AN ABSTRACT OF THE THESIS OF

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Title: THE PURIFICATION AND CHARACTERIZATION OF A RIBONUCLEASE FROM BOVINE AORTA

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Reports of investigations on nucleases in arterial tissue are few in number. The present study is concerned with the purification and characterization of an aortic ribonuclease.

An endonuclease (ARNase I) isolated from the bovine aorta has been purified 4611 fold by means of fractionation with ethanol, acid extraction, isoionic precipitation, BioRex 70 column chromatography and dialysis. A second fraction from BioRex 70 column chromatography, ARNase II, was partially purified 667 fold.

A pH optimum for ARNase I activity on 0.5% sodium RNA was observed at 7.5 and no activity was detected at pH 5.1. The enzyme exhibited a temperature optimum of 60°C.

Polyuridylic acid (poly U) was rapidly depolymerized by ARNase I, whereas polycytidylic acid (poly C) was only slowly hydrolyzed by the enzyme at a rate of 1/24 that exhibited on poly U. Polyguanylic acid, polyadenylic acid and polyinosinic acid were

resistant to enzymatic action.

ARNase II was 18 times more active on poly C and one-seventh as active on poly U as was ARNase I.

Cytidylyl-(3'-5')-cytidine and uridylyl-(3'-5')-cytidine were hydrolized by ARNase I with the products being 2', 3'-cyclic cytidylic acid and cytidine and 2', 3'-cyclic uridylic acid and cytidine, respectively. Adenylyl-(3'-5')-cytidine and guanylyl-(3'-5')-cytidine were not acted upon by the enzyme.

On incubation of sodium RNA with ARNase I, only 16% of the acid soluble nucleotides were present as oligonucleotides of three units or less.

These data show that ARNase I is an endonuclease with a specificity for the formation of pyrimidine phosphates during the hydrolysis of polynucleotides and a preference for uridylic acid residues.

The Purification and Characterization of a Ribonuclease from Bovine Aorta

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The bovine aortas used were supplied by the D. E. Nebergall Meat Company, Albany, Oregon without cost.

To my loving wife who has endured these years of limited living, played father, as well as mother, on far too many occasions and contributed much more than her wifely share to our family.

ABBREVIATIONS

Ribonucleic Acid	RNA
Ribonuclease	RNase
2', 3'-cyclic cytidylic acid	Cp!
2', 3'-cyclic uridylic acid	Up!
2', 3'-cyclic guanylic acid	Gp!
2', 3'-cyclic adenylic acid	Ap!
Cytidine	C
2', 3'-cytidylic acid	Cp
5'-cytidylic acid	pC
2', 3'-uridylic acid	Up
Cytidylyl(3'-5') cytidine	CpC
Uridylyl(3'-5') cytidine	UpC
Adenylyl(3'-5') cytidine	ApC
Guanylyl(3'-5') cytidine	GpC
Poly cytidylic acid	poly C
Poly uridylic acid	poly U
Poly adenylic acid	poly A
Poly inosinic acid	poly I
Poly guanylic acid	poly G

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INTRODUCTION

Nucleases, enzymes which catalyze the depolymerization of nucleic acids, are essential to all living matter. Depending upon the reaction catalyzed, the general term "nucleases" is divided into three main areas, those enzymes which react with 1) ribonucleic acid (RNA), 2) deoxyribonucleic acid (DNA), and 3) both ribonucleic acid and deoxyribonucleic acid. Within the group that reacts with only ribonucleic acid are the ribonucleases. RNase activity has been observed in a wide variety of plant and animal life. The enzymes and/or their modes of action are almost as varied as the life forms themselves, although some of the enzymatic characteristics seem to jump species lines and are shared by two or more RNases. Generally speaking, RNases from plant sources give as intermediates or products both purine and pyrimidine cyclic phosphates, whereas animal RNases produce pyrimidine cyclic phosphates and oligonucleotides terminating in pyrimidine cyclic phosphates. The cyclic phosphates may, in both cases, be hydrolyzed by the enzyme to the corresponding 3'-monophosphate. Many of the microbial RNases also give 3'-monophosphates as products of their enzymatic action. Usually the 3'-monophosphates of both purines and pyrimidines are

formed. In contrast, several of the microbial RNases are specific for the production of 5'-monophosphates. Some of the most intensely investigated and more highly purified RNases from animal, plant and microbial sources will be reviewed in the following pages.

The most widely studied of the RNases are found in the bovine pancreas. Two enzymes exhibiting RNase activity have been purified and characterized from the bovine pancreas, RNase A and RNase B (14). The degradation of RNA by these enzymes proceeds in three consecutive steps, 1) a nonhydrolytic change in the structure of the RNA molecule (8), 2) the rapid transesterification of the 3' phosphate of a pyrimidine residue from the 5' position of the adjacent nucleotide to the 2' position of the pyrimidine residue, thus forming a pyrimidine 2', 3'-cyclic phosphate and 3) the slow hydrolysis of the 2', 3'-cylcic phosphate to the 3' monophosphate. The amino acid composition of RNase A was determined by Hirs et al. (15). Later work by Plummer and Hirs (32) showed that RNase B had the same amino acid composition as RNase A. In addition it was observed that the two enzymes had the same specific activity and ultraviolet adsorbtion spectra. The difference between RNase A and RNase B was shown to be that RNase B was a glycoprotein and RNase A was observed to to contain no carbohydrates.

Ibuki et al. (17) isolated an RNase from cow's milk that is similar to pancreatic RNase A in its catalytic action on RNA and cyclic nucleotides. The milk RNase has a pH optimum of 7.6 which

is also similar to pancreatic RNase A (20). A RNase from bovine milk was also purified by Bingham and Zittle (4, 5). These investigators found the enzyme to be identical to pancreatic RNase A in its chromatographic and electrophoretic behavior and in its amino acid composition.

Bingham and Kalan (3) isolated a RNase from bovine milk that was determined to have an amino acid composition identical to bovine pancreatic RNase B. The polysaccharide content of the two molecules is different.

Maver and Greco (26) have partially purified RNases from bovine liver and spleen. Both acid and alkaline RNases were found in each of the two organs. The acid and alkaline RNases from the liver and the spleen hydrolyze RNA to give as final products both pyrimidine and purine 3' phosphates. No RNA core remains in the acid RNase reaction and only a small core remains in the alkaline RNase reaction. In the hydrolysis of RNA, the acid RNases have a preference for the production of 3'-adenylic acid over 3'-cytidylic acid, but the alkaline RNases produce a greater amount of 3'-cytidylic acid than 3'-adenylic acid.

A heat stable RNase from calf spleen has been reported by Kaplan and Heppel (18) to be purified 700 fold. The substrate specificity was reported to be the same as that of pancreatic RNase, but the two enzymes have different pH optima (6.0-6.5 for spleen and 7.6

for pancreatic). In addition, the two enzymes chromatograph differently on the ion exchanger XE-64.

Bovine aorta ribonuclease has been partially purified by Gamble et al. (11) This enzyme was found to be different with respect to catalytic activity from pancreatic RNase. The pH optimum of the partially purified preparation was reported to be around 7. Poly C and poly U were found to be depolymerized by aorta RNase, but poly I and poly A were resistant to hydrolysis by the enzyme. Aorta RNase differs from other RNases of bovine origin in that the final products of its catalytic action on poly C are oligonucleotides and 2', 3'-cyclic cytidylic acid.

Animals other than the cow have been employed as sources for RNase isolations. Liver RNases have been isolated from the rat. Both an acid RNase (36 fold purified), and alkaline RNase (24 fold purified) were reported by Zytko et al. (48) and de Lamirande (6). Acid rat liver RNase was observed to hydrolyze the 2', 3'-cyclic phosphates of adenosine and uridine, but the alkaline RNase was inactive towards both of the cyclic compounds.

Squid caecal RNase was detected to hydrolyze both purine and pyrimidine diester bonds to the corresponding 2', 3'-cyclic phosphate (9). Pyrimidine cyclic phosphates are resistant to further attack, but the 2'-3'-cyclic adenylic acid is slowly hydrolyzed in the presence of the enzyme.

Aquist and Anfinsen (1) showed the presence of eight fractions of RNase activity resulting from column chromatography of sheep pancreas preparations. Each of the eight fractions had the same isoionic point and their isoionic point was different from that measured under similar conditions for bovine pancreatic RNase.

Human urine and sperm contain several different RNases (13) which exhibit catalytic properties that differ from those of bovine pancreatic RNase.

Whereas most animal RNases have alkaline pH optima, the plant RNases exhibit acid pH optima around pH 5. Ryegrass RNase has been partially purified (38) and it was shown by Shuster, Khorna and Heppel (39) that it hydrolyzes RNA completely to the 2', 3'-cyclic phosphates which are in turn slowly hydrolyzed to the corresponding 3'-monophosphate.

Frisch-Niggemeyer and Reddi (10) and Reddi (33) have purified 100 fold a RNase from tobacco leaves. This enzyme hydrolyzes RNA completely to the cyclic monophosphates. The purine cyclic phosphates were reported to be hydrolyzed to their 3'-monophosphates and the pyrimidine cyclic phosphates were shown to be stable to any further reaction by the RNase.

Wilson (47) has purified 4, 100 times an RNase from corn meal. He found that when dinucleoside phosphates were employed as substrates, both purine and pyrimidine cyclic phosphates were formed and that only subsequent hydrolysis of the purine cyclic

phosphates occurred at any appreciable rate.

Soybean RNase purified by Merola and Davis (27) gave both purine and pyrimidine 2', 3'-cyclic phosphates and 3'-pyrimidine monophosphates as products in the hydrolysis of RNA.

One of the earliest of the plant RNases to be isolated was one purified 230 fold by Holden and Pirie (16) from pea leaves. Markham and Strominger (25) showed that this enzyme gives the cyclic product of both pyrimidines and purines as products from its action on RNA and that the purines are slowly hydrolyzed to the corresponding 3'-monophosphates.

Tuve and Anfinsen (45) purified 250 times a spinach RNase. The enzyme completely degrades RNA to the 3'-mononucleotides.

Several RNases which have proved to be quite different from other known enzymes have also been isolated from microbial sources. A heat sensitive, acid RNase from Bacillus subtilis was purified 150 fold by Nakai et al. (28). The enzyme hydrolyzes RNA to give mainly cyclic nucleotides as products.

Poly A, poly C, poly I, and poly U were degraded to their corresponding 3'-monophates by an intracellular RNase isolated from Tetralymena pyriformis by Laxorus and Scherbarum (21).

The 2',3'-cyclic phosphates of adenosine, guanosine, uridine and cytidine were the end products of the degradation of yeast RNA by RNase isolated from the insoluble particle fraction of Azotobacter

agilis (37). Another enzyme obtained from Azobacter agilis which was studied by Stevens and Hilmore (43) was found to give 5'-monophosphates as the products of degradation of RNA.

Among the most interesting of the RNases of microbial origin, T_1(36) and T_2(29), were isolated from Aspergillis oryzae cultures. T_1 shows a specificity for the hydrolysis of the guanosine 3', 5'-phosphodiester bond. T_2 has a much greater specificity for the phosphodiester bond of adenosine, but cytidylic and uridylic acids are also a product of the enzymatic hydrolysis.

Spahr and Hollingworth (41) purified 730 fold a latent RNase from Escherischia coli which hydrolyzes RNA to the corresponding 3'-mononucleotides. This enzyme differs from others in that it exhibits a greater specificity toward the 6-amino as compared to the 6-keto nucleotides. An enzyme which acts as both an endo- and an exonuclease has been purified from Escherichia coli by Spahr (40). This enzyme hydrolyzes RNA and gives as products 5'-monophosphates of all four nucleotides. A nucleotide core remains after the reaction is completed.

Yeast is the source of an endonuclease which has been purified 30 fold (22). The RNase was observed to hydrolyze homopolymers (poly A and poly U) to their corresponding 5'-mononucleotides.

Quite a different specificity is exhibited by an RNase isolated from Anacystis nidulans (30). This enzyme is specific for the

phosphodiester linkage in RNA which involves a ribose that is methylated in the 2' position. Produced by enzymatic action is the 5'-mononucleotide. The enzyme does not appreciably attack a normal phosphodiester bond of a non-methylated ribose.

As one can see from this listing of many RNases which have been at least partially purified, RNases are not limited to one form of life, but are quite widely found in nature. In addition, a unique degree of reactivity or specificity is not possessed by all RNases. This thesis is a report of the purification of a RNase found in the bovine aorta and a characterization of the enzyme with respect to its reactivity and specificity utilizing various ribonucleic acids.

MATERIALS

BioRex 70 (200-400 mesh) in the sodium form, Cellex E (ECTEOLA-cellulose) and BioGel P-2 were obtained from BioRad Laboratories.

Mann Research Laboratory, Inc., was the source of 2', 3'-guanylic acid (Gp), 2', 3'adenylic acid (Ap), 2', 3'cytidylic acid (Cp), 2', 3'-uridylic acid (Up) and p-nitrophenyl phosphate.

Reagent grade ammonium sulfate, sulfuric acid, isopropanol, ammonium hydroxide, acetic acid, ethanol and sodium chloride were acquired from the Baker and Adamson Company.

Polyuridylic acid (poly U), polycytidylic acid (poly C), polyadenylic acid (poly A), polyguanylic acid (poly G), polyinosinic acid (poly I) and guanylyl (3'-5') cytidine (GpC) were obtained from Miles Chemical Company.

The following chemicals were from Mallinckrodt Chemical Works: potassium monobasic and dibasic phosphate, trichloroacetic acid and potassium hydroxide.

D. E. Nebergall Meat Company of Albany, Oregon, supplied the aortas without cost.

Schwarz Bioresearch, Inc., was the source of cytidine 2', 3'-cyclic phosphate (Cp!).

Isobutyric acid used was from the J. T. Baker Company.

Nutritional Biochemical Corporation was the source of the sodium RNA.

Tris (7-9)(Tris (hydroxymethyl) aminomethane), Bovine Pancreatic RNase, type III A (90-95% homogeneous), DEAE-cellulose, 2'3'-cyclic uridylic acid (Up!) and 2', 3'-cyclic guanylic acid (Gp!) came from the Sigma Chemical Company.

Beta-alanine, uridylyl (3'-5') cytidine (UpC), cytidylyl (3'-5') cytidine (CpC), and adenylyl (3'-5') cytidine (ApC) were requisitioned from Calbiochem, while Matheson, Coleman and Bell were the suppliers of glycine used.

Cytidine (C) and 5'-cytidylic acid (pC) were purchased from P-L Biochemicals, Inc.

Polyacrylamide gel and ammonium persulfate were acquired from the E-C Apparatus Corporation.

