PURIFICATION AND PROPERTIES OF AN EXONUCLEASE (PHOSPHODIESTERASE I) FROM CUCUMIS MELO

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PURIFICATION AND PROPERTIES OF AN EXONUCLEASE (PHOSPHODIESTERASE I) FROM CUCUMIS MELO

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Major professor

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ABSTRACT

PURIFICATION AND PROPERTIES OF AN
EXONUCLEASE (PHOSPHODIESTERASE I) FROM CUCUMIS MELO

By

Richard E. Jagger, Jr.

An exonuclease was purified from Cucumis melo seeds to an extent of 3,400-fold with a recovery of about 14 percent of the total exonuclease activity present in the crude extract. The enzyme displays many of the properties typical of phosphodiesterase I activity. No contamination by nonspecific phosphodiesterase, endonuclease, phosphatase, nucleotidase, adenylic acid deaminase or adenosine deaminase was detected.

The enzyme was prepared by an expeditious procedure involving a heat step with a accompanying change in pH, followed sequentially by acetone fractionation, ammonium sulfate fractionation, Sephadex G-100 gel filtration and phosphocellulose chromatography.

The enzyme has a pH optimum of pH 9.3, with the activity decreasing quite sharply on either side of this pH. The stability of C. melo exonuclease was dependent both on temperature and on pH, optimal conditions being 4°C and pH 7.5.

The enzyme activity was stimulated by Mg^{++}, Ca^{++} and Ba^{++} and was destroyed or reduced in the presence of sulfhydryl compounds, fluoride ion, and EDTA.

Hydrolysis of denatured DNA and ribosomal RNA was shown to be exonucleotylic in nature. Using gel filtration techniques, the molecular weight of the enzyme was determined to be about 78,000 daltons.

An activation energy of 2,800 cal per mole was obtained for the hydrolysis of p-nitrophenyl-pT. The enzyme hydrolyzed the p-nitrophenyl esters of 5'-nucleotides, with p-nitrophenyl-pdG being hydrolyzed faster than p-nitrophenyl-pT or p-nitrophenyl-pdC, and with the hydrolysis of p-nitrophenyl-pdA being the slowest. The 5'-deoxyribotide esters were hydrolyzed more rapidly than the ribotide analogues.

Denatured DNA and ribosomal RNA were hydrolyzed much more slowly than were the p-nitrophenyl nucleotides. Hydrolysis of native DNA by the enzyme preparation could not be detected. The ribotide homopolymers of polynucleotides were hydrolyzed at a slow rate by the exonuclease. A strong inhibition by 5'-AMP of the hydrolysis of p-nitrophenyl-pT and p-nitrophenyl-pdG was observed and determined to be competitive in nature.

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Richard E. Jagger, Jr.

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INTRODUCTION

In 1903, Araki observed that a substance extracted from animal tissues caused changes in isolated nucleic acid (1). In the subsequent sixty-eight years, a wealth of information has been obtained about the detection of, the isolation of, and the nature of the substances which catalyze the chemical hydrolysis of nucleic acids. Enough material has been published to warrant at least one review article per year for the last few years dealing in part or entirely with the enzymes which degrade DNA and/or RNA. These enzymes have been called nucleases, polynucleotidases, RNases and DNases, to name a few. With the mountain of reports describing an enlarging number of enzymes of this nature, a meaningful classification of such enzymes was needed. Laskowski (2) has proposed nine criteria for use in classifying DNases, and Bernard (3) has modified it for classifying RNases. With these two reviews some order to the classification of degradative enzymes of nucleic acid has been achieved.

Among the enzymes considered in the reviews is a class of enzymes commonly known as phosphodiesterases. In a general sense phosphodiesterases are enzymes which hydrolyze the bond between a phosphoryl function and one of two ligands attached to it, the latter possessing an alcoholic function through which the phosphate ester bond is formed. It is usually, but not always, assumed that one of the two ligands to the phosphoryl group is a nucleoside.

Hydrolysis of both the inter-ribonucleotide and/or the

inter-deoxyribonucleotide linkages with the stepwise formation of 5'- or 3'-nucleotides is characteristic of a class of phosphodiesterases called exonucleases.

Phosphodiesterase however is still generally used interchangeably with exonuclease, presenting a small state of confusion. In this thesis the term phosphodiesterase is assumed to be completely analagous to the term exonuclease.

Phosphodiesterases have been found to be ubiquitous in nature, having been detected and isolated from E. coli (4, 5), bacteriophage λ (6), Erlich Ascites Tumor cells (7), snake venom (8), salmon testis (9), mammalian organs (10-13), and higher plants (14-16) for examples. Exonuclease I, II, and III from E. coli and an DNA exonuclease from λ degrade only DNA while other exonculeases from E. coli and from Erlich Ascites Tumor cells degrade only RNA. The phosphodiesterases (exonucleases) from other sources tend to be nonspecific toward the sugar moiety, and have been defined by Razzell (17) as phosphodiesterase I and phosphodiesterase II by a criteron based on the specificity of hydrolysis.

Phosphodiesterase I, of which venom phosphodiesterase (8) is an example, exhibits an absolute specificity for a nucleoside with a 5'-phosphoryl residue and a 3'-hydroxyl function. This enzyme liberates nucleoside-5'-phosphates from a number of substrates including DNA, RNA, coenzymes and a number of synthetic substrates, i.e. p-nitrophenyl nucleoside-5'-phosphates. The hydrolysis of these synthetic substrates demonstrates unequivocally the presence of phosphodiesterase I activity in the presence of other polynucleotidases (17).

The complementary exonuclease to phosphodiesterase I is

phosphodiesterase II. For enzyme activity the substrate must have a nucleoside 3'-phosphoryl residue and a free 5'-hydroxyl function. The typical synthetic substrate for this type of activity is either Tp-p-nitrophenyl* (18) or 2,4-dinitrophenyl-thymidine-3'-phosphate (9). The former has been used to demonstrate the unequivocal presence of this type of activity (17). Phosphodiesterase II hydrolyzes the Tp-p-nitrophenyl substrates to nitrophenol and 3'-TMP.

Phosphodiesterase II activity was originally found in extracts of calf spleen (19); the enzyme from this source has been purified and some of its properties have been reported (18, 20, 23, 24). The enzyme has also been found in animal tissues (10), salmon testis (9), and extracts from Lactobacillus acidophilus (21, 22). The pH optimum ranges from pH 5.5 to 7.8, and the enzyme activity is not inhibited by the presence of EDTA. The moiety attached through the 3'-phosphoryl linkage has considerable influence on the rates of hydrolysis. The rate of hydrolysis by enzymes from L. acidophilus and salmon testis is markedly lower when a nucleotide or oligonucleotide replaces the p-nitrophenyl group, as in TpTp, DNA or RNA. The effect is reversed for the spleen enzyme; that is, the rate of hydrolysis is greater for dinucleotides or RNA than that observed for Tp-p-nitrophenyl. The activity for both spleen and salmon is higher using Tp-2,4-dinitrophenyl than the p-nitrophenyl ester. The hydrolysis of oligonucleotides by Phosphodiesterase

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*The abbreviations used in this manuscript are those adopted by the Journal of Biological Chemistry. Other abbreviations are Tp-p-nitrophenyl, p-nitrophenyl thymidine-3'-phosphate; p-nitrophenyl-pT, p-nitrophenyl thymidine-5'-phosphate, p-nitrophenyl-pdC; p-nitrophenyl deoxycytidine-5'-phosphate; p-nitrophenyl-pdG, p-nitrophenyl deoxyguanosine-5'-phosphate; p-nitrophenyl-pdA; p-nitrophenyl deoxyadenosine-5'-phosphate; p-nitrophenyl-pA, p-nitrophenyl adenosine-5'-phosphate; p-nitrophenyl-pU, p-nitrophenyl uridine-5'-phosphate; m units, milliunits.

II begins at the 5'-terminus, sequentially liberating nucleoside-3'-phosphates (17). Little if any base preference is indicated. There exists, however, an unusual property of phosphodiesterase II worth mentioning at this point. With the three enzymes which have been purified, all possess nucleotide transesterification activity. The enzymes are able to produce significant amounts of longer oligonucleotides from TpT. The homogeneity of any of the three phosphodiesterase II preparations has not been established. Many of the properties of the enzyme activity are being investigated as new enzyme sources are being detected and the activities subsequently purified. Several reviews on phosphodiesterases describe some additional general properties (17, 20, 23-25).

Phosphodiesterase I, the most extensively investigated class of phosphodiesterases (exonucleases), was first reported by Uzawa in 1932, (26) as an activity contained in snake venom. The work produced in the following 28 years was principally concerned with the purification and properties of the activity from snake venom (8, 27-32) and from intestinal mucosa (19, 25). Since 1960, phosphodiesterase I was examined in numerous plant and animal sources including hog kidney and liver (33, 34), animal and human tissues (10), rat liver (12), rat intestinal mucosa (13, 35), peas, corn and potato (34, 36), malt (14), carrot (15, 37), and Avena leaf tissue (16). The venom of various snakes has been the principal source of the enzyme for biochemical investigations. Numerous approaches to purification have been attempted (38-46).

The reaction catalyzed by the exonuclease proceeds with the liberation of nucleoside-5'-monophosphates from substrates of the form

Xp-nucleoside. When X is the p-nitrophenyl group as in the case of p-nitrophenyl-pT, the hydrolysis yields p-nitrophenol and 5'-TMP. The enzyme will also hydrolyze bis-p-nitrophenyl-phosphate; so a nucleoside is not essential as part of the substrate. Although venom exonuclease possesses a pH optimum of pH 8.9 to 9.5, it has been shown to hydrolyze normally resistant XpXp type substrates at low pH, pH 5, the resistance of the 3'-phosphoryl moiety being lowered by the reduction of the negative charge at the 3'-phosphoryl groups (47, 48). The phosphodiesterase activity is enhanced by the presence of divalent metal ions, especially Mg^{++}, and is inhibited by metal chelators i.e. EDTA and also sulfhydryl compounds. Other general properties of the enzyme are discussed in several review articles (17, 20, 23, 49).

Previous results of an investigation by the author on phosphodiesterases (exonucleases) (50) showed that phosphodiesterase I activity with a pH optimum near pH 8.9 and a nonspecific phosphodiesterase activity with a pH optimum near pH 5.0 were present in extracts of all animal and plant sources tested. A preference in the rate of hydrolysis of p-nitrophenyl derivatives of pyrimidine nucleoside-5'-phosphates over those of purine nucleoside-5'-phosphates was shown by all tissue extracts. Two members of the family Cucurbitaceae, muskmelon and cucumber, showed an unusual activity based on the pyrimidine/purine ratios. The ratio is set up as the rate of hydrolysis of p-nitrophenyl-pdT + p-nitrophenyl-pdC/p-nitrophenyl-pdG + p-nitrophenyl-pdA. The pyrimidine/purine ratios were 2-fold higher than that of the average value of the plants tested. Because of this unusual activity and also because Cucumis melo served as a source for another nuclease in this laboratory, an investigation was undertaken to purify this enzyme activity and to examine its properties and specificity more closely.

MATERIALS AND METHODS

Enzyme Source

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

Enzyme Substrates

The p-nitrophenyl-thymidine-5'-phosphate and the nitrophenyl-thymidine-3'-phosphate used to compare hydrolysis rates were products of Raylo Chemical Company, Edmonton, Alberta, Canada. The p-nitrophenyl-thymidine-5'-phosphate used in the enzyme purification assays as well as sodium-p-nitrophenyl-phosphate, bis-p-nitrophenyl-phosphate, DNA from calf thymus (Type I), mononucleotides, and oligouridylic acid were products of Sigma Chemical Company, St. Louis, Missouri, The poly-nucleotides, Poly A, Poly U, Poly G and Poly C were obtained from Schwarz-Mann Bioresearch, Inc., Orangeburg, New York.

p-Nitrophenyl-cytidine-5'-phosphate, p-nitrophenyl deoxyadenosine-5'-phosphate, p-nitrophenyl-deoxyguanosine-5'-phosphate, p-nitrophenyl-adenosine-5'-phosphate and p-nitrophenyl-uridine-5'-phosphate were synthesized in this laboratory using the method of Borden and Smith (51).

"Crude" rRNA, a gift from Dr. R. C. Slabaugh, was prepared from rabbit recticulocyte ribosomes by the following procedures. Ribosome suspensions were treated with 6 M LiCl and 4 M urea in 0.05 M sodium acetate pH 5.6 and 0.05 M 2-mercaptoethanol. This mixture precipitated rRNA after a 16 hour 4°C incubation (52). The "crude" 10,000 x g rRNA

precipitate from the above incubation was dissolved in 0.01 M Tris Cl, pH 7.5. One-tenth volume of 20% potassium acetate pH 5.0 was then added followed by 2 volumes of absolute ethanol. After letting the mixture stand overnight at -30°C, the precipitate was collected by centrifugation. The precipitate was dissolved in water and lyophilized to a powder. The lyophilized powder was designated rabbit reticulocyte rRNA.

Enzymes

Alkaline phosphatase EC 3.1.3.1 (*E. coli*, BAPC), snake venom phosphodiesterase EC 3.1.4.1 (*Crotalus adamanteus*, VPH) and micrococcal nuclease EC 3.1.4.7 (*Staphoccus aureus*, NFCP) were obtained from Worthington Biochemical Corporation, Freehold, New Jersey.

Resins

Sephadex G-100 (40-120 μ) was purchased from Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey, and was prepared according to the instructions of the manufacturer. Cellulose phosphate cation exchange resin, 0.8 meq per gram, was obtained from Sigma Chemical Company and prepared in the following manner. The resin was suspended in water and the fines were removed. The process was repeated three times. The resin was then washed alternatively with 0.3 M KOH and water until both washes were colorless. The resin was then washed extensively with water and finally stored in water with .0017% Zepharin HCl as a bacteriostatic agent. The resin was resuspended in 0.01 M Tris acetate and washed with the same solution before packing in the column.

Reagents

All chemicals used in the course of this work were reagent grade materials purchased from Mallinckrodt Chemical Works, Fisher Scientific Co., or J. T. Baker Chemical Co. unless otherwise specified. Tris-(hydroxymethyl)aminomethane (Trisma Base, reagent grade) cacodylic acid and , monothioglycerol (crystalline, free acid) were products of Sigma Chemical Co. β-Mercaptoethanol was a product of Eastman Organic Chemicals, Rochester, New York. Thioglycolate was from Calbiochem, Los Angeles, California. Lanthanum nitrate (C. P.) was obtained from Sargent Welch Co., Detroit, Michigan. Acrylamide, N,N,N'N'-tetramethyl-enediamine (Temed) and N,N'-methylenebisacrylamide (Bis) were obtained from Canalco, Rockville, Maryland.

Protein Determinations

Throughout the purification of the enzyme, the concentration of protein was determined by the method of Lowry *et al.* (53). Bovine serum albumin (Fraction IV) was used as a reference standard. The absorbance determinations were made on a Bausch and Lomb Spectronic-20 colorimeter at 750 nm using an infrared filter. On purified muskmelon exonuclease, because of the extremely low concentrations of protein, the tannic acid method of Mejbaum-Katzenellenbogen and Dobryszyca (54) was used for estimating the protein concentration. The absorbance was determined at 650 nm in the Spectronic-20.

Phosphate Determinations

Phosphate determinations were carried out by a modification of the method of Dreisbach (55). Sample aliquots 0.5 ml were mixed with 0.5 ml

of water-saturated phenol. The aqueous and organic phases were separated by centrifugation (1,100 x g) for 10 minutes to separate the layers. The A_{310} of the organic (upper) phase was then determined in a Beckman DB Spectrophotometer in cuvettes fitted with glass caps. From the A_{310} values, a standard curve was constructed for each assay.

Exonuclease Assays

Assay I, used primarily for determining exonuclease activities at different conditions of pH, was a procedure similar to that of Razzell and Khorana (8). The incubation mixture in a final volume of 0.3 ml contained 0.25 μmoles of p-nitrophenyl-pT, 100 μmoles of an appropriate buffer, 12 μmoles of magnesium acetate and 100 μl of enzyme previously diluted with 0.01 M Tris-acetate pH 7.5 to an appropriate activity. This mixture minus enzyme was preincubated for 5 minutes at 37°C, then the enzyme was added. Aliquots (0.05 ml) were removed at timed intervals and added to 1.0 ml of 1.0 N NaOH. The absorbance was determined at 400 nm in a Beckman DB Spectrophotometer. The molar extinction of p-nitrophenol under these conditions was determined to be 17,100.

