PURIFICATION AND CHARACTERIZATION OF PANCREATIC DEOXYRIBONUCLEASES

By

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PURIFICATION AND CHARACTERIZATION OF

PANCREATIC DEOXYRIBONUCLEASE

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> "I bow before Him and am silent. The farther I push forward, the farther He withdraws His bounds. The more I reflect, the less I understand. The more I gaze, the less I see, and the less I see, the more certain am I that He exists; for if He does not exist there is nothingness everywhere, and who can conceive that nothingness exists?"
>
> "Now, all things are unknown to us, and first and foremost that which we think we know."
>
> — Maeterlinck, 1928

No one does anything entirely on their own, though while engaging the task it may seem that all rests on our own shoulders. It is necessary to acknowledge those who have helped to bear the task, share the burden, and delight in its completion.

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The loving encouragement and support of my wife, Darlene, was ever present. The sacrifices necessary to complete this work were accepted by her without complaint, and more than once, her love for me made a heavy burden easier to bear.

To my son, Ryan Walter, I give special thanks, for the light in his eyes is that of the future. There is no greater blessing I have received this past year than to watch him grow and to delight with him in the new discoveries of everyday things. God grant that I might look everyday at the world with the eyes of a child.

In closing, I would like to dedicate this work to the loving memory of my brother-in-law, Ronald W. Schoen, who died of cancer on February 14, 1979. Death is a door through which we pass into a realm beyond our comprehension. May God allow me to open the door with as much courage and dignity as Ron.

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ABBREVIATIONS

A	-	Adenine
A_{280}	-	Absorbance at 280 nm
Ala	-	Alanine
Asp	-	Aspartic acid
C	-	Cytosine
°C	-	Degrees centigrade
COOH	-	Carboxyl
cm	-	Centimeter
CM	-	Carboxymethyl
Con A	-	Concanavalin A
dA	-	Deoxyadenine
dC	-	Deoxycytosine
DEAE	-	Diethylaminoethyl
dI	-	Deoxyinosine
DNA	-	Deoxyribonucleic acid
DNase	-	Deoxyribonuclease
dT	-	Deoxythymine
G	-	Guanine
g	-	Gravity
Glu	-	Glutamic acid
Gly	-	Glycine
gm	-	Gram

His - Histidine

hr - Hour

Ile - Isoleucine

l - Liter

M - Molar

µg - Microgram

µl - Microliter

mA - Milliamp

mg - Milligram

ml - Milliliter

mm - Millimeter

mM - Millimolar

MW - Molecular Weight

N - Normal

NH_2 - Amino

nm - Nanometer

P - Phosphate

psi - Pounds per square inch

SDS - Sodium Dodecyl Sulfate

Ser - Serine

-SH - Sulfhydryl group

Thr - Threonine

Tyr - Tyrosine

U - Uracil

UV - Ultraviolet

V - Volts

X - Adenine, Cytosine, Guanine or Uracil

CHAPTER I

INTRODUCTION

In animals, two types of deoxyribonucleases are observed. One of these, designated DNase I (EC 3.1.4.5), has been found in secretory organs such as the pancreas and the parotid glands. DNase I is an endonuclease which hydrolyzes DNA by splitting phosphodiester bonds yielding 5' phosphate terminated polynucleotides with a free hydroxyl group on position 3'. DNases of type I have a pH optimum between pH 7.0 to 8.0 and require divalent metal ions (Mn^{+2} or Mg^{+2}) for activity. DNases of type II are lysosomal enzymes which occur in almost all cells. Hydrolysis of DNA by DNase II occurs optimally at acidic pH (pH 4.5 to 5.0) and yields 3' phosphate terminated polynucleotides. This research study is concerned only with DNases of type I.

DNase I isolated from bovine pancreas has been the most extensively studied. Bovine DNase exists in multiple forms (designated A, B, C and D); the multiplicity observed being due to a histidine residue replacing a proline residue in the primary sequence and the presence or absence of a sialic acid residue in the carbohydrate moiety. These differences allow the chromatographic and electrophoretic separation of the various forms.

DNase multiplicity occurs in other mammalian and plant species; however, the chemical basis for the observed multiplicity has not been established. The intracellular functions of the enzyme as well as the

biological significance of the multiplicity are not yet entirely understood. Further investigation of DNase from other sources is necessary in order to determine the function of this enzyme in the cellular environment and to understand the biological role of the observed multiplicity.

Further investigation requires a suitable purification method and separation procedure. My purpose in conducting this study was to devise suitable purification procedures for pancreatic DNase from two other mammalian sources and to develop a system that would enable rapid separation and detection of the multiple forms of DNase I.

CHAPTER II

LITERATURE REVIEW

The first observation of the enzymatic breakdown of nucleic acids was reported in 1903 by Araki (1) who discovered the liquefying action of crude preparations of trypsin on gels of thymus nucleic acid. In 1905, Sachs (2) demonstrated that the liquefying action was caused by an enzyme distinct from trypsin and presented evidence that trypsin rapidly inactivates the nuclease. Several unsuccessful attempts to separate the nuclease from the inactivating proteolytic enzymes were made by De la Blanchardiére (3) in 1913.

Researchers of this period were primarily interested in the investigation of the end products from the enzymatic digestion of nucleic acids and employed relatively crude tissue extracts as the source of the enzyme. It was not until the 1940's that purified preparations of DNase from bovine pancreas were independently obtained by Fischer (4), Laskowski (5), and McCarty (6). McCarty and Avery (7) used this purified DNase preparation in a landmark experiment to show that the transforming factor of Pneumococcus was DNA in nature, thus establishing DNase as an important enzymatic tool in nucleic acid research. The potential application of DNase in this area brought renewed efforts to further purify and to more completely characterize the structure and function of DNase.

General Properties of the Enzyme

The preparation of crystalline DNase by Kunitz (8) made it readily obtainable and initiated considerable study of DNase as both an enzyme and a protein. He determined that DNase was a soluble protein of the albumin type having an isoelectric point of pH 4.7. Lindberg (9), first studied the physical and chemical properties of DNase. He elucidated an approximate amino acid composition and showed that leucine was the NH_2-terminus of the protein. Using ultracentrifugation techniques, he also determined the molecular weight to be 31,000 gm/mole. Price et al. (10) verified Lindberg's results and ascertained the protein to be a single chain of about 270 residues containing two disulfide bonds. They also established DNase to be a glycoprotein which contained glucosamine and mannose in the carbohydrate moiety. Catley et al. (11), by digestion of chromatographically purified DNase with Pronase, determined that the carbohydrate side chain was a heptasaccharide consisting of 2 N-acetylglucosamine and 5 mannose residues attached at a single point on the peptide chain to an asparagine residue through an aspartamido-hexose linkage.

The amino acid sequence and the pairing of the half-cystine residues in bovine pancreatic DNase A was derived from the structural analyses by Liao, Salnikow, Moore and Stein (12, 13). They determined DNase to consist of a single polypeptide chain of 257 amino acid residues with two disulfide bonds (Figure 1). The disulfide bond essential for activity links residues 170 and 206, while the non-essential disulfide bond links residues 98 and 101. The carbohydrate moiety was attached to asparagine^{18}, and a threonine residue was

Figure 1. Amino Acid Sequence of Bovine Pancreatic DNase from Liao et al. (13). Lines joining the half-cystine residues indicate disulfide bonds.


CARB.
LEU-LYS-ILE-ALA-ALA-PHE-ASN-ILE-ARG-THR-PHE-GLY-GLU-THR-LYS-MET-SER-ASN-
ALA-THR-LEU-ALA-SER-TYR-ILE-VAL-ARG-ARG-TYR-ASP-ILE-VAL-LEU-ILE-GLU-GLN-VAL-
ARG-ASP-SER-HIS-LEU-VAL-ALA-VAL-GLY-LYS-LEU-LEU-ASP-TYR-LEU-ASN-GLN-ASP-ASP-
PRO-ASN-THR-TYR-HIS-TYR-VAL-VAL-SER-GLU-PRO-LEU-GLY-ARG-ASN-SER-TYR-LYS-GLU-
ARG-TYR-LEU-PHE-LEU-PHE-ARG-PRO-ASN-LYS-VAL-SER-VAL-LEU-ASP-THR-TYR-GLN-TYR-
ASP-ASP-GLY-CYS-GLU-SER-CYS-GLY-ASN-ASP-SER-PHE-SER-ARG-GLU-PRO-ALA-VAL-VAL-
LYS-PHE-SER-SER-HIS-SER-THR-LYS-VAL-LYS-GLU-PHE-ALA-ILE-VAL-ALA-LEU-HIS-SER-
ALA-PRO-SER-ASP-ALA-VAL-ALA-GLU-ILE-ASN-SER-LEU-TYR-ASP-VAL-TYR-LEU-ASP-VAL-
GLN-GLN-LYS-TRP-HIS-LEU-ASN-ASP-VAL-MET-LEU-MET-GLY-ASP-PHE-ASN-ALA-ASP-CYS-
SER-TYR-VAL-THR-SER-SER-GLN-TRP-SER-SER-ILE-ARG-LEU-ARG-THR-SER-SER-THR-PHE-
GLN-TRP-LEU-ILE-PRO-ASP-SER-ALA-ASP-THR-THR-ALA-THR-SER-THR-ASN-CYS-ALA-TYR-
ASP-ARG-ILE-VAL-VAL-ALA-GLY-SER-LEU-LEU-GLN-SER-SER-VAL-VAL-GLY-PRO-SER-ALA-
ALA-PRO-PHE-ASP-PHE-GLN-ALA-ALA-TYR-GLY-LEU-SER-ASN-GLU-MET-ALA-LEU-ALA-ILE-
SER-ASP-HIS-TYR-PRO-VAL-GLU-VAL-THR-LEU-THR

established as the COOH-terminus.

Effect of Metal Ions on DNase

Essential Role of Metal Ions on DNase Activity

Initial investigators of DNase discovered that DNase required divalent metal ions for activity (4, 14). Kunitz (15) showed that the concentration of Mg^{2+} needed for activity was proportional to the DNA concentration. This was later confirmed by Wieberg (16) and by Shack and Bynum (17) who suggested that the substrate for DNase may be a metal-ion DNA complex.

Several other divalent metal ions can activate DNase, although the highest enzymatic activity was achieved with Mg^{2+} plus Ca^{2+} or with Mn^{2+} (18). The synergistic effect of Ca^{2+} and Mg^{2+} was initially encountered by Wieberg (16) who demonstrated that the activity of DNase in the presence of both metal ions, at a Mg^{2+}:Ca^{2+} ratio of 10, was 4 to 5-fold greater than the sum of the activity of DNase with Ca^{2+} alone plus Mg^{2+} alone. The synergism of Ca^{2+} and Mg^{2+} suggested that DNase may have at least two requirements for metal ions one of which was fulfilled better by Ca^{2+} while the other was fulfilled better by Mg^{2+}.

At equivalent ionic strengths, Ca^{2+} and Mg^{2+} were found to have qualitatively different effects on the enzyme. Ca^{2+} was a weak activator (16), but was effective in stabilizing DNase and preventing proteolytic inactivation (10), and was necessary to promote disulfide bond reformation after reduction (19). Mg^{2+}, on the other hand, was a good activator, but did not stabilize DNase as effectively as Ca^{2+} (10).

Results of Price (20) supported the separate function hypothesis

of the synergistic effect of Ca^{2+} and Mg^{2+} on DNase activity. Characterization of Ca^{2+} and Mg^{2+} binding through gel filtration studies employing $^{45}Ca^{2+}$ showed that the enzyme can bind from 5 to 7 Ca^{2+} ions. Two Ca^{2+} ions were found to bind strongly to DNase at pH 7.5. Lowering the pH to 5.5 allowed Mg^{2+} or Mn^{2+} to compete for one Ca^{2+} binding site. The single strong Ca^{2+} binding site present at pH 5.5 was specific for Ca^{2+}. The specificity of this site for Ca^{2+} was not overcome by 0.05 M Mg^{2+} or Mn^{2+}. The DNase molecule was also observed to possess two Mg^{2+} binding sites, though the binding of Mg^{2+} to the enzyme was found to be considerably weaker.

Although DNase has a high affinity for divalent metal ions and an obligatory requirement of them for stabilization and activity, it was found that simple gel filtration at neutral pH removed Ca^{2+} (the most tightly bound) completely from the enzyme (19).

Douvas and Price (21) hypothesized that Mg^{2+} served primarily as a counterion, neutralizing the negative charge on the DNA substrate, while Ca^{2+} bound preferentially to, and had its various effects on the DNase molecule. Their data established that the optimal concentration of Mg^{2+} depended on the concentration of DNA-P. This hypothesis was supported by Price (22), who determined that Ca^{2+} was an absolute requirement for DNase activity, but the concentration of Ca^{2+} was not directly related to the substrate (DNA) concentration.

The activation of DNase by Mn^{2+} was observed to be equal to the level of activation produced by the synergistic effect of Ca^{2+} plus Mg^{2+} (18). Electron paramagnetic resonance studies by Jouve et al. (23) of the binding of Mn^{2+} to DNase showed that three Mn^{2+} binding sites were available at pH 7.5 at 2°C. Two of these sites bound Mn^{2+}

strongly, while the third site bound Mn^{2+} weakly. The two strong binding sites were not equivalent as Ca^{2+} competed with both, while Mg^{2+} competed with only one of them.

Mn^{2+} was also found to exert a number of effects on various interactions of DNase with other molecules. The binding of one Mn^{2+} ion to the enzyme was found by Price et al. (24) to facilitate the alkylation of a histidine residue in the active site of DNase. The concentration of Mn^{2+} needed to achieve one-half of the maximum rate of inactivation by iodoacetate implied the association of the metal ion with the weak site observed by Jouve et al. (23). It was also previously observed by Price et al. (10) that Mn^{2+} stabilized DNase against proteolytic inactivation, though not as effectively as Ca^{2+}. Jouve et al. (23) suggested that the site of this binding was probably the strong Mn^{2+} binding site competed for by Ca^{2+} ions.

The effect of metal ions on DNase catalytic activity also involved the ion's interaction with the substrate (DNA). Research on the interaction of divalent metal ions with nucleic acids elucidated two sites for metal binding on DNA molecules. The sites proposed were the phosphate moieties of the sugar-phosphate backbone and the electron-donor groups on the bases (25). Various metal ions were found to have different affinities for these sites. Specifically, Mg^{2+} was found to stabilize the DNA double helix by binding to the acidic phosphate groups, while Mn^{2+} was observed to introduce a variable amount of destabilization in the ordered DNA structure by binding simultaneously to the phosphate groups and the base rings (25). These findings were supported by the elucidation of two types of binding sites on DNA, with different affinities for metal ions. The strong Mn^{2+} binding

sites were competed for by Mg^{2+} ions and were probably the phosphate groups (23). The weak Mn^{2+} binding sites which were available only for Mn^{2+} ions were found to be the electron-donor groups of the base rings comprised mainly of quanine-cytosine pairs (23).

The strong activating effect Mg^{2+} and Mn^{2+} exert on the catalytic activity of DNase was proposed to be due primarily to their interactions with DNA, either as counterions or agents which destabilize the ordered structure of the DNA molecule to allow greater access for hydrolytic enzymes, or as agents that increase the DNase-DNA interaction (23, 25).

The Effect of Metal Ions on the Structure and Stability of DNase

Price et al. (10) reported that 5 mM Ca^{2+} would fully stabilize DNase against proteolytic attack, and that as little as 0.1 mM could reduce the rate of inactivation by one-half. The stabilizing effect of Ca^{2+} on the structure of DNase was proposed as a mechanism which may permit the enzyme, which is highly susceptible to proteolytic inactivation, to remain active in the presence of the proteases excreted along with it by the pancreas.

Ca^{2+} also exerted a profound effect on the reduction and reformation of the disulfide bonds present in native DNase (19). The two disulfide bonds of DNase were reduced quickly at pH 7.2 by mercaptoethanol without a denaturing agent. The inactive fully reduced form of DNase was very stable in the absence of divalent cations. The addition of 4 mM Ca^{2+} resulted in the reformation of the disulfide bonds and full regain of activity within minutes after addition. It was also found that the presence of Ca^{2+} during the reduction resulted

in an active enzyme containing one disulfide bond and two -SH groups. The enzyme remained active even after the -SH groups were carboxymethylated with iodoacetate. It was concluded that Ca^{2+} was necessary for the native tertiary structure of the protein to be assumed.

