THE PURIFICATION AND CHARACTERIZATION OF A NUCLEASE FROM THE SEEDS OF MUSKMELON

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LAWRENCE D. MUSCHEK
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This is to certify that the

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ABSTRACT

THE PURIFICATION AND CHARACTERIZATION OF A NUCLEASE FROM THE SEEDS OF MUSKMELON

by Lawrence D. Muschek

A nuclease, i.e. an enzyme capable of degrading both RNA and DNA, has been purified about 2900-fold from the seeds of muskmelon. The purification procedure includes ammonium sulfate fractionation, DEAE-cellulose and CM-cellulose column chromatography, and gel filtration on P-100.

Purified nuclease catalyzes the hydrolysis of denatured DNA, native DNA, RNA, and the 3'-phosphoryl linkages of 3'-AMP, 3'-GMP, and 3'-UMP, but not of 3'-CMP. The enzyme preparation also catalyzes the dephosphorylation of the corresponding 2'-deoxyribonucleoside-3'-monophosphates (except 3'-dCMP) but at a significantly slower rate.

The various activities are inseparable and remain in constant ratios to each other throughout the purification procedure subsequent to the DEAE-cellulose step. Furthermore, the activities remain together through polyacrylamide disc gel electrophoresis and in isoelectric focusing experiments.

The enzyme preparation is free of nonspecific phosphodiesterase activities as indicated by a lack of activity on a variety of 3'- and 5'-p-nitrophenyl nucleotide derivatives. Slight contamination of the preparation by nonspecific phosphomonoesterase, however, has been observed.

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The DNase activity is preferential towards denatured DNA, has two separate pH optima, 5.7 and 8.0, exhibits endonucleolytic hydrolysis at both pH optima, and at both pH's forms 5'-phosphoryl terminated products.

Activity on 3'-AMP is maximal at pH 8.0.

The RNase activity is maximal at pH 5.7, proceeds in an endonucleolytic manner at about twice the rate of that of the DNase, and forms 5'-phosphoryl terminated products.

Activity on native DNA appears to be endonucleolytic in nature and proceeds at pH 5.7 at about twice the rate observed at pH 8.0. The rate of hydrolysis of native DNA at pH 8.0 is less than 10 percent of that observed at the same pH with denatured DNA.

Cytosine mononucleotide is absent from hydrolyzates of denatured DNA at pH 8.0, and present only in minor amounts in hydrolyzates of both RNA and denatured DNA at pH 5.7.

All activities described migrate together through Sephadex G-100 with an elution pattern of a species having a molecular weight of about 50,000.

At pH 8.0 the enzyme preparation appears to be capable of hydrolyzing polyadenylic acid and polyuridylic acid, but not polycytidylic acid. The structures of the products of hydrolysis of the copolymer, polyuridylic-cytidylic acid, indicate that the 3' oxygen to phosphorus bond of cytidylate is not hydrolyzed.

An extensive hydrolysis of denatured DNA by the nuclease appears to form approximately equal quantities of mono-, di-,

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tri-, and tetranucleotides.

The enzyme preparation is able to hydrolyze poly-2'-O-methyladenylic acid to 5'-phosphoryl terminated mono- and oligonucleotides.

THE PURIFICATION AND CHARACTERIZATION OF A NUCLEASE FROM THE SEEDS OF MUSKMELON

By

Lawrence D. Muschek

A THESIS

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in partial fulfillment of the requirements
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1970

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Appreciation is also expressed to the National Institutes of Health for providing the funds to support this investigation.

L.D.M.

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Dedicated to the light of my life . . .

NANCY, WENDY, AMY, DEBBY . . .

and CHRISTIAN

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INTRODUCTION

> We wish to suggest a structure for the salt of deoxyribose nucleic acid (D.N.A.). This structure has novel features which are of considerable biological interest.

To say that the communication (1) introduced by these words set off a virtual bomb in the fields of biochemistry and genetics would certainly be an understatement. Watson and Crick's (1, 2) helical model for the structure of DNA made an impact that was felt not only by the chemist but also by the geneticist. Whenever a new and radical idea is introduced, feverish work ensues to attempt to support or disprove such an idea. Thus, the field of nucleic acid chemistry called for new technology to meet the needs of the biochemist who was attempting to delineate nucleic acid structure.

Certainly, the key to elucidating the functions of the nucleic acids lies partly in the determination of the primary, secondary, and higher orders of structure. When one considers the difficult question of the primary sequence of nucleotides along a DNA or RNA chain, it is not surprising to find that the first complete structural analysis of a nucleic acid, Holley and coworkers' (3, 4) elucidation of the primary structure of an alanine transfer RNA, was done on a molecule of merely 25,000 molecular weight and 77

nucleotides in length. The task becomes quite formidable for DNA molecules even from the viruses, because these have molecular weights which are several million in magnitude. Let us examine some of the problems associated with determining the primary sequence of a transfer RNA. Holley (3, 5) made good use of the chemical property of RNA which makes it susceptible to partial or total hydrolysis by base. Even with this tool, and others which made it possible to separate the products of hydrolysis, without the help of some enzymes of rather high specificity the task would have been much more difficult. For instance, his use of venom phosphodiesterase to degrade oligonucleotides (5) of a specific length to a mixture of products varying from one another by one nucleotide, greatly facilitated the determination of their primary sequence. Initially, the oligonucleotides with which Holley worked were formed by specifically acting endonucleases, enzymes that cleave nucleic acids preferentially at positions other than the termini. These endonucleases have been used for a great variety of structure determinations of RNA molecules. One of the biggest problems in the field of nucleic acid chemistry today is the fact that there are no known endonucleases which will degrade DNA with as precise a specificity as such endonucleases as ribonuclease T_1 (6).

The search for new and more useful tools of nucleic acid degradation has produced a multitude of various kinds of deoxyribonucleases. Thus a need for a system of classi-

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fication of these various enzyme types with regard to their specificity has prompted a worker (7) in the field to devise the following:

1. Specificity toward the sugar moiety.
2. Exo- versus endonucleolytic mode of action.
3. Cleaving the internucleotide bond on the 3'-P versus the P-5' side, and forming products bearing either 5' or 3' monophosphate.
4. Nature of bases adjacent to the susceptible linkage.
5. Specificity toward secondary structure of the DNA; native (double-stranded) versus the denatured (monostranded) DNA.
6. Inability to attack the DNA from the same species.
7. Nucleases incapable of hydrolyzing dinucleotides.
8. Exonucleases incapable of attacking either native or denatured DNA but capable of hydrolyzing oligonucleotides.
9. Ability of some endonucleases to hydrolyze both strands of the native DNA simultaneously at the same locus.

Classifications like the preceding must be out of necessity in a constant state of revision because new nucleases are being reported that have all possible combinations of properties. The only classification which has remained absolute is number 3.

It would be a pointless and rather lengthy task to attempt to review all of the outstanding nucleases that have been reported over the past few years. Suffice to say

that extremely unusual enzymes showing much promise as potential tools have been isolated from almost every living organism. For more information regarding nucleases from various sources the reader is referred to the following recent reviews (7, 8, 9).

This work has centered on enzymes derived from plants. Plants have been used for many years as potential sources for the purification of both RNases and DNases. Workers looking for either one or the other have, in the course of their work, discovered that a rather unusual combination of nuclease and nucleotidase activities purify as one species from a variety of plant sources. Generally speaking, these nucleases (attacking both RNA and DNA) appear to prefer the single stranded form of the substrate. Optimal activity is observed at pH's between 5 and 7. An additional activity, 3'-nucleotidase (often reported as 3'-AMPase) has been found to accompany many of the previously mentioned nuclease activities. Molecular weights have usually been observed to be in the 15-30,000 range. Plants which have been found to contain these nuclease: 3'-nucleotidase combinations are barley (10, 11), ryegrass (12), rice bran (13), mung bean (14-18), wheat (19), corn (20, 21), Penicillium citrinum (22, 23, 24), and Phoma cucurbita-cearum (25). The enzymes best characterized from this series are those from mung bean and wheat. No one has stated that the associated RNase:DNase:3'-nucleotidase activities are part of the same protein molecule; in fact, Walters and Loring have concluded

that the mung bean DNase and RNase:3'-nucleotidase reside in different proteins. This view has been challenged by Johnson and Laskowski (26). The final conclusion regarding this dilemma awaits more purified preparations of enzyme. Whatever conclusions appear, the fact remains that a number of these enzymes appear to exhibit some preference for certain nucleotide bases early in the stage of hydrolysis. It was for this reason that a number of other plants were surveyed for their content of DNase activity.

The association of deoxyribonuclease, ribonuclease, and 3'-nucleotidase seems common throughout all levels of complexity in the plant kingdom. The fact that these associated activities have been found in dormant seeds as well as in rapidly growing seedlings suggests a possible role in the process of DNA repair or replication.

This thesis reports work done originally on the purification of a DNase from the seeds of muskmelon. As will become apparent, RNase and 3'-nucleotidase activities were found to be closely associated with the DNase activity. Some of the properties and specificities of this association have been studied and are described.

EXPERIMENTAL PROCEDURE

Materials

Enzyme Source

Muskmelon seeds (*Cucumis melo*), Honey Rock variety, were purchased in a 100 pound quantity from Farm Bureau Services, Lansing, Michigan.

Nucleic Acids and Derivatives

DNA[1] from salmon sperm (Type III), DNA from calf thymus (Type I), various nucleotide and nucleoside derivatives, imidazole (Grade I), and glycine were purchased from Sigma Chemical Company. Exceptions to this were 3'-GMP[1], 3'-UMP, and 3'-CMP which were obtained from P-L Biochemicals, Inc. Polyadenylic acid (Poly A), polycytidylic acid (Poly C), and polyuridylic acid (Poly U), each containing 2.5 μmoles of polynucleotide phosphorus per mg of nominal polymer weight (apparent average molecular weight 10^5 to 10^6) were purchased from Schwarz Bioresearch, Inc. Poly U,C[1] was a generous gift from Dr. F. M. Rottman. The nucleotide derivatives 5'-p-adenylate, 5'-p-nitrophenyl cytidylate, 5'-p-nitrophenyl-2'-deoxyadenylate, and 3'-p-nitrophenyl thymidylate were prepared

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[1]The abbreviations are those specified by the Journal of Biological Chemistry. Also see the appendix. Reference to this footnote will be made frequently throughout the body of this thesis. Whenever it appears the Appendix should be consulted for an appropriate explanation.

and donated generously by Mr. R. E. Jagger. p-Nitrophenyl phosphate (disodium hexahydrate), Grade A; bis-(p-nitrophenyl) phosphoric acid; and sodium-p-nitrophenyl thymidine-5'-phosphate, Grade B were purchased from Calbiochem.

Enzymes

5'-Nucleotidase, Grade II was purchased from Sigma Chemical Company. Pancreatic RNase (5x crystallized) was purchased from Calbiochem. Snake venom phosphodiesterase (*Crotalus adamanteus*, VPH); phosphodiesterase II (Bovine spleen, SPH) and alkaline phosphatase (*E. coli*, BAPC) were purchased from Worthington Biochemical Corporation.

Resins

DEAE-cellulose (Cellex-D), 0.99 meq/g and P-100 (50-150 mesh) were purchased from Bio-Rad Laboratories. CM-cellulose (course mesh), 0.60 meq/g was purchased from Sigma Chemical Company. G-25 (100-270 mesh), G-100 (40-120 μ), and DEAE-Sephadex A-25, 3.5 ± 0.5 meq/g (40-120 μ) were obtained from Pharmacia Fine Chemicals Inc.

Reagents

All chemicals were reagent grade materials purchased from either J. T. Baker Chemical Company or Mallinckrodt Chemical Works unless otherwise specified. Lanthanum nitrate was purchased from E. H. Sargent and Company. Ammonium formate and Buffalo Black NBR were obtained from Allied Chemical Company. Tris(hydroxymethyl)aminomethane

(Trizma Base, reagent grade) was purchased from Sigma Chemical Company. Bromphenol Blue was purchased from Fisher Scientific Company. Acrylamide, N,N,N',N'-tetramethylethylenediamine (Temed), and N,N'-methylenebisacrylamide (Bis) were obtained from Canalco.

Methods

Preparation of Buffers

Two buffers were used during the course of the purification procedure. Buffer A was an ammonium acetate solution, 0.1 mM in zinc acetate, adjusted to pH 8.0 with 28.9 percent ammonium hydroxide. Buffer B was an ammonium acetate solution, 0.1 mM in zinc acetate, adjusted to pH 5.5 with glacial acetic acid. All designations of concentration refer to the concentration of ammonium acetate before pH adjustment was made.[2] All other buffers used in this study were made in a similar manner, e.g. 0.2 M Tris-HCl, pH 8.2 refers to a 0.2 M solution of tris(hydroxymethyl)aminomethane adjusted to pH 8.2 with 36 percent HCl. Triethylammonium bicarbonate (TEAB) was made from triethylamine (TEA) by the following procedure: A 520 ml quantity of cold[1] deionized water was mixed with 280 ml of cold[1], twice redistilled TEA. Carbon dioxide from a dry ice generator was bubbled through the solution which was stirred constantly in an ice

[2]The change in ammonium acetate concentration after the pH was adjusted was less than 0.1 percent.

bath. The rate of CO_2 evolution was increased after an hour and continued until the pH of a ten-fold diluted sample of solution was in the range of 7.8 ± 0.1. A 200 ml quantity of cold[1] deionized water was then added (total volume equaled 950 ml after the addition). A 2 M TEAB stock solution was obtained which, if stored at 4°, was stable for about two weeks.

Preparation of RNA from Yeast

All RNA used in this study, unless otherwise specified, was prepared by the method of Crestfield et al. (27). The yeast used was bought fresh for each preparation. The final nucleic acid product was stored at -10°.

Preparation of Dialysis Tubing

All dialysis tubing used in the enzyme purification procedure was routinely boiled for 1 hour in 0.2 M Na_2CO_3, 0.01 M EDTA solution with frequent stirring. This treatment was followed by washing with deionized water. The boiling procedure was repeated until the resulting wash liquid was colorless. At this point the tubing was washed extensively with deionized water to remove all Na_2CO_3 and EDTA, and stored in 50 percent ethanol at 4°.

Preparation of Ion Exchange Resins

DEAE-cellulose: A 40 g quantity of dry resin (Cellex-D, Bio-Rad) was suspended in 2 liters of deionized

water stirred mechanically in a 2 liter beaker. Fines were removed by allowing the resin to settle for 20 minutes, decanting the suspension above the settled resin and resuspending in 2 liters of deionized water. Three to four such cycles were sufficient to remove most of the smallest particles. All of the following washings were done with the aid of a sintered glass filter (2 liter capacity) fitted to a suction flask and water aspirator. The resin was suspended on the filter in two 2 liter batches of 0.1 N NaOH. Following the base wash, the resin was washed with 2 liters of deionized water. The base wash was repeated if the previous water filtrate exhibited any color. After washing the resin with water, 2 liters of 0.1 N HCl were added, the resin was rapidly suspended, and the HCl removed immediately. The resin was washed with 2 liter portions of deionized water until the pH of the filtrate was about 5 (using pH paper). A 2 liter portion of 0.1 N NaOH was then added and the resin was washed with deionized water until the filtrate pH equaled 5. The washed, settled resin usually amounted to about 500 ml. This resin was then washed with two 500 ml portions of 0.12 M Buffer A (see Preparation of Buffers) and stored at 4^{o} in the same buffer prior to loading into a column.

CM-cellulose: A 25 g quantity of dry resin was suspended in 2 liters of deionized water in a 2 liter beaker and fines were removed by a procedure similar to that used with DEAE-cellulose, except that with CM-cellulose the settling time was 7 minutes. The settled resin was then

resuspended and washed on a sintered glass filter, using suction, with 1 liter of 0.5 M NaCl, 0.5 N NaOH solution. Two liters of deionized water were used to wash the resin. An additional wash with NaCl-NaOH solution usually was required to remove the last traces of color appearing in the filtrate. A rapid washing cycle was then carried out with 1 liter of 0.1 N HCl followed immediately by 2 liters of deionized water. Finally, the 1 liter NaCl-NaOH wash was done and followed by washing with 6 liters of deionized water. If the resin was to be stored for a long period of time before use it was washed with 1 liter of 95 percent ethanol, dried at room temperature for 2 days, and stored in a closed container. Just prior to use in a column, the resin was suspended in 1 liter of 0.01 M Buffer B.

DEAE-Sephadex A-25: A 50 g quantity was allowed to swell with constant stirring in 1 liter of deionized water at 4° for 15 hours. Fines were removed by a method similar to that used with DEAE-cellulose except that the settling time for DEAE-Sephadex was 15 minutes. The resin was washed with 0.1 N NaOH (2 liters per wash cycle) until the acidified filtrate gave a negative test for chloride with silver nitrate solution (usually 5-6 wash cycles were sufficient for 50 g of resin). Base was removed by three 2 liter washes with deionized water. If the resin was to be used with ammonium formate, it was washed with 2 liters of 0.1 N formic acid followed by removal of the excess acid with two 1 liter washes of 1.0 M ammonium formate, pH 7.5. The resin was

then equilibrated with 0.01 M ammonium formate, pH 7.5 by repeated washing. If the resin was to be used with triethylammonium bicarbonate (TEAB) it was washed, following the last base and water wash, with 2 liters of 0.5 M TEAB, pH 7.8, followed by equilibration with 0.01 M TEAB, pH 7.8.

Thin-Layer Chromatography

For the rapid separation of individual deoxy- or ribomononucleotides the method of Randerath (28) was used. Essentially the procedure consisted of ascending thin-layer chromatography on polyethylenimine (PEI) cellulose sheets using acetic acid-LiCl solvents.

Paper Chromatography

The two-dimensional paper chromatographic method of Felix et al. (29) was used for the separation of nucleosides, nucleotides, and nucleoside diphosphates of the deoxy series. Chromatography in the first direction was accomplished by use of a solvent (solvent I) which was prepared by mixing 75 parts (by volume) of 95 percent ethanol and 30 parts (by volume) of 1 M ammonium acetate adjusted to pH 7.5. This solvent system separated three groups of compounds the migration rates of which decrease in the following order: nucleosides > nucleotides > nucleoside diphosphates. The four components of each group were separated in the second direction by use of a solvent (solvent II) composed of isopropanol, water, and a saturated solution of ammonium sulfate (2:18:80 by volume, respectively). Whatman No. 3 MM paper

(57 x 46 cm) was used for all separations. Further experimental details are given by Laskowski (30) concerning this method.

Polyacrylamide Disc Electrophoresis

The method of Ornstein (31) and Davis (32) was used for polyacrylamide gel electrophoresis. Columns, 0.5 x 11.5 cm, were filled to a height of 5 cm with separating gel containing acrylamide at 7 percent (w/v) concentration. The other components in the separating gel were Tris-HCl buffer, 0.370 M, pH 8.9; N,N,N',N'-tetramethylethylenediamine (Temed), 0.03 percent (v/v); ammonium persulfate, 0.07 percent (w/v); and N,N'-methylenebisacrylamide (Bis), 0.184 percent (w/v). This gel ran at pH 9.5. The stacking gel contained Tris-HCl buffer, 0.062 M, pH 6.8; Temed, 0.0575 percent (v/v); acrylamide, 2.5 percent (w/v), Bis, 0.625 percent (w/v); riboflavin, 0.5 mg percent and sucrose, 20 percent (w/v). The buffer used in the electrode compartments was 0.025 M in Tris and 0.192 M in glycine. Samples (0.5 ml) to be analyzed were made 10 percent (w/v) in sucrose and 0.001 percent (w/v) in Bromphenol Blue as tracking dye. Such samples were routinely layered onto the stacking gel by displacement of electrode buffer. A potential of 350-400 volts and a current of 5 MA per column was maintained for a period of approximately 2 hours, or until the tracking dye band was observed to reach the end of the gel. Immediately after turning off the current, the gels were removed from the columns and

either sliced and eluted for subsequent enzyme assays, or stained. The staining procedure consisted of soaking the gels in a 0.5 percent (w/v) solution of Buffalo Black in 7 percent (v/v) acetic acid for 1 hour. Destaining was accomplished by gentle stirring in several 500 ml portions of 7 percent acetic acid over a 24 hour period. Subsequently, the destained gels were stored in closed tubes in 7 percent acetic acid.

DNase Assays

When it was discovered that the hydrolysis of denatured DNA exhibited two separate pH optima, assay methods were developed to measure activity at both pH 8.0 (termed DNase B) and pH 5.7 (termed DNase A).

DNase B: This assay measures the depolymerization of heat-denatured DNA to products which are soluble in lanthanum nitrate-HCl reagent. The reaction mixture contained, in a total volume of 1.2 ml: 0.6 ml of 0.2 M tris(hydroxymethyl)aminomethane, pH 8.2[3], 0.5 ml of denatured DNA (15 minutes at 100°, followed by quick cooling to 0°) at a concentration of 40.0 (final conc. = 1.92 mg/ml) A_{260} units[1] per ml (determined following the heating procedure), and 0.1 ml of enzyme diluted sufficiently with 0.12 M Buffer A so as to obtain A_{260} values between 1.0 and 2.0. The combination of DNA and Tris buffer was brought to

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[3]Final pH at 37° with all components = 8.0.

37° and the reaction initiated by the addition of enzyme. Incubation of the mixture was carried out for 20 minutes at the end of which a 1.0 ml quantity of cold[1] lanthanum nitrate-HCl reagent (0.02 M $La(NO_3)_3$, 0.2 N HCl) was added. The mixture was stirred on a vortex mixer and maintained at 4° for 15 minutes. Centrifugation at 1,100 x g for 20 minutes at 4° was then used to separate the precipitate which had formed. The A_{260} of the supernatant solution was then determined in a Beckman DB Spectrophotometer. Absorbance readings above 1.0 were determined by diluting the solutions with water.

A unit of DNase B activity is defined as that amount of enzyme which in 20 minutes causes the formation of lanthanum nitrate-HCl soluble material amounting to 1.0 A_{260} unit[1] per ml of reaction mixture.

DNase A: This assay measures the same type of parameter as that in the DNase B assay except that the reaction mixture is maintained at pH 5.7. The only difference in reaction components is the substitution of 0.2 M imidazole, pH 5.9[4] for Tris. All other conditions as outlined under the DNase B assay are maintained in the assay for DNase A. A unit of DNase A activity is defined in the same terms as a unit of DNase B with the implied restriction of pH.

RNase Assay

This assay measures the depolymerization of yeast RNA to products which are soluble in lanthanum nitrate-HCl reagent.

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[4]Final pH at 37° = 5.7.

The reaction mixture contained, in a total volume of 1.2 ml, 0.6 ml of 0.2 M imidazole, pH 5.9[4], 0.5 ml of yeast RNA (see Methods for preparation) at a concentration of 40.0 A_{260} units[1] per ml, and 0.1 ml of enzyme diluted sufficiently with 0.12 M Buffer A so as to obtain A_{260} values between 1.0 and 2.0. The procedures employed were essentially identival to those described under the assay for DNase B. Solutions of RNA were made fresh for each assay by dissolving the nucleic acid (stored at -10°) in water at a concentration of 2.0-2.2 mg per ml and then diluting the mixture to a concentration of 40.0 A_{260} units[1] per ml.

A unit of RNase activity is defined in the same terms as a unit of DNase activity, i.e. that amount of enzyme which in 20 minutes causes the formation of lanthanum nitrate-HCl soluble material amounting to 1.0 A_{260} unit[1] per ml of reaction mixture.

3'-AMPase Assay

This assay measures the conversion of adenosine-3'-monophosphoric acid (3'-AMP) to free adenosine and inorganic phosphate. The extent of reaction is determined by measuring inorganic phosphate [by a modification of the method of Dreisbach (33)] after a suitable incubation of enzyme and 3'-AMP. The reaction mixture contained, in a total volume of 2.0 ml: 1.8 ml of 0.11 M tris(hydroxymethyl)aminomethane, pH 8.2, 0.10 ml of 0.04 M 3'-AMP, and 0.1 ml of enzyme diluted sufficiently with 0.12 M Buffer A so as to obtain

A_{310} values between 0 and 1.0. The combination of 3'-AMP and Tris was brought to 37° and the reaction initiated by addition of enzyme. A 0.5 ml aliquot of reaction mixture was removed immediately after the addition of enzyme and mixed with an equal volume of water-saturated liquid phenol.[5] Another 0.5 ml portion was removed after 15 minutes and treated in the same manner. The aqueous and phenol phases were separated by centrifugation at 1,100 x g for 5 minutes at room temperature. A determination of inorganic phosphate was then carried out on the aqueous (upper) phase according to the following procedure. A 0.1 ml aliquot of aqueous phase was added to 1.5 ml of a solution[6] composed of 2 N sulfuric acid: 8 percent ammonium molybdate (2:1, v/v) in screw-cap culture tubes. Two ml of xylene-isobutanol (65:35, v/v) were then added. The tubes were capped immediately and shaken 15 seconds, and centrifuged for 5 minutes at 1,100 x g at room temperature. The A_{310} of the upper (organic) phase was then determined in a Beckman DB spectrophotometer in capped cuvettes. The xylene-isobutanol solution described above was used in the reference cell. A standard curve relating A_{310} to inorganic phosphate was constructed for each assay. The inorganic phosphate standards were taken through the same extraction procedures as the aliquots of reaction mixtures containing enzyme and

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[5]This treatment eliminated the nonspecific binding of inorganic phosphate to protein.

[6]Made fresh daily.

and 3'-AMP. A unit of 3'-AMPase activity is defined as that amount of enzyme which causes the release of 0.1 μmole of inorganic phosphate per ml of reaction mixture in 15 minutes.

Protein Determination

Protein concentrations were determined by the method of Lowry *et al.* (34) using bovine serum albumen as a reference standard. Colorimetric readings were made at 750 mμ on a Beckman DB Spectrophotometer.

Determination of Average Chain Length of Mixed Oligonucleotides

A method for the determination of average chain length of a mixture of oligonucleotides was provided by the determination of the ratio of total to terminal phosphate. The terminal phosphate was removed by the action of alkaline phosphatase (35). The total phosphate was obtained in inorganic form by ashing the sample with sulfuric acid. Inorganic phosphate was then measured in both samples using the same assay procedure described previously for 3'-AMPase.

Assay for total phosphate: A modification of the method of Fiske and SubbaRow (36) was used for ashing the sample. A 0.5 ml sample of oligonucleotide solution having an A_{260} of 2-3 was heated over a flame with 1.0 ml of 5 N sulfuric acid in a 25 ml Folin-Wu tube until heavy white vapors appeared. After cooling, 2 drops of 2 N nitric acid were added and heating was continued until white vapors again appeared. If the sample still remained brown in color,

another drop of nitric acid was added. After cooling, 0.5 ml of H_2O was added to the colorless solution and the tube was placed in a boiling water bath for 5 minutes. A 0.10 ml aliquot of this mixture was then added directly to 1.5 ml of the ammonium molybdate-sulfuric acid solution previously described under 3'-AMPase assay. The same hydrolysis procedures were employed with inorganic phosphate standards ranging in concentration from 0.1-1.0 μmole per ml, and a standard curve relating A_{310} to phosphate concentration was constructed.[7]

Assay for terminal phosphate: The reaction mixture contained 1.0 ml of 0.2 M Tris, pH 8.2, 0.10 ml alkaline phosphatase (diluted to 20 units[8] per ml with 0.1 M Tris, pH 8.2), 0.25-0.50 ml of oligonucleotide solution, 40 A_{260} units[1] per ml, and water up to a final volume of 2.0 ml. This mixture was incubated at 37° for 4 hours. A 0.5 ml aliquot was then removed and mixed with 0.5 ml of water-saturated liquid phenol. After separation of the aqueous and organic phases by centrifugation (1100 x g for 5 minutes), a 0.1 ml aliquot of aqueous phase was assayed for inorganic phosphate as described previously under 3'-AMPase assay. The appropriate blanks lacking enzyme were included in order to compensate for any free phosphate present in the oligonuc-

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[7]It was found that this standard curve differed somewhat in slope from that obtained from the 3'-AMPase assay because of slight volume differences.

[8]As defined by Garin and Levinthal (35).

leotide mixture. Average chain length was then determined from the ratio of total phosphate to terminal phosphate.

EXPERIMENTAL RESULTS

Purification of Enzyme

The term "enzyme," as used throughout this description, refers to all associated nuclease and 3'-nucleotidase activities which subsequently will be shown to be inseparable.

Preparation of Muskmelon Seeds

It has been found that the purification procedure is most conveniently carried out on not more than 1 kilogram of seeds at a time.

One kilogram of dry seeds was washed in a 12 liter chromatography jar with distilled water over a one half hour period with frequent stirring. The wet seeds were then spread on a double layer of cheesecloth and dried in an oven overnight at a temperature of 35-40°.