METHODS

General Procedures

Activity Assay

The reaction solution was prepared by combining 0.1 ml of a pH 7.0, 0.1M potassium phosphate buffer, 0.1 ml of enzyme solution and 0.2 ml of a 1.0% sodium RNA. Two controls were run. In the first control, the sodium RNA blank, 0.1 ml water was substituted for 0.1 ml enzyme solution. In the second control, the enzyme blank, 0.2 ml water was substituted for 0.2 ml of 1.0% sodium RNA. The reaction solution was incubated for five minutes at 37° C in a shaking water bath. The reaction was stopped by the addition of 0.33 ml of a 10% trichloroacetic acid (TCA) solution. After allowing this mixture to stand on ice for five minutes, the precipitate was removed by centrifugation at 10,000 rpm for 15 minutes at 3° C. The supernatant was then immediately and quantitatively decanted into graduated centrifuge tubes and the volume was brought to 4.0 ml with water. The acid soluble nucleotides were measured at 260 mμ on a Beckman double beam spectrophotometer, Model DB. The reference sample consisted of a 0.83% TCA solution. Data are reported as the difference between the absorbance at 260 mμ of the acid soluble nucleotides of the reaction

solution and the sum of the absorbance at 260 mμ of the acid soluble material of the two controls.

A unit is defined to be that amount of protein required to cause a net absorbancy at 260 mμ of 1.00 under the assay procedure. Specific activity is expressed as units/mg protein.

The effluents of all columns in the purification of the enzyme were assayed according to this procedure with the exception that the reaction solutions were incubated for 15 minutes rather than five minutes.

Protein Determinations

Protein concentrations were measured by the method of Lowry et al. (23). Proteins eluted from columns were monitored by either direct spectrophotometric (280 mμ) readings or by the 280/260 absorbancy ratio method of Warburg and Christian (46).

Paper Chromatography

For ascending chromatography the solvent was a solution of 40 gms of ammonium sulfate in 100 ml of 0.1M phosphate buffer, pH 7.0. The nucleotides were spotted on Whatman No. 1 chromatography paper for use in this solvent system. Two different systems were employed for descending paper chromatograms: (1) isobutyric acid: 1M ammonium hydroxide: 0.2M EDTA

(100: 60: 0.8, v/v/v), (2) isopropanol:water: ammonia (70:30:0.35, v/v/v) with ammonia in the vapor phase (modified method of Markham and Smith (24)). Whatman No. 3 chromatography paper was used for descending systems. Chromatographed spots were located by an ultraviolet light mineral lamp. After chromatography the papers were allowed to air dry. Identifications of the chromatographed nucleotides were confirmed by eluting the spots with water and comparing their ultraviolet spectra (320-220 mμ) to standards. The data of Stanley and Bock (42) were also employed as comparisons.

Procedures for Enzyme Purification

Preparation of Crude Homogenate

Aortas from cattle two to three years of age were obtained immediately after sacrificing and placed on ice. All excess fat and connective tissue was removed with scissors. Aortic portions no longer than 12 to 14 inches in length were used. The aortas were rinsed with cold, distilled water, cut into small pieces and homogenized at high speed in a Waring blender for one minute with an equal weight of cold distilled water. The resulting homogenate was centrifuged at 5,000 rpm in a Servall refrigerated centrifuge for 40 minutes at 3°C. The solid debris was discarded and the supernatant was collected and frozen or used immediately. Surgical gloves were

worn at all times to prevent contamination of the aortas.

Precipitation with Ethanol

The crude homogenate was either centrifuged directly after preparation or thawed and then centrifuged at 13,000 rpm for 30 minutes at 3° C in a Servall centrifuge. Insoluble material was discarded. The supernatant was made 50% (v/v) with respect to ethanol by a slow addition of cold 95% ethanol (one liter ethanol/ 900 ml 13,000 rpm supernatant) to a rapidly stirred volume of supernatant. The resulting mixture was allowed to stand overnight at -16° C. This mixture was then centrifuged at 10,000 rpm for 30 minutes at 3° C. After removal of the supernatant, which was found to be inactive, the precipitate was washed successively with 85, 95 and 100% ethanol and diethyl ether. The preparation was allowed to dry in the air at room temperature. It was observed that the alcoholic precipitate could be stored with no loss of activity in the refrigerator until used.

Acid Extraction

Acid extraction of the 50% alcoholic precipitate was accomplished by homogenizing 200 mg of the precipitate/10 ml of 0.25 M sulfuric acid with the use of a teflon homogenizer. All insoluble material was removed by centrifugation at 10,000 rpm for 30 minutes

at 3° C. Additional fractionation occurred by bringing the acidic solution to pH 3.5 with 10M potassium hydroxide whereby inactive material was precipitated. During overnight dialysis versus cold, distilled water (4° C), a precipitate formed in the dialysis bag. Centrifugation (10,000 rpm, 30 minutes, 3° C) separated the insoluble material. This insoluble material was found to contain no significant activity. The dialyzed supernatant was lyophylized to dryness and stored in the freezer until used.

Fractionation on DEAE-Cellulose

Cellulose ion exchange columns were prepared by employing the basic concepts of Peterson and Sober (31). The cellulose was washed in a large excess of distilled water. After the cellulose was allowed to settle for 15 minutes, the excess water and fines were decanted. This procedure was repeated. The next two washes were made with 0.1M sodium hydroxide and 0.1M hydrochloric acid respectively. These solutions were removed by filtration. The cellulose was finally washed to a neutral pH with water.

DEAE-cellulose was equilibrated with excess 0.01M acetate buffer, pH 5.05 (initial buffer) and poured to give a final column size of 1.7 × 13.5 cm. The column equilibration and pouring was done in the cold room at 4° C. Column chromatography on DEAE-cellulose was carried out on a sample of lyophylized acid extract

(16 mg) in 0.05M Tris, pH 7.5, buffer. A stepwise increase in acetate ion concentration from 0.01-0.1M (pH 5.05) was employed to elute the protein from the column. The final elutant was 1M sodium chloride in 0.1M acetate, pH 5.05 buffer. A flow rate of 0.1 ml/minute was employed. Fractions of 3 ml were collected. The column was run at 4°C.

Fractionation on BioRex 70

BioRex 70 (200-400 mesh) in the sodium form was washed with excess 0.1M potassium phosphate buffer, pH 7.0. The fines which did not settle upon standing for 15 minutes were removed by decantation. Removal of fines was repeated one time. Columns of two sizes were prepared--1.7 × 10 cm and 2.5 × 41 cm. The prepared columns were washed with 200 ml and 1000 ml, respectively, of 2M sodium chloride in 0.1M potassium phosphate, pH 7.0 buffer and finally were washed free of salt by eluting the columns with 0.1M phosphate buffer pH 7.0 until the effluent was free of chloride ion. Earlier test samples were run on the smaller of the two columns and later experiments, which provided samples for enzymatic characterizations, involved the use of the larger column.

Once the column was free of chloride ion, lyophylized protein was dissolved in 0.1M potassium phosphate buffer, pH 7.0 (initial buffer), and placed on the ion exchanger. An equal volume of initial

buffer was used to wash the protein onto the column. The column was eluted with 40 ml of initial buffer followed by a 120 ml, 0-2M, linear gradient of sodium chloride in the initial buffer. Protein loads of 15-71 A_{280} units were applied to the smaller column, whereas loads of 110 to 209 A_{280} units were placed on the larger columns.

The flow rate on the smaller columns was 1 ml/minute and the larger columns flowed at the rate of 1.2 ml/minute. Fractions of 2.5 ml were collected at room temperature with the use of a Techicon automatic fraction collector.

Specificity Assays

Homopolymers as Substrates

When poly C, poly U, poly G, poly A and poly I were used as substrates for ARNase I, ARNase II and pancreatic RNase, 0.1, 0.1, 0.327, 0.327 and 0.327% (w/v) solutions, respectively, replaced the 1.0% sodium RNA. Incubation periods of five and 15 minutes for poly U, 5, 15, and 60 minutes for poly C, and 195 minutes for poly G, poly A and poly I were employed. In the case of poly U 1.5 ml of a saturated ammonium sulfate solution was used to stop the reaction. All other homopolymer reactions were stopped as previously described.

Dinucleoside Phosphates as Substrates

Dinucleoside phosphate activity of ARNase I was determined employing 0.1% solutions of UpC, CpC, GpC and ApC respectively, in place of 1.0% sodium RNA. The reactants were incubated for 24 hours in the reaction solution which contained 50 μg enzyme/ml. The reaction was stopped by immediately placing the incubated material on ice and then separating the protein from the nucleotides and nucleosides on a BioGel P-2 desalting column. Pancreatic RNase A was incubated with CpC under similar conditions. Substrate and enzyme blanks were also run as controls.

The products from hydrolysis of CpC were desalted and separated on BioGel P-2. Each peak was evaporated to dryness on a Buchi vacuum evaporator at 30° C. The dried samples were dissolved in 1 ml of water and applied to a previously prepared Cellex E column. The nucleotides were eluted in a linear gradient (0-0.20M) of lithium chloride in water (Tener (44)). Fractions of 6.2 ml were collected at a rate of 0.72 ml/minute.

The nucleic acid peaks were pooled, respectively, desalted and chromatographed on paper for identification.

Sodium RNA as a Substrate

A 90 minute assay of the action of ARNase I on 0.1% sodium RNA was carried out according to the procedure of Markham and Smith (24). The enzyme concentration was 1 μg protein/ml reaction solution, so that the initial reaction products would be studied. The reaction solution (8 ml) was incubated at 37° C in a shaking water bath. Material diffusing out of the dialysis membrane during incubation was collected in 100 ml of distilled water. At the end of 90 minutes, the reaction was stopped by the addition of 6.6 ml 10% TCA. After separation of the insoluble material by centrifugation, the acid soluble supernatant was measured spectrophotometrically at 260 mμ, concentrated and desalted on BioGel P-2. Absorbtion at 260 mμ of the dialysant was measured and the 100 ml volume was concentrated to 2 ml and desalted on BioGel P-2. The salt free fractions of dialysant and dialysate were separately pooled, concentrated and subjected to paper chromatography. The dialysant sample was chromatographed with the ammonium sulfate and the isobutyric acid: ammonium hydroxide: EDTA solvents. The dialysate sample was subjected to the isoproponol:water:ammonia solvent. The bands which were separated during chromatography were eluted in water and their spectra were recorded.

Miscellaneous Procedures

Purification of Sodium RNA

Purification of the commercial sodium RNA was achieved by adding 95% ethanol to a 2% solution of sodium RNA in 0.1M sodium chloride until the mixture became cloudy. The mixture was allowed to set overnight at -16° C. The resulting precipitate was removed by centrifugation at 3,000 rpm for 30 minutes at 3° C. The precipitate was then washed with 85, 95, and 100% ethanol and finally with diethyl ether. The purified sodium RNA was air dried at room temperature.

Discontinuous Vertical Gel Electrophoresis

Discontinuous vertical gel electrophoresis was run under alkaline pH conditions (stacking at pH 8.9) according to the method outlined in the E-C Bulletin Volume II, No. 1, 1966. Stock solutions were prepared as follows:

a. Running buffer, pH 8.9

Tris 46 gm

Conc. HCl approx. 4 ml

The Tris and concentrated HCl were dissolved in 500 ml distilled water. Diluted HCl was added in order to adjust the

solution to pH 8.9. The solution was brought to 1 liter with water.

b. Spacer Buffer, pH 6.7

Tris 7.5 gm

Conc. HCl approx. 4 ml

The buffer was prepared in the same way as was buffer A.

c. Electrode Buffer, pH 8.3

Tris 1.2 gm

Glycine 5.8 gm

Sufficient water was added to the Tris and glycine to make a final volume of 2 liters.

d. Plug and Running Gel Solution, 7.0%

The plug and running gel solution was prepared by dissolving 14.0 gm polyacrylamide and 0.2 ml N, N, N', N'-tetramethylethylenediamine (TMED) in sufficient buffer A to make 200 ml. Immediately preceding the pouring of the gel, 0.1 gm ammonium persulfate was added with vigorous stirring.

e. Spacer Gel Solution, 4.0%

Polyacrylamide (4.0 gm) and TMED (0.1 ml) were dissolved and brought to 100 ml by the addition of buffer B. Ammonium persulfate (0.1 gm/100 ml) was added with rapid stirring just before using.

The EC 410 cell was placed at a 45° angle and 80 ml of plug gel

solution was poured between the cooling plates. This gel was overlaid with 5 ml water and allowed to polymerize for 20 minutes. The water was removed and the cell was placed in a vertical position. After rinsing out any remaining water with running gel solution, 70 ml of running gel solution was poured between the cooling plates and the gel was overlaid with water. After 20 minutes the water was removed and the excess was rinsed away with spacer gel. The cell was placed horizontally, the slot former was inserted and 100 ml of spacer gel was added to the top of the running gel. The gel was allowed to set for 20 minutes. Electrode buffer was added to each of the electrode compartments, the excess spacer gel and slot former were removed and 0.1 ml of the sample mixture (protein solution plus 20% sucrose in Sephodex G200) was added to each sample slot. The voltage was maintained at 400v for one hour. When electrophoresis was completed, the gel slab was removed, stained in amido black stain (1.0% in 7% acetic acid) and destained on the EC 489 destainer.

For acidic pH conditions (stacks at pH 4.5) the method of Resifeld, Lewis and Williams (34) was adapted for use in the EC 470 vertical gel cell. Alterations of the Reisfeld technique are as follows:

a. An EC 470 vertical gell cell was used instead of small glass columns.

b. The small pore gel was 8% polyacrylamide and the large

pore gel was 4% polyacrylamide.

c. The large pore gel was polymerized by the addition of 0.1 gm ammonium persulfate/100 ml of gel solution. For the small pore solution, 0.14 gm ammonium persulfate plus 0.21 gm sodium sulfite/100 ml gel solution was added.

d. The protein samples were mixed with a 20% sucrose-Sephadex G-200 solution. This mixture was then applied to the sample slots.

e. Electrophoresis was carried out by applying a current of 180 ma for 90 minutes.

f. After electrophoresis was completed, the gels were stained for a three hour period.

g. The gels were destained in an EC 489 destainer.

BioGel P-2 Desalting Column Chromatography

BioGel P-2 (200-400 mesh), 18 gm, was equilibrated overnight with 300 ml water at room temperature. A 1.5 × 27.5 cm column was prepared by slowly adding the equilibrated gel to the column at room temperature. The column was washed with distilled water until a zero absorbancy at 260 mμ was obtained. Nucleotide solutions were adjusted to pH 7 and desalted on the column using water as the elutant. Fractions of 5.9 were collected at a rate of 1 ml/minute at room temperature.

Cellex E Column Chromatography of Nucleotides

Cellex E was pretreated in a manner similar to the pretreatment of the DEAE-cellulose. After the separation of fines, the acid and alkaline washes, the Cellex E was washed to a neutral pH with water. A thin slurry of Cellex E and water was slowly added to a column of 1 cm diameter until a height of 20 cm was reached. The column was washed with water until a zero absorbancy at 260 mμ was obtained. The lithium chloride method of Tener et al. (44) was used to elute the nucleotides from the column. Fractions of 6.2 ml were collected at a rate of 0.8 ml/minute at room temperature.

DNase Activity Assay

ARNase I was assayed for DNase activity at pH 5.0 and 7.0 according to the method of Kunitz (19). The enzyme was incubated at both pH's for 30 minutes.

