted on Sephadex G-15. The desalted enzyme preparation was made 5 ml in volume with equilibration buffer and applied to the calibrated Sephadex G-100 column. Enzyme activity was detected by the acid soluble oligonucleotide assay.

Commercial DNase I was dissolved in the equilibrating buffer and chromatographed to check the calibration of the column.
POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis was performed according to Davis (39). Gels were run in batches of eight.

DETERMINATION OF pH OPTIMUM

Three buffer systems were used to cover the range from pH 4.0 to 8.0. Each buffer was used at a concentration of 0.1 M; they were prepared and used the same day. All pH values were checked with a pH meter during the experiment. The enzyme solution used was a 10 fold purified preparation obtained from a DEAE 32 cellulose column.

AMMONIUM SULPHATE FRACTIONATION

Ammonium sulfate fractionations were performed on samples of crude enzyme extract. Ten ml of crude enzyme extract were pipetted into a Sorvall centrifuge tube and solid ammonium sulfate was added to give the required concentration. The weight of salt necessary was determined from the nomogram published by Dixon (40). The amounts of ammonium sulfate added are shown in the table below:
Table I. The amounts of solid ammonium sulfate added to 10 ml of 0.02 M Tris-HCl buffer to give the required percentage solutions.

<table>
<thead>
<tr>
<th>% SAT</th>
<th>g/10 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>1.44</td>
</tr>
<tr>
<td>30</td>
<td>1.75</td>
</tr>
<tr>
<td>35</td>
<td>2.09</td>
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<tr>
<td>40</td>
<td>2.42</td>
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<tr>
<td>45</td>
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<tr>
<td>50</td>
<td>3.12</td>
</tr>
<tr>
<td>55</td>
<td>3.50</td>
</tr>
<tr>
<td>60</td>
<td>3.90</td>
</tr>
</tbody>
</table>

The mixture was stirred to dissolve all the solid ammonium sulfate and then allowed to stand at 4°C for 30 minutes. The precipitate was collected by centrifugation at 10,000 x g for 10 minutes in a Sorvall RC-2B. The supernatant was removed and the precipitate was redissolved in 10.0 ml of Tris-HCl buffer. The protein concentration of the supernatant and precipitate solutions was determined by measuring the absorbance at 280 nm. DNase I activity was measured by gel assay and by the acid soluble oligonucleotide assay.

DETERMINATION OF THE NATURE OF THE PRODUCTS OF HYDROLYSIS

Ten mg of DNA were dissolved in 10 ml of M.E.S. buffer, pH 6.8, and mixed with 0.5 ml of 200 fold
purified enzyme in a test tube. The mixture was incubated at 37°C for 3 hours. The reaction was stopped by immersing the test tube in a boiling water bath for 10 minutes. The resulting cloudy solution was clarified by centrifugation.

A column of DEAE cellulose, DE-32, (1 x 10 cm), was packed at atmospheric pressure in 0.5 M ammonium carbonate and washed with distilled water according to the method of Tomlinson and Tener (41). Five ml of the clarified digest were added and washed in with 5 ml of distilled water. A linear gradient of 250 ml of 7 M urea containing 25 ml of 0.1 M Tris-HCl and 250 ml of 7 M urea containing 0.3 M NaCl and Tris buffer was applied. The elution of nucleotides was followed by measuring the A260 of the eluted fractions.

Selected peaks were desalted by ion exchange on DEAE cellulose as described by Tomlinson and Tener. The nucleotide was eluted with 2 M ammonium carbonate and the pooled material was repeatedly taken to dryness on a rotary evaporator and redissolved until no ammonium carbonate remained.

The desalted, purified material was assumed to be mononucleotide on the basis of its elution position.
It was digested with a preparation of 5' nucleotidase dissolved in 0.1 M sodium barbital buffer, pH 9.0 (42). The phosphate liberated was assayed by a modification of the method described by Fiske and Subba Row (43).

**PHOSPHATE ASSAY**

Each sample was made 2 ml in volume with glass distilled water and an equal volume of the assay reagent (6N H$_2$SO$_4$: H$_2$O: 2.5% ammonium molybdate: 10% ascorbic acid, 1:2:1:1, v/v/v/v) was added. The tubes were covered with parafilm, shaken, and incubated at 45° C for 20 minutes. The blue colour which appeared was estimated at 800 nm.