Homogenization of Seeds

Unless otherwise specified all procedures were carried out at 0-4°. The washed, dried seeds were broken up into a meal using a Hobart (Model 4612) meat grinder with strainer fitting containing 4 mm diameter openings. The meal was then divided into two equal parts and each was homogenized with 1 liter of cold[1] deionized water for 2 minutes in a

commercial Waring Blendor (1 gallon capacity). Following homogenization, the suspension was centrifuged at 13,200 x g for 30 minutes and the supernatant solution was filtered through glass wool to separate unwanted bits of floating material.

Ammonium Sulfate Fractionation

The filtered crude extract was brought to 87 percent of saturation by the addition of solid ammonium sulfate (previously passed through a standard 40 mesh screen) in the amount of 575 g per liter of extract. Addition of the ammonium sulfate was done slowly over a 1 hour period while the solution was being stirred mechanically. After stirring for 20 minutes the solution was centrifuged at 13,200 x g for 20 minutes. Following centrifugation the supernatant fluid was discarded and the precipitate was dispersed into 400 ml of 55 percent ammonium sulfate solution (made by combining 220 ml of saturated solution with 180 ml of deionized water). The suspension was stirred mechanically for 30 minutes after breaking up any lumps of precipitate resulting from the transfer procedure. The suspension was then centrifuged at 13,200 x g for 45 minutes. The supernatant solution, after being separated from the precipitate, was brought to 90 percent of saturation by the addition of solid ammonium sulfate in the amount of 253 g per liter of solution. After stirring the suspension for 15 minutes it was centrifuged at 27,000 x g for 30 minutes. The supernatant fluid

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was discarded and the precipitate dissolved in 100 ml of 0.06 M Buffer A. This solution was stored at 4° for approximately 12-15 hours before proceeding with dialysis. Dialysis was carried out in two 2.3 cm diameter cellulose bags (see Methods for washing procedure) for a total of 3 hours against three 2 liter portions of 0.12 M Buffer A. The contents of both bags were combined and centrifuged at 27,000 x g for 15 minutes in order to remove a small amount of precipitate which had formed during the dialysis.

DEAE-Cellulose Chromatography

The supernatant solution from the previous centrifugation was brought to room temperature[1] and diluted with an equal volume of deionized water, also at room temperature. The remaining manipulations concerned with DEAE-cellulose chromatography were carried out at room temperature. Following dilution the dialyzed ammonium sulfate fraction was applied to a DEAE-cellulose column (3.0 x 20.0 cm) previously equilibrated with 1 liter of 0.12 M Buffer A at a flow rate of 2.0 ml per minute regulated with a peristaltic pump. Following application of the ammonium sulfate fraction, the column was washed with the same buffer with which it was equilibrated until the optical density at 280 mμ of column effluent came down to 0.10 (time required 4 hours at a flow rate of 2.0 ml per minute). A gradient prepared from 500 ml each of 0.25 M Buffer A and 0.75 M Buffer A was applied to the column at a flow rate of 1.5 ml per minute. The collection of 10 minute fractions was begun immediately after

starting the Buffer A gradient.

The elution pattern of protein and enzyme activity is shown in Figure 1. Enzyme activity was located by assaying 0.10 ml portions of alternating fractions. The bulk of activity was consistently eluted between 0.30 and 0.40 M ammonium acetate. Fractions containing at least 0.50 units of DNase per ml (see DNase B assay under Methods) were pooled. About 60 percent of the activity applied to the column was found in the pooled material whereas less than 2 percent of the applied protein was present in this fraction.

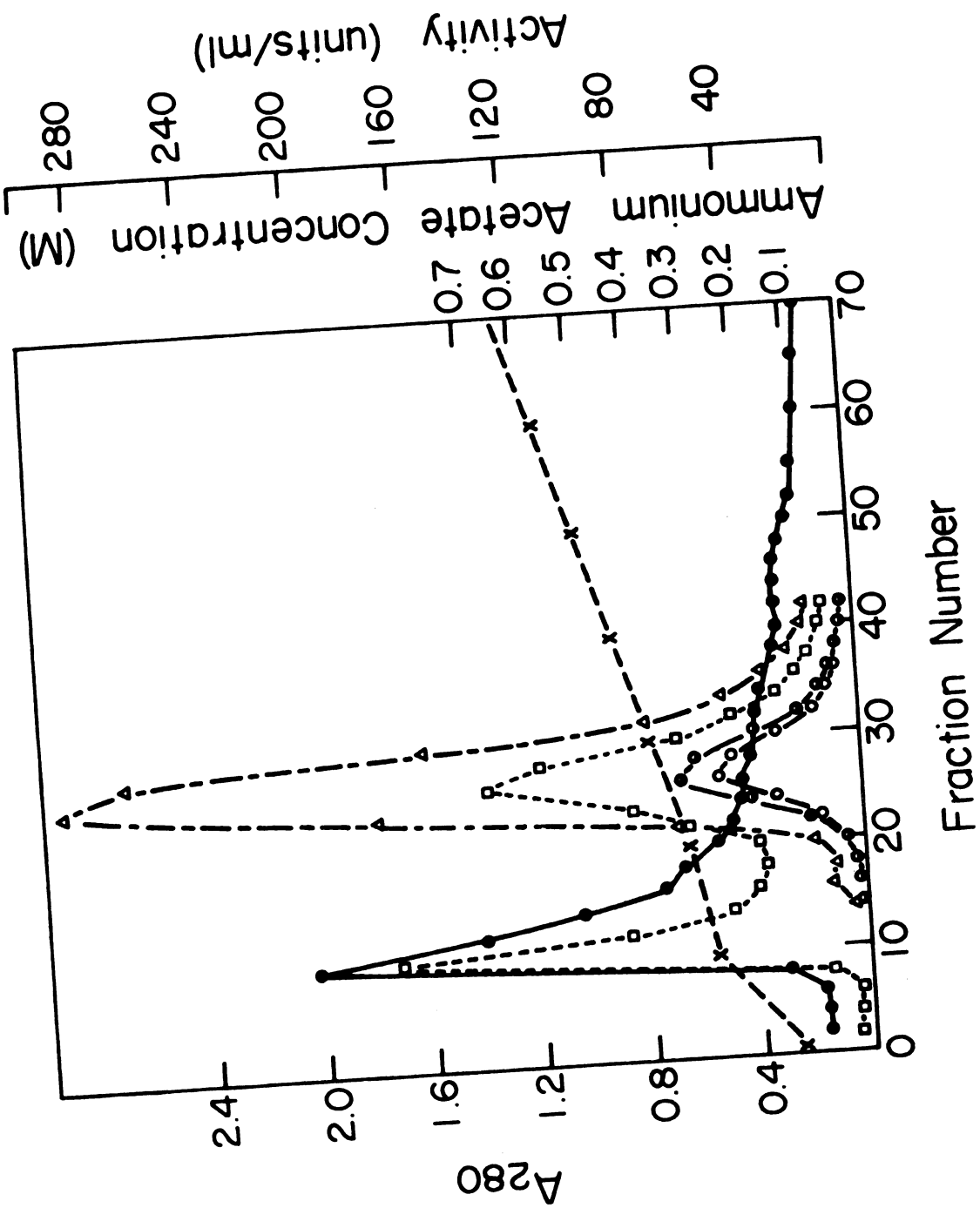
CM-Cellulose Chromatography

The pooled fraction from the DEAE-cellulose column was cooled to 4° and adjusted to pH 5.5 with 1 N acetic acid. Dialysis was then performed for a total of 6 hours against three 2 liter portions of 0.01 M Buffer B at 4°. The refractive index was routinely determined to provide a measure of the salt concentration[9] before applying the material to the CM-cellulose column. The dialyzed DEAE-cellulose fraction was then applied to a CM-cellulose column (2.2 x 30.0 cm), previously equilibrated with 1 liter of 0.01 M Buffer B at a regulated flow rate of 2.0 ml per minute. The column was washed with the same buffer for 1 hour at a flow rate of 2.0 ml per minute. A gradient prepared from 200 ml each of

[9]It was previously found that if the initial salt concentration was above 0.07 M the enzyme would pass through the column.

Figure 1: Elution Pattern from a DEAE-Cellulose Column of Protein, DNase, RNase, and 3'-AMPase Activity

The column, 3.0 x 20.0 cm, was equilibrated with 0.12 M Buffer A as described in the text. After applying the diluted, dialyzed ammonium sulfate fraction, the column was washed with 500 ml of 0.12 M Buffer A. A gradient from 0.25 to 0.75 M Buffer A was used to treat the column at a flow rate of 1.5 ml per minute. The total volume of the gradient was 1000 ml. Fractions were collected at 10 minute intervals. Enzyme activities were assayed as described in Methods. A_{280}, ●——●; DNase B, ●——●; DNase A, O——O; RNase, □---□ ; 3'-AMPase, △---△; Buffer B (Ammonium acetate) concentration, X---X.


A280
2.4
2.0
1.6
1.2
0.8
0.4
0
10
20
30
40
50
60
70
Fraction Number
Ammonium Acetate Concentration (M)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Activity (units/ml)
280
240
200
160
120
80
40

0.01 M and 0.40 M Buffer B was used at a flow rate of 1.0 ml per minute to elute the enzyme. Fractions were collected at 10 minute intervals. Figure 2 shows the elution pattern for protein and enzyme activity. The peak of activity was located in a manner similar to that used on the DEAE-cellulose column. All fractions containing any measurable DNase activity were pooled. The pooled fraction was adjusted to pH 9.0[10] with 1 N ammonium hydroxide before storage at 4°.

Chromatography on P-100 (DEAE-Cellulose)

The following procedure was carried out at room temperature.[1] A Bio-Gel P-100 column (2.0 x 70 cm) was constructed. A pad of glass wool 3-4 mm thick was placed on the resin and a 2.0 x 10 cm bed of DEAE-cellulose was poured on top of the glass wool. The column was equilibrated with 1 liter of 0.12 M Buffer A at a flow rate of 1.0 ml per minute. The CM-cellulose enzyme fraction, previously brought to room temperature after storage at 4° for 12-15 hours, was applied to the column at a flow rate of 1.0 ml per minute. Following application of the sample, the column was washed with 0.12 M Buffer A for 2 hours at a flow rate of 1.0 ml per minute. No enzyme activity could be detected in the pass-through or wash fractions. A 30 ml quantity of 0.75 M Buffer A was then applied to the column followed by

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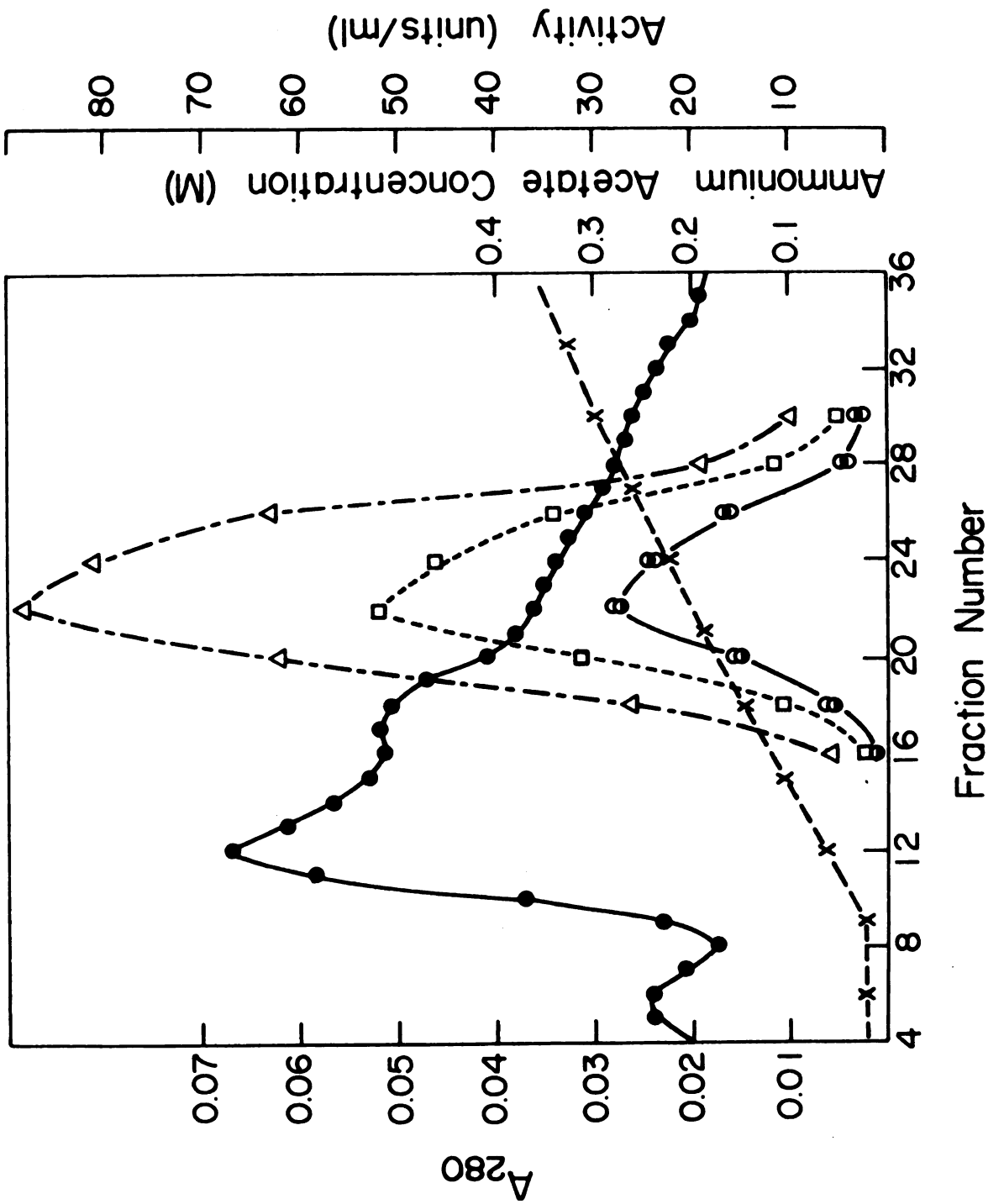
[10]It has been found that extensive loss of enzyme activity occurred if the CM-cellulose pooled material was stored at pH 5.5 for any amount of time; therefore, assays and pH adjustment were done as soon as possible.

Figure 2: Elution Pattern from a CM-Cellulose Column of Protein, DNase, RNase, and 3'-AMPase Activity

The column, 2.2 x 30.0 cm, was equilibrated with 1 liter of 0.01 M Buffer B. The dialyzed enzyme fraction from the DEAE-cellulose column was applied at a regulated flow rate of 2.0 ml per minute after which the column was washed for 1 hour with 0.01 M Buffer B at the same flow rate. A linear gradient established with 200 ml of 0.01 M and 200 ml of 0.40 Buffer B was used at a flow rate of 1.0 ml per minute to elute the enzyme. Fractions were collected at 10 minute intervals. Enzyme activities were assayed as described in Methods. A_{280}, ●——●; DNase B, ●——●; DNase A, O——O; RNase, □---□ ; 3'-AMPase, △---△ ; Buffer B concentration, X---X.


A280
0.07
0.06
0.05
0.04
0.03
0.02
0.01
4
8
12
16
20
24
28
32
36
Fraction Number
0.4
0.3
0.2
0.1
Ammonium Acetate Concentration (M)
80
70
60
50
40
30
20
10
Activity (units/ml)

the previously used 0.12 M Buffer A. Beginning at the time when the 0.75 M buffer was applied, five minute fractions were collected at a flow rate of 1.0 ml per minute. Figure 3[11] indicates the relative positions of elution of the enzyme activity and protein. The central six fractions from the enzyme peak were pooled and stored at 4°. This purification step routinely gave 80-90 percent recovery of enzyme activity originally applied to the column, an increase in specific activity to a value 5-6 times that of the CM-cellulose enzyme fraction, and a reduction in volume to less than one tenth of that of the CM-cellulose fraction. The enzyme preparation in this form was stored at -20° over a period of 6 months without any detectable loss of activity.

In order to have a sufficient quantity of enzyme preparation with which to do both characterization and specificity studies six 1 kilogram batches were purified up to and through the DEAE-cellulose step. The DEAE-cellulose fractions from three of these were combined and carried through the remaining procedures. This same treatment was repeated for the other three DEAE-cellulose fractions. At the conclusion of the purification, both batches (representing a total of 6 kilograms of dry seeds originally) were combined. This enzyme preparation was placed in storage in 1.0 ml quantities at -20°. Unless otherwise specified all

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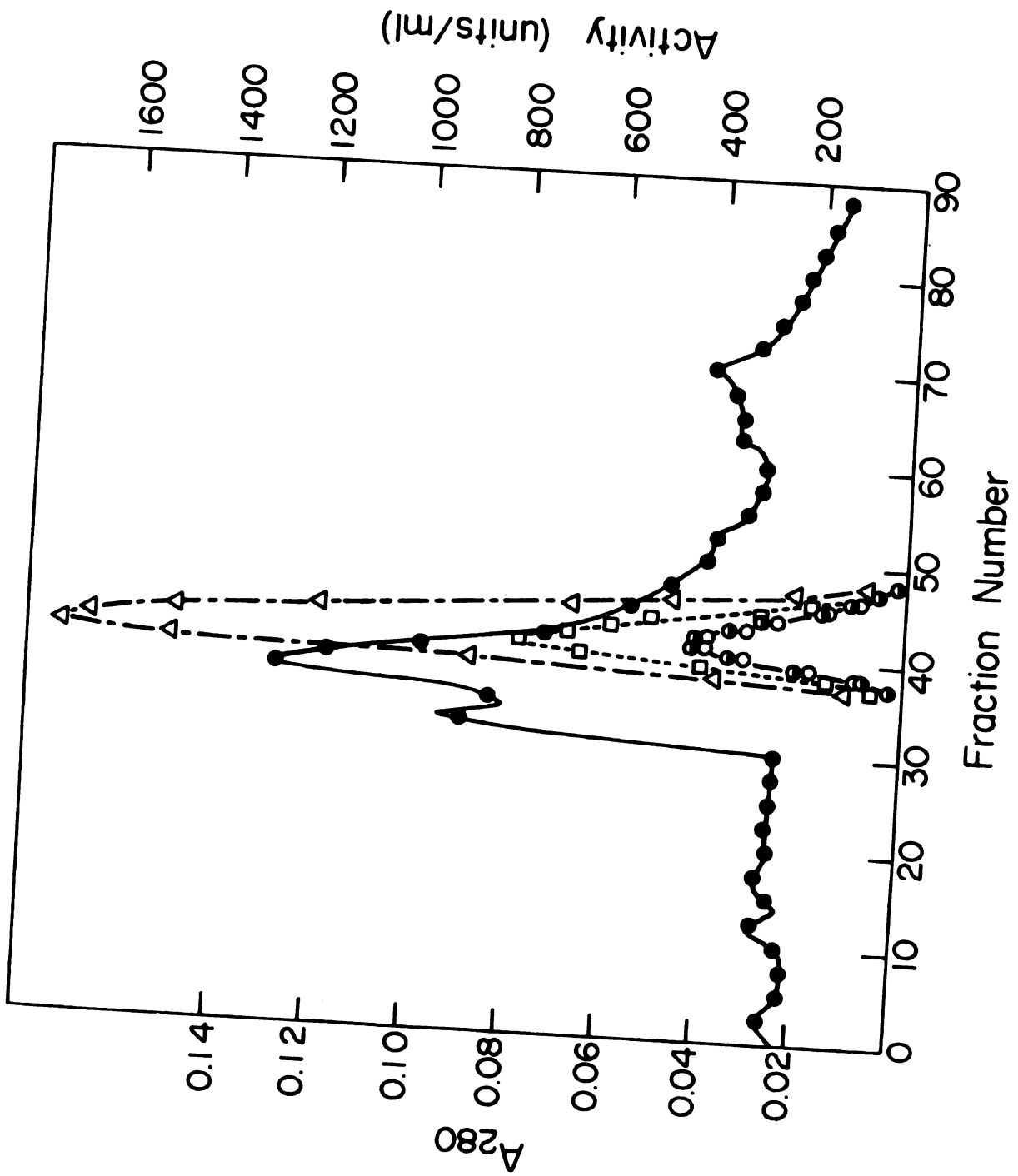
[11]Figure 3 summarizes the results of a 3 Kg. batch of enzyme.

Figure 3: Elution Pattern from a Bio-Gel P-100 DEAE-Cellulose Combination Column of Protein, DNase, RNase, and 3'-AMPase Activity (from a 3 Kg Preparation)

A Bio-Gel P-100 column, 2.0 x 70.0 cm, was constructed as described in the text. A DEAE-cellulose resin bed was poured on top of the P-100 resin to a height of 10 cm. Following equilibration of the column with 1 liter of 0.12 M Buffer A, the CM-cellulose enzyme fraction was applied at a flow rate of 1.0 ml per minute. The 0.12 M Buffer A was used to wash the column for 2 hours at the same flow rate. A 30 ml quantity of 0.75 M Buffer A was applied at a flow rate of 1.0 ml per minute. This treatment was followed by washing with 0.12 M Buffer A until 90 five minute fractions had been collected. Enzyme activities were assayed as described in Methods. A_{280}, ●——●; DNase B, ●——●; DNase A, O——O; RNase, □---□ ; 3'-AMPase, △---△.


A280
0.02
0.04
0.06
0.08
0.10
0.12
0.14
Activity (units/ml)
200
400
600
800
1000
1200
1400
1600
0
10
20
30
40
50
60
70
80
90
Fraction Number

results given in this thesis have been derived from the use of the 6 kilogram preparation of enzyme. Table I gives the results of a typical 1 kilogram preparation carried through all previously described manipulations.

Further Attempts to Separate the Four Activities

Isoelectric Focusing

Even though the four activities appeared to purify together as shown in the previous discussion it was desirable to have additional evidence bearing on the question of their inseparability. Thus, electrofocusing was used in an attempt to separate the activities. An LKB Model 8101 electrofocusing column (total capacity = 106 ml) was loaded with ampholyte solutions to give a final linear pH gradient of 3.0-10.0. A 1.0 ml sample of P-100 enzyme (200 DNase B units) was added after one half of the total ampholyte solution had been layered in the apparatus. A potential of 300 volts was applied to the column for a period of 48 hours using a Krohn-Hite Model UHR-240 constant voltage power supply. The column was emptied from the bottom by pumping water into the top at a flow rate of 1.0 ml per minute while collecting 1.0 minute fractions. During loading, electrofocusing, and unloading the apparatus was maintained at a temperature of 0.5°. The pH of every fifth fraction was determined while nuclease and 3'-AMPase were measured in alternate fractions. Data from the above determinations are summarized in Figure 4. It was evident that all activities were found in the same

TABLE I

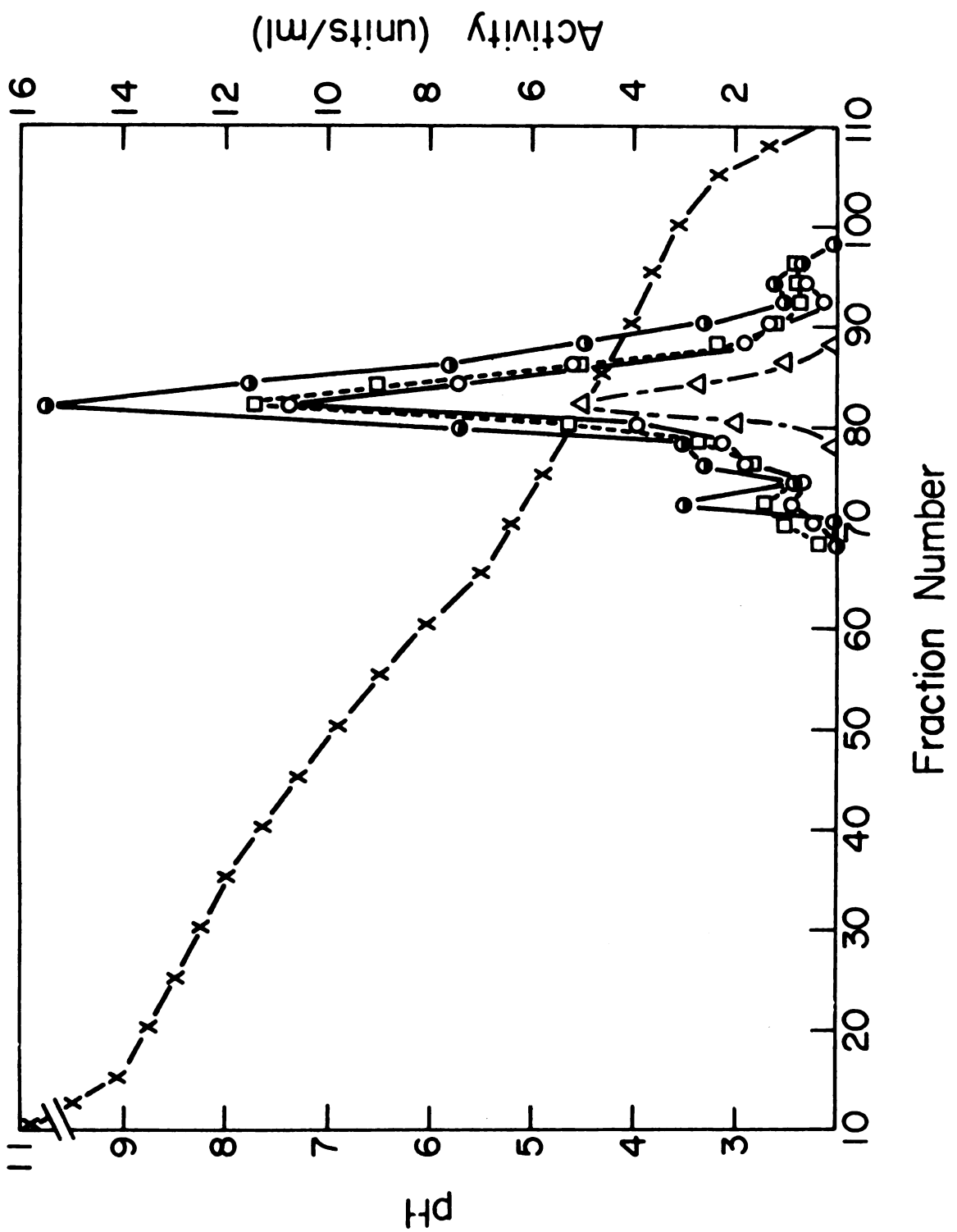
Summary of Purification of DNase[a] from 1 Kilogram (Dry Weight) of Muskmelon Seeds

Fraction	Total Units DNase B[a]	Total Protein (mg)	Specific Activity units/mg	Percent Yield DNase B
Crude	25,800	11,100	2.32	100
Ammonium Sulfate	8,620	2,085	4.14	33
DEAE-Cellulose	5,670	34.7	163	22
CM-Cellulose	3,200	3.17	1,010	12
P-100	2,750	0.41	6,700	11

[a]DNase activity assayed at pH 8.0.

Figure 4: Elution Pattern of DNase, RNase, and 3'-AMPase from an Electrofocusing Column

An LKB, Model 8101, electrofocusing column (total capacity = 106 ml) was loaded with ampholyte solutions to give a final linear pH gradient of 3.0-10.0. Muskmelon nuclease (1.0 ml of P-100 fraction, 200 DNase B units total) was added after one half of the total ampholyte solution had been layered in the apparatus. A potential of 300 volts was applied for a period of 48 hours during which time the apparatus was maintained at 0.5°. The column was emptied at a flow rate of 1.0 ml per minute while collecting 1.0 minute fractions. Assays for DNase, RNase, and 3'-AMPase were done as described in Methods. DNase B, ●——●; DNase A, O——O; RNase, □ ---□ ; 3'-AMPase, △---△ ; pH, X—X.


pH
Activity (units/ml)
Fraction Number
11
9
8
7
6
5
4
3
16
14
12
10
8
6
4
2
10
20
30
40
50
60
70
80
90
100
110

peak at a position corresponding to an isoelectric point of 4.4. The ratios of the various activities, however, did not conform to those previously observed in the latter stages of the purification scheme. This discrepancy was probably due to loss of activity by the enzyme preparation, a condition not surprising in light of previous observations of greatly decreased stability at pH values below 6.0. The RNase and 3'-AMPase determinations were performed 15 and 18 hours respectively later than the DNase measurements. An attempt was made to reactivate the enzyme by storing the peak tubes at 4° after adjusting the pH to 8.0 (with and without added zinc acetate, 0.1 mM), but this attempt proved unsuccessful.

Polyacrylamide Disc Electrophoresis

Two 0.5 ml quantities of P-100 enzyme each containing 22 μg of protein (100 DNase B units) were subjected to electrophoresis in separate columns using a 7 percent polyacrylamide gel at pH 9.5 (as described in Methods). Bromphenol blue was present in both samples to act as a tracking dye. When the dye band reached the end of the gel, the current was turned off. Both gels were removed, one was stained with Buffalo Black (as described in Methods), and the other was cut into sections each 2 mm thick. Each section was placed in 1.0 ml of 0.1 M Tris buffer, pH 8.2, and allowed to stand at 4° for 15 hours. At the end of the 15 hour period the gel slices were discarded and the eluates assayed for DNase, RNase, and 3'-AMPase (as described in Methods).

