THE PURIFICATION AND PARTIAL CHARACTERIZATION OF ASSOCIATED DEOXYRIBONUCLEASE, RIBONUCLEASE, AND 3'-NUCLEOTIDASE ACTIVITIES OF WHEAT SEEDLINGS

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ABSTRACT

THE PURIFICATION AND PARTIAL CHARACTERIZATION OF ASSOCIATED DEOXYRIBONUCLEASE, RIBONUCLEASE, AND 3'-NUCLEOTIDASE ACTIVITIES OF WHEAT SEEDLINGS

by Douglas M. Hanson

In a preliminary investigation of the deoxyribonuclease activities of a variety of plant sources it was observed that wheat seedlings contained a relatively high level of deoxyribonuclease activity towards denatured DNA. This thesis reports work done in an attempt to purify and to determine the specificity of this enzyme activity. It was hoped that the enzyme might exhibit a relative degree of base specificity and thus be useful in studies on the structure and base sequence of DNA.

A nuclease has been purified from germinating wheat seedlings to an extent of over 830-fold. The purified enzyme preparation hydrolyzes denatured DNA, rRNA, and the 3'-phosphoester linkage of 3'-AMP at similar rates. The preparation is free of detectable contaminating enzyme activities.

The deoxyribonuclease, ribonuclease, and 3'-nucleotidase activities have remained associated throughout a variety of purification procedures including chromatography on several ion-exchange resins, gel filtration, and polyacrylamide disc electrophoresis at pH 9.5 and 8.3. The ratios of the three activities remain essentially constant throughout the last four steps in the purification procedure

as well as throughout all of the above mentioned procedures. The three enzyme activities exhibit a great degree of similarity with respect to a variety of properties. It is tentatively concluded that the three associated enzyme activities are either properties of the same protein or of a very stable complex of two or more proteins.

The deoxyribonuclease activity is highly preferential for denatured DNA. The hydrolysis of denatured DNA was found to be endonucleolytic in manner by a number of criteria. The mononucleotides and presumably the oligonucleotides produced bear a 5'-phosphoryl group. At all stages of digestion investigated, dAMP was the predominate mononucleotide component. The dAMP level was always about 2-fold greater than any of the other three deoxymononucleotides. A very low level of dGMP was observed throughout the digestion and this level actually decreased as a percentage of the total mononucleotide fraction as the digestion proceeded. Essentially all of the deoxyguanylate residues could be accounted for in the oligonucleotide fraction. Thus it would appear that the wheat deoxyribonuclease exhibits a relative degree of base specificity with bonds involving deoxyadenylate residues being preferentially cleaved and bonds involving deoxyguanylate residues being relatively resistant.

The mode of action of the wheat ribonuclease on rRNA was found to be exonucleolytic and the mononucleotides produced appear to be the 2',3'-cyclic compounds.

The association of deoxyribonuclease, ribonuclease,

and 3'-nucleotidase activities seems common in plants and may also be common in other biological sources as well. The biological significance of this association is not clear, but the observation that these activities exist in relatively high levels in germinating, rapidly growing seedlings suggests that these enzymes may play a role in the process of DNA repair or replication.

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D. M. H.

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Dedicated

to

Lorraine and Michael

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INTRODUCTION

Friedrich Miescher, a postdoctoral student of Hoppe-Seyler, was the first to report the extraction of nucleic acid material from the cell (1). That was in 1871 and Miescher called the nuclear material that he had isolated from pus cells "nuclein." In 1889, Altmann (2) proposed the name "nucleic acid" for this material. The years following the discovery of "nuclein" were filled with attempts to determine the chemical composition and structure of this material. Since biochemists seem to have a knack for disrupting things, especially molecules, it was not long before the major components of the nucleic acid material were determined. As is usually the case for most biological materials which have been isolated, enzymes were soon discovered which were capable of degrading the newly discovered nucleic acid material. Thus in 1903, Araki (3) observed that extracts of tissues such as liver, thymus, and spleen had the power to liquefy gels of DNA. Levene and Medigreceanu (3a) introduced the collective term "nuclease" for all enzymes which were involved in the metabolism of nucleic acids or their degradation products or precursors. The pancreatic juice of dogs was also found to contain enzymes which caused the liquefaction of nucleic acid gels (4, 5). This liquefaction occurred without the liberation of nitrogenous bases or inorganic

phosphate. Feulgen (6) made the important observation that degradation of DNA by pancreas preparations stopped at the formation of oligonucleotides and did not yield many mononucleotides. Laskowski (7) and McCarty (8) achieved a considerable purification of the enzyme from pancreas and in 1950 Kunitz (9) crystallized the enzyme and introduced the term "deoxyribonuclease" for those enzymes which are capable of degrading DNA. The pancreatic deoxyribonuclease (DNase) was referred to as DNase I. The term DNase I is presently used to denote a whole class of enzymes from various sources which exhibit optimal activity at alkaline pH.

A DNase which has an optimum pH in the range of 4.5 to 5.5 was reported in spleen by Catcheside and Holmes (10). Maver and Greco (11, 12) reported that a large portion of the DNase activity of thymus gland was due to an enzyme which was different from DNase I-type enzymes. The spleen enzyme has been purified to homogeneity by Bernardi and Griffe (13) and has been called DNase II. DNase II-type enzymes, with acid pH optima, have been isolated and purified from a variety of sources.

Microorganisms have also been a source of a wide variety of DNase activities. These enzymes have recently been reviewed by Laskowski (14) and Lehman (15).

In 1942 Greenstein reported the presence of DNase activity in extracts of the embryos of corn, wheat, pumpkin, sunflower, and lima bean. These activities were capable of depolymerizing thymus DNA. The fact that these plant enzymes could degrade animal DNA was strong evidence that the previous

distinction which had been made between "plant" and "animal" DNA (16, see p. 3) was no longer tenable. It was also of interest that these DNase activities were relatively high in germinating, rapidly growing seedlings. Brawerman and Chargaff (17) partially purified a DNase from germinating barley, using commercial malt diastase as a starting material. The enzyme contained RNase and 3'-nucleotidase activities as well as DNase activity. This enzyme has been more extensively studied by Holbrook et al. (29). Shuster prepared a DNase from rye grass (18). This plant source was also a good source of 3'-nucleotidase activity. DNase activity has also been reported in soybean sprouts (19).

A DNase from mung bean sprouts was reported by Stockx and Van Parijs (20). It was also observed that an RNase and a 3'-nucleotidase activity were associated with the mung bean DNase (21). These activities have been further studied by Sung and Laskowski (22). The properties of these enzymes have been extensively studied by Walters and Loring (23, 24) who concluded that the DNase activity was separate from the RNase and 3'-nucleotidase activity because only the DNase activity decreased on storage of the enzyme preparation. Other interpretations of these results, however, are also possible.

Mukai (25) has reported the presence of a DNase with DNase I-type properties in rice bran. At least two distinct DNase activities exist in germinating garlic (26). Bjork has separated and partially purified two endonucleases from

potato tubers (27). Adams and Fairley have reported the purification of a DNase from muskmelon seed (28). This DNase, an endonuclease, appears to be closely associated with an RNase and a 3'-nucleotidase activities (see footnote 5).

A complete listing and discussion of all the DNases which have been isolated since their discovery in 1903 would fill several volumes due to the vast number and variety of activities which have been demonstrated. DNase activities have been reported in almost every possible type of biological source and it is a safe generalization to say that no source has been found which is completely devoid of DNase activity.

The classification of DNase activities was originally quite simple and four major properties of DNases were selected for the purpose of classification (30). These properties were:

1. Specificity toward the sugar moiety.
2. Exo- versus endonucleolytic mode of action.
3. Cleavage of the internucleotide bond on the 3'-P versus the 5'-P side, thus forming products bearing either 5'- or 3'-monophosphate.
4. Nature of the base adjacent to the susceptible linkage; i.e. base specificity.

While these criteria were sufficient for some years it soon became evident that several other properties of DNases should also be considered for the purpose of classi-

fication (14). These additional properties are:

5. Specificity toward the secondary structure of the DNA; i.e., native (double-stranded) versus denatured (single-stranded) DNA.
6. Inability to attack the DNA from the same species.
7. Inability to hydrolyze dinucleotides.
8. Inability to attack either native or denatured DNA but capacity of hydrolyzing oligonucleotides.
9. Ability to hydrolyze both strands of native DNA simultaneously at the same locus.

It is becoming evident that no classification of DNase activities can be absolute. The wide variety of enzymes which have been isolated indicate that many of these properties mentioned above overlap and that while a particular enzyme may be like other DNases in some of its properties it may be greatly different with respect to some other properties. Also the observation by a number of workers (14) that the specificity and mode of action of many DNases changes during the course of DNA hydrolysis further complicates the placement of a particular enzyme in a definite classification.

Of the criteria listed above only number 3, the cleavage of internucleotide bonds to produce products which bear either a 5'- or 3'-monophosphate terminus, remains absolute. To data no enzyme has been found which can split the internucleotide bond on either side of the phosphorus atom. For a long time it was felt that exo- versus endonucleolytic cleavage was a reliable criteria for classification. It now

appears, however, that under specific conditions an endonuclease may favor an exonucleolytic activity. This has been shown for micrococcal nuclease (31) and may explain why endonucleases such as the one from Neurospora crassa (32) and the wheat endonuclease reported in this thesis produce a significant amount of mononucleotides at all stages in the digestion of DNA. Many DNases have been shown to possess a distinct preference for DNA in either the native (double-stranded) state or for DNA in the denatured (single-stranded) state. Some of these enzymes which exhibit specificity toward the secondary structure of the DNA have recently been reviewed (14, see p. 181).

To underline the complexity of nuclease action let us consider the case of DNases which are relatively preferential for native DNA. It might be expected that the overall mode of action of all DNases of this category would be the same. That this is not the case has been decisively shown by Young and Sinsheimer (33), who compared the early action of DNase I and DNase II on native DNA. By studying under alkaline conditions the density gradient centrifugation patterns of DNA treated with these two enzymes, they were able to show a distinct difference in their mode of action. Splenic DNase II degraded the DNA by simultaneous cleavage of both polynucleotide chains at or near the same position. With DNase I, however, single-stranded cleavages occurred; and, on an average, only one in four cleavages resulted in scission of both chains at the same locus.

Subsequent studies have shown that similar mechanisms exist for other nucleases as well.

One of the major goals of nuclease research has been the isolation of specific nucleases which exhibit an absolute base specificity and can be used in the determination of base sequences in nucleic acids. A milestone event occurred in 1965 when Holley and co-workers reported the complete base sequence of alanine-sRNA (34, 35). This was determined with the extremely specific T_1-RNase. The same type of sequence determination is theoretically possible for DNA, however, DNases are notoriously non-specific. To date no enzyme has been found which exhibits the degree of base specificity observed with some of the RNases. Several enzymes have been reported which are relatively specific, at least in the early stages of DNA hydrolysis, so it is still possible that specific DNases will someday be isolated. The most specific DNases observed to date are all endonucleases. An endonuclease from the hepatopancreas of Octopus vulgaris (36) is preferential for the bond, $pX_{\overline{T}}pC$, where X is any deoxynucleoside residue. The mung bean DNase mentioned previously (22) has preferential activity for bonds involving deoxyadenylate residues, while the endonuclease I from potato tubers is preferential for both deoxyadenylate and deoxythymidylate moieties (37). The N. crassa endonuclease (32) is preferential for bonds involving deoxyguanylate and has the further specificity that bonds involving deoxycytidylate appear to be relatively resistant. It is

possible that meaningful base sequence analysis in DNA can someday be performed using several of these DNases in sequence.

The biological role of DNases has long been a subject of much speculation. Unfortunately, most of the information on this subject is still just that, speculation. In a recent review by Lehman (15), a large amount of circumstantial evidence is compiled in an attempt to demonstrate a role for DNases in the process of DNA repair and replication. Most of this circumstantial evidence was taken from work on bacteria and on phage-infected bacteria. A large number of DNase activities exhibiting a variety of modes of action have been shown to be induced upon infection of a bacteria by a phage particle. Many of these enzymes appear to be involved directly or indirectly in the process of the synthesis of the DNA of progeny phage. The observations mentioned earlier that the levels of many of the plant DNases were relatively high in germinating, rapidly growing plant seedlings also suggests a possible role for these enzymes in DNA repair and replication.

Since few plant sources have been studied in detail for specific DNase activities, an initial investigation was made to determine which plant sources were high in DNase activity. Wheat seedlings were found to be a relatively abundant source of DNase activity toward denatured DNA. This thesis describes the purification and partial characterization of this DNase activity. Some initial findings

on the specificity of the catalytic activity of the enzyme on denatured DNA are also reported. The DNase activity is associated with an RNase and a 3'-nucleotidase activity. Some properties of this complex of activities are described.

EXPERIMENTAL PROCEDURE

Materials

Genessee Wheat (Triticum aestivum L.) treated with the fungicide, methyl mercury dicyandiamide, was used for all enzyme preparations and was purchased from the Michigan Farm Bureau, Lansing, Michigan. All chemicals were standard reagent grade materials. Ammonium sulfate (J. T. Baker, Granular) was routinely sieved through a 40 mesh screen immediately before use.

Salmon sperm DNA[1] (Type III), 5'- and 3'-AMP, 2'- and 3'-UMP, Tris (Trizma base) buffer, snake venom 5'-nucleotidase, and N-ethylmaleimide were purchased from Sigma Chemical Company. Oligouridylic acid, pancreatic ribonuclease (5x crystallized), dithiothreitol, bis-p-nitrophenyl phosphate, iodoacetic acid, and iodoacetamide were purchased from Calbiochem. Acrylamide and bis-acrylamide for polyacrylamide gel electrophoresis were purchased from Canalco. DEAE-cellulose, ECTEOLA-cellulose, P-60, and P-30 were purchased from Bio-Rad.

DEAE-Sephadex A-25 was purchased from Pharmacia, and DEAE-cellulose chromatography paper (DE-81) from Whatman.

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[1]The abbreviations are those specified by the Journal of Biological Chemistry. The following additional abbreviations are used: TEAB, triethylammonium bicarbonate; dDNA, denatured DNA; TCA, trichloroacetic acid; DNase, deoxyribonuclease; RNase, ribonuclease.

Snake venom phosphodiesterase and alkaline phosphatase (BAPC grade) were purchased from Worthington. Orcinol and N-bromosuccinimide were products of Fisher Chemical Company. Diphenylamine and triethylamine were purchased from Eastman Kodak. The diphenylamine was twice crystallized from benzene before use in the colorimetric assay for DNA. Triethylamine was converted to triethylammonium bicarbonate by the following procedure. To 280 ml of twice redistilled triethylamine was added 520 ml of cold distilled water and the solution was thoroughly mixed in the cold. The solution was placed in an ice-bath and CO_2 from a dry ice generator was slowly bubbled through the solution with stirring. After about one hour the rate of CO_2 bubbling was increased and continued until the pH of the solution was between 7.4 and 7.8. The solution was then diluted to 1 liter with cold water. This gave a stock solution of 2 M triethylammonium bicarbonate which was then stored at 4^{o}. This solution was stable for only about two weeks at 4^{o}.

The 2', 3'-cyclic UMP was the generous gift of Dr. F. M. Rottman. The ryegrass 3'-nucleotidase was prepared in this laboratory by Dr. A. B. Adams according to the procedure of Shuster (18). The synthetic substrates, 5'-p-nitrophenyl thymidylate, 5'-p-nitrophenyl adenylate, and 3'-p-nitrophenyl thmidylate were prepared in this laboratory by R. E. Jagger. High molecular weight ribosomal RNA was prepared from commercial yeast by the method of Crestfield, Smith, and Allen (38).

Phosphocellulose (cation exchange resin, 0.9 meq per g) was a Sigma product. It was routinely prepared for chromatography as follows. The resin was suspended in water, allowed to settle for 20 minutes and suspended material removed by decanting. This process was repeated 5 or 6 times. The resin was then washed alternately with 0.1 N KOH and water until the KOH wash was colorless. The resin was next extensively washed with water and finally with 95% ethanol. The ethanol-washed cellulose was spread on sheets of aluminum foil and allowed to dry in the air at room temperature. This dry powder was then stored in a brown bottle. Immediately before use in a column the dry cellulose powder was suspended in sodium acetate buffer, 0.05 M pH 4.5. The other cellulose resins used in this work were prepared in a similar manner.

Dialysis tubing, a product of Union Carbide Corporation, was routinely boiled in a 0.2 M Na_2CO_3, 0.01 M EDTA for 1 hour, and then washed with water. This was repeated until the wash solution was no longer yellow (usually 3 to 4 boiling cycles). The tubing was then extensively washed with deionized water and stored in 50% ethanol at 4°.

The dialysis tubing used for the dialysis of the lanthanum nitrate-acid soluble products was size 23 and designated as "small pore" tubing. It was also prepared as described above.