**ASSAY FOR DNase I INHIBITOR**

Commercial DNase I was dissolved in Tris buffer, pH 7.5, and diluted to an activity of 5 units per ml as determined by the acid soluble oligonucleotide assay. Protein solutions suspected to contain inhibitors of DNase I were pipetted into Sorvall centrifuge tubes containing the solutions for the acid soluble oligonucleotide assay. The standardized DNase I solution was added and the tubes were incubated and processed as described previously.
RESULTS AND DISCUSSION

PREPARATION OF CRUDE ENZYME EXTRACT

Two different procedures have been adopted to disrupt the intestinal mucosa cells and solubilize the DNase activity. In the one case enzyme preparations were obtained by homogenizing the tissue for long periods of time at low speeds in an attempt to avoid foaming and possible denaturation. The second method involved short homogenization times and high speeds to effect cell breakage as quickly as possible. Dixon and Webb (44) state that many enzymes are denatured at surfaces, for this reason it is important to avoid the formation of foam.

Large amounts of foam were produced in the second method where high speed homogenization was employed; indeed more than half of the crude enzyme extract was present as a thick pink foam which took approximately fifteen minutes to settle.

Denaturation occurs when the protein molecule is opened either by unfolding or separation of the adjacent portions of polypeptide chains. This is
usually caused by the breaking of a large number of hydrogen bonds. Bovine DNase I has been shown (11) to possess a disulfide bond which is essential for activity and joins residues 170 and 206. Separation of the adjacent portions of the polypeptide chain in the carboxyl terminal end of DNase I cannot occur without prior reduction of the disulfide bridge. It is not known whether DNase I can be denatured at a surface if the essential disulfide bridge is intact. However, samples of acetone powder in buffered 7 M urea exhibited considerable DNase I activity suggesting that the enzyme is not easily denatured by unfolding.

The crude enzyme extracts prepared by low speed homogenization possessed both lower total activity and lower specific activity than the enzyme preparations obtained by the short high speed homogenization procedure. It seems likely therefore, that proteolysis rather than denaturation is the major cause of loss of DNase I activity in the preparations obtained by low speed homogenization.

**STABILITY OF DNase I PREPARATIONS**

Figure 5 shows a time course of the loss of
Fig. 5. Time course of the loss of DNase I activity from a crude enzyme preparation in 0.02 M Tris-HCl buffer, pH 7.2, containing no divalent metal ions. Activity was determined by the acid soluble oligonucleotide assay.
activity of a crude enzyme preparation stored at 4°C in 0.02 M Tris buffer. Enzyme activity declined rapidly and half the enzyme activity had disappeared within 8 hours. The crude enzyme preparation remained clear throughout the experiment and there was no evidence of precipitation. The addition of divalent metal ions helps maintain enzymic activity of the crude preparation (4). In the presence of 10 mM Mg++, DNase I activity was stable for at least 2 days at 4°C. However, storage of crude enzyme preparations at 4°C for more than 2 days generally resulted in bacterial contamination and subsequent loss of between 30% and 50% of the activity of the fresh preparations. For this reason the crude enzyme preparations were not stored prior to purification.

With increasing purification the enzymic activity was no longer stabilized by magnesium ion. Addition of 5 mM calcium ion, however, preserved activity for several days at 4°C. The more highly purified enzyme preparations, therefore, were stored in 0.1 M Tris buffer, pH 7.5, containing 5 mM calcium ion at 4°C or as a freeze dried powder at -20°C.
Ion exchange chromatography was adopted as the first step in the purification of DNase I. Crude enzyme extract (approximately 1500 mg of protein) was loaded onto a DEAE cellulose column, 2 x 25 cm, prepared as described previously. A large amount of protein containing 5% to 10% of the total eluted enzyme activity washed through the column without being retained. The absorbed protein was eluted with a linear phosphate gradient of 0.02 M to 0.25 M (Figure 6). Two major peaks of protein were fractionated. The first of these contained 90% to 95% of the eluted DNase activity. The second peak contained much protein but was devoid of enzyme activity. Figure 6 shows the relative areas of these three protein peaks. The amount of protein in the first and third peaks represents 90% of the total eluted protein. This protein was discarded. The theoretical purification based on 100% recovery of the enzyme was 10 fold. In general, however, the DEAE cellulose chromatography step afforded only a 6-8 fold purification, indicating that some enzyme activity was lost during the procedure.
Fig. 6. Chromatography of a crude enzyme extract of DNase I on DEAE cellulose column equilibrated with 0.02 M phosphate buffer, pH 6.8, containing 10 mM Mg++. Elution with a linear phosphate gradient 0.02 M to 0.25 M also containing 10 mM Mg++. Dotted line represents absorption at 280 nm, solid line represents units of DNase I activity, dotted and dashed line represents conductivity of elution gradient.
REMOVAL OF PHOSPHATE

The partly purified enzyme from the DEAE cellulose step was freeze-dried immediately after elution. This yielded a white powder containing large amounts of phosphate. The phosphate was removed by precipitation with calcium ion. This was necessary as calcium ion is required to preserve the activity of the more highly purified enzyme preparations. The amount of calcium ion added as a precipitating agent was crucial. Addition of excess calcium ion precipitated all the phosphate and removed the buffering capacity, which resulted in precipitation of protein and DNase I. This could be avoided by adding Tris buffer to the partly purified enzyme preparation before the addition of calcium ion.