Assay II, used for following enzyme activity during purification procedures and used for comparing the rates of hydrolysis of various p-nitrophenyl-pdX substrates, was essentially the procedure of Razzell and Khorana (18). The reaction was carried out in a 37°C constant temperature cell compartment on a recording spectrophotometer system consisting of a Hitachi Model 139 monochromator and a Gilford Model 220 absorbance indicator. The reaction mixture consisted of 0.25 μmoles of substrate (p-nitrophenyl-pT), 50 μmoles of Tris acetate pH 8.9 and 0.5 μmoles of magnesium acetate and enzyme in a 0.5 ml volume. The mixture minus

enzyme was preincubated 5 minutes at 37°C, the reaction being initiated by the addition of the enzyme solution. An increase in absorbance of 5.3 indicates the hydrolysis of 1.0 μmole/ml based on a molar extinction of 15,800 in the recording spectrophotometer at 400 nm. A unit is defined as the hydrolysis of one μmole of substrate/hour at 37°C. Specific activity is defined as units per mg of protein.

Nucleotidase Assay

The assay used to determine the release of inorganic phosphate from several isomers of adenosine monophosphate was set up as described below. The buffered nucleotide solution in a volume of 2.0 ml, formed from 1.9 ml of 0.11 M Tris acetate (either pH 7.2 or 8.9, adjusted with acetic acid) and 0.1 ml of 0.04 M nucleotide (5'-AMP, 3'-AMP or 2'-AMP), was mixed with 0.06 ml of enzyme. The final enzyme concentration was 14 units per ml. The assay mixture was incubated 60 minutes. Duplicate samples of 0.5 ml were removed at 60 minutes and assayed for inorganic phosphate by the modified method of Dreisbach. The zero time assays were handled similarly except that samples were withdrawn for phosphate determinations immediately after adding the enzyme.

Deaminase Assay

Adenosine deaminase and adenylic acid deaminase activities were measured by the increase in A_{285} nm by a procedure similar to that of Smiley, Berry and Suelter (56). Contained in 0.5 ml were 20 μmoles of sodium cacodylate buffer pH 6.8, 0.5 μmoles of substrate (5'-AMP or adenosine) and 0.5 μmoles of β-mercaptoethanol. The mixture was pre-incubated in a constant temperature cell of the recording

spectrophotometer at 37°C. After 5 minutes the reaction was initiated by the addition of 2.9 units of enzyme. The increase in absorbance at 285 nm was followed with time.

Gel Filtration

Experiments designed to establish whether the enzyme action on denatured DNA and ribosomal RNA was endo- or exonucleotic were performed by the method of Birnboim (57). The G-100 column, however, was replaced by a column (1.2 x 24 cm) of Bio-Rad P-6 polyacrylamide gel, Bio-Rad Laboratories, Richmond, California. The fractionation range is 1,000 to 6,000 daltons. The flow rate was maintained at 1 ml per minute with a peristalic pump and the elution pattern was monitored automatically with an ISCO Model UA-2 Ultraviolet Analyzer.

Isoelectric Focusing

The apparatus was an LKB Electrofocusing unit, type 8100, with a type 8101 column (110 ml volume). A density gradient of sucrose, in which Ampholine carrier ampholytes in the pH range from 3-10 were dissolved, was prepared according to the manufacturer's instructions. The sample was introduced when one-half of the gradient has been layered in the apparatus.

A potential of 300 volts was applied to the column for 36 hours at 4°C using a Krohm-Hite Model UHR-240 constant voltage power supply. The ampholyte solution was drained from the bottom of the column (the cathode) by pumping water into the top with a peristalic pump at a rate of about 2 ml per minute. One minute fractions were collected. The pH of each fraction was determined with a combination electrode on a Leeds and

Northrup pH Meter.

Polyacrylamide Gel Electrophoresis

Disc gel electrophoresis in acrylamide gels was carried out in 0.5 mm ID tubes and was essentially the procedure of Davis (58) except that the sample gel was eliminated. Samples of 0.5 ml were mixed with 0.05 ml of glycerol and placed on top of the spacer gel. A current of 5 ma per tube was applied to the gels for 3 hours at 4°C. The gels were stained via two methods. The activity stain was essentially the procedure of Sierakowska and Shugar (59) modified by Lerch (60). Gels were placed in a 10 ml solution of 0.1 M Tris buffer pH 8.9 containing 10 mg of Fast Red TR salt (Sigma) and 0.05 mg of α-naphthyl-thymidine-5'-phosphate. The enzyme activity is localized as a maroon precipitate formed by the coupling of the α-napthol released with the diazotate. The gels were also stained overnight for protein using Coomassie blue 0.3% in 10% TCA containing 30% methanol after the protein was fixed using 12.5% TCA. The gels were destained using 10% TCA containing 30% methanol and were stored in 10% TCA.

Paper Chromatography

The separation of oligonucleotides from bacterial alkaline phosphatase was carried out using descending chromatography on Whatman 3 MM in 95% ethanol, 1 M ammonium acetate, pH 7.5 (7:3, v/v). This system, solvent system I, was also used for the chromatography of the Poly U digest by muskmelon exonuclease.

EXPERIMENTAL RESULTS

Purification of the Exonuclease

Preparation of Cucumis melo Seeds

Muskmelon seeds (700-800 g) were placed in a three liter beaker and washed continuously for one-half hour with distilled water. The seeds were drained through a double layer of cheesecloth and dried in an oven for 3 hours at 35-40°C.

Preparation of Soluble Enzyme

Washed seeds (600 g) were allowed to imbibe for 12 hours in distilled water. They were subsequently drained through a double layer of cheesecloth and resuspended in 2 liters of cold (4°C) distilled water. The resuspended seeds were homogenized at 4°C in a commercial Waring Blendor (Model CB-5) for 15 seconds at 16,500 rpm followed by 45 seconds at 20,500 rpm. The crude homogenate obtained was centrifuged at 14,000 x g for 20 minutes in a Lourdes Centrifuge (Model A-2) equipped with a 1350 rotor. The supernatant liquid was then filtered through glass wool to remove unwanted suspended and floating material. The filtered supernatant liquid was designated the Step I enzyme fraction.

pH Adjustment and Heat Coagulation Precipitation

The Step I enzyme was made 0.1 M in ammonium acetate and the pH was carefully adjusted to pH 5.0 using glacial acetic acid. The solution

was heated to 60°C with occasional stirring in an 80°C hot water bath. The enzyme solution was then chilled in ice until the temperature had dropped below 20°C. Unwanted material was removed by centrifuging 20 minutes at 16,300 x g in a Sorvall RC-2B using a GSA rotor. All subsequent centrifugations were accomplished using a Sorvall refrigerated centrifuge. The supernatant liquid was again filtered through glass wool to remove floating material. This gave a clear, light yellow solution designated as the Step II enzyme fraction.

Acetone Fractionation

After chilling in a slated ice bath, Reagent grade acetone at -20°C equal to 30% of the enzyme volume was added dropwise (10 ml per minute) from a reservoir cooled with ice and dry ice via six polyethylene delivery tubes (0.085 inch I.D.) to the Step II enzyme. Upon completion of the addition of acetone, the mixture was stirred for an additional 15 minutes and then centrifuged 14,000 x g for 15 minutes. The precipitate was discarded. To the supernatant fluid acetone was again added so that the final acetone volume was equal to 60% of the starting enzyme volume. Upon completion of acetone addition, the suspension was stirred for 15 minutes. The enzyme was collected by repeated centrifugation at 4,000 x g in four 250 ml centrifuge bottles. The precipitate was taken up in 0.01 M Tris-acetate buffer, pH 8.0, containing 10^{-4} M magnesium acetate and resuspended with the use of a glass Potter-Elvejehm homogenizer. The resulting suspension was stirred 3 hours to ensure that the enzyme was dissolved completely. Centrifugation for 20 minutes at 40,000 x g (SS-34 rotor) removed the insoluble material. The clear, yellow supernatant fluid was designated as the Step III enzyme fraction.

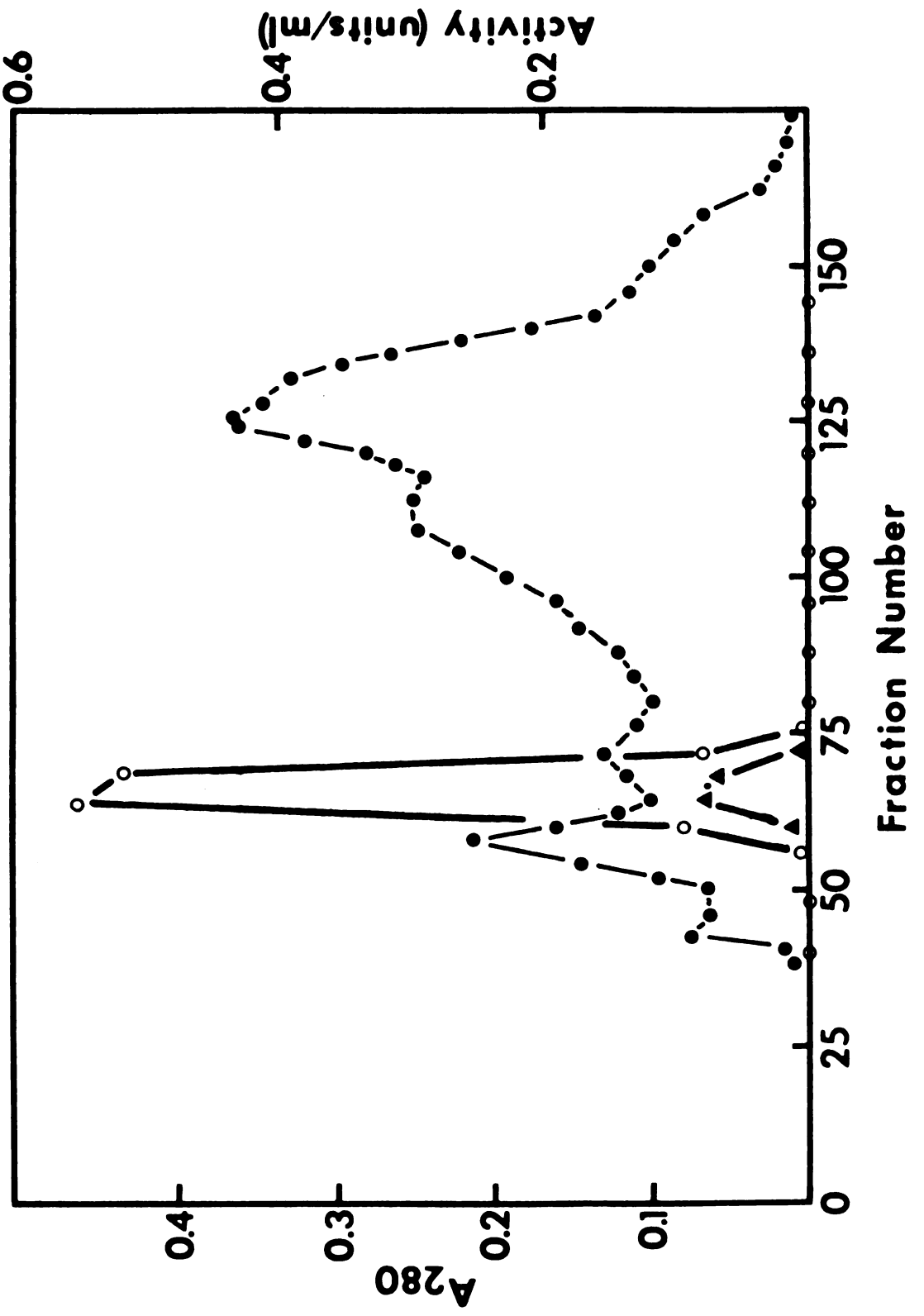
Ammonium Sulfate Fractionation

The Step III enzyme solution was adjusted to pH 5.0 with glacial acetic acid. Ammonium sulfate (.25 g/ml of Step III enzyme) was added slowly while the solution was being mechanically stirred. Additional ammonium sulfate was added until a 55% saturated solution of ammonium sulfate was obtained as determined by refractive index on a Bausch and Lomb Abbe-3L Refractometer. This procedure was necessary because of the varying amounts of salt carried over during the acetone precipitation step. After 15 minutes of stirring, the solution was centrifuged 40,000 x g for 20 minutes and the precipitate was discarded. Ammonium sulfate (.15 g per ml) was added to the supernatant as in the first precipitation. Additional ammonium sulfate was added until a 75% saturated solution of ammonium sulfate was again determined via refractive index. The suspension was centrifuged 40,000 x g for 20 minutes and the supernatant was discarded after assaying for activity. The clear, brownish tinged liquid was termed the Step IV enzyme fraction.

G-100 Sephadex Gel Filtration

The Step IV enzyme fraction was immediately layered on the top of a G-100 Sephadex column (5.0 x 93 cm) previously equilibrated with 0.01 M Tris acetate buffer, pH 7.5, containing 10^{-4} M magnesium acetate. Use of sucrose to increase the density of the sample was not required due to the amount of ammonium sulfate present in the enzyme solution. The column was eluted at room temperature overnight with the same buffer in a descending manner. Flow rate was adjusted 1 ml per minute, and 10 minute fractions were collected. Figure 1 from a typical preparation, shows the phosphodiesterase activity, nonspecific phosphodiesterase

Figure 1. Elution pattern of Sephadex G-100 Gel Filtration. To a column of G-100 Sephadex (5 x 93 cm) previously equilibrated with 0.01 M Tris acetate buffer, pH 7.5 containing 10^{-4} M magnesium acetate, a 10 ml sample containing the Step IV enzyme was applied. The column was eluted with the same buffer. Ten minute fractions of about 10 ml were collected. The fractions containing the enzyme were pooled to yield the Step V enzyme fraction. Phosphodiesterase activity, O—O; nonspecific phosphodiesterase activity ▲—▲; and absorbance 280 nm, ●—●—●.


A280
0.4
0.3
0.2
0.1
0
25
50
75
100
125
150
Fraction Number
Activity (units/ml)
0.6
0.4
0.2

activity and 280 nm absorbance profiles. The tubes which contained phosphodiesterase activity were pooled. This pooled fraction was designated as the Step V enzyme fraction.

Chromatography on Phosphocellulose

The pooled fraction from the G-100 Sephadex column was diluted 1:1 with 0.01 M Tris acetate, pH 7.6, and washed onto a 2 x 25 cm phosphocellulose column previously equilibrated at room temperature with 0.01 M Tris acetate buffer, pH 7.6. The elution rate was about 1 ml per minute and 15 minute fractions were collected. Following enzyme absorption onto the resin, the column was washed with 230 ml of the same buffer. Next a 500 ml linear gradient of 0 to 0.5 M NaCl in Tris acetate buffer was started. The elution rate was kept at about 1 ml per minute and the fraction collector was changed to collect 5 minute fractions. Typical phosphodiesterase activity and 280 nm absorbance profiles are shown in Figure 2. The fractions containing the second peak of phosphodiesterase activity were pooled and subsequently concentrated to a small volume using an Amicon Model 602 Diaflo cell with a PM-10 membrane (Amicon Corp., Lexington, Massachusetts). The concentrated fraction is the Step VI enzyme fraction. The results of a typical 600 g preparation is shown in Table I. The final specific activity was 13,080 with over a 3,000-fold enrichment. A 14.0% recovery of activity was obtained.

Figure 2. Elution pattern of the phosphocellulase ion exchange column. The phosphocellulose column (2 x 25 cm) was equilibrated with Tris acetate buffer pH 7.5. The Fraction V enzyme (120 ml) was applied and the column was washed with 230 ml of the Tris buffer. A linear gradient of 0.0 to 0.5 M NaCl was applied at a flow rate of about 1 ml per minute. Five minute fractions were collected. The total volume of the gradient was 500 ml. ●—● , absorbance at 280 nm; ○—○, exonuclease activity.