The following buffers were used throughout this work and are designated Buffer A and Buffer B in the text. Buffer

A, 0.05 M sodium acetate, pH 4.5, 1 mM in zinc acetate, 2 mM in cysteine; Buffer B, same as A except, 0.1 mM in Zinc acetate.

Methods

DNase Assay

The assay used here measures the conversion of denatured DNA to fragments which are soluble in lanthanum nitrate-HCl reagent. The reaction mixture contained, in the order of mixing, 0.05 ml of 30 mM dithiothreitol, 1 ml of denatured DNA (10 minutes at 100°, followed by quick cooling) at a concentration of 1 mg per ml, an aliquot of enzyme up to 0.1 ml, and sodium acetate buffer, 0.05 M, pH 5 to give a total volume of 1.5 ml. The mixture was incubated at 37° for 10 minutes and then placed on ice for 3 to 4 minutes. The next step was the addition of 1.5 ml of cold lanthanum nitrate-HCl reagent (0.02 M $La(NO_3)_3$, 0.2 M HCl). The mixture was stirred vigorously and allowed to stand at 4° for 10 minutes. This was followed by centrifugation at 1,100 x g for 20 minutes at 4°. The A_{260} of the supernatant fluid was determined in a Beckman DB Spectrophotometer.

A unit of enzyme is defined as that amount which catalyzes in 10 minutes the formation of lanthanum-acid soluble material with an A_{260} of 1.0 per ml of reaction mixture. This assay served as the basis for enzyme purification, and was used for all experiments bearing on the properties of

the DNase. It was observed that this assay was not proportional to enzyme concentration at high and low levels of enzyme. This is shown in Figure I. This created a problem in comparing the activity of different preparations or after different treatments of the enzyme. This problem was effectively overcome by always selecting aliquots of the enzyme solution which gave values in the range of 1 to 2.0 A_{260} per 10 minutes. Since this is the most linear range of the curve, values within this range are comparable and reproducible.

The activity for hydrolysis of native DNA was assayed by essentially the same procedure as described above with the following exceptions. The reaction mixture contained native salmon sperm DNA at a concentration of 1 mg per ml.

A second assay for the activity on denatured DNA was also employed in part of this investigation. The procedure is a modification of the procedure developed by Mead (65) to assay nuclease[2] and oligodeoxynucleotidyl transferase activities. In the original procedure labeled DNA was applied to DEAE-cellulose paper discs and the discs were subjected to washing with either 0.1 M NaCl in 2 M urea or 0.3 M NaCl in 2 M urea. The discs were then dried and the radioactivity remaining on the discs was determined. Since this procedure is time-consuming and involves many steps the author devised the following modification which is faster and does not

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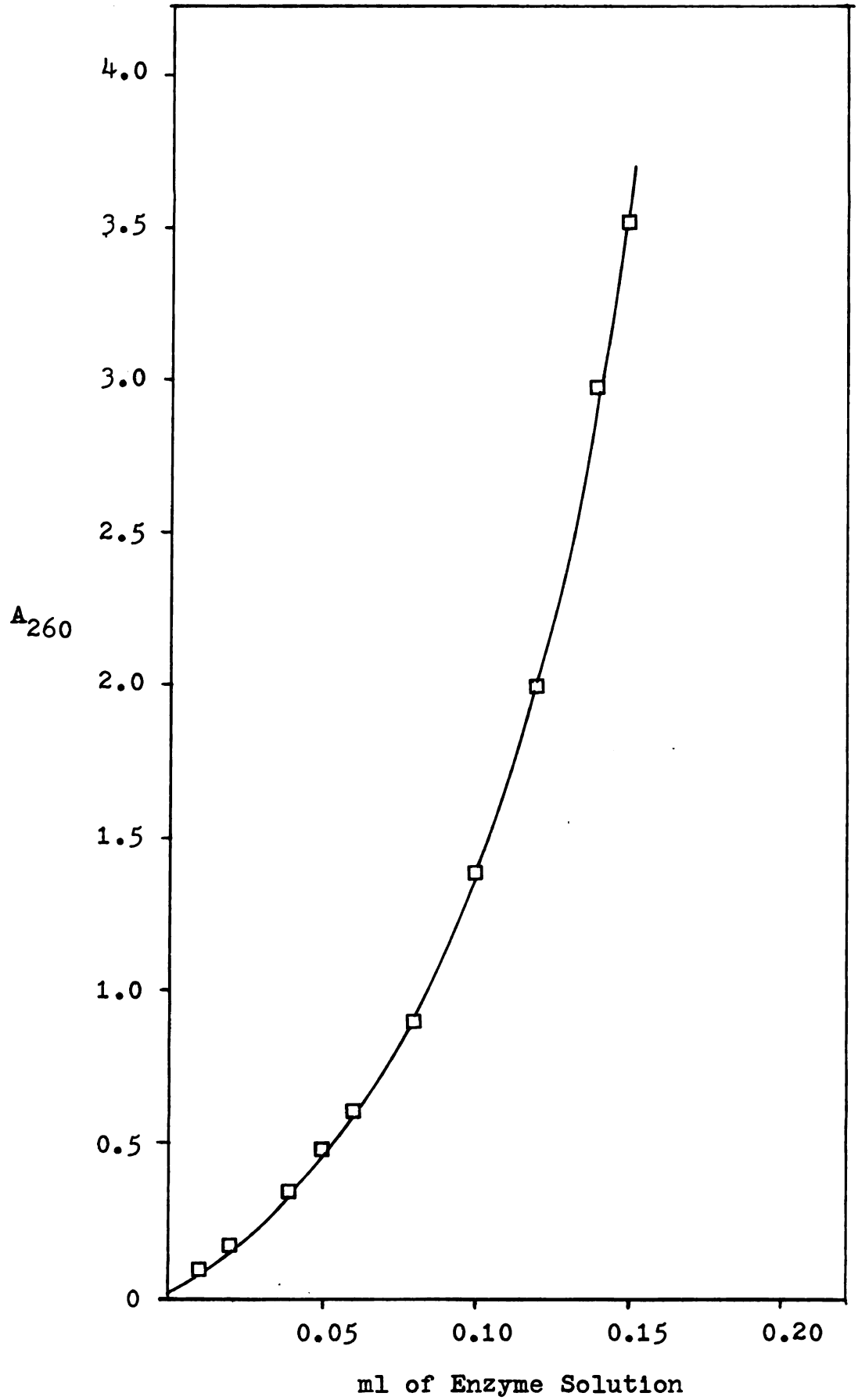
[2]C. G. Mead, personal communication.

Figure I: Non-linearity of Lanthanum nitrate-acid soluble product assay for DNase activity. Samples, as indicated, of a 1 to 50 dilution of the P-30 enzyme fraction were incubated under standard conditions at 37^{o} for 10 minutes as described in "Methods and Materials." In all subsequent assays aliquots of enzyme were chosen which gave A_{260} values in the range of 1 to 2 in 10 minutes since this appeared to be the most linear region of the curve. DNase, □——□.


4.0
3.5
3.0
2.5
A_{260}
2.0
1.5
1.0
0.5
0
0.05
0.10
0.15
0.20
ml of Enzyme Solution

require the use of labeled DNA. A slurry of DEAE-cellulose was used instead of the DEAE discs and the A_{260} of the salt washes was determined by direct measurement. Incubation mixtures containing 2 ml of denatured DNA (0.5 mg per ml), 0.05 ml of 50 mM dithiothreitol, an aliquot of enzyme up to 0.2 ml, and sodium acetate buffer, 0.05 M, pH 5 to a final volume of 2.5 ml were incubated at 37° for 10 minutes. At the end of the incubation the mixture was placed in ice-water for 5 minutes. A sample (1.0 ml) was removed and placed in each of two conical centrifuge tubes to which had been added 0.5 ml of a DEAE-cellulose slurry (60 mg DEAE per ml). The tubes were mixed and allowed to stand at 4° for 5 minutes. To one tube was added an equivalent volume (1.5 ml) of 0.2 M NaCl, 4 M urea solution and to the other tube an equal volume of 0.6 M NaCl, 4 M urea solution. The final salt concentrations were 0.1 M and 0.3 M NaCl and 2 M in urea respectively. These tubes were mixed repeatedly over a period of 30 minutes at room temperature and then centrifuged at 1,100 x g for 10 minutes. The A_{260} of the supernatant fluid was determined directly in a DB Spectrophotometer.

A unit of enzyme is defined as that amount which catalyzes in 10 minutes the formation of material which was eluted from the DEAE-cellulose by 0.3 M NaCl with an A_{260} of 1.0 per ml of incubation mixture. It was determined using C^{14}-dGMP that the 0.1 M wash removes more than 98% of this mononucleotide from the DEAE-cellulose. Undoubtedly this

wash elutes also dinucleotides and possibly tri- and tetranucleotides as well. Since the oligonucleotides eluted by the 0.3 M NaCl wash presumably range in size up to much larger compounds, the difference between the A_{260} of the two wash solutions is a relatively good measure of endonuclease activity. Thus this assay can be used to distinguish exo- from endonuclease activity and to obtain a rough estimate of the relative proportion of the two activities when they are both present in the same sample.

RNase Assay

This assay was essentially the same as that described above for the DNase activity with the following exceptions. The reaction mixture contained 1 ml of rRNA at a concentration of 1 mg per ml, and the incubation was carried out for 30 minutes at 37°.

A unit of enzyme is defined as that amount which catalyzes the formation of lanthanum-acid soluble material with an A_{260} of 1.0 per ml of incubation mixture in 30 minutes. The RNase activity exhibited the same non-proportionality to enzyme concentration at high and low enzyme levels as did the DNase activity. This was corrected for the RNase activity in the same manner as described for the DNase activity.

3'-Nucleotidase Assay

The assay used here measures the release of P_i from 3'-AMP, and is a modification of the method of Dreisbach (39).

The reaction mixture contained, in the order of mixing, 0.05 ml of 50 mM dithiothreitol, 1 ml of 3'-AMP (0.75 mg per ml), an aliquot of enzyme up to 0.1 ml, and sodium acetate buffer, 0.05 M, pH 5 to give a total volume of 2.5 ml. The mixture was incubated at 37° for 15 minutes and then placed on ice for 3 to 4 minutes. An aliquot (0.5 ml) was removed and placed in a 1 x 10 cm screw cap culture tube that contained 3 ml of 1 N H_2SO_4 and 0.4 ml of 8% ammonium molybdate. To each tube was added 2 ml of xylene: isobutanol (65:35 v/v). The tubes were capped, shaken for 20 seconds and centrifuged at 1,100 x g for 10 minutes at 4°. The A_{310} of the supernatant fluid was determined against an appropriate blank in a DB Spectrophotometer. This assay was linear to an A_{310} value of 1.0, representing 0.2 μmoles of P_i.

Assays of the cruder enzyme fractions or of large enzyme aliquots which contained a relatively high concentration of protein were complicated by the formation of a precipitate at the interphase of the two solutions and by the trapping of relatively large amounts of P_i in this precipitate. To circumvent this problem these samples were routinely deproteinated with phenol by the following procedure. At the end of the incubation period the incubation mixture was cooled on ice and then 2 ml of 88% phenol (water saturated) was added and the mixture was stirred with a Vortex Jr. Mixer. The solution was allowed to stand at room temperature for 5 minutes, mixed again, and finally centrifuged at 1,100 x g for 10 minutes. A sample (0.5 ml of the aqueous

(top) phase was removed and assayed for P_i as described above. Appropriate controls were always carried through the phenol extraction procedure. It is essential that the phenol be completely water saturated otherwise reduction in the volume of the aqueous phase and resulting error in the P_i determination will occur.

A unit of 3'-nucleotidase activity is defined as that amount which catalyzes the release of 0.1 μmole of P_i per ml of incubation mixture in 15 minutes.

Gel Filtration

The gel filtration experiments to determine the mode of action of the enzyme activities were performed by the method of Birnboim (66). The columns, P-100 for DNase activity and P-6 for RNase activity, were both 1.5 x 10 cm and elution was carried out at 1 ml per minute flow rate. The flow rate was maintained by means of a peristaltic pump and the elution pattern was monitored automatically with an ISCO model UA-2 recording UV monitor.

Assay for TCA-soluble Ribose and Deoxyribose

Assays for TCA-soluble ribose and deoxyribose were performed as follows. A sample (1 ml) of the standard incubation mixture was placed in 1.2 ml of cold 20% TCA in a conical centrifuge tube. The contents of the tube were mixed and allowed to stand at 4° for 15 minutes. Next the material was centrifuged at 1,100 x g for 20 minutes at 4°. An aliquot (0.2 ml) of the TCA supernatant was removed and

assayed for ribose by the standard orcinol method (67). A second aliquot (1 ml) of the TCA supernatant was removed and assayed for deoxyribose by the standard diphenylamine (Dische) method (67). The amount of TCA-soluble ribose and of deoxyribose was determined from the appropriate standard curves. Since deoxyribose reacts with orcinol to some extent it was necessary to run a standard curve for DNA as well as RNA in the orcinol test. In samples containing DNA and RNA, the necessary correction for the amount of TCA-soluble deoxyribose was made in the manner described by Schneider (67).

Paper Chromatography

Chromatography on DEAE-cellulose paper (DE-81) was used to separate cyclic-UMP and 2'- or 3'-UMP. The chromatograms were developed in a solvent system of 0.1 M ammonium formate for 4 hours. The R_f values for 2',3'-cyclic-UMP, 2'-UMP and 3'-UMP in this system were 0.55, 0.29, and 0.28 respectively. The two-dimensional chromatography system of Sulkowski and Laskowski (68) was employed for separation of the dinucleotides produced on digestion of denatured DNA by the wheat DNase. Samples of the dinucleotide fraction were spotted on Whatman 3 mm paper and developed in the first direction for 72 hours in a solvent (solvent I) containing ethanol-I M ammonium acetate, pH 7.5 (75:30, v/v). In a second direction the paper was developed for 20 hours in a solvent (solvent II) containing saturated ammonium sulfate-isopropanol-water (80:2:18, v/v). Solvent I was also used for the separation of the mononucleotides from rRNA digestions after treatment with 3'- and 5'-nucleotidase and

alkaline phosphatase. In this case, however, the chromatograms were developed for only 20 hours in this solvent.

DEAE-Sephadex Chromatography

The hydrolysis products from the digestion of denatured DNA were separated on DEAE-Sephadex. A column, 3 x 67 cm, was packed with DEAE-Sephadex A-25 and washed with several liters of 0.14 M triethylammonium bicarbonate (TEAB) at pH 8. A 5 hour digest of 500 mg of denatured DNA was applied to the column (see text). The column was washed with about 1 liter of 0.14 M TEAB. A linear gradient from 0.14 to 0.3 M TEAB was then applied to the column, the total volume of the gradient was 4 liters. A second linear gradient from 0.3 to 0.46 TEAB in a total volume of 8 liters was next applied. At the end of the second gradient the column was washed with 1 M TEAB. All elutions were at a flow rate of 1 ml per minute and 20 ml fractions were collected. Approximately 95% of the A_{260} units applied to the column were recovered. Figure 11 shows a typical elution pattern from this column. The first three peaks; I_a, I_b, and I_c contained dCMP:dTMP, dAMP and dGMP respectively. This separation of mononucleotides was not expected, however, it proved to be very beneficial in studying the distribution of the mononucleotides produced by the DNase activity. It was also found that the same separation of deoxymononucleotides could be achieved in a batchwise treatment of DEAE-Sephadex. Digests of denatured DNA were applied to a DEAE-Sephadex column, 2.2 x 15 cm, which had been equilibrated with 0.14 M TEAB. The column was then

washed with 300 ml of 0.14 M TEAB. Elution of the mononucleotides was then carried out by washing the column with 0.18 M TEAB. The elution pattern of the mononucleotides was the same as that observed when a gradient was applied to the column. The mononucleotides in each peak were positively identified by their characteristic absorption spectra, R_f values in solvent II, and conversion to the corresponding nucleoside on treatment with alkaline phosphatase and chromatography in solvent I. Furthermore, the elution pattern of the 4 known deoxymononucleotides in this procedure was identical to that observed with DNA digests. The recovery of each of the 4 known deoxymononucleotides was between 95 and 100%. Since dTMP and dCMP did not separate in this system it was necessary to determine the relative amounts of these nucleotides in the peak by spectrophotometric analysis of the binary mixture. This was performed as described by Loring (69). The batch-wise separation of the mononucleotides on DEAE-Sephadex provides a rapid means of quantitatively determining the relative distribution of mononucleotides produced at various stages in the digestion of DNA by a DNase. The procedure has the further advantage that the solvent is volatile and can be removed by repeated flash evaporation, yielding a product which is essentially salt free.

Protein Determination

Protein concentrations were determined by the method of Lowry *et al.* (40) using bovine serum albumin as a reference standard. The determinations were made using the red

filter of a Klett-Summerson model 800-3 colorimeter.