The addition of too little calcium ion removed insufficient phosphate. When samples treated in this way were chromatographed on Sephadex G-100, a white precipitate formed which blocked the gel column. Such columns had a very poor flow rate and the results obtained were variable.

CHROMATOGRAPHY ON G-100

Following the removal of phosphate, the enzyme
preparation was further purified on Sephadex G-100. DNase I was retarded by the gel and separated from the bulk of the protein as illustrated in Figure 7. A large peak of material absorbing at 280 nm was eluted just before the salt peak. Since this material was eluted slightly before the salt it was probably retained because of its molecular dimensions and not because of ionic interactions with the gel. The average molecular weight of this fraction must be approximately 5,000 daltons or less since its elution volume is only slightly less than the total volume of the column. This material probably consists of amino acids and short polypeptides.

The void volume ($V_0$) of the preparative Sephadex G-100 column was determined using Blue Dextran 2000. The partition coefficient $K_{av}$ was calculated from the well known relation of Laurant and Killander (53):

$$K_{av} = \frac{V_a - V_o}{V_t - V_o}$$

for $V_e$ values from several preparations. The value of $K_{av}$ obtained was used to estimate roughly the molecular weight of DNase I from a plot of $K_{av}$ vs M.W. The molecular weight of DNase I was found to be 40,000 daltons. This is somewhat higher than the value
Fig. 7. Chromatography of a DNase I preparation on Sephadex G-100 column equilibrated with 0.01 M Tris-HCl buffer, pH 7.5, containing 5 mM Ca++. Dotted line represents absorption at 280 nm, solid line represents units of DNase I activity, dotted and dashed line represents conductivity of eluant.
obtained by analytical gel filtration and suggests that ionic interactions with the gel played no part in the separation.

**RECHROMATOGRAPHY ON DEAE 32**

The partly purified material was further purified by rechromatography on DEAE cellulose in phosphate buffer. A small amount of enzyme came through the column on application of the sample. Reduction of the amount of protein applied did not eliminate this fraction, suggesting that it was not due to overloading of the column. Catley et al (40) have shown that the carbohydrate moiety of bovine DNase IA is attached to the enzyme through an amide bond involving the \( \beta \)-COOH of aspartic acid. It was possible that the enzyme which does not absorb to the DEAE cellulose is DNase which has lost its sugar group. Such a molecule would contain an extra negative charge due to the \(-\text{COOH}\) group. This assumes that the amide is removed with, or subsequent to, the removal of the sugar groups.

When all the unbound enzyme had been washed from the column a gradient of 0.02 M to 0.10 M phosphate was applied. A single peak of protein was eluted. All the remaining DNase I activity was
found in the leading edge of this peak as is shown in Figure 8. It was essential to add calcium ion to the enzyme solution immediately after elution from the column in order to stabilize the preparation. Over 90% of the eluted enzyme activity disappeared when the preparation was stored for 12 hours in 0.02 M phosphate buffer containing 10 mM magnesium ion. A summary of the purification of DNase I is presented in Table II.

Table II. Typical results for a three step preparation of DNase I from rat intestinal mucosa. The crude enzyme preparation was chromatographed on DEAE cellulose, concentrated and applied to Sephadex G-100 and rechromatographed on DEAE cellulose.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Units of Activity</th>
<th>Total Protein</th>
<th>Specific Activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>462</td>
<td>1524 mg</td>
<td>0.3</td>
</tr>
<tr>
<td>DEAE 32</td>
<td>341</td>
<td>136 mg</td>
<td>2.5</td>
</tr>
<tr>
<td>G 100</td>
<td>240</td>
<td>20 mg</td>
<td>12.0</td>
</tr>
<tr>
<td>DEAE 32</td>
<td>60</td>
<td>1.1 mg</td>
<td>60.0</td>
</tr>
</tbody>
</table>

The enzyme was purified 200 fold relative to the crude preparation. The yield was 15%.
Fraction No.

Fig. 8. Chromatography of a DNase I preparation on a DEAE cellulose column equilibrated with 0.02 M phosphate buffer, pH 6.8, containing 10 mM Mg++. Elution with a linear phosphate gradient 0.02 M to 0.10 M also containing 10 mM Mg++. Dotted line represents absorption at 280 nm, solid line represents units of DNase I activity, dotted and dashed line represents conductivity of elution gradient.
PROPERTIES OF DNase I

Some of the characteristics of the purified and partly purified enzyme preparations were investigated. The enzyme exhibited maximal activity at pH 6.8 (Fig. 9); it was more than 10 times as active at pH 6.8 as at pH 5.0.