Several effects on the structure of DNase were observed on binding of divalent metals to sites on the DNase molecule. Ca^{2+} and Mg^{2+} induced positive difference spectra due to red shifting of the spectrum of DNase (27). The effect was probably due to a conformational change upon binding of the divalent metal ion which caused movement of tryptophan and tyrosine residues to the hydrophobic interior of the protein. This finding was supported by evidence from solvent perturbation studies of DNase where the binding of Ca^{2+} to DNase resulted in complete loss of tryptophan exposure and a 62% reduction in the exposure of tyrosine to the solvent (27). Further, it was observed by Hugli and Stein (28) that binding of Ca^{2+} to DNase protected the catalytically essential tyrosine from nitration by tetranitromethane.

Poulos and Price (29) observed significant changes in the circular dichroism (CD) and optical rotatory dispersion (ORD) spectra of DNase upon binding of Ca^{2+} to the enzyme. Binding of Ca^{2+} led to a more ordered structural arrangement of the polypeptide backbone. It was reported that this structural change did not involve large alterations in a shape or volume of the protein as no change in the sedimentation coefficient of the protein was observed on binding of Ca^{2+}. They also noted that Mn^{2+} gave only a 30% increase in ellipticity at the 215 nm minimum in the CD spectrum relative to that observed with Ca^{2+}, while Mg^{2+} caused no change in ellipticity at that wavelength. This indicated that there was less structural interaction between

Mn^{2+} and Mg^{2+} and the DNase molecule than between Ca^{2+} and the enzyme. The direct interactions of the various divalent metal ions with the DNase molecule provide a structural basis for the differential effects of these ions on the activity and specificity of DNase.

The Effect of Metal Ions on the Mechanism and Specificity of DNase Catalytic Activity

The enzymatic hydrolysis of double stranded DNA was found to occur by either a mechanism in which both strands were cleaved during one encounter with the enzyme (single hit) or by a mechanism in which the enzyme cleaved only one strand per encounter (double hit). Results by Thomas (30), and independently by Schumaker et al. (31), showed that DNase I placed primarily single strand cleavages in DNA with Mg^{2+} as the activating metal ion. Subsequently, Melgar and Goldthwait (32, 33) showed that the single strand cleavage mechanism (double hit) could be converted to a double strand cleavage mechanism (single hit) by changing the divalent metal ion from Mg^{2+} to Mn^{2+}.

The shift in mechanism from double to single hit kinetics by the substitution of Mn^{2+} for Mg^{2+} was most probably due to the fact that hydrolysis between some of the deoxynucleotide residues could occur with Mn^{2+} but not with Mg^{2+}. Conclusive evidence for the specificity imposed by divalent metal ions on the mechanism of DNase hydrolysis of DNA was found by Bollum (34) from the study of hydrolysis of complementary deoxyhomopolymers by DNase. In the presence of Mg^{2+}, the extent of hydrolysis of the poly dA strand of the poly dA:poly dT homopolymer was less than that of the poly dT strand. When the substrate was poly dI:poly dC, only the poly dI strand was hydrolyzed

in the presence of Mg^{2+} as the activating metal ion. The addition of trace amounts of Ca^{2+} or the substitution of Mn^{2+} as the activating metal ion led to the cleavage of both the dI and the dC strand.

Junowicz and Spencer (35, 36) by analyzing the terminal nucleosides of oligonucleotides released by DNase after varying periods of hydrolysis showed that the specificity of the enzyme varied not only according to what divalent metal ion was used for activation of the enzyme, but also during the time course of the reaction. With either activation by Ca^{2+} or by Mg^{2+} plus Ca^{2+}, the distribution of terminal nucleosides of oligonucleotides released by DNase at any period of hydrolysis was the same; the specificity of the enzyme was found to remain constant throughout the time course of the reaction. However, in the presence of Mn^{2+} or Mg^{2+} alone, the terminal nucleosides of oligonucleotides released by DNase when partial and extensive hydrolysates were compared were found to be different.

Sufficient information to explain the mechanism of the specificity changes in the hydrolysis of DNA by DNase dependent on the activating metal ion was not available. However, the observations were found to support the concept of different functions for the activating divalent metal ions on the action and specificity of DNase.

Inhibition of DNase

To date, none of the DNases isolated from various sources have been shown to participate in any of the replication or repair processes associated with cellular functions of DNA requiring controlled hydrolysis of specific portions of the DNA molecule. Rather, DNase was found to extensively hydrolyze DNA, and it was postulated that

DNase was therefore functioning on the physiological level as a digestive enzyme. However, specific inhibitors of DNase were found which may constitute important regulating factors in controlling or modifying the activity of DNase at the molecular level.

The presence of a protein inhibitor of DNase was first observed by Dabrowska et al. (37), who found that tissue extracts of the crop gland of a pigeon had an inhibitory effect on the activity of DNase. Further study by Cooper et al. (38), showed the presence of protein inhibitors in a number of normal and pathological human and animal tissue extracts. Gupta and Herriott (39) demonstrated that the cellular elements of blood contained measurable quantities of a DNase inhibitor which was also found to be present in a latent condition in blood serum.

In 1970, Lindberg and Skoog (40) purified the inhibitor from calf thymus and determined that the interaction between the DNase and the inhibitor gave rise to a stable complex consisting of one molecule of each of the protein species. Lazarides and Lindberg (41) demonstrated that the widely occurring DNase I inhibitor was actin. Formation of the stable actin:DNase I complex resulted in the inhibition of the biological properties of both proteins; DNase could no longer hydrolyze DNA, and actin could no longer form high molecular weight polymers.

Recently, it was reported that the actin:DNase I complex was dissociated and the inhibitory effect of actin on DNase was reversed by interaction with plasma membranes. Rohr and Mannberg (42) reported that free and membrane-bound 5' nucleotidases were responsible for the dissociation of the complex with subsequent reactivation of the biological activities of both proteins. Grazi and Magri (43) confirmed

these findings and further described an independent mechanism whereby phosphorylation of actin by interaction with liver plasma membranes resulted in the loss of the inhibitory effect of actin on DNase.

The physiological significance of these interactions has not been determined. However, it was suggested that the actin:DNase I complex and the various factors that affect the association of it may operate in DNA metabolism by regulating DNase activity or these factors may regulate the function of actin, or the regulation of both proteins may be interrelated (41).

In addition to the actin inhibitor of DNase, a number of non-proteinaceous inhibitors were found to exist. Among these were compounds such as ethylenediamine tetraacetate (EDTA) and fluoride, which were found to exert their inhibitory action through their ability to remove by coordination or precipitation the activating divalent metal ions (16).

A novel inhibitor for DNase I, 2-nitro-thiocyanobenzoic acid (NTCB) was recently discovered by Liao, Wadano and McKenzie (44, 45). Though no cysteine residues were found to be present in DNase, incubation of DNase with NTCB in the presence of Ca^{2+} at a pH above 7.5 resulted in irreversible inactivation of the enzyme with resultant peptide bond cleavage at four points in the primary sequence. These cleavages occurred at the amino ends of Thr^{14}, Ser^{40}, Ser^{135} and Ser^{72}. The reagent was found to be specific for DNase I as it did not inactivate proteolytic enzymes such as lysozyme or chymotrypsin under the same conditions that produced inactivation in bovine pancreatic DNase and did not inactivate other nucleases such as ribonuclease, snake venom phosphodiesterase, or spleen acid DNase (DNase

II). However, it did inactivate malt DNase, and was therefore proposed as a specific inhibitor for DNase I.

Characterization of Various DNases

Bovine Pancreatic DNase

Soon after bovine DNase was obtained in crystalline form (8), doubts concerning its homogeneity were expressed. It was first established by Price et al. (10) who employed chromatography on sulfoethyl-Sephadex at pH 4.7, that bovine pancreatic DNase was partially separated into two active components (A and B) having almost identical properties. The major form (A) was shown to be homogenous by gel electrophoresis at pH 8.4 and gel filtration on Sephadex G-75, and its amino acid composition was found to be consistent with the molecular weight of 31,000 gm/mole determined previously by Lindberg (9).

Bovine DNase was further resolved into four components on phosphocellulose at pH 4.7 by Salnikow et al. (46). The three main fractions, DNases A, B and C were present in approximately 4:1:1 molar proportion, while DNase D was a minor component. All four forms were found to possess comparable specific activities.

In order to ensure that the observed multiplicity was not due to the formation of active degradation products as a result of the acid extraction procedure, lyophilized pancreatic fluid, after chromatography on DEAE-cellulose, was resolved into three active components when chromatographed on phosphocellulose at pH 4.7. Rechromatography of the respective fractions on phosphocellulose

identified them as DNases A, B and C.

The three major forms were isolated in sufficient quantities for comparison of their chemical properties. The amino acid analyses showed DNases A and B to have essentially identical amino acid compositions; however, DNase C had one less histidine and one more proline residue. Analyses of the composition of the carbohydrate moiety showed DNases A and C to have identical neutral carbohydrate moieties, while the carbohydrate moiety of DNase B contained one residue of sialic acid. The three DNases were found to have the same NH_2-terminal leucine and COOH-terminal threonine residues.

Peptide maps obtained by tryptic-chymotryptic hydrolysates of DNases A, B and C were used by Salnikow and Murphy (47) to verify the substitution of a single residue of proline in DNase C for histidine in DNase A. They found one peptide in DNase C that moved to a significantly less basic position relative to that in DNase A. Isolation and subsequent sequencing of this peptide demonstrated that DNase C differs from DNase A in a single substitution of His^{118} by a proline in the linear sequence of the protein. A point mutation of the CAX→CCX type in the DNase gene was proposed as the explanation for the proline-for-histidine replacement in DNase C. Comparison of the peptide map of DNase B with that of DNase A showed a unique acidic glycopeptide which further verified the presence of sialic acid in the carbohydrate moiety of DNase B.

The minor component of the four DNases in bovine pancreas, DNase D, was purified and characterized by Liao (48). The DNases were found to be strongly absorbed on DEAE-cellulose at pH 8.0 and could be eluted relatively specifically by increasing concentrations of Ca^{2+} in the

eluting buffer. The proteolytic activity present in the initial sample could be removed by this chromatographic procedure which allowed the isolation of the minor component, DNase D, in stable form.

Subsequent amino acid analyses coupled with peptide mapping of the four forms showed that the amino acid sequence of DNase D was indistinguishable from that of DNase C. However, analysis of the carbohydrate moiety showed that DNase D contained one galactose and one sialic acid residue not present in DNase C.

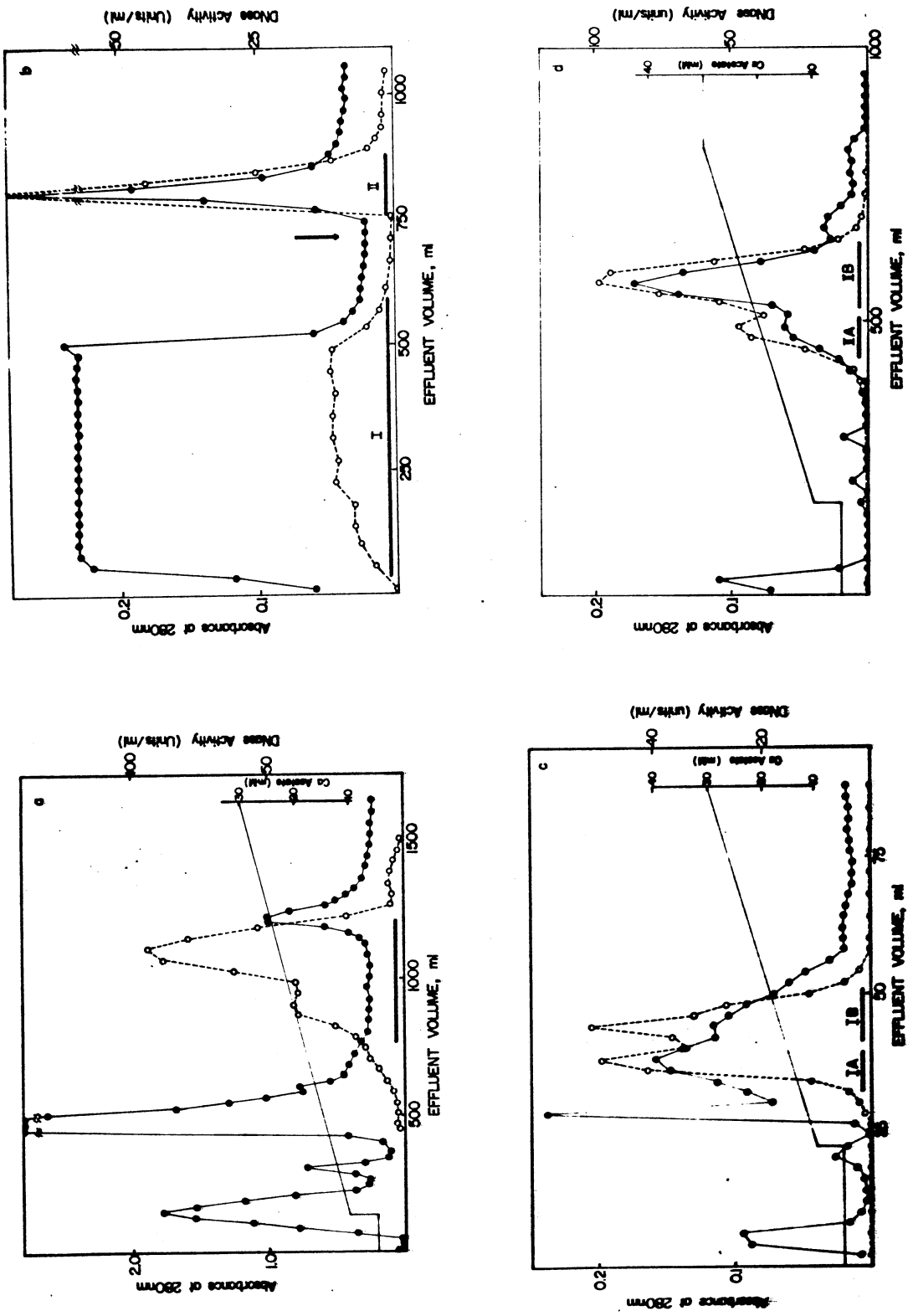
The multiplicity of bovine pancreatic DNase was therefore due to the synthesis of two amino acid sequences of DNase differing by a single substitution of proline in DNases C and D for His^{118} in DNases A and B with and without the addition of a sialic acid and a galactase residue in the carbohydrate moieties (DNases B and D were the sialoglycoproteins). These differences accounted for the chromatographic and electrophoretic properties of the four forms.

Ovine DNase

Wadano et al. (49) developed a procedure which utilized chromatography on CM-cellulose and Con A-agarose to resolve ovine pancreatic DNase into its respective components. The results of the chromatographic separation are illustrated in Figure 2. Chromatography of the pool from the initial CM-cellulose chromatography on Con A-agarose resulted in the separation of the enzyme into an unbound (I) and bound (II) component. Rechromatography of each component on individual CM-cellulose columns resolved each into two active fractions, yielding a total of four active forms.

The two fractions recovered from rechromatography on CM-cellulose

Figure 2. Purification of Ovine DNase by Wadano et al. (49). Bars indicate pooled active fractions; solid lines indicate the absorbance at 280 nm; broken lines indicate the DNase activity. (a) Chromatography of the $(NH_4)_2SO_4$ fraction on a CM-cellulose column, 1.5 X 42 cm. Elution was performed with 500 ml each of 0.01 and 0.03 M calcium acetate buffer, pH 4.7. (b) Chromatography of the active fraction from (a) on a Con A-agarose column, 2.5 X 20 cm. The column was equilibrated and eluted with 0.1 M Tris-HCl, pH 7.0, and 10 mM in $CaCl_2$. The arrow indicates the change of elution to 5% glucose in the same buffer. (c) Rechromatography of fraction I from (b) on a CM-cellulose column, 0.9 X 18 cm. Elution was performed with 200 ml each of 0.01 and 0.03 M calcium acetate buffer, pH 4.7. (c) Rechromatography of fraction II from (b) on a CM-cellulose column, 0.9 x 18 cm. Elution was performed with 200 ml each of 0.01 and 0.03 M calcium acetate, pH 4.7.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IA
IB

of the unbound component on Con A-agarose (IA and IB) were determined by SDS gel electrophoresis to be contaminated by other proteins. The fractions obtained from rechromatography on CM-cellulose of the bound component on Con A-agarose (IIA and IIB) were found to be homogenous by SDS gel electrophoresis as each was composed of a single band representing a molecular weight of 31,000 gm/mole which was identical to that found in bovine DNase. Further similarities between the bovine and ovine DNases were made apparent by end-group analyses of the ovine enzyme which indicated leucine to be the NH_2-terminal residue and threonine to be the COOH-terminus in fraction IIB of ovine DNase.