Polyacrylamide Disc Electrophoresis

Polyacrylamide gel electrophoresis was performed according to the method of Ornstein (41) and Davis (42). A column, 0.9 cm in diameter, composed of a 4 cm layer of 10% running gel and a 1.5 cm layer of stacking gel was routinely employed. A sample of enzyme (0.05-0.2 ml) containing 200 to 400 μg of protein was applied to the gel column. A potential of 340 volts and a current of 5 MA was applied for a period of 4 hours at 4°. Electrophoresis at pH 9.5 was in a buffer system containing 0.005 M Tris and 0.04 M glycine. The buffer system for electrophoresis at pH 8.3 was 0.034 M L-asparagine adjusted to pH 8.3 with 1 M Tris.

RESULTS

Purification of Enzyme

The procedures described below were devised originally for purification of the endonuclease. As will become evident later, 3'-nucleotidase and ribonuclease activities were found to accompany the endonuclease throughout the purification steps. In the following discussion, therefore, use of the term enzyme refers to the combination of all three activities.

Growth of Wheat Seedlings

The dry wheat seed was spread evenly on wet newspaper and then rolled into cylindrical form. These rolls were incubated at 30-32° for 48 hours in the dark. The seedlings were then removed from the paper and used immediately for enzyme preparation.

Homogenization of Seedlings

Unless otherwise stated all operations were carried out at 4°. In a typical purification (Table I_a, I_b, I_c) 7 kilograms of freshly germinated seedlings were homogenized with 6 liters of sodium acetate buffer, 0.05 M, pH 4.5, for 1 minute in a Commercial Waring Blendor. The homogenate was squeezed through cheesecloth to remove the bulk of the insoluble material. The filtrate was centrifuged at 5,800 x g for

Table I_a

Summary of purification of DNase from 7-kilogram of wheat seedlings

Fraction	Total protein	DNase		
		Total activity	Specific activity	Yield
	mg	units	units/mg	%
Crude extract	71,780	35,520	0.5	100
Ammonium sulfate[a]	6,400	11,520	1.8	32.4
Ethanol[b]	720	24,990	34.7	70.3
75° Treatment	551	11,049	20.1	31.1
Phosphocellulose	42.6	5,148	120.1	14.4
Phosphocellulose-P-30[c]	17.6	5,775	328.1	16.2
Concentrated P-30	10.5	4,400	418.0	12.2

[a]These assays are thought to give low values, varying among different preparations, because of retention of ammonium sulfate which is inhibitory to the enzyme activities.

[b]Assays of the ethanol fraction were found to be poorly reproducible.

[c]An increase in all three enzyme activities was routinely observed in this step.

Table I_b

Summary of purification of RNase
from 7-kilogram of wheat seedlings

Fraction	Total protein	RNase		
		Total activity	Specific activity	Yield
	mg	units	units/mg	%
Crude extract	71,780	436,600	6.1	100
Ammonium sulfate[a]	6,400	135,040	21.1	30.9
Ethanol[b]	720	24,000	33.3	5.4
75° Treatment	551	9,570	17.4	2.2
Phosphocellulose	42.6	3,960	93.0	0.9
Phosphocellulose-P-30[c]	17.6	5,280	300.0	1.2
Concentrated P-30	10.5	4,400	419.1	1.0

[a]These assays are thought to give low values, varying among different preparations, because of retention of ammonium sulfate which is inhibitory to the enzyme activities.

[b]Assays of the ethanol fraction were found to be poorly reproducible.

[c]An increase in all three enzyme activities was routinely observed in this step.

Table I_c

Summary of purification of 3'-nucleotidase from 7-kilogram of wheat seedlings

Fraction	Total protein	3'-Nucleotidase		
		Total activity	Specific activity	Yield
	mg	units	units/mg	%
Crude extract	71,780	218,300	3.1	100
Ammonium sulfate[a]	6,400	30,080	4.7	13.7
Ethanol[b]	720	22,800	31.7	10.4
75° Treatment	551	9,860	17.9	4.5
Phosphocellulose	42.6	4,686	110.0	2.1
Phosphocellulose-P-30[c]	17.6	5,335	303.1	2.4
Concentrated P-30	10.5	4,050	385.7	1.8

[a]These assays are thought to give low values, varying among different preparations, because of retention of ammonium sulfate which is inhibitory to the enzyme activities.

[b]Assays of the ethanol fraction were found to be poorly reproducible.

[c]An increase in all three enzyme activities was routinely observed in this step.

10 minutes and the clear supernatant fluid was decanted.[3]

Ammonium Sulfate Fractionation

The freshly prepared crude extract (3,700 ml was brought to 57% of saturation by slowly adding 1,239 g of solid ammonium sulfate that had been passed through a 40 mesh screen. The solution was stirred for 30 minutes and was then centrifuged at 15,000 x g for 20 minutes. The precipitate was discarded and the supernatant solution was adjusted to 75% of saturation with 419 g of ammonium sulfate. The suspension was stirred an additional 30 minutes and then centrifuged at 15,000 x g for 30 minutes. The precipitate was dissolved in 600 ml of sodium acetate buffer, 0.05 M, pH 4.5, by vigorous mechanical stirring for about one hour at 4°.

Ethanol Fractionation

To 640 ml of the ammonium sulfate fraction was added 960 ml of 95% ethanol at 4°. The ethanol was added from a separatory funnel over a period of about 15 minutes into the vortex of the stirred solution. The suspension was stirred for another 10 minutes and centrifuged at 15,000 x g for 20 minutes. The precipitate was discarded and 384 ml of 95% ethanol was added as above to the clear supernatant

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[3]The material had to be carried from the initial homogenation through the heat step as quickly as possible to minimize loss of activity. Normally this was accomplished within 12 hours. The intermediate fractions could be stored without appreciable loss of activity, however, in the presence of 3 M urea, a condition known to prevent the aggregation and precipitation of wheat proteins (43). Such storage was not regularly utilized because of problems caused by urea in later stages of purification.

solution. After stirring for 10 minutes the solution was centrifuged at 15,000 x g for 30 minutes. Excess ethanol was removed from the precipitate with a stream of nitrogen until the fragrance of ethanol could no longer be detected. The precipitate was dissolved in 300 ml of Buffer A.

Some preparations retained more ammonium sulfate than others, leading to decreased recoveries of enzyme activity in the heat step. Accordingly, the ethanol fraction was diluted until the apparent ammonium sulfate concentration was below 0.1 M as determined from the refractive index of the enzyme solution in comparison with the refractive indices of ammonium sulfate solutions of known concentration.

Heat Fractionation

The ethanol fraction was split into 2 portions of about 150 ml. Each portion was placed in a 1 liter flask to give a large surface area for the heating procedure. The flasks were then placed in a 95° water bath until the temperature rose to 75°. They were then transferred to a 75° water bath and maintained at this temperature for 10 minutes with shaking. The flasks were then placed in ice water until the temperature decreased to below 10°. The solutions were centrifuged at 25,000 x g for 45 minutes. The pellet of precipitated protein had a tendency to break up into small clumps which floated out with the supernatant solution on decanting. For this reason the supernatant

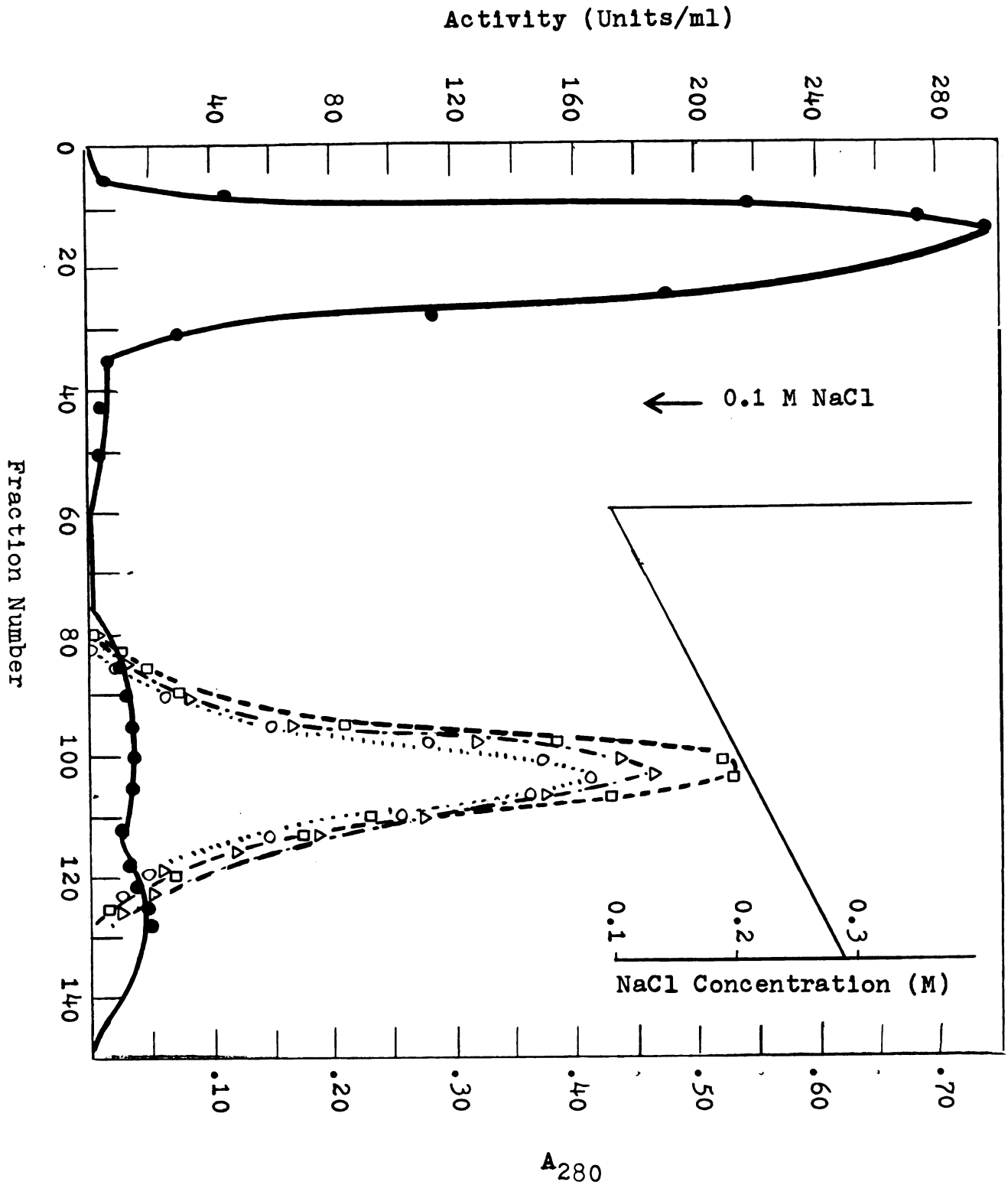
solutions was routinely filtered through Whatman No. 1 filter paper at 4° under gravity flow. This gave a combined volume of 290 ml of a clear fluid that was slightly yellow in color.

Phosphocellulose Chromatography

A column 1.8 x 26 cm was packed with 6.5 g dry weight of phosphocellulose resin which had been equilibrated with 500 ml of Buffer A. The column was extensively washed with this buffer and then the fraction from the heat step was applied at a rate of 1 ml per minute with a peristaltic pump regulating the flow rate. The column was then washed with 200 ml of the same buffer, and with 200 ml of Buffer A which was also 0.1 M in NaCl. This was followed by a linear salt gradient from 0.1 to 0.3 M NaCl in the same buffer, the total volume of the gradient being 1,200 ml. Fractions of approximately 20 ml were collected at a flow rate of 1 ml per minute.

The elution pattern of protein from the phosphocellulose column is shown in Figure 2. Approximately 85% of the protein was recovered in the pass through and wash fraction; however, only little enzyme activity was found in this material. The DNase activity was eluted between 0.18 and 0.25 M NaCl. Those fractions (95 through 112) which were purified from 16 to 52 fold over the previous step were pooled. The pooled material represented about 50% of the activity applied to the column and about 15% of the total original activity.

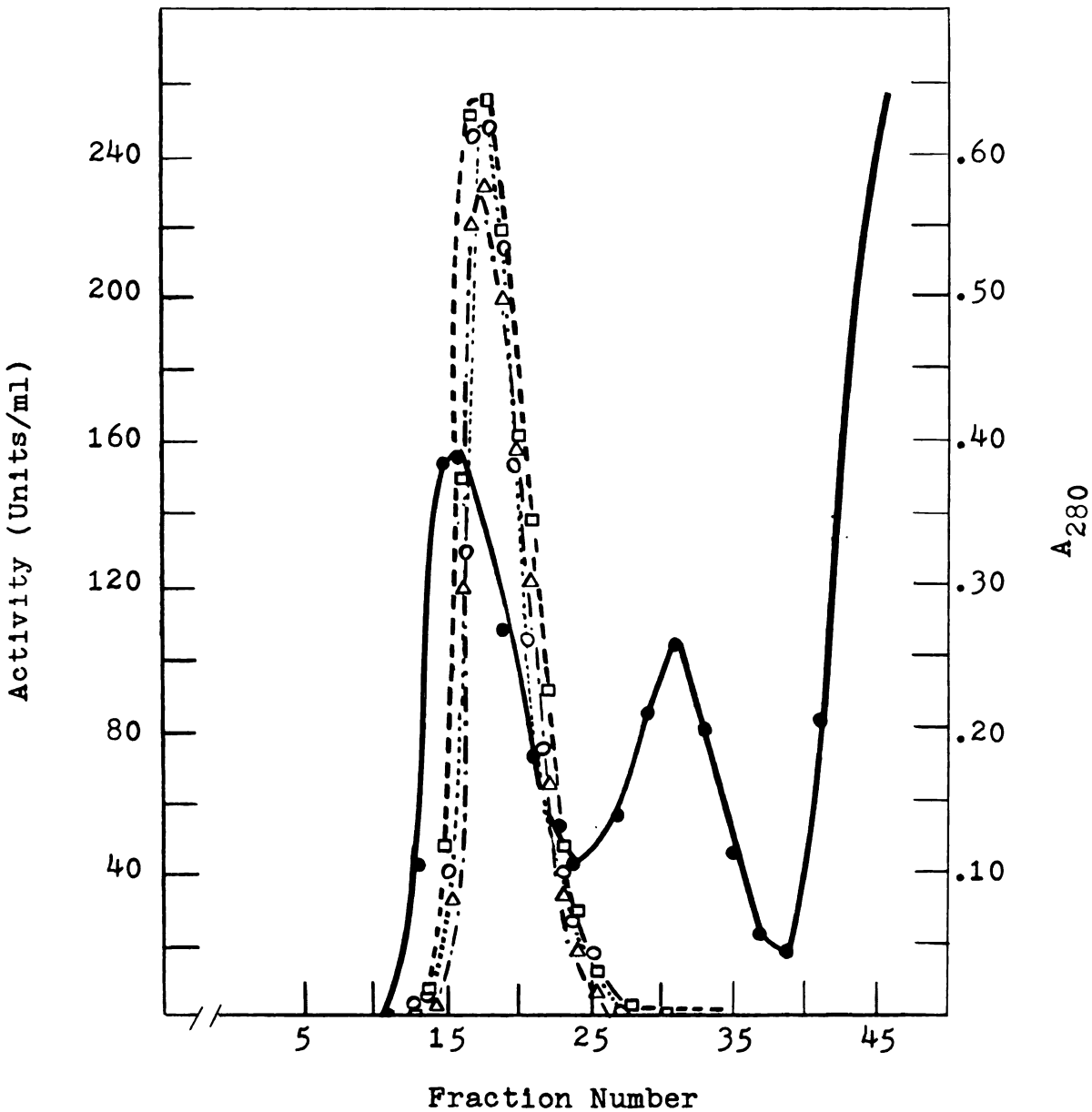
Figure 2: Elution pattern from a phosphocellulose column of protein, DNase, RNase, and 3'-nucleotidase activity. The column, 1.8 x 26 cm, contained 6.5 g (dry weight) of resin. The heat fraction (290 ml) was applied and the column was washed with 200 ml of Buffer A, then with 200 ml of Buffer A containing 0.1 M NaCl. A linear gradient from 0.1 to 0.3 M NaCl was applied at flow rate of 1 ml per minute, the total volume of the gradient was 1,200 ml. Fractions of approximately 20 ml were collected. The enzyme activities were assayed as described in "Methods and Materials." Protein, ●——●; DNase, □---□; RNase, O···O; 3'-nucleotidase, △-·-△.