Figure 5 indicates the staining pattern for protein and the relative position of the enzyme activities found in the slices. It is evident that even at this degree of purity the enzyme preparation contained a number of different proteins. The four enzyme activities, however, were found to be associated with one protein band (the slowest moving one) and were evidently distributed through this band in a constant ratio to one another. Subsequent experiments utilizing up to 100 μg of protein per sample gave essentially the same staining pattern as that observed in Figure 5.

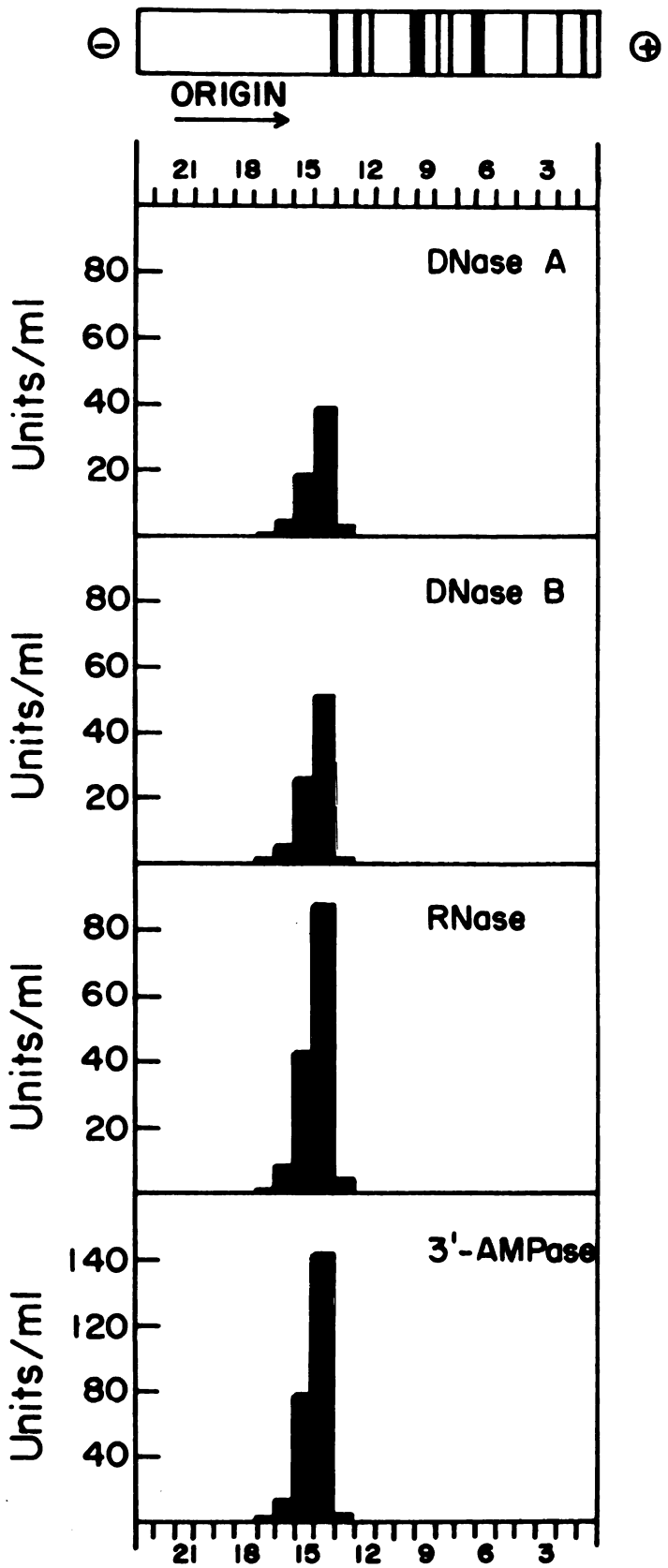
Properties of the P-100 Enzyme Preparation

Contaminating Enzyme Activities

In order to determine whether or not significant amounts of contaminating phosphomono- or phosphodiesterase activities were present in the enzyme preparation following the P-100 purification step, aliquots of the P-100 fraction, each containing 2.0 units of DNase B activity (specific activity of 4,550), were tested with various substrates at pH's 4.5, 7.0, and 9.0 for 24 hours at 37°. No detectable hydrolysis was observed with bis-p-nitrophenyl phosphate, 5'-p-nitrophenyl uridylate, 5'-p-nitrophenyl thymidylate, 5'-p-nitrophenyl-2'-deoxyadenylate, or 3'-p-nitrophenyl thymidylate. The four common 2'-deoxynucleoside-5'-monophosphates (dAMP, dGMP, dCMP and TMP) were also not hydrolyzed under the above conditions. A slight amount of

Figure 5: Staining Pattern of Protein and Distribution of Enzyme Activities in a Polyacrylamide Gel After Electrophoresis

Two 22 ug quantities of P-100 enzyme (each containing 100 units of DNase B) were subjected to electrophoresis in 7% polyacrylamide gels at pH 9.5 (as described in Methods). One gel was stained for the presence of protein and the other cut into sections each 2 mm thick. Each section, after soaking in 0.1 M Tris buffer, pH 8.2, for 15 hours, was assayed for DNase, RNase, and 3'-AMPase (as described in Methods).


⊖
⊕
ORIGIN
21 18 15 12 9 6 3
DNase A
DNase B
RNase
3'-AMPase
Units/ml
80
60
40
20
140
120
80
40
21 18 15 12 9 6 3

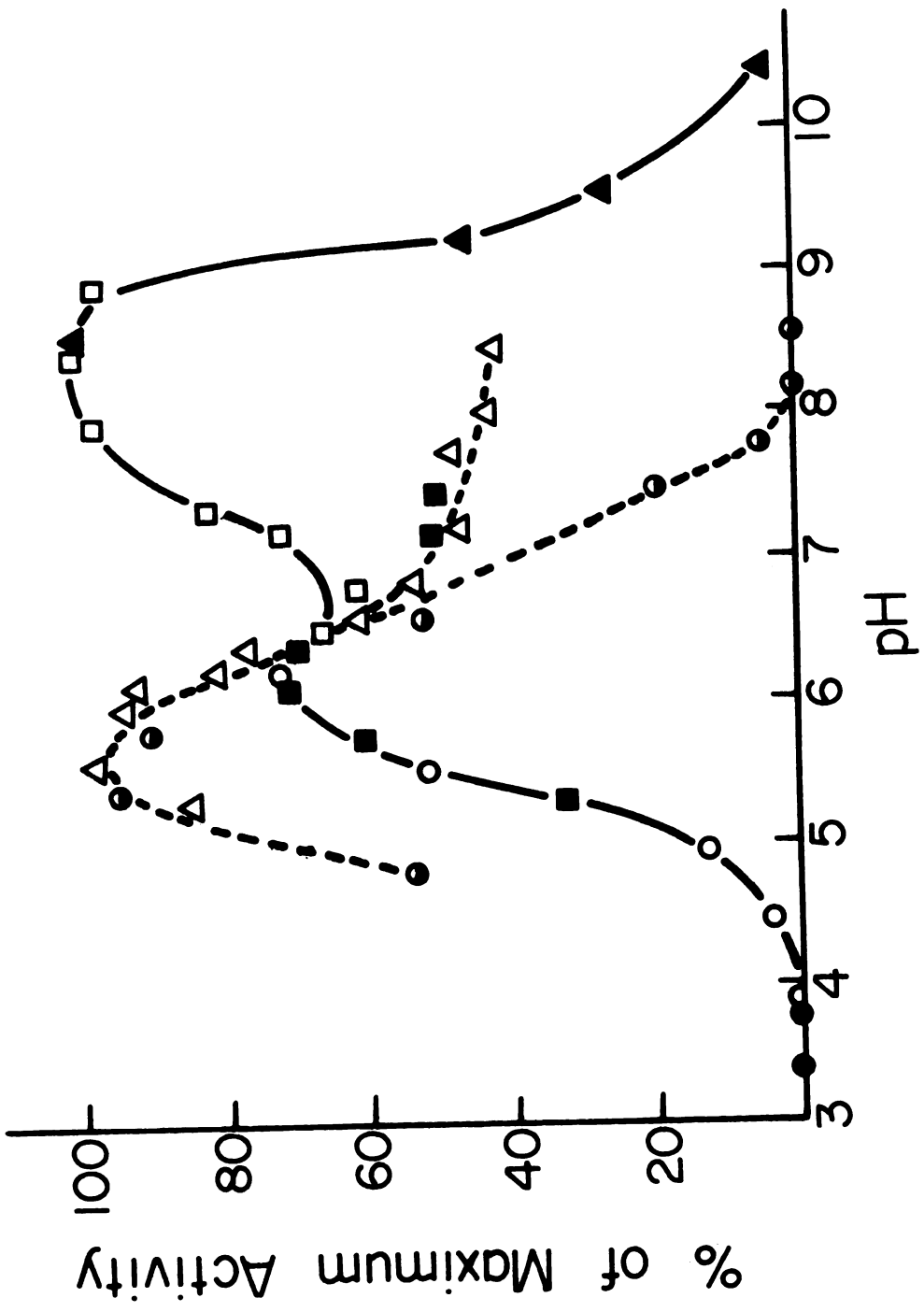
hydrolysis of p-nitrophenyl phosphate was observed. Further discussion concerning other phosphomonoesterase activity is found under Specificity Studies. During the course of extensive hydrolysis of denatured DNA no nucleosides nor inorganic phosphate could be found. Thus, it has been concluded that 5'-nucleotidase and phosphodiesterase activities were absent from the enzyme preparation following chromatography on P-100.

pH Optima of DNase Activity

Figure 6 indicates the relationship between pH and DNase activity as measured by the standard lanthanum nitrate-HCl assay described under DNase Assays in Methods. Each reaction contained 0.65 ml of 0.2 M buffer, 0.5 ml denatured DNA (as described in Methods), and 0.05 ml of P-100 enzyme preparation previously diluted to a concentration of 20 DNase B units per ml with 0.12 M Buffer A. Measurements of pH were made on the substrate-buffer mixture at 37°. Reactions showing the highest activity underwent a pH change of less than 0.1 over the 20 minute incubation period. The existence of two separate pH optima, first observed with cacodylate and Tris buffers, was substantiated when reactions were carried out in imidazole and histidine buffers in the pH 5 to 7 range. Another important result was the great difference between the activities of the enzyme in the presence of histidine and imidazole buffers at pH 8.0-8.5. A similar relationship has also been observed with the 3'-AMPase

Figure 6: pH-Activity Curve for DNase

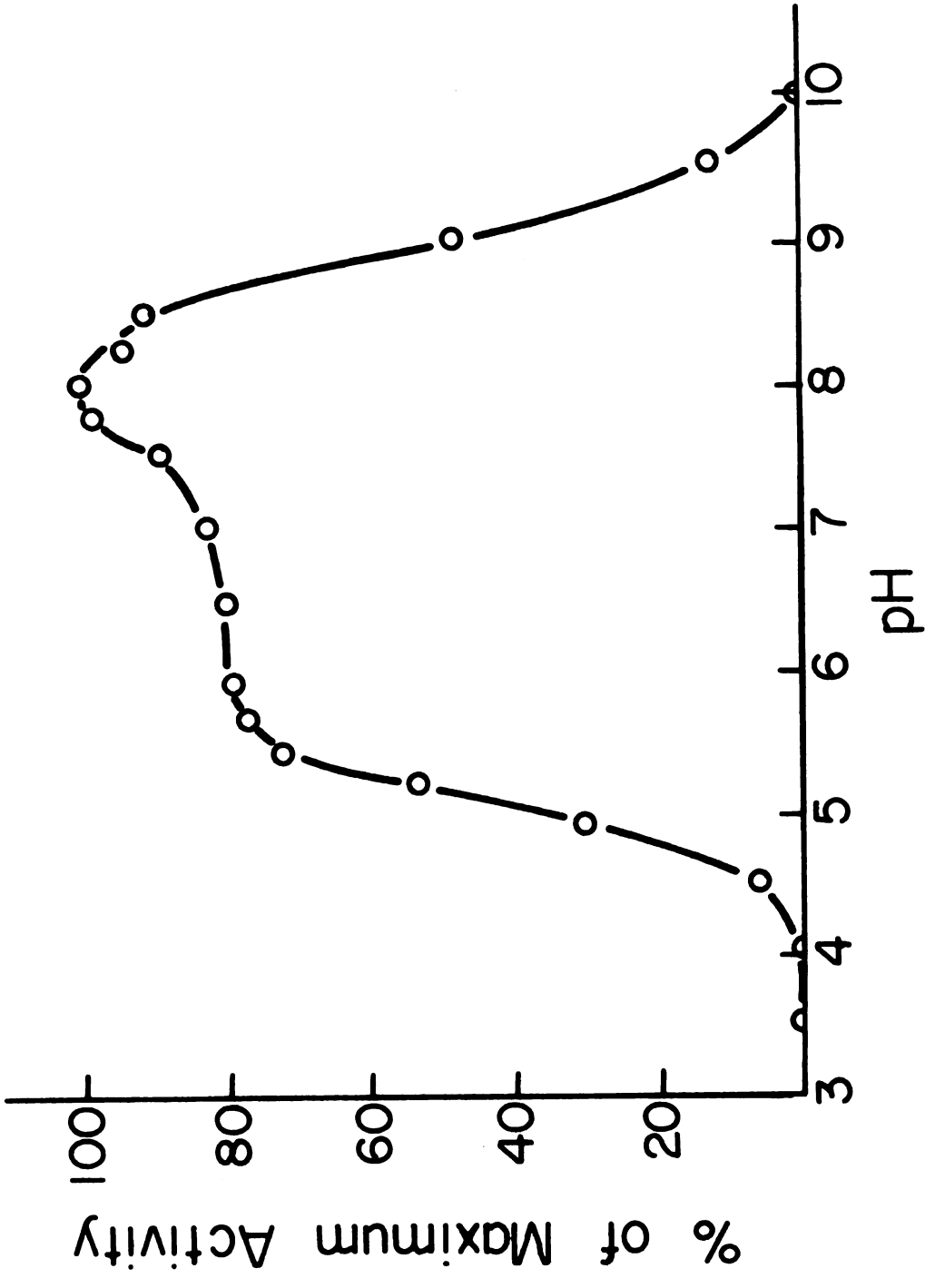
Assays for DNase activity using heat-denatured DNA were performed as described in Methods. Final buffer concentration was 0.108 M in each case. Buffer counter ions were either sodium or chloride. Buffers: citrate, ●——●; acetate, O——O; cacodylate, ■——■; tris, □——□; glycine, ▲——▲; imidazole, △---△; histidine, ⊖---⊖.


% of Maximum Activity
100
80
60
40
20
3
4
5
6
7
8
9
10
pH

activity (see Figure 10). In order to rule out buffer effects (note in Figure 6 the shift of the optimum to a lower pH and the stimulation of activity with imidazole and histidine as compared to cacodylate), DNase activity was measured in the absence of buffers by use of a pH stat (E. H. Sargent and Co.). The instrument was used principally to maintain the pH and temperature of a reaction. Aliquots were withdrawn and assayed at the beginning and end of the incubation period. In a final volume of 6.0 ml, a typical reaction contained: 5.0 ml denatured DNA at a concentration of 1 mg per ml in 0.12 M NaCl (18.0 A_{260} units[1] per ml after denaturation), 0.1 ml of P-100 enzyme previously diluted to a concentration of 40 DNase B units per ml with 0.10 M NaCl, 0.1 to 0.2 ml of 0.01 N HCl or 0.01 N NaOH to bring the pH to an appropriate value, and water to 6.0 ml. All reactions were incubated at 37° for 20 minutes after adding enzyme. Aliquots of 1.2 ml were removed immediately after adding enzyme and at the end of 20 minutes. These aliquots were added to 1.0 ml of cold[1] lanthanum nitrate-HCl reagent and treated as described under DNase Assays in Methods. Figure 7 indicates the relative activities obtained at various pHs. It is evident that the enzyme does exhibit DNase activity over a rather wide range of hydrogen ion concentration. The results shown in Figure 7 are qualitatively similar to the cacodylate-Tris buffer curves presented in Figure 6.

Figure 7: pH-Activity Curve for DNase Using a pH Stat

Each point represents the results of a lanthanum nitrate-HCl assay for DNase (as described under DNase assays in Methods) on 1.2 ml aliquots of a reaction mixture containing the following: 5.0 ml denatured DNA, 1.0 mg per ml in 0.12 M NaCl, 0.1 ml of P-100 enzyme (previously diluted to a concentration of 40 DNase B units per ml with 0.10 M NaCl), 0.1-0.2 ml of 0.01 N NaOH or HCl for adjustment of pH, and water to a final volume of 6.0 ml. Incubations following the addition of enzyme were carried out for 20 minutes at 37°. Aliquots of 1.2 ml were assayed immediately after the addition of enzyme and at the end of the incubation period by precipitation with 1.0 ml of cold lanthanum nitrate-HCl reagent as described under DNase assays in Methods.


% of Maximum Activity
100
80
60
40
20
3
4
5
6
7
8
9
10
pH

pH Optimum of RNase Activity

The relationship between RNase activity and pH for various buffers is shown in Figures 8 and 9. Conditions used are described in Methods under RNase Assay. One modification made, however, was that the enzyme was diluted to a concentration one fourth that used in the DNase pH optima study. The absolute activities at the maxima in Tris and imidazole buffers were equal. As with the DNase, the RNase exhibits activity over a rather wide pH range. As with the DNase activity, the pH optimum of RNase activity shifts from about 6.5 to 5.5 when imidazole or histidine is substituted for cacodylate buffer.

pH Optimum of 3'-AMPase Activity

Figure 10 shows the effect of varying pH on 3'-AMPase activity. The assays were done in accordance with the details given under 3'-AMPase Assay. Enzyme concentrations were equal to those used in the determination of DNase pH optima (Figure 6).

Effect of Metal Ions

In order to investigate the relative effects of various metals on DNase, RNase, and 3'-AMPase activities a series of experiments were carried out using the assay conditions outlined in Methods for the appropriate activity. All metals were added as the chloride salts to the incubation mixtures just before the addition of enzyme. The final concentration was 10^{-3} M (except for Na, Li, and K ions

Figure 8: pH-Activity Curve for RNase

Assays for RNase activity were performed as described under RNase Assay in Methods. Buffer concentration was maintained as described in the legend for Figure 6. The enzyme solution was diluted to a concentration of 5.0 DNase B units per ml prior to use because of the higher activity with RNA as compared to denatured DNA. Buffers: citrate, ●——●; acetate, O——O; cacodylate, ■——■ ; tris, □——□ ; glycine, ▲——▲.

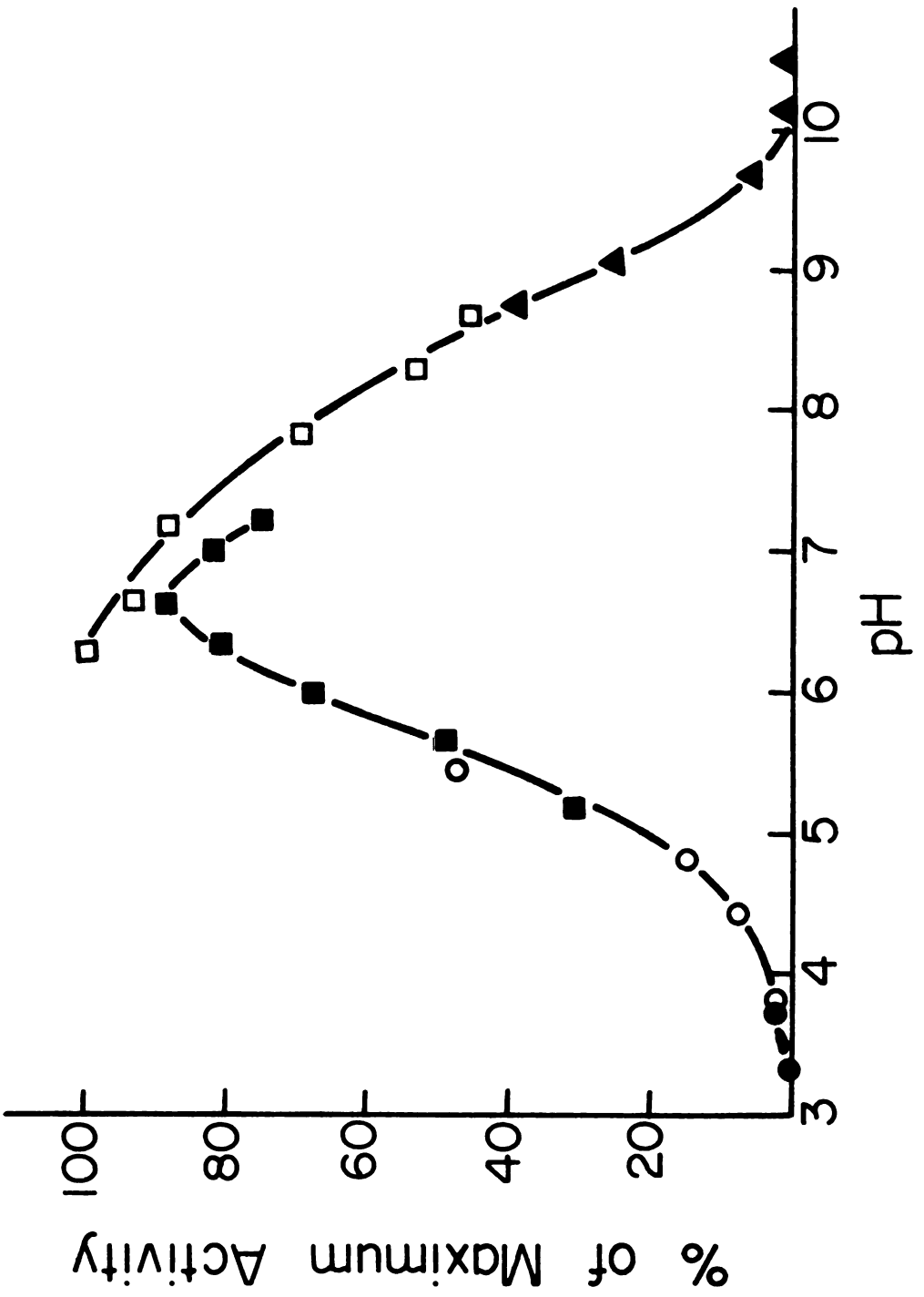

% of Maximum Activity
100
80
60
40
20
3
4
5
6
7
8
9
10
pH

Figure 9: pH-Activity Curve for RNase

Assays for RNase activity were performed as described in Methods. All other conditions were as described in the legend for Figure 8. Buffers: imidazole, △——△; histidine, ●——●.

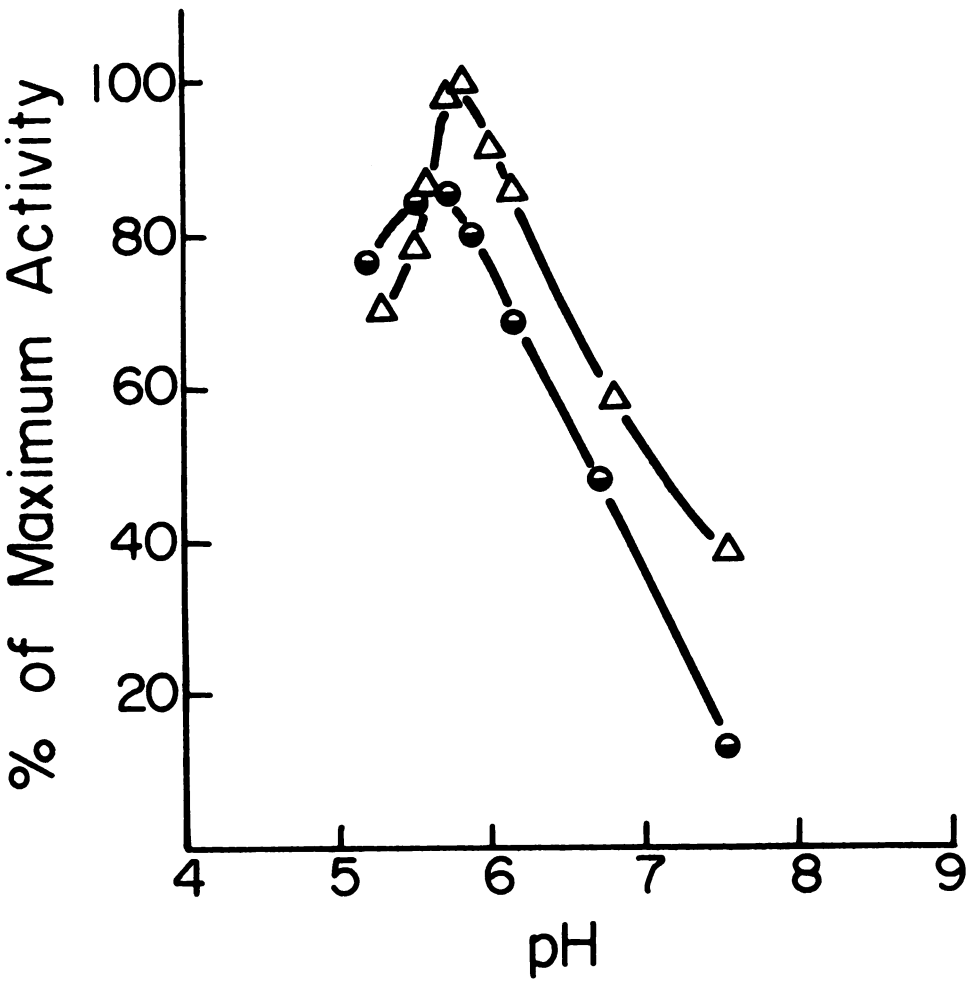
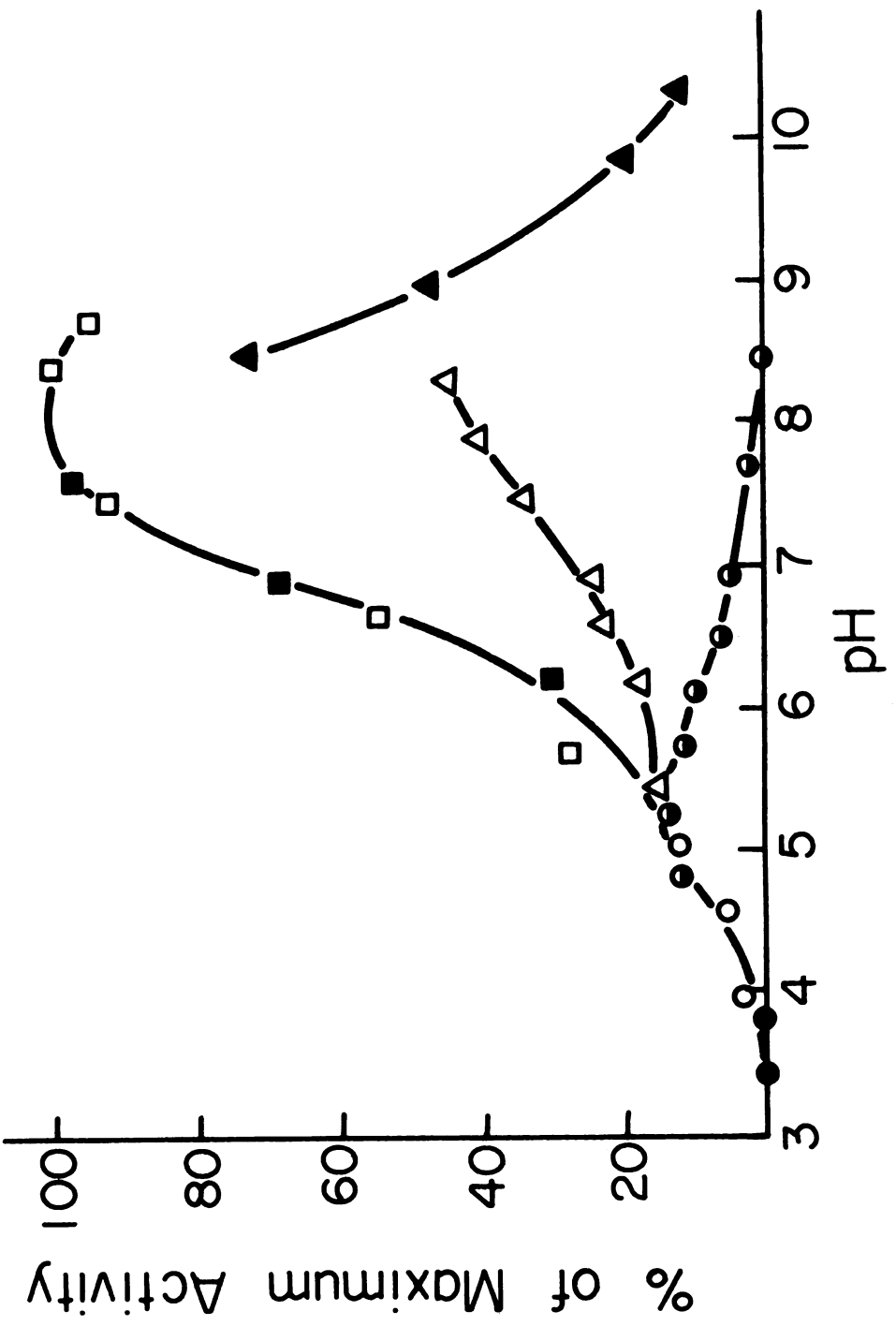

% of Maximum Activity
100
80
60
40
20
4
5
6
7
8
9
pH

Figure 10: pH-Activity Curve for 3'-AMPase

Assays for 3'-AMPase activity were performed as described under 3'-AMPase Assay in Methods. The conditions of enzyme and buffer concentration were the same as those in the DNase pH-activity study (Figure 6). Buffers: citrate, ●——●; acetate, O——O; cacodylate, ■——■; Tris, □——□; glycine, ▲——▲; histidine, ⊖——⊖; imidazole, △——△.