The divalent metal ion requirements of the enzyme were investigated. Divalent metal ions were essential for activity, the order of effectiveness in activating enzyme activity, being $\text{Mn}^{++} > \text{Mg}^{++} > \text{Ca}^{++}$. An equimolar mixture of $\text{Mg}^{++}$ and $\text{Ca}^{++}$ was almost as effective an activator as $\text{Mn}^{++}$. Figure 10 illustrates the results obtained with the 200-fold purified DNase I. As may be seen from the diagram, $\text{Ca}^{++}$ is a particularly weak activator of the enzyme.

Enzyme activity was inhibited in the presence of $\text{K}^+$ or $\text{Na}^+$ (47) or haemoglobin (48). Samples of commercial DNase I were run as controls in these experiments and found to be inhibited to a comparable extent as shown in Figure 11. Thus, the results with rat intestinal mucosa enzyme confirm those obtained by other investigators (47, 48) using bovine pancreatic DNase I.
Fig. 9. The effect of pH on the activity of 10 fold purified DNase I. The pH range was covered by acetate, Tris and M.E.S. buffers, all 0.1 M.
Fig. 10. The effect of metal ions on the activity of 100 fold purified DNase I. All measurements of activity were carried out by the acid soluble oligonucleotide assay in 0.1 M M.E.S. buffer, pH 6.8.
Fig. 11. The effect of Na⁺, K⁺ and haemoglobin on rat DNase I. The open circles represent activity of the rat enzyme, the triangles represent activity of the bovine pancreatic enzyme.
The time course of the action of DNase I on DNA was investigated. The results are shown in Figure 12. The curve obtained was sigmoidal in form which is not predicted by present knowledge on the mode of action of DNase I. The curve displays an initial lag which is an artifact of the acid soluble oligonucleotide assay. When an endonuclease such as DNase I attacks at random a long strand of DNA it is expected that the first few scissions of the DNA by the enzyme will not produce oligonucleotides short enough to be acid soluble. This means that the activity of the enzyme indicated by the assay will be lower than the actual activity.

With longer incubation time the curve plateaus; this is probably due to autoretardation. Autoretardation of the DNase I reaction has been frequently observed with the bovine enzyme.

**PRODUCTS OF HYDROLYSIS - 5' OR 3'?**

Calf thymus DNA was digested with 200 fold purified DNase I and the digest chromatographed by the method of Tomlinson and Tener (41). The mixture
Fig. 12. Time course of the action of DNase I on DNA. Assay tubes contained 0.3 mg of DNA dissolved in 2.9 ml of M.E.S. buffer, pH 6.8, containing 10 mM Mn++].
of oligonucleotides was resolved into peaks corresponding to 1, 2, or 3 degrees of polymerization.

Part of the mononucleotide material was desalted by ion exchange on DEAE cellulose after the method of Tomlinson and Tener (41). This material was digested by the method of Dixon (42) with 5' nucleotidase from Crotalus Adamanteus venom and analyzed for phosphate by the procedure described previously (see Materials and Methods). The results obtained are shown below in Table III.

Table III. Nucleotides were produced by the action of DNase I on DNA and separated by ion exchange chromatography on DEAE cellulose in 7 M urea. The nucleotides were purified, digested with 5' nucleotidase and the digest assayed for phosphate.

<table>
<thead>
<tr>
<th>Digest Number</th>
<th>Nucleotide</th>
<th>5'AMP</th>
<th>5' Nucleotidase</th>
<th>Water</th>
<th>PO₄²⁻</th>
<th>μmoles/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>0.11</td>
<td></td>
</tr>
</tbody>
</table>

The 5' nucleotidase liberated phosphate from the mononucleotide fraction, demonstrating that this fraction
contains phosphate in the 5' position. The amounts of mononucleotides and 5' AMP used, as measured by absorbance at 260 nm, were roughly equal. This result, taken together with the observation that the amounts of phosphate liberated from each fraction by 5' nucleotidase were almost equal, suggests that most, or all, of the mononucleotides were present as 5' monoesters. Unfortunately, this was not tested by digestion with 3' nucleotidase.

CHARACTERIZATION OF THE ENZYME

Based on the criteria originally listed by Laskowski(18), the enzyme purified from rat intestinal mucosa may be characterized as a DNase I type enzyme. It has a neutral pH optimum and requires divalent metal ions for activity. Laskowski proposed four major criteria for the classification of nucleases:

1) Specificity toward the sugar moiety.
   Incubation of partly purified DNase I with RNA under standard conditions produced no increase in A_{280}.