Activity (Units/ml)
40
80
120
160
200
240
280
0
20
40
60
80
100
120
140
Fraction Number
0.1 M NaCl
0.1
0.2
0.3
NaCl Concentration (M)
.10
.20
.30
.40
.50
.60
.70
A280

Concentration of Phosphocellulose Fraction and P-30 Chromatography

The pooled material from the phosphocellulose column (330 ml) was diluted with Buffer B until it was 0.1 M in NaCl as determined from the refractive index of the solution. This usually required at least an equal volume of buffer. A column (2.2 x 76 cm) of Bio-Gel P-30 was packed under gravity flow and a pad of wet glass wool 1 mm thick was placed on top of the gel bed. A bed of phosphocellulose (2.2 x 3 cm, 1 g dry weight of resin) was layered on top of the glass wool pad. This combination column was then washed with 300 ml of Buffer B. The enzyme fraction was applied to the column at a flow rate of 1 ml per minute and then the column was washed with Buffer B until the effluent was free of NaCl (about 320 ml). The column was washed with 45 ml of Tris-maleate buffer, 0.05 M, pH 6.5, 0.1 mM in zinc acetate, 2 mM cysteine, 0.7 M NaCl, at a flow rate of 0.5 ml per minute. The high concentration of salt and the pH change were found to be sufficient to elute the enzyme from the phosphocellulose as a very sharp yellow band which then entered the P-30 gel in a very small volume. The column was then washed with Buffer B at 1 ml per minute. As shown in Figure 3, the enzyme activity was eluted in fractions 15 through 23. Ultraviolet absorbing material, presumably the buffer components, began to appear in fraction 41 and NaCl in fraction 48.

Figure 3: Elution pattern from a combination phosphocellulose P-30 column of protein, DNase, RNase, and 3'-nucleotidase activity. A column, 2.2 x 76 cm, was packed with P-30 and a 1 mm pad of wet glass wool was placed on top of the bed of gel. A bed of phosphocellulose (2.2 x 3 cm, 1 g dry weight of resin) was layered on top of the pad. The column was washed with 300 ml of Buffer B. The diluted phosphocellulose fraction (660 ml) was applied to the column at a flow rate of 1 ml per minute. The column was washed with Buffer B until free of NaCl. The protein was eluted with 45 ml of Tris-maleate buffer, 0.05 M, pH 6.5, 0.1 mM in zinc acetate, 2 mM in cysteine, 0.7 M in NaCl, at a flow rate of 0.5 ml per minute. The column was then washed with Buffer B. The three enzyme activities were eluted from the column in fractions 15 through 23. Buffer components began to elute in fraction 41 and NaCl in fraction 48. The enzyme assays were performed as described in "Methods and Materials." Protein, ●——●; DNase, □ ---□ ; RNase, 0•••0; 3'-nucleotidase, △-·-△ .


Activity (Units/ml)
240
200
160
120
80
40
.60
.50
.40
.30
.20
.10
A_{280}
5
15
25
35
45
Fraction Number

Concentration of the P-30 Fraction

Fractions 15 through 23 (55 ml) from the P-30 column were pooled and concentrated with a rotary flash evaporator, adjusting the vacuum to minimize foaming. This step was carried out at 28° and usually took about 1 hour. The volumn of the P-30 fraction was routinely reduced by a factor of 10. However, the solution was never allowed to go completely to dryness as this resulted in a greater than 90% loss of enzyme activity.

The concentrated fraction was then dialyzed for a total of 15 hours against two 1 liter portions of Buffer B, changing the buffer after the first six hours. The average degree of purification of the concentrated P-30 fraction was about 830-fold and the average recovery was about 12% of the total DNase activity present in the crude homogenate. This fraction was used in most of the experiments to be discussed below and will be designated as the P-30 fraction.

Preparation of Enzyme for Storage

The dialyzed concentrated enzyme solution was routinely filtered through a sterile Millipore Filter (GSWP 025, 0.22 μ) to minimize bacterial contamination. One or two ml portions were placed in sterile, screw-cap culture tubes. These tubes of "sterile" enzyme solutions were then stored either at -20° or 4° until use.

Further Attempts at Separation of the Three Activities

Batch Treatment with Various Adsorbents

In an attempt to find an ion-exchange resin or adsorbent which would effect a separation of the three enzyme activities the following experiment was performed. A total of 8 ion-exchange resins (phosphocellulose, carboxymethylcellulose, DEAE-cellulose, DEAE-Sephadex, ECTEOLA-cellulose, Dowex-1, Dowex-50, and Amberlite-50) and 2 adsorbents (woodcellulose, and Bentonite) were used in a batch-wise manner to treat enzyme solutions at pH 4.5, 6.5, and 8.0. After 15 minutes of mixing the resins were centrifuged and the supernatant solutions assayed for the three enzyme activities. Within the accuracy of the assay procedures, no separation of the three activities was found. The activities either did not bind to the resin or were bound to the same degree.

DEAE-Cellulose Chromatography at pH 8

A column of DEAE-cellulose, 1.5 x 10 cm, was packed and washed with 0.025 M Tris-chloride, pH 8.0, 0.1 mM in zinc acetate. Enzyme from the P-30 fraction was applied to the column in the same buffer at a rate of 1 ml per minute. A linear salt gradient from 0 to 0.5 M NaCl (total volume of 200 ml) was then applied to the column. The three enzyme activities appeared in a single peak at 0.12 M NaCl concentration. This peak also showed a tail of activity on the higher salt side of the peak. This tail was essentially the same for all three activities.

ECTEOLA-Cellulose Chromatography

The P-30 fraction was also chromatographed on an ECTEOLA-cellulose column (1 x 10 cm) with Tris-maleate buffer, 0.05 M pH 6.5, 2 mM in cysteine, 0.1 mM in zinc acetate. A linear salt gradient from 0 to 0.5 M NaCl was applied and again the three activities appeared as a single peak at approximately 0.1 M salt concentration.

pH Gradient on Phosphocellulose

Treatment of the enzyme preparation with phosphocellulose at pH 6.5 indicated that the three activities were not retained by the resin at this pH. Since this resin gave a good purification at pH 4.5 when a salt gradient was applied to the column it was decided to study the behavior of the activities in response to a pH gradient. The P-30 enzyme at pH 4.5 was added to a 1 x 10 cm column of phosphocellulose previously equilibrated with Buffer B. A pH gradient consisting of 50 ml each of Buffer B and 0.05 M Tris-maleate buffer, pH 6.9, 0.1 mM in Zn^{++}, 2 mM in cysteine was applied to the column at a flow rate of 1 ml per minute. The three activities were eluted from the column at approximately pH 5.5 as a single peak, with the pattern of the three activities throughout the peak again being essentially the same.

Chromatography on Bio-Gel P-60

A 2 x 80 cm column of Bio-Gel P-60 (50-150 mesh) was packed under gravity flow in Buffer B. A sample of enzyme

from the phosphocellulose fraction was applied and chromatographed at a rate of 1 ml per minute in the same buffer. The enzyme was retained by this gel as shown in Figure 4. The three activities were obtained in a single peak at about the same elution position as pancreatic RNase dimer. This suggests a possible molecular weight of about 30,000. A small peak of material adsorbing at 280 mμ was also found which was retained to a greater extent by the P-60 column. This material was, however, completely devoid of any enzyme activity.

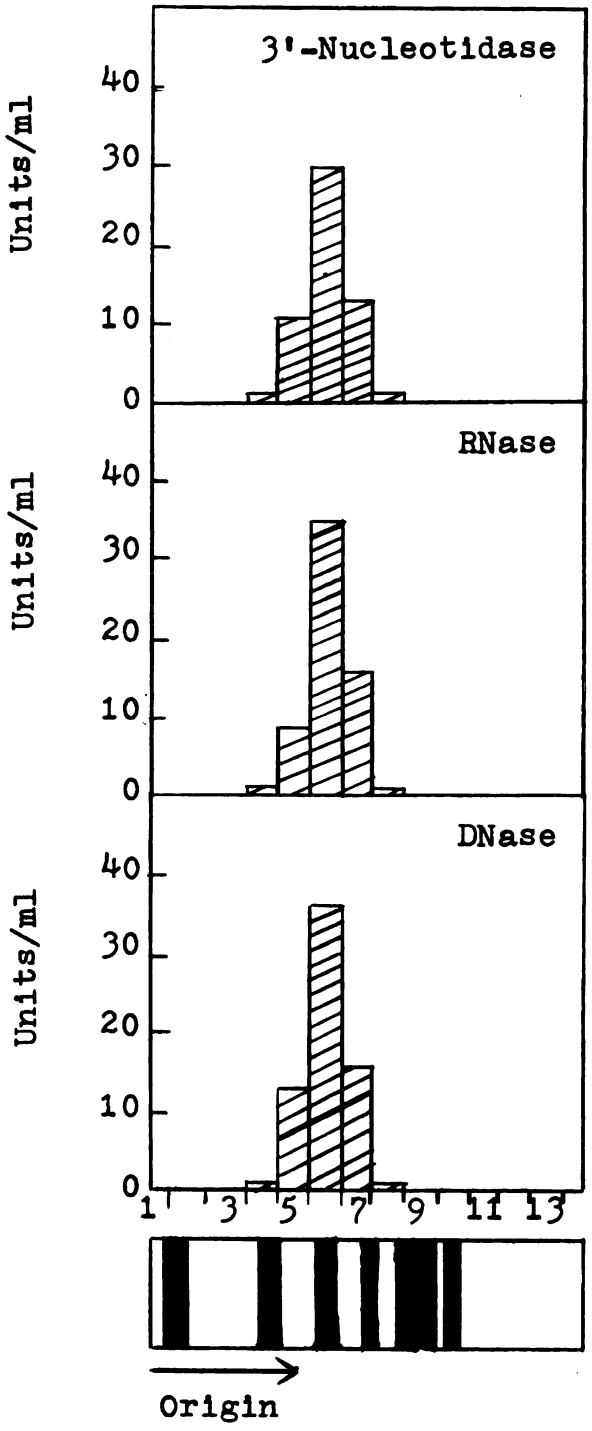
Polyacrylamide Disc Electrophoresis

An aliquot of the P-30 fraction containing up to 400 μg of protein was examined by electrophoresis using a 10% polyacrylamide gel at pH 9.5, as described in "Methods and Materials." Half the gel was stained with Coomassie Blue Stain by the method of Chrambach et al. (44). The other half of the gel was cut into 4 mm segments. Each segment was placed in 1 ml of Tris-chloride, 0.025 M, pH 8 and allowed to stand overnight at 4°. The solutions were then assayed for the three enzyme activities. Figure 5 shows the staining pattern of the proteins and the distribution of the three activities within the gel. Judging from the number of bands obtained, the preparation still contains a number of protein species; however, the three enzyme activities were found to be associated with a single band and were distributed through this band in essentially identical

Figure 4: Elution pattern from a P-60 column of protein, DNase, RNase, and 3'-nucleotidase activities. A column, 2 x 80 cm, of Bio-Gel P-60 was packed and washed with Buffer B. A sample (10 ml) of the phosphocellulose fraction was applied and chromatographed in Buffer B at a flow rate of 1 ml per minute. Enzyme assays were performed as described in "Methods and Materials." Protein, ●——●; DNase, □---□; RNase, O•••O; 3'-nucleotidase, Δ-•-Δ.

Activity (Units/ml)
200
160
120
80
40
0
10
20
30
40
50
60
70
80
90
Fraction Number
A_{280}
.50
.40
.30
.20
.10

Figure 5: Staining pattern of protein and distribution of enzyme activity within a polyacrylamide gel after electrophoresis. A sample (0.2 ml) of the P-30 fraction containing 400 μg of protein was applied to a 10% polyacrylamide gel as described in "Methods and Materials." Electrophoresis at pH 9.5 was performed at 4° for 4 hours with an applied current of 5 MA. The gel was cut in half, one half was stained with Coomassie Blue stain, and the other half was cut into 4 mm segments. Each segment was placed in 1 ml of Tris-chloride buffer, 0.025 M, pH 8 and allowed to stand overnight at 4°. The solutions were assayed for enzyme activity as described in "Methods and Materials."


3'-Nucleotidase
RNase
DNase
Units/ml
40
30
20
10
0
1
3
5
7
9
11
13
Origin

fashion. A similar result was obtained upon electrophoresis at pH 8.3.

It is apparent that a significant purification can be achieved by electrophoresis. Experiments applying preparative electrophoresis to the P-30 fraction are now in progress.

Properties of the Enzyme Preparation

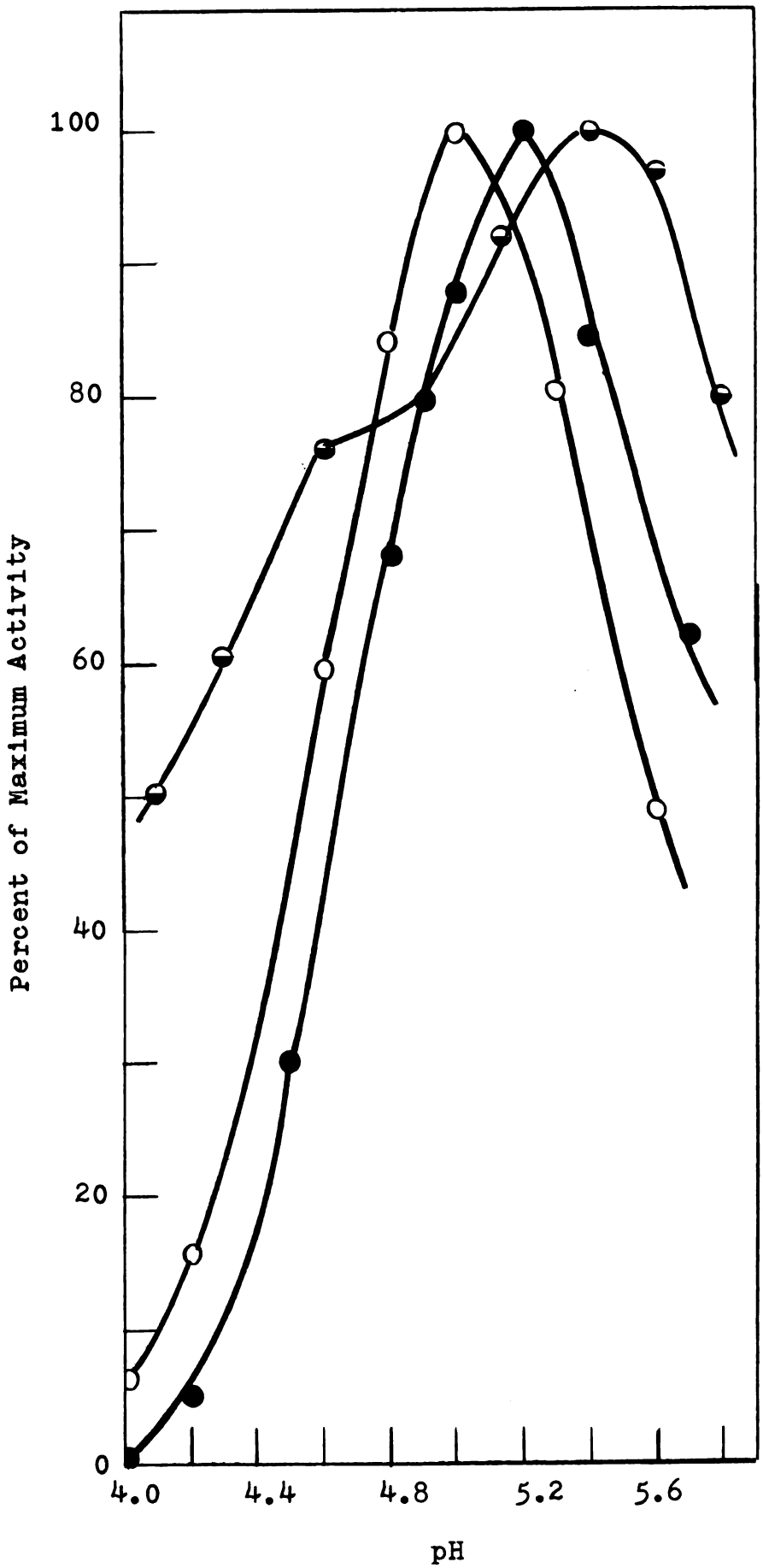
Contaminating Enzyme Activities

Aliquots of a P-30 fraction, each aliquot containing 8.8 DNase units with a specific activity of 418, gave no detectable hydrolysis at pH 5 of 5'-AMP, bis-p-nitrophenyl phosphate, 5'-p-nitrophenyl thymidylate, or 5'-p-nitrophenyl adenylate after 24 hour incubation at 37°. These assay conditions represent a ten-fold higher enzyme concentration and a 144-fold longer incubation period than that used under the normal assay conditions for DNase activity. Under the same conditions a slight amount of hydrolysis of p-nitrophenyl phosphate and of 3'-p-nitrophenyl thymidylate was observed. On examination of DNA digests, however, neither 3'-nucleotides, nucleosides, nor P_i could be detected. From these results it was concluded that the purified enzyme preparations contained little if any 5'-nucleotidase, phosphodiesterase, and non-specific phosphomonoesterase activities.

pH Optimum

Figure 6 shows the similar pH-activity curves, in the absence of added Zn^{++}, for the DNase, RNase, and 3'-nucleo-

Figure 6: pH-activity curve. The assays were performed as described in "Methods and Materials." All points are for 0.05 M sodium acetate buffer adjusted to the appropriate pH with either 1 M acetic acid or KOH. DNase, O——O; RNase, ●——●, 3'-nucleotidase, ●——●.