The amino acid compositions of ovine DNase IIA and bovine DNase A were compared and it was found that 12 possible amino acid residue differences existed between them (Table I). The best pairing of the residue changes from bovine to ovine was constructed assuming conservation of amino acids and single base changes. The pairings were: Glu → Asp, Thr → Ile, Tyr → Ser, Ala → Gly, 2His → 2Pro. Previously, Salnikow et al. (46) showed that bovine DNase C has five histidine and ten proline residues instead of six histidine and nine proline residues as found in bovine DNase A, making only five residue substitutions between bovine DNase C and ovine DNase fraction IIA. Since ovine DNase was not stable in Ca^{2+}-free solutions, chromatography on phosphocellulose which was used to separate the four forms of bovine DNase (46), was not applicable in the separation of ovine DNase because of the absence of Ca^{2+} in the eluting buffer. Therefore, a direct comparison of the multiple forms of ovine and bovine DNase was not possible until further more detailed characterization of ovine

TABLE I

AMINO ACID COMPOSITIONS OF OVINE DNASE

	Ovine[a]		Bovine[b]
	Fraction IIA	Fraction IIB	DNase A
Aspartic acid	32.9 (33)	33.3 (33)	32
Threonine	14.1 (14)	13.8 (14)	15
Serine	30.9 (31)	31.3 (13)	30
Glutamic acid	18.0 (18)	18.4 (18)	19
Proline	11.3 (11)	10.7 (11)	9
Glycine	10.2 (10)	10.0 (10)	9
Alanine	20.9 (21)	21.1 (21)	22
Half-Cystine	nd (4)[c]	3.8 (4)	4
Valine	23.9 (24)	23.9 (24)	24
Methionine	4.2 (4)	3.9 (4)	4
Isoleucine	12.0 (12)	12.0 (12)	11
Leucine	22.8 (23)	22.3 (22)	23
Tyrosine	13.7 (14)	13.5 (14)	15
Phenylalanine	10.5 (11)	10.7 (11)	11
Histidine	4.1 (4)	3.9 (4)	6
Lysine	9.3 (9)	9.2 (9)	9
Tryptophan	nd (3)[c]	2.3 (3)	3
Arginine	11.3 (11)	11.7 (12)	11

[a]Results were expressed as the calculated number of residues per enzyme molecules from Wadano et al. (49). The possible number of residues is in parentheses.

[b]The values for bovine DNase A are established in its sequence study which showed a total of 257 amino acid residues in the protein (13). The total number of residues in ovine DNase is also assumed to be 257.

[c]Not determined; residues were assumed from homology between fraction IIA and fraction IIB.

DNase could be performed.

Ovine DNase fraction IIB was shown from CM-cellulose chromatography (Figure 2d) to be slightly more basic than fraction IIA. Comparison of the amino acid composition of these two fractions (Table I) showed fraction IIB may have one more arginine and one less leucine residue as compared with fraction IIA, a difference which could also be explained by a single base mutation (Leu (CUX) → Arg (CGX)) in the DNase gene.

All four fractions of ovine DNase found to be devoid of sialic acid in the carbohydrate moiety. The inability of fractions IA and IB to bind to Con A-agarose suggested that these fractions may contain a carbohydrate moiety not bound by Con A-agarose or may be completely devoid of carbohydrates.

Mouse DNase

Several tissues of the mouse were found by Ball and Rutter (50) to possess DNase I activity. However, it was found that high levels of DNase I activity were present in the parotid salivary gland and in salivary secretions, while the rodent pancreas exhibited very little DNase I activity. The various DNases isolated by them were characterized on the basis of catalytic activity, electrophoretic patterns on cellulose acetate strips at pH 9.5, acid lability, and heat stability.

Mouse parotid DNase I closely resembled the bovine pancreatic DNase in many of its properties, including pH necessary for optimal activity, requirements for metal ion, and acid stability. Electrophoresis of the mouse parotid enzyme on cellulose acetate strips at

pH 9.5 with DNase activity staining using DNA-containing agar gels upon which the strips were impressed, and which were then stained with methyl green dye, revealed the mouse parotid gland extracts to consist of three closely spaced bands. In comparison, in the same electrophoretic system, bovine pancreatic extracts were shown to contain two major and three minor bands. The mouse parotid enzyme was therefore shown to also consist of multiple forms.

The DNase activity extracted from mouse pancreas was found to be acid labile and to further possess inhibitory activity toward bovine pancreatic DNase I which could be eliminated by acidification of the mouse pancreatic homogenate to pH 3.0.

The mouse liver was found to contain DNase I activity. The DNase from mouse liver was also found to be acid labile, as acidification of the homogenate to pH 3.0 resulted in loss of approximately 93% of the original activity present. The mouse liver DNase I could also be distinguished from mouse parotid DNase I by heat-stability tests. The mouse parotid preparation was found to be stable to 50°C, while the mouse liver preparation was found to lose approximately 80% of its activity at 45°C. Similar thermal inactivation was also reported for bovine liver DNase, while the bovine pancreatic DNase retained activity to 55°C.

Antonoglou and Georgatsos (51) reported mouse liver DNase to contain five multiple forms on the basis of ion exchange and gel permeation experiments on various sub-cellular fractions. They determined the DNase I activity to consist of two cationic forms, one of which was present in the nuclear and mitochondrial fractions and another in the supernatant fractions; two weakly anionic forms,

distributed in the mitochondrial and supernatant fractions, and one anionic form in both the nuclear and the supernatant fractions. The possibility of overlap in the distribution of the various forms was not accounted for, nor was electrophoretic demonstration of the multiplicity performed. However, it is evident from their experiments that multiple forms of DNase I bearing different ionic properties were present in mouse liver.

Rat Parotid DNase

Ball (52) purified DNase from the parotid gland of the rat. Chromatography on sulfoethyl-Sephadex performed in the manner of Price et al. (10) showed it to be homogenous, but the existence of multiple forms was demonstrated by disc gel electrophoresis of the purified DNase at pH 8.3. The presence of a minor band with DNase activity was detected in the purified preparation. Electrophoresis of crude parotid homogenates demonstrated the presence of the same two enzymatically active protein bands detected in the electrophoresis of the purified enzyme. The nature of the structural differences between these two forms was not determined.

Bovine Parotid DNase

Experimental evidence suggested that an inverse relationship existed between DNase activity in the pancreas and DNase activity in the parotid (53). For example, the mouse had a high level of parotid DNase activity, and a negligible level of pancreatic DNase activity (50), while the bovine species had a high level of pancreatic DNase activity and a reduced level of parotid DNase activity (54). In spite

of this low level of activity, Lunblad et al. (54) were able to purify the enzyme and partially characterize its structural and catalytic components.

Chromatography of the extracted enzyme after gel filtration on sulfopropyl-Sephadex at pH 4.7 resolved the enzymatic activity into several fractions. The major component, designated as parotid DNase A, was subjected to further chemical analyses. It was found that the parotid enzyme was similar to the pancreatic enzyme as it had an alkaline pH optimum, an obligate requirement for divalent cations, and it was found that Ca^{2+} protected the enzyme from inactivation by proteolytic enzymes. The amino acid composition of the parotid DNase A resembled that of the pancreatic enzyme very closely. Subsequent determination of the NH_2-terminal sequence identified 12 residues of the first 17 residues with certainty, and all 12 of these were identical to those present in the analogous position in the pancreatic enzyme. However, immunological differences between the parotid and pancreatic enzymes were demonstrated previously (55), and it was found in the present study that the parotid DNase A had a higher molecular weight of 35,000 gm/mole relative to the pancreatic DNase which has a molecular weight of 31,000 gm/mole (9, 13).

Human DNase

Funakoshi et al. (56) purified human pancreatic DNase I to apparent homogeneity. It had essentially properties similar to those of bovine DNase. The molecular weight determined by gel filtration was approximately 30,000 gm/mole. Maximum activity of the purified enzyme was observed at pH 7.2-7.6, and divalent cations were required for

activity. A discrepancy between the two enzymes was observed in the mode of DNA hydrolyses. Bovine pancreatic DNase I produces single-strand breaks (double-hit kinetics) in double-stranded DNA in the presence of Mg^{2+} (30, 31), but double-strand cleavages (single-hit kinetics) in the presence of Mn^{2+} or Mg^{2+} plus Ca^{2+} (32, 33). Human pancreatic DNase I was found to only produce single-stand breaks; no modification of the mechanism of cleavage occurred with a change in divalent cation present in the assay medium.

Recently, Love and Hewitt (57) have partially purified and characterized the major electrophoretic form of serum DNase and the major DNase from human pancreas. The physical properties and enzymatic characteristics of these two human DNases were found to be essentially the same and also similar to the bovine enzyme. Multiple forms of each type were demonstrated by DNA-polyacrylamide gel electrophoresis at pH 9.3. The molecular weight of the human DNase varied from 33,000-38,000 gm/mole in serum and 35,000-38,000 gm/mole in pancreatic tissue. The pI range was 3.9-4.3 for human DNase from both sources.

A notable difference between the human DNase and the bovine enzyme was observed in the ability of Ca^{2+} to restore activity after inactivation by mercaptoethanol. The activity of bovine pancreatic DNase can be restored by Ca^{2+} (19), whereas the human DNase activities cannot. Also, Price (22) has shown that bovine pancreatic DNase exhibits activity with only Ca^{2+} present in the reaction media, whereas the human DNases are inactive without Mg^{2+} under all pH conditions.

They also confirmed the finding of Funakoshi et al. (56) that human DNases from both sources exhibited predominantly double-hit kinetics under all reaction conditions, but like the bovine enzyme, human DNases

were found to form double strand cleavages more rapidly in the presence of Ca^{2+} than in its absence.

Porcine DNase

Little characterization of the porcine enzyme has been accomplished. In 1946, Laskowski (58) reported the partial purification of DNase from porcine pancreas, and that same year devised a method yielding an average 7-fold purification from the crude material (59). In the same study he confirmed the activating effect of Mg^{2+} (14) and showed that NaCl in high concentrations inhibited the enzymatic activity of porcine DNase. He also reported the interesting finding that porcine DNase was not as stable as the bovine enzyme in acid, necessitating a reduction from 0.25 N H_2SO_4 as originally suggested by Kunitz and Northrop (60) in the extraction of bovine DNase, to 0.2 N H_2SO_4 for the porcine DNase extraction. He also observed that the acid extraction procedure was advantageous because of protection of the DNase from the action of proteolytic enzymes and in allowing greater ease in centrifugation of the initial crude homogenate.

Malt DNase

A DNase similar to bovine pancreatic DNase was isolated and purified from malt barley by Liao (61). DEAE-cellulose chromatography at pH 8.0 resolved the enzyme into four distinct forms, designated as malt DNases A, B, C and D in order of elution. The two forms present in the greatest amount (A and B) were further purified by rechromatography on DEAE-cellulose using a $CaCl_2$ gradient to resolve the active

enzyme from contaminating proteins that co-eluted during the first DEAE-cellulose chromatography employing a Tris-HCl gradient. Subsequent analyses of the two fractions on disc and SDS gel electrophoresis showed them to be homogeneous. The molecular weights estimated by the SDS electrophoretic procedure were near 32,000 gm/mole, or slightly larger than the bovine pancreatic enzyme. The pH-activity profile of the barley malt enzyme in the presence of either Mn^{2+} or Mg^{2+} is similar to the bovine DNase and exhibits a pH optimum at 7.5. Also, Ca^{2+} was shown to protect the DNase from inactivation, though the instability of malt DNase was probably not due to proteolytic inactivation as malt DNases A and B were stable in crude or purified form in the presence of trypsin or chymotrypsin in the absence of Ca^{2+} for 8 hours at room temperature.

Amino acid compositions of malt DNases A and B showed many similarities to that of the bovine pancreatic enzyme with major differences in the amounts of glutamic acid, proline and glycine. Comparison of the A and B forms of malt DNase showed the A form to contain 5 more residues than the B form. The presence of an extra lysine residue in malt DNase A probably accounted for its elution ahead of malt DNase B on DEAE-cellulose chromatography.

Characterization of malt DNases C and D was not accomplished due to the small amount of each and the apparent heterogeneity they exhibited. The chemical basis for the observed multiplicity of malt DNase has not been determined.

The products of the hydrolyses of DNA by malt and bovine pancreatic DNase were indistinguishable, and further hydrolysis by snake venom phosphodiesterase of the products from digestion of DNA by malt DNase

confirmed the malt enzyme to be a 5' phosphate producer. This fact coupled with the pH optimum, metal ion requirements and inactivation by NTCB established it as a DNase I type nuclease.

CHAPTER III

MATERIALS AND METHODS

Frozen ovine and porcine pancreas were purchased from Pel-Freez. Fresh porcine pancreas was obtained from the Meat Science Department at Oklahoma State University. Bovine pancreatic DNase (DP grade) was obtained from Worthington; four forms of bovine DNase (A, B, C, and D) were prepared from it according to Salnikow et al. (46). Calf thymus DNA (Type V), Con A, Con A-Agarose, phenylmethylsulfonyl fluoride (PMSF), acrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), and Sephadex G-25 (200-300 μ) and G-100 (40-120 μ) were from Sigma Chemical Co. N,N'-Methylenebisacrylamide (Bis) was from Eastman Kodak Co. CM-cellulose (CM-52) and DEAE-cellulose (DE-52) were purchased from Whatman Ltd. Ampholines were from Bio-Rad.

DNase Assay

DNase activity was determined by a modification of the hyperchromicity assay of Kunitz (8) as described by Liao (48). One unit of enzyme is defined as the activity that causes the increase of one absorbance unit at 260 nm per minute per ml of assay medium at 25°C. Specific activity is designated as units of one ml of enzyme solution per absorbance of that solution at 280 nm (units/A_{280}). Solutions of enzyme were diluted 10-100 fold with 0.01 M Calcium Acetate buffer, pH 4.7 for all assays and measurements of A_{280}.

Preparation of Con A-Agarose

When necessary, Con A-agarose was prepared as described by Steinemann and Stryer (62). Agarose was first activated by CNBr, followed by the addition of Con A; the remaining active groups were blocked by treatment with 1.0 M glycine for two hours at pH 9.0 (63). The binding capacity of the Con A-agarose to bind glycoproteins was measured with purified bovine DNase A. Normally, 1.0 ml of gel bed volume of Con A-agarose containing 2 mg of Con A was capable of binding 0.2-0.3 mg of DNase A.

Preparation of DEAE-Cellulose

The pre-swollen ion-exchanger (DE-52) was stirred into five volumes of water and allowed to settle. The supernatant was decanted and this procedure was repeated two times. The DEAE-cellulose was then stirred into two volumes of Tris-HCl buffer, pH 8.0 at a final concentration of 0.02 M and allowed to stand for one hour. The supernatant was decanted, and the procedure was repeated. The ion-exchanger was finally suspended in two volumes of the same buffer and autoclaved for 15 minutes at 18 psi. The DEAE-cellulose solution was stored at 4°C until needed.

The ion-exchanger was equilibrated after pouring into an appropriate column by running the initial (equilibrating) buffer through the column until the pH and the conductivity of the effluent was equal to the conductivity of the initial buffer.

Preparation of CM-Cellulose

The pre-swollen CM-cellulose (CM-52) was suspended in five volumes of distilled water and allowed to settle. The water was decanted and the procedure was repeated. The ion-exchanger was then suspended in two volumes of water to which was added 50% NaOH to a final concentration of 0.5 N. This solution was allowed to stand for one hour. The supernatant was decanted and the CM-cellulose was washed with five volumes of distilled water. The washings were repeated until the pH of the suspension approached neutrality. 0.1 M calcium acetate buffer, pH 4.7 was added to a final concentration of 0.005 M, the pH was determined by a pH meter with a glass electrode, and the suspension was titrated to pH 4.7 with glacial acetic acid if necessary. After standing for one hour, the supernatant was decanted and replaced with two volumes of 0.005 M calcium acetate buffer, pH 4.7. The suspension was stored at 4°C until needed. No autoclaving was necessary with CM-cellulose.