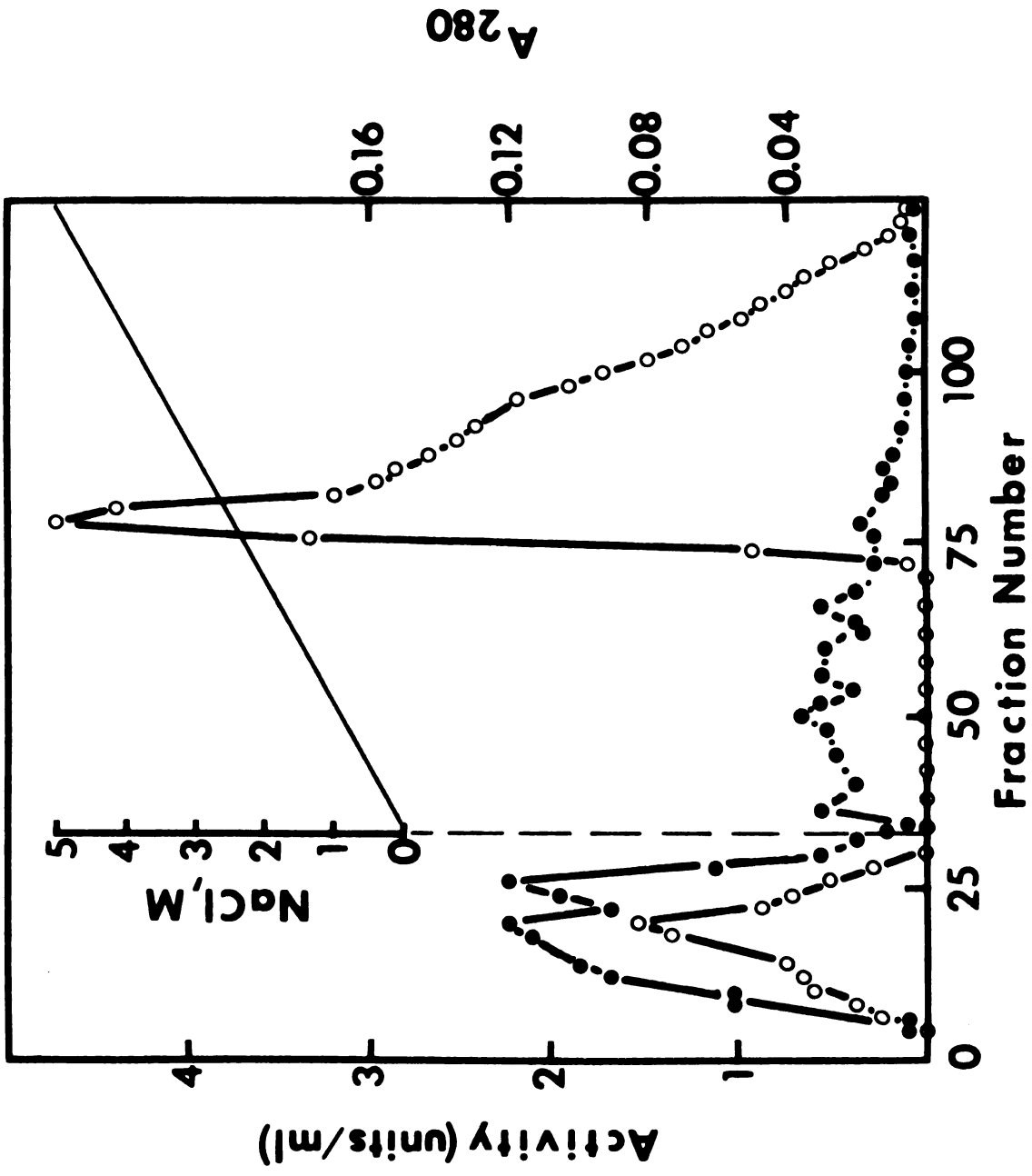

A280
0.16
0.12
0.08
0.04
NaCl,M
0
1
2
3
4
5
Activity (units/ml)
0
1
2
3
4
0
25
50
75
100
Fraction Number

TABLE I

Summary of the Purification of Exonuclease from 600 g (dry weight) of Muskmelon Seeds

Fraction	Volume	Total Activity units	% Yield	Specific Activity units/mg protein	Fold Enrichment
I. Crude	1,550	27,600	100	3.79	1
II. Heat and pH change	1,450	27,200	99	7.48	2
III. Acetone fractionation	28.5	23,900	87	25.5	6.7
IV. Ammonium sulfate	9	20,200	73	37.3	9.8
V. G-100 Sephadex	120	14,800	54	1,236	325
VI. Cellulose phosphate	15	3,920	14	13,000	3,440

Comments on the Isolation Procedure

Unlike the preparations of phosphodiesterase from animal sources, the muskmelon exonuclease was found not to be associated with particulate matter in that ultracentrifugation did not pellet the enzyme activity. Activity in the crude homogenate would not precipitate using conventional ammonium sulfate fractionation techniques, seemingly because of the large amount of lipid at this stage. In an attempt to remove the lipid, acetone fractionation was introduced. Although fractionation of the enzyme with acetone did yield a small increase in specific activity, the procedure was poorly reproducible. The use of a pH adjustment followed by a period of heating of the enzyme solution described by Harvey, Malsman and Nussbaum (15) was attempted as an alternative purification step. A 2-fold increase in specific activity was normally obtained when the pH of the crude homogenate was made 0.1 M in sodium acetate, adjusted to pH 5.0, and heated to 60°C. The ionic strength, the pH and the temperature were optimized for best recovery with the highest specific activity. Centrifugation of the solution after cooling in ice produced a clear supernatant devoid of any particulate or lipid-like material. After this step, the acetone fractionation, which followed, could be reproduced extremely well.

Knowledge of the pI obtained from isoelectric focusing and the use of the refractometer allowed the use of ammonium sulfate fractionation at this stage of purification. Prior results of the 55 to 75% ammonium sulfate cut was a type of Russian roulette, the activity never coming down in the same place twice. The readings of the refractive index demonstrated that varying amounts of salt were being carried through the acetone fractionation, and thus corrections for the amount of

ammonium sulfate to be added could be made. Another advantage of ammonium sulfate fractionation was the reduction in volume of the enzyme solution. This was essential in order to obtain a small sample for application to the G-100 column. Ammonium sulfate fraction was performed at pH 5.5 near the pI of 5.3 to ensure adequate precipitation of the enzyme.

The Sephadex G-100 column provided the greatest purification of the steps used in the purification scheme. Table I shows a 40-fold increase in specific activity of the pooled G-100 fraction over that of the ammonium sulfate step. This may be somewhat misleading since ammonium sulfate exerts an inhibitory influence on enzyme activity. At a concentration of 0.1 M $(NH_4)_2SO_4$, there is 20% inhibition of the exonuclease. Only one peak of exonuclease activity on p-nitrophenyl-pT at pH 8.9 or bis-p-nitrophenyl-phosphate at pH 8.9 was eluted from the Sephadex G-100 column. Although the other fractions were assayed with aliquots of enzyme 10-fold higher than those normally used, no other activity was detected.

In early purification schemes, the pooled G-100 peak was concentrated and applied to a column of hydroxylapatite. The activity was eluted with a sodium phosphate gradient. At best, a 2-fold increase in activity with 40% recovery was observed. Chromatography on CM Sephadex at pH 5.0 gave only a 10% increase in activity with poor yields. The enzyme was not retained by DEAE in the pH range 7.0-9.0. A 1.5-fold purification with a 30% yield occurred. Preparative gel electrophoresis using the Canalco preparative polyacrylamide unit was attempted but only a 1.1-fold purification was achieved with 36% recovery. This may have been, in fact, an observation similar to that seen with a more purified preparation when it was subjected to an electrogradient on the electrofocusing

column where there was no recovery of enzyme activity.

The procedure which was found to yield significant purification was that using a phosphocellulose column. Early in the development of the isolation procedure, phosphocellulose was examined as a possible purification step. Phosphocellulose chromatography at pH 7.5 was not used because of the considerable amount of activity which appeared in the wash fractions in pilot columns. As can be also seen in Figure 2, between 40 and 50% of the applied activity usually appeared in the wash fractions. Because of the loss of almost one-half of the activity with phosphocellulose this step was not incorporated into the purification procedure. A later re-examination of the use of phosphocellulose on the scaled up procedure was performed. Again, about half of the applied activity came out in the wash fractions, but the majority of the protein was eluted also in the wash fraction. No activity was eluted early in the sodium chloride gradient but protein concentration being determined as A_{280} returned to almost baseline values. Little if any protein was found in the fractions of the gradient when the remaining activity was finally eluted. The exonuclease activity was eluted initially as a sharp peak followed by slow decrease in activity with elution volume. This elution pattern of activity was typical with the use of phosphocellulose and it was also seen when higher salt gradients were used. Concentration of the fractions containing activity with a Diaflo ultrafiltration cell provided a rapid means of reducing the volume of the pooled sample with little loss of activity. This loss of activity from earlier experiments was shown to be about 5 to 10%.

Properties of Muskmelon Exonuclease

The Enzyme Assay

The enzymatic hydrolysis of p-nitrophenyl-pT by muskmelon exonuclease was linear over the time period used and was also proportional to enzyme concentration (Figure 3). The enzyme aliquots used in the assay system were selected such that between 0.0015 and 0.02 μmoles of p-nitrophenol were released in a 10 minute period. These values are equivalent to the hydrolysis of .6% and 8.0% of the substrate respectively in the 0.5 ml assay volume. The linearity of the assay was retained over a 10-fold change in enzyme concentration.

Effect of pH on Enzyme Activity

The pH-activity curve for muskmelon exonuclease is shown in Figure 4. The exonuclease was most active in the alkaline region, having a fairly sharp pH optimum at 9.3 in NH_4HCO_3 buffer using p-nitrophenyl-pT as substrate. Tris acetate buffer produces a slight depression of activity compared with ammonium bicarbonate buffer at the same pH. Below pH 7.5 there is no appreciable activity which can be measured in imizazole acetate or ammonium cacodylate buffer. Unless otherwise specified, assays were performed at pH 8.9 in Tris buffer for comparison with data obtained by others on alkaline exonucleases (Phosphodiesterase I).

pH Effect on the Stability of the Exonuclease

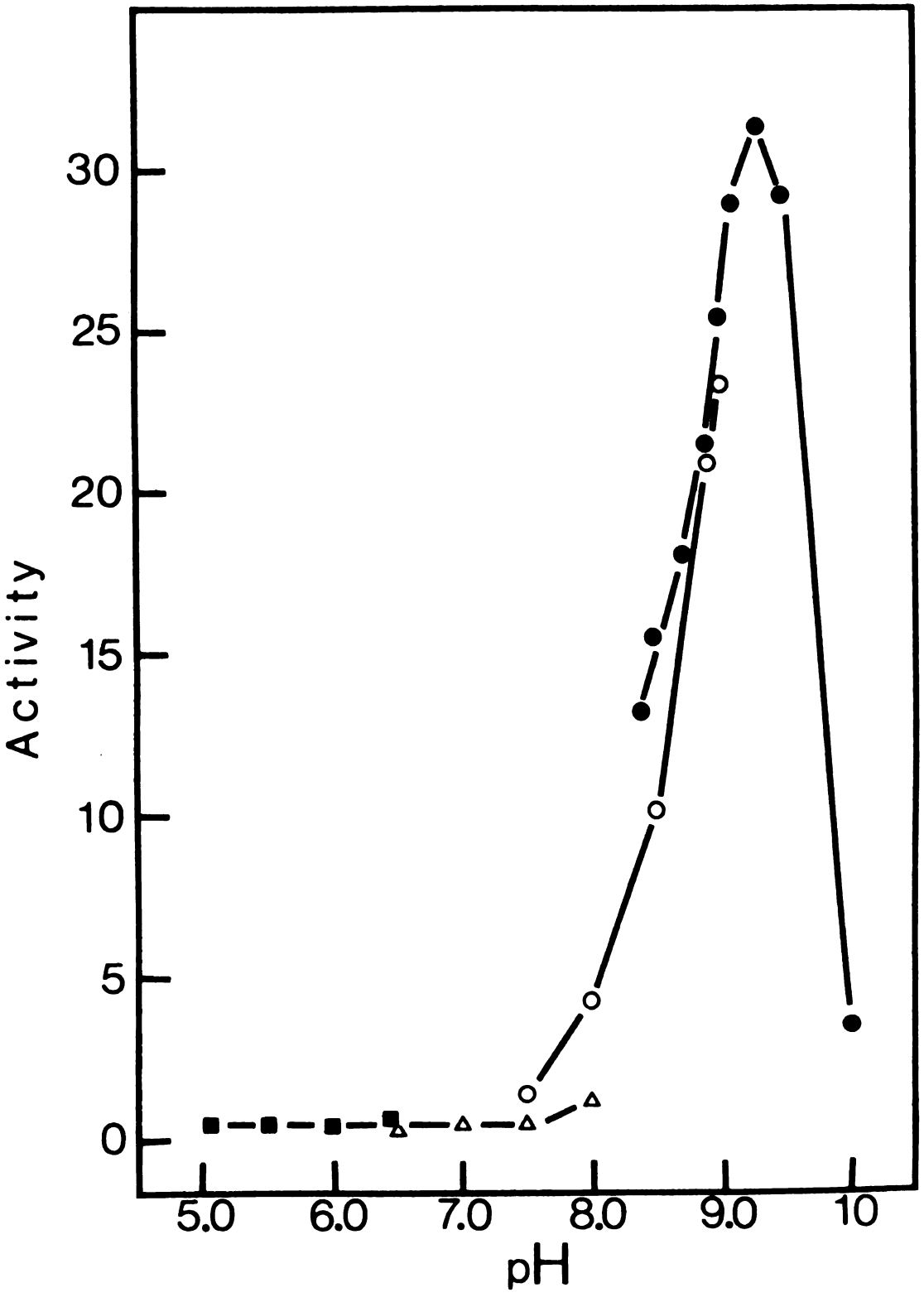
In an attempt to determine if muskmelon exonuclease would be more stable at a particular pH, assays were performed to compare enzyme stability with pH. The buffers (ammonium acetate, ammonium cacodylate, and Tris acetate) were prepared at various pH's and a concentration of

Figure 3a. p-Nitrophenyl-pT hydrolysis by C. melo exonuclease as a function of time. The reaction mixtures of 0.5 ml volumes contained 0.25 μmoles Tris acetate pH 8.9, 0.05 μmoles of magnesium acetate were mixed with 0.25 μmoles of p-nitrophenyl-pT. This mixture was preincubated 5 minutes at 37°C; the reaction was initiated by the addition of enzyme (50 milliunits). The increase in absorbance at 400 nm was followed with time.

Figure 3b. p-Nitrophenyl-pT hydrolysis by C. melo exonuclease as a function of enzyme concentration. The reaction mixtures were the same as described above. Values for initial velocities, the change in the absorbance at 400 nm in a 10-minute period, were calculated from plots similar to those in Figure 3a.

a
$\Delta A_{400\ nm}$
0.1
0.2
0.3
0.4
0
10
20
30
Time (minutes)
b
$\Delta A_{400\ nm}$ per 10 minutes
0.1
0.2
0.3
0.4
0
20
40
60
80
µl of enzyme

Figure 4. Effect of pH on exonuclease activity. All points obtained were of duplicate assays of the Step VI enzyme fraction. Assays were performed as described in Methods under Assay I. All buffers were adjusted to the desired pH at 37°C. Counter ions of the buffers used were either ammonium or acetate. Buffers: ■—■, cacodylate; △—△, imidazole; ○—○, Tris; ●—●, bicarbonate. Activity is expressed as μmoles of p-nitrophenol released per hour.