Alkaline Phosphatase Activity Assay

The method of Garin and Leventhal (12) was used to measure the ARNase I preparation for alkaline phosphatase activity. The enzyme was incubated with p-nitrophenyl phosphate for 30 minutes.

RESULTS

Purification of Enzyme

Fractionation with Ethanol

As a result of the fractionation of 2250 ml crude homogenate by ethanol (50% v/v), 29 gms of dried precipitate were obtained. The dried precipitate was stored in the refrigerator. Storage periods of up to six months caused no loss of activity by the enzyme.

Acid Extraction

The dried alcoholic precipitate was extracted with sulfuric acid as previously described. While adjusting the pH of the acidic solution to pH 3.5, a precipitate formed. This insoluble material was removed and determined to have no activity. After complete neutralization, the solution was assayed to be 202 fold purified. At this point in the purification scheme, a 3.5 fold increase in the total number of units of activity was noted (Table 1). The total number of activity units of the neutralized acid extract was considered to be 100%.

Table 1. Purification of ARNase I and ARNase II.

	Purification Step	Volume (ml)	Units	Total Units	% Yield	Specific Activity	Fold Purification
I	Crude Homogenate	2250	.5	11,474		.45	1
II	Acid Extraction - Neutralization	1072	3.7	39,661	100	92	202
III	Dialyzed Acid Extract	1262	2.7	34,062	86	133	295
IV	BioRex 70						
	Peak I-A	13.6	32.0	4352	11	46	104
	Peak I-B (ARNase II)	27.0	48.0	12,960	33	300	667
	Peak II Supernatant	39.4	8.9	3507	9	330	733
	ARNase I	14.0	41.5	5810	15	2075	4611

Dialysis of Acid Extracts

The next step in the purification scheme was dialysis of the neutralized acid extract against cold, distilled water. During dialysis a precipitate formed within the dialysis bag. It was removed from the soluble solution and found to contain only a small trace of activity. The supernatant contained 86% of the activity and was purified 295 fold. In order to concentrate the large volume of dilute protein solution, the dialized acid extract was lyophylized to dryness. No loss of activity was observed as a result of lyophylization.

DEAE-Cellulose Chromatography

A solution of lyophylized protein in buffer was subjected to DEAE-cellulose column chromatography (Figure 1). The elution pattern shows two peaks of activity. The first active peak (fractions 5-10) came off in the initial buffer (0.01M acetate, pH 5.05) whereas the second peak (fractions 155-165) was removed by 1M sodium chloride in 0.1M acetate, pH 5.05 buffer. The former peak exhibited a three fold increase in purification. The latter peak gave no increase in activity. There was a 100% recovery of protein and an 83% recovery of activity from the column. This chromatographic procedure was abandoned since the BioRex 70 column gave essentially the same recovery of activity, but a greater increase in purification.

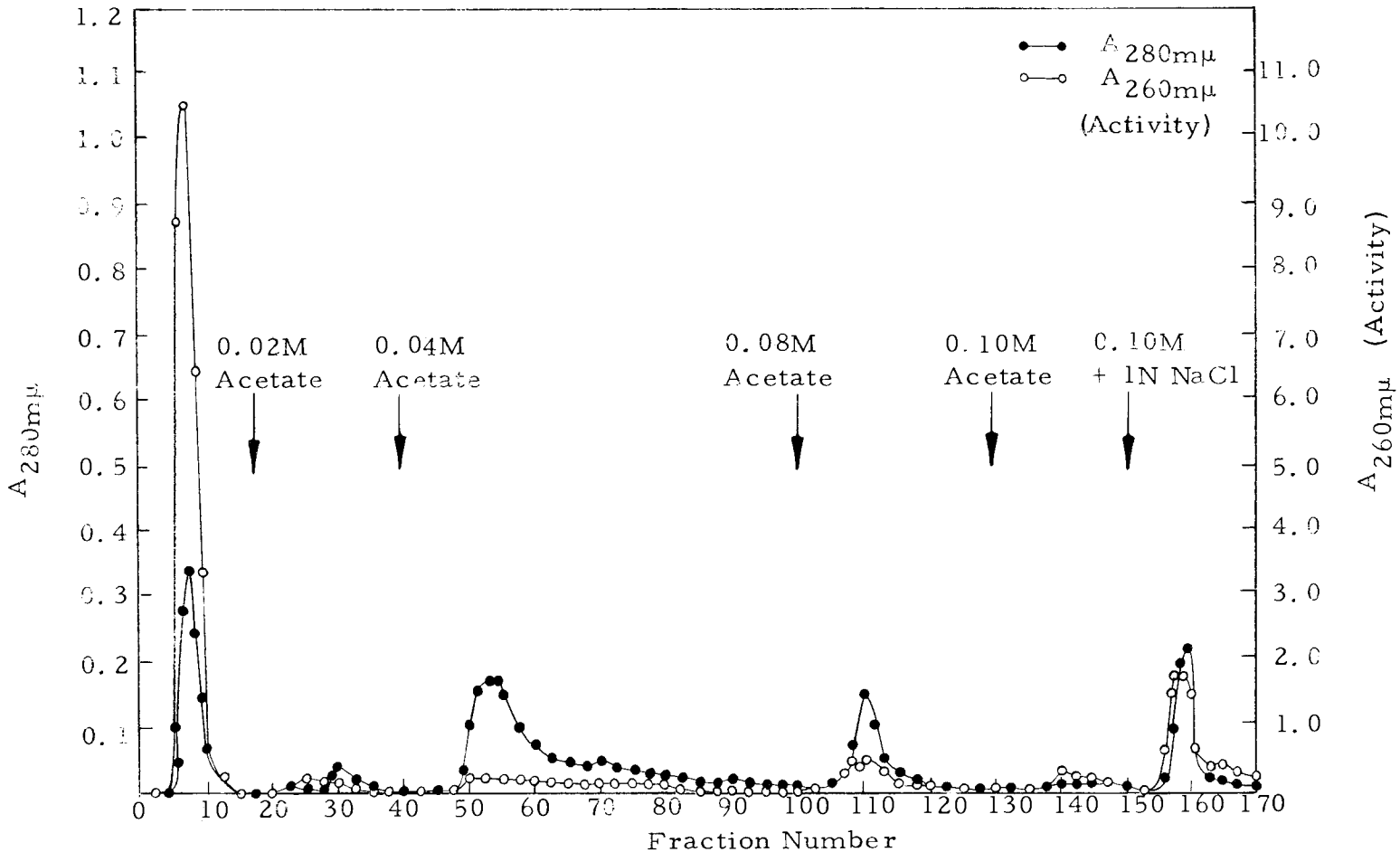

Figure 1. DEAE-cellulose column chromatography of dialized acid extract.

BioRex 70 Column Chromatography

When a solution of dissolved lyophylized protein was subjected to column chromatography on BioRex 70, a weakly acidic cation exchanger, the activity was separated into two major fractions (Figure 2). The first of the two major fractions was eluted by the initial buffer (0.1M phosphate, pH 7.0) in fraction numbers 15-28 (Peak I). No effort was made to further fractionate the material in tubes 15-28. It was, however, separated into two parts, tubes 15-19 (Peak I-A) and tubes 20-28 (Peak I-B or ARNase II). Tubes 15-19 and 20-28 were pooled, respectively, and dialyzed against cold, distilled water. An 11% yield of total activity and a 104 fold increase in activity was observed in Peak I-A. Peak I-B (ARNase II) showed a 33% yield of total activity and a 667 fold purification.

The second major peak of activity was in tubes 60-73 (Peak II). Peak II was much more strongly bound to the column than was Peak I and was eluted from the column over a range of 1.4 to 1.9M sodium chloride. On smaller columns (1.7 × 10 cm) Peak II came off over a range of 0.8 to 1.4M sodium chloride. Tubes 60-73 were pooled and dialyzed against cold distilled water. As a result of dialysis, material fell out of solution. After assaying the Peak II supernatant solution it was found to contain 9% of the total activity and was 330 fold purified. The precipitate was found to be soluble in 0.5M sodium

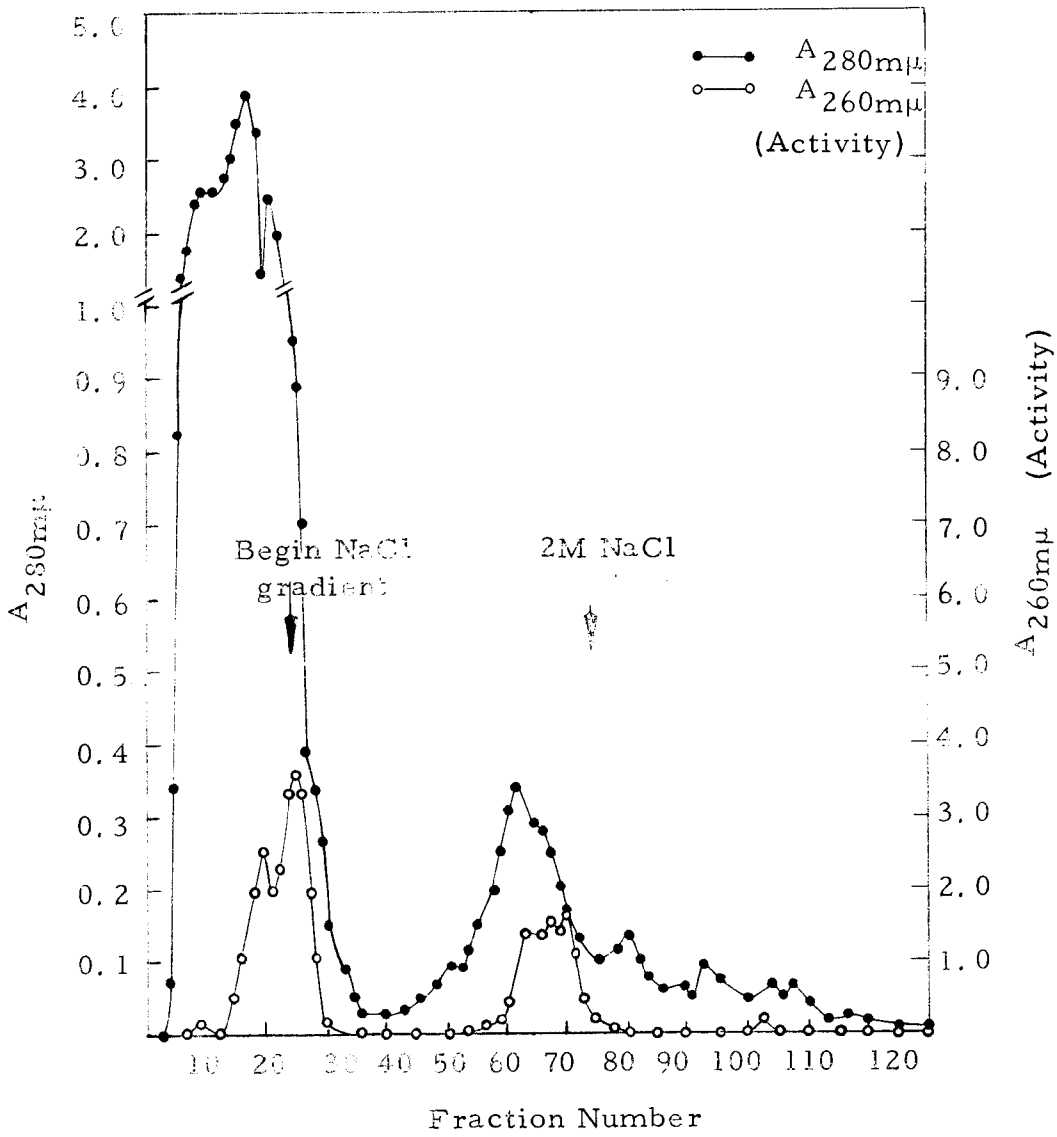

Figure 2. BioRex 70 column chromatography of dialyzed acid extract.

chloride. This solution, which contains ARNase I, represents a yield of 15% of the total activity. The enzymatic activity of ARNase I corresponds to a 4, 611 fold purification.

Summing the activity of the two major peaks from the BioRex 70 column gives a 68% yield of initial activity. This one step in the purification scheme gave a 79% yield of activity. When measuring column recovery with respect to A_{280} units, a 110% recovery was calculated.

Homogeneity Analysis

Discontinuous Vertical Gel Electrophoresis

The migration of ARNase I on polyacrylamide discontinuous gel electrophoresis under acidic conditions was compared to pancreatic RNase and chymotrypsinogen (Figure 3). It was observed that ARNase I ran slightly faster than pancreatic RNase which, in turn, ran faster than chymotrypsinogen. Only one band of protein was observed in the case of ARNase I. ARNase II (Figure 4), under acidic conditions, separated into several bands, none of which migrated as far as did ARNase I. Under alkaline conditions (Figure 5), no bands were observed when ARNase I was applied to the gel and ARNase II separated into several bands.

Top: Samples from left to right (1, 2) 12 μg Chymotrypsinogen, (3) 15 μg Pancreatic RNase in 0.5M NaCl, (4) 15 μg ARNase I, (5) 15 μg Pancreatic RNase, (6, 7) 15 μg ARNase I, (8) 23 μg Chymotrypsinogen.

Bottom: Left - 20 μg ARNase I, Right - 20 μg Pancreatic RNase.