% of Maximum Activity
100
80
60
40
20
3
4
5
6
7
8
9
10
pH

which were 10^{-2} M). The appropriate controls containing metal ions but without enzyme and/or substrate were included in order to detect invalid A_{260} or A_{310} readings. The P-100 enzyme preparation used was diluted to a concentration of 10 DNase B units per ml with 0.12 M Buffer A. The final concentration of zinc in the DNase and RNase assays, therefore, was 4×10^{-7} M, and in the 3'-AMPase assay, 2.4×10^{-7} M. These levels were assumed to be insignificant compared to the levels of added metals. Table II summarizes the results obtained. None of the metal ions led to major increases in enzyme activity, although the divalent cations, Mg^{++}, Ba^{++}, Ca^{++}, and Sr^{++} stimulated DNase B and RNase activity 7-20 percent, and similar increases in RNase and 3'-AMPase activity were found with Li^{+}, Na^{+}, and K^{+}. The other divalent metal ions tested were generally inhibitory to all activities of the preparation.

It must be pointed out, however, that these changes in enzymatic activity brought about by the addition of mono- or divalent cations could reflect interactions of the metals with substrates, especially in the case of polynucleotides (9), rather than direct effects on the enzyme involved.

Ratios of DNase A, RNase, and 3'-AMPase to DNase B Through Purification

In order to ascertain whether or not the four activities mentioned above purify together, measurements of each activity were made following each step in the purification procedure. Table III indicates the relationships, expressed

Add

a A
ca

b Th
exc

c All
3'-
0.0

TABLE II

Effect of Cations on Activity

Addition to Assay[b]	Percent of Activity in Absence of Cation			
	DNase B[a] pH 8.0	DNase A[a] pH 5.75	RNase[a] pH 5.7	3'-AMPase[a] pH 8.0
None[c]	100	100	100	100
$MgCl_2$	112	104	107	97
$BaCl_2$	114	98	107	102
$SrCl_2$	120	104	113	98
$CaCl_2$	123	91	114	127
$MnCl_2$	63	77	57	25
$ZnCl_2$	34	66	37	6
$CoCl_2$	52	78	51	77
$FeCl_2$	18	35	12	72
$NiCl_2$	37	62	45	83
$CdCl_2$	36	56	29	49
$CuCl_2$	82	43	27	115
LiCl	98	90	117	114
KCl	94	84	123	125
NaCl	96	92	122	123

[a]All reactions were performed at the pH optima values indicated for each type of activity.

[b]The final concentration of the additives were 0.001 M except for LiCl, KCl, and NaCl which were 0.01 M.

[c]All reactions contained 0.01 M ammonium acetate (except 3'-AMPase in which the ammonium acetate concentration was 0.006 M) because of the enzyme addition.

TABLE III

Ratios of DNase A, RNase, and 3'-AMPase to DNase B Through Purification

Fraction	Ratio of Specific Activities			DNase B Specific Activity
	DNase A / DNase B	RNase / DNase B	3'-AMPase / DNase B	
Crude	1.16	145	1.92	2.32
Ammonium Sulfate	0.758	23.2	3.45	4.14
DEAE-Cellulose	0.759	2.02	4.33	163
CM-Cellulose	1.00	2.05	4.27	1,010
P-100	0.920	1.73	4.11	6,700

as the ratio of each specific activity to that of DNase B, obtained from the same preparation as that used for the data in Table I. As is evident, the ratios involving DNase A and 3'-AMPase change in the same relative proportions throughout most of the purification scheme. The ratio involving RNase, however, does not stabilize in the last step of the purification. This could reflect the presence of some RNase activity not associated with the other nuclease activities in the preparation.

Molecular Weight Determination

In order to estimate the molecular weight of the P-100 enzyme preparation, the gel filtration method of Andrews (37, 38) was used. A 2.5 x 46 cm column of Sephadex G-100 (40-120 μ bead size) was equilibrated with 0.12 M Buffer A at a flow rate of 0.5 ml per minute at 4°. All samples were applied to the column in a volume of 2.0 ml of equilibration buffer containing 10 mg of sucrose. The material loaded on the column was layered beneath the equilibration buffer above the resin bed. Buffer flow was maintained at a constant rate by the use of a peristaltic pump; fractions were collected at 10 minute intervals. Various combinations of standard proteins were chromatographed to give a calibration curve relating elution volume to the known molecular weight of each standard. The variation of elution volume of consecutive passes of the same protein through the column was in the order of 3 percent. Table IV lists the various standard

TABLE IV

Protein Standards Used in Molecular Weight Determination

Protein Standard	Source	Molecular Weight ($\times 10^{-3}$)	Parameter Measured	Amount Used	Elution Volume (ml)
Cytochrome c (Horse Heart)	Sigma Type VI	12.4	A_{410}	2.0 mg	147
Micrococcal Nuclease	Sigma Grade IV	16.8	A_{260} (a)	0.1 mg	137
DNase I (Beef Pancreas)	Sigma DN-ST	31.0	A_{260} (b)	0.5 mg	128
Hemoglobin (Rabbit Reticulocytes)	(c)	64.5	A_{410}	1.8 mg	110
Alkaline Phosphatase (*E. coli*)	Worthington BAPC	80.0	A_{400} (d)	0.05 mg	93.0
Lactate Dehydrogenase (Rabbit Muscle)	Worthington LAD	135	A_{280}	2.1 mg	71.0

[a]DNase activity measured using the standard $La(NO_3)_3$-HCl assay at pH 8.0.

[b]DNase activity measured using the standard $La(NO_3)_3$-HCl assay at pH 8.0 except that nDNA was used instead of dDNA.

[c]Gift from Mr. Ching Jer Chern.

[d]Alkaline phosphatase activity measured by the method of Garen and Levinthal (35).

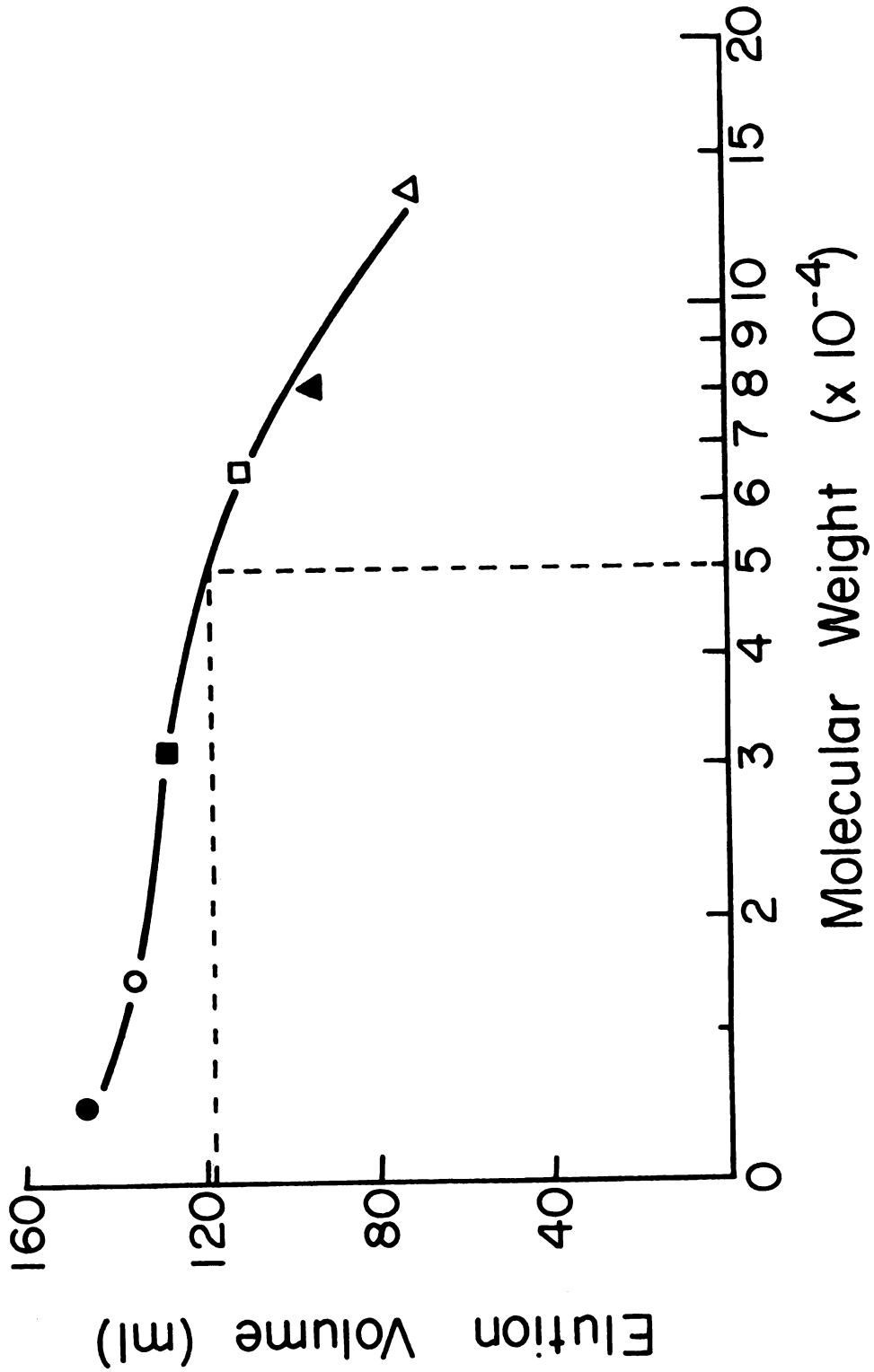
proteins used in constructing the calibration curve shown in Figure 11. As can be seen in Figure 11 the extrapolated value for the molecular weight of the P-100 purified muskmelon nuclease is about 50,000.

Stability of P-100 Enzyme Preparation

It has been previously shown (see Purification) that the P-100 enzyme preparation is unusually stable to storage at -10° for relatively long periods of time. In addition, less than 15 percent of the DNase activity was lost in 6 months when P-100 enzyme preparations were stored at 4° or lyophilized and kept at -10°. An experiment designed to find a way to abolish DNase activity indicated that at pH 7.5 in the presence of 1 mM magnesium chloride and substrate levels of dDNA the P-100 enzyme DNase activity was not affected by exposure to temperatures of 100° for up to 15 minutes. At pH 10, however, the DNase activity was destroyed. Preliminary work on the purification scheme also showed that following the ammonium sulfate fractionation the DNase activity was particularly stable when heated to a temperature of 80° for 20 minutes if two specific conditions were maintained. These conditions were the presence of 1 mM zinc chloride at pH 9.0. Maximum stability was observed only when both parameters of pH and zinc concentration were maintained. This heat treatment would have been retained as part of the purification procedure were it not for the fact that this procedure appeared to significantly

Figure 11: Calibration Curve for Molecular Weight Determination on Sephadex G-100

A 2.5 x 46.0 cm column of Sephadex G-100 was equilibrated with Buffer A at a flow rate of 0.5 ml per minute at 4°. Various combinations of standard proteins (amounts shown in Table IV) were applied to the column in a total volume of 2.0 ml of equilibration buffer made 0.5 percent (w/v) in sucrose. The elution volume of each protein was determined by extrapolating to the center of the peak when its concentration parameter was plotted against fraction number. Standard proteins: cytochrome c, ●; micrococcal nuclease, O; pancreatic DNase, ■; hemoglobin, □; alkaline phosphatase, ▲; lactic dehydrogenase, △; muskmelon nuclease, ---.


Elution Volume (ml)
160
120
80
40
0
2
3
4
5
6
7
8
9
10
15
20
Molecular Weight (x 10-4)

reduce the yield of DNase activity eluted from the DEAE cellulose column in the subsequent step as compared to preparations not heated. Dialysis for an extended period of time also appeared to decrease DNase activity. When a 4 ml sample of P-100 enzyme was dialyzed against 2 liters of 0.10 M sodium chloride at pH 8.0 for 27 hours a 50 percent decrease in total DNase activity was observed as compared to an undialyzed control. Complete loss of enzyme activity was also observed when a 1.0 ml sample of P-100 enzyme was passed through a 1.0 x 15.0 cm Bio-Gel P-2 column previously equilibrated with 0.01 M sodium chloride at pH 5.7. Attempts to reactivate the enzyme by addition of zinc acetate (0.1 mM final concentration) and/or adjustment of the pH to 8.0 were unsuccessful.

Studies on Enzyme Specificity

Relative 3'-Nucleotidase Activities

Since nuclease preparations from other plant sources have been shown to exhibit phosphatase activity toward not only 3'-AMP but 3'-UMP, 3'-GMP, and 3'-CMP as well, it was desirable to see whether or not the muskmelon nuclease also demonstrated such a range of specificity. The four 3'-phosphoryl mononucleotides were used as substrates in a reaction containing components as described in Methods for the 3'-AMPase Assay. Each of the four substrates was present at a final concentration of 2 mM in the assay. Equal amounts of P-100 enzyme were added to each reaction. Aliquots were removed

at two incubation times, 15 and 30 minutes, in order to check for linearity of inorganic phosphate production with time. Under the conditions where half of the 3'-AMP had been hydrolyzed in 30 minutes no hydrolysis of 3'-CMP could be detected. The relative rates of hydrolysis, expressed as percent of maximum activity with 3'-AMP equal to 100, for all four mononucleotides tested is given in Table V.

The next question to be considered was whether or not the corresponding 2'-deoxynucleoside-3'-monophosphates were hydrolyzed by the muskmelon nuclease preparation. Work with the nuclease from mung bean (26) indicated that the 3'-nucleotidase of that enzyme was sugar-specific in that only 3'-ribonucleotides were hydrolyzed. Preliminary experiments with the muskmelon nuclease using enzyme and substrate concentrations comparable to those used in the study summarized in Table V indicated that no breakdown of 2'-deoxyribonucleoside-3'-monophosphates occurred. However, in order to be certain, the levels of enzyme were increased forty-fold, and incubation times were maintained at 15 hours. Reaction mixtures of 0.10 ml total volume contained either imidazole, 0.10 M, pH 5.9 or Tris-HCl, 0.10 M, pH 8.2 plus mononucleotide at 1 mM final concentration. After the incubation period, all mixtures were frozen in a dry ice-ethanol bath and lyophilized to dryness. The dry residue in each case was dissolved in 0.04 ml of H_2O, spotted on Whatman No. 1 paper, and chromatographed in solvent I for 16 hours. Mixtures without enzyme were included as controls in the incuba-

TABLE V

Relative 3'-Nucleotidase Activities Toward Ribonucleoside-3'-Monophosphates

Mononucleotide Assayed[a]	Percent of Maximum Activity
3'-AMP	100
3'-GMP	73
3'-UMP	34
3'-CMP	0

[a]Assays were carried out as described in Methods for 3'-AMPase. Incubation times of 15 and 30 minutes were used. Liberation of inorganic phosphate was linear with time up to 30 minutes. Activities were calculated from the data obtained in the 15 minute incubation.

tion and chromatographic procedures. The four 2'-deoxynucleosides were also chromatographed in lanes adjacent to each corresponding nucleotide. Table VI summarizes the results found. At pH 8.0 there definitely was hydrolysis of 3'-dAMP, 3'-dGMP, and 3'-TMP but not of 3'-dCMP. The only nucleotide dephosphorylated at pH 5.7 was 3'-dAMP, and even that was not completely hydrolyzed under the conditions used.

Activity Toward Cyclic Mononucleotides

RNases from various sources (9) have been shown to catalyze the hydrolysis of nucleoside-2',3'-cyclic monophosphates or the corresponding 3',5'-cyclic derivatives. Because a number of these enzymes have been found in plant tissues, it was necessary to determine whether or not the muskmelon nuclease preparation exhibited such activity. Since it was already known that the muskmelon nuclease formed 5'-phosphoryl terminated products upon hydrolysis of both RNA and DNA (see later sections on the determination of phosphate position in mononucleotides from hydrolyzates of RNA and dDNA), the presence of cyclic phosphodiesterase activity would indicate that the enzyme preparation was contaminated by at least one other RNase. Reactions containing the following substrates - adenosine-3',5'-monophosphate, adenosine-2',3'-monophosphate, guanosine-3',5'-monophosphate, cytidine-2',3'-monophosphate, and uridine-2',3'-monophosphate - were incubated 15 hours at 37° under the same conditions of substrate and enzyme concentration as those involving the

TABLE VI

Relative 3'-Nucleotidase Activities Toward 2'-Deoxyribonucleoside-3'-Monophosphates

Mononucleotide Tested[b]	pH 5.7	pH 8.0
3'-dAMP	±[a]	+
3'-dGMP	-	±
3'-TMP	-	+
3'-dCMP	-	-

[a] + signifies complete conversion to the 2'-deoxynucleoside

± signifies partial conversion to the 2'-deoxynucleoside

- signifies no detectable conversion to the 2'-deoxynucleoside.

[b] All substrates were present at a final concentration of 1 mM. Each reaction contained 2.0 units of DNase (8.2 units of 3'-AMPase) in a final volume of 0.10 ml. Reactions were incubated at 37° for 15 hours.

2'-deoxyribonucleoside-3'-monophosphates. Chromatography of the reactions in Solvent I[12] for 15 hours indicated that no hydrolysis of the cyclic mononucleotides had occurred as a result of prolonged exposure to the muskmelon nuclease preparation.

Relative Hydrolysis Rates of Native DNA, Denatured DNA, and RNA

The earliest work done on the purification of DNase activity from muskmelon seeds involved the use of native DNA exclusively (39). When it became apparent that heat-denatured DNA and RNA were hydrolyzed more rapidly than native DNA, an experiment was conducted to demonstrate the relative rates of hydrolysis of all three substrates. Since activity toward denatured DNA exhibited two separate pH optima, rates of hydrolysis were measured at both pHs for an additional means of comparison. Reactions containing native DNA[13], denatured DNA or RNA at concentrations specified in Methods under the appropriate assay, but ten-fold greater in volume, were brought to 37°. A 1.0 ml aliquot was removed immediately, and at various time intervals, after adding 1.0 ml of P-100 enzyme (previously diluted to a concentration of 20 DNase B units per ml with 0.12 M Buffer A). One ml of cold[1] $La(NO_3)_3$-HCl reagent was mixed

[12]Cyclic mononucleotides migrate in Solvent I with R_f values midway between the normal phosphomonoesters and their corresponding nucleosides.

[13]The concentration of native DNA was adjusted to equal that of denatured DNA on a weight per volume basis.

immediately with each reaction aliquot and the mixture treated as described for the appropriate assay in Methods. Appropriate blanks not containing enzyme were included and incubated in a parallel fashion. Figure 12 summarizes the data obtained. An additional experiment using calf thymus DNA instead of DNA from salmon sperm gave almost identical results to those shown in Figure 12.

Mode of Action on RNA

The method of Birnboim (40) was used to characterize hydrolysis products in order to determine whether the mode of action of the P-100 enzyme preparation was endo- or exonucleolytic in nature. Essentially, the method consisted of separating the products of early stages of hydrolysis on a gel filtration column. Exonucleases characteristically degrade polynucleotides by stepwise removal of mononucleotides from one end of the chain. Endonucleases, on the other hand, make scissions in the polynucleotide chain at points other than the ends thus giving mainly oligonucleotide products of decreasing chain length as hydrolysis proceeds. In this experiment it was desirable to test the method with enzymes which were known to be either exo- or endonucleases. Figures 13a-13c summarize the results obtained from three separate reactions containing the components described in the legends. For those reactions containing enzymes (see Figures 13b and 13c), 0.5 ml aliquots were removed at various times after addition of the enzyme. The aliquot was pipetted

Figure 12: Relative Hydrolysis Rates of Native DNA (nDNA), Denatured DNA (dDNA), and RNA

Reactions contained either native or denatured salmon sperm DNA, or RNA at concentrations described in Methods. Reaction volumes were 10 times greater than those described under the appropriate assays in Methods. Aliquots of 1.0 ml were removed at various times after the addition of 1.0 ml of P-100 enzyme (previously diluted to a concentration of 20 DNase B units per ml with 0.12 M Buffer A). These aliquots were treated with 1.0 ml of $La(NO_3)_3$-HCl reagent as described in Methods for the appropriate assay. Substrate-pH: RNA-pH 5.7, △——△; dDNA-pH 5.7, □——□; dDNA-pH 8.0, ■——■; nDNA-pH 5.7, O——O; nDNA-pH 8.0, ●——●.

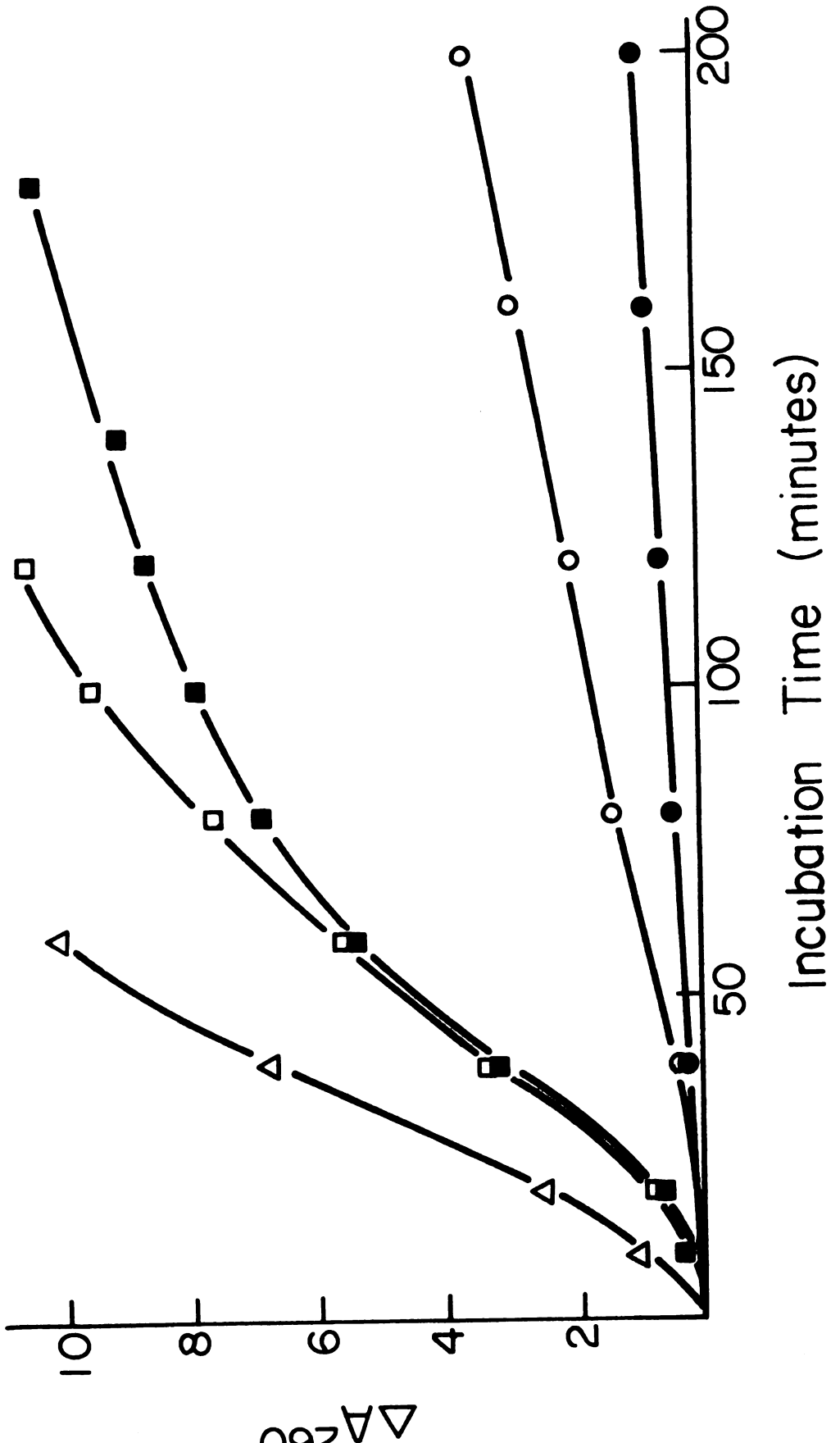

ΔA260
10
8
6
4
2
50
100
150
200
Incubation Time (minutes)

Figure 13a: Chromatography on Sephadex G-25 of a Mixture of RNA and 5'-AMP

Figure 13b: Chromatography on Sephadex G-25 of a Reaction Containing RNA and Venom Phosphodiesterase, a Known Exonuclease, at Various Stages in the Hydrolysis

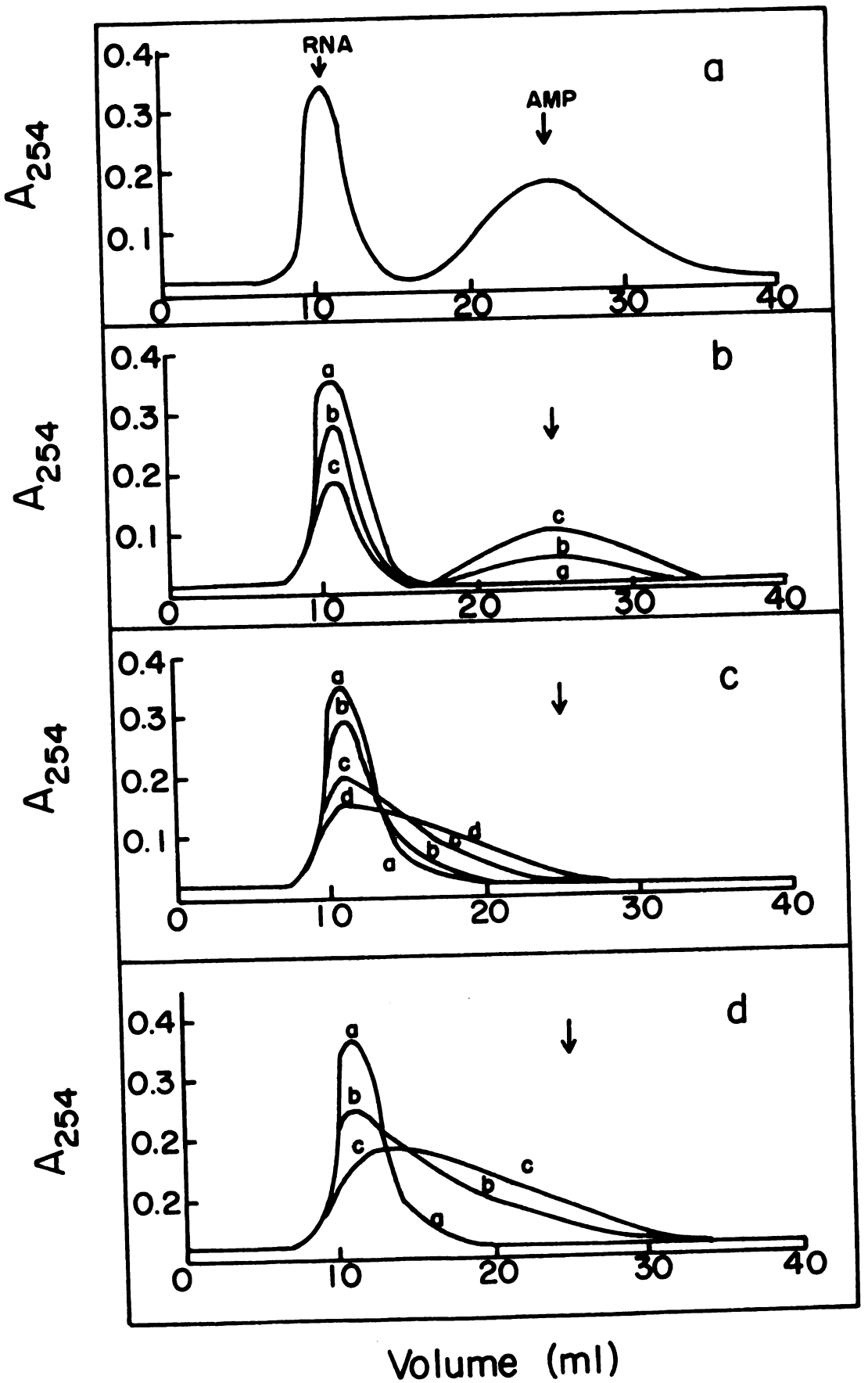
The reaction contained 8.0 ml RNA at a concentration of 20.0 A_{260} units per ml in 0.1 M Tris-HCl, pH 8.8, 0.8 ml of 0.36 M magnesium chloride, and 0.8 ml of venom phosphodiesterase, 2.3 mg per ml. Each time a 0.5 ml aliquot was removed for chromatography on Sephadex G-25, a 1.0 ml aliquot was simultaneously removed and treated with 1.0 ml of cold lanthanum nitrate-HCl reagent as described in Methods under RNase Assay. The various elution profiles are thus expressed in terms of the percent of total nucleic acid converted to lanthanum nitrate-HCl soluble products as determined by the A_{260}. a) 0%; b) 47%; c) 71%.