Preparation of Sephadex G-25 and G-100

The dry Sephadex was allowed to swell in five times the final bed volume of water for 24 hrs. The supernatant was decanted and the gel was rinsed twice with the same volume of water, decanting slowly each time to remove the fines. The swollen gel was then stored in two times the final bed volume of water containing 0.02% NaN_3 to inhibit bacterial growth. The gel was stored at 4°C until required.

The Sephadex was equilibrated before pouring into the column by suspending the gel in the appropriate initial buffer. After the gel

was poured into the column it was washed with at least one column volume of the same buffer.

Rapid Equilibrium Dialysis

This method allows monitoring of both external and internal concentration parameters during the dialysis process. Also, the constant agitation of the solution inside the dialysis tubing greatly reduces the time necessary for both solutions to reach equilibrium.

A dialysis bag tied at one end was gently stretched over the lip of an open-ended tube and fastened in place with two O-rings. A paddle stirrer attached to a variable speed motor was positioned on the inside of the bag, taking care that it did not contact the sides of the bag upon rotation. The solution to be dialyzed was placed inside the bag to approximately one cm over the lip. Two glass balls were added to the inside of the bag to keep it taunt. The dialysis apparatus was then placed in a suitable container with the dialyzing solution. Gentle rotation of the paddle inside the bag was initiated, as was stirring of the dialyzing solution with a magnetic stirring bar. At 30 minute intervals, samples of the dialysate were removed and assayed for DNase activity and the conductivity was also measured and compared with a sample of the dialyzing solution.

SDS Polyacrylamide Gel Electrophoresis

SDS gel electrophoresis was performed according to the procedure of Weber and Osborn (64) with minor modifications. The 10% polyacrylamide SDS gels were prepared one day in advance as follows: for five 80 mm long gels 5 mm in diameter, 5 ml of gel buffer (7.8 gm

$NaH_2PO_4 \cdot H_2O$, 20.4 gm Na_2HPO_4, and 2 gm SDS per liter), 4.5 ml acrylamide solution (22.2 gm acrylamide, 0.6 gm N,N'-methylene bis acrylamide per 100 ml), 0.5 ml ammonium persulfate solution (10 mg/ml of water) and 10 µl of TEMED were mixed together. Two ml of this solution was added to each tube which was then overlaid with water.

To prepare the sample for electrophoresis, 50-100 µl of the sample (concentration approximately 1 mg/ml in water) was mixed with 5 µl of 0.05% bromophenol blue in water, one drop of glycerol, and 20 µl of 10% SDS. If the protein sample was too concentrated, 50 µl of 0.01 M sodium phosphate buffer pH 7.0 was added. The DNase samples were normally run in duplicate, one sample prepared as above, and the duplicate prepared in the same way except for the addition of 5 µl of mercaptoethanol to the sample mixture. The addition of mercaptoethanol results in the reduction of the two disulfide bonds present in DNase, and allows for the detection of any cleavages of the enzyme molecule.

In preparation for a run, the water layer was removed from the top of the gels and the gels were placed in the electrophoresis apparatus. 50-100 µl of the sample mixture was placed on the gel with a pipette, and the sample was carefully overlaid with running buffer (gel buffer diluted 1:2 with water). Electrophoresis was performed at a constant current of 8 mA per gel with the positive electrode in the lower chamber. Electrophoresis proceeded until the marker dye reached the bottom of the gel. The gels were then removed from the glass tubes by rimming with a syringe filled with cold water, and using a pipette bulb to exert pressure. The gels were cut in the middle of the marker dye band and stained with a solution of 0.1% Coomassie Brilliant Blue R in 5% methanol and 10% acetic acid in water. The

gels were then destained in a solution of 5% methanol and 10% acetic acid in water with one change. Mild heat (≃ 60°C) and stirring were used to facilitate staining and destaining.

Molecular Weight Determination

To determine the molecular weight of an unknown DNase sample, a bovine standard (usually DNase A) was run at the same time as the unknowns. The molecular weight of the unknown DNase was estimated by comparing its mobility relative to the mobility of the bovine standard which has a known molecular weight of 30,072 gm/mole (9, 13).

Isoelectric Focusing

Isoelectric focusing was performed by the method described by Awdeh et al. (65) and Bio-Rad instructions on horizontal polyacrylamide gel electrofocusing (66) modified by Kim and Liao (67) in our laboratory to make the procedure applicable for the separation of the multiple forms of DNase.

The polyacrylamide slab gels (3.5 X 20 cm) were cast between two glass plates separated by spacers approximately 1 mm thick. The narrow ampholyte range gel (pH 4.0-6.0) was prepared as follows: 0.4 ml of Bio-Lyte 4/6 was added to 2 ml of monomer concentrate (24.25 gm acrylamide and 0.75 gm Bis in 100 ml of water), 2 ml of 2.5% (w/v) glycerol in water, 5.6 ml of water and 0.5 ml of 0.01% Riboflavin in water. This solution was mixed by gentle inversion and then pipetted between the two glass plates. Polymerization was initiated by irradiating the plate for one hour with a UV light. After polymerization was complete, the side spacers were removed and the top plate was gently lifted off

the gel. Samples were then pipetted onto filter paper squares (Whatman 3 mm) approximately 5 mm on a side and placed on the gel one fourth the total distance from the negative pole and approximately 0.5 cm apart and from each edge. The glass plate was then inverted onto two carbon electrodes in a covered chamber with a small amount of water below the electrodes to prevent the gel from drying out. Electrofocusing was performed for 18 hours with a constant voltage of 500 V at 4°C. After the electrofocusing was completed, the gel was cut across at each electrode and then measured. A guide strip approximately 0.5 cm wide was cut from one side of the gel and cut into equal segments. These squares were then sequentially placed into individual tubes and one ml of distilled water was added. After standing for 12 hours at 4°C, the pH of each was determined and a pH profile of the gel constructed.

The remaining portion of the gel was stained in a solution of 0.05% Coomassie Brilliant Blue R in 27% ethanol and 10% acetic acid containing 0.5% $CuSO_4$. After staining, the gel was destained in 12% ethanol and 7% acetic acid containing 0.5% $CuSO_4$. The inclusion of $CuSO_4$ in the staining and destaining solutions was to prevent any background staining of the ampholytes (68). The migration distance of the protein band was measured from the negative electrode and the isoelectric point of the particular protein was determined from the pH profile previously constructed. A bovine standard mixture (DNase A, B, C, and D) was normally run with each sample as a control and reference.

Discontinuous Polyacrylamide Gel Electrophoresis at Acidic pH

This technique was developed to separate the multiple forms

of DNase. The method used was modified from that of O. Gabriel (69). The 10% polyacrylamide tube gels were prepared by mixing one part gel buffer (pH 4.5 buffer stock solution, 31.2 gm β-alanine and 8.0 ml glacial acetic acid in 100 ml of water diluted 1:10), 2 parts acrylamide stock solution (40.0 gm acrylamide and 1.0 gm Bis per 100 ml of water), 4 parts ammonium persulfate solution (0.07 gm per 100 ml of water), and 2 µl of TEMED per gel. The above solution was mixed by gentle inversion and 2 ml of the solution was pipetted into each tube and overlaid gently with water.

After the gels polymerized (30 min - 1 hr), the water was removed from the top of the gel and the gels were placed in an electrophoresis apparatus filled with running buffer (stock pH 4.5 buffer diluted 1:100 with water). Samples were prepared by mixing 5-20 µl of sample (usually 1 mg/ml in 2 mM $CaCl_2$), 20 µl of 50% (w/v) glycerol in water, and 5 µl of methyl green tracking dye in small test tubes. The samples were pipetted onto their respective gels and gently overlaid with the running buffer. Electrophoresis was initiated with a constant current of 1 mA per gel which was increased to 2 mA per gel after the tracking dye had completely entered the gel. The electrophoresis was allowed to continue for 5 hours after which the gels were removed from the tubes and stained as described in the section on SDS gel electrophoresis.

Enzyme Activity Staining Agar

This technique was developed as a sensitive method for the detection of DNase activity on polyacrylamide gels. The method was modified from that of Rosenthal and Lacks (70) for use in the low pH discontinuous gel electrophoresis system.

Following electrophoresis, the gel was placed in a quartz test tube (0.8 X 9.0 cm) and laid in a horizontal position. To 5 ml of melted 2% agar containing 10 mM $MgCl_2$ and 5 mM $CaCl_2$ was added 5 ml of DNA solution (20 µg/ml) containing 1 µg/ml of ethidium bromide. The agar was cooled to 55°C and pipetted around the gel. After the agar had solidified, the tube was incubated at room temperature and checked at 15 minute intervals under a long UV light source for the presence of DNase bands which would appear as clear bands in a fluorescent pink-orange background. Normally DNase activity could be visualized after 15-30 minutes of incubation.

Amino Acid Analysis

For extracting proteins from stained polyacrylamide gels and subsequent amino acid analysis, the procedure was adopted from that described by Bernabeu et al. (71) for the extraction of ribosomal proteins as modified by Liao and Wadano (45). The stained band was cut and the destaining solution inside the gel was replaced by soaking in water for two hours with one change. The wet gel was weighed to estimate the amount of water present, and twice the volume of glacial acetic acid was added. After 6 hours, the acid was transferred to a hydrolysis tube and the gel was extracted twice with the same volume of 66% acetic acid as that of the glacial acetic acid. The extent of extraction was estimated by the removal of the Coomassie Blue dye from the gel. All extracts were combined with the first solution and taken to dryness in a vacuum desicator.

The protein was hydrolyzed in 200 µl of 6 N HCl containing 0.25% phenol, and 0.5% thioglycolic acid in evacuated sealed tubes at 110°C

(12). Amino acid analysis was performed on the nanomole scale with an analyzer (72), modified for use with a 2.8 mm bore column (73).

CHAPTER IV

RESULTS AND DISCUSSION

Purification of Ovine DNase

Continued study of oving DNase resulted in modification of the purification procedure developed earlier in our laboratory by Wadano et al. (49). The development of a purification technique to improve the yield of DNase was necessary if further structure and function analyses were to be performed. Also, the parameters experimentally developed in this purification may serve to elucidate and perfect techniques used in the purification of other DNases.

Initial Preparation

From previous experiments, it was observed that the final yield of ovine DNase from the initial preparation of the enzyme before chromatography was variable. Development of a reproducible method that could be performed in one day for the initial preparation of pancreatic ovine DNase was desirable. The following procedure fulfilled these requirements.

%00 gm of ovine pancreas, thawed to room temperature, was homogenized in an Omnimixer at maximum speed for two minutes with one volume of cold distilled water. The container was washed with a second volume of cold distilled water, the washing and homogenate were combined and stirred for one hour at 4°C. The suspension, designed as crude

homogenate, was filtered through two layers of cheesecloth to remove remaining tissue, then assayed for DNase activity and protein concentration as described in materials and methods. The solution was brought to 0.25 $(NH_4)_2SO_4$ saturation by the slow addition, with stirring, of 144 gm/l of reagent grade solid $(NH_4)_2SO_4$. The solution was allowed to stand for 30 minutes at 4°C, then was titrated to pH 3.0 by the addition at room temperature of 0.5 N H_2SO_4. The solution was then centrifuged at 27,000 x g for 30 minutes at 4°C. This and all subsequent centrifugation were performed in a Sorvall (model RC-2B) centrifuge with an SS-34 rotor. The precipitate was discarded and the clear supernatant was brought to 0.5 $(NH_4)_2SO_4$ saturation by the addition of 158 gm/l of reagent grade solid $(NH_4)_2SO_4$. The solution was again allowed to stand for 30 minutes at 4°C and then was centrifuged as previously described. The supernatant was discarded, the tubes were washed quickly with distilled water to remove any excess residual $(NH_4)_2SO_4$ from the sides of the tubes, and the precipitate was suspended in approximately 30 ml of 0.005 M Calcium Acetate buffer, pH 4.7. After stirring for 30 minutes at 4°C, the solution was centrifuged, the remaining precipitate was discarded, and the supernatant was assayed for DNase activity and protein concentration. The solution was stored frozen (-20°C) until needed. Table II shows the results of a typical initial preparation procedure.

This method was reproducible and gave good yields (65-75%). The 0.25-0.5 $(NH_4)_2SO_4$ fractionation coupled with acid titration to pH 3.0 resulted in a five-fold purification of the crude homogenate yielding a specific activity of 4.5. Further experiments with the initial preparation showed heat treatment of the crude homogenate at 40°C for 90 minutes

TABLE II

SUMMARY OF PURIFICATION OF OVINE DNASE

Purification Step	Total Volume ml	Activity units/ml	Total Activity units x 10^3	Activity Recovered %	Specific Activity units/A_{280}	Purification x-fold
1) Initial Preparation						
A) Crude Homogenate	243	74	18.0	100	0.9	1
B) 0.25-0.5 $(NH_4)_2SO_4$ precipitate at pH 3.0	33	369	12.2	68	4.5	5
2) Sephadex G-25[a]	139	179	24.9	49	5.6	6.5
3) CM-Cellulose Chromatography	92	214	19.7	28	18.0	20
4) Sephadex G-25	30	547	16.4	23	35.0	39
5) DEAE-Cellulose Chromatography						
A) Pool I	85.5	22	1.9	2.6	272	302
B) Pool II	51	15	0.8	1.1	217	241
C) Pool III	91	63	5.7	8.0	525	583
D) Pool IV	125	17	2.1	3.0	387	430

[a]Values shown were the total result from Sephadex G-25 chromatography of the combination of three $(NH_4)_2SO_4$ fractionations.

was beneficial as it caused an increase of approximately seven-fold in purification.

Equilibration in the Buffer for CM-Cellulose Chromatography

The initial preparation required the removal of the residual $(NH_4)_2SO_4$ and the equilibration of the enzyme solution at the pH and ionic strength necessary for CM-cellulose chromatography. Changing the conditions of the enzyme solutions was the most critical procedure throughout the purification process. Previously, dialysis was used to change the ionic strength and pH of the solution. Unfortunately, the results were variable with the recovery of activity being 30% or less. A method using Sephadex G-25 to equilibrate the solution was devised to eliminate the difficulties encountered at this stage of purification.

The typical procedure was to apply 30 ml of the initial enzyme preparation to a Sephadex G-25 column (2.5 X 45 cm) equilibrated at room temperature with 0.005 M Calcium Acetate, pH 4.7. After the sample had entered the gel, the column was washed with the same buffer until 30 fractions (5 ml each) were collected. The conductivity and protein concentration of alternating fractions were determined, and the fractions containing protein and equilibrated to acceptable ionic strength were pooled. The total DNase activity, protein concentration, and conductivity of the pooled fractions were measured.

Experimentally, this method was found to be very reproducible and yielded an average of 75% of the activity applied to the column with a slight increase in specific activity. A single column could be run in

less than one hour, and after a one liter wash with the buffer, the column could be reused, on the average for a total of four separations before repacking.