2) Endonucleolytic vs Exonucleolytic Mode of Action
   This was not tested directly but a lag in the action of DNase on DNA was observed. This suggests an endonucleolytic mode of
action since an exonuclease would produce acid soluble mononucleotides immediately.

3) **Specificity of Cleavage to Form 5' or 3' Monoesters**
   This was investigated and the enzyme was found to produce mainly 5' monoesters. However, the possibility that some 3' material was produced was not eliminated.

4) **Base Specificity**
   The base specificity of the rat enzyme was not investigated as the base specificity of DNase I is thought to change throughout the reaction. Numerous investigators have attempted to demonstrate a base specificity of DNase I but none have been successful.

   More recently, Laskowski (2) has suggested that these criteria for DNase I should be replaced by a single criterion, namely reaction with a protein inhibitor of DNase I.

   The various fractions from the first chromatographic step on DEAE cellulose were tested for the presence of a DNase I inhibitor as previously described. No evidence for the presence of a DNase I inhibitor was found. Attempts to prepare the inhibitor from rat spleen were unsuccessful.
AMMONIUM SULFATE FRACTIONATION

The results of ammonium sulfate fractionation of the crude enzyme preparation are shown in Figure 13. Most of the enzyme precipitated between 30% to 50% saturation of ammonium sulfate. This gave a two fold purification and a 75% yield. This step was not used as part of the purification procedure.

The results obtained differed significantly from those of Lee and Zbarsky (50). They found that most of the DNase I activity precipitated between 15% to 20% saturation of ammonium sulfate. This gave a 64% yield and a 20 fold purification. They also found that 85% of the protein in the crude enzyme extract was precipitated at 10% saturation of ammonium sulfate. Such results are unprecedented for extracts prepared from whole mammalian cells.

MOLECULAR WEIGHT

The molecular weight of the rat enzyme was estimated by gel filtration on Sephadex G-100. Early attempts to establish the molecular weight of the enzyme using samples containing salt gave widely differing molecular weight estimates ranging up to 40,000 daltons. In the final experiments, two, desalted,
Fig. 13. Fractionation of crude enzyme preparation by ammonium sulfate. Horizontal bars represent protein in supernatant, unshaded boxes represent protein in precipitate, curved lines represent enzyme activity.
partly purified samples of DNase I gave identical elution profiles. The peak of activity eluted in the same tube in each run. These results were further checked with a sample of commercial DNase I which also eluted in exactly the same position as the rat enzyme.

Although the principles of gel filtration have been known since 1956 when Lathe and Ruthven (51) separated polysaccharides and proteins on starch gels, no rigorous theoretical treatment exists. It is generally agreed that the total volume $V_t$ of a gel column is given by:

$$V_t = V_o + V_i + V_m$$

where $V_o$ is the volume outside the gel grains, $V_i$ is the volume inside the gel grains and $V_m$ is the volume of the gel matrix. For molecules able to penetrate part of the inner volume:

$$V_e = V_o + K_d \cdot V_i$$

where $V_e$ is the elution volume and $K_d$ is a constant. Porath (52) assumed that the accessible volume inside the gel particle was cone shaped and predicted that:

$$\sqrt[3]{K_d} \ll \sqrt{M.W.}$$

where M.W. is the molecular weight.
It is usual in preparing calibration curves for molecular weight determinations on Sephadex to plot:

\[ K_{av} \text{ vs } \log \text{M.W.} \]

\( K_{av} \) was defined by Laurant and Killander (53) as:

\[ K_{av} = \frac{V_e - V_o}{V_t - V_o} \]

and is an empirical relationship.

In the present work, the data obtained have been analyzed by both of the above methods. Analysis of the data by the method of Porath (52) indicates a molecular weight of 29,700 daltons, whereas analysis by the method of Laurant and Killander (53) indicates a molecular weight of 30,500 daltons.

Lieberman et al (54) calculated that the molecular weight of DNase I from the small intestine of rat was between 32,00 and 35,000 daltons. Commercial DNase IA has a molecular weight of 30,072 daltons (11); this value corresponds with that obtained for the DNase I from rat intestinal mucosa.
GEL ELECTROPHORESIS

A DNase I preparation, which had been partly purified on DEAE 32, was chromatographed on Sephadex G-100 as previously described. Samples of DNase I were taken from the leading edge, centre, and trailing edge of the enzyme peak together with a sample of the enzyme preparation applied to the G-100 column and analyzed by gel electrophoresis. Figure 14 shows the optical density trace obtained for the various fractions. It was expected that fractions taken from the leading edge of the enzyme peak would be comprised mainly of proteins possessing molecular weights greater than that of DNase I. Separation by polyacrylamide gel electrophoresis is dependent on charge and molecular weight. Thus, since the protein sample constituted a single fraction from an anion exchange column, it might be expected to contain proteins of similar charge. Fractionation of this sample by gel electrophoresis, therefore, would be primarily dependent on molecular weight differences. If this assumption is correct, the proteins in the fractions from the leading edge of the peak should produce bands close to the
origin of the gel. The protein species in the fractions from the middle of the enzyme peak should migrate towards the centre region of the gel while those in the fractions from the trailing edge should move to the end of the gel. This was observed as may be seen by inspection of Figure 14.