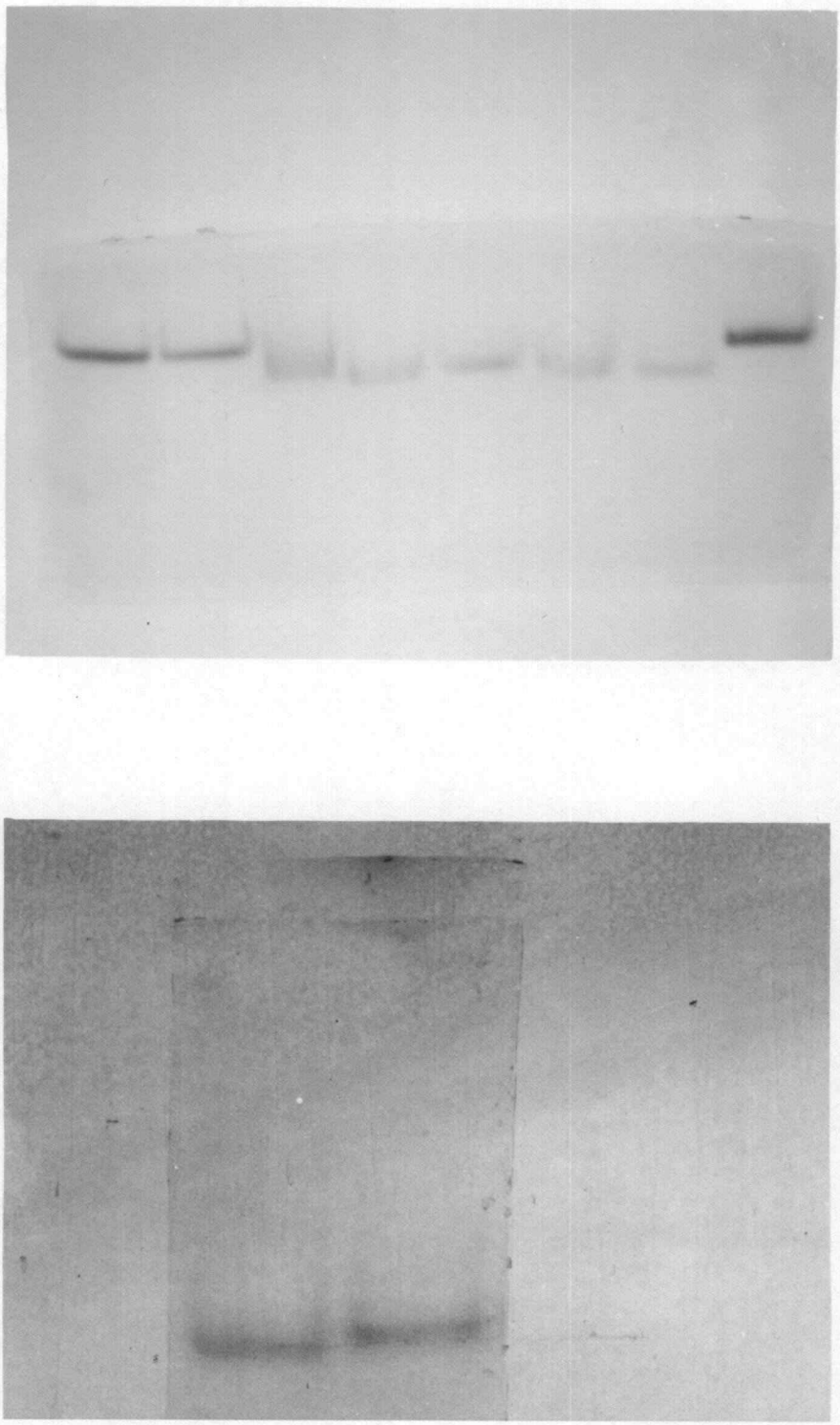

Figure 3. Comparative acid gel electrophoresis of ARNase I, pancreatic RNase and chymotrypsinogen.

Samples from left to right (1, 2) 8 μg, PRNase, (3) 8 μg ARNase I, (4) 40 μg Peak II supernatant, 11 μg, (5) 40 μg Peak I B (ARNase II) (6) 70 μg Peak I A (7) 50 μg dialyzed acid extract (8) 80 μg crude homogenate.

Samples from left to right (1, 2) 8 μg PRNase, (3) 8 μg ARNase I, (4) 40 μg Peak II supernatant, 11 μg, (5) 40 μg Peak I B (ARNase II) (6) 70 μg Peak I A (7) 50 μg dialyzed acid extract (8) 80 μg crude homogenate.

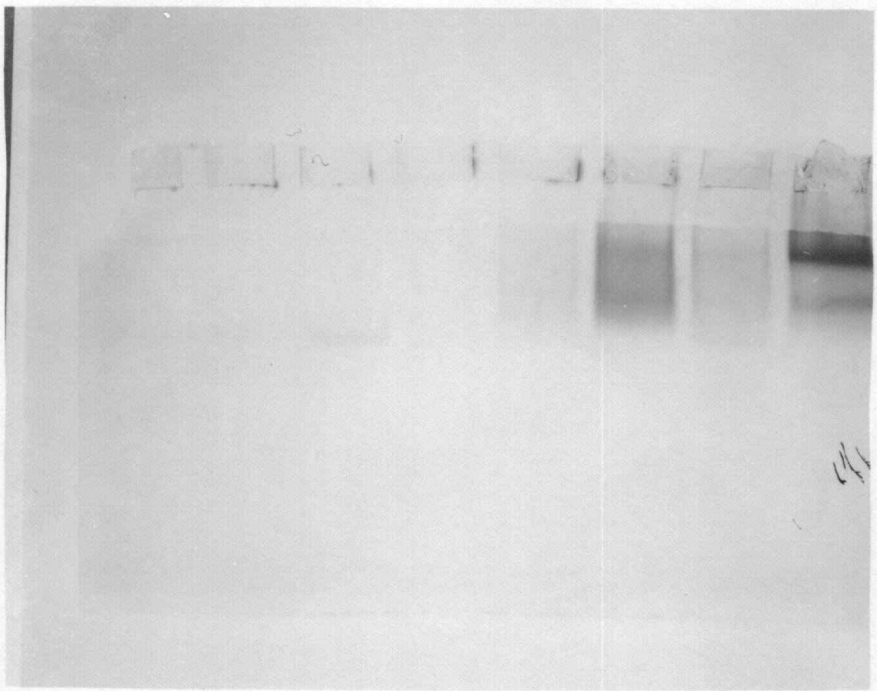

Figure 4. Comparative acid gel electrophoresis of ARNase I with partially purified fractions.

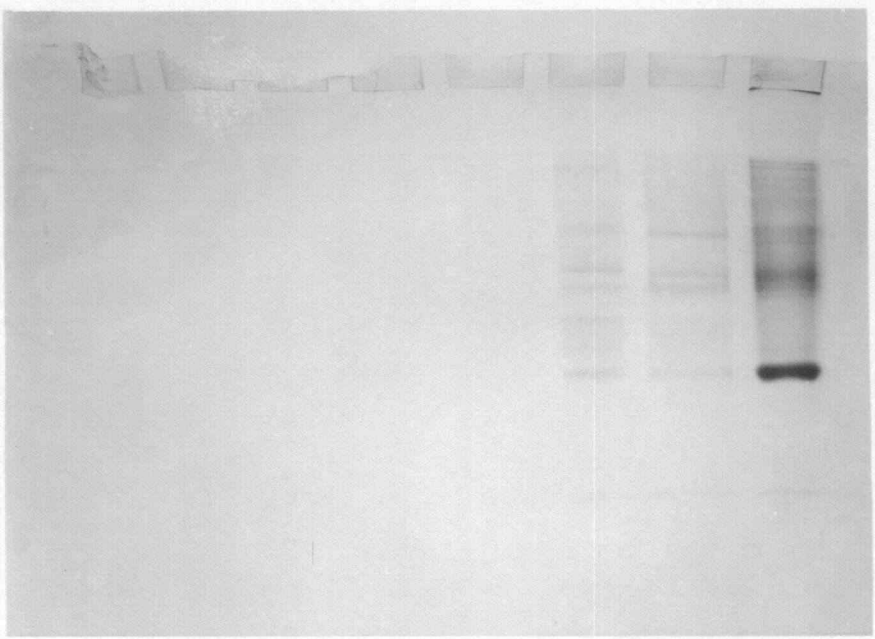

Figure 5. Comparative alkaline gel electrophoresis of ARNase I with partially purified fractions.

DNase and Alkaline Phosphatase Activities

ARNase I was found to be free of any DNase activity when assayed at pH 5.0 and 7.0 for 30 minutes. No alkaline phosphate activity was observed when the enzyme preparation was incubated for 30 minutes with p-nitrophenyl phosphate.

Reaction Parameters

All of the activity assays were run according to the procedure as outlined in Methods with the exception that the parameter being studied was allowed to vary.

Effect of Reaction Time on Activity

ARNase I solutions were diluted in order to give a final concentration of 1 μg protein/ml of reaction solution. It can be seen from the results plotted in Figure 6 that the production of acid soluble nucleotides under these conditions is linearly related to the reaction time over a period from zero to eight minutes.

Effect of pH on Activity

An ARNase I concentration of 1 μg protein/ml reaction solution was employed in the assay. In assaying activity versus pH, the following buffers were substituted for 0.1M phosphate, pH 7.0: 0.1M

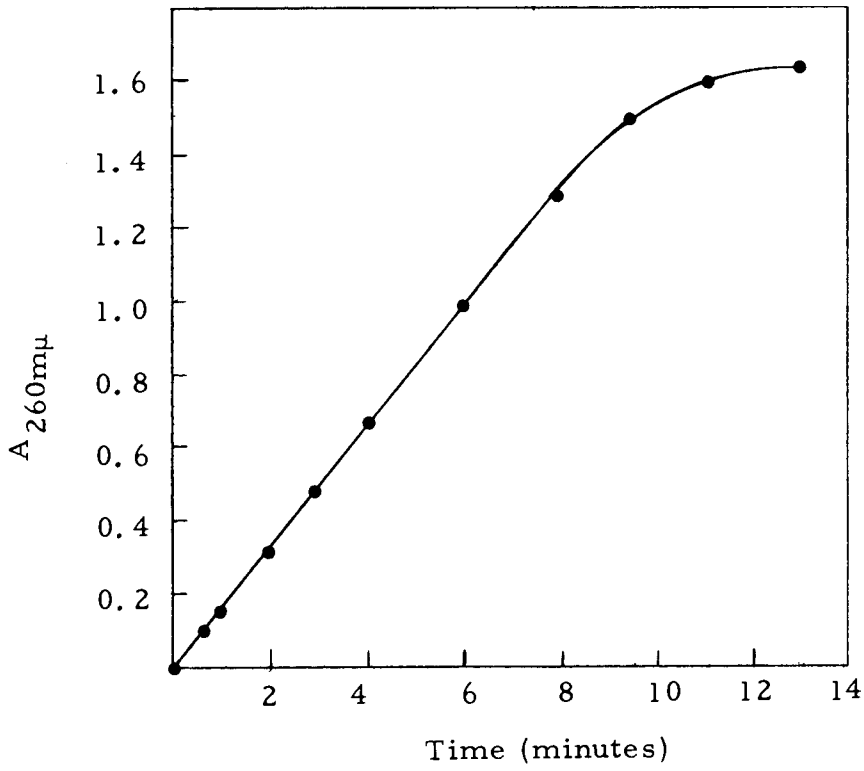

Figure 6. Sodium RNA hydrolysis by ARNase I as a function of time.

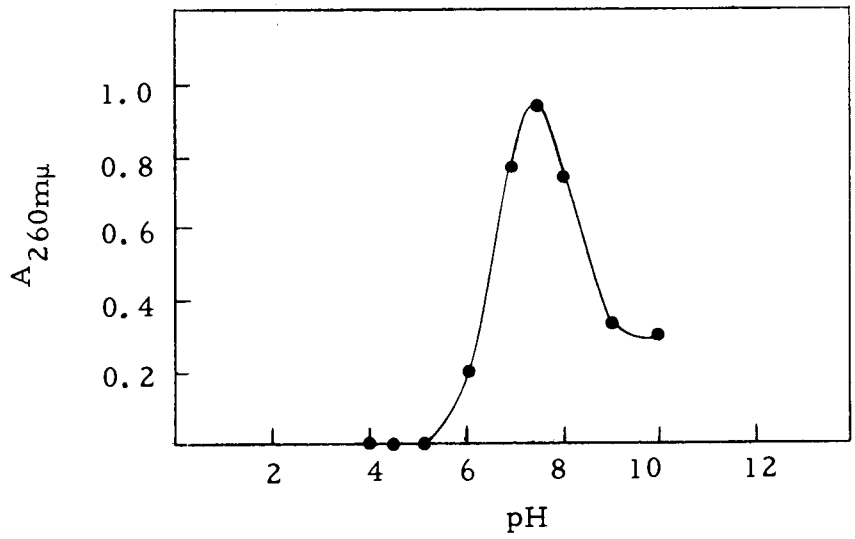

Figure 7. Sodium RNA hydrolysis by ARNase I as a function of pH.

acetate (pH 4.0-5.1), 0.1M phosphate (pH 6.2-7.5), 0.1M Tris-HCl (pH 8.0-8.8), and 0.1M glycine (pH 9.8). The data (Figure 7) show that the enzyme has a pH optimum at 7.5 and no activity at pH 4.0-5.1. At pH 9.8 the enzyme retains 35% of its optimal activity measured at pH 7.5.

Effect of Enzyme Concentration on Activity

When measuring activity as a function of ARNase I concentration (Figure 8), it was observed that a linear relationship exists over a range from 0.25-1.50 μg protein/ml of reaction solution.

Effect of Temperature on Activity

All reagents except the enzyme were brought to the desired temperature. At zero time, a solution of 1 μg ARNase I/ml reaction solution was added to the temperature equilibrated solution. Optimum temperature for the reaction is approximately 60° C (Figure 9). At 95° C the enzyme exhibits only 2% of the activity measured at 60° C.

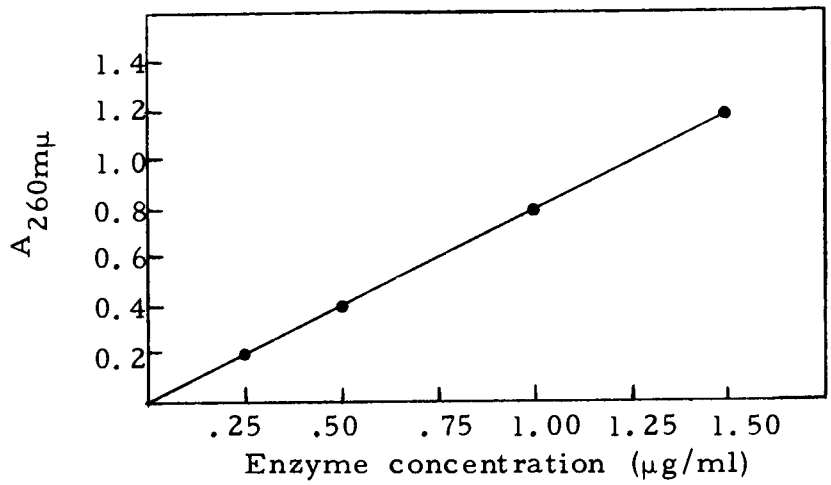

Figure 8. Sodium RNA hydrolysis by ARNase I as a function of enzyme concentration.

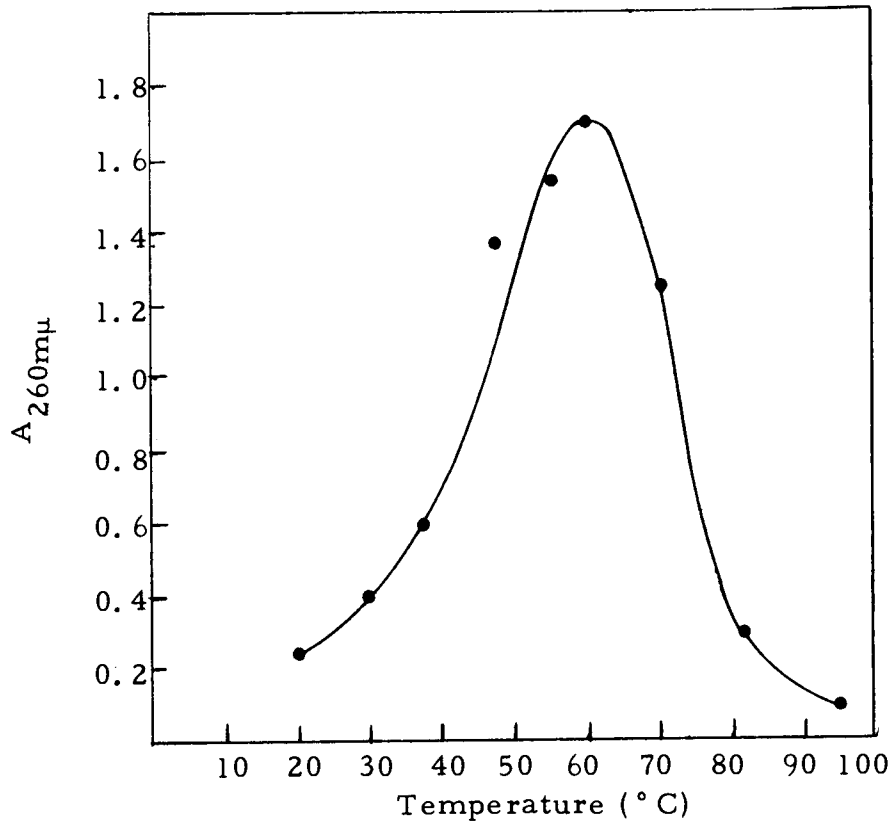

Figure 9. Sodium RNA hydrolysis by ARNase I as a function of temperature.