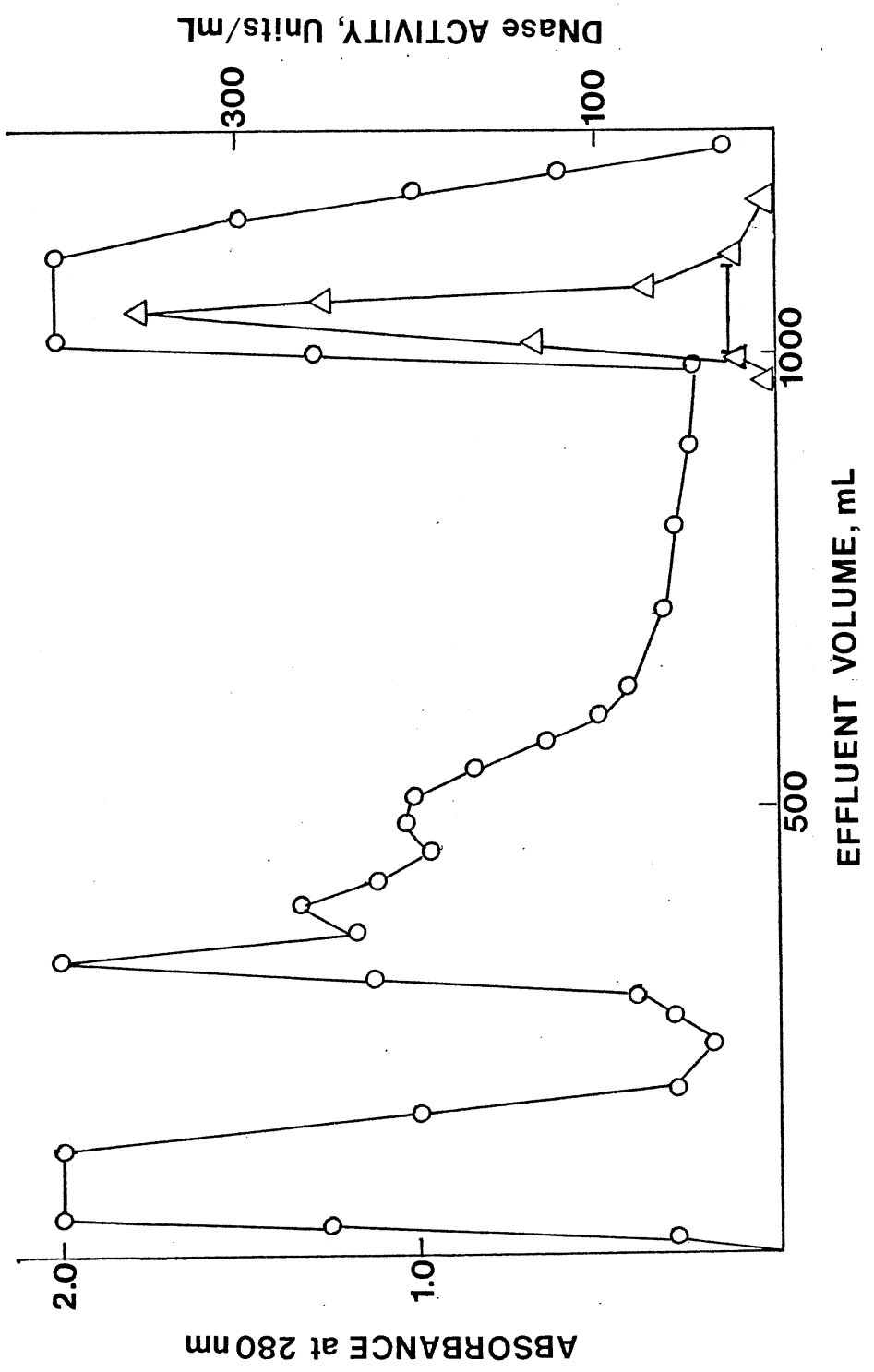
Experiments using cold ethanol precipitation adapted from the method of Kunitz (8) to concentrate the sample were attempted at this stage of purification. The results of these experiments were variable, irreproducible, and very often showed that less than 20% of the original activity was recovered. The variation may be due to a number of conditions, for example, residual $(NH_4)_2SO_4$ concentration in the solution, and the temperature at which the enzyme is precipitated. Ethanol precipitation is not recommended at this stage of purification.

CM-Cellulose Chromatography

The equilibrated enzyme solution (combined pool of three extractions) was applied to a CM-cellulose column (1.5 X 20 cm) equilibrated at 4°C with 0.005 M calcium acetate buffer, pH 4.7. The column was washed with equilibrating buffer until the absorbance at 280 nm (A_{280}) of the effluent declined and stabilized. In most cases, approximately one liter of buffer was needed. The column was then washed with 250 ml of 0.05 M calcium acetate buffer, pH 4.7. Alternating fractions were assayed for DNase activity. The active fractions appeared in the 0.5 M washing and were combined in a single pool. Figure 3 shows a typical chromatographic result. Table II provides a summary of the results from this procedure.

Results of previous CM-cellulose chromatography (49) showed that two active fractions were partially separated when the column was developed with a linear gradient. Since the two fractions were

Figure 3. Chromatography of Ovine DNase on CM-Cellulose. Open circles indicate absorbance at 280 nm; triangles indicate DNase activity; bar indicates pooled active fraction.


DNase ACTIVITY, Units/mL
300
100
1000
500
EFFLUENT VOLUME, mL
ABSORBANCE at 280 nm
2.0
1.0

eventually combined as one at this stage of purification, stepwise washing was used to elute the active fractions as a single peak. This was advantageous for subsequent purification due to the reduced volume. A four-fold purification over the previous step was obtained by this procedure.

Equilibration in the Buffer for DEAE-Cellulose Chromatography

Transition of the solvent of the active fraction from CM-cellulose to that used in DEAE-cellulose chromatography usually resulted in an appreciable loss of enzymatic activity. The results from dialysis were not reproducible, and in some cases near total inactivation of the enzyme in the dialysate occurred. Equilibration with Sephadex G-25 also resulted in similar difficulties. However, Sephadex G-25 was still the preferred method because the process was rapid and could be easily monitored.

At this stage in purification, it was important that a serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF) (74) be present because of the increased catalytic activity of serine proteases at alkaline pH. PMSF was the reagent of choice as it did not form insoluble products with $CaCl_2$ and it was more convenient to use than diisopropyl phosphofluoridate (DFP) (10).

The recovery of enzymatic activity from the Sephadex G-25 column could be greatly improved if the column was used only once. Repeated use of the same column, even after extensive washing resulted in a steady decline of recovered activity. The cause of this has not been determined. Experimentally, the loss of activity was accompanied by

a loss of the total protein applied. Also, large amounts of precipitated material were observed in the gel matrix upon unpacking the column. The fact that a proteolytic inhibitor was present seems to rule out the possibility of protease digestion of DNase. It may suggest that perhaps some type of aggregation of DNase with another substance which causes precipitation of the proteins due to the sudden change in pH and ionic strength was responsible for the observed results.

In practice, prior to gel filtration, the active fraction from CM-cellulose chromatography was treated with 5-10 mg of PMSF for one hour by stirring at room temperature. The solution was then titrated to pH 7.0 with 1.0 M Tris-OH and rotary evaporated to approximately 20 ml. The solution was assayed for enzymatic activity and protein concentration and then placed on a Sephadex G-25 column (2.5 X 45 cm) equilibrated at room temperature with 0.02 M Tris-HCl, pH 8.0, 0.002 M in $CaCl_2$. The column was washed with the same buffer until 30 fractions (5 ml each) were collected. The conductivity and A_{280} of alternating fractions were determined. The fractions containing protein and equilibrated to acceptable ionic strength were pooled. A result with good recovery of activity is summarized in Table II. A two-fold purification was obtained with this treatment. In this particular instance, the recovery of activity was high. This illustrates the potential usefulness of this technique, even though a few preparations did not yield as good a recovery of activity.

DEAE-Cellulose Chromatography

The equilibrated sample from Sephadex G-25 was loaded onto a DEAE-

cellulose column (1.5 X 45 cm), equilibrated at 4°C with 0.02 M Tris-HCl pH 8.0, 0.002 M in $CaCl_2$. The column was washed with 175 ml of equilibrating buffer. A linear gradient was applied (500 ml each of 0.002 M-0.01 M $CaCl_2$ in 0.02 M Tris-HCl buffer, pH 8.0). The effluent was assayed for DNase activity and its A_{280} was measured. The active fractions were pooled as shown in Figure 4.

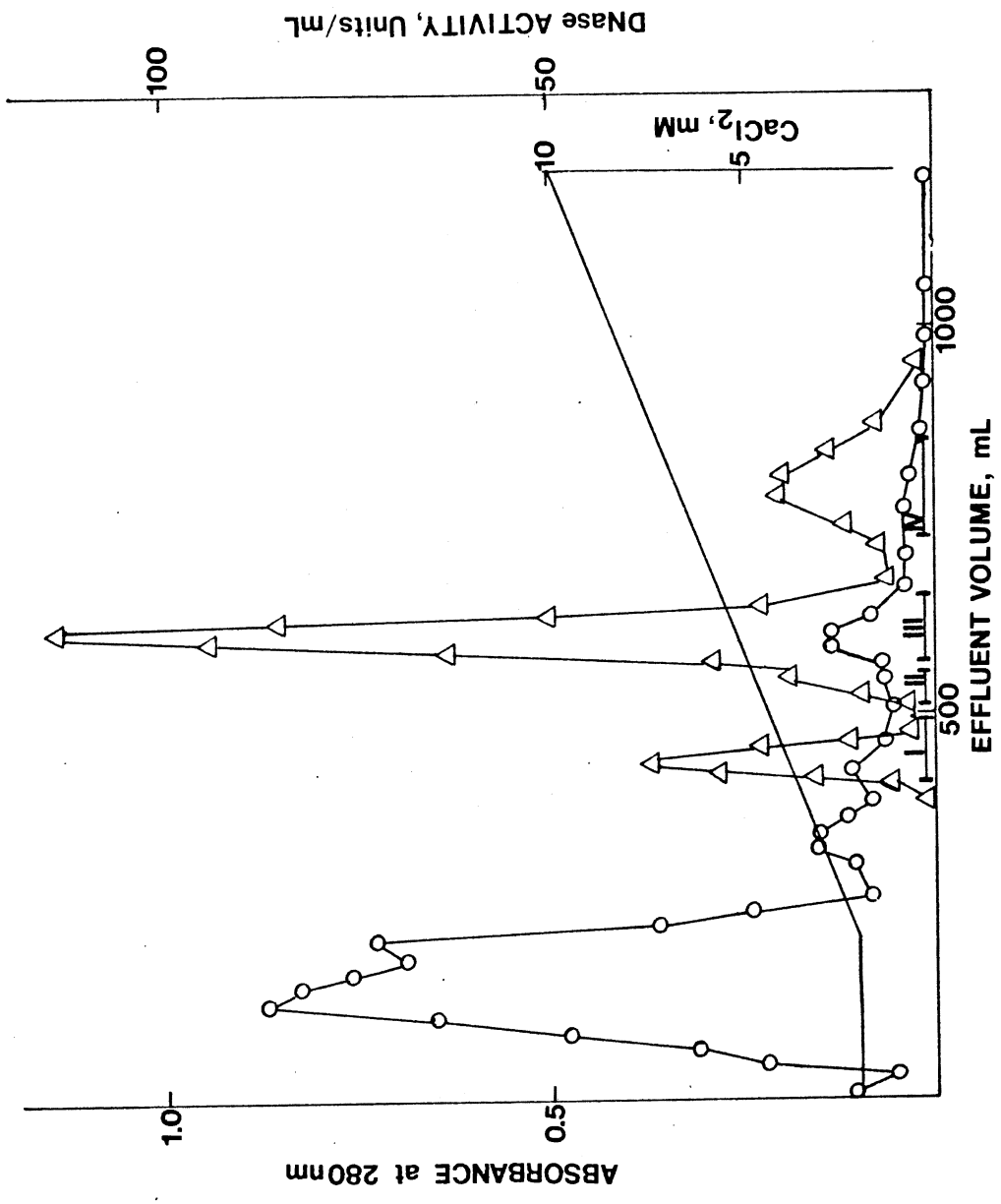
DEAE-cellulose chromatography separated ovine DNase into four active components. The four forms were eluted at a Ca^{2+} concentration between 3.8 mM and 6.8 mM. The purification achieved varied from 12-30 fold over the previous step (Table II). The specific activities vary from 217 units/A_{280} (pool II) to 525 units/A_{280} (pool III) which approaches that of a purified DNase (600-1000 units/A_{280}). The total yield was approximately 12.5% of the original activity.

The separation of ovine DNase into four active components on DEAE-cellulose lends support to the experimental findings of Wadano et al. (49) who previously separated ovine DNase into four forms using CM-cellulose and Con A-agarose chromatography. However, no comparisons could be made as to which of the active fractions from this DEAE-cellulose chromatography related to the multiple forms they observed from their chromatographic procedures.

Purification of Porcine DNase

Purification and characterization of porcine DNase was initiated because comparisons of the enzymes from bovine and porcine species may show a greater difference than when the bovine and ovine DNases were compared since the porcine and bovine species are not similar.

Figure 4. Chromatography of Ovine DNase on DEAE-Cellulose. Open circles indicate absorbance at 280 nm; triangles indicate DNase activity; solid line indicates $CaCl_2$ concentration; bars indicate pooled active fractions.


ABSORBANCE at 280nm
1.0
0.5
DNase ACTIVITY, Units/mL
100
50
$CaCl_2$, mM
10
5
500
1000
EFFLUENT VOLUME, mL
I
II
III
IV

Extraction of Porcine DNase

The method of initial preparation of the enzyme was modified from that used by Wadano et al. (49) for ovine DNase, adapted from the method of Kuntz (8). 125 gm of porcine pancreatic tissue was thawed at room temperature and then homogenized in an Omnimixer with one volume of cold distilled water at maximum speed for two minutes. The Omnimixer container was washed with an additional volume of cold distilled water and the washing and tissue homogenate were combined. Calcium Acetate buffer, pH 4.7, was added to a final concentration of 0.01 M along with approximately 5-10 mg of PMSF. The solution was stirred gently overnight at 4°C. The precipitate was discarded and the supernatant was filtered through Schleicher and Schuell shark skin and then Whatman #5 paper. The final filtrate, designated as crude homogenate, was assayed for DNase activity and protein concentration as previously described. Solid reagent grade $(NH_4)_2SO_4$ was added slowly with stirring at 4°C to 0.3 saturation (176 gm/1) and stirred for 30 minutes at 4°C. The solution was centrifuged at 27,000 X g for 30 minutes at 4°C, the precipitate was discarded, and the supernatant was assayed for DNase activity and protein concentration. At this point, the pH was measured to ensure that the individual preparation did not deviate excessively from the normal range (experimentally observed to fall within the range of pH 5.2-5.6), and the solution was brought to 0.55 $(NH_4)_2SO_4$ saturation by the addition of 162 gm/1 of solid reagent grade $(NH_4)_2SO_4$. The solution was stirred slowly at 4°C, and then centrifuged at 27,000 X g for 30 minutes at 4°C. The supernatant was discarded, and the 0.55 $(NH_4)_2SO_4$ saturated precipitate was suspended in a minimum volume of 0.005 M

Calcium Acetate buffer, pH 4.7; stirred for 30 minutes at 4°C, and then centrifuged at 27,000 X g for 30 minutes at 4°C to remove residual insoluble material. The clear supernatant was assayed for DNase activity and protein concentration and frozen (-20°C) if not used immediately. Table III summarizes the purification steps. At this stage of purification, the enzyme was stable in the buffer and when frozen would retain a high percentage of its activity for many months. The recovery of activity from the original tissue homogenate was 68% with a purification of approximately five-fold yielding a specific activity of 6.2.

Initially, it was discovered that the porcine DNase was extremely sensitive to low pH environments. Sensitivity of porcine DNase to these conditions were alluded to by Laskowski (58, 59) in his early work on DNase purification. Whereas both bovine (8) and ovine (49) pancreatic DNase exhibited good stability in acidic conditions, acidification of the homogenized porcine pancreas solution with 0.5 NH_2SO_4, even in the presence of 0.3 $(NH_4)_2SO_4$ saturation, resulted in poor yields at pH 4.0 and complete loss of enzymatic activity at pH 3.0. However, $(NH_4)_2SO_4$ fractionation could be accomplished without acid titration, though higher concentrations of $(NH_4)_2SO_4$ were required to precipitate the enzyme (0.3-0.55 $(NH_4)_2SO_4$ saturation). Also, heat treatment of the initial crude homogenate, which increased the specific activity in the ovine procedure, did not yield an increase in purification with porcine DNase.