Figure 13c: Chromatography on Sephadex G-25 of a Reaction Containing RNA and Pancreatic RNase, a Known Endonuclease, at Various Stages in the Hydrolysis

The reaction contained 8.0 ml RNA at a concentration of 20.0 A_{260} units per ml in 0.1 M Tris-HCl, pH 7.5, 0.8 ml water, and 0.8 ml pancreatic RNase, 0.2 µg per ml. Aliquots of 0.5 and 1.0 ml were removed at various times after the addition of enzyme and treated as described in the legend for Figure 13b. The elution profiles represent the following stages of hydrolysis: a) 0%; b) 32%; c) 63%; d) 77%.

Figure 13d: Chromatography on Sephadex G-25 of a Reaction Containing RNA and P-100 Purified Muskmelon Nuclease at Various Stages in the Hydrolysis

The reaction contained components as described in Methods under RNase Assay except that the volume of each component was increased ten-fold. Aliquots were removed at various times and treated as described in the previous figure legends. The elution profiles represent the following stages in the hydrolysis: a) 0%; b) 35%; c) 72%.


RNA
AMP
a
b
c
d
A_{254}
0.4
0.3
0.2
0.1
0
10
20
30
40
Volume (ml)

into a test tube containing 0.05 ml of glacial acetic acid to terminate the reaction. A half ml of 0.1 M sodium acetate, pH 4.5, was then added and 0.5 ml of this mixture was applied to a 1.2 x 13 cm Sephadex G-25 column previously equilibrated with 0.1 M sodium acetate, pH 4.5, at a regulated flow rate of 1.0 ml per minute. As can be seen in Figures 13b the typical elution pattern for an exonuclease was obtained from venom phosphodiesterase, an enzyme previously shown (41) to be an exonuclease. Using Figure 13a as a reference, it can be seen that the only products eluting from the column (in Figure 13b) were mononucleotides. In Figure 13c, however, the elution profile indicates that as hydrolysis proceeds products are formed which vary in size from mononucleotides up to the large polynucleotides in the unhydrolyzed, excluded peak. This is a typical elution profile for an endonuclease (pancreatic RNase). Figure 13d summarizes the results obtained when the muskmelon nuclease (P-100 fraction) was tested. Conditions used were identical to those used in the assay for RNase (see Methods) except that the volume of each reaction component was increased ten-fold. The P-100 enzyme preparation was diluted to a concentration of 4.0 DNase B units per ml before use. As is evident from the results of Figure 13d, the muskmelon nuclease appeared to degrade RNA in an endonucleolytic fashion.

Mode of Action on Denatured DNA (dDNA)

The same method (40) as that described directly above was used to determine the mode of action of the P-100 enzyme preparation on heat denatured DNA. Because of the presence of two DNase pH optima, it was desirable to test the enzyme at both pH 5.7 and 8.0. Reactions containing dDNA, P-100 enzyme, (20 DNase B units per ml), and either Tris or imidazole buffer in the amounts described in Methods under assays for DNase B and DNase A, respectively (except that the volume of each component was ten-fold greater) were incubated at 37°. Aliquots of 0.5 and 1.0 ml were removed at various times and treated as described in the previous section (see Mode of Action on RNA). Figures 14b and 14c summarize the results obtained from reactions at pH 5.7 and 8.0 respectively. The enzyme appeared to act endonucleolytically on dDNA at both pH optima.

Mode of Action on Native DNA (nDNA)

Since the P-100 enzyme preparation exhibited significant activity on native DNA, especially at pH 5.7, (see Relative Hydrolysis Rates of Native DNA, Denatured DNA, and RNA and Figure 12) it was thought important to characterize the mode of action on this substrate as well. Therefore, reactions containing native DNA (at concentrations comparable to those used in the previous study on dDNA), P-100 enzyme (20 DNase B units per ml), and either Tris or imidazole buffer in amounts described in Methods under assays for

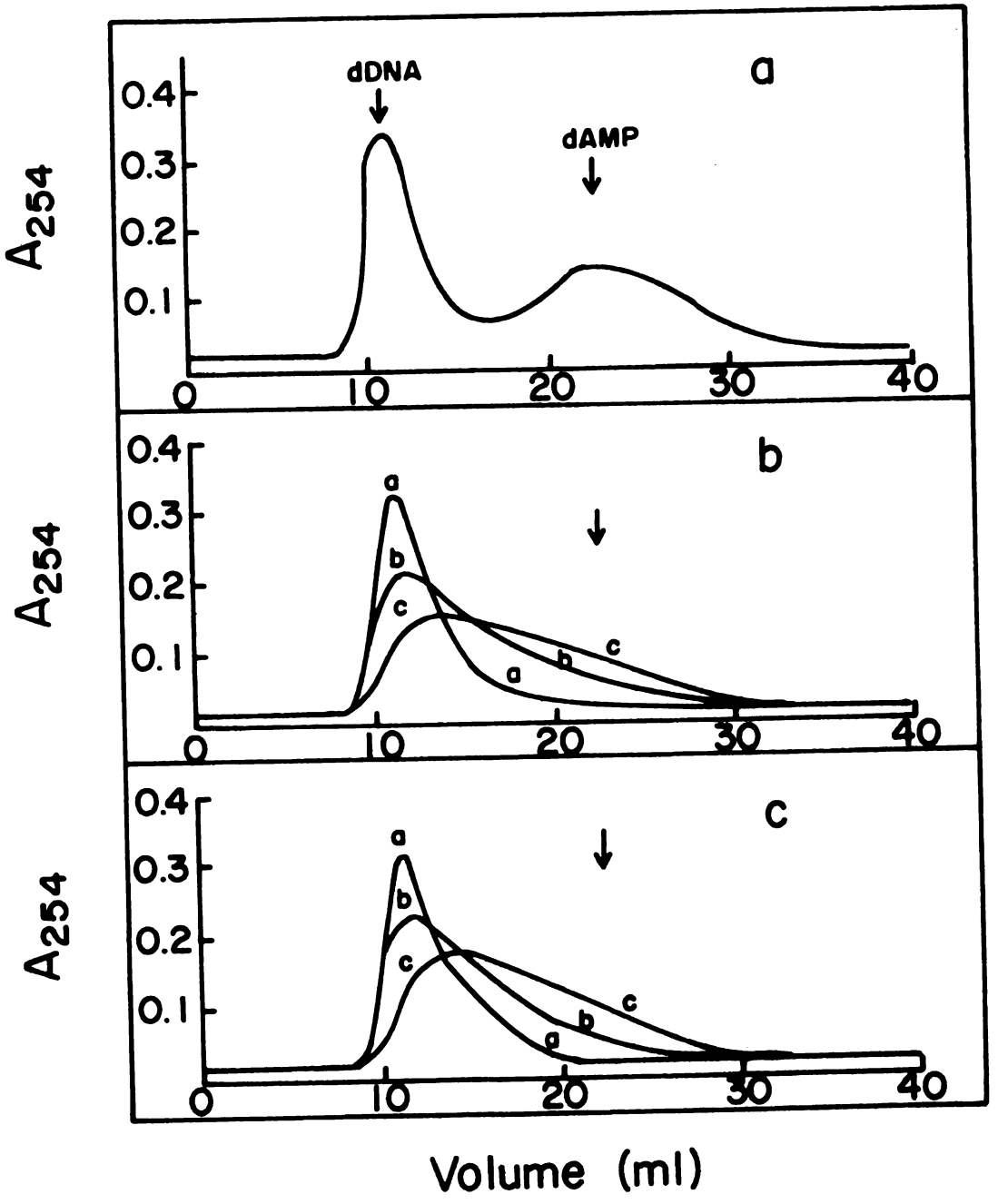
Figure 14a: Chromatography on Sephadex G-25 of a Mixture of dDNA and dAMP

Figure 14b: Chromatography on Sephadex G-25 of a Reaction, at pH 5.7, Containing dDNA and P-100 Enzyme at Various Stages in the Hydrolysis

The reaction contained components as described in Methods under DNase A assay except that the volume of each component was increased ten-fold. Aliquots were removed at various times and treated as described in the text (see Mode of Action on RNA). The elution profiles represent the following stages in the hydrolysis: a) 0%; b) 54%; c) 91%.

Figure 14c: Chromatography on Sephadex G-25 of a Reaction, at pH 8.0, Containing dDNA and P-100 Enzyme at Various Stages in the Hydrolysis

The reaction contained components as described in Methods under the assay for DNase B except that the volume of each component was increased ten-fold. Aliquots were removed at various times and treated as described in the text (see Mode of Action on RNA). The elution profiles represent the following stages in the hydrolysis: a) 0%; b) 45%; c) 87%.


dDNA
dAMP
a
b
c
A_{254}
0.4
0.3
0.2
0.1
0
10
20
30
40
Volume (ml)

DNase B and DNase A, respectively (except that the volumes were ten-fold greater) were incubated at 37°. Aliquots were removed at various times and treated as described in the discussion titled Mode of Action of RNA except that each aliquot to be chromatographed was brought to 100° for 10 minutes and quickly cooled in ice in order to denature the DNA[14]. Figures 15b and 15c indicate that at pH 5.7 and 8.0, respectively, the enzyme degrades nDNA in an endonucleolytic manner.

Determination of Phosphate Position in Mononucleotides Obtained From a Hydrolyzate of RNA

Preliminary experiments with the products of hydrolysis of RNA by the P-100 enzyme preparation indicated that mononucleotides as well as oligonucleotides were formed upon extensive[15] degradation. Treatment of these products with 5'-nucleotidase (42) (which dephosphorylates only nucleoside-5'-monophosphates) produced significant amounts of inorganic phosphate and nucleosides. Therefore, it was desirable to determine whether or not all mononucleotides produced in a hydrolysis of RNA were the 5'-phosphoryl derivatives.

A reaction containing 25 ml of yeast RNA (see Methods: Preparation of RNA from Yeast) at a concentration of 2 mg per ml; 30 ml of 0.2 M imidazole, pH 5.9; and 5.0 ml of P-100 enzyme previously diluted to a concentration of 20 DNase B

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[14]Native DNA was found to decrease the flow rate of the Sephadex G-25 column.

[15]Complete conversion of substrate to lanthanum nitrate-HCl soluble products when measured by the RNase assay as described in Methods.

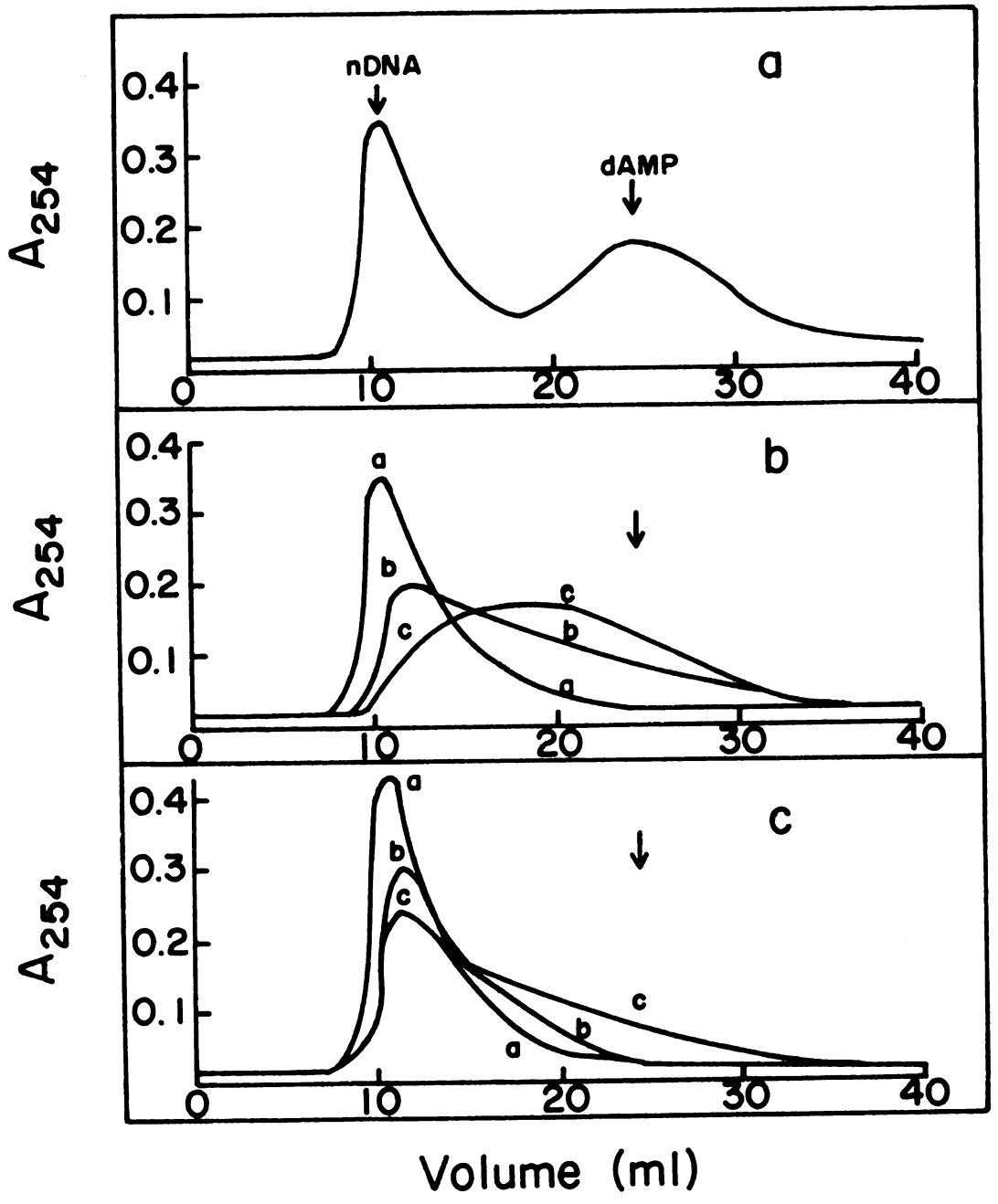
Figure 15a: Chromatography on Sephadex G-25 of a Mixture of nDNA and dAMP

Figure 15b: Chromatography on Sephadex G-25 of a Reaction, at pH 5.7, Containing nDNA and P-100 Enzyme at Various Stages in the Hydrolysis

The reaction contained components as described in Methods under DNase A assay except that the DNA was not denatured and the volume of each component was increased ten-fold. Aliquots were removed at various times and treated as described in the text. The elution profiles represent the following stages in the hydrolysis: a) 0%; b) 55%; c) 83%.

Figure 15c: Chromatography on Sephadex G-25 of a Reaction, at pH 8.0, Containing nDNA and P-100 Enzyme at Various Stages in the Hydrolysis

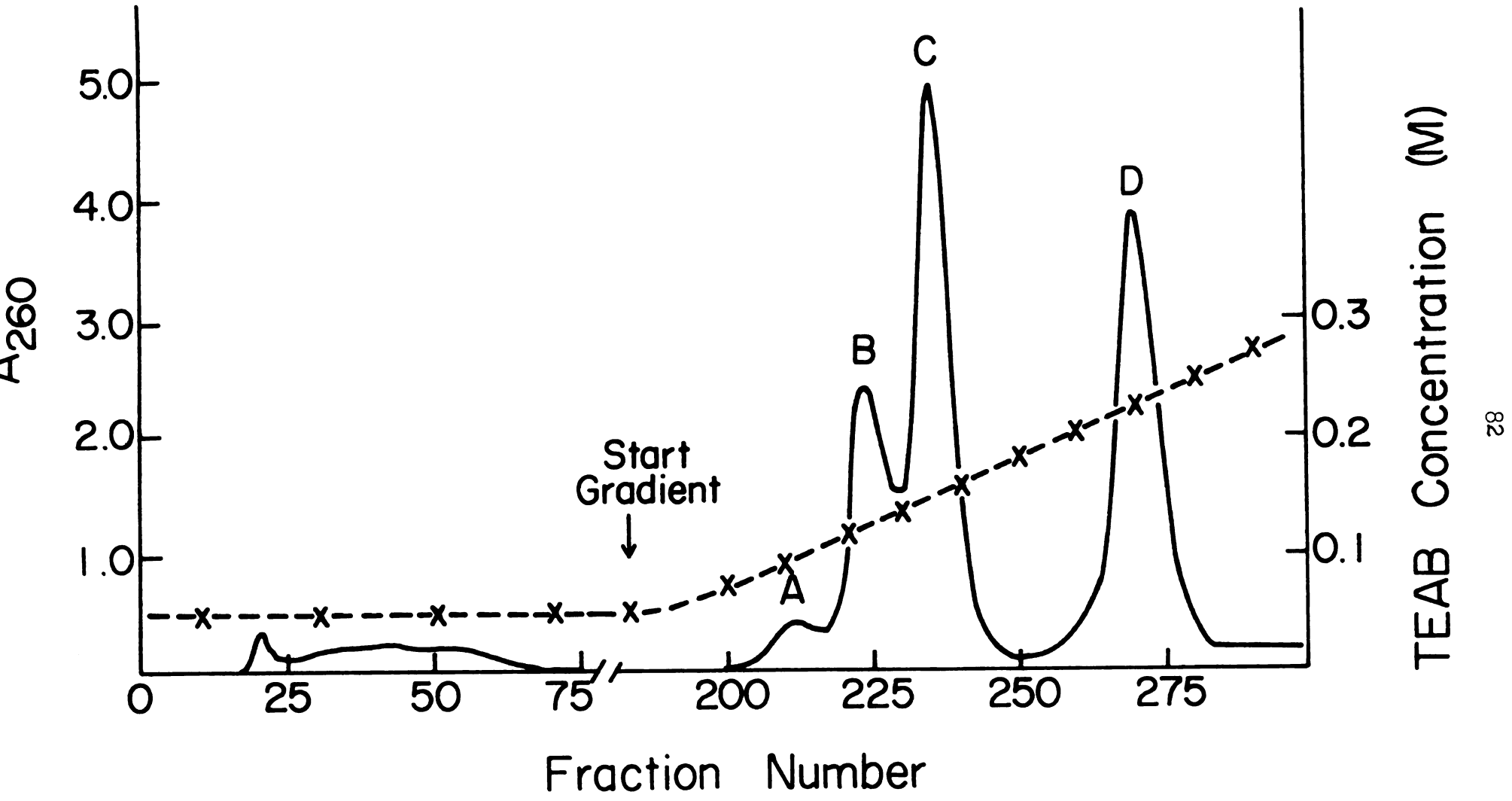
The reaction contained components as described in Methods under DNase B assay except that the DNA was not denatured, and the volume of each component was increased ten-fold. Aliquots were removed at various times and treated as described in the text. The elution profiles represent the following stages in the hydrolysis: a) 0%; b) 21%; c) 43%.


nDNA
dAMP
a
b
c
A_{254}
0.4
0.3
0.2
0.1
0
10
20
30
40
Volume (ml)

units per ml with 0.12 M ammonium acetate, pH 8.0; was incubated at 37° for 90 minutes. At the end of the incubation period a 0.6 ml aliquot was removed, added to 0.5 ml of cold[1] lanthanum nitrate-HCl reagent, and treated as described in Methods under RNase assay. No visible precipitate formed following centrifugation. The A_{260} of the mixture was 12.4. These results indicated that all substrate in the reaction had been converted to lanthanum nitrate-HCl soluble products. The hydrolyzate from which the aliquot was taken was adjusted to pH 8.0 with NaOH and diluted to 120 ml with deionized water in order to prepare it for chromatography on DEAE-Sephadex. The diluted hydrolyzate was applied to a 2.2 x 14 cm DEAE-Sephadex A-25 column (see Methods: Preparation of Ion Exchange Resins) previously equilibrated with 2 liters of 0.05 M triethylammonium bicarbonate (TEAB), pH 8.0, (see Methods: Preparation of Ion Exchange Resins) at a flow rate of 1.0 ml per minute. The collection of 5 minute fractions was begun immediately. After all the sample had been applied, the column was washed with 0.05 M TEAB, pH 8.0, until the A_{260} of the effluent was 0.025. The column was then treated with a gradient prepared from 500 ml each of 0.05 M and 0.30 M TEAB, both at pH 8.0, at a flow rate of 1.0 ml per minute. The A_{260} elution profile determined during application of the sample, washing, and gradient elution, is shown in Figure 16. The center three fractions from each peak, A-D in Figure 16, were pooled. TEAB was removed from each pooled fraction by repeated evaporation to dryness on

Figure 16. Chromatography on DEAE-Sephadex A-25 of a Hydrolyzate of RNA by P-100 Enzyme

A reaction containing 25 ml of yeast RNA (see Methods: Preparation of RNA from Yeast) at a concentration of 2 mg per ml; 30 ml of 0.2 M imidazole, pH 5.9; and 5.0 ml of P-100 enzyme previously diluted to a concentration of 20 DNase B units per ml with 0.12 M ammonium acetate, pH 8.0; was incubated at 37° for 90 minutes. At the end of the incubation period a 0.6 ml aliquot was removed and treated with cold lanthanum nitrate-HCl reagent as described in the text. It was found from this test that all substrate was converted to lanthanum nitrate-HCl soluble products under the above conditions. The hydrolyzate was adjusted to pH 8.0 with NaOH and diluted to 120 ml with deionized water. The diluted hydrolyzate was applied to a 2.2 x 14 cm DEAE-Sephadex A-25 column (see Methods: Preparation of Ion Exchange Resins) previously equilibrated with 2 liters of 0.05 M TEAB, pH 8.0 (see Methods: Preparation of Ion Exchange Resins) at a flow rate of 1.0 ml per minute. The collection of 5 minute fractions was begun immediately. After all the sample had been applied, the column was washed with 0.05 M TEAB, pH 8.0, until the A_{260} of the effluent was 0.025. The column was then treated with a gradient prepared from 500 ml each of 0.05 M and 0.30 M TEAB, both at pH 8.0, at a flow rate of 1.0 ml per minute. The A_{260} of each fraction was determined in a Beckman DB spectrophotometer.


5.0
4.0
3.0
2.0
1.0
A_{260}
0
25
50
75
200
225
250
275
Fraction Number
Start
Gradient
A
B
C
D
0.3
0.2
0.1
TEAB Concentration (M)

a Buchler flash-evaporator at 40°. Material from each peak amounting to 0.2 A_{260} unit was spotted on a polyethylenimine (PEI) cellulose coated sheet (9 x 15 cm) adjacent to the four known ribonucleoside-5'-monophosphates. Thin layer chromatography was performed as described in Methods. The material from each peak in Figure 16 gave one spot which migrated with an R_f value identical to the following standard mononucleotides: Peak A, CMP; Peak B, UMP; Peak C, AMP; and Peak D, GMP. The spectral characteristics of material from each peak, measured at pH 2, supported the identification made using thin layer chromatography. With the above evidence proving that peaks A-D (Figure 16) contained only mononucleotides, the pooled, desalted fractions from all four peaks were themselves pooled in a total volume of 2.0 ml giving what was termed a "mixed-mononucleotide" fraction. This mixed-mononucleotide fraction was then subjected to hydrolysis by alkaline phosphatase and 5'-nucleotidase under the following conditions. Each reaction contained 1.0 ml of 0.2 M Tris-HCl buffer, pH 8.2, 0.2 ml of mixed-mononucleotide at a concentration of 61.0 A_{260} units per ml, 0.2 ml of either 5'-nucleotidase (Sigma, Grade II), 60 µg per ml or alkaline phosphatase (Worthington, BAPC), 2.1 mg per ml and water to a final volume of 2.0 ml. The appropriate control reactions lacking enzymes or substrate were included as well as were reactions containing 5'-AMP. All reactions were incubated at 37° for one hour. At the end of the incubation period a 0.5 ml aliquot was removed,

treated with 0.5 ml of phenol, and assayed for inorganic phosphate by the procedure described in Methods under 3'-AMPase assay. Virtually 100 percent of the inorganic phosphate released from the mixed-mononucleotide by alkaline phosphatase was released by 5'-nucleotidase. The same relative amounts of inorganic phosphate were found in those reactions containing 5'-AMP. Thus, all mononucleotides isolated from a hydrolyzate of RNA by the P-100 enzyme were the 5'-phosphoryl derivatives. The assumption can also be made that the oligonucleotide products were terminated by a 5'-phosphate.

Determination of Phosphate Position in Mononucleotides Obtained From a Hydrolyzate of Denatured DNA at pH 5.7

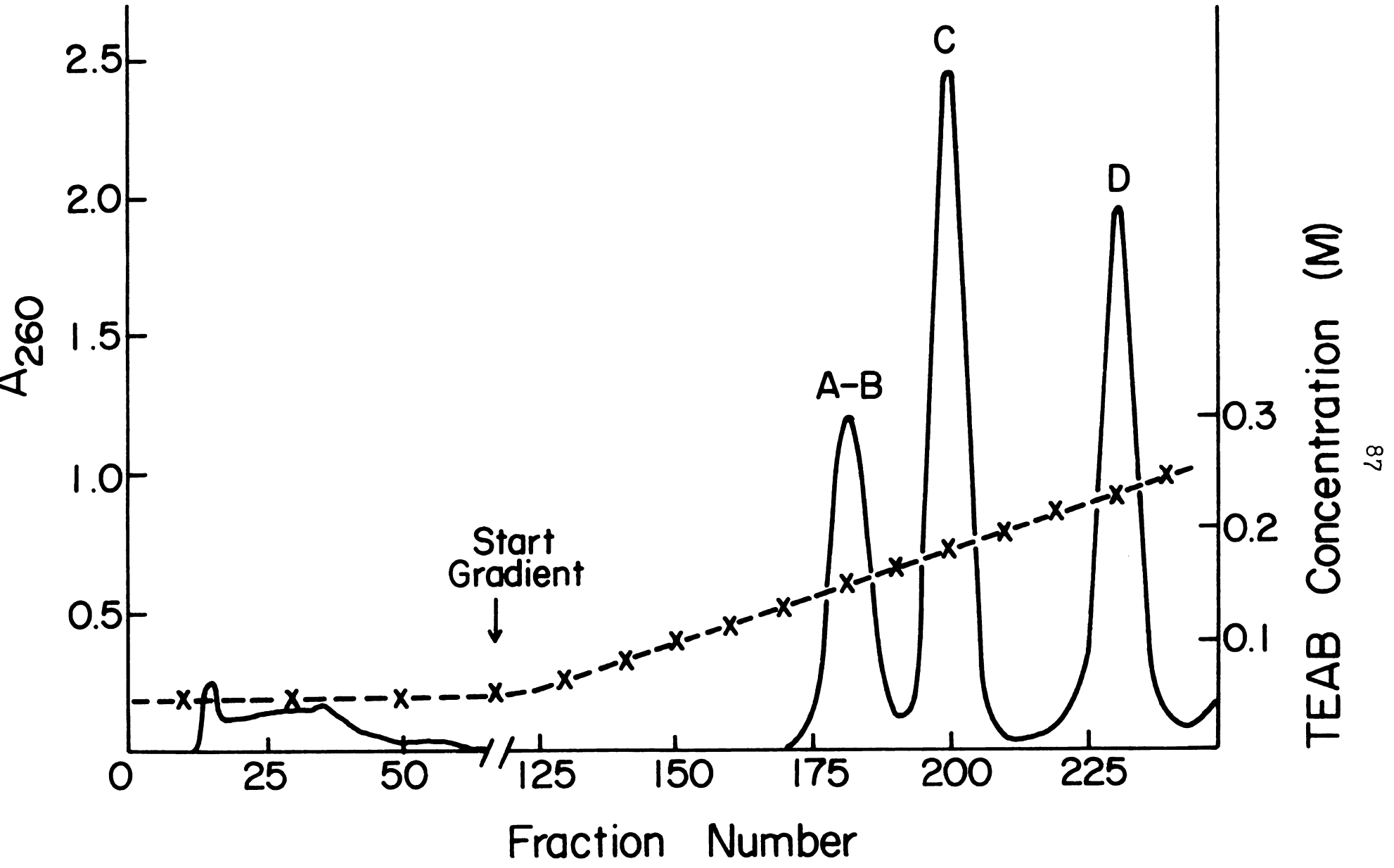
In order to determine the nature of the phosphate position in mononucleotides from a hydrolyzate of dDNA at pH 5.7 an experiment similar to that utilized for the mononucleotides from RNA was performed.