Specificity

Homopolymers as Substrates

Reactions using poly C, poly U, poly A, poly I, and poly G as substrates were run. The data in Table 2 give the results of the hydrolysis of these homopolymers by ARNase I, ARNase II and pancreatic RNase. ARNase I is 24 times as active on poly U as on poly C when measured at 1μg enzyme/ml reaction solution for five minutes. Under these conditions ARNase I is also just as active as pancreatic RNase on poly U. ARNase I is inactive toward poly A, poly I and poly G when reacted for three hours 15 minutes at an enzyme concentration of 50μg/ml. Pancreatic RNase was shown to react with poly A under these same conditions, but not with poly I or poly G. ARNase II was measured to be seven times more active on poly C as on poly U at 1μg protein/ml for five minutes.

Dinucleoside Phosphates as Substrates

CpC, UpC, ApC and GpC were reacted individually as substrates for ARNase I in a 24 hour incubation. In each case the ARNase I concentration was 50 μg/ml. Products were either desalted on a BioGel P-2 column or spotted for paper chromatography

Table 2. Homopolymers as substrates.[1]

Enzyme	Substrate	Enzyme Concentration (μg/ml)	Time (minutes)	$A_{260m\mu}$
ARNase I	Poly C	1	5	0.042
			15	0.116
			60	0.51
	Poly C	7	5	0.442
			15	0.83
	Poly U	1	5	1.01
	Poly I	50	195	0.004
	Poly G	50	195	0.004
	Poly A	50	195	0.000
	NaRNA	1	5	0.280
ARNase II	Poly C	1	5	0.79
	Poly U	1	5	0.12
			15	0.68
PRNase	Poly C	1	5	0.87
	Poly U	1	5	0.99
	Poly I	50	195	0.000
	Poly G	50	195	0.002
	Poly A	50	195	0.79

[1] Concentrations of homopolymers were - poly C - 0.1%, poly U - 0.1%, poly I - 0.33%, poly G - 0.33%, poly A - 0.3 %, NaRNA - 0.1% shown as a comparison.

directly after incubation. During the 24 hour incubation there was no measureable hydrolysis of the substrates in the absence of enzyme.

CpC. Chromatography on BioGel P-2 of the CpC plus ARNase I reaction solution separated the products into two peaks, fractions 5-7 (Peak I) and 8-10 (Peak II) (Figure 10). On desalting the pancreatic RNase reaction solution, the products of its action on CpC were also separated into two peaks, fractions 6-8 (Peak III) and 9-11 (Peak IV) (Figure 11). Each of the four peaks was separately pooled, concentrated and chromatographed on Cellex E. Standard solutions of CpC, Cp, C, pC, and Cp! were chromatographed in a similar manner. The elution patterns of the standards and peaks I, II, III and IV are shown in Figures 12 and 13. By comparison to the standards, peaks II and IV correspond to standard C. Peak I was eluted at a fraction number similar to standards CpC and Cp!. Peak III was separated into two peaks, the faster moving peak IIIa chromatographed similarly to standards Cp! and/or CpC. The slower peak IIIb corresponds to standards Cp and/or pC. The identities of the Cellex E fractions were confirmed by paper chromatography in the following manner:

After Cellex E chromatography, peaks I and II (from ARNase I reaction) and peaks III and IV (from pancreatic RNase reaction) were subjected to paper chromatography with the ammonium sulfate

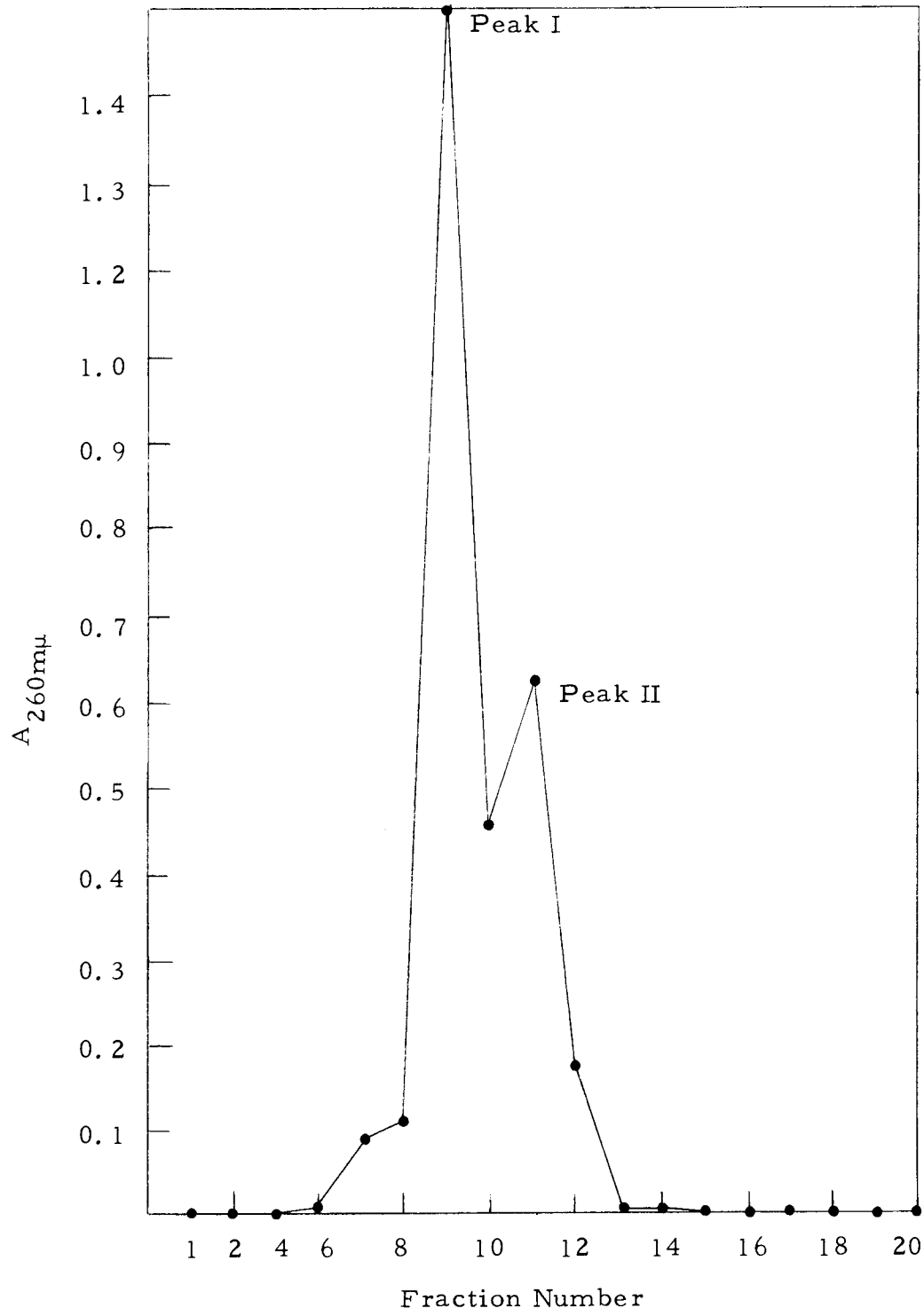

Figure 10. BioGel P-2 column chromatography of CpC-ARNase I hydrolysis products.

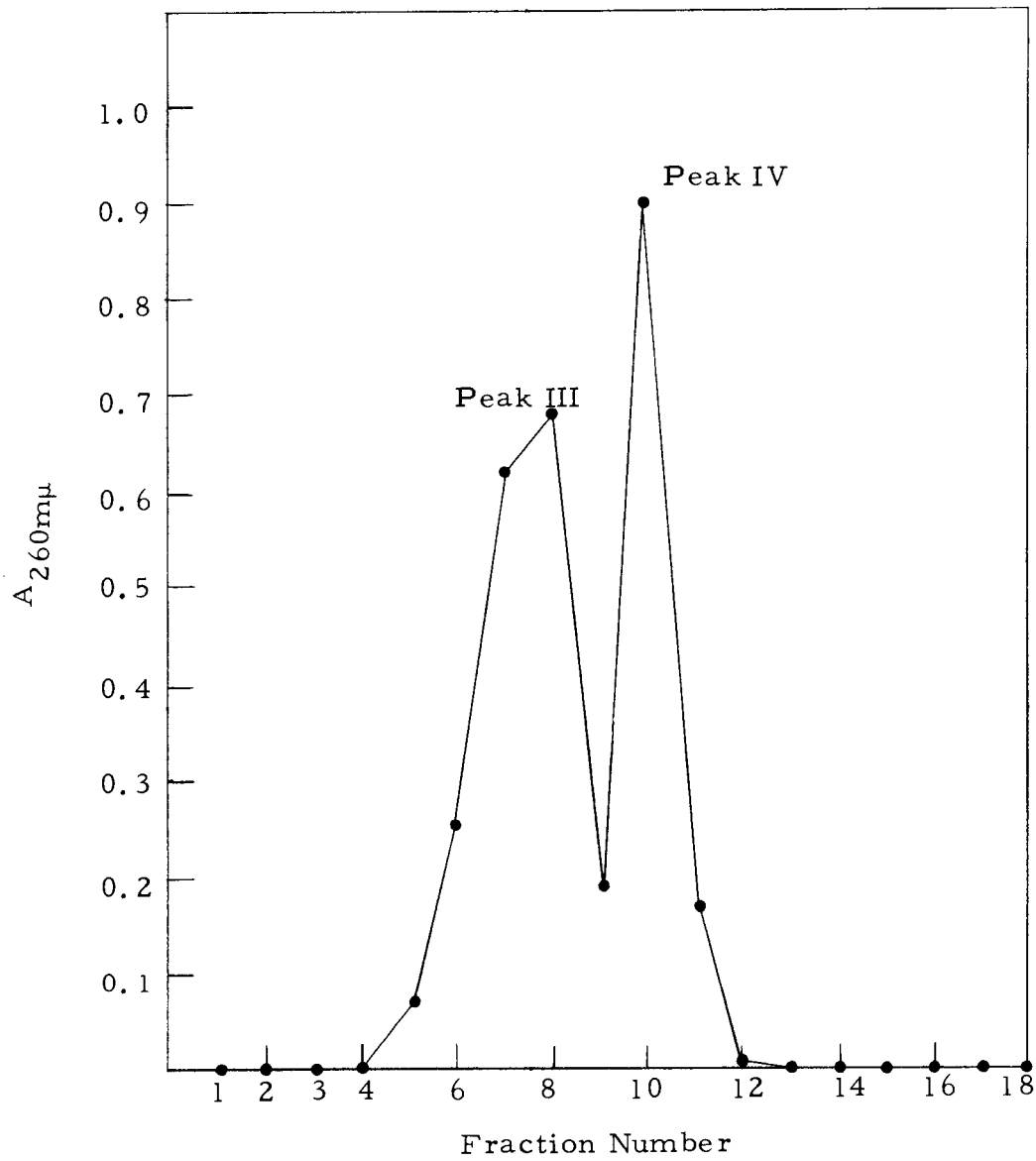

Figure 11. BioGel P-2 column chromatography of CpC-Pancreatic RNase hydrolysis products.

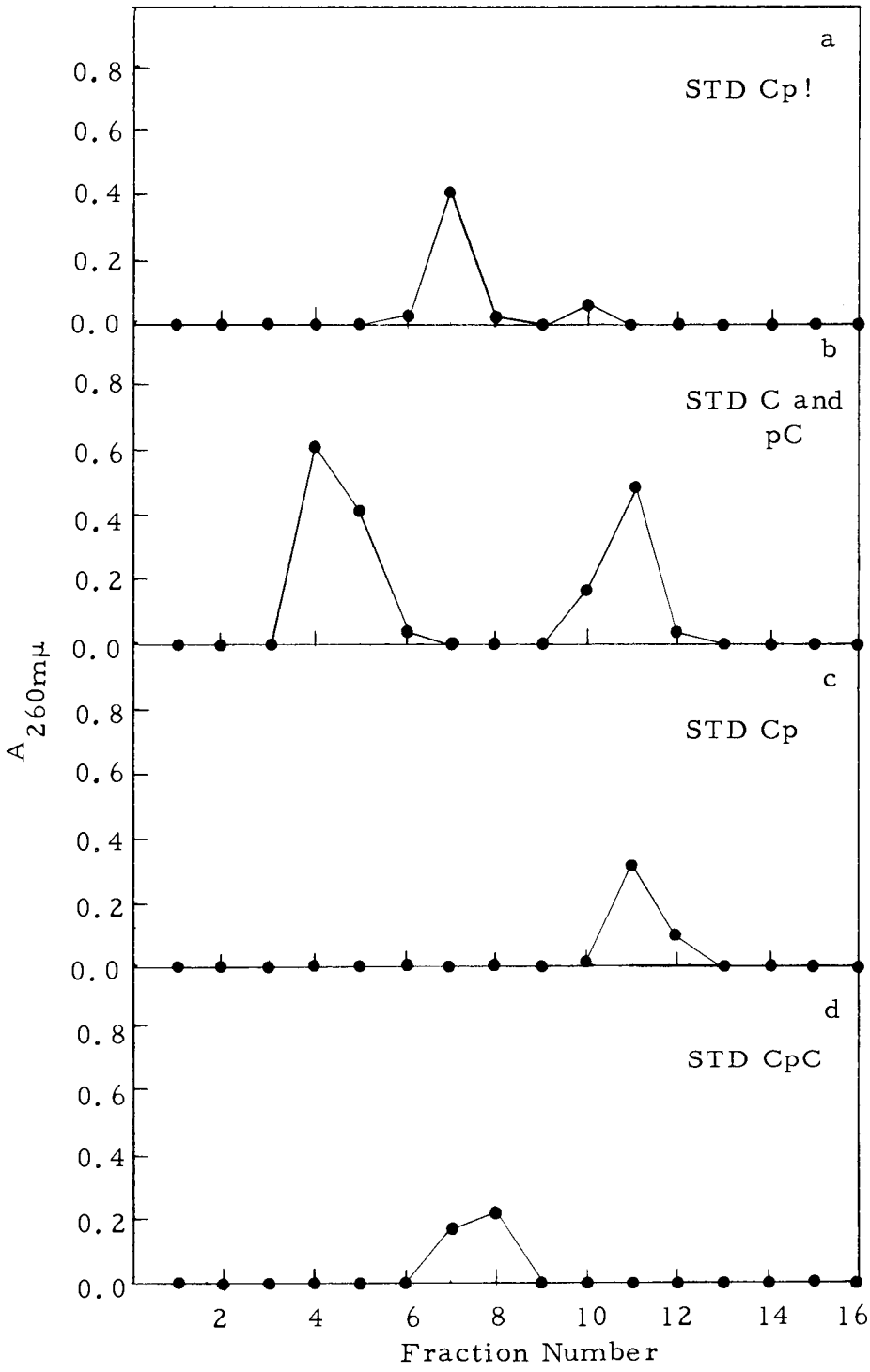

Figure 12. Cellex E column chromatography of standards (a) Cp!, (b) C and pC, (c) Cp, (d) CpC.