The uniqueness of porcine DNase was made apparent by the fact that it was extremely difficult to continue from this initial preparation with purification techniques that were successful in the purification of

TABLE III

SUMMARY OF THE PURIFICATION OF PORCINE DNASE WITH $(NH_4)_2SO_4$

Purification Step	Total Volume ml	Activity units/ml	Total Activity units x 10^3	Activity Recovered %	Specific Activity units/A_{280}	Purification x-fold
1) Crude Homogenate	449	88	40	100	1.2	1
2) 0.3 $(NH_4)_2SO_4$ Supernatant	458	82	38	95	2.0	1.6
3) 0.55 $(NH_4)_2SO_4$ Precipitate	103	258	27	68	6.2	5.2

bovine and ovine pancreatic DNases. CM-cellulose chromatography used in the purification of ovine DNase (49) resulted in poor recoveries in the purification of porcine DNase. Phosphocellulose chromatography, employed by Salnikow et al. (46) to purify bovine DNase, was unsatisfactory as significant breakthrough of DNase activity occurred during sample loading. This was not the result of improper equilibration of the starting solutions or the result of exceeding the binding capacity of the ion-exchanger. Inability of porcine DNase to bind to phosphocellulose indicates that it is an acidic protein. Therefore, the ion-exchange media of choice would be that of the anion exchange type.

Equilibration for DEAE-Cellulose Chromatography

Attempts to adjust the porcine enzyme solution to conditions necessary for DEAE-cellulose chromatography (0.02 M Tris-HCl pH 8.0 + 0.002 M $CaCl_2$) resulted in drastic loss of enzymatic activity. Dialysis of the 0.55 $(NH_4)_2SO_4$ precipitate suspended in 0.005 M Calcium Acetate buffer, pH 4.7 either by normal or rapid equilibrium methods resulted in the loss of over 60% of the original activity even in the presence of PMSF or DFP. Equilibration with Sephadex G-25 was not reproducible; the recovered activity declined at each successive run, and the column rapidly become unuseable due to the amount of precipitated material in the gel matrix. Titration of the 0.55 $(NH_4)_2SO_4$ precipitate suspension to pH 8.0 with 1.0 M Tris-OH resulted in the immediate loss of more than 70% of the enzymatic activity. However, titration of the crude homogenate to pH 8.0 with 1.0 M Tris-OH caused a negligible loss of enzymatic activity. The final purification procedure was thus developed to eliminate the $(NH_4)_2SO_4$ fractionation step.

Chromatography of Porcine DNase on DEAE-Cellulose

Previous studies by Keller et al. (75) of the chromatographic sequence of the major components of bovine pancreatic juice using DEAE-cellulose chromatography as a primary separation method, and the use of DEAE-cellulose by Liao (48) to separate and purify the multiple forms of bovine DNase led to the subsequent development of a purification procedure for porcine DNase utilizing DEAE-cellulose chromatography as the major purification tool. The present approach is to chromatograph a solution isolated from the crude tissue homogenate, titrated to pH 8.0, in the presence of Ca^{2+} on DEAE-cellulose, without the use of $(NH_4)_2SO_4$ fractionation. The final purification procedure is summarized in Table IV.

200 gm of porcine pancreas was minced coarsely with scissors and suspended in two volumes of cold distilled water with approximately 5-10 mg of PMSF. Mincing the tissue was preferred over homogenization, as the latter resulted in too much suspended material that could not be cleaved from the extraction by centrifugation or filtration. The preparation was stirred gently overnight at 4°C and then strained through cheesecloth to remove the pancreatic tissue. The resulting solution was centrifuged at 27,000 X g for one hour at 4°C, the precipitate was discarded, and the supernatant was filtered through Schleicher and Schuell shark skin and then through Whatman #5 paper. The filtrate, designated as crude homogenate, was assayed for DNase activity and protein concentration. $CaCl_2$ was added to a final concentration of 0.002 M and the solution was titrated to pH 8.0 with 1.0 M Tris-OH. Centrifugation at 27,000 X g for 30 minutes at 4°C was per-

TABLE IV

SUMMARY OF THE PURIFICATION OF PORCINE DNASE

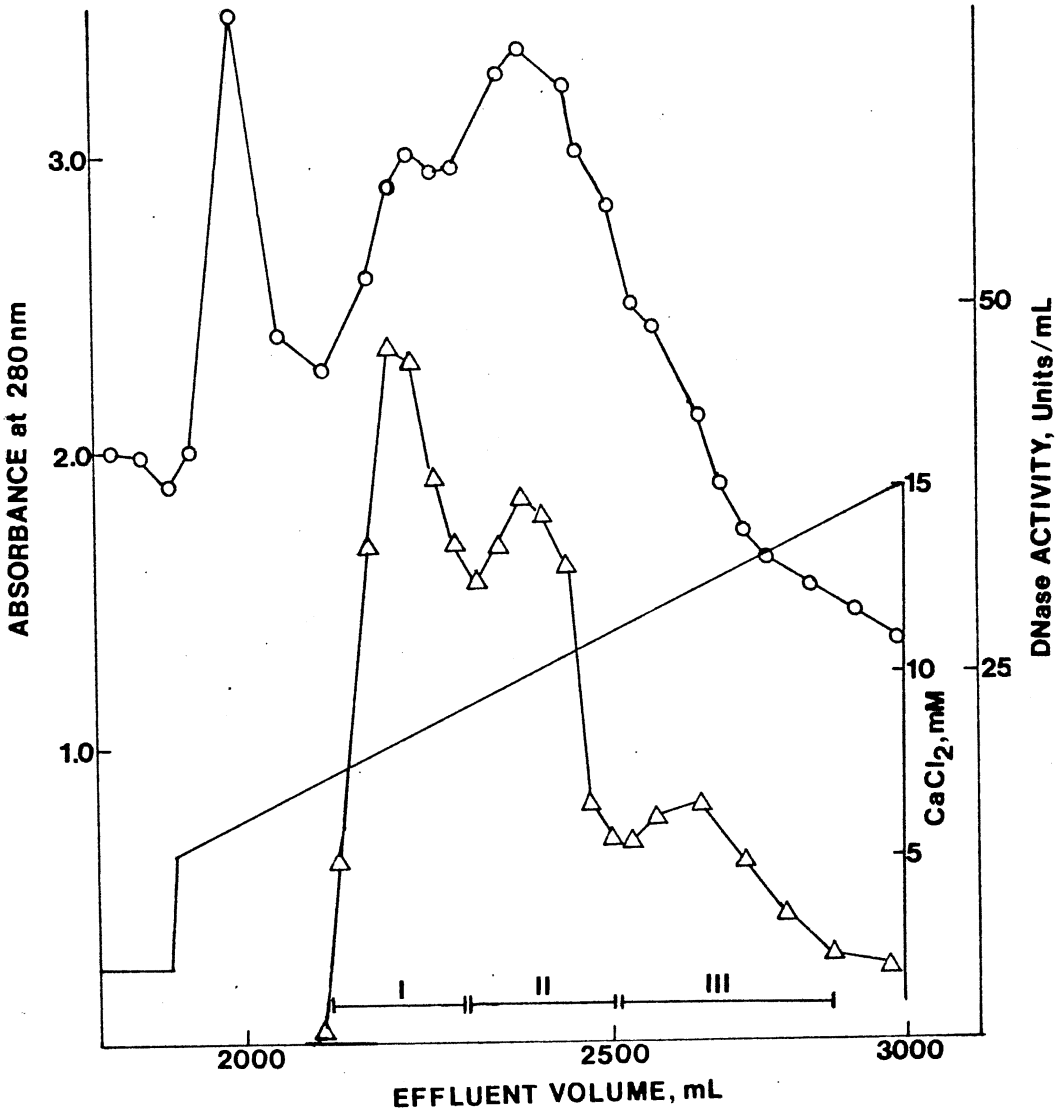
Purification Step	Total Volume ml	Activity units/ml	Total Activity units x 10^{-3}	Activity Recovered %	Specific Activity units/A_{280}	Purification x-fold
1) Crude Homogenate	315	76	24	100	0.6	1
2) pH 8.0 Supernatant	305	74	22	92	0.8	1.3
3) DEAE-Cellulose Chromatography						
A) Pool I	202	36	7.3	30	13.3	22
B) Pool II	156	35	5.5	23	10.8	18
C) Pool III	375	12	4.5	19	6.3	10.5
4) Rechromatography on DEAE-Cellulose						
A) Pool I	48	47	2.2	9	101	168
B) Pool II	15	202	3.0	12.5	68	113
5) Con A-Agarose Chromatography						
A) Pool I	7	33	0.2	1	124	206
B) Pool II	15	32	0.5	2	196	327

formed to remove any insoluble material. The clear supernatant, designated pH 8.0 supernatant, was then assayed for DNase activity and protein concentration. The conductivity of the enzyme solution was read and compared against that of the DEAE-cellulose initial buffer. The enzyme solution was diluted with cold distilled water to the same ionic strength as the initial buffer and loaded onto a DEAE-cellulose column (2.5 X 20 cm) equilibrated at 4°C with 0.02 M Tris-HCl pH 8.0, 0.002 M in $CaCl_2$. After the sample was loaded onto the column, it was washed with 500 ml of the initial buffer, and eluted with a linear gradient comprised of 500 ml each of 0.02 M Tris-HCl pH 8.0 with 0.005 M and 0.015 M $CaCl_2$ respectively. The chromatogram is shown in Figure 5.

Porcine DNase was resolved into three active peaks by this procedure. The purification achieved was 22, 18, and 10.5-fold, yielding specific activities of 13.3, 10.8, and 6.3 units/A_{280} respectively. The purification achieved was satisfactory, as the main purpose of this initial chromatography was to remove a large amount of contaminating materials. Also, since very little preparatory purification was conducted prior to the chromatography a very crude sample of very large volume was applied. The total recovery of the activity applied was 72% of the original sample. The active peaks eluted at Ca^{2+} concentrations of approximately 8, 9.8 and 12 mM which indicates that porcine DNase is much more tightly bound to DEAE-cellulose than bovine or ovine DNase. This strongly supports the previous results of the cation Exchange chromatography experiments which demonstrated the acidic nature of this protein.

The significant differences in Ca^{2+} concentration required to elute

Figure 5. Chromatography of Porcine DNase on DEAE-Cellulose. Open circles indicate absorbance at 280 nm; triangles indicate DNase activity; solid line indicates $CaCl_2$ concentration; bars indicate pooled active fractions. Chromatogram shows only gradient elution of porcine DNase activity.


ABSORBANCE at 280nm
3.0
2.0
1.0
50
25
DNase ACTIVITY, Units/mL
15
10
5
$CaCl_2$, mM
I
II
III
2000
2500
3000
EFFLUENT VOLUME, mL

the active peaks indicates that there are at least three distinct forms of porcine DNase present. The incomplete resolution of the various forms makes it impossible to detect more isozymes.

Rechromatography on DEAE-Cellulose

The three active peaks were pooled separately, assayed for DNase activity and protein concentration, then rotary evaporated to approximately 30 ml and centrifuged in a clinical centrifuge at maximum speed for 10 minutes at room temperature to remove any precipitated material. Each pool was placed in dialysis tubing with a few crystals of PMSF and dialyzed against water (one liter) for 90 minutes, then against two liters of 0.02 M Tris-HCl pH 8.0, 0.002 M $CaCl_2$ for 12 hours with one change of solution. The pools were assayed for DNase activity and protein concentration. At this time, pool III was frozen as it was the minor fraction with a lower specific activity. The conductivity of pool I and II was determined and compared with that of the initial buffer. Cold distilled water was added to each pool to adjust them to the proper ionic strength. The samples were then loaded onto separate identical DEAE-cellulose columns (0.9 X 2.0 cm) equilibrated at 4°C with 0.02 M Tris-HCl pH 8.0, 0.002 M in $CaCl_2$. After washing each with 100 ml of equilibrating buffer, a linear Tris-HCl gradient of 100 ml each of 0.02 M-0.1 M Tris-HCl pH 8.0, 0.002 M in $CaCl_2$, was initially applied. This failed to elute any of the samples, and it was necessary to apply a second linear gradient with increased Tris-HCl concentration from 0.1 M-0.2 M (100 ml each) containing 0.002 M $CaCl_2$ to effect elution. The major active peak from each chromatography was pooled and assayed for DNase activity and protein concentration. The chromatogram of each chromatography

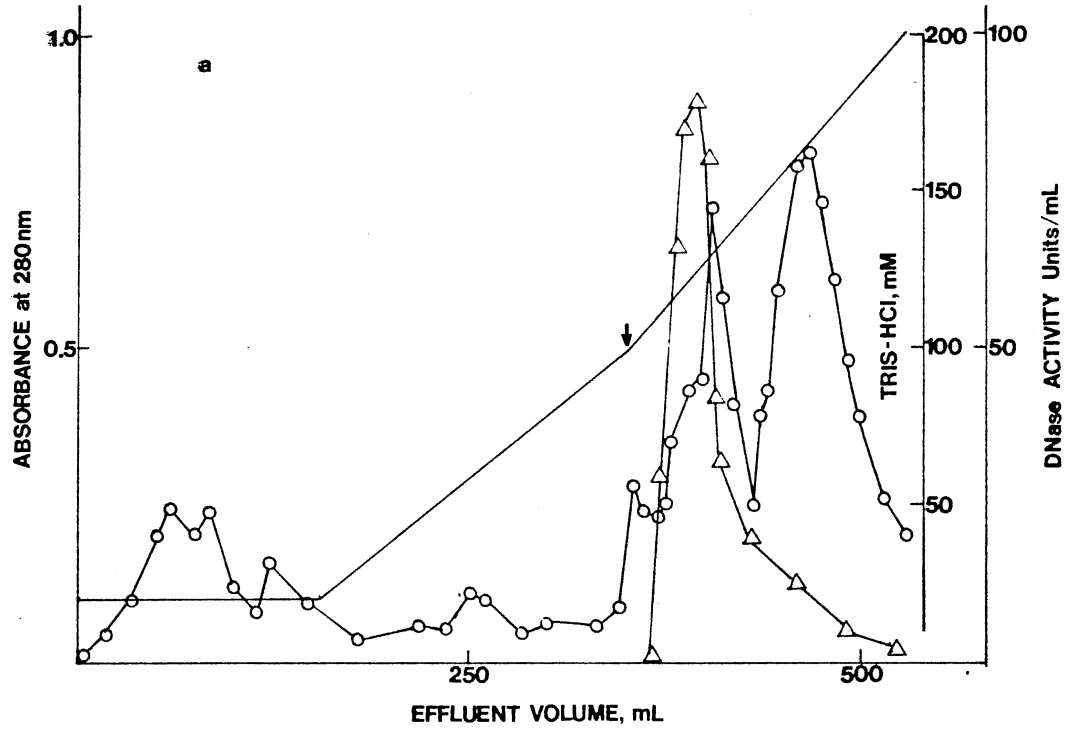
is shown in Figure 6.

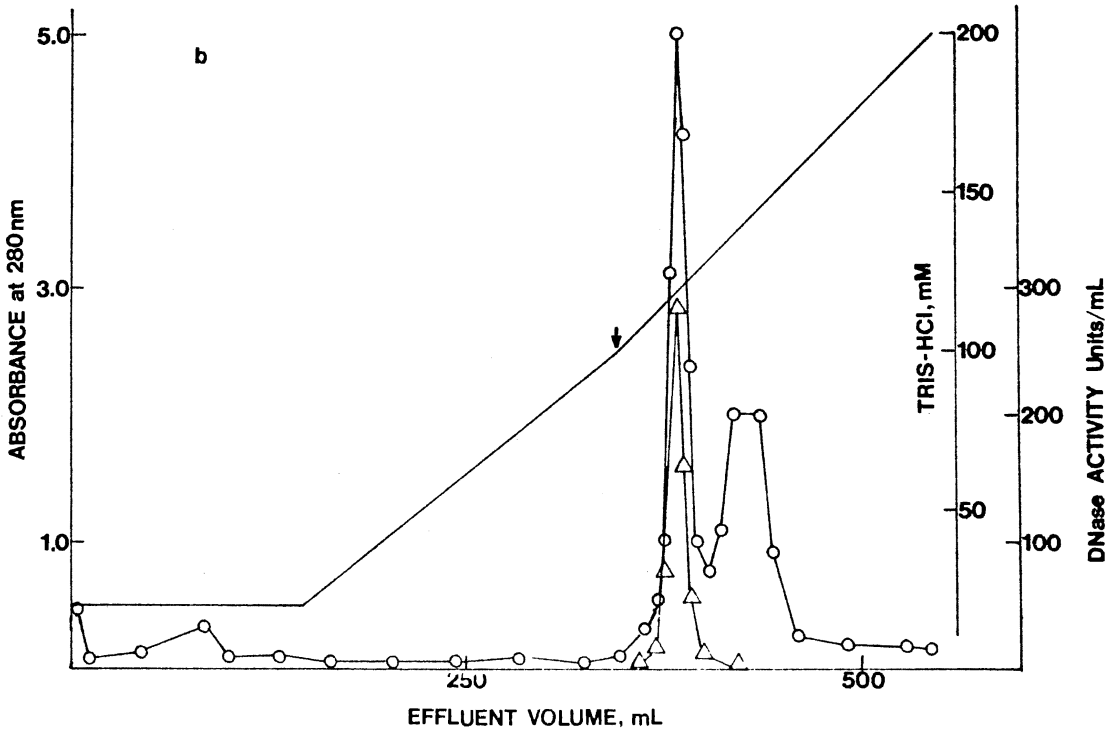
Rechromatography and DEAE-cellulose of pool I and II from the initial chromatography resulted in a 168 and 113-fold purification which yielded specific activities of 101 and 68 units/A_{280} respectively. Increased concentration of Tris-HCl relative to bovine or ovine DNase was required to elute the activity from both columns. Pool I required a Tris-HCl concentration of approximately 0.123 M and pool II required a concentration of 0.119 M Tris-HCl, indicating that both were more tightly bound to the DEAE-cellulose than either the bovine or ovine enzymes. More than one active peak was observed from the rechromatography of pool I, indicating that minor quantities of other multiple forms were present. Only one active peak was noted from the rechromatography of pool II. In both cases, significant amounts of contaminating proteins that co-eluted with the respective active peaks in the initial DEAE-cellulose chromatography utilizing a linear Ca^{2+} gradient were separated from them by this chromatographic procedure with a Tris-HCl linear gradient of increasing ionic strength. The recovery of activity applied to the columns was 30% and 54% for pool I and II respectively.