Since the total activity of each fraction and the total protein applied to each gel were known, it should be possible to identify one band as the enzyme band. Assuming that each band represents one protein and that the area under each band of the optical density traces is proportional to the amount of protein present, then \( A_e \propto E \) and \( A_e/E = K \) where \( A \) is the area of any protein band, \( A_e \) is the area of the enzyme protein band, \( E \) is the amount of enzyme present and \( K \) is a constant. The ratio \( A_n/E \) is calculated for \( n = 1 \) to \( n = \) the total number of protein bands on the gel for each gel of the series. One band should display a constant ratio of band area to enzyme activity for all the gels; this band is expected to be the enzyme band. Tables IV and V below show the results obtained.
Table IV. Activity determined by the acid soluble oligonucleotide assay and optical density at 280 nm of the samples of purified enzyme eluted from Sephadex G-100 which were subjected to gel electrophoresis.

<table>
<thead>
<tr>
<th>Fraction #</th>
<th>$A_{280}$</th>
<th>$A_{260}$ (assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>1.28</td>
<td>0.01</td>
</tr>
<tr>
<td>11</td>
<td>0.47</td>
<td>0.31</td>
</tr>
<tr>
<td>12</td>
<td>0.48</td>
<td>0.76</td>
</tr>
<tr>
<td>14</td>
<td>0.23</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Table V. Relative intensity of each protein band as determined by area under optical density traces for each of several bands from gels corresponding to fractions 11 and 12.

<table>
<thead>
<tr>
<th>Band #</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area</td>
<td>787</td>
<td>863</td>
<td>535</td>
<td>172</td>
</tr>
<tr>
<td>Relative Intensity</td>
<td>1.0</td>
<td>1.1</td>
<td>0.68</td>
<td>0.25</td>
</tr>
<tr>
<td>$A_n/E$</td>
<td>3.1</td>
<td>3.4</td>
<td>2.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Area</td>
<td>180</td>
<td>432</td>
<td>504</td>
<td>86</td>
</tr>
<tr>
<td>Relative Intensity</td>
<td>1</td>
<td>2.32</td>
<td>2.71</td>
<td>0.46</td>
</tr>
<tr>
<td>$A_n/E$</td>
<td>1.32</td>
<td>3.1</td>
<td>3.6</td>
<td>0.6</td>
</tr>
</tbody>
</table>
Fig. 14. Optical density traces of polyacrylamide gels. Gels were run at 150 V for 2 hours, stained with Coomassie Blue and destained electrophoretically.
These results suggest that either band 6 or 8 is the enzyme band. It was not possible from these data to establish which is the enzyme band. Indeed, the data obtained cast doubt on the assumption that each band represents a single protein species. Inspection of Figure 14B shows a split peak which is not resolved in Figure 14C.

An attempt to determine the exact location of the enzyme was made by assaying DNase I activity in situ in the gels. DNA was added to acrylamide gels and samples containing DNase I were subjected to electrophoresis. After electrophoresis these gels were incubated at 37° C for 24 hours and developed in 0.5 N HCl. A broad diffuse zone of clearing was observed covering the last third of the gel. Other attempts to localize DNase I activity have also failed. Flanagan (60) embedded a polyacrylamide gel in a DNA-agar gel prepared by the method previously described. The system was incubated for 24 hours at 37° C and developed in 0.5 N HCl. No DNase I activity was detected. The experiment was repeated with the polyacrylamide gels split lengthwise and then embedded in the DNA-agar gel, but no enzyme activity could be detected.
Figures 15A and 15B show optical density scans of ten fold purified Dnase I (15A) and 300 fold purified DNase I (15B). The 300 fold purified sample shows only one major peak corresponding to either peak 5 or peak 6 on Figure 14A. On the basis of these data it seems probable that band 6 represents the DNase I enzyme.
Fig. 15. Optical density traces of polyacrylamide gels. Fig. 15A shows a sample of enzyme 10 fold purified by a single chromatography on DEAE cellulose. Fig. 15B shows a sample of enzyme purified 300 fold.
INTERACTIONS BETWEEN CALCIUM ION AND DNase I

The interactions between calcium ion and DNase I are of considerable interest. In the presence of 10^{-4} M calcium ion, the bovine enzyme is stabilized against proteolysis (4). Despite the important role played by calcium ion in the stabilization of DNase I, this cation is not the most effective activator of the enzyme. Price et al (7) have suggested therefore that the bovine enzyme possesses several binding sites for metal ions. Binding of calcium ion at one site causes a change in the shape of the enzyme molecule which reduces its susceptibility to proteolytic attack. This site is specific for calcium ion.