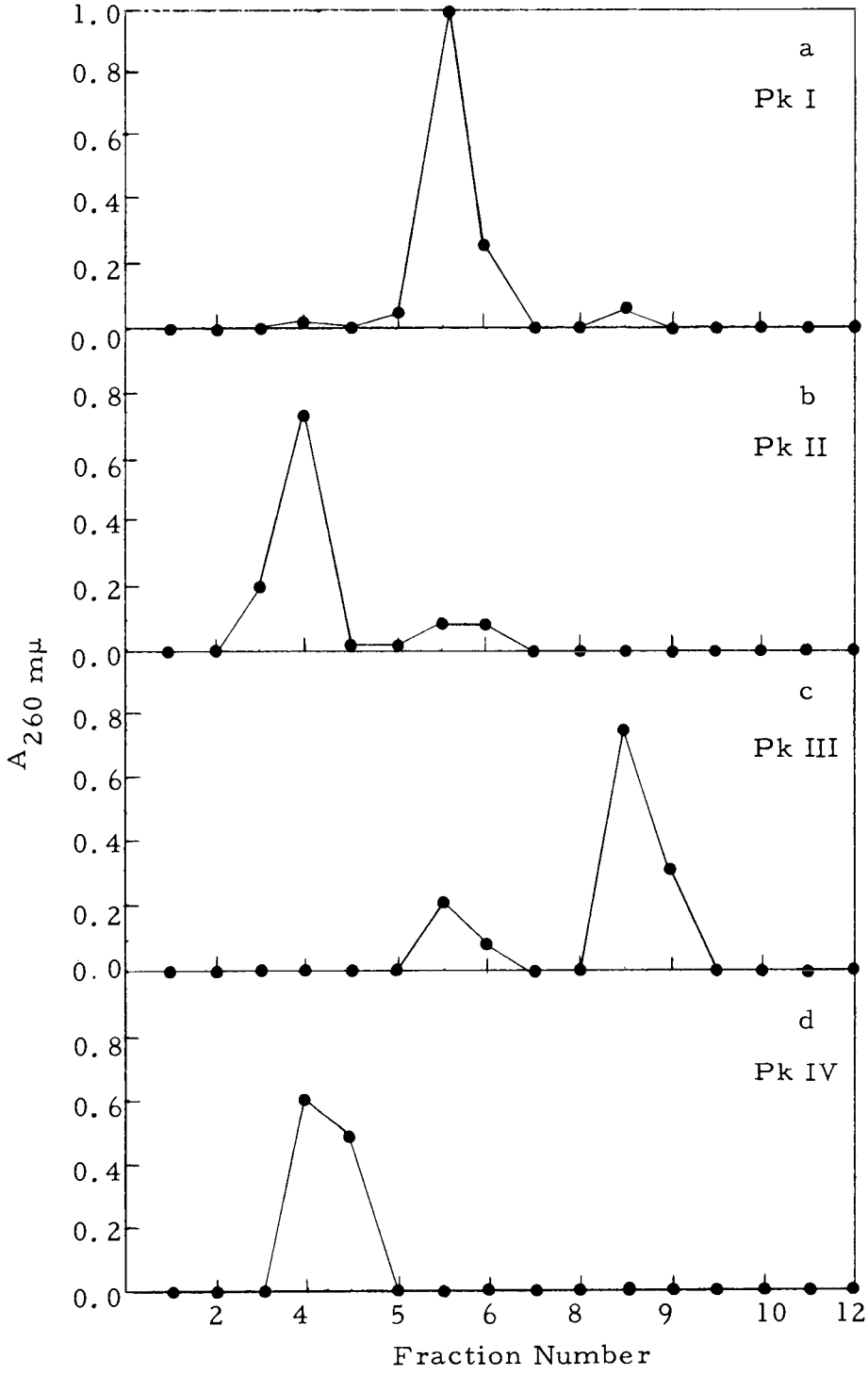

Figure 13. Cellex E column chromatography of BioGel P-2 Peaks (a) I, (b) II, (c) III, (d) IV.

solvent (Figure 14). Peaks II and IV were determined to be cytidine. This is in agreement with the data from Cellex E chromatography. Peak IIIb was identified to be 2' and/or 3'-cytidylic acid. Peak IIIa was found to migrate with a similar R_f as did standard CpC and 2'-3'-cyclic cytidylic acid. CpC and 2', 3'-cyclic cytidylic acid cannot be separated by this chromatographic procedure. It is important to point out that in this solvent system no 2' or 3'-cytidylic acid or 5'-cytidylic acid was detected in Peak I of CpC-ARNase I hydrolysis.

Further investigation of the reaction products of CpC plus ARNase I was undertaken by paper chromatography in the isopropanol:water:ammonia solvent system. Reaction products were chromatographed directly after incubation. Spotted were standards C, Cp, Cp! and CpC (50 μg each), CpC - ARNase I reaction solution (30μg) plus standard CpC (50μg) and CpC - ARNase I reaction solution (30μg). The compounds in the CpC-ARNase I hydrolysate were determined to be CpC, C, Cp! and Cp (Figure 15). A second chromatogram which confirmed the presence and position of Cp and Cp! was run in the same solvent system. In this experiment (Figure 16), a mixture of the CpC reaction products plus standard Cp! (50 μg) and a mixture of CpC reaction products plus standard Cp (50 μg) were co-chromatographed. In the co-chromatography, the location of standards was possible because the standards represented, in each case, over 60% of the ultraviolet absorbing material originally

Top: Samples from left to right, (1) Standard C, (2) Peak I, (3) Standard Cp!, (4) Peak I, (5) Peak I, (6) Standard CpC, (7) Peak IIIa, (8) Standard Cp, (9) Peak IIIb, (10) Standard pC.

Bottom: The samples were chromatographed in ammonium sulfate - 0.1M phosphate buffer, pH 7.0 solvent

Bottom: samples from left to right (11) Standard C, (12) Standard CpC, (13) Standard pC, (14) Standard Cp, (15) Standard Cp!, (16) CpC reaction substrate blank, (17) CpC plus ARNase I reaction solution, (18) ARNase I protein blank, (19) Peak II, (2) Peak IV, (21) PRNase protein blank. The samples were chromatographed in ammonium sulfate-0.1M phosphate buffer, pH 7.0 solvent.

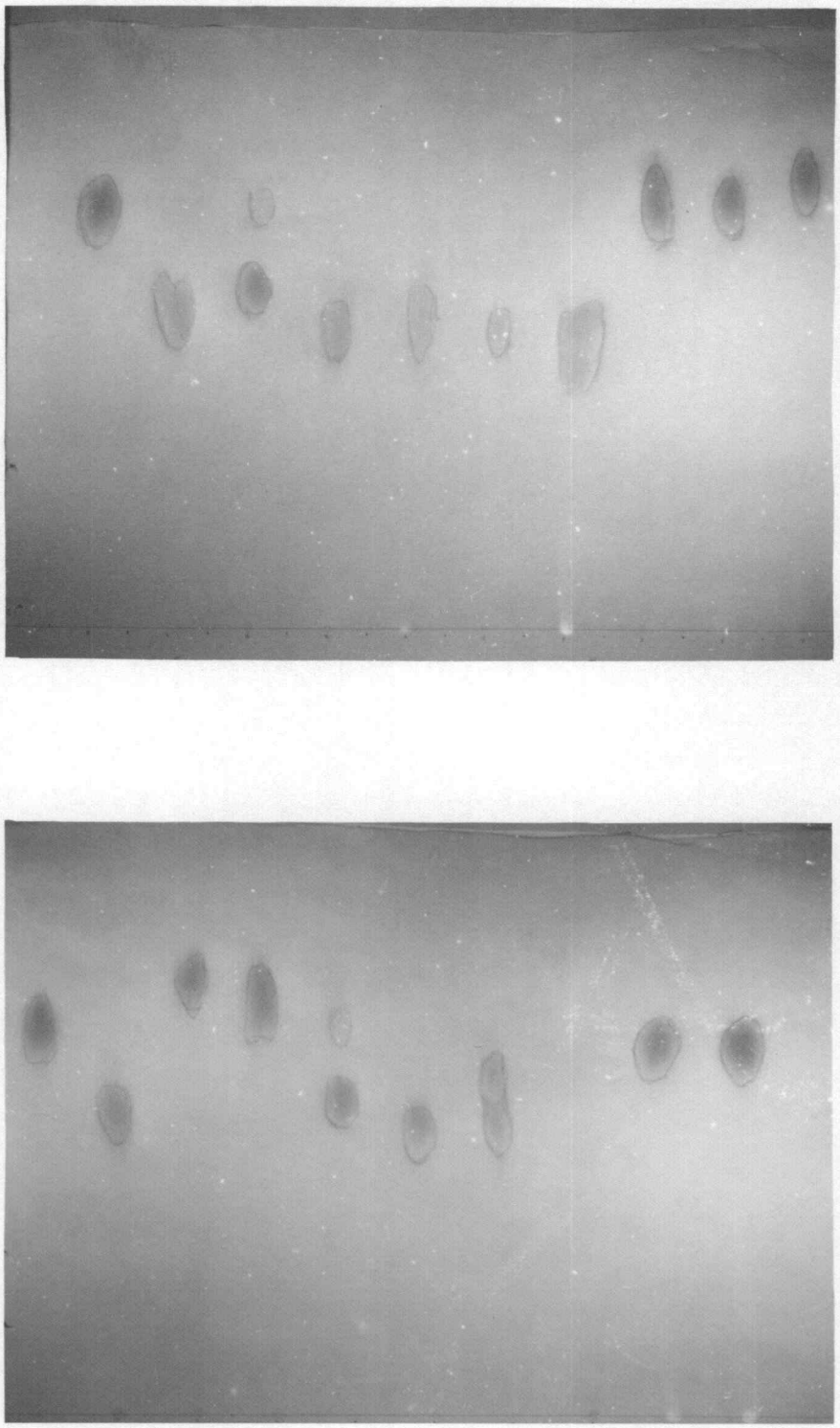

Figure 14. Ascending paper chromatography of Cellex E chromatographed BioGel P-2 peaks I, II, III and IV of CPC-ARNase I and CpC-pancreatic RNase hydrolysis.

Samples from left to right: (1) Mixed standards Cp and C, (2) Standard Cp!, (3) Standard CpC, (4) Mixed standards - CpC, Cp!, Cp, C, (5) Standard CpC plus ARNase I - CpC hydrolysate, (6) ARNase I - CpC hydrolysate. The samples were chromatographed in the isopropanol water:ammonia solvent.

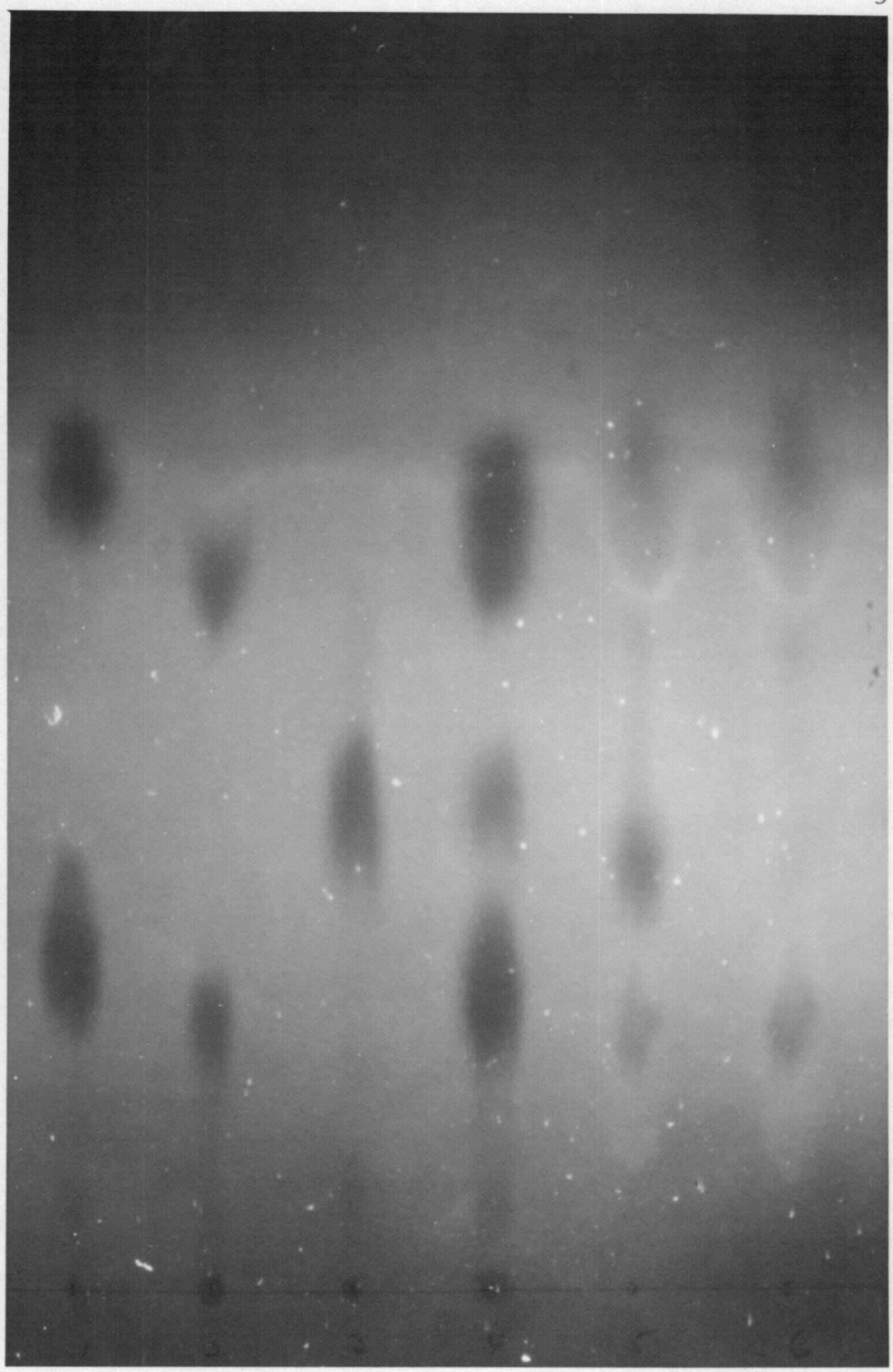

Figure 15. Descending paper chromatography of CpC-ARNase I hydrolysis products.