Con A-Agarose Chromatography

Specific activities of the pools from Step 4 of Table IV were still far from those of purified DNases (600-1000 units/A_{280}). In a previous purification of ovine DNase (49), the affinity of Con A-agarose for glycoproteins such as DNase was used as a rapid method to separate DNases from contaminating proteins. An advantage of this technique is that the samples could be applied directly from the previous chromatography without further manipulation since the ionic strength of the

Figure 6. Rechromatography of Porcine DNase on DEAE-Cellulose. Open circles indicate absorbance at 280 nm; triangles indicate DNase activity; solid line indicates Tris-HCl concentration. a) Rechromatography of pool I from DEAE-cellulose chromatography (Figure 5); b) Rechromatography of pool II from DEAE-cellulose chromatography (Figure 5).


a
1.0
0.5
ABSORBANCE at 280nm
200
150
100
50
TRIS-HCl, mM
100
50
DNase ACTIVITY Units/mL
250
500
EFFLUENT VOLUME, mL


b
5.0
3.0
1.0
ABSORBANCE at 280nm
200
150
100
50
TRIS-HCl, mM
300
200
100
DNase ACTIVITY Units/mL
250
500
EFFLUENT VOLUME, mL

solution was not a critical parameter.

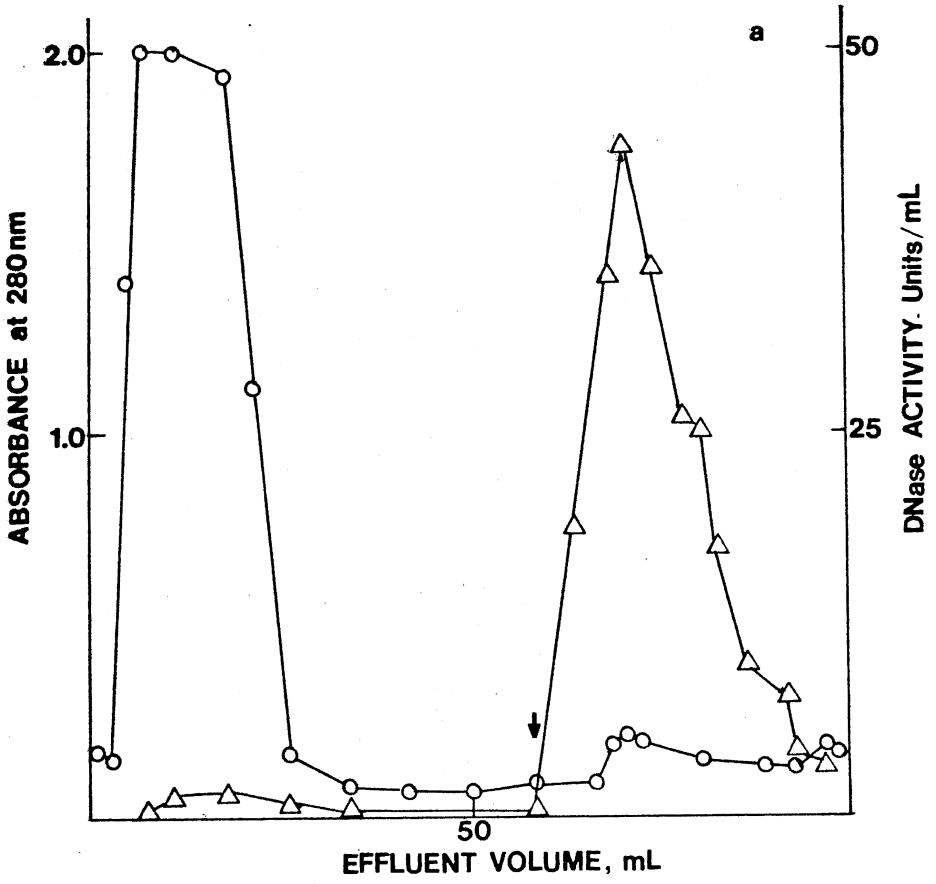
To test the effectiveness of this method, pool II was applied directly to a Con A-agarose column (0.9 X 6 cm) equilibrated at 4°C with 0.1 M Tris-HCl pH 8.0, 0.002 M in $CaCl_2$. After washing with 50 ml of the equilibrating buffer, elution with the same buffer containing 5% glucose was performed. The active fractions were pooled and assayed for total DNase activity and protein concentration. The results from this procedure are shown in Figure 7a. A 327-fold purification yielded a specific activity of 196. However, only 16% of the activity applied to the column was recovered. No further activity was recovered from the column even after such treatments as elution with 5% glucose in 0.01 M Calcium Acetate buffer, pH 4.7 or 3.0 M guanidine-HCl.

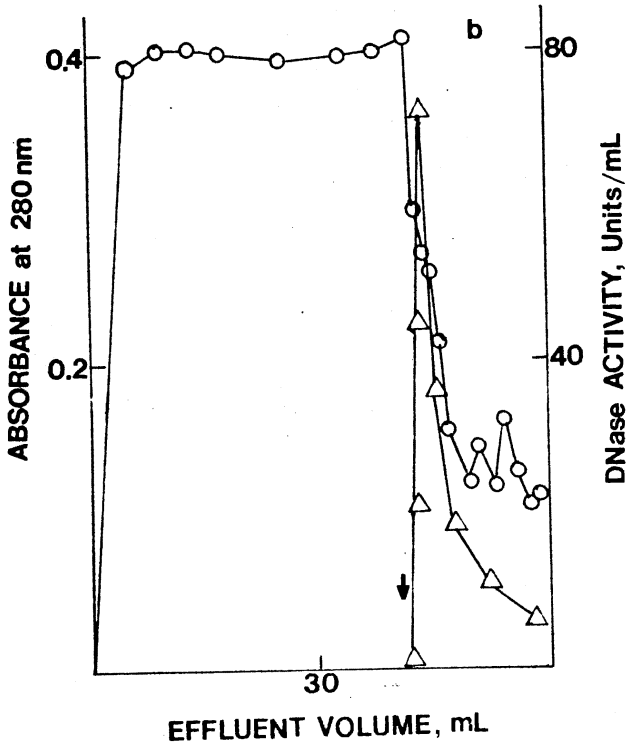
Further experiments showed that a reduced bed volume of Con A-agarose was sufficient to bind the entire sample previously applied. Therefore, a pipette column with two ml total gel volume equilibrated as in the previous experiment was used in an attempt to further purify pool I. The sample was loaded without further manipulation, and the column was then washed with approximately 5 ml of the equilibrating buffer and elution was attempted as before with the same buffer containing 5% glucose. The results of this chromatography are shown in Figure 7b. The purification achieved was 206-fold yielding a specific activity of 124. The total activity recovered from the column was 20% of that initially applied.

SDS Polyacrylamide Gel Electrophoresis

Pool II from Table IV was placed in dialysis tubing with a few crystals of PMSF and dialyzed extensively against two liters of water

Figure 7. Con A-Agarose Chromatography of Porcine DNase. Open circles indicate absorbance at 280 nm; triangles indicate DNase activity; arrow indicates the change of elution to 5% glucose in the same buffer. a) Chromatography of pool II from DEAE-cellulose chromatography (Figures 5 and 6b); b) Chromatography of pool I from DEAE-cellulose chromatography (Figures 5 and 6a).


a
2.0
1.0
ABSORBANCE at 280nm
50
25
DNase ACTIVITY. Units/mL
50
EFFLUENT VOLUME, mL


b
0.4
0.2
ABSORBANCE at 280nm
80
40
DNase ACTIVITY, Units/mL
30
EFFLUENT VOLUME, mL

with three changes for 18 hours at 4°C. The dialysate was lyophilized, and the dry powder stored at -20°C.

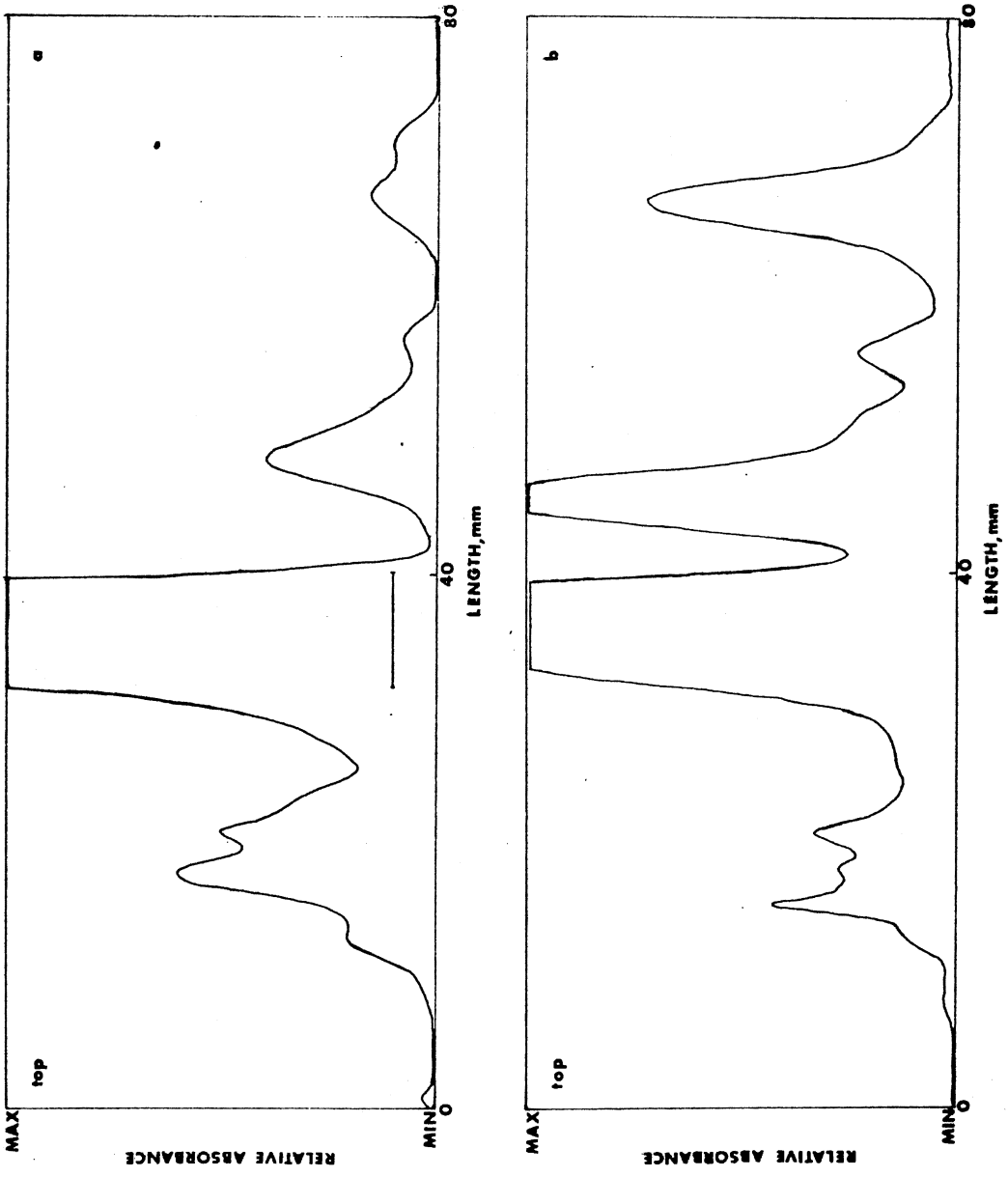
The lyophilized protein was then dissolved in one ml of water and SDS gel electrophoresis was performed as outlined in materials and methods. The gel scans (Figures 8a and b) show a major band with contaminating proteins of higher and lower molecular weights. A second SDS gel electrophoresed with bovine DNase A as a standard in a separate gel was run to verify that the major band from the previous gel was a DNase. The bovine standard migrated approximately the same distance as the major band in the first gel, which was then assumed to correspond to porcine DNase. In the presence of mercaptoethanol which reduces the two disulfide links in native DNase (19), the major band in Figure 8a decreased (Figure 8b) while the lower molecular weight protein bands increased, perhaps indicating the presence of cleavage products in the porcine DNase sample.

The molecular weight of porcine DNase estimated by comparing the migration of its relative to that of bovine DNase A (M.W. = 30,072 g/mole) was approximately the same molecular weight as that of the bovine standard.

Isoelectric Focusing

Determination of the isoelectric point by the technique of Kim and Liao (67) verify the acidic nature of the porcine enzyme. The isoelectric point of porcine DNase from pool II was found to be approximately 4.5, relative to bovine DNase standards of 5.2 to 4.8. (This is the range from DNase A to DNase D.)

Figure 8. SDS Polyacrylamide Gel Scans of Porcine DNase. a) Sample without addition of mercaptoethanol; bar indicates portion of gel cut for extraction of the protein for amino acid analysis; b) sample with mercaptoethanol present.


a
top
MAX
MIN
0
40
80
LENGTH, mm
RELATIVE ABSORBANCE
b
top
MAX
MIN
0
40
80
LENGTH, mm
RELATIVE ABSORBANCE

Discontinuous Polyacrylamide Gel Electrophoresis at Acidic pH

Results of polyacrylamide gel electrophoresis at pH 4.5 are shown in Figure 9. Porcine DNase (from pool II) migrated slightly further than bovine DNase D toward the negative electrode, indicating that it has more net positive charge at pH 4.5. This would appear to contradict the fact that it has a more acidic pI (more negative charge) than bovine DNase D. However, from the amino acid composition (Table V), it can be seen that the acidity of the porcine enzyme is due to the presence of more acidic residues relative to bovine DNase rather than the absence of basic residues. The larger numbers of glutamic acid residues in porcine DNase may be responsible for the behavior of the protein in this gel system. The pKa of the acidic side chain of glutamic acid is 4.25. The pH of the gel system (pH 4.5) may be close enough to the pKa of some of the glutamic acid residues, due to their local environment, for some of them to be protonated. This would increase the net positive charge on the molecule which would result in the increased migration of the particular protein.

Amino Acid Analysis

The major DNase band (Figure 8a) observed in SDS gel electrophoresis was cut out, the enzyme extracted, and the amino acid composition of the extracted porcine DNase was determined as described in materials and methods.

The amino acid composition of porcine DNase (pool II) was compared with the amino acid composition of ovine DNase (49) and bovine DNase A

Figure 9. Polyacrylamide Discontinuous Gel Electrophoresis at Acidic pH of Porcine DNase. A, B, C and D represent bovine DNases A, B, C and D respectively; P represents porcine DNase. Negative pole was at the bottom of the tubes.