Activity
30
25
20
15
10
5
0
5.0
6.0
7.0
8.0
9.0
10
pH

0.10 M. Two and four-tenths enzyme units were diluted to 2.0 ml in ammonium acetate pH 4.0 and pH 5.0, ammonium cacodylate pH 5.0, pH 6.0, and pH 7.0, Tris acetate pH 7.0, 8.0 and 9.0, and ammonium acetate pH 9.0 and pH 10.0, and were incubated for 2, 4, 8, 16, 32 and 64 hour periods at 37°C. Enzyme activity was determined at each of the periods by assaying at pH 8.9. The results show that storage for even short periods of time at pH 4.0 (2 hours) completely destroys enzyme activity. Most buffers at the various pH values tested caused a small initial drop in activity over a 12 hour period. Yet after 12 hours, there was little if any change in activity with time for any of the buffer systems, except for ammonium acetate buffer at pH 10.0 which displayed a continuous decline of activity after an initial activation. Enzyme incubated in Tris-acetate buffer or ammonium acetate buffer at pH 9 and Tris-acetate buffer at pH 7 or 8 retained the highest levels of activity.

Isoelectric Point of Muskmelon Exonuclease

The isoelectric point of the muskmelon exonuclease was determined early in the purification procedure using the LKB 8100 electrofucusing apparatus. This information was needed to ensure more complete precipitation in the subsequent acetone and ammonium sulfate fractionation procedures. With Step II enzyme, a single zone of activity at pI = 5.3 was obtained using a pH 3 to 10 gradient. In later experiments using Step IV enzyme, no activity could be recovered from the electrofocusing column even after 260 units were initially applied.

Effect of Temperature on Muskmelon Exonuclease

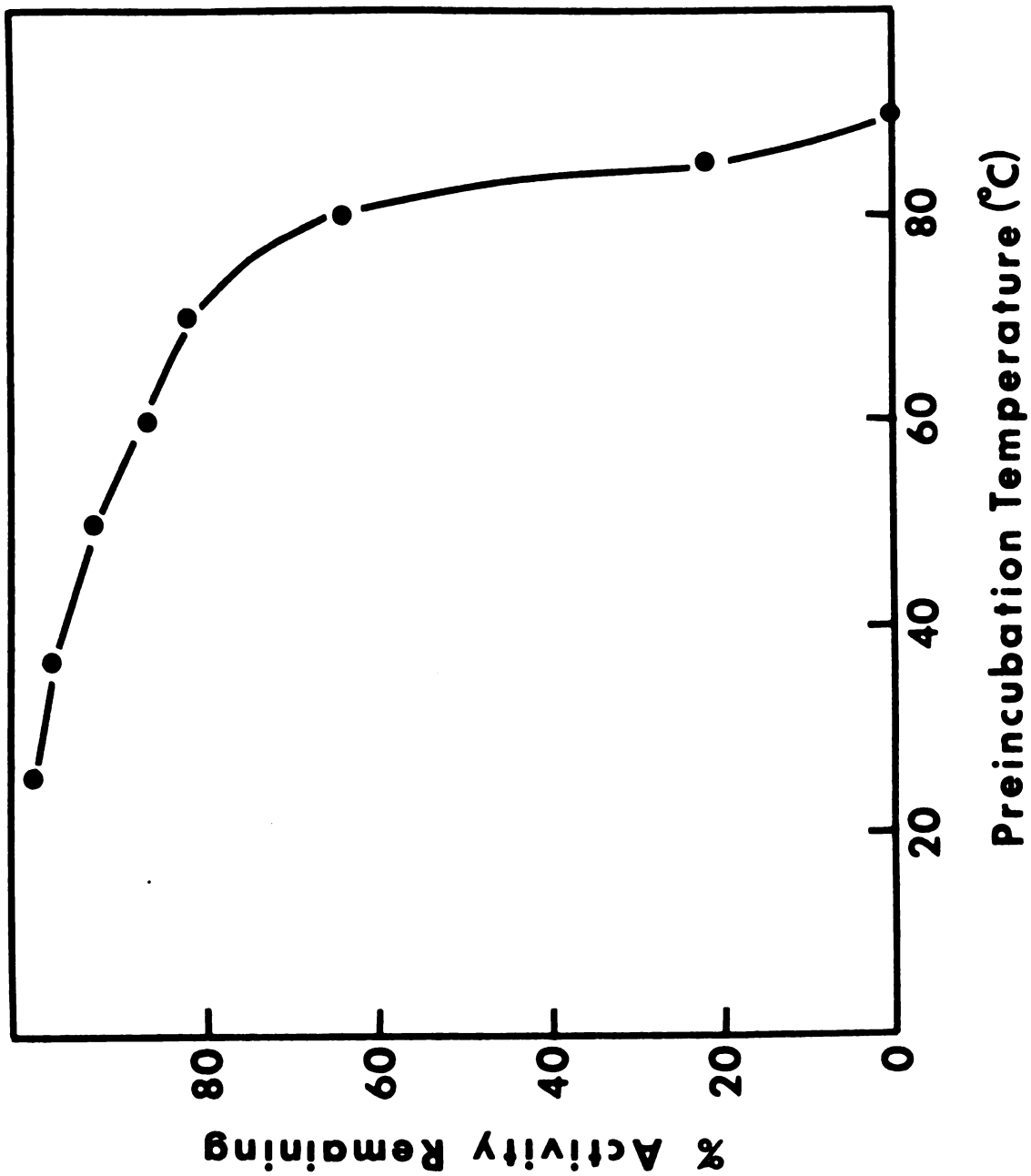
To determine the effect of temperature on exonuclease activity at different pH's, the following experiment was devised. One-hundred units of enzyme were diluted to 5.0 ml in 0.1 M Tris buffers at various pH's and heated to 80°C for 15 minutes. The pH's used were 7.5, 8.0, 8.5, and 9.0. When the samples were chilled in ice and subsequently assayed a pH dependent loss of activity was observed. At pH 7.5, no loss of activity was seen in the 15 minute incubation but at higher pH's a 4% to 17% loss was seen after the 15 minute incubation. When a 0.1 M carbonate-bicarbonate buffer was used, 12% and 34% decreases in activity were seen at pH 8.5 and 9.0. The incubations at pH 9.0 and 10.0 in carbonate-bicarbonate buffer led to 87% and 100% losses of activity respectively. Thus the enzyme appears to be much less stable at a given pH in a carbonate-bicarbonate buffer than in a Tris acetate buffer.

To investigate the effect of temperature on exonuclease activity at pH 8.9, aliquots containing 33 units of the enzyme were diluted to 10.0 ml with Tris acetate 0.1 M pH 8.9. One ml volumes were incubated for 30 minutes at various temperatures (24°, 37°, 50°, 60°, 70°, 80°, 85°, and 90°C). After cooling in ice, the various samples were assayed using assay II described in Methods and Materials. The results are shown in Figure 5. Above 70°C there was marked loss of activity after the 30 minute incubation period. At 90°C there was no detectable activity remaining.

Effect of Storage and Dialysis

Aliquots (0.1 ml) of a 20-fold diluted Step VI enzyme were placed in 0.6 ml test tubes under three conditions: refrigeration at 4°C,

Figure 5. Effect of temperature on exonuclease activity. Muskmelon exonuclease (33 units) was diluted to 10 ml with 0.10 M Tris acetate buffer pH 8.9. Aliquots of 1.0 ml each were incubated for 30 minutes at 7 different temperatures. The samples were then cooled in ice for 5 minutes after which they were assayed as in Assay II. The results are graphed as percent activity remaining based on the room temperature sample as 100%.


% Activity Remaining
0
20
40
60
80
Preincubation Temperature (°C)
20
40
60
80

frozen at -25°C and standing room temperature. The 0 time sample possessed an activity of 640 units per ml. After 10 days at these conditions, the samples were brought to room temperature and assayed using Assay II. The refrigerated sample had an activity of 540 units per ml. Both the frozen sample and the room temperature sample possessed activities of 500 units per ml. There is very little difference in the conditions of storage since the values of 540 and 500 units per ml amounted to a 16% and 22% respective loss of activity in 10 days.

Dialysis against pH 7.5 Tris buffer (0.01 M) for 48 hours produced no detectable loss of activity over the nondialyzed sample. In this experiment a lower concentration of enzyme was used. Triplicate samples (1.0 ml) containing 19.5 units of enzyme were placed in small dialysis tubing and dialyzed 48 hours. There was a decrease of 8.8% of the starting activity, but the nondialyzed sample also had a decrease in activity of 8.8%, yielding 17.8 units of enzyme. There was no decrease in activity of the dialyzed sample over the control sample.

Effect of Activators and Inhibitors on Exonuclease Activity

The effects of metals, activators, and inhibitors upon exonuclease activity is shown in Table II. All additions gave a final concentration of 10^{-3} M with the exception of ammonium sulfate whose final concentration was 0.10 M (this was also made pH 8.9 with NH_4OH before addition to prevent a pH change). Sixty-five m units (20 µl) of exonuclease were added to the assay to initiate the reaction. Among the cations tested, only Mg^{++}, Ca^{++}, and Ba^{++} evoked a stimulatory effect on activity. Co^{++}, Zn^{++}, and Hg^{++} exhibited a marked inhibitory effect displaying greater than 25% inhibition. Sr^{++}, Mn^{++}, Cd^{++}, Ni^{++}, and Cu^{++} yielded slight

TABLE II

Effect of Activators and Inhibitors on Exonuclease Activity

Reactions were carried out as described in Materials and Methods under Assay II, except that the enzyme reaction contained millimolar concentrations of the metal listed. Addition of Step VI enzyme initiated the reaction.

Addition (10^{-3} M final concentration)	% of Control
none	100
$MgCl_2$	106
$CaCl_2$	107
$BaCl_2$	109
$CoCl_2$	68
$SrCl_2$	99
$MnCl_2$	93
$ZnCl_2$	68
$CdCl_2$	81
$NiCl_2$	79
$CuCl_2$	75
$HgCl_2$	72
NaF	16
NaCl	100
$(NH_4)_2SO_4$*	79

*0.1 M final concentration.

inhibitory effects on exonuclease activity ranging from 1% to 25%.

A monovalent anion, fluoride, provided the strongest inhibition of activity, 84%, whereas chloride had no effect. Ammonium sulfate (0.1 M) displayed an 21% inhibition of activity unlike the ammonium sulfate activation seen with the malt enzyme (14).

Effect of EDTA upon Activity

Characteristically EDTA and other metal chelators are inhibitors of phosphodiesterase I activities (8, 13, 15). In order to determine whether EDTA exhibited an inhibiting influence upon muskmelon exonuclease activity, measurements of activity in the presence of EDTA were performed. In a volume of 400 µl the following were contained: 50 µmoles of Tris acetate buffer pH 8.9 EDTA of varying concentrations, and 31.9 milliunits of enzyme. This was incubated for 5 minutes at 37°C in a constant temperature compartment of the spectrophotometer. The enzyme reaction was initiated upon the addition of 0.25 µmoles of p-nitrophenyl-pT (100 µl). Activity was followed as the increase in A_{400} with time. Figure 6 shows the inhibitory effect of EDTA as a function of concentration. EDTA in extremely low concentrations (2×10^{-5} M) inhibited enzyme activity completely. Fifty percent inhibition was displayed by 7×10^{-6} M EDTA. This data suggests that EDTA binds to a metal which is essential for enzyme activity.

Reversal of EDTA Inhibition by Metals

The protocol for the experiment to examine the possible reversal of EDTA inhibition by metals is shown in Table IIIa. Based on the data

Figure 6. Inhibition of exonuclease activity by EDTA. The assays were performed as described in the text with constant amounts of substrate (p-nitrophenyl-pT) and enzyme (31.9 milliunits).

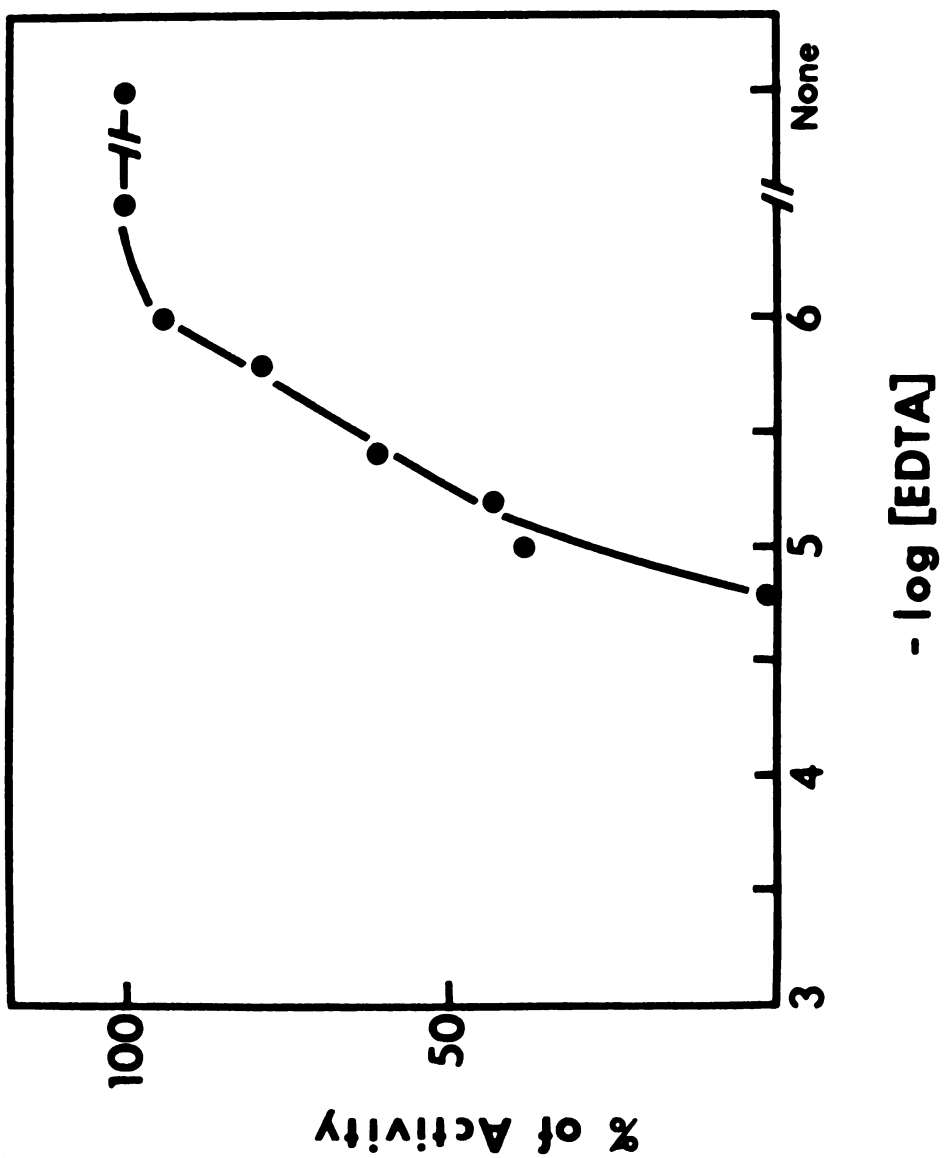

% of Activity
100
50
3
4
5
6
None
- log [EDTA]

TABLE IIIa

Protocol for Experiment Displaying Metal Ion Reversal of EDTA Inhibition

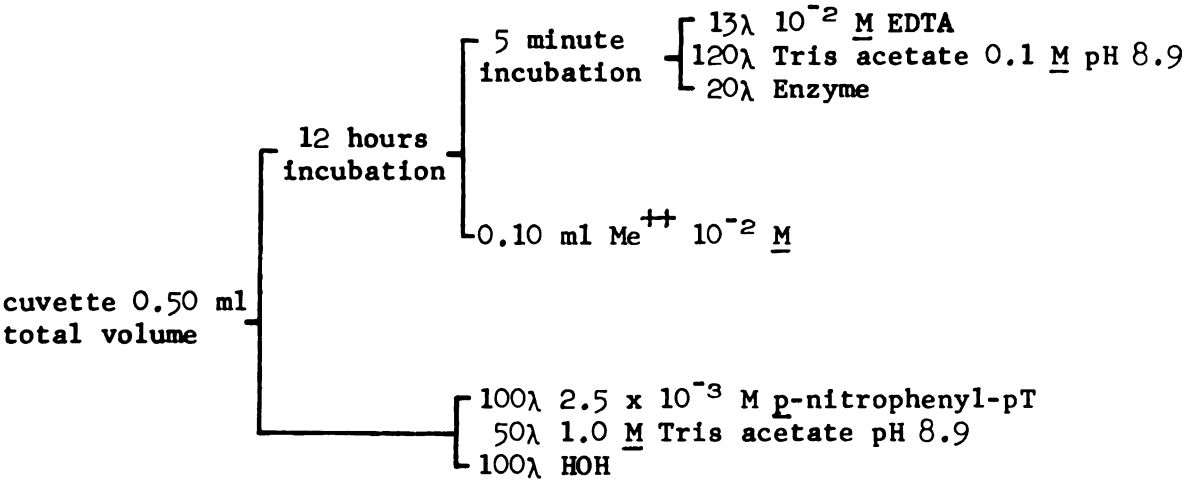


TABLE IIIb

Effect of Divalent Metal Ions in Restoration of EDTA Inhibited Exonuclease Activity[1]

Addition	m units of activity	% of Control
none[2]	0	0
$MgCl_2$	35.6	119
$NiCl_2$	0	0
$CdCl_2$	0	0
$SrCl_2$	36.6	122
$CoCl_2$	21.2	71
$MnCl_2$	1.9	6
$CuCl_2$	0	0
$BaCl_2$	2.8	9
$CaCl_2$	33.7	112
$ZnCl_2$	0	0
Control[3]	30.0	100

[1]Assays were performed as described in the text and outlined in the above protocol.
[2]No metal ion addition.
[3]Neither EDTA nor metal was added.

from the above assays, .01 μmoles of EDTA is needed to inhibit 31.9 milliunits of enzyme in a 0.5 ml assay volume. With a 6.5-fold scale-up in enzyme, a value of 0.065 μmoles was calculated to insure inhibition by EDTA. Subsequent addition of 1.00 μmoles of Me^{++} would yield an 61-fold excess of metal ions.