Left - CpC-ARNase I hydrolysate plus standard Cp!

Right - CpC-ARNase I hydrolysate plus standard Cp. The samples were chromatographed in the isopropanol water:ammonia solvent.

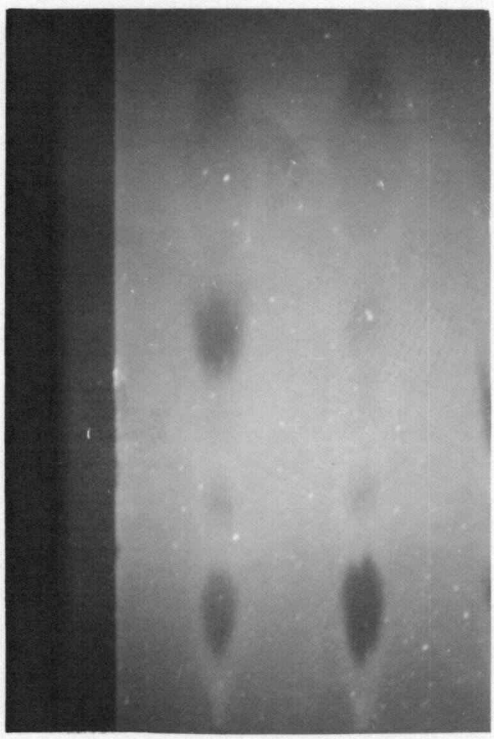

Figure 16. Co-chromatography of CpC-ARNase I hydrolysis products.

spotted. These data show that the slowest moving spot corresponds to Cp and the spot which is third from the bottom is Cp!. Figure 15 locates CpC between these two spots and C to be the fastest moving of all. C migrates to a position on the secondary solvent front. It should be stated that standard Cp! is quite significantly hydrolyzed during chromatography in this basic solvent. Since no Cp was detected in the Cellex E chromatography of peak I, nor was any Cp observed in paper chromatography in the ammonium sulfate solvent system, it is, therefore, determined that any significant Cp in the chromatography by the basic solvent is due to hydrolysis of Cp! by the solvent. The compounds present in the final incubation mixture were determined to be unreacted CpC and the hydrolysis products Cp! and C.

Figure 13a shows a minor peak from the Cellex E chromatography of Peak I which is eluted in a position corresponding to standard Cp and/or pC. This peak represents less than 3% of the total products and was present in insufficient amounts for further analysis by paper chromatography. These data show that the compounds present in the final incubation solution after the hydrolysis of CpC by ARNase I were as follows: 1) CpC - 26%; 2) 2', 3'-cyclic cytidylic acid - 34%; 3) cytidine - 37%; and 4) 2', 3' or 5' - cytidylic acid - 3%. The pancreatic RNase reaction went to 76% completion.

UpC. Chromatography on BioGel P-2 of the reaction products of UpC plus ARNase I incompletely separated the products into two fractions (Figure 17). The ultraviolet spectra from 320-220 mμ were taken of the two fractions and it was observed that fraction A (tubes 7, 8) had a spectrum which was characteristic mainly of uridine, whereas fraction B's (tubes 9, 10) spectrum was due almost exclusively to the presence of cytidine. The two fractions were separately pooled, concentrated and chromatographed on paper in the ammonium sulfate solvent system. Figure 18 shows that fraction B migrated a distance corresponding to standard C. Fraction A gave an overlapping spot corresponding to standards UpC, Up!, and C. In this solvent system, one cannot separate completely UpC from Up!, nor Up! from C. Standard Up can be separated from standard Up!. No spot in the reaction product migrated to an area corresponding to Up. These data suggest the presence of UpC, Up! and C in fraction A.

It was observed that chromatography on BioGel P-2 resulted in the elution of the reaction solution's buffer salts in fraction A. No significant alteration in migration occurs when nucleotides containing small amounts of salt are chromatographed in the ammonium sulfate solvent system, but a slower rate of migration does take place in the isopropanol: water: ammonia solvent system as a result of the salt. Since fraction A was known to contain small amounts of buffer

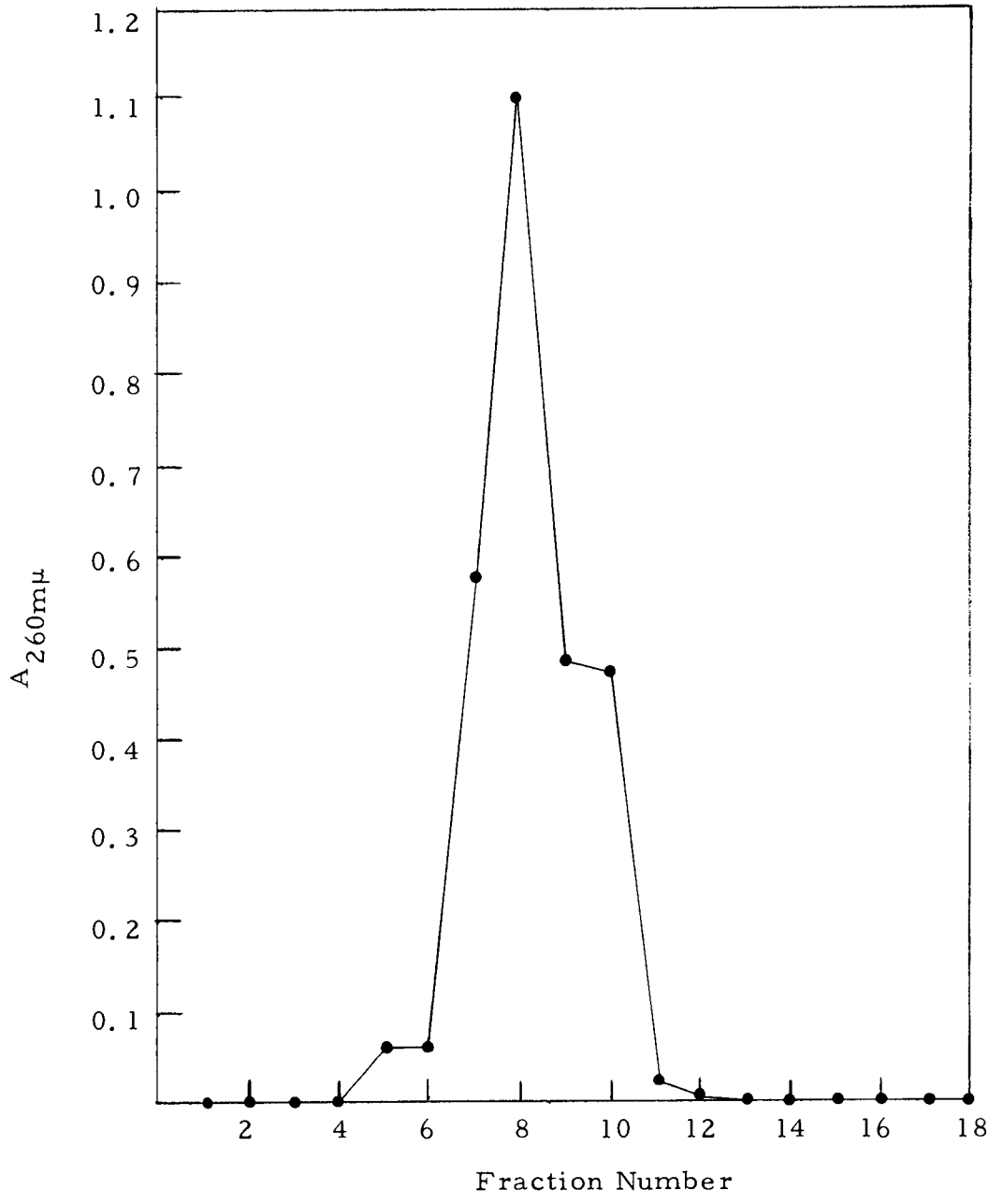

Figure 17. BioGel P-2 column chromatography of UpC-ARNase I hydrolysis products.

Spotted from left to right: (1) Fraction A, (2) Standard Up!, (3) Standard Up, (4) Standard C, (5) Fraction B, (6) Standard UpC, (7) UpC reaction substrate blank. The ammonium sulfate-0.1M phosphate buffer, pH 7.0 solvent was used.

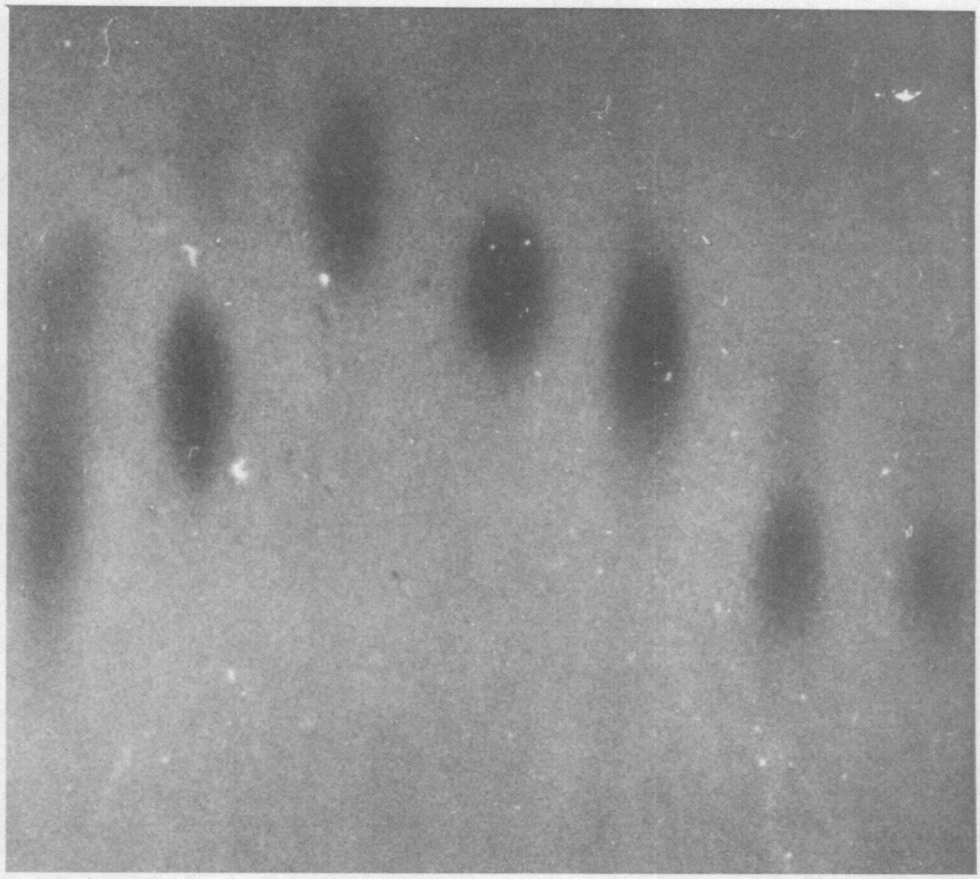

Figure 18. Ascending paper chromatography of BioGel P-2 fractions A and B, from UpC-ARNase I hydrolysis.

salts and since the migration of nucleotides in the isopropanol: water: ammonia solvent is influenced by these salts, cochromatography of standards and fraction A was employed to identify the components of fraction A.

Chromatographed were standards Up, Up! and UpC, fraction A, a mixed spot of fraction A and standard UpC, a mixed spot of fraction A plus standard Up! and a mixed spot of fraction A plus standard Up (Figure 19). In each case 50μg of the standard and approximately 30 μg of fraction A were spotted. In the cochromatography, the location of the standards was possible because the standards represented, in each case, over 60% of the ultraviolet absorbing material originally spotted. Upon chromatographic separation of each mixed sample, the spot which contained the overwhelming majority of 260 mμ absorbing material was determined to be the location of the standard. Fraction A separated into four separate spots when subjected to paper chromatography in the isopropanol: water: ammonia solvent. The three lowest spots were identified by cochromatography to be, from the bottom up, (1) Up (2) UpC and (3) Up! (Figure 19). Chromatographed under identical conditions standard C was shown to migrate with the secondary solvent front (Figure 15), thus the fastest moving spot in fraction A, which was located at the secondary solvent front, was determined to be C.

The chromatographically separated spots of fraction A were

Top: Samples from left to right: (1) Standard Up, (2) Standard Up!, (3) Standard UpC, (4) Mixed Up, Up!, UpC, (5) Standard UpC plus fraction A, and (6) Fraction A.

Bottom: Left - Fraction A plus standard Cp!, Right - Fraction A plus Standard Cp. The samples were chromatographed in the isopropanol water:ammonia solvent.

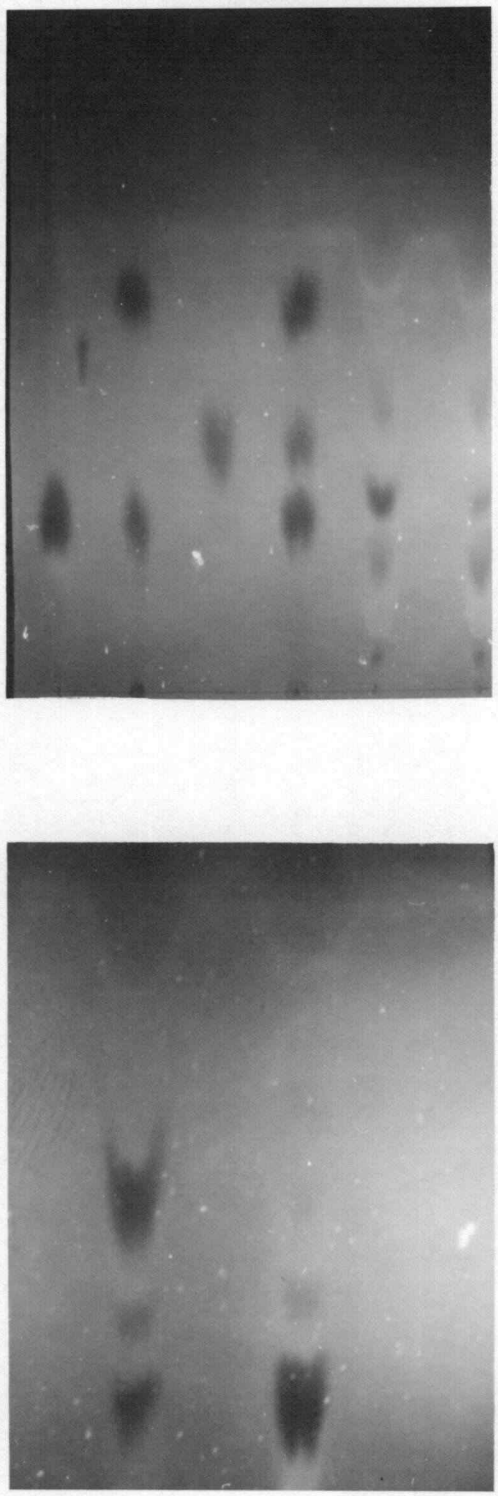

Figure 19. Co-chromatography of UpC-ARNase I hydrolysis products.

eluted and their spectra were recorded. The spectrophotometric confirmation, in each case, agreed with the chromatographic identification of the components of fraction A.