A reaction containing 25 ml of denatured DNA (heated at 100° for 15 minutes, quickly cooled to 4° in an ice-water bath) from salmon sperm (Sigma, Type III) at a concentration of 2.0 mg per ml; 30 ml of 0.2 M imidazole, pH 5.9; and 5.0 ml of P-100 enzyme previously diluted to a concentration of 20 DNase B units per ml with 0.12 M ammonium acetate, pH 8.0; was incubated at 37° for 2.5 hours. At the end of the incubation period 0.6 ml of reaction mixture was assayed as described in Methods for DNase A. It was found that all substrate was converted to lanthanum nitrate-HCl soluble products under the above conditions. The hydrolyzate was

then adjusted to pH 8.0 with NaOH and diluted to 120 ml with deionized water. This diluted hydrolyzate was applied to a 2.2 x 15 cm DEAE-Sephadex A-25 column (see Methods: Preparation of Ion Exchange Resins) previously equilibrated with 2 liters of 0.05 M TEAB, pH 8.0, (see Methods: Preparation of Ion Exchange Resins) at a flow rate of 1.0 ml per minute. The collection of 5 minute fractions was begun immediately. After all the sample had been applied, the column was washed with 0.05 M TEAB, pH 8.0, until the A_{260} of the effluent was 0.025. The column was then treated with a gradient prepared from 500 ml each of 0.05 M and 0.30 M TEAB, both at pH 8.0, at a flow rate of 1.0 ml per minute. The A_{260} elution profile determined during application of the sample, washing, and gradient elution, is shown in Figure 17. Previous chromatography of the four common 5'-phosphoryl-2'-deoxymononucleosides under the same conditions as used in this experiment, showed that 5'-dCMP and 5'-TMP were eluted together in the first peak, while 5'-dAMP and 5'-dGMP, respectively, were eluted immediately after the pyrimidines (dCMP and TMP) in two well separated peaks, all with 95-100 percent recoveries. The center three fractions from each peak (A-B, C, and D in Figure 17) were pooled and TEAB was removed by flash-evaporation as in the previous experiment utilizing the mononucleotides from RNA. Thin layer chromatography (see Methods) of the material from each peak along with the determination of spectral characteristics, at pH 2, indicated that peak A-B contained mostly (>75 percent) TMP, peak C contained only

Figure 17: Chromatography on DEAE-Sephadex A-25 of a Hydrolyzate of Denatured DNA by P-100 Enzyme at pH 5.7

A reaction containing 25 ml of denatured DNA (heated at 100° for 15 minutes, quickly cooled to 4° in an ice-water bath) from salmon sperm (Sigma, Type III) at a concentration of 2.0 mg per ml; 30 ml of 0.2 M imidazole, pH 5.9; and 5.0 ml of P-100 enzyme previously diluted to a concentration of 20 DNase B units per ml with 0.12 M ammonium acetate, pH 8.0; was incubated at 37° for 2.5 hours. At the end of the incubation period a 0.6 ml aliquot was assayed as described in Methods for DNase A and it was found that all substrate was converted to lanthanum nitrate-HCl soluble products under the above conditions. The diluted hydrolyzate (120 ml) was applied to a 2.2 x 15 cm DEAE-Sephadex A-25 column (see Methods: Preparation of Ion Exchange Resins) at a flow rate of 1.0 ml per minute. The collection of 5 minute fractions was begun immediately. After all the sample had been applied, the column was washed with 0.05 M TEAB, pH 8.0, until the A_{260} of the effluent was 0.025. The column was then treated with a gradient prepared from 500 ml each of 0.05 M and 0.30 M TEAB, both at pH 8.0, at a flow rate of 1.0 ml per minute. The A_{260} of each fraction was determined in a Beckman DB spectrophotometer.


2.5
2.0
1.5
1.0
0.5
A_{260}
A-B
C
D
Start
Gradient
0.3
0.2
0.1
TEAB Concentration (M)
0
25
50
125
150
175
200
225
Fraction Number

dAMP, and peak D contained only dGMP. The material from the central portions of peaks A-B, C, and D were combined in a total volume of 2.0 ml and termed mixed-mononucleotide. This mixed-mononucleotide fraction was then subjected to hydrolysis by alkaline phosphatase and 5'-nucleotidase under the same conditions as those used previously with the mixed-mononucleotide fraction obtained from RNA. The A_{260} of the mixed-mononucleotide fraction from dDNA was 37.0. It was found that 5'-nucleotidase released 96 percent of the inorganic phosphate, from the mixed-mononucleotide fraction, that was released by alkaline phosphatase. This result indicated that the mononucleotides released from dDNA at pH 5.7 by the P-100 enzyme were the 5'-phosphoryl derivatives.

Determination of Phosphate Position in Oligonucleotides Obtained From a Hydrolyzate of Polyadenylic Acid by P-100 Enzyme

In a hydrolyzate of poly-2'-O-methyladenylic acid (poly A^m) produced by the P-100 enzyme preparation (see Hydrolysis of Poly-2'-O-methyladenylic Acid for further details) a significant amount of 2'-O-methyladenosine and oligonucleotides lacking phosphate at either terminus was present. One or both of the following may have been the cause of the presence of dephosphorylated products in the hydrolyzate: a nuclease producing 3'-phosphoryl terminated products which possibly would have in turn been dephosphory-

lated by the 3'-nucleotidase activity[16] or a separate phosphatase capable of removing 5'-phosphates from mono- and oligonucleotides. Because of the extreme conditions of both incubation time and amount of enzyme needed to give significant degradation of the poly A^m, the effect of any nonspecific phosphomonoesterases or other nucleases present would be greatly magnified. For this reason it was desirable to characterize the shorter oligonucleotides from a partial hydrolysis of polyadenylic acid (poly A) to see whether or not they contained dephosphorylated products. Reaction mixtures composed of 0.60 ml poly A, 1.0 mg per ml (15.8 A_{260} per ml); 0.30 ml of 0.20 M Tris-HCl, pH 8.2; 0.060 ml of P-100 enzyme previously diluted to concentrations of 20 and 40 DNase B units per ml with 0.12 M Buffer A; and 0.24 ml of water were incubated at 37°. Aliquots of 0.40 ml were removed after 1 and 2 hours of incubation. These aliquots, as well as the remaining 0.40 ml which was incubated for a total of 3 hours, were frozen immediately upon removal, and lyophilized. Each residue was dissolved in 0.04 ml of water and spotted on Whatman 3MM paper for chromatography in n-propanol:29 percent ammonium hydroxide: water solvent system (55:10:35, respectively, v/v)[17]. The

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[16] Dr. M. Laskowski, Sr. has, by personal communication, stated that the 3'-nucleotidase from mung bean is capable of dephosphorylating 3'-phosphoryl terminated dinucleotides.

[17] This solvent system is capable of resolving small oligonucleotides of homopolymers from 1-5 in length when used with Whatman No. 1 or 3MM paper.

solvent was permitted to migrate for a period of 18 hours after which the paper was dried. It was evident from the distribution of U.V. absorbing spots observed that 2 or 3 hours incubation at an enzyme concentration of 40 DNase B units per ml, and 3 hours incubation at an enzyme concentration of 20 DNase B units per ml gave the most desirable degree of hydrolysis, i.e. all of the substrate was converted to mono- and oligonucleotides up to tetranucleotide in size. The above conditions outlined as optimal for the production of di-, tri-, and tetranucleotides were then used with three separate reaction mixtures. Chromatography on Whatman 3MM paper in the solvent system described previously (n-propanol:ammonium hydroxide:water) was again used to separate the products of hydrolysis. A reaction lacking enzyme and containing 5'-AMP instead of poly A was also chromatographed in order to have a marker for mononucleotide. A series of four to five spots was observed on the chromatograms from each reaction that contained enzyme. This series corresponded to the following products[18] of hydrolysis, arranged in order of decreasing R_f: mononucleotide (pA) > dinucleotide (pA_2) > trinucleotide (pA_3) > tetranucleotide (pA_4) > pentanucleotide (pA_5). All spots containing di-, tri-, and tetranucleotide were eluted from the paper by the method of Davis, et. al. (43). The eluants from spots of corresponding R_f's were combined in a total

[18]Dr. F. M. Rottman, personal communication.

volume of 2.0 ml. The following amounts of each type of oligonucleotide were recovered: pA_2, 5.41 A_{260} units[1]; pA_3, 5.71 A_{260} units; pA_4, 4.82 A_{260} units. Each oligonucleotide solution was lyophilized and the residue dissolved in a sufficient amount of water to give a concentration of 20.0 A_{260} units per ml. In order to determine whether or not the isolated oligonucleotides contained 3'-phosphoryl termini or, alternatively, no phosphoryl termini, each was completely hydrolyzed by snake venom phosphodiesterase under the following conditions: a typical reaction mixture contained 0.025 ml of Tris-succinate, 0.15 M, pH 6.0; 0.025 ml of magnesium acetate, 0.012 M; and 0.020 ml of venom phosphodiesterase (Worthington, VPH), 0.5 mg per ml, potency of 0.3. These reactions were incubated at 37° for 3 hours. (The above condition of pH has been described by Richards and Laskowski (44) to be optimal for the hydrolysis of 3'-phosphoryl terminated oligonucleotides by venom phosphodiesterase.) Aliquots of 0.010 ml were removed before adding enzyme and at the end of the incubation period. In preparation for thin layer chromatography each aliquot was spotted in a separate lane along the longer side of a 20 x 10 cm sheet of PEI-cellulose (MN-Polygram CEL 300 PEI, Brinkman Instruments, Inc.). Preliminary experiments using 0.75 M lithium chloride (45) as solvent showed that a mixture of adenosine, 5'-AMP, and pA_2, pA_3, or pA_4 were separated by ascending thin layer chromatography on PEI-cellulose with the following R_f values: adenosine, 0.53;

5'-AMP, 0.32; pA_2, 0.18; pA_3, 0.15; and pA_4, 0.13. Experiments designed to determine the lowest level of adenosine that could be detected on a PEI-cellulose sheet after thin layer chromatography in 0.75 M lithium chloride indicated that this value was 0.003 A_{260} unit. All reactions tested by the chromatographic method indicated that the three types of oligonucleotides were completely degraded to a compound giving a single spot with an R_f equal to that of 5'-AMP. No spots with $R_f \leq 0.2$, indicative of unhydrolyzed oligonucleotide or nucleoside diphosphates, could be detected, nor could spots having an R_f equal to that of adenosine be seen. Taking into account that a 0.010 ml aliquot of reaction mixture contained 0.067 A_{260} unit it is evident that approximately 5 percent (0.003 A_{260} unit) of this amount could have been detected as adenosine, if this nucleoside had been produced in the course of hydrolysis by venom phosphodiesterase. These results indicate two facts. First, the oligonucleotide products of hydrolysis of poly A were terminated on the 5' end by a phosphate and on the 3' end by a free hydroxyl group. Second, if there were dephosphorylated oligonucleotides present, they were present in amounts less than 10 percent of the total dinucleotide, 15 percent of the total trinucleotide, and 20 percent of the total tetranucleotide.

Susceptibility of Polyadenylic Acid, Polyuridylic Acid, and Polycytidylic Acid to Hydrolysis by P-100 Enzyme

When it was found that the P-100 enzyme preparation exhibited great differences in its ability to dephosphorylate the various nucleoside-3'-monophosphates (see Relative 3'-Nucleotidase Activities) it was reasoned that this difference might have some bearing on the ability of the enzyme to hydrolyze polyribonucleotides containing only one type of purine or pyrimidine base. Walters and Loring (46) have suggested that the differences observed in 3'-nucleotidase activities of a partially purified nuclease from mung bean might have some bearing on the specificity of the accompanying RNase activity. With this reasoning in mind the following experiment was conducted. Reaction mixtures were prepared to contain 110 microliters of 0.20 M Tris-HCl buffer, pH 8.2; 10 microliters of P-100 enzyme (200 DNase B units per ml); and 100 microliters of either polyadenylic acid (poly A), polyuridylic acid (poly U), or polycytidylic acid (poly C), each at a concentration of 1.0 mg per ml in water. These mixtures were incubated at 37° for 1 hour. The appropriate controls lacking enzyme were included in the incubation procedure. At the end of the incubation period 10 microliter aliquots of each reaction were spotted 2 cm from the shorter side of a 14 x 11 cm piece of MN-Polygram CEL 300/UV_{254} (Brinkman Instruments, Inc.). The three corresponding nucleoside-5'-monophosphates (AMP, UMP, and CMP) were spotted in positions adjacent to appropriate reactions

containing polynucleotide and enzyme. The sheet was developed in a closed tank for 3 hours by ascending thin layer chromatography using the solvent, n-propanol:29 percent ammonium hydroxide:water (55:10:35, respectively, v/v). The solvent migrated to a distance of 11 cm from the origin in the 3 hour period. After drying the chromatogram the U.V. absorbing spots were marked. The following results were observed. Each standard mononucleotide migrated with an R_f of $\sim$0.45. The positions spotted with reactions containing polynucleotide but lacking enzyme each exhibited one spot which had not migrated from the origin (indicating no degradation[19] of the polynucleotide). The positions spotted with reactions containing poly A plus P-100 enzyme and poly U plus P-100 enzyme each exhibited one spot which had migrated to a position adjacent to the standard mononucleotide spots. The position spotted with the reaction containing poly C plus P-100 enzyme, however, displayed one spot which had not migrated from the origin. These results indicate that the P-100 enzyme preparation was capable of hydrolyzing poly A and poly U completely to mononucleotides, but was not capable of hydrolyzing poly C.

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[19]On thin-layer plates with this solvent partial degradation of a polynucleotide results in streaking of U.V. absorbing material from the origin to the mononucleotide position.

Characterization of the Products of Hydrolysis of Polyuridylic-Cytidylic Acid (Poly U,C) Produced by P-100 Enzyme

The preceding section along with the one describing differences in 3'-nucleotidase activities have suggested that the 3' oxygen to phosphorus bond of cytidylic acid is not hydrolyzed by the P-100 enzyme preparation. In order to test this hypothesis more rigorously, a ribonucleotide copolymer containing uracil and cytosine (base ratio of 2:1, respectively) was subjected to extensive hydrolysis by P-100 enzyme under the following conditions. Poly U,C[20] (3.4 ml at a concentration of 82.1 A_{260} units[1] per ml) was thoroughly mixed with 3.4 ml of redistilled, water-saturated phenol. The phases were separated by centrifugation at 1100 x g for 10 minutes at room temperature. Three ml of aqueous phase was recovered and made 0.2 M in potassium acetate by the addition of 0.33 ml of the 2 M salt solution. The polynucleotide was precipitated at 4° by the addition of 6.0 ml of 95 percent ethanol. Centrifugation at 27,000 x g for 10 minutes was used to separate the precipitate. This was subsequently dissolved in 2.0 ml of water after discarding the supernatant solution. The ethanol precipitation was repeated and the precipitate dissolved in 2.0 ml of water. This solution was then lyophilized to dryness to remove the last traces of ethanol. The white, fibrous

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[20] A generous gift from Dr. F. M. Rottman.

residue was dissolved in 3.0 ml of water. It displayed an A_{260} of 67.0. A reaction mixture containing 0.747 ml of poly U,C (67.0 A_{260} units per ml); 1.25 ml of 0.20 M Tris-HCl, pH 8.2; 0.25 ml of water (pentachlorophenol, 0.5 µg per ml); and 0.25 ml of undiluted P-100 enzyme was incubated in a closed container at 37° for 16 hours.[21] The reaction mixture was diluted with 2.5 ml of water before applying it to a 1.2 x 32 cm column of DEAE-Sephadex A-25 at a flow rate of 0.8 ml per minute. The total A_{260} in the sample was 49.2 units[1]. The resin was prepared in the formate form (see Methods: Preparation of Ion Exchange Resins) and equilibrated with 0.05 M ammonium formate, pH 7.5. A 100 ml quantity of 0.05 M ammonium formate, pH 7.5, was used to wash the column. Column effluent was continuously monitored using an Isco Model UA ultraviolet optical unit recording A_{254}. Fractions of 5 ml each were collected throughout the application of the sample, washing, and gradient elution. Following the washing procedure, the column was treated with a gradient prepared from 500 ml each of 0.05 M and 0.50 M ammonium formate, both at pH 7.5 at a flow rate of 0.8 ml per minute. The elution profile determined from the A_{260} of each fraction during the course of the gradient elution is shown in Figure 18. At the termination of the 0.05-0.5 M gradient, the column was

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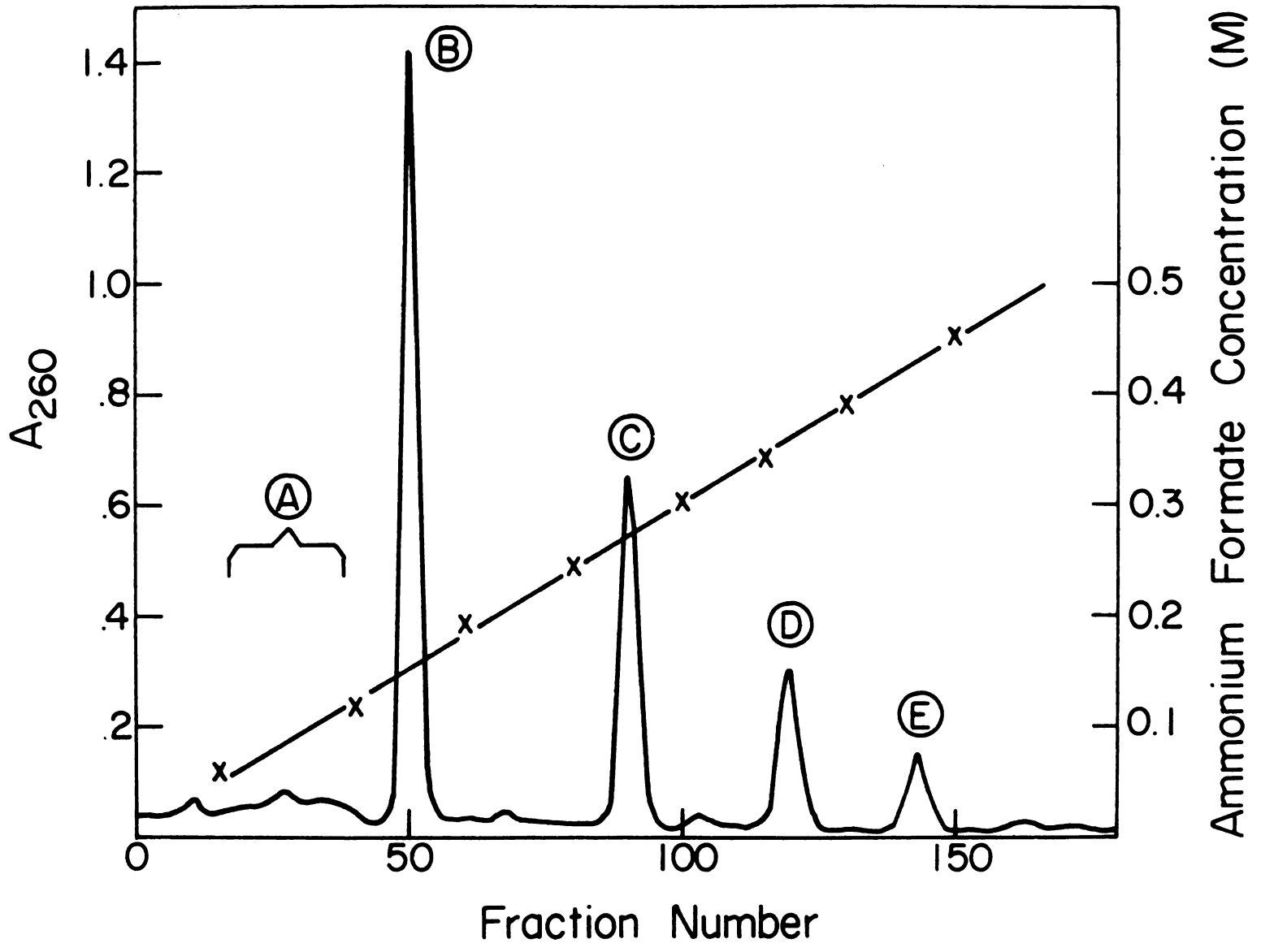
[21]Preliminary experiments using thin layer chromatography on PEI-cellulose indicated that the hydrolysis had reached a limit under these conditions.

Figure 18: Elution Pattern from a DEAE-Sephadex A-25 Column of the Products of Hydrolysis of Poly U,C by P-100 Enzyme

A reaction mixture containing 0.747 ml of poly U,C (67.0 A_{260} units[1] per ml); 1.25 ml of 0.20 M Tris-HCl, pH 8.2; 0.25 ml of water (pentachlorophenol, 0.5 µg per ml); and 0.25 ml of undiluted P-100 enzyme was incubated in a closed container at 37° for 16 hours. The reaction mixture was diluted with 2.5 ml of water before applying it to a 1.2 x 32 cm column of DEAE-Sephadex A-25 at a flow rate of 0.8 ml per minute. Before application of the reaction mixture, the column was equilibrated with 5 liters of 0.05 M ammonium formate, pH 7.5. After adding the sample the column was washed with 100 ml of 0.05 M ammonium formate, pH 7.5. The column was then treated with a gradient prepared from 500 ml each of 0.05 and 0.5 M ammonium formate, both at pH 7.5, at a flow rate of 0.8 ml per minute. At the termination of the gradient, the column was washed with 200 ml of 1.0 M ammonium formate, pH 7.5. Fractions of 5 ml each were collected during the course of sample application, washing, and gradient elution. The A_{260} of each fraction was determined in a Beckman DB spectrophotometer.


A260
1.4
1.2
1.0
.8
.6
.4
.2
0
50
100
150
Fraction Number
Ammonium Formate Concentration (M)
0.5
0.4
0.3
0.2
0.1
A
B
C
D
E

washed with 200 ml of 1.0 M ammonium formate, pH 7.5. This washing step resulted in the elution of a small peak as indicated by the A_{254} monitor. The fractions containing this material were pooled and termed "1.0 M Wash" (Fraction F). The following fractions were pooled and each pooled group given a letter designation as shown in Figure 18; Peak A, fractions 17-38; Peak B, 47-54; Peak C, 87-94; Peak D, 116-123; and Peak E, 140-147. Ammonium formate was removed from each fraction (A-F) by repeated lyophilization; the residue was dissolved in approximately 5 ml of water for each cycle. After all ammonium formate was removed, the remaining residue in each fraction was dissolved in water and brought to 1.0 ml in volume. The A_{260} of each fraction was determined. A percentage yield was calculated for each fraction from this value and the total of 49.2 A_{260} units[1] applied to the DEAE-Sephadex A-25 column. These results are shown in Table VII. In order to characterize each fraction with regard to 5' and 3' terminal nucleotides, each fraction (A-E) was dephosphorylated using alkaline phosphatase. Separate reaction mixtures for each fraction were incubated at 37° for 3 hours. Each reaction mixture contained 1-4 A_{260} units[1] from a specific fraction, 0.11 ml of alkaline phosphatase (Worthington, BAPC) at a concentration of 0.92 mg per ml (20 units[8] per mg) in 0.2 M Tris-HCl, pH 8.2, and water up to 1.0 ml. Each reaction was lyophilized following the incubation period. The residue was dissolved in 0.04 ml of water and the entire

TABLE VII

Characterization of the Products of Hydrolysis of Polyuridylic-Cytidylic Acid (Poly U,C) by P-100 Enzyme

Fraction[a]	Total A_{260} Units Recovered	Percent of Total[b] A_{260} Units Applied to A-25 Column
A	1.92	3.91
B	17.4	35.4
C	10.9	22.2
D	5.40	11.0
E	2.26	4.60
F (1.0 M Wash)	2.22	4.51
		81.6 Total Recovered

[a]See Figure 18.

[b]49.2 A_{260} units applied to DEAE-Sephadex column.

amount was spotted on Whatman No. 1 paper. Chromatography for 14.5 hours in Solvent I (see Methods: Paper Chromatography) along with standard UMP, CMP, uridine and cytidine gave the following results. The reaction mixture containing fraction A gave one very light spot under U.V. which migrated near the solvent front faster than either standard mononucleotides or nucleosides. Fraction B material showed one spot with R_f equal to that of uridine. Fraction C reaction mixture exhibited one spot with R_f of 0.17, i.e. significantly less than either standard mononucleotide. Material from Fraction D migrated just off the origin, while material from Fraction E appeared not to have moved. Chromatography in Solvent I was used for two reasons: first, to get some idea of the nature (mono- or oligonucleotide) of the material in each fraction; and second, to separate each dephosphorylated compound from the alkaline phosphatase (which remains at the origin)[17]. The spots containing dephosphorylated material from Fractions C and D were eluted from the paper with water. The eluant from each was divided into two equal parts, one half to be completely hydrolyzed with venom phosphodiesterase and the other to be hydrolyzed with potassium hydroxide. The residues from C and D to be hydrolyzed with KOH were dissolved in 0.050 ml of water. An equal volume of 0.2 N KOH was added to each and the reactions were heated in a boiling water bath for 20 minutes. The salt concentration was reduced by perchloric acid treatment as described by

Davidson and Smellie (47). Thin layer chromatography of C on PEI-cellulose (see Methods: Thin-Layer Chromatography) revealed one mononucleotide[22] spot migrating like 3'-CMP. The only nucleoside component in base-hydrolyzed C was shown by paper chromatography in Solvent I to be uridine. The material resulting from KOH hydrolysis of D appeared to contain one mononucleotide, 3'-CMP, when chromatographed on PEI-cellulose. Again, the only nucleoside component, as shown by paper chromatography in Solvent I, in base-hydrolyzed D was uridine. In order to verify the possible structures for C and D suggested by the above results, the other halves of dephosphorylated C and D fractions were subjected to complete hydrolysis by venom phosphodiesterase. Venom phosphodiesterase is known to hydrolyze oligonucleotides by stepwise liberation of nucleoside-5'-monophosphates from the 3'-hydroxyl terminus. If the oligonucleotide is dephosphorylated (both termini), the nucleotide residue on the 5' terminus appears as a nucleoside. Reaction mixtures containing 0.05 ml of either dephosphorylated C or D (2.0 A_{260} units); 0.01 ml of Tris-acetate, 0.3 M, pH 8.8; 0.01 ml of 0.3 M magnesium acetate; 0.01 ml of venom phosphodiesterase (Worthington, VPH), 0.5 mg per ml at a potency of 0.3; and 0.02 ml of water were incubated for 21 hours at 37°. Identical reaction mixtures lacking oligonucleotide but containing instead either 5'-CMP and 5'-UMP or the nucleosides, cytidine and uridine, were incubated under the

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[22]Nucleosides migrate with the solvent front.

same conditions. Chromatography of the reaction mixtures after the incubation period on paper in Solvent I, and also on PEI-cellulose, gave the following results: the reaction mixture containing hydrolysis products from dephosphorylated C contained only 5'-UMP and cytidine, whereas the D reaction mixture exhibited spots corresponding to 5'-UMP, 5'-CMP, and cytidine, but no uridine. Because at least two possible oligonucleotide structures, differing in chain length by at least one nucleotide, were possible for D, the spots on the paper chromatograph of base-hydrolyzed D were removed and eluted, each with 1.5 ml of 0.01 N HCl. Since the 3' terminus was shown to be uridine, and the only other component from base-hydrolysis was 3'-CMP, the molar ratio of uridine to 3'-CMP would indicate the chain length and; the most plausible structure of D. The ratio of uridine to 3'-CMP was found to be 1:2.7. This indicated that peak D (Figure 18) was a mixture of the tri- and tetranucleotides pCpCpU and pCpCpCpU. A summary of the characterization of peaks C and D is shown in Table VIII. Peak B was rather conclusively shown both by thin layer chromatography on PEI-cellulose and by the determination of spectral characteristics to contain only 5'-UMP. Peaks E and F, because of the limited amount of material available, were not characterized beyond the point of determining spectral characteristics. Evidently, E and F contained a high proportion of cytidylic acid judging from the position of their maximum absorption in the U.V. The 280:260 ratios

TABLE VIII

Summary of the Characterization of Peaks "C" and "D" From DEAE-Sephadex A-25 Column (Figure 18)

Dephosphorylated Oligonucleotide	Products from KOH Hydrolysis	Products from VPH Hydrolysis	Proposed Structure
C [XpY]	Cp + Ur	Cr + pU	CpU
D [$(Xp)_nY$]	Cp + Ur	Cr + pC + pU	CpCpCpU + CpCpU [a]

Xp = 3'-mononucleotide

pX = 5'-mononucleotide

Xr = nucleoside

[a] Proposed to be the mixture since the molar ratio of Ur to Cp was found to be 1:2.7.

observed for peaks B-F increased steadily from 0.48 (pH 7.0) for B to 1.06 for F, another indication that the proportion of cytidylic acid to uridylic acid was increasing. Thus, the tacit assumption is made that these later peaks are of the same general structure as C and D, i.e. $(pC)_n pU$.