100
80
60
40
20
0
Percent of Maximum Activity
4.0
4.4
4.8
5.2
5.6
pH

tidase activities. Maximal activity for DNA hydrolysis by the P-30 fraction was between pH 4.8 and 5.3, for RNA hydrolysis between 4.9 and 5.5, and for 3'-AMP hydrolysis between 4.8 and 5.8. The 3'-nucleotidase curve also had a shoulder at about pH 4.7. The meaning of this is not clear at present. If similar experiments are conducted with Zn^{++} present at 1 mM concentration the three curves were unchanged below pH 5.1. Above this pH, all three activities were markedly decreased. A precipitate is apparent under these conditions.

Effect of Sulfhydryl Compounds

The three enzyme activities were found to require the presence of sulfhydryl compounds for maintenance of maximal activity at pH 4.5. Table II shows the decrease in the activities after dialysis overnight at 4° against buffer lacking sulfhydryl compounds. At pH 8, however, the presence of such compounds caused complete loss of all three activities in approximately 24 hours at 4°. A time course of inactivation by 1 mM dithiothreitol at pH 8 is shown in Table III for the three activities. The curves were found to be very similar with 50% inactivation being reached at 2.3, 2.1, and 2.9 hours after dithiothreitol addition for the DNase, RNase, and 3'-nucleotidase activities respectively. This effect at pH 8 appears to be a general sulfhydryl effect as can be seen in Table IV. Essentially the same results were obtained regardless of the sulfhydryl source, i.e. dithiothreitol, mercaptoethanol, or cysteine, provided that the total sulfhydryl concentration was the same in each case. All three

Table II

Effect of the removal of Zn^{++} or cysteine on DNase, RNase and 3'-nucleotidase activities at pH 4.5

Samples (1.0 ml) of enzyme were placed in dialysis tubing and dialyzed against two 2-liter changes of buffer at 4^{o} for 15 hours. The control buffer was Buffer B. The other buffers were the same as B but lacked the component indicated. The assay procedures were those described in "Methods and Materials."

Compound deleted	Percent of Original Activity Remaining		
	DNase	RNase	3'-Nucleotidase
Control	100	100	100
Zn^{++}, cysteine	7	29	9
Cysteine	80	90	63
Zn^{++}	71	65	66

Table III

Time course of dithiothreitol inactivation of the three enzyme activities at pH 8

To a sample (4.0 ml) of enzyme in 0.025 M Tris-chloride buffer at pH 8 was added 80 µl of 50 mM dithiothreitol to give a final concentration of 10^{-3} M dithiothreitol. The enzyme solution was allowed to stand at 4° and samples (0.4 ml) were removed at the times indicated below. These samples were assayed as described in "Materials and Methods."

Hours after addition	Percent of Original Activity Remaining		
	DNase	RNase	3'-Nucleotidase
0	100	100	100
1	78	66	81
3	41	41	49
6	24	24	20
12	9	9	3
24	1	1	0

Table IV

Effect of various sulfhydryl compounds at pH 8

Samples (0.5 ml) of enzyme in 0.025 M Tris-chloride buffer at pH 8 were allowed to stand at 4° for 15 hours in the presence of the sulfhydryl compound at a final concentration of 10^{-3} M, except that the molarity of the dithiothreitol was half this value. The samples were then assayed as described in "Methods and Materials."

Sulfhydryl addition	Percent of Original Activity Remaining		
	DNase	RNase	3'-Nucleotidase
None	100	100	100
Dithiothreitol	3.4	1.5	1.4
Cysteine	8.9	20.6	0.9
Mercaptoethanol	8.9	10.3	2.8

activities were stable to storage at pH 8 in the absence of sulfhydryl compound to the same extent that they were stable at pH 4.5 in the presence of sulfhydryl compound.

Titration curves for activity versus the sulfhydryl concentration over the range of 2×10^{-2} to 10^{-5} M were essentially the same at pH 4.5 for the three activities. Similar curves were obtained regardless of whether dithiothreitol or cysteine was used as the sulfhydryl compound provided that the total sulfhydryl concentration was the same.

Removal of cysteine from the inactivated enzyme preparation by dialysis against 0.05 M Tris-chloride, pH 8.0 caused no detectable reactivation of the DNase activity. Dialysis of this same material against Buffer B resulted in approximately doubling the activity in 24 hours at 4°. This increase, however, represented only about 5% of the initial DNase activity. Whether additional increase in activity occurs with longer periods of time at pH 4.5 was not determined.

The point in the purification procedure at which cysteine or dithiothreitol is added was found to be very critical. Routinely the sulfhydryl compound was first added to the system after the ethanol fractionation and before the heat step. In the heat step and subsequent procedures the enzyme was not stable in the absence of sulfhydryl compounds. It was found, however, that addition of sulfhydryl to any step prior to the ethanol fractionation resulted in a com-

plete loss of enzyme activity in only a few hours at 4°. The explanation of this observation is obscure, but one possibility is that sulfhydryl compounds activate one or more proteases which are removed by the ethanol step.

Effect of Zinc Ion

The addition of zinc acetate to a final concentration of 1 mM in the assay mixture caused about a 20% increase in DNase activity with the less purified fractions (up to and including the heat step). Addition of Zn^{++} at this concentration to the assay mixture of subsequent, more highly purified fractions led to as much as a 25% decrease in DNase activity. Slight inhibition of the purest fractions was found with Zn^{++} at concentrations as low as 10^{-5} M. For this reason Zn^{++} was not routinely added to the assay mixtures. The stability of the three enzyme activities to storage and to subsequent treatments was, however, strongly dependent on the presence of Zn^{++} in the enzyme medium. Removal of the Zn^{++} from Buffer B resulted in about the same degree of loss of all three activities in 15 hours at 4°. This is shown in Table II. The figure also shows that removal of both Zn^{++} and cysteine gave a much greater decrease of the activities than did removal of the Zn^{++} or cysteine alone. Because of this stabilizing effect of Zn^{++}, the purification steps subsequent to ethanol fractionation and storage of the enzyme were carried out in buffer that was at least 10^{-4} M in Zn^{++}. Attempts to reactivate enzyme preparations from which both Zn^{++} and cysteine were removed by

dialysis at pH 4.5, by restoring both of these substances were only partly successful. About 35% of both DNase and RNase activities could be regained. The nucleotidase activities were not examined in this experiment. At pH 8 added Zn^{++} did not appear to be required for stability of the three activities.

Stability of Enzyme to Storage

The heat fraction was stable in Buffer A at 4° for several weeks with no appreciable loss of DNase activity. The phosphocellulose fraction has been stored in Buffer B for up to 5 months at either 4° or -20° with less than 5% loss of the three enzyme activities. Repeated freezing and thawing of the phosphocellulose fraction was observed to result in a small but significant loss of activity. To minimize this loss, the enzyme solution was divided into portions of 1-2 ml and stored in sterile culture tubes until use.

Effect of Inhibitors

Table V shows a typical set of data for the inhibition of the DNase and RNase activities by KH_2PO_4. The effect of phosphate was at least qualitatively the same for both the DNase and RNase activities. Approximately 50% inhibition of the DNase activity was obtained at a phosphate ion concentration of 1×10^{-3} M whereas 5×10^{-3} M phosphate ion was needed to inhibit the RNase to the same extent.

Table V

Effect of inhibitors on DNase and RNase activities

Reactions were carried out at pH 4.5 as described in "Methods and Materials" with inhibitors added to the reaction mixture at 4° five minutes before adding enzyme.

Inhibitor	Molar concentration	Percent of Original Activity Remaining	
		DNase	RNase
None	---	100	100
KH_2PO_4	1×10^{-5}	117	96
	1×10^{-4}	78	86
	1×10^{-3}	53	78
	1×10^{-2}	0	3
	1×10^{-1}	0	0
None	---	100	100
NaF	1×10^{-5}	78	86
	1×10^{-4}	108	62
	1×10^{-3}	70	69
	5×10^{-3}	21	38
	1×10^{-2}	0	15

A similar effect was obtained when sodium fluoride was used as the inhibitor. As indicated in Table V fluoride ion affected both DNase and RNase activities in much the same way, with 50% inhibition occurring at approximately 2×10^{-3} M and 3×10^{-3} M respectively. The effect of both of these inhibitors on the 3'-nucleotidase activity could not be assessed because of interference with the assay procedure.

EDTA Effect

The effect of EDTA on the DNase and RNase activities was examined over the range of concentration of EDTA from 10^{-8} to 2×10^{-2} M. The titration curves are shown in Figure 7 and demonstrate a region of concentration between about 10^{-5} M and 10^{-2} M EDTA in which the degree of inhibition was found to be constant for each activity. Note that the RNase activity was inhibited more strongly at intermediate concentrations of EDTA than was the DNase activity. The curves do, however, show the same overall pattern for both the activities. No explanation for the unusual shape of this curve is readily available.

Effect of Metal Ions

The effect of various metal ions on the three enzyme activities was studied with the results shown in Table VI. A P-30 fraction with a specific activity of 418 was used for this study. The metals (all as chlorides) were added to the incubation mixture to a final concentration of 10^{-3} M. The solutions were allowed to stand at 4° for 5 minutes before

Figure 7: Effect of EDTA on activity of DNase and RNase. EDTA was added to the incubation mixtures to the final concentrations indicated and the mixtures were allowed to stand at 4° for 5 minutes. An aliquot of enzyme was added and the assays were performed as described in "Methods and Materials." The activity in the absence of added EDTA was taken as control. DNase, O——O; RNase, ●——●.

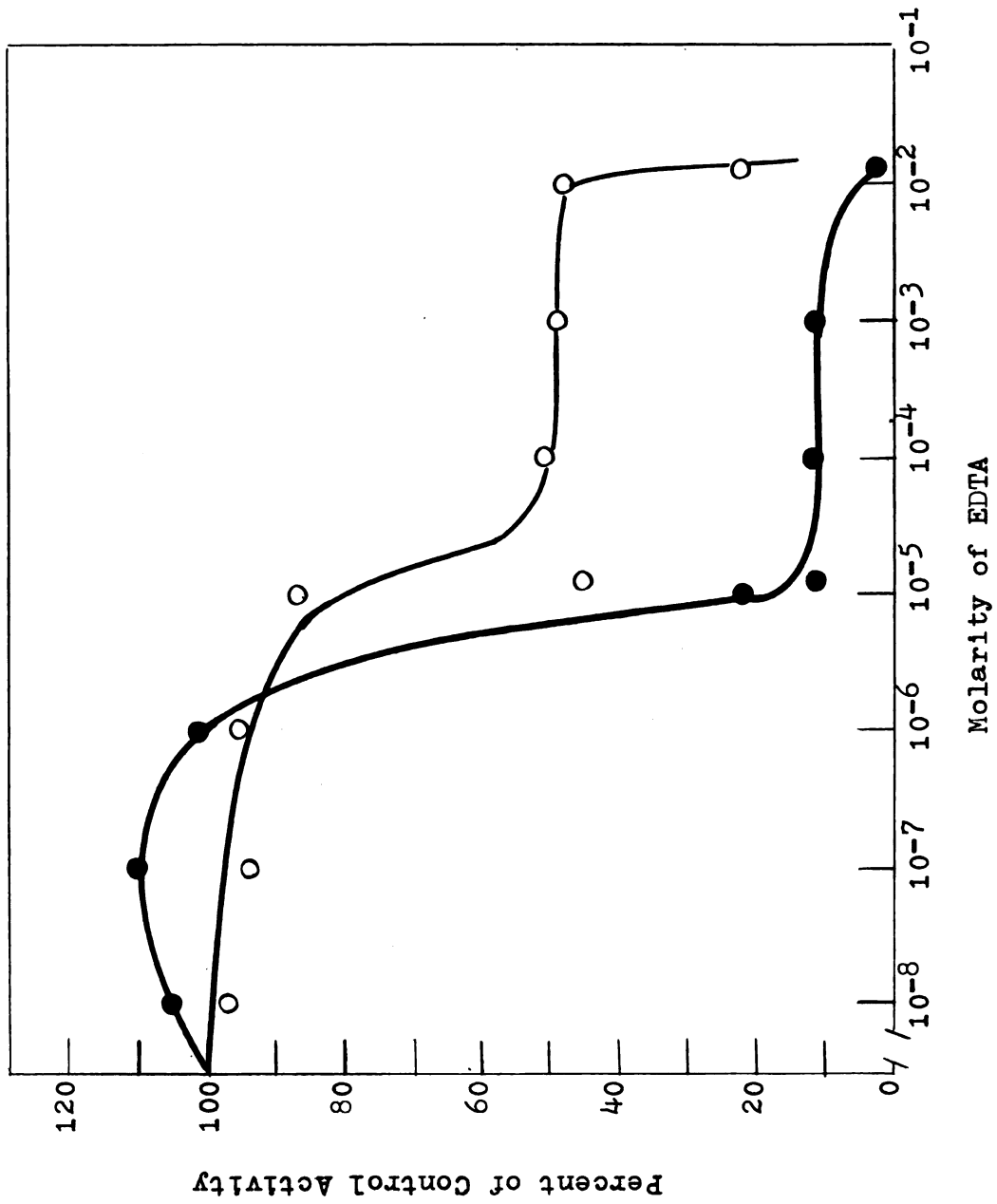

Percent of Control Activity
120
100
80
60
40
20
0
10-8
10-7
10-6
10-5
10-4
10-3
10-2
10-1
Molarity of EDTA

Table VI

Effect of cations on activity

Reactions were carried out at pH 5, as described in "Methods and Materials," with cations added as indicated five minutes before adding enzyme.

Addition to assay[a]	Percent of activity in absence of cation		
	DNase	RNase	3'-Nucleotidase
None	100	100	100
$MgCl_2$	92	133	89
$MnCl_2$	68	71	76
$ZnCl_2$	76	85	88
$CoCl_2$	54	39	66
$CaCl_2$	93	109	91
$FeCl_2$	61	56	--
$NiCl_2$	58	54	79
$CdCl_2$[b]	9	3	52
NaCl	113	107	80
KCl	115	128	92
NH_4Cl	103	113	91

[a]The final concentrations of the additives were 10^{-3} M except for NaCl, KCl and NH_4Cl which were 10^{-2} M.

[b]The experiments with Cd^{++} may not be valid in that obvious precipitates formed in all cases.

adding enzyme, incubating the mixture and assaying for enzymatic activity.

The DNase was inhibited to some extent by all the divalent ions tested, although only slightly by Mg^{++} and Ca^{++}. Relatively strong inhibition was obtained with Mn^{++}, Zn^{++}, Co^{++}, Fe^{++}, Ni^{++}, and Cd^{++}. The RNase activity was stimulated by Mg^{++} and Ca^{++} and relatively strongly inhibited by Mn^{++}, Co^{++}, Fe^{++}, Ni^{++}, and Cd^{++}. The 3'-nucleotidase activity was also relatively strongly inhibited by Mn^{++}, Co^{++}, Ni^{++}, and Cd^{++}. The experiments with Cd^{++} may not be valid in that obvious precipitates formed in all cases.

The addition of 10^{-2} M KCl, NaCl, or NH_4Cl to the incubation mixture resulted in a slight stimulation of the DNase and RNase activities. These same salts caused a slight inhibition of the 3'-nucleotidase activity.

In general the various ions resulted in qualitatively the same changes in rates of hydrolysis for all three activities. The stimulatory effect of Ca^{++} and Mg^{++} on the RNase activity may be due to an effect of these metals on the substrate and not necessarily an effect on the enzyme itself.

Effect of Amino Acid Modifying Reagents

The effect of a series of compounds which are known to react with specific amino acid residues of a protein was determined with the P-30 enzyme fraction. An aliquot of enzyme in Tris-chloride buffer, 0.025 M, pH 8, 0.1 mM in zinc acetate was treated with a final concentration of 10^{-3} M modifying reagent and allowed to stand at 4^{o} for 12 hours.

The treated enzyme was then assayed for each of the three enzyme activities in the standard manner. Appropriate controls were also performed to correct for any effects of the modifying reagents on the various substrates or assay procedures.

Table VII presents the data resulting from these experiments. At pH 8 all three enzyme activities were essentially completely destroyed by N-bromosuccinimide. None of the enzyme activities were decreased by treatment with iodoacetic acid, iodoacetamide, N-ethylmaleimide, or sodium arsenite. The enhancement of the RNase activity by all of these compounds is at present not readily explainable.

Temperature Effect and Heat Inactivation

The three activities were found to possess very similar heat stabilities. The phosphocellulose fraction was used for this study. This represented enzyme which had been previously heated to 75° during purification. Aliquots of enzyme (0.5 ml) were placed in conical centrifuge tubes and heated at selected temperatures for 8 minutes. The tubes were then placed in ice-water to achieve rapid cooling. Above 85° all three activities were essentially completely destroyed in 8 minutes. The temperature values for 50% reduction of activity for the DNase, RNase, and 3'-nucleotidase activities were 76°, 75°, and 75° respectively. Stimulation of all three activities

Table VII

Effect of some common amino acid modifying reagents on the enzyme activities

Samples (0.5 ml) of enzyme in 0.025 M Tris-chloride buffer at pH 8, 0.1 mM zinc acetate and 10^{-3} M in modifying reagent were left at 4° for 12 hours. The samples were then assayed as described in "Methods and Materials."