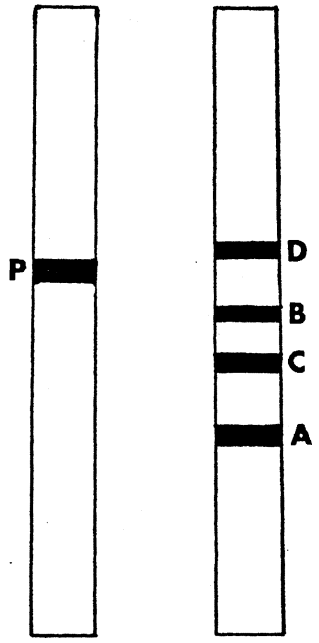

P
D
B
C
A

TABLE V

COMPARISON OF AMINO ACID COMPOSITIONS OF BOVINE, OVINE AND PORCINE DNASE

	Bovine[a] DNase A	Porcine[b] Fraction II	Ovine[c] Fraction IIA
Aspartic acid	32[d]	34	33
Threonine	15	14	14
Serine	30	22	31
Glutamic acid	19	25	18
Proline	9	10	11
Glycine	9	10	10
Alanine	22	20	21
Half-cystine	4	nd[f]	4
Valine	24	18	24
Methionine	4	2	4
Isoleucine	11	14	12
Leucine	23	25	23 (22)[e]
Tyrosine	15	9	14
Phenylalanine	11	8	11
Histidine	6	6	4
Lysine	9	10	9
Tryptophan	3	nd[f]	3
Arginine	11	14	11 (12)[e]

[a]Data from sequence study by Liao et al. (13).

[b]Data obtained from acid hydrolysis of the protein extracted from polyacrylamide gels.

[c]Data from results of Wadano et al. (49).

[d]Results expressed as number of residues per enzyme molecules. Total number of residues in porcine DNase is also assumed to be 257.

[e]Numbers in parentheses indicate number of residues in ovine DNase fraction IIB.

[f]These residues were not determined.

(13) in Table V. Since this analysis is only an approximate composition, very few definite conclusions can be drawn from it. However, this analysis agrees with other experimentally determined parameters of porcine DNase. The excess of acidic amino acid residues, especially glutamic acid, relative to both bovine and ovine DNases was expected from the results of the various chromatographic procedures. The inability to bind to phosphocellulose and the strong binding to DEAE-cellulose both suggest that porcine DNase is an acidic protein. The instability of porcine DNase at low pH, relative to bovine and ovine DNases, might thus be explained by the presence of more acidic amino acid residues in the primary sequence. It is possible that in native porcine DNase, unprotonated (charged) carboxyl groups are necessary to maintain the proper conformation of the active enzyme. Lowering the pH of the solution would protonate these groups and cause a conformational change resulting in an inactive enzyme form.

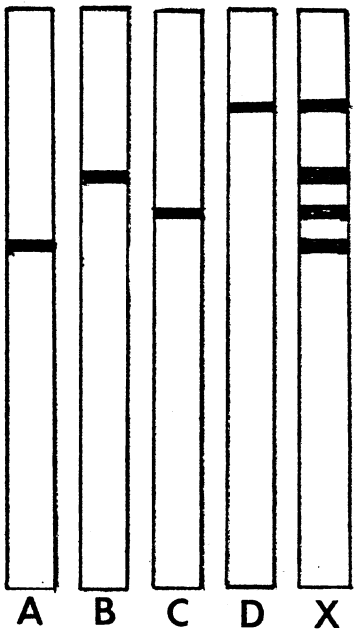
Discontinuous Polyacrylamide Gel Electrophoresis at Acidic pH

This system was investigated as a tool that could rapidly demonstrate multiple forms of DNase. Coupled with activity staining, it may be possible to compare the electrophoretic mobility and banding patterns of DNase from a variety of species or various tissues and fluids of the same species. It was hoped in this manner that insight might be gained concerning the biological function and significance of multiplicity.

Tube Gel System

Figure 10 shows the electrophoretic separation of bovine DNases A,

Figure 10. Separation of Multiple Forms of Bovine Pancreatic DNase on Polyacrylamide Discontinuous Gel Electrophoresis at Acidic pH. A, B, C and D represent DNases A, B, C and D respectively; X is the mixture of four forms. Negative pole was at the bottom of the tubes.


A
B
C
D
X

B, C, and D using the tube gel system as described in materials and methods. The order of migration from negative electrode to positive electrode was A, C, B, D. The observed pattern of multiplicity was different in order from that of phosphocellulose chromatography in that B and C were reversed, but was consistent with the results obtained by Salnikow et al (46) from electrophoresis on cellulose acetate strips at pH 4.7. The separation of the various forms in this system is based on the relative total charge differences of the various DNase isozymes due to amino acid substitutions in the primary sequence and the presence or absence of sialic acid residues in the carbohydrate moieties. The charge differences due to these factors are shown for the bovine enzyme in Table VI. Bovine DNase A lacks sialic acid residues in the carbohydrate moiety and has a histidine residue substituted for a proline residue in the primary sequence. This gives it a total positive charge at pH 4.5 relative to the other isozymes causing it to migrate the most distance toward the negative electrode. Bovine DNase D has a sialic acid residue in the carbohydrate moiety and a proline residue substituted for a histidine residue in its primary sequence. This results in a net negative charge at pH 4.5 relative to the other multiple forms causing it to migrate the least toward the negative electrode. Bovine DNase B has a sialic acid residue present in the carbohydrate moiety and a histidine residue substituted for a proline residue in its primary sequence giving it a net charge at pH 4.5 of zero. Bovine DNase C has no sialic acid present in the carbohydrate moiety, and has a proline residue substituted for a histidine residue in its primary sequence resulting also in a net charge of zero at pH 4.5. Though both bovine DNase B and C have no known charge differences, the migration rate of

TABLE VI

ELECTROPHORETIC SEPARATION OF BOVINE DNASE MULTIPLE FORMS: SEPARATION DUE TO RELATIVE TOTAL CHARGE AT PH 4.5

Isozyme	One Residue of Sialic Acid in Carbohydrate Moiety	Charge Due To Sialic Acid at pH 4.5	Amino Acid Residue-118[a]	Charge at Residue-118 at pH 4.5	Resultant Net Charge From Amino Acids and Carbohydrate Moiety
A	No	0	His	+1	+1
B	Yes	-1	His	+1	0
C	No	0	Pro	0	0
D	Yes	-1	Pro	0	-1

[a]His - Histidine residue

[b]Pro - Proline residue

DNase B toward the negative electrode is more retarded in this system than DNase C. The reason for the retarded migration rate of bovine DNase B relative to DNase C is not clear at this time, especially in view of the fact that DNase B appears more basic (greater positive charge) on phosphocellulose chromatography at pH 4.7.

Activity Staining of Tube Gels

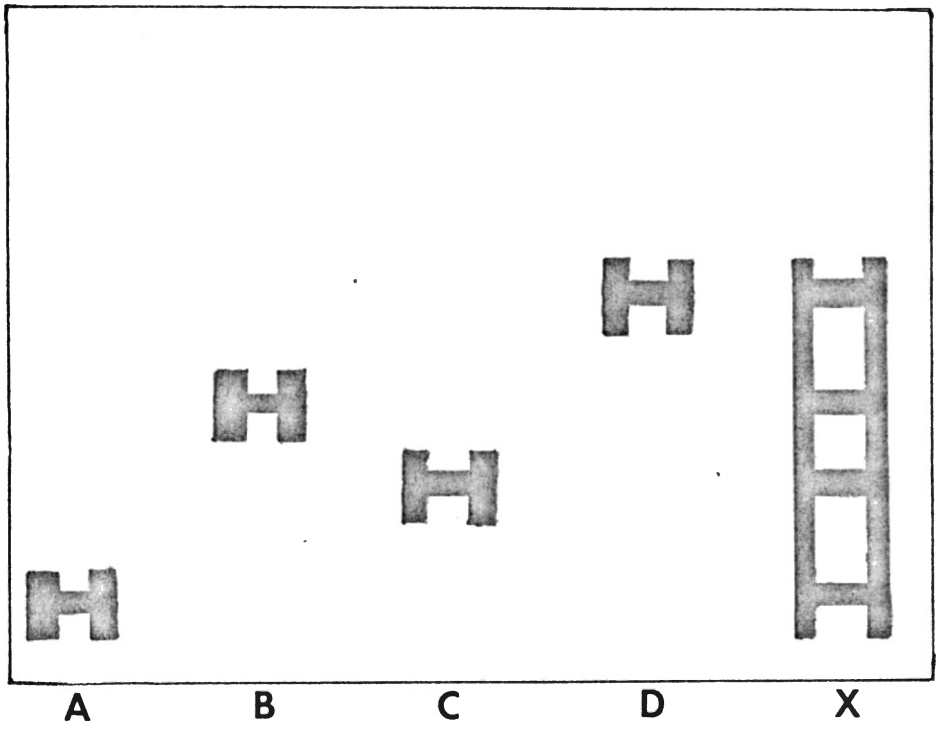
The activity staining agar technique outlined in the materials and methods chapter was applied to this system using purified bovine DNase A. A clear wide band was observed in the fluorescent background of ethidium bromide stained DNA in the same position as on a control gel run at the same time but stained in the usual fashion with Coomassie Brilliant Blue R. The appearance of a wide band was due to the fact that the agar was poured around a tube gel 5 mm in diameter and the enzyme activity was required to diffuse from the gel through the surrounding thickness of solidified agar (approximately three millimeters). The staining technique could be considerably improved by running the electrophoresis on polyacrylamide slab gels and then overlaying a thin sheet of agar over the gel. The diffusion would be negligible and the results could be visualized much more rapidly.

Slab Gel System

Application of this electrophoretic system to polyacrylamide slab gels failed to produce sharp bands. Innumerable gels were run varying the buffer concentration, acrylamide concentration, polymerization time, sample concentration and size, applied power, running time, temperature, and sample well configuration parameters. Though excellent separation of

the multiple forms could be achieved, the system was plagued by the recurrence of "H" banding patterns (Figure 11). As the value of this system resides in the sensitivity of the staining technique, the inability to produce sharp bands with the slab gel system severely limits the usefulness of this system. The diffusion inherent in the agar staining technique using tube gels would mean that narrowly separated isozymes would be detected as single bands. The resolving power of the electrophoretic system would be negated by diffusion during the staining process. On the other hand, the sensitivity of the thin agar sheet activity staining method would be negated by the "H" bond electrophoretic pattern which would cause bands to be poorly resolved.

Figure 11. Separation of Multiple Forms of Bovine Pancreatic DNase on Polyacrylamide Discontinuous Slab Gel Electrophoresis at Acidic pH. A, B, C and D represent bovine DNase A, B, C and D respectively; X is the mixture of four forms. Negative pole was at the bottom of the slab.


A
B
C
D
X

CHAPTER V

SUMMARY AND CONCLUSIONS

Continued purification and subsequent characterization of DNase is necessary if the physiological role of the enzyme and the biological function of the observed multiple forms is to be determined. The representative enzyme of this type, bovine pancreatic DNase, has been extensively characterized, and therefore is the model by which succeeding DNase I type enzymes are compared. The purpose of this investigation was to further purify and characterize DNase from ovine and porcine pancreas and to develop an electrophoretic system to enable rapid separation and detection of the multiple forms of DNase.

A reproducible purification procedure utilizing acid extraction, heat treatment and $(NH_4)_2SO_4$ fractionation was devised to initially isolate the enzyme from ovine pancreas. The enzyme was then further purified by chromatography on CM-cellulose and separated into four active components on DEAE-cellulose. Previous results by Wadano et al. (49) demonstrated the existence of four multiple forms of ovine DNase; the separation of the enzyme into four multiple forms on DEAE-cellulose lends support to their experimental findings. However, no direct comparisons could be made as to which of the active fractions from this chromatographic procedure relate to the multiple forms they observed until further studies determine the chemical basis for the multiplicity.

For the same reason, few comparisons can be drawn between the

multiple forms of ovine DNase separated on DEAE-cellulose and those of the bovine enzyme separated by Liao (48) using a similar procedure. However, the ovine enzyme appears to be more tightly bound to the ion-exchanger than the bovine enzyme as 2-3 times more Ca^{2+} was required to elute the ovine DNase from the column relative to that required to elute the bovine enzyme. The reason for this increased absorption was not apparent.

DNase activity in the porcine pancreas was first reported by Laskowski (58, 59) in 1946. No further purification or characterization of DNase from this source has been reported since then. Initial attempts to purify this enzyme showed it to be sensitive to low pH conditions, a fact alluded to by Laskowski (59). It was found possible to devise an extraction procedure that did not utilize acid treatment. A method using $(NH_4)_2SO_4$ fractionation was initially proposed, and though acceptable yields were obtained (65-70%) and the method was reproducible, further attempts to purify the enzyme from this solution were unsatisfactory. Chromatographic procedures utilizing cation-exchange media such as CM-cellulose and phosphocellulose suggested that porcine DNase was an acidic protein as satisfactory absorption of the enzyme to these types of ion-exchangers was not obtainable. For these reasons, a purification method employing DEAE-cellulose chromatography without prior $(NH_4)_2SO_4$ fractionation was devised. Three active fractions were partially resolved by this procedure. The active components were eluted at Ca^{2+} concentrations of approximately 8, 9.8 and 12 mM which indicated that porcine DNase was more tightly bound to DEAE-cellulose than either the bovine or ovine enzymes and further demonstrated the acidic nature of this protein. Continued purification by rechromatography on DEAE-

cellulose and affinity chromatography on Con A-agarose resulted in the isolation of one of the three forms in sufficient quantity to permit partial characterization of the enzyme. SDS gel electrophoresis showed this fraction to still be heterogeneous. However, from co-migration of the major band from the porcine sample with that of a bovine DNase A standard and from the fact that porcine DNase comprised the majority of protein present, it was assumed that it represented the porcine enzyme. This also indicated that the molecular weight of the porcine DNase approximated that of the bovine enzyme.

Amino acid analyses of the protein extracted from the major band of the SDS gel furnished a crude approximate composition. Due to the approximate nature of this analysis, few conclusions concerning the comparison of porcine DNase with that of the bovine or ovine enzyme could be drawn. However, it was found to verify the increased acidity of this protein and it could also be determined that the acidic nature of porcine DNase was due to an excess of acidic residues, especially glutamic acid, rather than a lack of basic residues relative to the bovine or ovine DNases. Further verification of the increased acidity of the porcine enzyme was provided by isoelectric focusing electrophoresis which showed porcine DNase to have an isoelectric point of approximately pH 4.6 relative to a bovine standard mixture which exhibited isoelectric points in the range of pH 5.2-4.8 (DNase A to DNase D).

The chemical basis for the observed multiplicity was not determined, and until further purification and characterization of porcine DNase further conclusions concerning its structure cannot be made. It can be stated that porcine DNase is significantly different from either bovine

or ovine DNase. This was confirmed by the parameters necessary to effect purification and from the approximate characterizations of the structure made in this study. Porcine DNase is a more acidic protein than either the bovine or ovine enzyme; whether this acidity is due to structural differences in the amino acid composition or carbohydrate moiety cannot be determined without further experimentation.

A system employing discontinuous polyacrylamide gel electrophoresis at pH 4.5 was devised to separate the multiple forms of DNase. In a tube gel system, separation of the four forms of bovine DNase (A, B, C and D) was demonstrated. The separation of the multiple forms was based on differences in the total charge on each at pH 4.5, due to differences in the amino acid or carbohydrate moiety composition.

An enzyme activity staining method was devised to increase the sensitivity of this technique. In the tube gel system it was found that the bands produced by this method were not well defined due to diffusion of the enzyme through the activity staining agar. A slab gel electrophoretic system was proposed to eliminate this problem. However, this system was plagued by formation of "H" band patterns regardless of the parameters or conditions employed. This would negate the sensitivity of the thin agar sheet enzyme activity staining method as the bands would be poorly resolved.

Initially, this system and staining technique were proposed as a method whereby the multiple forms of DNase could be separated and detected from crude tissue extracts. The problems encountered in the slab gel system reduce the sensitivity and therefore the value of this technique. Unless further experimentation resolves the problems encountered, it will not be applicable for use on unpurified enzyme extracts.

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