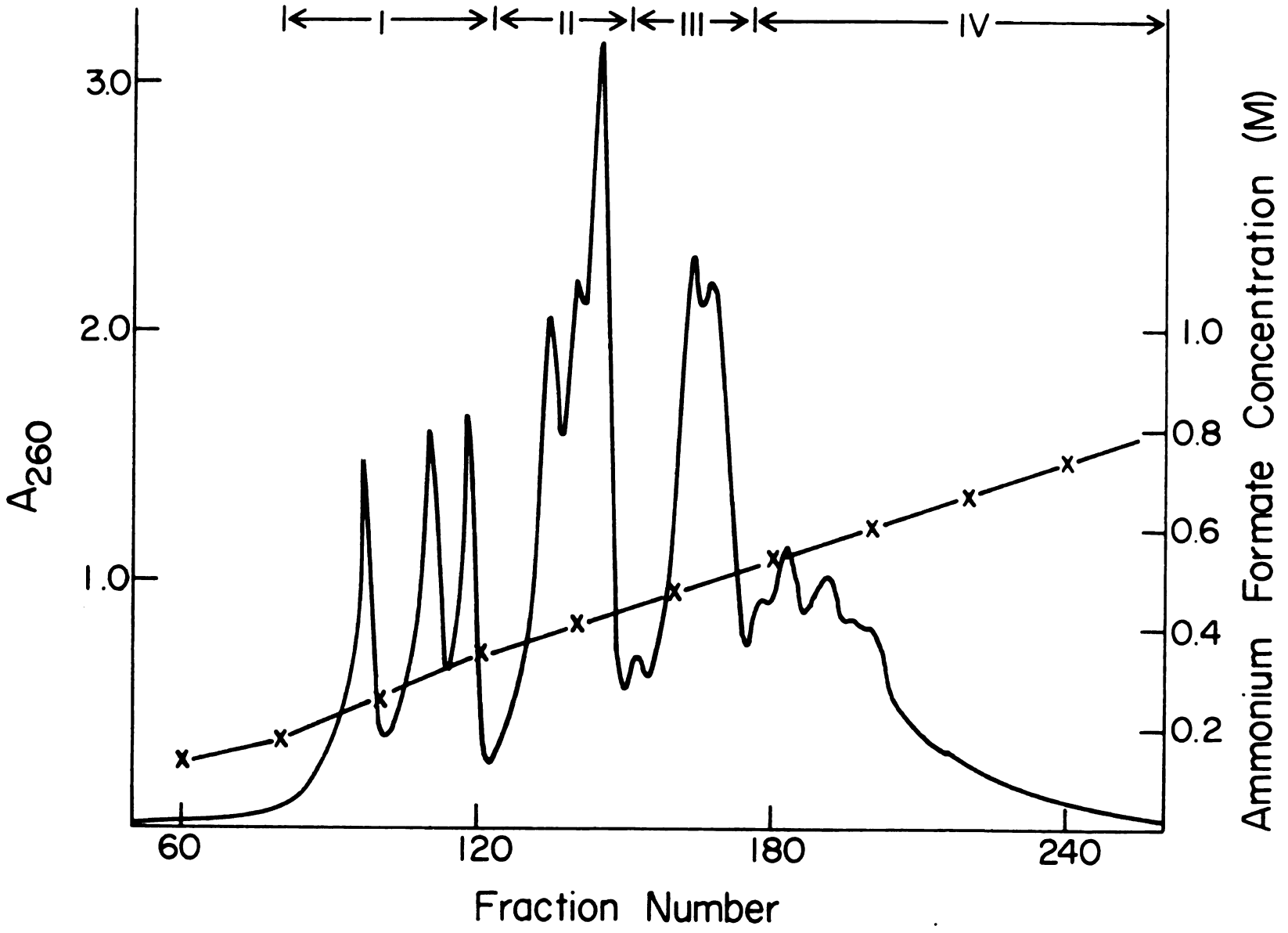
Characterization of the Products of Hydrolysis of Denatured DNA Produced by P-100 Enzyme at pH 8.0

Because of the apparent degree of specificity exhibited by the P-100 enzyme preparation with respect to the various polyribonucleotides tested it appeared desirable to determine whether or not polydeoxyribonucleotides were hydrolyzed in a similar way. For this reason, the following experiment was performed. A 100 mg quantity of DNA from salmon sperm was dissolved in water at a concentration of 2.5 mg per ml by gentle stirring at 4° over a 15 hour period. The DNA was denatured by heating at 100° for 30 minutes followed by rapid cooling in an ice-water bath. The volume was then brought to 50.0 ml with water. This 50 ml quantity of dDNA was combined with 60 ml of 0.2 M Tris-HCl, pH 8.2, and 10 ml of P-100 enzyme (previously diluted to a concentration of 20 DNase B units per ml with 0.12 M ammonium acetate, pH 8.0). The reaction mixture was incubated at 37° for 9 hours. Aliquots of 0.5 ml were removed at 20 minute intervals and treated with 0.5 ml of cold lanthanum nitrate-HCl reagent in order to follow the progress of the reaction. The production of lanthanum nitrate-HCl soluble, UV-absorbing materials was found to reach a limit, indicating the lack of susceptibility

of the degradation products to further hydrolysis, at about 180 minutes. This time was arbitrarily tripled in deciding on a total incubation time of 9 hours. The reaction mixture (117 ml) was diluted to 336 ml with water after the incubation period. This diluted sample, containing a total of 1880 A_{260} units, was applied to a 2.5 x 48 cm DEAE-Sephadex A-25 column previously equilibrated with 0.01 M ammonium formate, pH 7.5, at a flow rate of 1.5 ml per minute. Following application of the sample, the column was washed with 1200 ml of 0.01 M ammonium formate, pH 7.5. A gradient prepared from 2000 ml each of 0.01 M and 1.0 M ammonium formate, both at pH 7.5, was used as the eluting agent at a regulated flow rate of 1.3 ml per minute. Fractions were collected at 10 minute intervals. The elution pattern, determined by A_{260}, is shown in Figure 19. After the 0.01 M-1.0 M gradient had gone to completion, a second gradient prepared from 1000 ml each of 1.0 M and 2.0 M ammonium formate, both at pH 7.5, was established. No A_{260} above a base line value of 0.01 could be detected in fractions collected during the course of elution by the 1.0-2.0 M gradient. Previous experiments in which hydrolyzates of dDNA were fractionated on DEAE-Sephadex showed that mononucleotides were eluted in the first three peaks; i.e. the first peak contained a mixture of the two pyrimidine mononucleotides, the second was dAMP, and the third was dGMP. Material from the center of each of the first three peaks (see Figure 19) was chromatographed on PEI-cellulose with

Figure 19: Elution Pattern from a DEAE-Sephadex A-25 Column of the Products of Hydrolysis of dDNA Produced by P-100 Enzyme at pH 8.0

A reaction mixture containing 50 ml of dDNA (heated at 100° for 30 minutes followed by rapid cooling to 4°), 60 ml of 0.2 M Tris-HCl, pH 8.2, and 10 ml of P-100 enzyme (previously diluted to a concentration of 20 DNase B units per ml with 0.12 M ammonium acetate pH 8.0) was incubated at 37° for 9 hours. After the incubation period the reaction mixture was diluted to 336 ml with deionized water. This diluted sample, containing a total of 1880 A_{260} units, was applied to a 2.5 x 48 cm DEAE-Sephadex A-25 column previously equilibrated with 0.01 M ammonium formate, pH 7.5, at a flow rate of 1.5 ml per minute. Following application of the sample, the column was washed with 1200 ml of 0.01 M ammonium formate, pH 7.5. A gradient prepared from 2000 ml each of 0.01 M and 1.0 M ammonium formate, both at pH 7.5, was used as the eluting agent at a regulated flow rate of 1.3 ml per minute. Fractions were collected at 10 minute intervals. After the 0.01-1.0 M salt gradient had gone to completion, a second gradient prepared from 1000 ml each of 1.0 M and 2.0 M ammonium formate, both at pH 7.5, was used as the eluting agent. No A_{260} above a base line value of 0.01 could be detected in fractions collected during the course of elution by the 1.0-2.0 M gradient.


I
II
III
IV
3.0
2.0
1.0
A_{260}
1.0
0.8
0.6
0.4
0.2
Ammonium Formate Concentration (M)
60
120
180
240
Fraction Number

the four common 2'-deoxynucleoside-5'-monophosphates as reference standards. The second and third peaks were found to contain dAMP and dGMP, respectively. The first peak contained only TMP. Because these three peaks were not well separated from one another, they were pooled and termed Fraction I (as shown in Figure 19). The remaining tubes were pooled at points in the elution profile shown in Figure 19 to give Fractions II, III, and IV. All fractions collected during the washing step were pooled and termed "Wash-Thru." The A_{260} of Fractions I-IV and the Wash-Thru was determined. These values are shown in Table IX. In order to remove the volatile ammonium formate, each fraction was flash-evaporated to a volume of approximately 50 ml. Repeated cycles of lyophilization were then used to remove the remainder of the salt. Each residue was then dissolved in 1-2 ml of water at a concentration which ranged from 138-220 A_{260} units per ml. These concentrated fractions were stored at -10° when not in use. The first type of characterization to be done was a determination of the relative molar proportions of mononucleotides present in Fraction I. Two dimensional paper chromatography on Whatman 3MM sheets in Solvents I and II (see Methods: Paper Chromatography) was used to separate the various mononucleotides. The four common 2'-deoxynucleoside-5'-monophosphates and 2'-deoxynucleosides were also chromatographed as reference standards. All U.V. absorbing spots were cut from the chromatographs and the compounds eluted with 0.01 N

TABLE IX

Relative Amounts of Products of Hydrolysis of dDNA Recovered From a DEAE-Sephadex A-25 Column

Fraction	Volume (ml)	A_{260}	Total A_{260} Units
Wash-Thru	1580	0.023	36.3
I	560	0.650	364
II	370	1.42	525
III	325	1.38	448
IV	1070	0.423	453
			1826.3 Total Recovered

$$\frac{1826.3 \text{ Recovered}}{1880 \quad \text{Applied}} \times 100 = 97.2 \text{ Percent Recovery}$$

Material eluted from a DEAE-Sephadex A-25 column was pooled into four fractions (Fractions I-IV, Figure 19) plus a Wash-Thru fraction consisting of all eluate collected from the beginning of the application of hydrolyzate to the beginning of the 0.01-1.0 M gradient elution. The A_{260} of each fraction was determined on a Beckman DB spectrophotometer.

HCl. The recovery of standard mononucleotides was found to be in the range of 92-110 percent. Table X summarizes the relative amounts of mononucleotides found in Fraction I (Figure 19). In order to determine the average size of the products in each of Fractions I-IV, the procedure in Methods was used (see Determination of Average Chain Length of Mixed Oligonucleotides). The values obtained by this procedure are shown in Table XI along with data pertaining to the percent of the total hydrolyzate recovered in each fraction. In order to verify that oligonucleotides produced from dDNA at pH 8.0 by P-100 enzyme were, in fact, 5'-phosphoryl terminated, the following digestion with venom phosphodiesterase was performed. Reaction mixtures consisting of 0.02 ml of either Fraction II (220 A_{260} units per ml) or Fraction III (182 A_{260} units per ml); 0.01 ml of 0.3 M Tris-acetate, pH 8.8; 0.01 ml of 0.3 M magnesium acetate; 0.03 ml of venom phosphodiesterase, 0.5 mg per ml in water, potency 0.3; and 0.03 ml of water were incubated at 37° for 14.5 hours. Reaction mixtures lacking oligonucleotide but containing instead 2'-deoxynucleoside-5'-monophosphates and 2'-deoxynucleosides were treated in the same way. Following the incubation period all reactions were lyophilized and chromatographed on Whatman 3MM paper using Solvent I. All spots absorbing under U.V. light were removed, and the substances eluted with 0.01 N HCl. The A_{260} and A_{270} values of the eluates were determined, leading to the following result. It was found that 3.73 percent

TABLE X

Relative Amounts of Mononucleotides in Fraction I (Figure 19)

Mononucleotide	Percent of Total Mononucleotides
dAMP	28.5
dGMP	31.4
TMP	40.1
dCMP	Less than 2 percent

A 0.058 ml quantity of Fraction I (138 A_{260} units per ml) was chromatographed on Whatman 3MM paper in Solvents I and II as described in Methods. The four common 2'-deoxynucleoside-5'-monophosphates were also chromatographed as reference standards. All U.V. absorbing spots were removed from the chromaotgraphs and eluted with 0.01 N HCl. The recovery of standard mononucleotides was found to be in the range of 92-110 percent.

TABLE XI

Distribution of Products in a Hydrolyzate of dDNA

Fraction[a]	Average Chain Length	Percent of Total Hydrolyzate Recovered
I	1.04	20.4
II	2.08	29.4
III	2.96	25.0
IV	3.98	25.2

[a]Fraction numbers as given in Figure 19.

Material from each of Fractions I-IV was assayed for the ratio of total to terminal phosphate as described in Methods (see Determination of Average Chain Length of Mixed Oligonucleotides). The percent of the total hydrolyzate in each fraction was calculated from data shown in Table IX.

(measured by A_{270}) and 4.15 percent (measured by A_{260}) of the total venom phosphodiesterase hydrolyzate of Fraction II appeared as nucleoside. Likewise, 2.74 percent (A_{270}) and 3.18 percent (A_{260}) of the total hydrolyzate of Fraction III appeared as nucleoside. Taking into account the average chain length of each fraction these figures indicate that 7.5-8.3 percent of Fraction II was dinucleoside monophosphates (XpX), and 8.2-9.5 percent of Fraction III was trinucleoside diphosphates (XpXpX). Even with these results, the experiment still shows that principally 5'-phosphoryl terminated oligonucleotides were produced from dDNA at pH 8.0 by the P-100 enzyme.

The Hydrolysis of Poly-2'-O-Methyladenylic Acid by P-100 Enzyme

When it was found that the P-100 enzyme preparation acted endonucleolytically on both RNA and DNA to give 5'-phosphoryl terminated products, it was thought desirable to attempt to hydrolyze the polynucleotide poly-2'-O-methyladenylic acid (Poly A^m). Because this polymer exhibited unusually high viscosities at pH values below 6.0, it was decided to use pH 8.0 as one of the conditions for hydrolysis (since the P-100 enzyme exhibited significant RNase and DNase activities at this pH). A reaction containing 0.04 ml poly A^m, 27.5 A_{257} units per ml in water; 0.05 ml Tris-HCl, 0.2 M, pH 8.2; and 0.01 ml of undiluted P-100 enzyme was incubated at 37°. Aliquots of 0.01 ml were removed at various times and spotted on a 14 x 20 cm MN 300 cellulose

thin layer sheet for chromatography in the n-propanol:29 percent ammonium hydroxide:water solvent system described in the section, Susceptibility of Poly A, Poly U, and Poly C to Hydrolysis by P-100 Enzyme. The mononucleotide 2'-O-methyladenosine-5'-monophosphate (5'-A^mMP) and the corresponding nucleoside 2'-O-methyladenosine (A^m) were also included on the chromatogram as reference standards. Their R_f values were 0.57 and 0.86 respectively. It was evident by the appearance of the chromatogram after chromatography that even at 0.5 hour incubation a significant amount of the polymer had been degraded by the P-100 enzyme. By 2.0 hours products of hydrolysis as small as the mononucleotide (5'-A^mMP) were being formed. At 4.0 hours incubation, most of the U.V. absorbing material was adjacent to and just behind the mononucleotide area, however, it also was evident that larger products still remained in the hydrolyzate because U.V. material was still visible all the way to the origin. By 13 hours of incubation a single streaked spot was observed to extend from about half the distance between 5'-A^mMP and the origin to a position adjacent to the 5'-A^mMP spot. No A^m (nucleoside) was observed in the hydrolyzate, but there was a spot, which became visible at about 2.0 hours incubation, that migrated approximately half way between the nucleoside and mononucleotide. The intensity of this spot increased with longer incubation times indicating that greater amounts of this product were being formed with time.

Two separate methods were then employed for the separation of oligonucleotides of adenylic acid up to a chain length of four so that a more accurate indication of the amounts of smaller products being formed could be made. These methods were ascending thin layer chromatography on PEI-cellulose using 0.8 M Tris-HCl, pH 8.4, as solvent and paper electrophoresis (48) on DEAE-cellulose strips in 0.1 M Tris-phosphate, pH 6.6. In both of these systems migration rates of the individual compounds decreased in the order mononucleotide > dinucleotide > trinucleotide > tetranucleotide.

It was apparent after using the previously described separation techniques that much larger amounts of P-100 enzyme along with longer incubation times than those used with poly A were necessary to achieve the same stage of hydrolysis with poly A^m.

In order to prepare 10-20 A_{260} units of the 5'-phosphoryl terminated tri- and tetranucleotides of 2'-O-methyladenylic acid (49) the following experiment was performed. A reaction mixture consisting of 6.90 ml poly A^m (20.0 A_{260} units per ml); 3.45 ml of 0.10 M Tris-HCl, pH 8.2; 3.45 ml of undiluted P-100 (200 DNase B units per ml); and 3.45 ml of water containing pentachlorophenol at a concentration of 0.5 µg per ml was incubated at 37° for 47 hours. Another 1.72 ml of P-100 enzyme was added and the reaction mixture was incubated for another 21 hours at 47°. The reaction mixture was then diluted to 100 ml with deionized water and applied to a 1.5 x 33 cm column of Whatman Advanced DE-23

which had previously been converted to the formate form with ammonium formate. The total A_{260} units applied to the column was 195.4. Following application of the sample the column was washed with 300 ml of 0.01 M ammonium formate, pH 7.5. The column was then treated with a gradient prepared from 800 ml each of 0.01 M and 1.0 M ammonium formate, both at pH 7.5. Fractions of 400 drops each were collected throughout the washing and gradient elution procedures. After the 0.01 M-1.0 M salt gradient had gone to completion, the column was washed with 100 ml of 2 M ammonium formate. Figure 20 shows the A_{260} elution pattern obtained during the 0.01-1.0 M salt gradient elution. No significant increase was observed in the A_{260} of fractions collected during either the application of the sample or the washing procedure. The fractions containing the highest concentration of products, as indicated by A_{260}, were pooled as separate peaks (see Figure 20 and Table XII). The A_{260} observed in each of these pooled peaks is shown in Table XII. Electrophoresis on DEAE-cellulose in 0.1 M Tris-phosphate buffer, pH 6.6 of material from peaks II-V indicated that each peak contained at least two components. These components were separated by paper electrophoresis on Whatman No. 40 using 0.1 M ammonium bicarbonate, pH 7.8. The characterization of each component separated by electrophoresis was then carried out by a determination of the ratio of nucleoside to mononucleotide produced by venom phosphodiesterase after dephosphorylation by alkaline phosphatase.

Figure 20: Elution Pattern from a Whatman Advanced DE-23 Column of the Products of Hydrolysis of Poly-2'-O-Methyladenylic Acid Produced by P-100 Enzyme

A reaction mixture consisting of 6.90 ml of poly A^m (20.0 A_{260} units per ml); 3.45 ml of 0.10 M Tris-HCl, pH 8.2; 3.45 ml of undiluted P-100 enzyme (200 DNase B units per ml); and 3.45 ml of water containing pentachlorophenol at a concentration of 0.5 µg per ml was incubated at 37° for 47 hours. Another 1.72 ml of P-100 enzyme was added and the reaction mixture was incubated for another 21 hours at 47°. The reaction mixture was then diluted to 100 ml with deionized water and applied to a 1.5 x 33 cm column of Whatman Advanced De-23. The total A_{260} units applied to the column was 195.4. Following the application of the sample the column was washed with 300 ml of 0.01 M ammonium formate, pH 7.5. The column was then treated with a gradient prepared from 800 ml each of 0.01 M and 1.0 M ammonium formate, both at pH 7.5. Fractions of 400 drops each were collected throughout the washing and gradient elution procedures. After the 0.01 M-1.0 M salt gradient had gone to completion, the column was washed with 100 ml of 2 M ammonium formate. The A_{260} of each fraction was determined in a Beckman DB spectrophotometer.

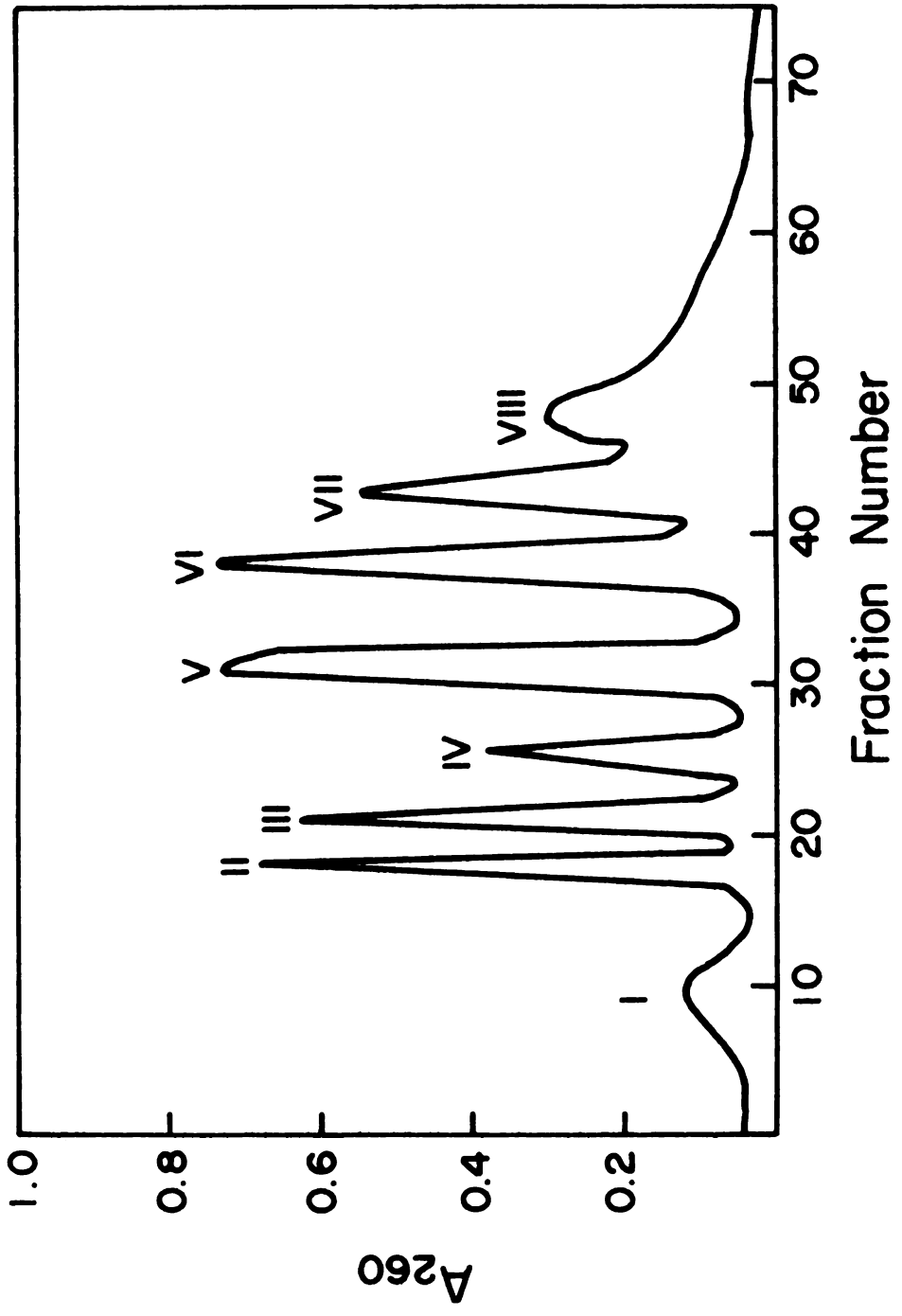

A260
1.0
0.8
0.6
0.4
0.2
10
20
30
40
50
60
70
Fraction Number
I
II
III
IV
V
VI
VII
VIII

TABLE XII

Recovery From a Whatman Advanced DE-23 Column of the Products of Hydrolysis of Poly A^m as Determined by A_{260}

Peak Number[a]	Fractions Pooled	Total A_{260}
I	5-14	12.7
II	17-18	15.2
III	21-23	17.8
IV	25-26	11.1
V-A	30	3.6
V-B	31-33	26.2
VI	37-40	28.0
VII	42-44	18.7
VIII	47-50	23.3
2 M Wash	(all fractions)	11.0
		167.6 Total Recovered

[a]See Figure 20.

As can be seen by the data in Table XIII[23] a significant percentage of the total hydrolyzate consisted of oligonucleotides lacking a terminal phosphate group.

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[23]The author thanks Dr. F. M. Rottman for generously providing this data.

TABLE XIII

Characterization of the Products of Hydrolysis of Poly-2'-O-Methyladenylic Acid Eluted from a Whatman Advanced DE-23 Column

Peak Number	Identity	Total A_{260} Recovered
I	$A_pA^m + A^m$	N.D.[a]
II	$pA^m + A^m$	N.D.
III	$A_pA_pA^m$	16.53
IV	pA_pA^m	8.78
V-A	$pA_pA_pA^m$	0.812
	$(A_p)_3A^m$	3.43
V-B	$pA_pA_pA^m$	19.22
	$(A_p)_3A^m$	10.90
	$(A_p)_2A^m$	2.74
VI	$(pA^m)_4$	20.56
	$(A_p)_4A^m$	14.29
VII	$(pA^m)_5$	13.33
	$(A_p)_5A^m$	6.51
VIII	$(pA^m)_6$	16.12
	$(A_p)_6A^m$	4.47
2 M Wash		N.D.

[a]Not Determined.

The material in each peak was resolved by electrophoresis on Whatman No. 40 paper using 0.1 M ammonium bicarbonate, pH 7.8. The characterization of each component was performed by a determination of the ratio of nucleoside to mononucleotide produced by venom phosphodiesterase after dephosphorylation by alkaline phosphatase.

DISCUSSION

The nuclease from muskmelon seeds described heretofore has been purified about 2900 X. Recoveries of total DNase activity have ranged between 10 and 15 percent of that measured in crude extracts. Some problems have been encountered when nuclease activities were measured in crude homogenates because there was enough material absorbing strongly at 260 mμ to give relatively high control values in the lanthanum nitrate-HCl assay. Assays for activity in crude homogenates were always done within 24-48 hours after preparation since noticable changes in the solution, such as precipitation, took place on prolonged storage at 4°.

Fractionation with ammonium sulfate was attempted in the normal way by adding increasing amounts of the salt and separating the various fractions precipitated by centrifugation. A much better separation of DNase from non-specific phosphodiesterase [as determined by the liberation of p-nitrophenol from bis-(p-nitrophenyl) phosphate] occurred, however, when the ammonium sulfate fractionation was done in the reversed procedure: i.e. precipitating first with a high concentration of the salt, and then dissolving the precipitate in a solution of lower ammonium sulfate concentration.[24] The final solution in the ammonium sulfate

[24] Dr. A. B. Adams, personal communication.

fractionation before dialysis could be stored as long as a week at 4° without detectable losses in DNase activity. The dialysis preceding DEAE-cellulose chromatography consistently resulted in about a 10 percent decrease in total activity which could not be accounted for in terms of the small amount of precipitate formed during this procedure.

Chromatography on DEAE-cellulose represented the best step in the purification procedure as far as overall purification was concerned. The enzyme activities were consistently eluted from the resin between 0.3 and 0.4 M ammonium acetate, indicating a strong affinity of the enzyme for the resin at pH 8.0. The presence of a second peak of RNase activity preceding the nuclease peak indicates that at this point in the purification the nuclease preparation is still contaminated with some other RNase not associated with the nuclease:3'-nucleotidase complex.

Suspicions regarding the presence of contaminating RNase following the DEAE-cellulose chromatography were confirmed when significant amounts of RNase activity could be measured in the CM-cellulose pass-through fraction (before gradient elution of the nuclease). No detectable DNase (A or B) nor 3'-AMPase was present in this same pass-through solution. This apparent separation of contaminating RNase activity, however, was not reflected in a significant change in the RNase to DNase B specific activity ratio. The nuclease appears to have a relatively low affinity for CM-cellulose at pH 5.5 as judged by the low salt concentration

required to elute it from the resin. This treatment, however, provided a rather good purification even though only about 50 percent of the total activity applied to the column was recovered. The loss in activities evidently was not due to the separation of nuclease activities since no other nuclease nor 3'-AMPase could be detected in the pass-through volume or at higher salt elutions up to 1.0 M. The other possibility remains, however, that separation could have occurred but the separated nuclease may have been inactive and hence, not detected. Instability to storage at low pH (4-6) has been consistently observed with the muskmelon nuclease, and has also been reported in the case of the mung bean nuclease (18).

Chromatography on the Bio-Gel P-100 DEAE-cellulose combination column resulted in two beneficial accomplishments: i.e. the volume following chromatography on CM-cellulose was decreased to about one tenth and a five- to six-fold increase in specific activity over that of the CM-cellulose fraction was obtained. Most of the ammonium acetate (0.75 M) used to elute the enzyme from the DEAE-cellulose was removed during the subsequent pass through the P-100 resin. This method utilizing a small pore resin such as P-2 could find great use in the concentration of large volumes of solutions containing enzymes or nucleic acids provided an appropriate ion exchange resin could be found. Enzyme preparations following P-100 chromatography could be stored at -10° or 4° over a period of several

months without more than 10-15 percent loss of DNase activity. These same preparations appear to survive lyophilization and storage at -10° in the anhydrous state.