For each metal ion examined, 160 milliunits of enzyme was mixed with 0.065 moles of EDTA and 50 μmoles of Tris acetate, pH 8.9, in a volume of 600 μl. After incubating this solution for 5 minutes at 37°C, 0.40 ml of the metal ion, 10^{-2} M, was added. This 1.0 ml mixture was incubated for 12 hours at 37°C. The concentrations of EDTA and Me^{++} during this incubation were .065 mM and 4.0 mM, respectively. Two-hundred-fifty μl of the metal-EDTA enzyme mixture was mixed in a cuvette containing 0.25 μmoles of p-nitrophenyl-pT, 50 μmoles of Tris acetate buffer, pH 8.9, in a total volume of 0.250 ml. The concentrations of EDTA and Me^{++} in the final reaction mixture were 0.016 μM and 100 μM, respectively. The increase of absorbance at 400 nm was followed with time.

The results of the metal ion reactivation of enzyme activity is shown in Table IIIb. The enzyme control having a value of 30 milliunits was carried through the identical manipulations except neither EDTA nor metal ions were added. The case where no metal was added displayed complete inhibition. Mg^{++}, Sr^{++}, and Ca^{++} were able to restore enzyme activity to 35.6, 36.5, and 33.7 milliunits, respectively. Cobalt restored 21.2 milliunits of activity while divalent Ni^{++}, Cd^{++}, Mn^{++}, Cu^{++}, Ba^{++}, and Zn^{++} restored little or no activity.

Effect of Sulfhydryl Compounds

The effect of the sulfhydryl compounds tested upon exonuclease activity is shown in Table IV. Cysteine, thioglycolate, thioglycerol, β-mercaptoethanol, and dithiothreitol were the reagents used. The assay was assay II with the exception that various sulfhydryl reagents were added to the preincubation mixture, the addition of enzyme (.095 units) initiating the reaction. All sulfhydryl solutions which were tested inhibited enzyme activity. The inhibition displayed by these sulfhydryl compounds is reflected in the concentration of sulfhydryl needed to evoke a 50% inhibition. Fifty percent inhibition was observed at 6.8×10^{-5} M for cysteine, 4.0×10^{-4} M for dithiothreitol, 5.4×10^{-3} M for β-mercaptoethanol, 5.7×10^{-3} M for thioglycerol and 2.0×10^{-2} M for thioglycolate. Cysteine inhibited the enzyme strongly while the other compounds inhibited enzyme activity to varying degrees.

Specificity of Muskmelon Exonuclease on p-nitrophenyl Substrates

The specificity assays using p-nitrophenyl derivatives were carried out at pH 8.9 and pH 5.5. Assay II was used except thatthe reaction mixture was carried out in a final volume of 1 ml, doubling the amount of all components so that the final concentrations were the same as in Assay II. In the pH 8.9 assays for most of the substrates, the components were preincubated at 37°C for 5 minutes and the reaction was then initiatedby the addition of 72 milliunits of enzyme (0.05 ml). The assays with p-nitrophenyl-phosphate, bis-p-nitrophenyl-phosphate, and p-nitrophenyl-thymidine-3'-phosphate at this enzyme concentration showed very little hydrolysis. Accordingly experiments were performed with these substrates using 2.9 units of enzyme, a 40-fold increase. A multiplier of 0.025 was

TABLE IV

Effect of Sulfhydryl Compounds on Activity

Condition of the assays are as described in the text. Values reported are the rates of hydrolysis expressed in μmoles of p-nitrophenyl-pT hydrolyzed per hour. Where values are omitted, assays were not performed.

Addition	Activity			
	Final Molar Concentration of Sulfhydryl Compound in Assay			
	10^{-2} M	10^{-3} M	10^{-4} M	10^{-5} M
Cysteine	--	.000 (00)*	.021 (22)	.095 (100)
Thioglycolate	.089 (94)	.093 (98)	.095 (100)	--
Thioglycerol	.019 (20)	.089 (94)	.091 (96)	--
β-Mercaptoethanol	.018 (19)	.091 (96)	.095 (100)	.095 (100)
Dithiothreitol	--	.026 (27)	.084 (88)	.095 (100)

*The second value given is the percent of the control value.

used to adjust the observed rates of hydrolysis for only a rough comparison with the 72 milliunit enzyme assays. The assays at this enzyme concentration may have been outside the range where the rate of hydrolysis of the substrate is proportional to enzyme concentration.

The relative rates of hydrolysis of the p-nitrophenyl derivatives are tabulated in Table V. The rate of hydrolysis of p-nitrophenyl-pdG was the highest, 87 μmoles per hour, at pH 8.9. The rates of hydrolysis for the other deoxynucleotide derivatives, dC, dT, and dA were 76, 72, and 11 m μmoles per hour, respectively at this pH. The ribonucleotide derivatives were hydrolyzed more slowly, p-nitrophenyl-pU being hydrolyzed at a rate comparable to one-half that for p-nitrophenyl-pT and p-nitrophenyl-pA being hydrolyzed at a rate of only 7% that of p-nitrophenyl-pT. Bis-p-nitrophenyl-phosphate, Tp-p-nitrophenyl, and p-nitrophenyl-phosphate gave extremely low rates of hydrolysis. A 40-fold increase in enzyme concentration in the enzyme assay gave values of 140, .023 and 0 mμmoles of substrate hydrolyzed per hour. A 40-fold increase in the hydrolysis of p-nitrophenyl-pT would yield a value of 2880 mμmoles of substrate hydrolyzed per hour. Table V shows, in parentheses, the rates of hydrolysis of the compounds normalized to 72 milliunits of enzyme.

The pH 5.5 assays were performed to detect possible contaminating phosphodiesterase and phosphatase activities. The substrate, in an amount of 0.25 μmoles was mixed with 50 μmoles of sodium acetate buffer at pH 5.5 in a total volume of 0.5 ml. No magnesium or metal ions were added. Again 2.9 units of enzyme were added to this mixture following a 5 minute preincubation at 37°C. The reaction was terminated after 20 minutes by the addition of 0.5 ml of 1.0 M NaOH. The absorbance at 400 nm was read in a Beckman DB spectrophotometer. The pH 5.5 assays

TABLE V

Specificity of Muskmelon Exonuclease on p-Nitrophenyl-Substrates

The conditions of the assays are discussed in the text. The activity values reported are rates of hydrolysis of the p-nitrophenyl esters obtained using 72 milliunits of exonuclease. The figures in parentheses are rates of hydrolysis obtained with 2.9 units of exonuclease which have been normalized to a 72 milliunit value.

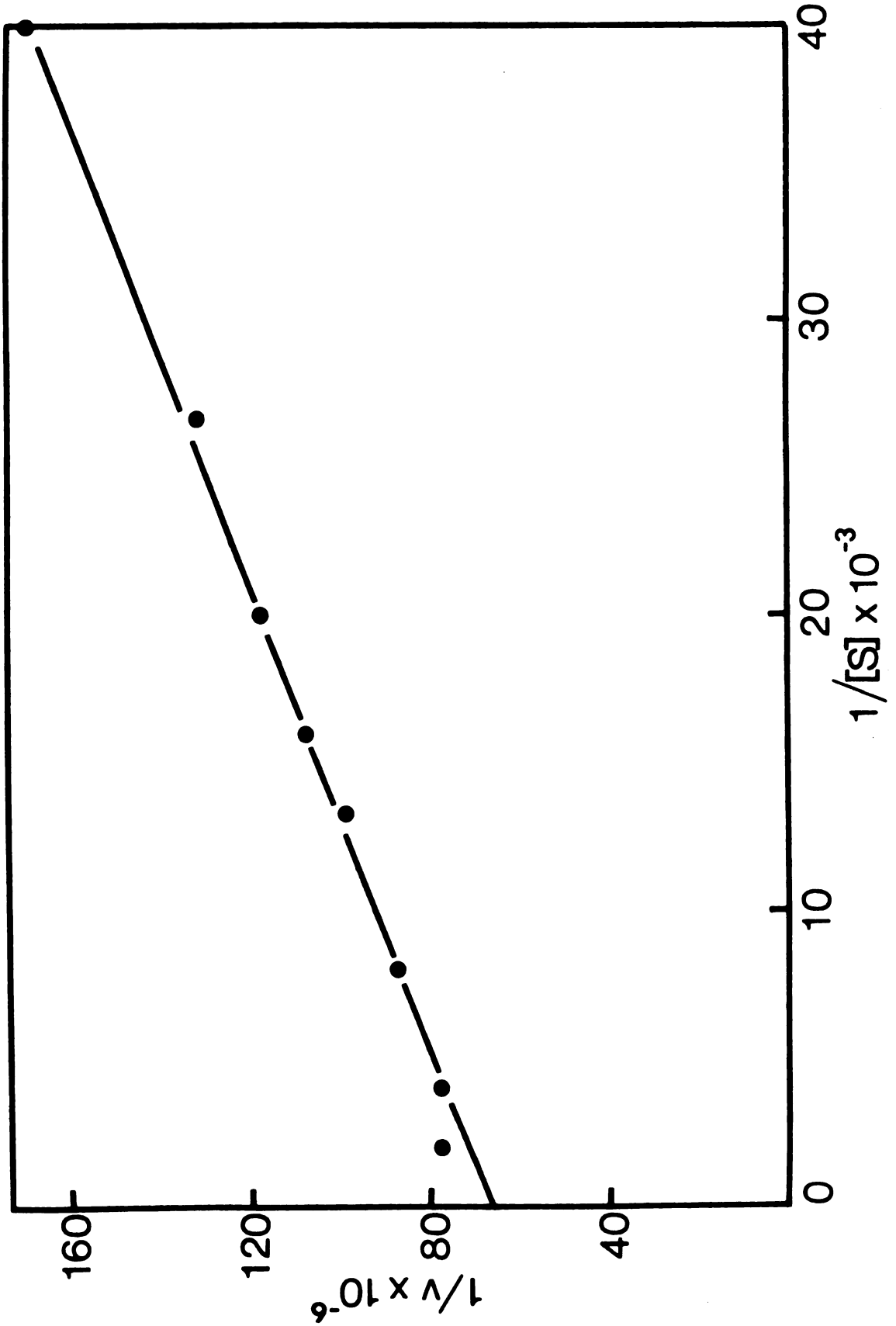
Substrate	Activity at pH 8.9	
	mμmoles per hour	% of p-nitrophenyl-pT
p-nitrophenyl-pdT	72	100
p-nitrophenyl-pdC	76	105
p-nitrophenyl-pdG	87	121
p-nitrophenyl-pdA	11	15
p-nitrophenyl-pA	5	7
p-nitrophenyl-pU	36	50
Bis-p-nitrophenyl-phosphate	3 (3.5)	4 (3)
Tp-p-nitrophenyl	0 (0.6)	0 (0.1)
p-nitrophenyl-phosphate	0 (0)	0 (0)

were fixed point assays and cannot be compared directly to the pH 8.9 assays. The 80-fold increase in enzyme concentration over the 72 milliunit/ml concentration used in the hydrolysis of p-nitrophenyl-pT at pH 8.9 was able to cause some hydrolysis of the p-nitrophenyl-phosphate, p-nitrophenyl-pT, Tp-p-nitrophenyl and bis-p-nitrophenyl-phosphate at pH 5.5. The values for the rates of hydrolysis of these esters are 0.36, 72, 1.8, and 7.8 mμmoles per hour respectively under the conditions described. It is interesting, however, that there was some hydrolysis even though the rates were low. This has been reported by other investigators for similar activities (16, 48).

Kinetic Characteristics

The assay substrate, p-nitrophenyl-pT, used in the bulk of the experiments was shown to be degraded by muskmelon exonuclease to 5'-dTMP and p-nitrophenol by use of paper chromatography system I. Experiments were performed with Step VI exonuclease to determine the kinetic parameters of the reaction. The K_m value obtained from the Lineweaver-Burk plot (61) shown in Figure 7 was found to be 3.6×10^{-5} M. The V_{max} was 14,900 μmoles per hour per mg of protein. Kinetic parameters for hydrolysis of the other deoxynucleotide-nitrophenyl substrates were also determined: p-nitrophenyl-pdG, V_{max} is 18,900 μmoles per hour per mg protein and K_m is 1.4×10^{-4} M; p-nitrophenyl-pdA, V_{max} is 8,660 μmoles per hour per mg protein and the K_m is 2.4×10^{-5} M; and p-nitrophenyl-pdC, V_{max} is 16,600 μmoles per hour per mg protein and the K_m is 3.8×10^{-5} M. These also reflect the rates of reaction seen in Table V. The values of the K_m's differ by less than a factor of 1.6 with the exception of that for p-nitrophenyl-pG which is about 4-fold higher than the

Figure 7. The relationship of substrate concentration to reaction velocity as shown by a Lineweaver-Burk plot for muskmelon exonuclease. The assay was basically enzyme assay II described in Methods except that all components were doubled (volume = 1.0 ml). The substrate concentration was varied from 0.025 mM to 0.75 mM. The mixture was preincubated at 37°C, the reaction being initiated by addition of 60 munits of Step VI enzyme. The velocity is expressed in μmoles of substrate hydrolyzed per hour per mg of enzyme.


160
120
80
40
1/v x 10⁻⁶
0
10
20
30
40
1/[S] x 10⁻³

three others.

The Effect of Nucleotides on the Rate of Hydrolysis

The effect of various nucleotides on the rate of hydrolysis of p-nitrophenyl-thymidine-5'-phosphate is summarized in Table VI. The largest degree of inhibition was displayed by adenosine-5'-phosphate. At a concentration of mM 5'-AMP, no hydrolysis of p-nitrophenyl-pT could be detected. All derivatives or analogues of 5'-AMP tested yielded a slight to a marked inhibition of activity. At millimolar concentrations, deoxyadenosine-5'-phosphate, the closest analogue of 5'-AMP, produced 98% inhibition. Other moieties attached to the basic 5'-AMP nucleotide decreased the degree of inhibition, i.e., AMP>ADP>ATP and AMP>DPN. The presence of a 2'- or 3'-phosphate attached to adenosine lowered the amount of inhibition as evidenced by the appreciable rate of hydrolysis of p-nitrophenyl-pT in the presence of 2'- or 3'-AMP. Similarly, TPN^+ caused only half the inhibition displayed by DPN^+ at millimolar concentrations. Oxidized flavin adenine dinucleotide (FAD) is an exception in that it caused very strong inhibition at 10^{-4} M, being equal to that exerted by 5'-AMP at the same concentration.

The product of the hydrolysis of p-nitrophenyl-pT, 5'-dTMP, lowered the hydrolysis rate to 34% of the control. The addition of 5'-dCMP or 5'-dGMP gave values of 39% and 47% of the control value, respectively.