Modifying reagent	Percent of original activity remaining		
	DNase	RNase	3'-Nucleotidase
None	100	100	100
N-bromosuccinimide	1	1	0.5
Iodoacetic acid	101	126	100
N-ethylmaleimide	101	139	101
Iodoacetamide	101	119	100
Sodium arsenite	101	125	100

between 65° and 70° was also observed. This may be similar to the temperature effect observed by Eley and Roth (45) with the chicken pancreas nuclease. Our results are shown in Table VIII.

The results of experiments examining the effect on enzyme activity of the duration of heating at 70° were also found to be very similar for the three enzyme activities (Table IX).

The Q_{10} values for the increase of the three activities in the range of 27° to 47° were determined. The RNase activity exhibited the highest Q_{10} value of 4.2 to 4.5. The DNase and 3'-nucleotidase activities exhibited values that were somewhat lower, near 3.4 and 2.1 respectively.

Ratio of Enzyme Activities During Purification

The ratios of the total RNase and 3'-nucleotidase activities to the activity of the DNase at various stages of the purification procedure are presented in Table X. It is purely fortuitous that these ratios are both near one, since each enzyme activity is calculated in arbitrary units dependent, for example, on incubation time. Clearly, both ratios range only from 1.0 to 1.3 throughout these various procedures.

Effect of DNA and RNA on RNase and DNase Activities

The following experiment was performed to determine whether the addition of rRNA to the denatured DNA assay would inhibit the DNase activity. Since the rRNA would

Table VIII

Enzyme activity with respect to heat treatment at various temperatures

Samples (0.5 ml) of enzyme were heated at the temperatures indicated for 8 minutes as described in the text. The samples were assayed as described in "Methods and Materials." The control values for unheated enzyme are taken at 100%.

	Percent of control activity remaining		
Temperature	DNase	RNase	3'-Nucleotidase
65°	75	78	64
70°	96	87	92
75°	61	53	51
80°	14	17	15
85°	1	0	0

Table IX

Enzyme activity with respect to duration of heating at 70°

A sample (2.5 ml) of enzyme in Buffer B was heated in a water bath at 70°. Samples were removed at the times indicated and were rapidly cooled in ice-water. These samples were then assayed as described in "Methods and Materials."

Minutes at 70°	Percent of zero time activity remaining		
	DNase	RNase	3'-Nucleotidase
10	70	73	84
20	61	62	71
30	42	44	52
40	36	35	39
60	18	15	10
90	9	6	0

Table X

Comparison of the ratios of RNase and 3'-nucleotidase activities to that of DNase after various procedures

The ratios are of the specific activity of DNase to RNase or to 3'-nucleotidase activity, respectively.

Procedure	Ratio[a]	
	DNase: RNase	DNase: 3'-Nucleotidase
Ethanol fractionation	1.0	1.1
Heat fractionation	1.2	1.1
Phosphocellulose (salt gradient)	1.3	1.1
P-30 column	1.1	1.1
P-60 column	1.3	1.0
Phosphocellulose (pH gradient)	1.0	1.1
DEAE-cellulose, pH 8	1.2	1.1
ECTEOLA-cellulose, pH 6.5	1.0	1.3
Gel electrophoresis	1.1	1.2

[a]The average value for these ratios was 1.1 for both the DNase: RNase and DNase: 3'-Nucleotidase.

presumably also be hydrolyzed during the DNase assay it was not possible to use the lanthanum nitrate-acid soluble product assay for this experiment. For this reason the DNase activity was followed by measuring the release of TCA-soluble deoxyribose as determined by the diphenylamine reaction as described in "Methods and Materials." The release of TCA-soluble deoxyribose material was linear up to 20 minutes as shown in Table XI. During this same period the addition of an amount of rRNA equilivalent to the amount of denatured DNA in the reaction mixture was found to cause approximately 42% inhibition of the DNase activity. After 60 minutes incubation the hydrolysis of denatured DNA in the presence of RNA had reached 99% of the maximum value which was obtained in 30 minutes without added RNA.

A similar experiment was performed to determine the effect of added denatured DNA on RNA hydrolysis. In this experiment the release of TCA-soluble ribose measured by the orcinol reaction was used as a measure of RNase activity. The orcinol assay was performed as described in "Methods and Materials" and the appropriate correction for the orcinol reaction with deoxyribose was made for all values. These results are also shown in Table XI. It was found that the addition of an equivalent amount of denatured DNA caused a 60 to 70% inhibition of the RNase activity during the initial stages of rRNA hydrolysis. After 60 minutes incubation the inhibition had dropped to approximately 42%.

Table XI

Effect of DNA and RNA on RNase and DNase activities

DNase and RNase assays were performed in the standard manner with the following exceptions. To the DNase incubation mixture was added an equivalent amount (1 mg) of rRNA and to the RNase mixture was added an equivalent amount (1 mg) of denatured DNA. At the times indicated 1 ml of each reaction mixture was placed in 1.2 ml of cold 20% TCA. The mixture was allowed to stand at 4° for 15 minutes and then was centrifuged for 20 minutes at 1,100 x g at 4°. Aliquots, 0.2 ml for ribose determination and 1.0 ml for deoxyribose determination, were removed and assayed for TCA-soluble ribose or deoxyribose as described in "Methods and Materials." Control values for the μg of each sugar made TCA-soluble at each period of time in the absence of added nucleic acid are taken as 100%. All values for TCA-soluble ribose in the RNA digests containing DNA were corrected for the contribution of TCA-soluble deoxyribose to the orcinol color reaction.

Minutes of incubation	Effect of RNA on DNase	Effect of DNA on RNase
	Percent of control activity	Percent of control activity
10	57	31
20	58	36
30	77	41
60	99	58

Longer periods of digestion of RNA in the presence of denatured DNA were not investigated.

Effect of Sulfhydryl Compound and 8 M Urea on Gel Electrophoresis Pattern

The inactivation of the three activities by sulfhydryl compounds at pH 8 and the stabilizing effect of Zn^{++} suggested that the enzyme might be composed of two or more subunits. The staining pattern of the proteins after gel electrophoresis was examined for samples of the P-30 fraction which had been treated both with dithiothreitol, and with dithiothreitol and 8 M urea at pH 8. The samples of the P-30 fraction (0.2 ml) were brought to 1 mM in dithiothreitol and solid urea was added to one of the samples to a final concentration of 8 M. These samples were allowed to stand at 4^{o} for 18 hours and then were subjected to electrophoresis on 10% polyacrylamide gel as described in "Methods and Materials." The gels were stained with Coomassie Blue as described previously. The staining pattern of the sample treated with dithiothreitol at pH 8 showed a marked reduction in the intensity of the band corresponding to the active band in untreated samples. In the sample which was treated with both dithiothreitol and 8 M urea this band was completely absent. The effect of 8 M urea alone was not investigated.

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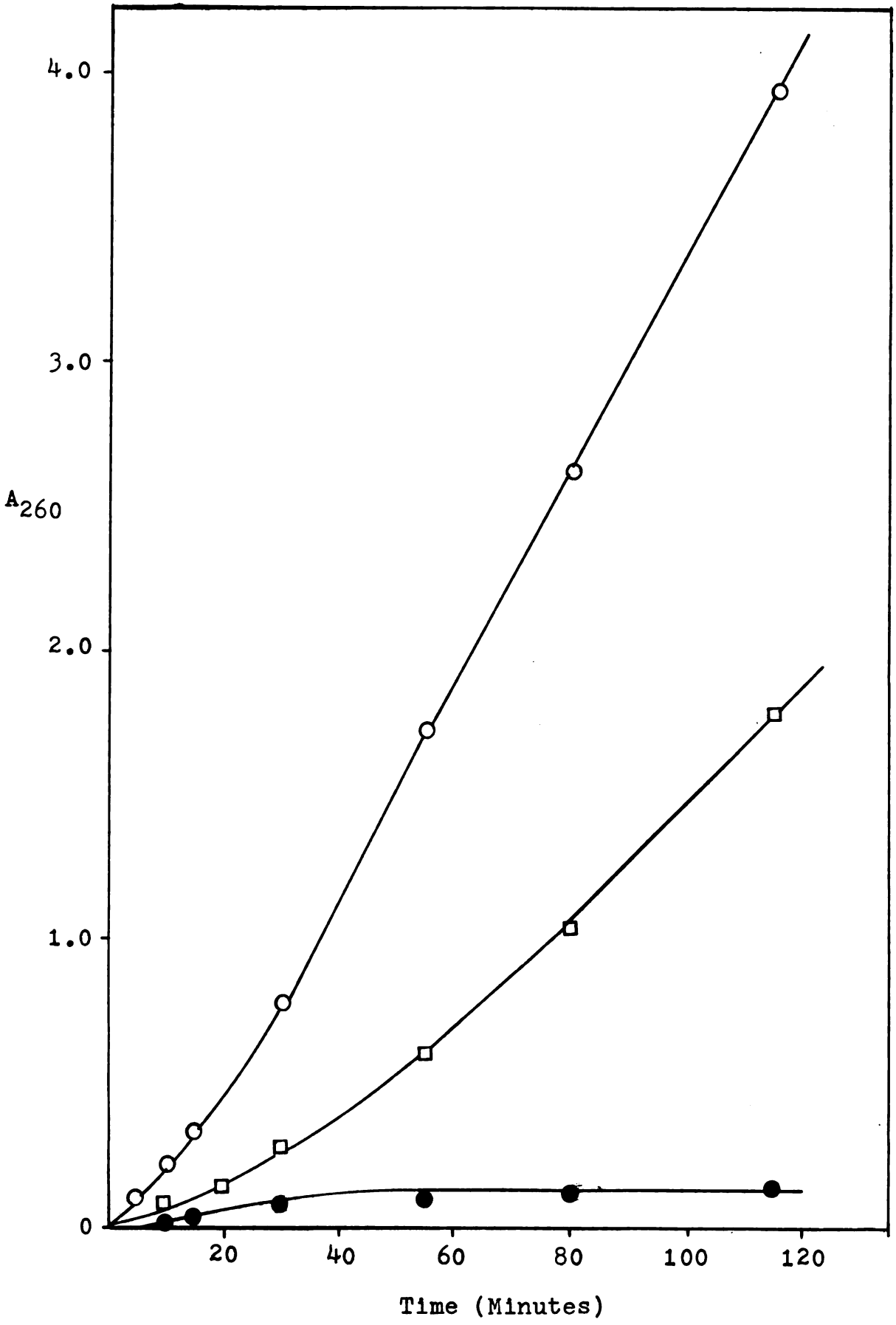
Studies on Enzyme Specificity

Activity Toward Native and Denatured DNA

One of the initial findings with crude wheat extracts was the relatively high level of activity toward denatured DNA as well as towards native DNA. The purification procedure for the wheat nuclease under investigation here was developed to maximize the activity toward denatured DNA. A time course of the hydrolysis of native and denatured DNA by purified wheat preparation is shown in Figure 8.

After an initial lag period, the hydrolysis of denatured DNA was linear with time. A similar lag period was also evident in the hydrolysis of native DNA. In early stages, the apparent rate of hydrolysis of native DNA was an appreciable fraction of that for denatured DNA--10% in the 15 to 25 minute period. However, the rate of liberation of lanthanum nitrate-acid soluble materials from native DNA decreased with time, so that in the 80 to 100 minute period, the rate was only 1.3% of that observed for the denatured DNA. No decrease in the rate of hydrolysis of the denatured DNA was apparent by the end of the experiment. Controls incubated in the absence of enzyme for the same periods of time showed essentially no increase in lanthanum nitrate-acid soluble material with either native or denatured DNA, thus demonstrating the absence of exogenous nuclease activity at pH 4.5 in the salmon sperm DNA preparation. From these results it appears that the DNase activity is highly prefer-

Figure 8: Time course of hydrolysis of denatured and native DNA and of rRNA as measured by the lanthanum nitrate-acid soluble product assay. Each substrate was incubated under standard conditions, as described in "Methods and Materials," with 0.3 units of enzyme. Reactions were terminated at the times indicated by placing the reaction mixture in ice-water and then the standard assay procedure was performed. Activity on: denatured DNA, O——O; native DNA, ●——●; rRNA, □——□.


4.0
3.0
2.0
1.0
0
A_{260}
20
40
60
80
100
120
Time (Minutes)

ential for denatured DNA, although this preference may not be absolute.

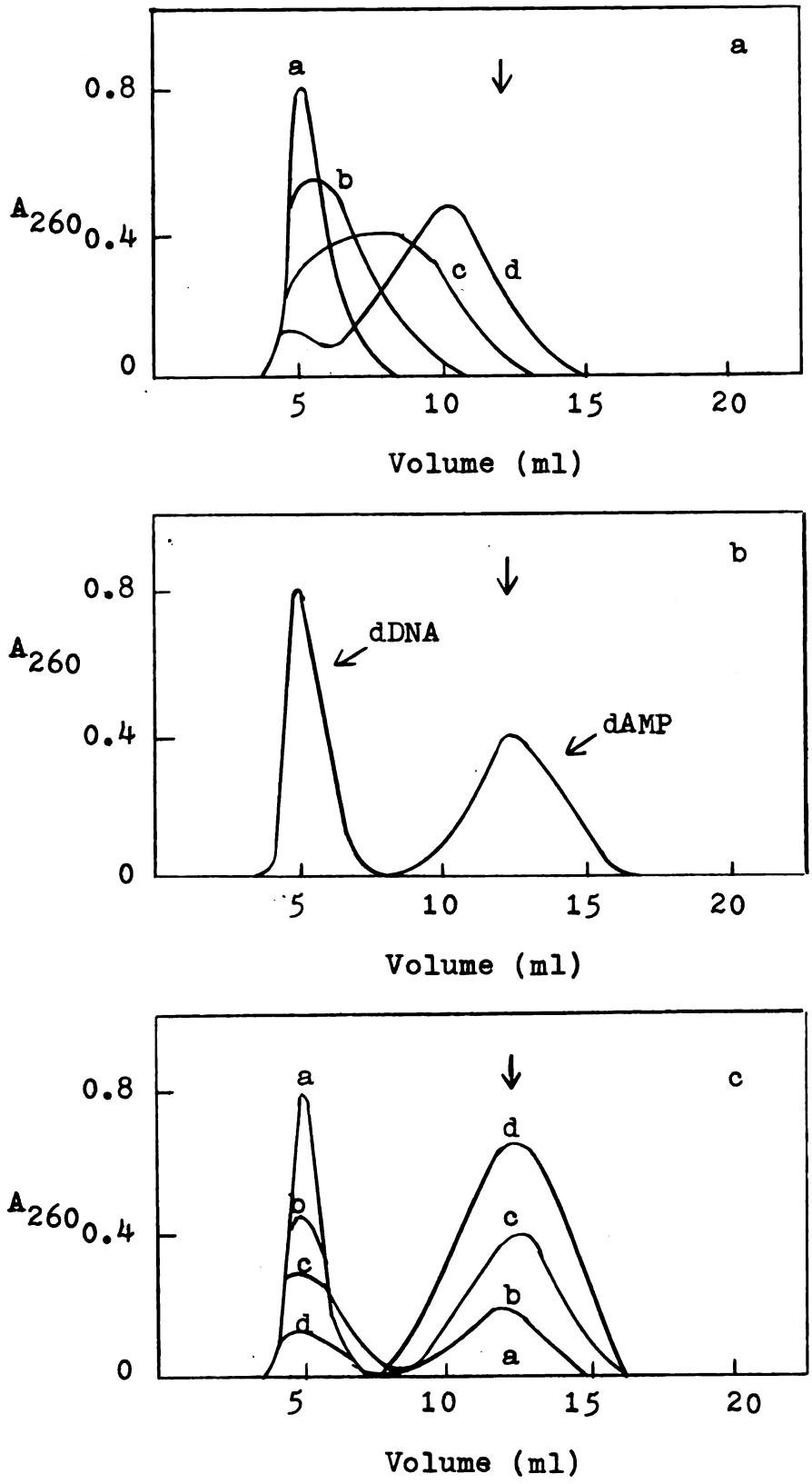
Mode of Action on Denatured DNA

In order to determine whether the hydrolysis of denatured DNA proceeded by an endo- or exonucleolytic mechanism, the following experiment was performed according to the method of Birnboim (66). A column, 1.5 x 10 cm, was packed with Bio-Gel P-100 in 0.15 M NaCl, 0.015 M sodium citrate at pH 7 and then washed with about 200 ml of the same solution. The column was attached to an ISCO ultraviolet monitor and a peristaltic pump was employed to regulate the flow rate at 1 ml per minute. A sample containing 500 μg of denatured DNA and 1 mg of 5'-dAMP in 0.5 ml of sodium acetate buffer, 0.05 M, pH 5, was chromatographed on the column. The elution pattern obtained gave the reference positions for denatured DNA and mononucleotides. Denatured DNA was incubated under standard assay conditions with 0.2 units of wheat DNase for various periods of time. At each time period 0.5 ml of reaction mixture was transferred to a conical centrifuge tube and heated at 100° for 3 minutes to inactivate the enzyme. The sample was cooled on ice and chromatographed on the P-100 column. The elution patterns at various incubation times are shown in Figure 9a and the standards are shown in Figure 9b. A similar experiment was performed with snake venom phosphodiesterase (Cortalus adamanteus venom) to determine the elution pattern from a known exonuclease. The incubations were the same as above except 50 μg of phosphodiesterase was added

Figure 9: a. Chromatography on P-100 of denatured DNA at various stages in its digestion by wheat DNase. Denatured DNA was incubated under standard assay conditions with 0.2 units of wheat DNase at 37°. Aliquots (0.5 ml) were removed at the times indicated and placed in a boiling water bath for 3 minutes. The sample was then cooled and chromatographed on a P-100 column, 1.5 x 10 cm, as described in "Methods and Materials." The elution patterns shown are for incubation periods of a) 0 minutes; b) 3 minutes; c) 6 minutes; d) 20 minutes. The arrow marks the position of the known mononucleotide, dAMP.

b. Chromatography on P-100 of a mixture of denatured DNA (dDNA) and mononucleotide (dAMP).