The enzyme preparation following P-100 chromatography exhibited no activity when a variety of p-nitrophenyl nucleotide derivatives were tested at pH 4.5, 7.0, and 9.0. The enzyme also appeared to be free of 5'-nucleotidase activity as indicated by the absence of inorganic phosphate in 24 hour incubations with the four 2'-deoxynucleoside-5'-monophosphates. A slight but significant hydrolysis of p-nitrophenyl phosphate at the higher pH range (7-9) was observed, however. The significance of this finding will become apparent later in the discussion regarding the characterization of products of hydrolysis of dDNA and poly A^m.

Initially, work with extracts of muskmelon seeds centered on the purification of DNase activity assayed at pH 7.5. It became evident, however, that RNase and 3'-nucleotidase activities were closely associated with the DNase activity. This close association of these three types of activities has also been observed in mung bean sprouts (14-18), rice bran (13), ryegrass (12), barley (10, 11), the fungus Penicillium citrinum (22-24), wheat seedlings (19), and corn (20, 21). When DNase, RNase, and 3'-AMPase were assayed at pH 7.5 the ratios of the specific activities of RNase and 3'-AMPase remained constant throughout the latter three steps of the purification. A thorough study of the

activities as a function of pH, however, revealed an additional DNase activity.

The first series of experiments testing the relationship of pH and DNase activity, using dDNA, revealed what appeared to be two pH optima (using Tris-HCl, cacodylate, and acetate buffers). These occurred at pH 6.0 and pH 8-9. When imidazole and histidine buffers were substituted for cacodylate and Tris, a striking difference in the activity at the higher pH was noted. A reasonable explanation for this effect might be that at higher pH a metal ion was required for activity and histidine, rather than imidazole, could have removed this metal from the enzyme by chelation. In the presence of histidine and imidazole the pH optimum occurred at about pH 5.7, and was comparable in magnitude to the optimum at pH 8-9 in Tris. Phosphate and citrate buffers used in the pH 5-7 range were strongly inhibitory. The activity observed with these buffers was only 10 percent of that observed with histidine or imidazole. When the pH-activity relationship for DNase was investigated, using a pH stat , with 0.10 M NaCl as the only major ionic species present, a curve was obtained which was very similar in shape to the curve resulting from the use of Tris, cacodylate and acetate buffers. This result conclusively proved the existence of two pH optima with respect to DNase activity on denatured DNA in the absence of buffer effects.

The RNase exhibited activity over a rather wide range of pH values when Tris and cacodylate buffers were utilized.

As in experiments with DNase activity the substitution of imidazole and histidine buffers resulted in a sharp optimum of activity at pH 5.7. This was the first evidence besides the purification data that these two activities were very intimately related.

Results from the experiments with 3'-AMPase indicated that maximal activity occurred between pH 7.5 and 8.5. The effects of imidazole and histidine at pH 8.0 were much like those observed for DNase at pH 8.0, i.e. very low levels of activity with histidine, and approximately half maximal (activity in the presence of Tris-HCl = 100 percent) with imidazole. A small peak of activity appeared to exist in the pH 5.0-5.5 range with histidine.

For the sake of convenience the lower DNase optimum (pH 5.7) was termed DNase A (acidic) and the higher one (pH 8.0) DNase B (basic). The RNase was assayed at pH 5.7 and the 3'-AMPase, at pH 8.0. Once the pH optima were determined, a more valid measure of the ratios of the various activities through the purification could be made. It was found, for instance, that levels of RNase in crude and ammonium sulfate fractions were much higher when assayed in imidazole at pH 5.7 than when measured at pH 7.5 in Tris buffer. When assays were performed at the pH optima at each stage in the purification, it was evident that the ratios of DNase A, RNase, and 3'-AMPase to DNase B did not vary significantly in the last three steps of the procedure. The degree of purification through these last three steps

was greater than forty-fold. It would seem reasonable to assume that if there were more than one protein entity responsible for the four activities present in the preparation, that a variety of separation techniques such as chromatography on DEAE-cellulose, CM-cellulose, and Bio-Gel P-100 would result in appreciable variation in the ratios of specific activities.

Two additional experiments were performed in an effort to separate the four activities. The isoelectric focusing treatment appeared to confirm the conclusion regarding the close association of all four activities, although the apparent lack of stability at the isoelectric point (pH 4.4) and the determination of the different activities at different time intervals after recovery from the column caused the ratios of activities to vary greatly from those observed in the P-100 enzyme preparation. Another possible explanation for the great variation in specific activity ratios in this experiment would be that RNase and 3'-nucleotidase may in part have been separated from the DNase but, once separated, were inactive and thus not detectable.

The other experiment performed in an effort to effect a separation was polyacrylamide disc gel electrophoresis. As shown in Experimental Results, all activities were localized in one protein band, and the ratios of activities through this band remained essentially constant. Since the four activities were found to migrate more slowly than any

other detectable protein, the possibility exists that a still greater purification might be feasible with the use of preparative disc electrophoresis. Experiments designed to provide mg quantities of P-100 enzyme are now in progress to further investigate the possible use of preparative electrophoresis. In light of the results obtained from the purification procedure and the isoelectric focusing and electrophoresis experiments, it must be concluded that DNase A, DNase B, RNase, and 3'-AMPase either reside in a single protein species or are part of a complex which is held together extremely tightly.

There is much evidence to indicate that zinc plays a major role in the stabilization of these associated activities in the pH range of 4-6. This fact has been well documented for the nuclease: 3'-nucleotidase activities of mung bean (18), P. citrinum (22, 23), and wheat seedlings (19). Although zinc has been observed to be inhibitory at a concentration of 10^{-3} M in assays it appears to greatly stabilize the muskmelon nuclease at high temperatures at 10^{-3} M. It therefore is likely that zinc plays some role in maintaining a stable conformation of the protein (or protein complex).

Another rather interesting difference between the muskmelon nuclease and many of the other plant nucleases was the finding that the molecular weight, determined on Sephadex G-100, was about 50,000. Most of the plant nuclease: 3'-nucleotidase enzymes cited previously have

been observed to be smaller species migrating on gel filtration columns like proteins with molecular weights of 15-30,000. It is intriguing to speculate on the possibility that the additional DNase pH optimum (at pH 8.0) might represent an additional protein subunit amounting to a molecular weight integral of 15-20,000. The possibility exists that methods of isolation may play a large part in determining the nature of the final product, especially if this product consists of a complex of protein subunits. For example, consider the hypothetical situation where a nuclease:3'-nucleotidase complex dissociates at pH values below 6.0 to give inactive subunits. This dissociation at low pH is blocked, or retarded by the presence of zinc. If this hypothetical situation were true, then it is possible that some of the differences observed among the nuclease: 3'-nucleotidase enzymes from different plant sources might simply be caused by the pH at which the initial extraction procedure was done.

The effects of cations on the muskmelon nuclease and 3'-nucleotidase activities appear to be complex. Certainly no cation affected all four activities in the same way. Some trends, however, are significant. The divalent cations of the alkaline earth series led to minor increases in DNase B and RNase activities. Calcium appeared to stimulate 3'-AMPase to some extent as well. The monovalent cations, sodium, lithium, and potassium brought about slight increases in RNase and 3'-AMPase, but were without much

effect on DNase A or B. The other divalent cations tested seemed to cause inhibition of all four activities. The relative magnitude of inhibition by these divalent cations seems to be similar for the DNase B and RNase activities (see Table II for summary).

It is rather difficult to draw conclusions regarding the nature of the interaction of metals in the various reactions. The experiment was originally done to see if any of the more common cations functioned to bring about major increases in any or all of the enzyme activities. Apparent stimulation of nuclease activities by metal ions is often brought about by effects of the metal on the conformation of the polynucleotide substrate (9) rather than by direct effects on the enzyme itself. One metal effect on the muskmelon nuclease which may have importance regarding the specificity of the endonuclease is that of zinc. As indicated in Table II moderate inhibition of the nuclease activities by zinc was contrasted with almost complete inhibition of the 3'-AMPase. In a previous discussion (see Susceptibility of Poly A, Poly U, and Poly C to Hydrolysis by P-100 Enzyme) the relative 3'-nucleotidase activities were implicated as possibly expressing the specificity of bond cleavage in the polynucleotide. A possibly related observation is that the presence of zinc (10^{-4} - 10^{-3} M) during the course of hydrolysis of high molecular weight RNA by both mung bean (46) and wheat seedling (19) nuclease: 3'-nucleotidases appeared to greatly increase the percentage

of mononucleotides in the hydrolyzate over hydrolyzates not containing zinc. These two results seem to indicate that zinc has the ability to change the specificity of the nuclease:3'-nucleotidase to one involving fewer endo- and more exonucleolytic kind of scissions. Alternatively, zinc may function in some way to facilitate the hydrolysis of di- and trinucleotides, thus giving a higher percentage of mononucleotides in the hydrolyzate compared to hydrolyzates lacking zinc.

Certainly, with an enzyme system as complex as this one, much work on the properties of the various activities remains to be done. The main thrust, however, of this author's work has been toward an elucidation of the possible specificities of the RNase and DNases.

When it became evident that the muskmelon nuclease exhibited 3'-nucleotidase activity, a study designed to compare the activity on the four ribonucleoside-3'-monophosphates was undertaken. The pH optimum of 3'-AMPase (pH 8.0) was chosen as a standard condition under which the other three nucleotides would be tested. A striking difference in relative rates was observed as shown in Table V. This same order of reactivity (A > G > U > C) has been observed with the mung bean 3'-nucleotidase (18). Under the conditions used in the muskmelon 3'-nucleotidase assay, no detectable hydrolysis of 3'-CMP occurred (as little as 2 percent hydrolysis could have been detected with the phosphate assay employed). This result along with later ones indicating

that CMP was either totally absent or present in only very minor amounts in hydrolyzates of dDNA and RNA directed the work toward elucidation of the details of enzyme specificity.

When the 2'-deoxynucleoside-3'-monophosphates were tested as possible 3'-nucleotidase substrates under conditions of substrate and enzyme concentrations similar to those used with the ribonucleoside-3'-monophosphates no hydrolysis could be detected. When the enzyme concentration was increased forty-fold and the incubation time was lengthened from 0.5 hour to 15 hours, however, hydrolysis of the compounds took place. The most important finding, however, was that 3'-dCMP was not degraded and 3'-dGMP was only partially degraded. This result points up another difference between the muskmelon nuclease:3'-nucleotidase and the analogous enzyme from mung bean. Johnson and Laskowski (26) have stated that only 3'-ribonucleotides were hydrolyzed, thus conferring sugar specificity at least on the nucleotidase activity. The muskmelon enzyme, however, appears to hydrolyze both ribo- and 2'-deoxyribonucleotides although there is a vast difference in the relative rates of hydrolysis. The total absence of detectable hydrolysis of 2'-deoxycytidine-3'-phosphate is the most interesting result in light of the previous findings with cytidine-3'-phosphate.

In order to test the muskmelon nuclease preparation for the presence of RNases or cyclic phosphodiesterases the enzyme preparation was exposed to various cyclic phosphodiester derivatives of the mononucleotides (both 3',5'- and

2',3'-cyclic mononucleotides). Under conditions of nucleotide concentration, enzyme concentration, and incubation time identical to those used with the 2'-deoxynucleoside-3'-monophosphates no significant hydrolysis of the cyclic nucleotides could be detected either at pH 5.7 or pH 8.0. A slight amount of nucleoside formation evident in the reactions containing enzyme was attributed to dephosphorylation of the 3'-phosphoryl nucleosides which were produced in low concentrations by nonenzymatic breakdown of the cyclic derivatives. This nonenzymatic breakdown to the phosphomonoesters was detected, in reactions lacking enzyme, as a more slowly migrating spot in Solvent I.

The earliest work on the purification of the muskmelon nuclease utilized native DNA as substrate with the hope that a DNase specific for double stranded DNA could be isolated. Experiments with the enzyme purified approximately 100-fold indicated that denatured DNA was hydrolyzed much more rapidly than was the double stranded form. When a more detailed study was undertaken with enzyme purified through the P-100 step ($\sim$ 2900-fold) it became apparent that some striking differences existed in the hydrolysis rates of various polynucleotide substrates. As the kinetic study in Figure 12 shows, RNA is hydrolyzed about twice as fast as denatured DNA. The relative rates of hydrolysis of denatured DNA at pH 5.7 and 8.0 appear to be equal up to about 60 percent conversion of the substrate to lanthanum nitrate-HCl soluble products. At this point the rate at pH 8.0 drops below that

measured at pH 5.7. This slowing in the rate of reaction has been described by Vanecko and Laskowski (50) as "auto-retardation," i.e. intermediates in the reaction are progressively more resistant substrates as their size decreases. The rate of the reaction slows down considerably and can be restored only by the addition of large amounts of enzyme. Such an observation was made with the muskmelon nuclease while using a pH stat to follow the progress of a reaction at pH 8.0. One explanation for the apparent difference in rates above 60 percent conversion of substrate at pH 5.7 and 8.0 could be the charge density on a small oligonucleotide. Since the primary phosphate has a pK_a of 0.7-1.6 and the secondary phosphate a pK_a of 6.0-6.5 short oligonucleotides would have a larger charge to size ratio at pH 8.0 than at pH 5.7. The fact that the additional charge at pH 8.0 would be located at the terminus adds to its interfering effect the shorter the oligonucleotide gets especially in the case of endonucleolytic type cleavages. In a long polynucleotide an endonuclease would rarely encounter a terminal phosphate as far as the points of preferential cleavage are concerned.

The rate of release of lanthanum nitrate-HCl soluble products from native DNA at pH 8.0 appears to be less than 10 percent of that of denatured DNA. The ratio of these rates at pH 5.7, however, increases quite significantly to about 20 percent. This same difference in the reactivity of native DNA at pH 5.7 and 8.0 was observed with DNA from calf thymus. Such a difference could also be rationalized by the

same reasoning of substrate ionization as was used for denatured DNA.

The great difference in reactivity of denatured DNA and native DNA is potentially useful in a variety of situations. It is doubtful, however, that the muskmelon nuclease exhibits a strict specificity for single stranded DNA, since with salmon sperm DNA, conversions of greater than 50 percent of the native DNA substrate to lanthanum nitrate-HCl soluble products were observed at pH 8.0. A strict judgment on this question must await experiments using DNA which has been shown by other criteria to contain no single stranded breaks or regions. Mikulski, Johnson, and Laskowski (51) have reported that a highly purified preparation of mung bean nuclease is capable of specifically degrading only denatured DNA in a mixture of denatured and native DNA.

It was encouraging from the standpoint of potential specificity to find that the nuclease activities on RNA at pH 5.7, on denatured DNA at pH 5.7 and 8.0, and also on native DNA at pH 5.7 and 8.0 exhibited an endonucleolytic mode of action. The gel filtration method used, however, does have one inherent disadvantage. The presence of relatively large amounts of exonuclease could be masked by the presence of small amounts of endonuclease the hydrolysis products of which spill over into the position at which mononucleotides are eluted from the column (see Figures 13-15). The mode of hydrolysis of poly A at pH 8.0 was also observed to be endonucleolytic since paper chromatographic evidence

indicated that di-, tri-, and tetranucleotides were formed at early stages in the reaction.

Experiments utilizing the action of alkaline phosphatase, a nonspecific phosphatase, and 5'-nucleotidase, a specific phosphatase which removes phosphate from only 5'-phosphoryl mononucleotides, conclusively demonstrated that muskmelon nuclease forms nucleoside-5'-monophosphates from RNA at pH 5.7, and the corresponding 2'-deoxy derivatives from dDNA at the same pH. Furthermore, the use of snake venom phosphodiesterase confirmed the presence of 5'-phosphoryl terminated oligonucleotides in hydrolyzates of poly A produced by the muskmelon enzyme at pH 8.0. Because of the presence of 3'-nucleotidase activity relatively large amounts of nucleosides would be present in hydrolyzates of RNA if ribonucleoside-3'-monophosphates were formed. This was not observed. The only contradiction to this reasoning would be the possible inhibition of 3'-nucleotidase by the presence of nucleoside-5'-monophosphates. At present no data are available to either confirm or deny this possibility.

It was surprising to find that when polyadenylic acid, polyuridylic acid, and polycytidylic acid each were tested as possible substrates for the muskmelon nuclease, hydrolysis of only the first two could be detected. This finding strengthened the suspicion, acquired from data on the relative 3'-nucleotidase activities, that the CpY bond was particularly invulnerable to the action of muskmelon nuclease.

The reaction mixtures were buffered at pH 8.0 instead of 5.7 in order to take advantage of the specificity observed with the 3'-nucleotidase at pH 8.0. Other experiments also showed that poly A could be hydrolyzed to 5'-AMP at pH 5.7 by the P-100 enzyme preparation.

The next logical question to ask was what would happen if one attempted to hydrolyze a polyribonucleotide containing cytidylic acid plus one other type of nucleotide. The hypothesis regarding the CpY bond would predict that all oligonucleotide products should be of the general structure $(pC)_npY$ where n is any number equal to or greater than 1.0. Of course, no CMP should be found in a limit[24] hydrolyzate if the CpY bond is invulnerable. The other nucleotide is expected to be present in hydrolyzates as the free mononucleotide since the occurrance of the YpY sequence should allow for the formation of pY (it must be remembered that 5'-phosphoryl terminated products are formed by the muskmelon nuclease).

The results obtained from the characterization of the products of hydrolysis of polyuridylic-cytidylic acid (poly U,C) support the previously described hypothesis. Chemical and enzymatic characterization of 84 percent of the recovered products indicated that UMP, pCpU, $(pC)_2pU$, and $(pC)_3pU$ were

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[24]"Limit" hydrolysis is here defined as one which has been permitted to proceed to the point where it is thought that all vulnerable phosphodiester bonds have been hydrolyzed.

the major components in the hydrolyzate. Another 10 percent of the recovered material, which was eluted from the DEAE-Sephadex A-25 column, had spectral characteristics that indicated the presence of high proportions of cytidylic acid. Table VIII summarizes the chemical and enzymatic methods of characterization used on the major oligonucleotide species.

Another observation regarding recovery of the characterized species was that the amount of each obtained was in agreement with the expected probabilities of occurrence of those sequences in a random copolymer as calculated by the method of Nirenberg, et. al. (52). For example, a polynucleotide containing uridylic acid:cytidylic acid in a molar ratio of 2:1 respectively should contain 44.5 percent of its weight as the nucleotide sequence UpU (0.666 x 0.666), 29.6 percent as UpUpU, 22.2 percent as CpU, 7.4 percent as CpCpU, and 3.7 percent as CpCpC. As can be seen in Table VII and VIII the major proportion of products have been recovered in amounts approximating these predicted frequencies.

The fractionation and partial characterization of hydrolysis products produced from denatured DNA by the muskmelon nuclease have brought to light some important properties of this enzyme. First, this experiment, as well as a number of others done previously under similar conditions, have shown that the nuclease converts about 20 percent of the total substrate to mononucleotides. The composition of this mononucleotide fraction, however, is unusual in that no dCMP

has been detected. Taking into account the observations made with respect to the 3'-nucleotidase activities on 2'-deoxyribonucleoside-3'-monophosphates one might predict the same sort of specificity for the hydrolysis of dDNA, i.e. inability of the enzyme to attack the dCpdY bond, thus producing no free cytidylic acid except in those cases where the DNA chains have cytidylic acid on the 3'-terminus. Most experiments in which calculations of the relative amounts of each mononucleotide were done showed that the purines were produced in about equal amounts; very often the amount of dGMP was slightly greater than that of dAMP. This result seems to be in contrast to those found with the nuclease:3'-nucleotidases of wheat (19) and mung bean (16) where dAMP was always found to be the mononucleotide in excess. The determination of average chain length of the fractions eluted from DEAE-Sephadex A-25, immediately after the mononucleotides together with the A_{260} values for these fractions indicates that roughly equal quantities of di-, tri-, and tetranucleotides were formed. It is difficult to say whether or not these products represent the extreme limit of the reaction in terms of size since as was previously mentioned the phenomenon of autoretardation could be operative in the latter stages of digestion.

Probably one of the most important, as well as the most discouraging results to come out of the work on the characterization of hydrolysis products of dDNA was the finding that approximately 7-8 percent of the dinucleotide,

and 8-9 percent of the trinucleotide fractions appeared to lack terminal phosphate groups. The most plausible reason for this result would be that the enzyme preparation must be contaminated with a slight amount of nonspecific phosphatase capable of removing phosphates from either 5'-phosphoryl mononucleotides or oligonucleotides. These dephosphorylations would only become evident when the products of hydrolysis (5'-phosphoryl terminated mono- and oligonucleotides) were incubated with the enzyme preparation for relatively long periods of time. Reactions in which the substrate was hydrolyzed rather rapidly, and in which only small amounts of enzyme were required, were found to contain no detectable dephosphorylated products (see Determination of Phosphate Position in Oligonucleotides Obtained from a Hydrolyzate of Polyadenylic Acid). The suspicion concerning nonspecific phosphatase contamination in the P-100 enzyme preparation was confirmed with assays on slices from a polyacrylamide gel electrophoretic separation of the muskmelon nuclease preparation. With p-nitrophenyl phosphate as substrate, two or possibly three regions of phosphatase activity were detected, regions not associated with the nuclease: 3'-nucleotidase band. The major amount of p-nitrophenyl phosphate phosphatase, however, was observed in the nuclease: 3'-nucleotidase band.

In the course of elucidating the specificity of the muskmelon nuclease it was found that the enzyme was capable of hydrolyzing the phosphodiester bonds of the polynucleotide

poly-2'-O-methyladenylic acid with the formation of 5'-phosphoryl terminated mono- and oligonucleotides ranging up to the heptanucleotide in length. This enzyme preparation is the first one to the author's knowledge capable of degrading this polynucleotide with the formation of 5'-phosphoryl terminated oligonucleotides. A report in the literature (53) concerning a specific RNase capable of degrading only 2'-O-methylated polynucleotides has since been retracted by the senior author[17]. The potential usefulness of the muskmelon nuclease in this latter case was complicated by the contaminating phosphatase activity in the preparation. Since the polymer was degraded much more slowly than was normal poly A, an incubation period amounting to 68 hours was needed. As is indicated in Table XIII, these extreme conditions produced dephosphorylated oligonucleotides comprising over 50 percent of the total oligonucleotide fraction. This fact necessarily complicated the isolation of the desired 5'-phosphoryl terminated tri- and tetranucleotides. A report of the use of these isolated tri- and tetranucleotides is presently being published (48).

SUMMARY

A nuclease from the seeds of muskmelon has been purified about 2900-fold with recoveries of DNase activities ranging between 10 and 15 percent of that measured in crude extracts.

The purified preparation hydrolyzes native DNA, denatured DNA, RNA, and the 3'-phosphoryl bond of 3'-AMP, 3'-GMP, and 3'-UMP but not of 3'-CMP.

Included in the purification procedure is an ammonium sulfate fractionation, column chromatography on DEAE- and CM-cellulose and gel filtration on a combination column of Bio-Gel P-100 and DEAE-cellulose.

The DNase activity on denatured DNA exhibits two pH optima, one at 5.7 (DNase A) and the other at 8.0 (DNase B). RNase activity is optimal at pH 5.7, and 3'-AMPase exhibits optimal activity at pH 8.0.

A close association of all four activities: DNase A, DNase B, RNase, and 3'-AMPase has been observed to exist through a variety of treatments. The ratios of DNase A, RNase and 3'-AMPase to DNase B do not vary significantly in the last three steps of the purification procedure. No separation of the activities is effected by either isoelectric focusing or polyacrylamide disc gel electrophoresis at pH 9.5. At this stage of purification the enzyme preparation exhibits 10 protein bands on polyacrylamide disc gel electrophoresis.

The slowest migrating band contains all four activities.

The four activities migrate together through Sephadex G-100 like a protein species having a molecular weight of about 50,000.

Cations affect the nuclease and 3'-nucleotidase activities in a complex manner. No cation affects all four activities to the same degree. Cations of the alkaline earth series cause 7-20 percent increases in DNase B and RNase activities. The monovalent cations, sodium, lithium, and potassium bring about similar increases in RNase and 3'-AMPase but are without much effect on DNase A or B. All other divalent cations cause varying degrees of inhibition of all four activities. The relative magnitude of inhibition caused by these divalent cations seems to be similar for the DNase B and RNase activities.

At pH 8.0 the order of reactivity of the nucleoside-3'-monophosphates is 3'-AMP > 3'-GMP > 3'-UMP. Under the conditions used in the 3'-nucleotidase assay no detectable hydrolysis of 3'-CMP occurs. Hydrolysis of three of the 2'-deoxynucleoside-3'-monophosphates occurs but at a significantly slower rate. 2'-Deoxycytidine-3'-monophosphate is not dephosphorylated.

When reacted with cyclic phosphodiester derivatives of the mononucleotides the nuclease preparation is unable to catalyze any significant hydrolysis at either pH 5.7 or pH 8.0 of various cyclic phosphodiester derivatives of the

mononucleotides, namely the 3',5'- and 2',3'-cyclic mononucleotides.

RNA is hydrolyzed by the nuclease about twice as rapidly as denatured DNA at pH 5.7. The relative rates of hydrolysis of denatured DNA at pH 5.7 and 8.0 are nearly equal up to about 60 percent conversion of the substrate to lanthanum nitrate-HCl soluble products. At this point the rate at pH 8.0 drops below that measured at pH 5.7.

The rate of hydrolysis of native DNA at pH 8.0 appears to be less than 10 percent of that of denatured DNA. The ratio of these rates at pH 5.7, however, increases quite significantly to about 20 percent.

Muskmelon nuclease appears to act endonucleolytically on RNA at pH 5.7, on denatured DNA at both pH 5.7 and 8.0, and on native DNA at both pH 5.7 and 8.0.

At their pH optima, all three nuclease activities form 5'-phosphoryl terminated products.

At pH 8.0 the nuclease preparation hydrolyzes polyadenylic acid and polyuridylic acid but not polycytidylic acid. There is strong evidence to indicate that at pH 8.0 polyuridylic-cytidylic acid (poly U,C) is hydrolyzed to a mixture of products containing UMP and oligonucleotides of the following general structure: $(pC)_n pU$ where n is any number equal to or greater than 1.0.

The partial characterization of the products of hydrolysis produced from denatured DNA by the muskmelon nuclease at pH 8.0 has indicated that about 20 percent of

the substrate is converted to mononucleotides, 30 percent to dinucleotides, and the remainder to equal amounts of tri- and tetranucleotides. The mononucleotide fraction lacks dCMP. The other three mononucleotides occur in the following percentages (of total mononucleotides isolated): TMP, 40.1; dGMP, 31.4; and dAMP, 28.5.

The enzyme preparation is suspected of being contaminated with nonspecific phosphomonoesterase activity because about 7-8 percent of the dinucleotides and 8-9 percent of the trinucleotides isolated from an extensively hydrolyzed sample of denatured DNA appear to lack terminal phosphate groups. This same enzyme preparation, however, appears to be free of nonspecific phosphodiesterase activities.

The muskmelon nuclease preparation is capable of hydrolyzing poly-2'-O-methyladenylic acid to 5'-phosphoryl terminated mono- and oligonucleotides.

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APPENDIX

Definition of Terms

A_{260} unit: A solution with an A_{260} of 1.0 (1.0 cm path length) is defined to contain 1.0 A_{260} unit per ml.

dDNA: Denatured deoxyribonucleic acid

nDNA: Native deoxyribonucleic acid

poly A^m: poly-2'-O-methyladenylic acid

poly U,C: polyuridylic-cytidylic acid

cold: 0-4° centigrade

room temperature: 23° centigrade

Commercial Locations

Allied Chemical Company: Morristown, New Jersey, U.S.A.
Baker Chemical Company: Phillipsburg, New Jersey, U.S.A.
Bio-Rad Laboratories: Richmond, California, 94804, U.S.A.
Brinkman Instruments, Inc.: Westbury, New York, 11590, U.S.A.
Calbiochem: Los Angeles, California, 90054, U.S.A.
Canalco: Rockville, Maryland, 20852, U.S.A.
Fisher Scientific Company: Fair Lawn, New Jersey, U.S.A.
Mallinckrodt, St. Louis, Mo., U.S.A.
Pharmacia Fine Chemicals, Inc.: Uppsala, Sweden
P-L Biochemicals, Inc.: Milwaukee, Wis. 53205, U.S.A.
Sargent and Company: Detroit, Michigan, U.S.A.
Schwarz Bioresearch, Inc.: Orangeburg, New York, 10962, U.S.A.
Sigma Chemical Company: St. Louis, Mo., 63178, U.S.A.
Worthington Biochemical Corporation: Freehold, New Jersey, 07728, U.S.A.