When p-nitrophenyl-pdG was used as substrate, no hydrolysis of p-nitrophenyl-pdG could be detected at mM concentration of 5'-AMP. Addition of deoxynucleotides 5'-TMP, 5'-dGMP, 5'-dCMP, and 5'-dAMP at mM final concentrations gave rates of hydrolysis of 67%, 35%, 26% and 0% of the control rate, respectively. Addition of the two isomers

TABLE VI

The Effect of Nucleotides on the Rate of Hydrolysis of p-Nitrophenyl-pT

The assay was essentially that described in assay II in Materials and Methods, 100 milliunits of enzyme were added to initiate reaction. Additions of nucleotides and nucleotide derivatives were made such that a final concentration of 10^{-3} M or 10^{-4} M was obtained. Activity is expressed in μmoles of substrate hydrolyzed per hour.

Addition	Enzyme activity at two inhibitor concentrations			
	10^{-3} M		10^{-4} M	
	Activity	% of Control	Activity	% of Control
none	.090	100		
5'-dGMP	.042	47		
5'-dCMP	.035	39		
5'-dTMP	.031	34		
5'-dAMP	.002	2	.017	19
5'-AMP	.000	0	.007	7
ADP	.008	8	.038	43
ATP	.014	16	.047	53
3'-AMP	.073	81	.071	79
2'-AMP	.061	68	.066	73
DPN	.003	3	.016	18
TPN	.052	58	.071	79
FAD	-	-	.007	7

of 5'-AMP, 2'-AMP and 3'-AMP gave rates of hydrolysis equal to 71% and 82% of the control.

Type of Inhibition Displayed by 5'-AMP

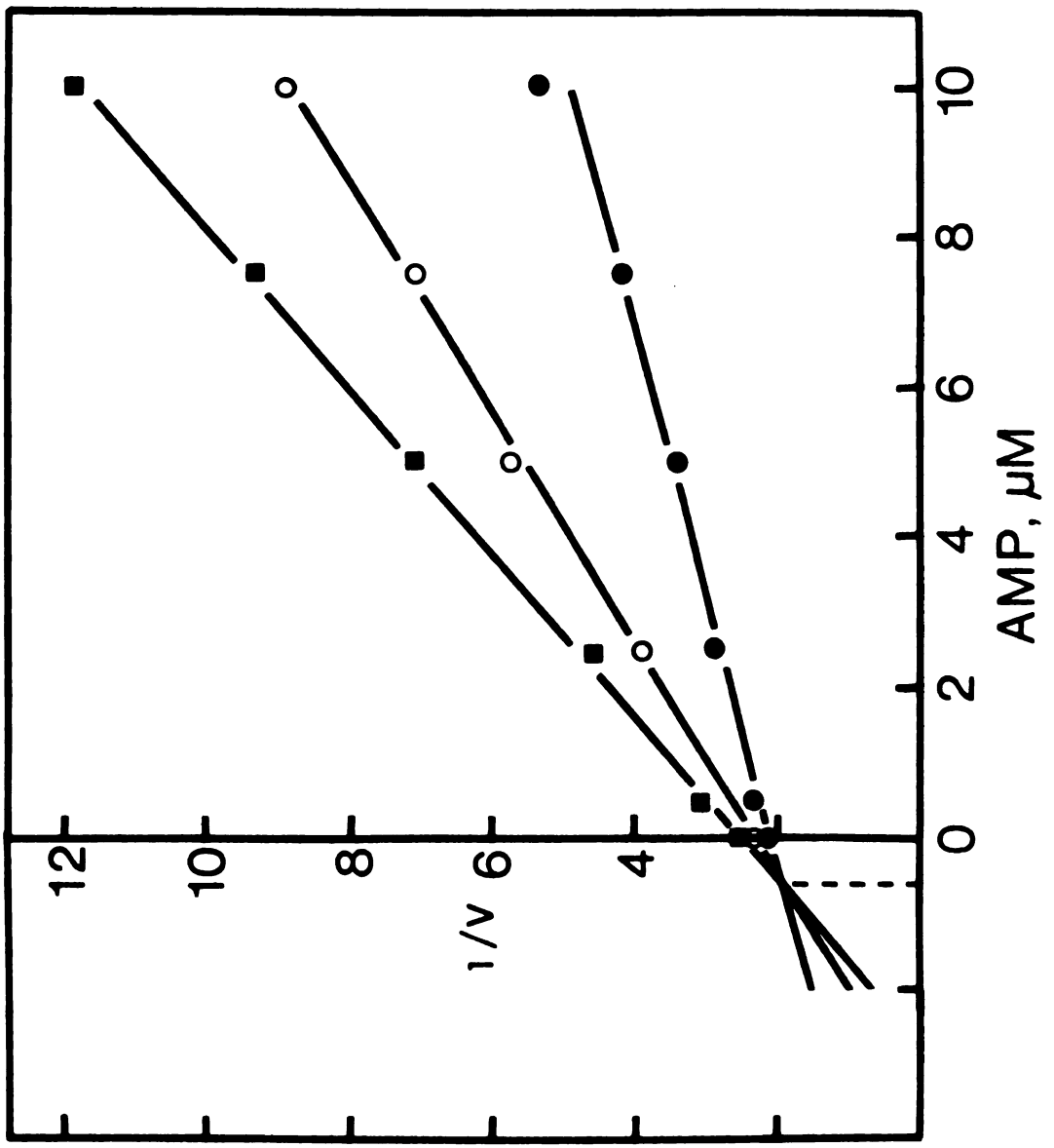
Because of the marked inhibition exerted by 5'-AMP, determination of the type of inhibition exerted became desirable.

The assay used had a final volume of 1.0 ml containing 100 μmoles of Tris acetate pH 8.9, 1.0 μmoles of magnesium acetate and substrate, p-nitrophenyl-pT, of three different concentrations: 0.5 mM, 0.25 mM, or 0.125 mM. Each mixture was incubated 5 minutes at 37°C, and the reaction was initiated by the addition of 100 milliunits of enzyme. The inhibitor (5'-AMP), when present, was at various concentrations from 0.1 mM to 7.5 μM. The data obtained was plotted as shown in Figure 8, the method described by Dixon (62). The Dixon plot indicated a clear case of competitive inhibition. The K_i for 5'-AMP using p-nitrophenyl-pT as substrate was 6×10^{-7} M.

Hydrolysis of Polynucleotides

To determine if there is a marked difference in the rates of hydrolysis of different polynucleotides, natural and synthetic polynucleotides were subjected to hydrolysis by muskmelon nuclease. To a 7.8 ml solution of each of the synthetic polynucleotide substrates (1 mg/ml), 0.78 ml of Tris acetate 1.0 M pH 8.9, 0.08 ml of 1.0 M $MgCl_2$, and 750 units (0.5 ml) of enzymes were added. At 20 minute periods 0.6 ml aliquots were removed and mixed with 0.6 ml of cold lanthanum nitrate-HCl reagent (0.02 M $La(NO_3)_3$, 0.2 M HCl). This was chilled on ice for 10 minutes and then centrifuged 20 minutes. The

Figure 8. Competitive inhibition by 5'-AMP of the hydrolysis of p-nitrophenyl-pT by the exonuclease from C. melo. The conditions of the assay are described in the text. Substrate concentrations of p-nitrophenyl-pT were ●---●, 0.5 mM; O---O, 0.25 mM; and ■---■, 0.125 mM. The enzyme used was the Step VI enzyme. Velocity is expressed as μmoles of p-nitrophenyl released per hour.


12
10
8
6
4
1/v
0
2
4
6
8
10
AMP, μM

absorbance at 400 nm of the supernatant solution was determined in a Beckman DB Spectrophotometer. The assays for the hydrolysis of DNA, denatured DNA and ribosomal RNA (rRNA) were similar except that 90 units of enzyme was used and time periods were 15 minutes. Initial velocities were calculated based on changes in A_{260} nm with time. The calculation of the millimicromoles of acid soluble nucleotide released was based on the extinction coefficients of the various bases. The extinction coefficient for calf thymus DNA was based on the following nucleotide composition of DNA: dAMP and TMP, 28.5%; dGMP and dCMP, 21.9% (63). The percentages of each nucleotide multiplied by its respective extinction coefficient gave an average extinction value of 11,100 per mole of DNA nucleotide. An extinction coefficient for ribosomal RNA based on the base ratios of rRNA of E. coli (64) when calculated gave an average extinction which deviated less than 1% from the value obtained for DNA. Thus, 11,100 was also used in calculations for rRNA.

The results in Table VII, show denatured DNA was the preferred substrate, releasing 2,500 millimicromoles of acid soluble nucleotide per hour under conditions of the assay. The rate of hydrolysis of rRNA was 53% that of dDNA while native DNA showed no increase in A_{260} nm over the blank value after 2 hours. The hydrolysis of the synthetic polynucleotides ranged from 0.9% to 14.8% that of denatured DNA. Poly G formed a fibrous DNA like precipitate in the buffer at pH 8.9 and there was no appreciable hydrolysis over blank by muskmelon exonuclease on this polymer.

TABLE VII

Hydrolysis of Polynucleotides

The assays are described in the text. The values for the rates of hydrolysis of native DNA, denatured DNA and rRNA have been normalized for an additional of 750 units of enzyme. A value for the extinction coefficient of calf thymus DNA and rabbit reticulocyte rRNA was calculated to be 11,100.

Substrate	mμmoles of acid soluble nucleotide released per hour	%
denatured DNA (calf thymus)	2,500	100
native DNA (calf thymus)	0	0
rRNA (rabbit reticulocytes)	1,320	52.8
Polyadenylic acid	23.5	0.9
Polycytidylic acid	118	4.7
Polyuridylic acid	369	14.8

Contaminating Activities

The Step VI enzyme was assayed for contaminating adenosine monophosphate nucleotidase and deaminase activities. The nucleotidase assay used was that described in Methods and Materials. The 60-minute assay was performed with enzyme concentrations of 14 units per ml and substrate concentrations of 2 mM. The possible hydrolysis of 5'-AMP, 3'-AMP and 2'-AMP by phosphatases or nucleotidases was examined at pH 8.9 and pH 7.2. The results show that there was essentially no release of inorganic phosphate, less than 0.02 μmoles per hour, at the above enzyme concentration. This value was normalized to less than one percent of the rate of hydrolysis of p-nitrophenyl-pT found in Table V.

The deaminase assays were run in cacodylate buffer at pH 8.9 using mM 5'-adenylic acid and mM adenosine concentrations. Addition of 2.9 units of enzyme to the 37°C preincubated assay mixture produced no detectable increase in absorbance at 285 nm for 30 minutes. Under these conditions, the deamination of 0.1 μmole of AMP or adenosine would have caused an increase of absorbance of 0.03 at 285 nm.

Mode of Degradation of Denatured DNA and Ribosomal RNA

To establish that the mode of attack by C. melo exonuclease was in fact an exonucleolytic rather than an endonucleolytic type of degradation, the method of Birnboim (57) was used except that Bio-Rad P-6 polyacrylamide resin replaced Sephadex G-100. Reaction mixtures contained 8 ml of denatured DNA or ribosomal RNA (20 A_{260} units per ml), 0.8 ml Tris acetate 1.0 M pH 8.9 and 0.08 ml magnesium acetate 0.36 M. The reactions were initiated by the addition of enzyme and incubated at 37°C. At various intervals after initiation of hydrolysis, 0.5 ml of the reaction

mixture was removed and added to 0.5 ml of the lanthanum nitrate-HCl reagent as described in the Experimental Results section under Hydrolysis of Polynucleotides. At the same time, 0.5 ml was also removed and added to 0.05 ml of glacial acetic acid to stop the reaction. This mixture was then diluted with 0.5 ml of 0.1 M sodium acetate. A half milliliter of this solution was withdrawn for chromatography on the P-6 polyacrylamide column. The elution patterns obtained from the U.V. monitor at various stages of hydrolysis are shown in Figure 9. The stages were determined by using the lanthanum nitrate-HCl assay and the results are expressed as a percentage of the total acid soluble products.

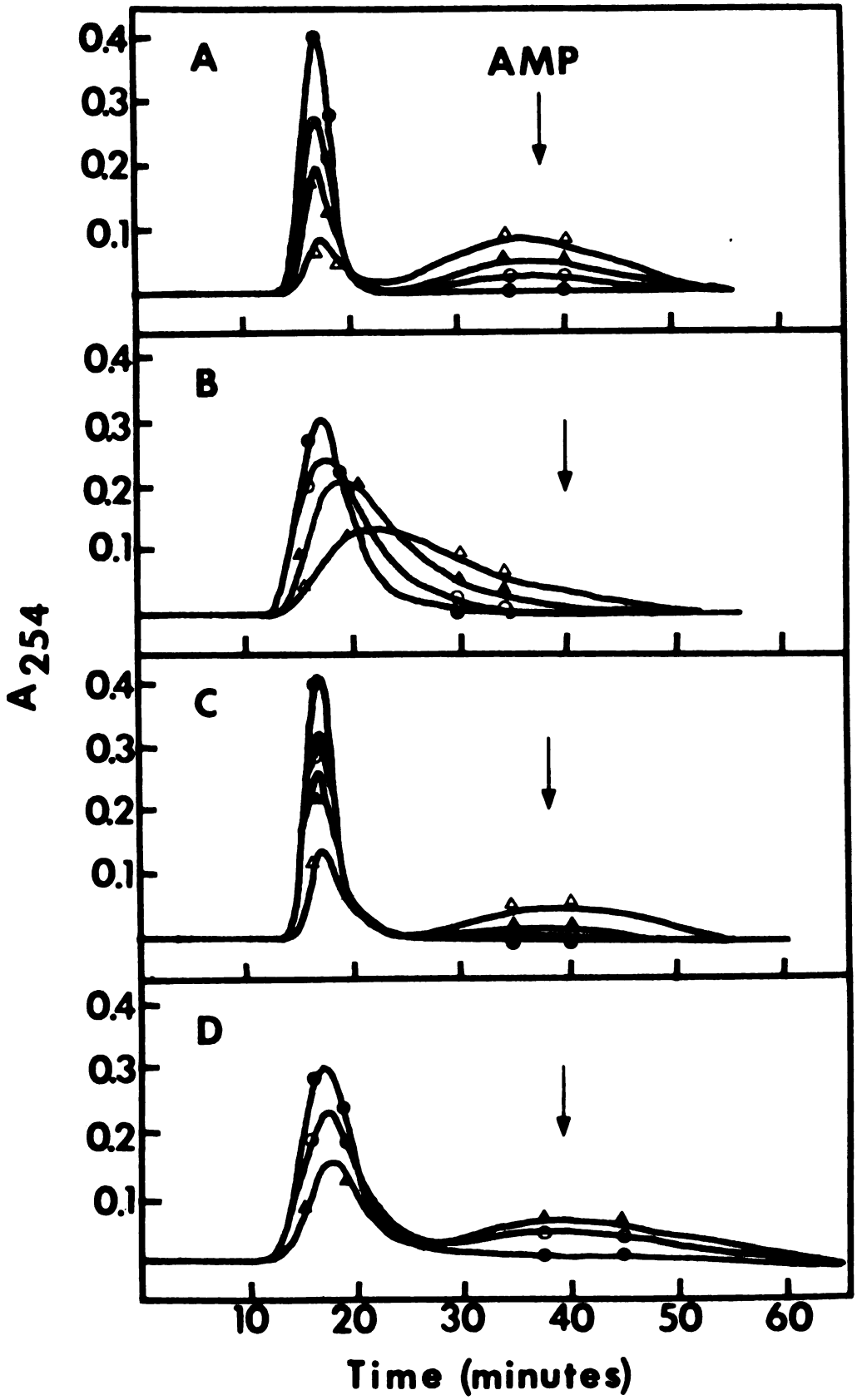
After typical patterns of exonucleolytic and endonucleolytic digestion of denatured DNA were established using venom phosphodiesterase (Figure 9a) and micrococcal nuclease from S. aureus (Figure 9b), a digestion of denatured DNA with exonuclease from muskmelon was performed. The resulting pattern, shown in Figure 9c, was similar to that produced by venom phosphodiesterase rather than the micrococcal endonuclease, in that intermediate sized products were absent. Thus on denatured DNA the mode of action of the muskmelon enzyme appeared to be an exonucleolytic type of digestion. The conditions for the digestion of ribosomal RNA were identical to those used for denatured DNA except that the enzyme concentration was increased 50%. Again, after appreciable degradation of RNA by the muskmelon exonuclease, the characteristic double peak appearance could be seen in the U.V. monitor tracings indicating an exonucleolytic type of degradation of ribosomal RNA.