This chromatographic procedure shows the components of fraction A to be UpC, Up and Up!, but since no Up was observed in the ammonium sulfate paper chromatography of fraction A and since significant hydrolysis of standard Up! (Figure 19) occurs in this basic solvent, it was concluded that the presence of Up in the chromatographic separation of fraction A was due entirely to hydrolysis by the basic solvent.

In separate experiments, ARNase I was incubated with UpC for one hour at an enzyme concentration of 50μg/ml reaction solution. Less than 12% of the UpC was hydrolyzed. ARNase I at a concentration of 6μg/ml reaction solution gave no detectable products when incubated for a period of $3\frac{1}{2}$ hours.

ApC and CpC

When ApC and GpC were reacted under the same conditions as were UpC and CpC, no reaction was detected to have taken place (Figure 20).

Spotted left to right: (1) Standard ApC, (2) ApC-ARNase I substrate blank, (3) ApC-ARNase I incubation solution, (4) Standard GpC, (5) GpC-ARNase I substrate blank, (6) GpC-ARNase I incubation solution. The ammonium sulfate-0.1M phosphate buffer, pH 7.0 solvent was used.

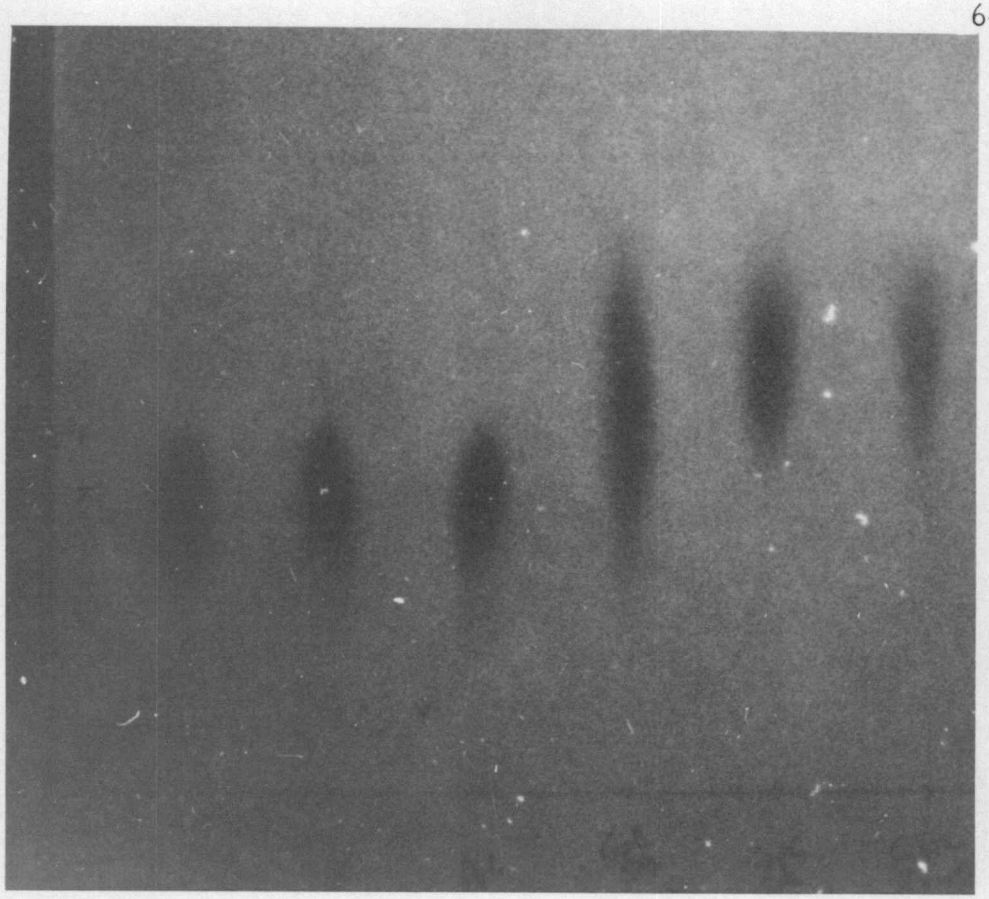

Figure 20. Ascending paper chromatography of ApC-ARNase I and GpC-ARNase I hydrolysis products.

Sodium RNA as a Substrate

The initial reaction products of ARNase I's catalytic action on sodium RNA was studied. It was calculated that 13% of the total available nucleotides in the reaction solution were present in the TCA soluble fraction. By chromatography in the isopropanol:water: ammonia solvent of the acid soluble nucleotides (Figure 21) followed by spectrophotometric measurement of the bands which were eluted with water, it is shown that 84% of the nucleotides remained at or near the origin. Markham and Smith (24) indicate that trinucleotides migrate in the solvent, therefore the nucleotide material at the origin must be at least four nucleotide units long. Material in the dialysant (outside of the dialysis membrane) was insufficient in quantity to give any identifiable mononucleotides.

These data indicate that under the indicated reaction conditions only a relatively small number of phosphodiester bonds are cleaved by the enzyme in the early stages of the reaction.

Left to Right - (1, 2) sodium RNA-ARNase I, 90 min hydrolysate from inside dialysis membrane, (3) mixture of standards Cp, Gp, Up and Ap. The samples were chromatographed in the isopropanol water:ammonia solvent.

Figure 21. Descending paper chromatography of sodium RNA-ARNase I hydrolysis products.

DISCUSSION

ARNase I has been purified 4611 fold when compared to the specific activity in the crude homogenate. The purification involved an ethanolic precipitation of the crude homogenate, acid extraction, isoionic precipitation, BioRex 70 column chromatography and dialysis. The enzyme was found to contain no alkaline phosphatase or DNase activity and appeared to be electrophoretically homogeneous by acid discontinuous polyacrylamide gel electrophoresis.

Noted in the first purification step was a 3.5 increase in the activity of the acid extracted solution when compared to the crude homogenate. This phenomenon indicates that either an inhibition of activity in the crude homogenate or an activation of activity in the acid extracted material occurs. Other investigators have observed alterations of activity between fractionation steps. Dickman, Morrill and Trupin (7) observed that extraction of mouse pancreas with sucrose solutions or with phosphate buffer gave results which were different from those due to extraction of the pancreas by acid. Column chromatography on IRC 50 of the sucrose or phosphate extracts separated the activity into three peaks (I, II, III). Chromatography in a similar manner of acid extracted material gave separation into only two peaks. Upon treatment with acid, peak I is converted to a mixture of peaks II and III. During this conversion the RNase activity of Peak I was

increased two to three fold. These data suggest that acid treatment causes a change in the structure of the enzyme and that this change is concurrent with an increase in activity. Roth and Hurley (35) have reported that an RNase inhibitor which is present in rat liver is likely to be an acidic protein. Binding of the inhibitor to the RNase was suggested to be by means of ionic interactions as well as by hydrogen bonding and Van der Waals attractions. The active site was not involved in the inhibitor-enzyme binding.

Considering the data of Dickman, Morrill and Trupin (7) and of Roth and Hurley (35), one could suggest that in the case of bovine aorta homogenate an inhibitor was present and that the inhibitor was removed by one of the purification steps between the crude homogenate and the neutralized acid extract. The most probable purification step which would account for this phenomenon would be the acid extraction procedure.

During the purification of the RNase activity from bovine aorta, two separate enzyme preparations were obtained. Column chromatography on DEAE-cellulose and BioRex 70 shows that the two enzymes (ARNase I and II) exhibit a large variation in ionic charge. Differences other than ionic charge, which resulted in the chromatographic separation of the two enzymes, were observed when both enzymes were reacted on poly C and poly U. ARNase I shows a 24 fold greater activity on poly U than on poly C while ARNase II

is seven times more active on poly C than on poly U. Comparing the activities of ARNase I to ARNase II at the same level of protein concentration reveals that ARNase I is 18 times less active towards poly C, but seven times more active towards poly U than is ARNase II. These comparisons were made with a purified preparation of ARNase I and a yet impure sample of ARNase II. The difference in purity might account for the lower activity of ARNase II on poly U, but increased purity in ARNase II would enlarge the activity difference between the two enzymes on poly C. ARNase I was measured to be as active as pancreatic RNase on poly U, but 21 times less active on poly C. The enzyme was inactive on poly A, poly G and poly I when measured at enzyme concentrations 50 times greater than that necessary for the degradation of poly C or poly U. Pancreatic RNase, at this same level of enzyme concentration, hydrolyzed poly A, but not poly I and poly G. Beers (2) indicated that pancreatic RNase was capable of depolymerizing poly I. Gamble et al. (11) showed that an enzyme concentration of 180 μg of pancreatic RNase/ml hydrolyzed poly I. Since the reaction conditions employed in the present study were similar to those of Gamble et al., except for the enzyme concentration (Gamble et al. 180 μg/ml, Lewis 50 μg/ml) and since it was observed in the data of Gamble et al. that a much greater concentration of enzyme was required to hydrolyze poly I than poly A, the difference in the results may be due to the

difference in enzyme concentration. By comparing the rate of reactivity of ARNase I on homopolymers with sodium RNA it can be seen that the enzyme is four times more active on poly U, but seven times less active on poly C than on sodium RNA.

A detailed look at the initial products formed in the reaction of ARNase I plus sodium RNA showed that only 13% of the RNA was hydrolyzed. Of the hydrolyzed products the large majority (84%) were at least four nucleotide units in length. The homopolymer and sodium RNA data indicate that a very select specificity is employed by ARNase I and, therefore, there is limited cleavage of phosphodiester linkages in sodium RNA by the enzyme. The homopolymer data show that the preference in specificity is for the hydrolysis of phosphodiester bonds involving a 3'-uridylic acid residue. The presence of di-, tri- and other oligonucleotides in the hydrolysate of sodium RNA after incubation with ARNase I suggests that ARNase I is an endonuclease.

The effects of pH, temperature, time and enzyme concentration on the activity of ARNase I were studied. ARNase I has a pH optimum (7.5) which is similar to most of the animal RNases. In contrast to bovine pancreatic RNase A, but in agreement with aorta RNase isolated by Gamble et al. (11), no activity is observed for ARNase I at pH 5. There is a slight difference in the pH optima of ARNase I and the partially purified aorta RNase of Gamble et al.

which was reported to be around pH 7. When comparing the temperature optima of ARNase I and the aorta RNase, a deviation between the two is noticed (ARNase I - 60°, aorta RNase around 50°). The difference in the pH and temperature optima of the two aortic enzyme preparations may be only apparent since the aorta RNase of Gamble et al. was not in a pure form. Under the reaction conditions employed, ARNase I activity was found to be directly related to the incubation time for a range of acid from zero to eight minutes. After ten minutes the rate of formation of acid soluble nucleotides. starts to decline rapidly and begins to approach a maximum. The enzyme also exhibits a linear relationship with respect to enzyme concentration when observed over a range of 0.25 to 1.50 μg protein/ml reaction solution.

Studies on the products from the action of ARNase I on the dinucleoside phosphates CpC, UpC, ApC and GpC revealed that no reaction took place on ApC or GpC. These findings are consistent with the fact that the enzyme was also inactive on poly A and poly G. A reaction did occur when CpC and UpC were emplcyed as substrates. These results were obtained after a 24 hour, exhaustive hydrolysis in an incubation solution which contained an enzyme concentration of 50 μg/ml reaction solution (50 times that necessary to depolymerize poly U and poly C). With both substrates, CpC and UpC, the nucleotide produced by the enzyme's actions was found to be

the 2', 3'-cyclic phosphate. When UpC was reacted with ARNase I for one hour at an enzyme concentration of 50 μg/ml reaction solution, only 12% of the UpC was hydrolyzed. No reaction was observed when 6 μg enzyme/ml reaction solution were incubated with UpC for $3\frac{1}{2}$ hours. It is to be noted that ARNase I is much less active on UpC than on poly U or poly C. Three differences exist between poly U (poly C) and UpC, (1) poly U (poly C) is a much larger molecule, (2) UpC contains uracil and cytidine whereas poly U (poly C) contains only uracil (cytidine), and (3) the 3' end of poly U (poly C) is a phosphate. Any one or combination of the three differences could account for the observed lower reactivity on UpC than on poly U (poly C).

The data reported in this thesis show that a ribonuclease (ARNase I) has been purified from the bovine aorta. This ribonuclease was found to differ from bovine pancreatic ribonuclease in several respects, the most notable being the products formed upon the hydrolysis of dinucleoside phosphates and the relative reactivity on poly U. ARNase I also differs from ARNase II isolated from the same initial preparation.

SUMMARY

By means of an alcoholic precipitation of the crude homogenate of bovine aortas, followed by acid extraction, pH 3.5 isoionic precipitation, dialysis, BioRex 70 column chromatography with subsequent dialysis, ARNase I was purified 4611 fold. A second active fraction from the BioRex 70 column chromatography, ARNase II, was purified 677 fold.

No DNase or alkaline phosphatase activity was detected in the ARNase I preparation. By analysis with discontinuous gel electrophoresis ARNase I appeared to be electrophoretically homogeneous.

When reacted with 1.0% sodium RNA, ARNase I showed a pH optimum of 7.5 and a temperature optimum of 60° C. The rate of reaction was observed to be linear with respect to time from zero to eight minutes. The activity varied directly with the enzyme concentration over a range of 0.25 to 1.50 μg enzyme/ml of reaction solution.

Poly U was rapidly depolymerized by ARNase I whereas poly C was only slowly reacted upon by the enzyme. ARNase II hydrolyzed poly C 18 times faster, and poly U seven times slower than did ARNase I. By comparison to pancreatic RNase, ARNase I exhibited activity on poly U equal to pancreatic RNase. When activities on poly C were compared, ARNase I was 21 times less active than

pancreatic RNase. Poly G, poly A and poly I were resistant to enzymatic attack.

Dinucleoside phosphates, CpC and UpC, were hydrolyzed by ARNase I. The products of reaction were Cp! and C, and Up! and C, respectively. GpC and ApC were not acted upon by the enzyme.

As a result of the depolymerization of sodium RNA by ARNase I, the vast majority of oligonucleotides in the acid soluble fractions were four or more units long.

This study indicates that ARNase I is an endonuclease which is different from ARNase II and from pancreatic RNase. ARNase I exhibits a specificity for the formation of pyrimidine phosphates during the hydrolysis of polynucleotides. The enzyme shows a preference for the phosphodiester bonds of uridylic acid over cytidylic acid residues.

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