DNase I also requires metal ions for catalytic activity. However, since the enzyme is not appreciably activated by the calcium ion concentration which protects it from proteolysis, a second binding site has been postulated. Magnesium or manganese ions at concentrations of 5 \times 10^{-3} M strongly activate the enzyme, whereas a similar concentration of calcium ion has little effect on enzyme activity.
Mg\textsuperscript{++} at a concentration of 1 mM is a weak activator while 0.2 M Ca\textsuperscript{++} is ineffective as an activator of the enzyme. In contrast, a mixture of these two ions, at the concentrations stated, strongly activates the enzyme. In the presence of 10\textsuperscript{-3} M Mg\textsuperscript{++}, a concentration of 5 \times 10\textsuperscript{-4} M calcium ion activates, whereas in the presence of higher concentrations of Ca\textsuperscript{++} DNase I activity is inhibited, as is shown in Figure 18.

To study further the inhibition by Ca\textsuperscript{++}, Lineweaver-Burk plots were derived for a number of calcium ion concentrations. As shown in Figure 16, all the lines intersect at the same point on the 1/v axes, indicating that calcium ion is a competitive inhibitor. The data obtained were analyzed by the classical Lineweaver and Burk equation (55) as follows:

\[
\frac{1}{V} = \frac{K_m}{V_m}(1 + \frac{I}{K_i})\frac{1}{S} + \frac{1}{V_m}
\]

Where \(V\) is the reaction velocity, \(K_m\) is the Michaelis-Menten constant, \(V_m\) is the maximal reaction velocity, \(I\) is the concentration of inhibitor and \(S\) is the concentration of magnesium ion. For \([I] = 0\), the slope of the line in the double reciprocal plot is \(K_m/V_m\). Thus, it is possible to estimate how strongly calcium ion binds to the DNase I-DNA system when it
Fig. 16. Lineweaver Burk plot showing the results obtained with calcium ion at different concentrations.
Fig. 17. Plot of $K_i$, the inhibitor constant for $Ca^{++}$ acting as a competitive inhibitor, against the concentration of $Ca^{++}$. 
Fig. 18. Stimulation of DNase I activity by Ca\(^{++}\) in the presence of 1.0 mM Mg\(^{++}\). Activity was determined by the acid soluble oligonucleotide assay.
is acting as a competitive inhibitor. The values of $K_i$ obtained by substitution in the equation above are tabulated in Table VI.

Table VI. Values obtained for $K_i$, the inhibitor binding constant, for different concentrations of Ca$^{++}$ in the DNase I-DNA system.

<table>
<thead>
<tr>
<th>Concentrations of Ca$^{++}$</th>
<th>5 mM</th>
<th>10 mM</th>
<th>15 mM</th>
<th>20 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Values obtained for $K_i$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>by substitution in the Lineweaver Burk equation</td>
<td>$5 \times 10^{-3}$</td>
<td>$2.8 \times 10^{-3}$</td>
<td>$2 \times 10^{-3}$</td>
<td>$6 \times 10^{-4}$</td>
</tr>
<tr>
<td></td>
<td>$2.5 \times 10^{-3}$</td>
<td>$1.4 \times 10^{-3}$</td>
<td>$1.35 \times 10^{-3}$</td>
<td>$4.6 \times 10^{-4}$</td>
</tr>
<tr>
<td></td>
<td>$2 \times 10^{-3}$</td>
<td>$1.9 \times 10^{-3}$</td>
<td>$.86 \times 10^{-3}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$1.8 \times 10^{-3}$</td>
<td>$1.05 \times 10^{-3}$</td>
</tr>
<tr>
<td>Average value for $K_i$</td>
<td>$3.3 \times 10^{-3}$</td>
<td>$1.97 \times 10^{-3}$</td>
<td>$1.32 \times 10^{-3}$</td>
<td>$5.3 \times 10^{-3}$</td>
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The results indicate that the extent of binding of calcium ion increases with increasing concentrations of this metal ion. The dependence of $K_i$ on the concentration of calcium ion is illustrated in Figure 17 where $K_i$ is plotted against Ca$^{++}$. The curved plot may be explained in either of two ways. Calcium ion may exert its effect by binding to either DNase I or DNA.