Figure 9A. Chromatography on Bio-Rad P-6 of denatured DNA at stages in its digestion by venom phosphodiesterase. The reaction mixture contained 100 μg of venom phosphodiesterase per ml. The A_{254} elution profiles correspond to the percent hydrolysis as determined by acid soluble products in the lanthanum nitrate-HCl assay: a) 0%, ●; b) 28%, ○; c) 46%, ▲; and d) 83% △.

Figure 9B. Chromatography on Bio-Rad P-6 of denatured DNA at stages in its digestion by micrococcal nuclease of S. aureus. The reaction mixture contained 50 μg per ml of micrococcal nuclease. The A_{254} elution profiles correspond to the percent acid soluble products: a) 4%, ●; b) 12%, ○; c) 40%, ▲; and 70%, △.

Figure 9C. Chromatography on Bio-Rad P-6 of denatured DNA at stages in its digestion by C. melo exonuclease. The reaction mixture contained 400 units of exonuclease per ml. The A_{254} elution profiles correspond to the percent acid soluble products: a) 0%, ●; b) 16_2, ○; c) 29% ▲; and d) 63%, △.

Figure 9D. Chromatography on Bio-Rad P-6 of ribosomal RNA at stages in its digestion by C. melo exonuclease. The reaction mixture contained 400 units of micrococcal nuclease. The A_{254} elution profiles correspond to the percent acid soluble products: a) 0%, ●; b) 37%, ○; and c) 65%, ▲.


A
AMP
B
C
D
0.4
0.3
0.2
0.1
A254
10
20
30
40
50
60
Time (minutes)

Hydrolysis of Oligouridylic Acid

Oligouridylic acid (25 μmoles) was treated with 10 units of alkaline phosphatase in 0.05 ml of 0.02 M Tris acetate pH 8.9 for 12 hours at 37°C. The hydrolysis mixture was then spotted on Whatman 3 MM paper and chromatographed for 56 hours as described in the Methods section. The spot containing the majority of the A_{260} units was cut out and eluted using ammonium bicarbonate, 0.01 M, pH 8.5. The eluant was concentrated to dryness using a Buchler rotary evaporator. No trace of alkaline phosphatase could be detected in the sample using p-nitrophenyl-phosphate as substrate.

Six micromoles of diphosphorylated oligo-U was dissolved in 0.2 ml of 0.05 M Tris acetate, pH 8.9, and 100 units of (40 μl) exonuclease were added. The reaction mixture was incubated at 37°C. At intervals of 30 minutes, 10 μl aliquots of the mixture was removed and spotted on Whatman 3 MM paper. Chromatography with appropriate standards was carried out in a descending manner for 16 hours using 95% ethanol: 1 M ammonium acetate (pH 7.5) (7:3, v/v). The chromatogram showed that in early stages of hydrolysis, only spots corresponding to UMP could be detected. No dinucleotide or trinucleotide spots could be visualized. Samples incubated for 3 and 4 hours produced ultraviolet absorbing material corresponding to uridine, UMP, a light spot localized between uridine and UMP (probably UpU) and long streak of products near the origin (most probably intermediate sized oligonucleotides). Spots corresponding to UMP in the 24 hour digest were converted to a faster migrating material when treated with 5'-nucleotidase.

The chromatograms obtained above reinforce an exonucleolytic type degradation which produces mononucleotides. No di- or trinucleotides were present in early stages of hydrolysis. The direction of sequential

cleavage by C. melo exonuclease was suggested by the data to be from 3' to 5'. If initial cleavage of the dephosphorylated oligouridylic acid had been from the 5' end releasing 5'-products, uridine would have been the first product of the reaction. Hydrolysis from the 3' to 5' end would yield only mononucleotides as early products which agrees with the experimental data.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was used for two reasons. It was desirable to have an estimation of the contamination by additional proteins of the protein containing exonuclease activity and also to determine if there were more than one band of activity which could be resolved by gel electrophoresis. The gels were prepared and run as described in Methods and Materials. Samples of 100 μg of protein (0.1 ml) were prepared by concentrating the Step V enzyme in dialysis tubing using Sephadex G-100 to absorb the moisture. Aliquots of 0.1 ml were applied to gels which were to be stained for protein and 0.020 ml aliquots were applied to gels which were to be stained for activity. After electrophoresing, the gels stained with Coomassie blue showed one broad dark band of protein and six light bands which migrated faster in the gel than the former. The activity stain showed a maroon double band of activity in the region of the slowly migrating dark band. This may suggest more than one band of diesterase activity, either by contamination by another exonuclease, or by resolution of two isozymes of the exonuclease activity. Polyacrylamide gels showed no protein bands when electrophoresed using concentrated Step VI enzyme. Only 20 μg of protein was applied in the sample and this result was anticipated. The activity

stain showed only one intensely colored band which migrated slowly in the gel.

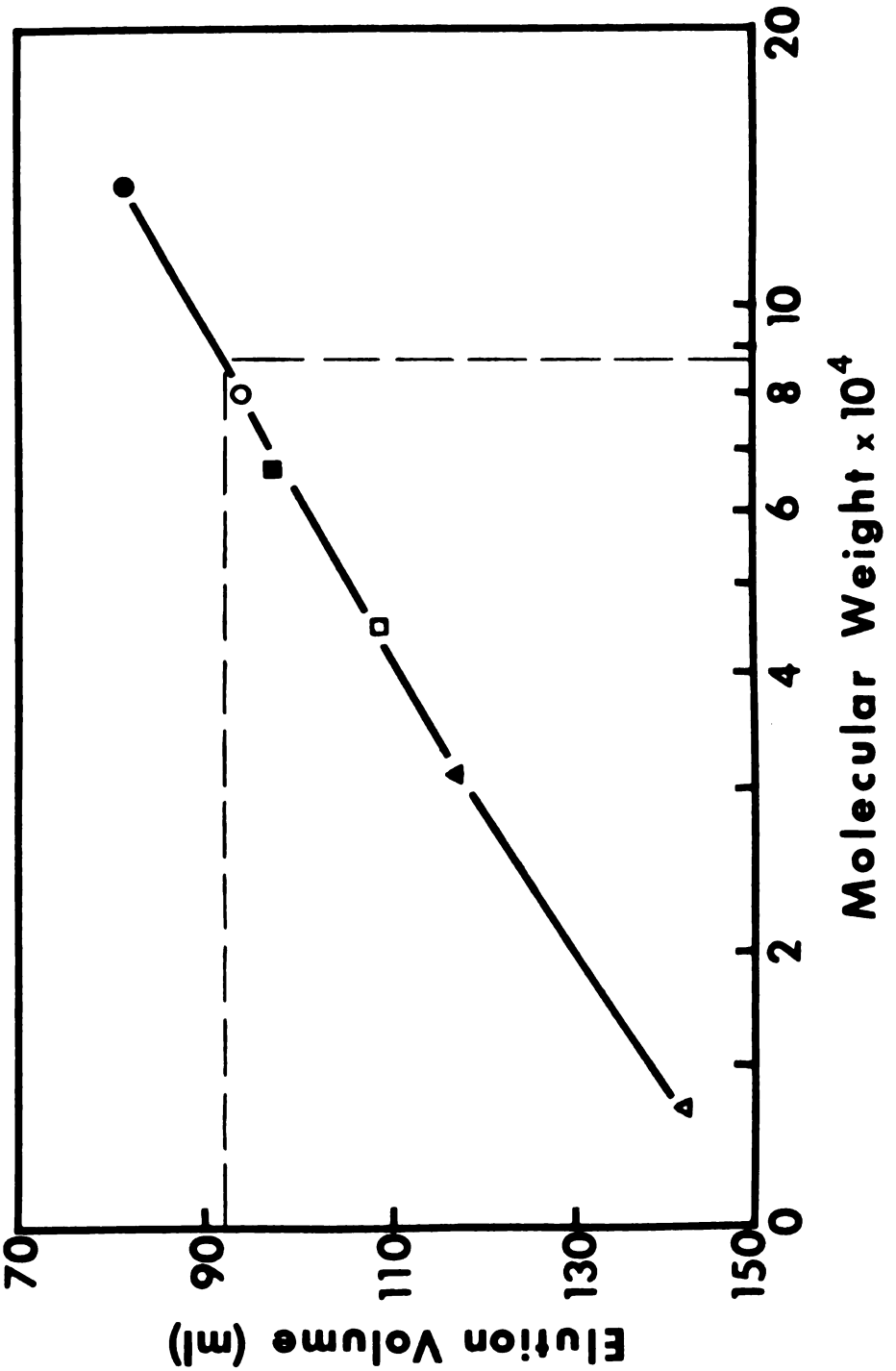
Determination of the Molecular Weight

Estimations of the molecular weight of the exonuclease from Cucumis melo were obtained from the gel filtration method of Andrews (65). A column (2.5 x 38 cm) of G-100 Sephadex (40-120 μ bead size) was prepared and equilibrated with 0.02M Tris acetate buffer pH 7.1 containing 10^{-4} M magnesium acetate. A flow rate of about 0.5 ml per minute was established by the use of a peristaltic pump. Fractions of 2.55 ml were collected at 5 minute intervals. The sample in a volume of 0.50 to 0.75 ml was layered under the top buffer above the resin bed and eluted with the above Tris buffer, the sample containing several standard proteins of known molecular weight. There was less than 2% variation in the elution volume of the same protein using consecutive passes through the column. Based on the known molecular weight of the standards used, extrapolation from the curve shown in Figure 10 gives a value of molecular weight of muskmelon exonuclease of 87,000. The molecular weight estimation via the gel filtration method has a maximum uncertainty value of ± 10% for globular proteins in the range of 10,000 to 150,000 daltons.

Determination of the Activation Energy of Muskmelon Exonuclease

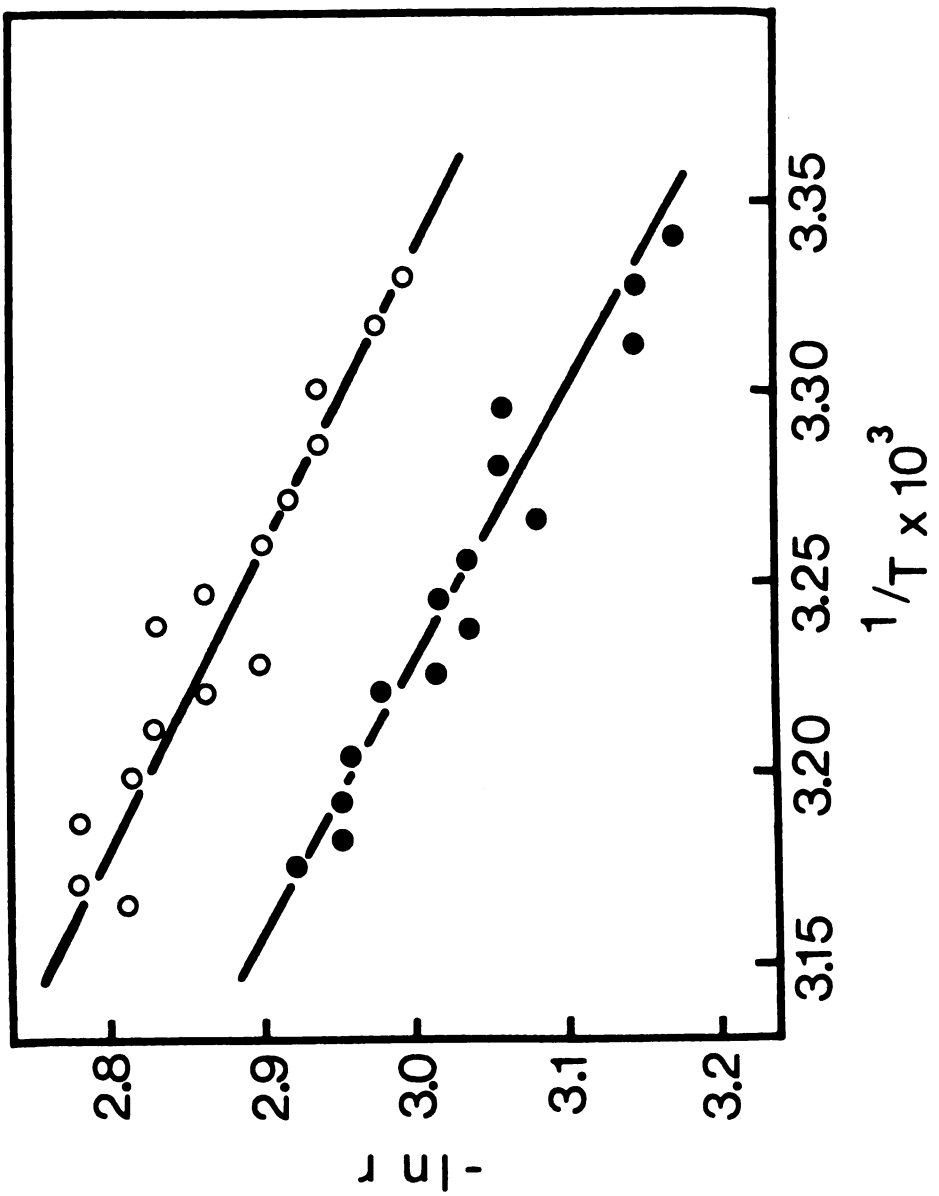
The method used to determine the activation energy was the procedure of Wilson (66). The reaction mixture of 1.0 ml contained 100 μmoles of sodium bicarbonate buffer pH 9.5, .10 μmoles of magnesium acetate, and 0.50 μmoles of p-nitrophenyl-pT. The assay mixture was pre-equilibrated at room temperature in a quartz microcuvette. The reaction was initiated

Figure 10. Determination of molecular weight by gel filtration. The semi-log plot of molecular weight of standard proteins versus the elution volume of that protein is shown. Procedure is described in the text. Standard of known molecular weight were △, cytochrome c, 12,400 (69); ▲, DNase I, 31,000 (70); □, ovalbumin, 45,000 (71); ■, bovine serum albumin, 67,000 (72); ○, alkaline phosphatase, 80,000 (73); and ●, lactate dehydrogenase, 135,000 (74). The muskmelon exonuclease (a Step VI preparation) had an elution volume of 92 ml giving it an estimated molecular weight of 87,000.


Elution Volume (ml)
70
90
110
130
150
0
2
4
6
8
10
20
Molecular Weight x 10^4

by the addition of aliquots of enzyme (130 milliunits per ml) as indicated in Figure 11. The cell was immediately stoppered with a Teflon plug equipped with the MT1 microprobe of a BAT-4 microprobe thermometer (Bailey Instrument Co., Saddle Brook, New Jersey). The cell was then placed in the sample compartment of a Turner Model 330 Spectrophotometer, thermostatted at 45°C. The output of the microprobe thermometer was followed with time on a Sargent SR recorder while the absorbance at 400 was followed on a Sargent SRL recorder. The reaction was followed through an increase of approximately 20°C in temperature, about 5 minutes. Rates of reaction were determined by drawing tangents to the absorbance curve at points corresponding to known temperatures. The data, rates of hydrolysis, and temperatures were analyzed by the CDC 6500 computer programmed to fit ln r vs. 1/T to the "best" straight line, determined by the least squares criterion. The Ea was calculated from the slope of this line. The energy of activation determined from this procedure gave values of 2.5 K_{cal} per mole and 2.8 K_{cal} per mole for 60λ and 50λ aliquots of the enzyme respectively as seen in Figure 11. Bicarbonate buffer system was used for this determination because of the larger change in pH with changes in temperature using Tris buffer.

Figure 11. Arrhenius plot for muskmelon exonuclease. Data for the curve was obtained from the analysis of the curves as explained in the text. Determinations shown are of two differing enzyme concentrations 50λ, O---O; and 60λ, ●---●. The line is the least squares "best" line through the data points. The correlation coefficient for the two enzyme concentrations are 0.890 and 0.937 for 60λ and 50λ, respectively.