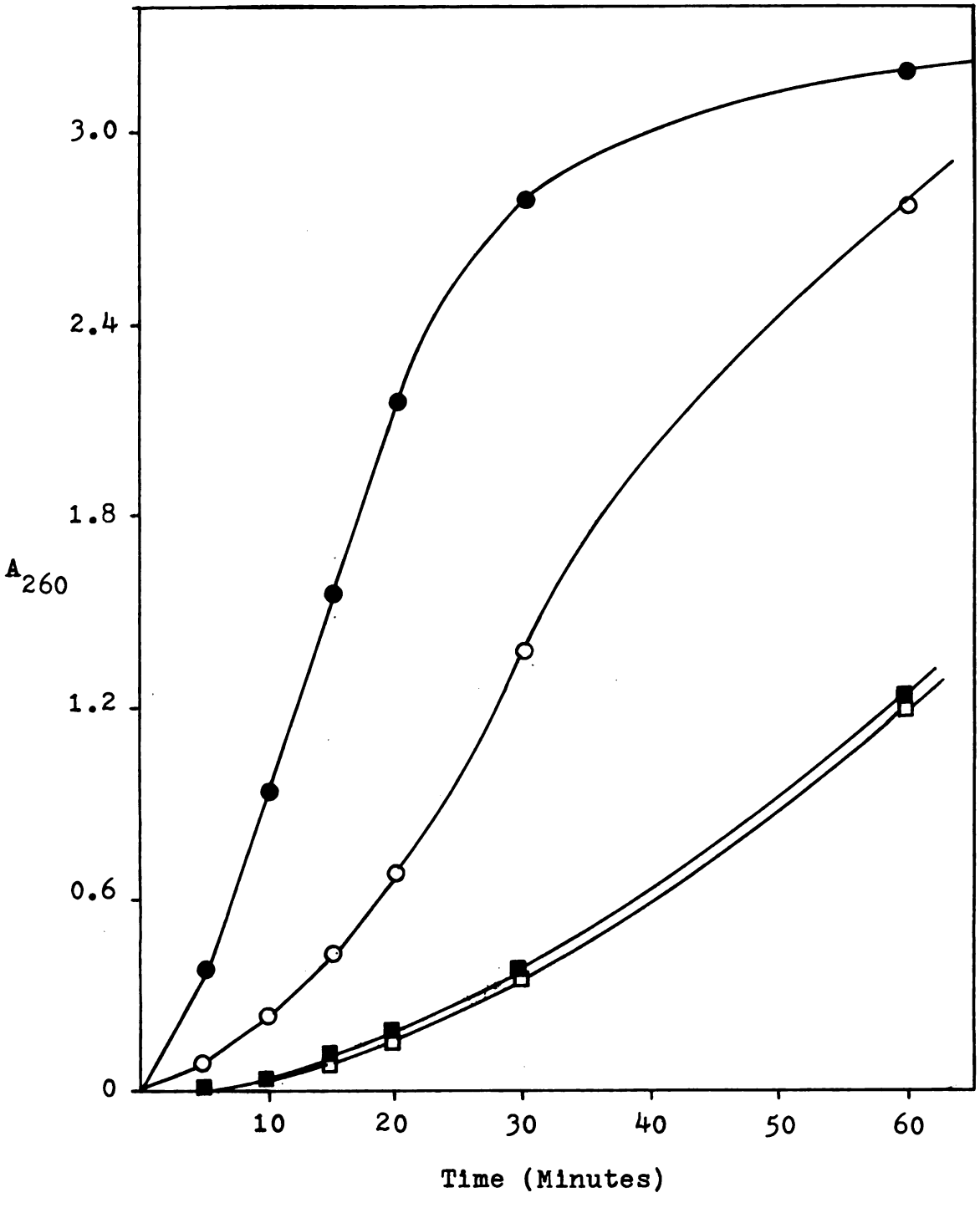
c. Chromatography on P-100 of denatured DNA at various stages in its digestion by snake venom phosphodiesterase, a known exonuclease. Incubation conditions were the same as above except that the buffer was 0.05 M Tris-chloride, pH 8.8, 0.03 M in $MgCl_2$ and 50 μg of snake venom phosphodiesterase was added to the reaction mixture. At the times indicated samples (0.5 ml) were removed and placed in a boiling water bath for 3 minutes, then cooled and chromatographed on the P-100 column. The elution patterns shown are for incubation periods of a) 0 minutes; b) 1 minute; c) 10 minutes; d) 60 minutes. The arrow marks the position of the known mononucleotide, dAMP.


a
0.8
A_{260} 0.4
0
a
b
c
d
5
10
15
20
Volume (ml)
b
0.8
A_{260}
0.4
0
dDNA
dAMP
5
10
15
20
Volume (ml)
c
0.8
A_{260} 0.4
0
a
b
c
d
d
c
b
a
5
10
15
20
Volume (ml)

instead of the wheat enzyme and longer incubation periods were required. The elution patterns obtained for venom phosphodiesterase hydrolysis of denatured DNA are shown in Figure 9c. The decrease in the height of the denatured DNA peak and the appearance of a mononucleotide peak is exactly what would be expected from an exonuclease producing mainly mononucleotides. In contrast the patterns from the wheat enzyme digest shows a decrease in the height of the denatured DNA peak and broadening of the peak in intermediate stages. This is what would be expected and is reported (66) as the pattern for an endonuclease which produces a variety of oligonucleotide fragments of all different sizes.

The DEAE assay procedure was also employed to study the mechanism of action of the DNase activity on denatured DNA. This procedure, as described in "Methods and Materials", should distinguish between an exo- and endonucleolytic attack on denatured DNA. Assuming that the 0.1 M salt wash removes mainly mononucleotides and short oligonucleotides from the DEAE, then for an exonuclease these two washes should give similar A_{260} values during the initial stages of hydrolysis. For an endonuclease the absorbancy of the 0.3 M wash should be much higher than the 0.1 M wash, since mainly larger oligonucleotides are being formed. Figure 10 shows a time course of the hydrolysis of denatured DNA by the wheat DNase as measured by the DEAE assay. After an initial lag the absorbancy of the 0.3 M wash was linear with time up to an A_{260} of about 2.0 which represents about 60% hydrolysis of

Figure 10: Time course of hydrolysis of denatured DNA by wheat DNase and snake venom phosphodiesterase as measured by the DEAE assay. Samples were incubated under standard conditions with 0.5 unit of wheat DNase or in 0.05 M Tris-chloride buffer, pH 8.8, 0.03 M $MgCl_2$ with 50 μg of snake venom phosphodiesterase. The curves for both salt washes (see "Methods and Materials") are shown: hydrolysis by the wheat DNase, 0.1 M NaCl wash, O——O; 0.3 M NaCl wash, ●——●; hydrolysis by venom phosphodiesterase, 0.1 M NaCl wash, □——□; 0.3 M NaCl wash, ■——■.

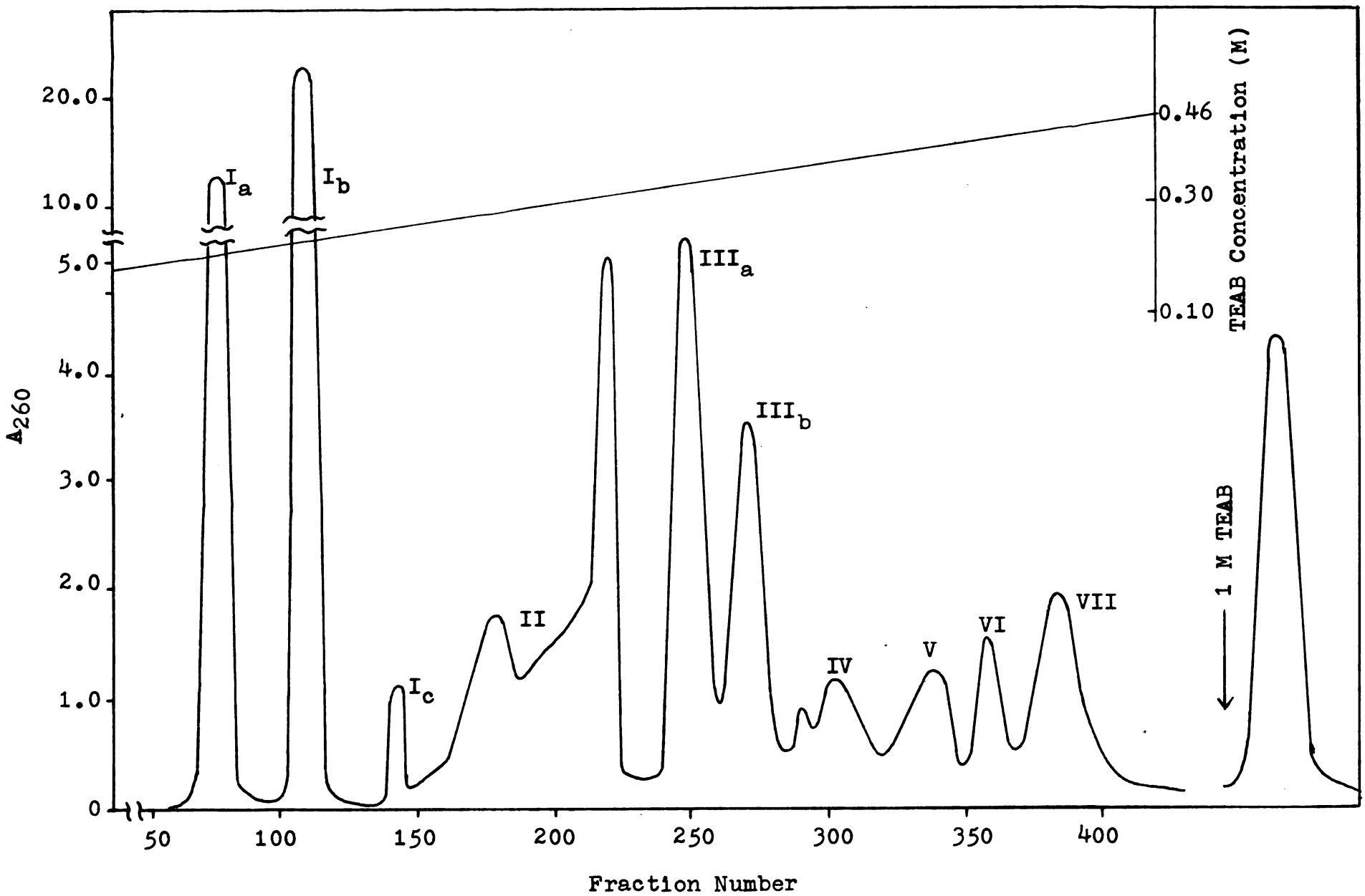

3.0
2.4
1.8
1.2
0.6
0
A_{260}
10
20
30
40
50
60
Time (Minutes)

the DNA. The 0.1 M wash exhibited a longer lag period and its absorbancy was lower than that of the 0.3 M wash curve at all incubation times, approaching it on long incubation. Figure 10 also shows a similar time course of hydrolysis of denatured DNA by snake venom phosphodiesterase. As would be expected for an exonuclease the two salt washes gave almost identical curves. The production of a large proportion of oligonucleotides throughout the digestion is taken as evidence for the endonucleolytic cleavage of denatured DNA by the wheat enzyme.

To further substantiate this a large scale digest of denatured DNA was chromatographed on a DEAE-Sephadex A-25 column (3 x 67 cm) as described in "Methods and Materials." The digest, containing 500 mg of denatured DNA in 500 ml of 0.05 M sodium acetate buffer, pH 5.0, 1 mM in zinc acetate, 1 mM in dithiothreitol, was incubated with 100 units of wheat DNase at 37° for 5 hours. The digestion was stopped by cooling in ice water and 28 ml of 0.1 M EDTA was added to give a final concentration of about 5 mM EDTA. This addition of EDTA was necessary to prevent the precipitation of zinc hydroxide when the solution was neutralized. To the solution was added 23.2 ml of 2 M triethylammonium bicarbonate and the pH was adjusted to 8.0 with 2 N NH_4OH. The digest was then applied to the DEAE-Sephadex column at a flow rate of 1 ml per minute. The column was extensively washed with 0.14 M triethylammonium bicarbonate at pH 8.0. A linear gradient from 0.14 to 0.3 M triethylammonium bicarbonate

was applied to the column, the total volume of the gradient was 4 liters. A second linear gradient from 0.3 to 0.46 M triethylammonium bicarbonate was then applied with a total volume of 8 liters. At the end of this gradient, the column was washed with 1 M triethylammonium bicarbonate. Figure 11 shows the elution pattern from the DEAE-Sephadex column. Peaks I_a, I_b and I_c are mononucleotides and described in "Methods and Materials." Peak II contains a mixture of at least 8 dinucleotides which were separated on paper chromatography in the 2 dimensional solvent system as described in "Methods and Materials." The peaks following peak II are assumed to be longer oligonucleotides in the order tri-, tetra-, pentanucleotide etc., as previously reported for this system (70). Assay of the digest chromatographed here revealed that the digestion had proceeded to about 75% conversion of the denatured DNA to lanthanum nitrate-acid soluble material. A similar experiment in which the digest was chromatographed on DEAE-Sephadex after only 1 hour's incubation gave a similar elution pattern. However, the proportion of material in the higher oligonucleotide peaks was much greater. No P_i or nucleosides were observed in either the 1 or the 5 hour digestion of denatured DNA. Thus the wheat DNase produced, as would be expected of an endonuclease, a variety of oligonucleotides of varying chain length in the hydrolysis of denatured DNA.

Figure 11: Elution pattern from a DEAE-Sephadex A-25 column of mono- and oligonucleotides produced during the hydrolysis of denatured DNA by wheat DNase. A column, 3 x 67 cm, of DEAE-Sephadex A-25 was packed and washed with 0.14 M triethylammonium bicarbonate (TEAB) at pH 8. A 5 hour digest of denatured DNA (500 mg) with 100 units of wheat DNase, as described in "Methods and Materials," was applied to the column and the column was washed with a liter of 0.14 M TEAB. A linear gradient from 0.14 to 0.3 M TEAB was applied to the column; the total volume of the gradient was 4 liters. This was followed by a second linear gradient from 0.3 to 0.46 M TEAB in a total volume of 8 liters. A flow rate of 1 ml per minute was maintained with the use of a peristaltic pump and fractions of approximately 20 ml were collected. Peak I_a contained dTMP and dCMP. Peak I_b and I_c contained dAMP and dGMP respectively.


20.0
10.0
5.0
4.0
3.0
2.0
1.0
0
A_{260}
I_a
I_b
I_c
II
III_a
III_b
IV
V
VI
VII
1 M TEAB
0.46
0.30
0.10
TEAB Concentration (M)
50
100
150
200
250
300
350
400
Fraction Number

Determination of Phosphate Position in Mononucleotides Obtained from Denatured DNA Hydrolysis

The following experiment was performed to determine whether the mononucleotides produced on hydrolysis of denatured DNA were the 3'- or 5'-isomers. Samples containing either 10 mg of material from the dAMP or 3 mg of material from the dCMP: dTMP peak eluted from the DEAE-Sephadex column (see next section) were prepared in 0.08 M Tris-chloride buffer, pH 8.2, 0.008 M in $MgCl_2$. Aliquots of these solutions were treated with approximately 5 units of either *E. coli* alkaline phosphatase, snake venom 5'-nucleotidase or rye grass 3'-nucleotidase for 90 minutes at 37°. The samples were then assayed for P_i as described in "Methods and Materials" for the 3'-nucleotidase assay. Standard 3'- and 5'-AMP (ribo-) were also used as controls. Table XII shows the data from this experiment. The values are expressed as the percentage of P_i released by alkaline phosphatase assuming that the enzyme would cleave all the phosphomonoester bonds present regardless of their position in the mononucleotide. Thus 5'-nucleotidase was observed to release essentially the same amount of P_i, if not more, from the mononucleotides of the DNA digest as was released by alkaline phosphatase. The 3'-nucleotidase released only about 1% of the P_i from dAMP and about 9% from the dCMP: dTMP mixture. Both nucleotidases were found to release about 1.6% of the P_i from the opposite nucleotide. This probably was caused by slight contamination of these enzymes by non-specific phosphatase

Table XII

Identification of phosphate position in mononucleotides from the hydrolysis of denatured DNA

Samples of the dAMP and dCMP: dTMP mixture separated from a 5 hour digest of denatured DNA with wheat DNase were treated with 5 units of either alkaline phosphatase, 3'-nucleotidase, or 5'-nucleotidase at 37° for 90 minutes. The samples were then assayed for P_i as described in "Methods and Materials." The values for the percent of P_i liberated by each nucleotidase are based on 100% for alkaline phosphatase, assuming that this enzyme hydrolyzed all of the phosphomonoester bonds.