1) If Ca$^{++}$ binds to the enzyme the competitive inhibition may be explained as follows:

DNase I activity requires the binding of two metal ions at different sites. Optimal activity is obtained
when one site is occupied by calcium and the other by magnesium. Increasing the concentration of calcium increases the number of enzyme molecules which have both sites filled by calcium. This bi-calcium complex is only slightly active catalytically and so the total reaction velocity $V$ is decreased. However, since the bi-calcium complex possesses some catalytic activity, the velocity $V$ is greater than the expected velocity for the complex containing both magnesium and calcium acting alone. Since the Lineweaver Burk equation was derived for a dead end inhibitor, hydrolysis due to the bicalcium complex causes an increase in the value of $V$ over the predicted value and a decrease in $K_1$ with increasing concentration of metal ion.

2) If calcium ion exerts its effects by binding to DNA, then some ratio of calcium ion to magnesium ion must be optimal for enzyme activity to account for the observed results. This ratio is about 2:1 (Mg:Ca). If the concentrations of both metal ions are raised, the enzyme is still optimally activated, if the ratio remains 2:1. Comparison of figures 18 and 19 illustrate this point. Inhibition by calcium thus appears to be due to departure from this
In the presence of 5 mM Ca$^{++}$, activity was determined by the acid soluble oligonucleotide assay.

Fig. 19. Stimulation of DNase I activity by Mg$^{++}$ in the presence of 5 mM Ca$^{++}$. Activity was determined by the acid soluble oligonucleotide assay.
optimum ratio. The significance of the ratio is not understood.

The data obtained are not sufficient to decide between these alternatives. However, Price (8) has calculated an average dissociation constant of $1.4 \times 10^{-5}$ M for the two strong metal ion binding sites of the bovine enzyme. A number of weaker sites also exist. In the present study, the values obtained for $K_1$ ranged from $3 \times 10^{-3}$ M to $5 \times 10^{-4}$ M. This weak binding provides indirect evidence that calcium ion binds to the DNA rather than the enzyme.
DNase I activity associated with a 105,000 x g supernatant obtained from an homogenate of rat intestinal mucosa tissue was purified and characterized. The properties of this enzyme were compared with those of the bovine pancreatic DNase I, which has been intensively characterized.

1) A 3 step column chromatography procedure was employed to obtain an approximately 200 fold purification of the crude enzyme preparation. On several occasions a 400 fold purification was achieved.

2) Ammonium sulfate fractionation effected only a 2 fold purification of the crude enzyme preparation. This method, therefore, was not included in the routine purification procedure. The discrepancy between this result and the findings of Lee and Zbarsky (50) may be due to differences in technical detail, for example, control of pH.

3) The lability of the intestinal mucosa DNase I increased dramatically during the purification procedure. While magnesium ions were effective in stabilizing the crude enzyme extract, only calcium ions stabilized the most purified enzyme. Protease activity was detected in the enzyme preparations.
by the casein digestion method of Kunitz (61). The specific activity of protease increased on purification of the DNase I, indicating that the protease was being co-purified.

4) Calcium ions activated as well as stabilized DNase I activity. It was of interest, therefore, to study the binding of metal ions in the DNase I-DNA system. This was done indirectly by examining the effects on DNase I activity of a Ca\(^{++}\) plus Mg\(^{++}\) activating system in which the concentration of each metal ion was varied independently of the other. It was found that calcium ion behaved as a competitive inhibitor of DNase I activity. The inhibitor constant, \(K_i\), was estimated to be \(5 \times 10^{-3}\) M. This value differs significantly from the \(K_d\) value (dissociation constant for calcium binding to DNase I) determined by gel filtration and suggests that the metal ions are binding to the DNA.

5) On the basis of its pH optimum, inactivation by iodoacetate, sensitivity to inhibition by Na\(^+\) or haemoglobin and molecular size as estimated by gel filtration on Sephadex G-100 the DNase I enzyme from rat intestinal mucosa was identical with the bovine pancreatic DNase I. It is possible, therefore, that
these enzymes are homologous or differ only in a small number of amino acid residues.

6) If highly purified DNase I from rat intestinal mucosa were available a tryptic "fingerprint" could be prepared and compared directly with a similar "fingerprint" of the bovine pancreatic DNase I to determine whether the enzymes are indeed homologous. Any differing peptides could be readily identified since the sequence of pancreatic DNase I is known (11). If different peptides did exist, these could be analysed. Thus a complete sequence of DNase I from rat intestinal mucosa could be obtained with a minimum of experimental effort.

Unfortunately, the partially purified enzyme described here is not homogeneous. Gel electrophoresis of a preparation which had been purified 300 fold demonstrated the presence of one dominant protein band together with several minor bands. Preparative gel electrophoresis of this partially purified enzyme preparation followed by gel filtration on Sephadex G-100 might yield a DNase I preparation of rat intestinal mucosa suitable for sequencing studies.
BIBLIOGRAPHY

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