-ln r
2.8
2.9
3.0
3.1
3.2
3.15
3.20
3.25
3.30
3.35
1/T x 10^3

DISCUSSION

In early experiments dealing with an examination of phosphodiesterase activities in higher plants, it was found that two members of the family Cucurbitacea possessed an activity which differed significantly from that of the other sources tested. For these preparations, pyrimidine derivatives were found to be hydrolyzed more rapidly than the purine analogs. One of these unusual enzymes, that of Cucumis melo, was selected for further study.

An exonuclease or phosphodiesterase I from Cucumis melo, the fourth enzyme of this type from higher plants which has been isolated and characterized, has now been purified about 3,400-fold. The preparation has a specific activity of 13,000 units per mg protein, thus being one of the more active preparations of exonuclease.

Two steps in the isolation procedure were major steps leading to the purification of the enzyme to its present specific activity. Removal of the lipid-like material by lowering the pH of the crude enzyme solution and heating to 60°C permitted the subsequent use of ammonium sulfate fractionation and column chromatography in the purification scheme. The phosphocellulose column apparently separated two exonuclease activities. The exonuclease activity which was not eluted in the wash fractions was eluted with NaCl, yielding a preparation which contained an extremely small amount of protein. The enzyme differs from all other purified phosphodiesterase I in that the enzyme does not bind to DEAE cellulose in the pH range from 7.5 to 8.5. A major attribute of the

C. melo enzyme is that the step VI enzyme can be prepared expeditiously from the imbibed seeds in 2 days using only two column procedures.

Values for protein concentration obtained by the Lowry method of protein determination were shown to be somewhat unreliable at low protein concentration in the presence of Tris buffer. This was due to the extremely shallow slope of the protein standard curve in Tris buffer. The average value for protein concentration using the method of Lowry on Step VI enzyme was 50 μg per ml. The tannic acid method of protein determination displayed no interference from Tris buffer even at low concentration. Values for the Step VI protein concentration was 20 μg per ml, the method being linear below the 10 μg per ml level. The enzyme demonstrates properties typically characteristic of phosphodiesterase type I (17).

The pH activity curve for muskmelon exonuclease is similar to that obtained for other phosphodiesterases from plant and animal sources in that the pH activity curve appears to be quite symmetrical around the optimum pH 9.3 and falls off quite sharply on either side of this pH. At pH 8.9, the pH of the assay conditions, the activity drops to 69% of that at the pH optimum. In Tris acetate buffer at pH 8.9, this activity is only slightly lower. Since the initial use of p-nitrophenyl-pT with venom phosphodiesterase in 1959, hydrolysis of this compound at pH 8.9 has become a standard assay for comparison of enzymes with various sources. For this reason the assays for muskmelon exonuclease were also performed at pH 8.9. There was essentially no activity at pH's below pH 7.5 in the buffers tested.

Stability as well as activity was pH dependent, but the C. melo enzyme like the other exonucleases from plant sources was more heat

stable than its animal counterparts. At 37°C the enzyme activity was destroyed completely at pH 4.0 after 2 hours. The pH of optimal stability ranged from pH 7.0 to 9.0 for 37°C incubation. After 15 minutes incubation at 80°C, the enzyme in Tris acetate buffer at pH 7.5 retained all of its activity. In Tris at higher pH's and in sodium carbonate-bicarbonate buffers (in the pH range of 8.5 to 10.0) considerable amounts of activity were lost. No attempt was made to determine the change in pH from 24°, the temperature at which the buffers were prepared, to 80°C. The significant negative temperature coefficient of Tris buffer and the loss of CO_2 from a bicarbonate-carbonate buffer system at 80°C may have played a large role in the loss of activity seen. At the pH of the enzyme assay, pH 8.9, the enzyme lost a high percentage of activity when the temperature was raised above 70°C, until at 90°C no detectable activity remained.

Exonucleases (phosphodiesterase I) have been shown to hydrolyze a spectrum of substrates, ranging from polynucleotides to substituted nucleoside components. C. melo exonuclease exhibited a high rate of hydrolysis for p-nitrophenyl nucleoside-5'-phosphates in comparison with oligonucleotide substrates. A preference in the rate of hydrolysis of p-nitrophenyl-pdG over the rate of hydrolysis of p-nitrophenyl-pdC and p-nitrophenyl-pT, and a low rate of hydrolysis of p-nitrophenyl-pdA was observed. In contrast to the two phosphodiesterases from other sources which have been examined (8, 34), ribotide derivatives of p-nitrophenol were hydrolyzed by the C. melo enzyme more slowly than their analogous deoxyribonucleotide derivatives. There was however no apparent correlation between the rate of hydrolysis and the K_m for the substrates tested.

Rates of hydrolysis of p-nitrophenyl esters other than p-nitrophenyl

nucleoside-5'-phosphates were extremely low. The nonspecific phosphodiesterase substrate, bis-p-nitrophenyl-phosphate, gave a rate only 4% that of p-nitrophenyl-pT. The hydrolysis of Tp-p-nitrophenyl and p-nitrophenyl-phosphate could not be measured at an enzyme level of 72 milliunits. Even upon a 40-fold increase in enzyme concentration above 72 milliunits, no nonspecific phosphatase activity could be detected using p-nitrophenyl-phosphate as substrate. There was an extremely small but measurable hydrolysis of p-nitrophenyl esters at pH 5.5 which agrees with reports describing exonucleases from other sources. This is quite unusual since this pH is almost 5 pH units from the pH optima of the enzyme. Use of this unique property has been made by Richards and Laskowski with venom exonuclease at pH 5.0 for the hydrolysis of XpXp to X and pXp, a reaction which at pH 8.9 is highly unfeasable due to the inhibition of the reaction by the ionized 3'-phosphoryl group (48).

The addition of various nucleoside monophosphates to the assay mixture lowered the rates of hydrolysis with p-nitrophenyl-pT or p-nitrophenyl-pdG, the only substrates tested. Low rates of hydrolysis of both esters were seen in the presence of adenosine-5'-monophosphate and deoxyadenosine-5'-monophosphate, the greater inhibition being offered by 5'-AMP. The ability of 5'-AMP to strongly inhibit the enzymatic activity of phosphodiesterases has only been suggested in an earlier report on kidney phosphodiesterase I. The type of inhibition exerted by 5'-AMP was determined to be competitive in nature with the aid of a Dixon plot. The low value for the K_I, 6×10^{-7} M, suggested that a strong complex exists between the enzyme and 5'-AMP. Observations with C. melo enzyme represents the only examination in some detail of this phenomenon. This also may explain the low rate of hydrolysis of

p-nitrophenyl-pdA and p-nitrophenyl-pA by C. melo exonuclease. The products of these reactions, 5'-dAMP and 5'-AMP, are probably bound tenaciously to the enzyme at or near the active site, inhibiting new incoming substrate from entering the region of the catalytic center and resulting in a low rate of hydrolysis. All ligands attached to 5'-AMP lower the inhibitory effect exhibited by adenylic acid itself. The presence of the phosphoryl group in the 2'- or 3'-position of the ribose sugar rather than the 5'-position also lowers the inhibition.

The rate of hydrolysis of polynucleotide was lower than that seen with the p-nitrophenyl derivatives. This is an observation which agrees with data of workers using exonuclease from other sources. Oligonucleotides, polynucleotides, and even dinucleotides were hydrolyzed at a lower rate than the respective p-nitrophenyl-nucleotides tested (8, 15, 16). A marked preference for heat denatured DNA was shown by the enzyme. Native DNA was not hydrolyzed under the assay conditions. Rabbit reticulocyte rRNA was hydrolyzed at about one-half the rate of denatured DNA. The slower hydrolysis of rRNA may have been due in part to the secondary structure attributed to ribosomal RNA. However, the low rate of hydrolysis of RNA compared with DNA was similar to the results obtained earlier with p-nitrophenyl derivatives of ribo- vs deoxyribotides, suggesting that the deoxyribotide moiety is indeed a preferred substrate. The rates of hydrolysis of the synthetic polymers were lower than these found for either natural polymer. Poly A was hydrolyzed at about one-third the rate for Poly U. This again is consistent with the results obtained earlier with the p-nitrophenyl esters of AMP and UMP. Poly C was hydrolyzed comparatively much more slowly than Poly U. This may have been due to the existence of considerable secondary structure

in Poly C at the pH of the assay conditions.

A possible explanation for the slower hydrolysis of the presumably natural substrates compared with the more rapid hydrolysis of p-nitrophenyl nucleotides can be proposed based on differences in the rate of the release of products following hydrolysis. The active center of the enzyme probably has at least two binding sites. The first is for the nucleoside-5'-phosphoryl group (8) and a second for the group or moiety attached to the 5'-nucleotide. For synthetic substrates, this group would be p-nitrophenol and for more "natural" substrates the group would be a nucleoside unit of an oligonucleotide. Binding at the first site would be similar for either substrate. It may be that the nonionized nitrophenyl group binds at site 2 with a binding constant similar to that of the nucleoside unit of an oligonucleotide. Upon hydrolysis, however, the negatively charged nitrophenoxide may be bound less strongly and therefore released more rapidly from site 2 than the non-charged nucleoside moiety. The release process may be the rate determining step in the reaction. In this connection it may be significant that the hydrolysis of pT pT is faster than the hydrolysis of TpT for enzymes from two different sources (8, 15). Again this is a situation in which a charged group in site 2 gives a higher rate of hydrolysis.

C. melo exonuclease benefits from the addition of certain divalent metal ions, i.e. Mg^{++}, Ca^{++}, and Ba^{++}. EDTA, a metal chelator, had an inhibitory effect upon activity of the exonuclease, a concentration of 2×10^{-5} M completely inhibiting enzyme activity. Fluoride ion, also a complexing agent, reduced enzyme activity 84% at mM concentration. If the fluoride was acting as a metal complexing agent, rather than binding to the enzyme itself as seen with adenylic acid deaminase (67), this

would be additional evidence that the exonuclease is a metal enzyme and that tightly bound metal ions are necessary for enzymatic activity. Inhibition of enzymatic activity by EDTA was restored by incubation of the enzyme with Mg^{++}, Sr^{++}, or Ba^{++}. The inhibition of exonuclease activity by sulfhydryl reagents reported with venom and rat intestinal mucosa exonucleases was also seen with the C. melo enzyme. The inhibition exerted by these compounds may again be by formation of chelators, since these complexes are formed by sulfhydryl compounds (64).

The stimulation of enzymatic activity by ammonium sulfate as reported for the malt enzyme (14) was not seen with C. melo exonuclease, an inhibition of activity being observed. There was no detectable contamination of the exonuclease by adenosine deaminase or adenylic acid deaminase. Commercial preparation of spleen exonuclease, however, are known to contain a contaminating deaminase activity (68). No phosphatase or nucleotidase activity could be detected in Step VI exonuclease when assayed at pH 7.2 or pH 8.9 with 5'-, 3'-, or 2'-AMP or with p-nitrophenyl-phosphate at pH 8.9. The hydrolysis of bis-p-nitrophenyl-phosphate at pH 8.9 was most probably an indication of the nonspecific nature of the enzyme activity rather than a separate contaminating phosphodiesterase. Phosphodiesterase II activity was not detected using Tp-p-nitrophenyl as substrate.

The enzyme activity has an exonucleolytic attack on denatured DNA, ribosomal RNA and oligouridylic acid. This was expected since this has been shown previously with similar phosphodiesterases from other plant and animal sources. The direction of hydrolysis appears to be from the 3' to the 5' end, 5'-mononucleotides being liberated. No production of di- or trinucleotide could be seen. An estimate for the molecular weight

of C. melo exonuclease via gel filtration was 78,000. This is much larger than the molecular weights for most nucleases but is somewhat smaller than the molecular weight of .98 to 1.15 x 10^5 obtained for carrot phosphodiesterase I (37). The activation energy of the muskmelon enzyme was about 2,800 cal per mole. This is quite small in comparison with the activation energy of most enzymatic reactions which range from about 8,000 cal per mole for citrate synthetase to 12,000 cal per mole for brain hexokinase (66).

The exonuclease from C. melo like the enzymes from Avena leaves (16) and carrot (15) is apparently not compartmentalized as has been found with the acid hydrolases nor is it bound as part of a small particle complex. Rather, the enzyme is most probably located in the cytosol of the plant cell. It is difficult at this time to postulate a biological role for the enzyme which has any experimental support. If no inhibitory control mechanism is in operation, the enzyme may be performing a continuous catabolic function within the cell.

Native DNA usually located in the nucleus, in mitochondria, or in chloroplasts resists hydrolysis by the enzyme, but single stranded DNA which may enter the cytosol from a compartmentalized source or from the environment would be readily degraded. Ribosomal RNA is not available for hydrolysis since it is usually an integral part of the ribosome. Messenger RNA and single stranded viral RNA may be excellent substrates for the exonuclease providing that the 3' terminus is free, particularly after partial degradation by an endonuclease. Transfer RNA which may be partly degraded from its 3' hydroxyl terminus by exonuclease has a repair system which may replace the terminal cytidylic and adenylic acid units.

The purified preparation of exonuclease from C. melo shows no

detectable contamination by nucleotidase, nonspecific phosphodiesterase, endonuclease, adenosine deaminase or adenylic acid deaminase activities. Accordingly, the enzyme may be a useful tool in nucleic acid biochemistry, particularly as an acid in sequence determination of oligonucleotides.

SUMMARY

An exonuclease from C. melo seeds has been purified to an extent of 3,400-fold with a recovery of about 14% of the total exonuclease activity present in the crude extract. The enzyme displays many of the properties typical of phosphodiesterase I activities. No contamination by endonuclease, nonspecific phosphodiesterase, phosphatase, nucleotidase or deaminase activities has been detected.

The enzyme was prepared by an expeditious procedure involving a heat step with a change in pH, acetone fractionation, ammonium sulfate fractionation, Sephadex G-100 gel filtration and phosphocellulose chromatography.

The enzymatic activity shows a pH optimum of pH 9.3 with activity decreasing quite sharply on either side of this pH. The stability of the activity was found to be dependent both on temperature and on pH, optimal conditions being 4°C at pH 7.5. The enzyme activity was stimulated by the addition of Mg^{++}, Ca^{++} and Ba^{++}. Enzymatic activity was reduced or destroyed by the presence of sulfhydryl compounds, fluoride ions, and chelating agents. The C. melo exonuclease was sensitive to extremely small amounts of EDTA. Reversal of the EDTA inhibition was obtained with Mg^{++}, Ca^{++} and Sr^{++}.

The purified enzyme readily hydrolyzed p-nitrophenyl esters of 5'-nucleotides. p-Nitrophenyl-pdG was hydrolyzed much faster than p-nitrophenyl-pT or p-nitrophenyl-pdC which hydrolysis of p-nitrophenyl-pdA was extremely slow. 5'-Deoxyribonucleotide esters of p-nitrophenol

were hydrolyzed faster than their ribotide analogues. Esters of p-nitrophenol other than 5'-mononucleotides were hydrolyzed at a rate of less than 4% of that seen for the hydrolysis of p-nitrophenyl-pT.

Denatured DNA and ribosomal RNA, presumably more natural substrates, were hydrolyzed at a rate much slower than that seen for the p-nitrophenyl-5'-deoxynucleotides. Native DNA was not hydrolyzed under the assay conditions. Of the homopolymers examined, Poly U was hydrolyzed much faster than Poly A or Poly C. Poly G was not hydrolyzed under the conditions of the assay. A possible explanation for the rapid hydrolysis of the p-nitrophenyl-5'-nucleotides in comparison with oligonucleotides was proposed.

An exceedingly strong inhibition by 5'-AMP of the hydrolysis of p-nitrophenyl-pdG and p-nitrophenyl-pT was observed and determined to be competitive in nature. An extremely small K_i for 5'-AMP was found.

Hydrolysis of denatured DNA and ribosomal RNA by the C. melo enzyme was shown to be exonucleolytic in nature. The molecular weight of the enzyme was determined to be about 78,000 using gel filtration. The activation energy obtained for the hydrolysis of p-nitrophenyl-pT was about 2,800 cal per mole.

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