	Percent of P_i released by alkaline phosphatase	
Substrate	5'-Nucleotidase	3'-Nucleotidase
dAMP	118	1.1
dCMP:dTMP	99	8.7
Known 5'-AMP	107	1.6
Known 3'-AMP	1.6	102

activity. Approximately 9% of the P_i was released from the dCMP: dTMP by 3'-nucleotidase, however, 5'-nucleotidase released 99% of the P_i. The cause of the apparent discrepancy between these two figures is not clear. It is apparent that at least the majority, if not all, of the mononucleotides bear a 5'-phosphoryl group. It was not possible to examine the deoxyguanylate because of the relatively low level of production of this mononucleotide during the hydrolysis. Thus, the wheat DNase appears to produce 5'-mononucleotides on hydrolysis of denatured and it is tacitly assumed that the oligonucleotides bear 5'-termini as well.

Mononucleotide Production from Denatured DNA at Various Stages of Digestion

As mentioned previously it was observed on chromatography of the digestion products from denatured DNA hydrolysis that a very small amount of deoxyguanylate was present in the digests. This observation prompted the following experiment to determine the relative proportion of the 4 mononucleotides at different stages of digestion. Solutions containing 40 mg of denatured DNA in 0.05 M sodium acetate buffer, pH 4.5, 1 mM in zinc acetate, 1 mM in dithiothreitol were incubated with 40 units of wheat DNase at 37° for 15, 30, 60 and 300 minutes. At the end of the incubation period each solution was cooled in ice-water and 2.5 ml of 0.1 M EDTA was added. The solution was allowed to stand for 5 minutes at 4° and 1.3 ml of 2 M triethylammonium bicarbonate was added and the pH was adjusted to 8.0 with 2 N NH_4OH. This material was then

applied to a DEAE-Sephadex column, 2.2 x 15 cm, and a batch-wise treatment with 0.18 M triethylammonium bicarbonate was performed as described in "Methods and Materials." Table XIII contains the data from a typical experiment. Since dCMP and dTMP appear in the same peak it was necessary to determine the concentration of these compounds by a spectrophotometric analysis of the binary mixture as described in "Methods and Materials." At all periods of digestion investigated dAMP was the most prevalent mononucleotide. The level of dAMP was always about 2-fold greater than any of the other 3 deoxynucleotides. The level of dAMP was increased throughout the digestion while the levels of dTMP and dCMP remained essentially constant at all early stages of digestion. In contrast, the level of dGMP decreased from about 24% of the total mononucleotide fraction at 15 minutes to only 5% at 60 minutes and to only about 2% after 300 minutes. It should be noted that these values are the percentage of the total mononucleotide fraction represented by each mononucleotide. Thus, while dGMP decreased in percent of the total mononucleotide fraction the actual number of μmoles of all mononucleotides, including dGMP, increased at all periods of digestion. Since the recovery of known dGMP solutions from the DEAE-Sephadex column is approximately 100 percent (see "Methods and Materials") it is felt that this low level of dGMP is real and not an artifact of the separation procedure. Furthermore, on total base analysis of all the peaks obtained from the DEAE-Sephadex column

Table XIII

Composition of the mononucleotide fraction at various stages in the digestion of denatured DNA

Solutions containing 40 mg of denatured DNA in 0.05 M sodium acetate buffer, pH 4.5 1 mM in zinc acetate, 1 mM in dithiothreitol were incubated with 40 units of wheat DNase at 37° for 15, 30, 60 and 300 minutes. The reaction was terminated by cooling and 2.5 ml of 0.1 M EDTA and 1.3 ml of 2 M triethylammonium bicarbonate (TEAB) was added. The pH was adjusted to 8. The samples were then applied to columns of DEAE-Sephadex A-25, 2.2 x 15 cm, and the columns were washed with 0.14 M TEAB. The mononucleotides were separated by a batch-wise treatment of the columns with 0.18 M TEAB as described in "Methods and Materials." Percentage values for mononucleotides are based on the μmoles of each nucleotide produced at each time period.

Minutes of incubation	Percent hydrolysis of dDNA[a]	Percent of total products as mononucleotides	Nucleotide distribution as Percent of total mononucleotides			
			dAMP	dTMP	dCMP	dGMP
15	11.9	1.5	40	24	13	24
30	29.3	2.4	50	24	12	12
60	54.5	5.8	61	23	10	5
300	72.0	32.1	45	25	28	2

[a]dDNA refers to denatured DNA.

described previously it was possible to account for approximately 100 percent of the deoxyguanylate residues in the original denatured DNA. Thus it appears that the remaining deoxyguanylate residues are contained in the longer oligonucleotides.

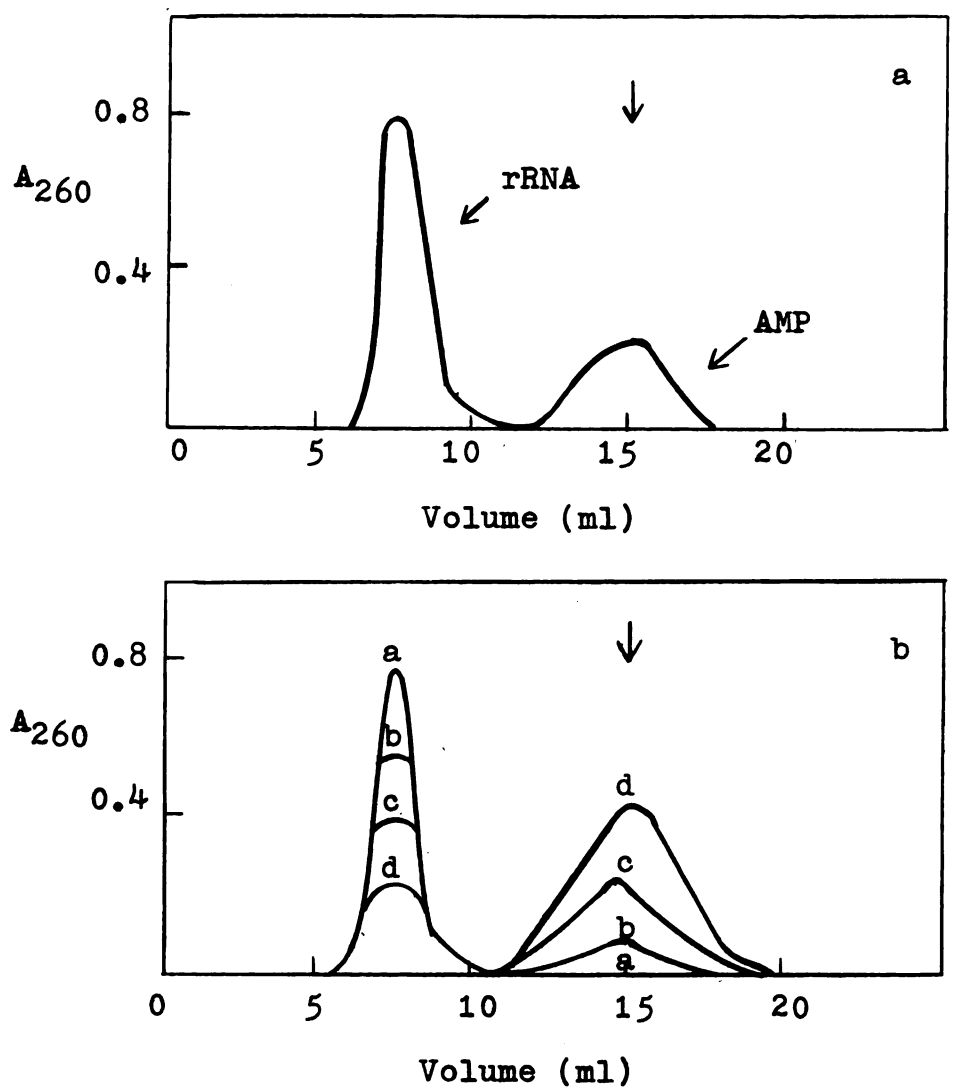
Activity Towards rRNA

A time course of rRNA hydrolysis by the wheat RNase is shown in Figure 8. After an initial lag the reaction proceeds in a linear manner.

In order to gain insight into the mode of action of the wheat RNase on rRNA a gel filtration experiment similar to that described above for denatured DNA was performed. A column, 1.5 x 25 cm, of Bio-Gel P-6 was packed and washed as described for the P-100 column. It was necessary to use P-6 Bio-Gel for this experiment instead of P-100 because of the lower molecular weight of the rRNA. The rRNA was incubated in 0.05 M sodium acetate buffer, pH 4.5, 1 mM in dithiothreitol, 1 mM in zinc acetate, with 0.6 units of enzyme for various periods of time. At each time interval an 0.4 ml sample was removed and heated to 100° for 3 minutes to inactivate the enzyme. This sample was then chromatographed on the P-6 column. The elution pattern at various stages of digestion is shown in Figure 12b. This is the type of pattern that one would expect from an exonuclease and was, indeed, the type of pattern observed with venom phosphodiesterase on denatured DNA (see Figure 9c). The elution

Figure 12: a. Chromatography on P-6 of a mixture of rRNA and mononucleotide, AMP.

b. Chromatography of P-6 of rRNA at various stages in its digestion by wheat RNase. Incubations were performed under standard conditions with 0.6 units of enzyme. Aliquots (0.4 ml) were removed at the times indicated and placed in a boiling water bath for 3 minutes. The samples were then cooled and chromatographed as above. The elution patterns shown are for incubation periods of a) 0 minutes; b) 15 minutes; c) 30 minutes; d) 60 minutes. The arrow indicates the position of the known mononucleotide, AMP.


a
0.8
A_{260}
0.4
rRNA
AMP
0
5
10
15
20
Volume (ml)
b
0.8
A_{260}
0.4
a
b
c
d
d
c
b
a
0
5
10
15
20
Volume (ml)

profile of rRNA and mononucleotide (AMP) standards are shown in Figure 12a. Thus it would appear that the wheat RNase activity acts predominately in an exonucleolytic manner on rRNA.

Effect of Zinc Ion on rRNA Hydrolysis

The following experiment was performed to determine the relative percent of lanthanum nitrate-acid soluble material which was dialyzable. Ribosomal RNA (2 ml) was hydrolyzed under normal assay conditions with 2.5 units of wheat RNase for 30 minutes in a total volume of 3 ml. The reaction was terminated by the addition of 3 ml of cold lanthanum nitrate-HCl reagent. The mixture was allowed to sit at 4° for 10 minutes and then was centrifuged for 20 minutes at 4°. To the clear supernatant fluid containing the lanthanum nitrate-acid soluble digestion products was added 1.0 ml of 0.1 M EDTA and the pH was adjusted to approximately 6 with 2 N NH_4OH. A sample (5 ml) of the neutralized supernatant fluid was placed in "small pore" dialysis tubing (#23) and dialyzed against 30 ml of distilled water at 4° for 5 hours. A control containing the 4 known deoxymononucleotides which had been taken through the lanthanum nitrate-HCl treatment was also dialyzed to determine when dialysis equilibrium had been achieved. The A_{260} of the dialysis fluid was determined and the percentage of the lanthanum nitrate-acid soluble products which were dialyzable was calculated. The results of this experiment were found to be different in the presence and absence of zinc. These results are shown

in Table XIV. In the absence of Zn^{++} approximately 50% of the lanthanum nitrate-acid soluble material was dialyzable. In the presence of 1 mM Zn^{++}, however, 80% of the lanthanum nitrate-acid soluble material produced in the same period of incubation was dialyzable. Because of the inhibitory effect of Zn^{++}, this sample reached only about 85% of the level of hydrolysis obtained without Zn^{++}. Thus it would appear that while a lower level of hydrolysis is obtained in the presence of Zn^{++}, a larger percentage of the hydrolysis products are dialyzable and presumably are mononucleotides.

Identification of Mononucleotides Produced from rRNA Digestion

A total of 10 mg of rRNA was digested with 10 units of wheat RNase at 37° for 5 hours. The mononucleotides were separated from the digestion mixture on DEAE-Sephadex by a batch-wise elution with 0.18 M triethylammonium bicarbonate as described in "Methods and Materials." All 4 ribonucleotides were present in about the same amounts in the digest. The mononucleotides were then treated with 5'- and 3'-nucleotidase as described previously for the mononucleotides from the DNA digest. Aliquots of the nucleotidase treated material were spotted on Whatman 3 mm paper and chromatographed in solvent I as described in "Methods and Materials." No conversion of any of the mononucleotides to the corresponding nucleosides was observed with either 5'- or 3'-nucleotidase as determined by their position on the chromato-

Table XIV

Effect of Zn^{++} on the hydrolysis of rRNA by wheat RNase

Samples of rRNA (2 mg) were incubated in a volume of 3 ml with 2.5 units of wheat RNase for 30 minutes at pH 4.5 in the presence and absence of 1 mM Zn^{++}. The reaction was terminated by the addition of 3 ml of cold lanthanum nitrate-HCl reagent. The mixture was allowed to stand at 4° for 10 minutes and then was centrifuged at 4° for 20 minutes at 1,100 x g. To the supernatant fluid was added 1 ml of 0.1 M EDTA and the pH was adjusted to 6. Samples (5 ml) were placed in "small pore" dialysis tubing and dialyzed against 30 ml of distilled water for 5 hours at 4°. The A_{260} of the dialysis fluid was determined.

Molarity of Zn^{++}	Percent of hydrolysis without Zn^{++}	Percent of products dialyzable
Control[a]	---	100
0	100	50
1 mM	85	80

[a]Solution containing the 4 known deoxymononucleotides.

gram. Treatment of the mononucleotides with alkaline phosphatase and chromatography in this solvent system revealed a conversion of the purine mononucleotides to nucleosides but was without effect on the pyrimidine mononucleotides. These results suggested that the RNase forms 2',3'-cyclic mononucleotides as hydrolysis products, and then converts the cyclic purine nucleotides to the 2'-mononucleotides. Experiments were performed using the homo-polymer oligo-uridylate as substrate to see if cyclic-UMP could be identified as the mononucleotide produced. A solution containing 2.5 mg of oligo-uridylate in 0.25 ml of 0.05 M sodium acetate buffer, pH 5, 2 mM in cysteine was incubated with 2 units of RNase at 37°. At zero, 15, 30 and 60 minutes an aliquot (50 µl) was removed and placed in a boiling water bath for 3 minutes to inactivate the enzyme. The sample was then spotted on DEAE-cellulose chromatography paper (DE-81) along with appropriate standards and chromatographed in 0.1 M ammonium formate solvent for 4 hours. At all stages of incubation the predominate mononucleotide component had an R_f value of 0.55. The R_f values in this solvent system for known 2',3'-cyclic-UMP, 2'-UMP and 3'-UMP were 0.54, 0.29 and 0.28 respectively. Thus it was tentatively concluded that the enzyme produced predominately 2',3'-cyclic-UMP upon digestion of oligo-uridylate. A small amount of material was also present at a position approximately that of 2'- or 3'-UMP.

As a further verification of the production of cyclic-uridylate, a sample of oligo-uridylate was hydrolyzed as

described above for a total of 120 minutes with 2 units of wheat RNase. The digestion mixture was split into 2 portions. One portion was taken to dryness in a rotary flash evaporator. The material was then dissolved in 20 µl of 80% acetic acid and allowed to stand at room temperature (about 25°) for 2 hours. The acetic acid digest was then flashed to dryness and suspended in 20 µl of water and taken to dryness. This process was repeated 3 times. The sample was finally dissolved in 20 µl of 95% ethanol and flashed to dryness to remove any residual traces of acetic acid. The material was dissolved in 10 µl of water and spotted on DEAE-cellulose paper. This hydrolysis condition, 80% acetic acid at room temperature for 2 hours, should have been sufficient to convert the 2',3'-cyclic-UMP to a mixture of 2'- and 3'-UMP but should not have hydrolyzed any oligonucleotides such as UpU or UpUp etc.[4] As controls, both known 2',3'-cyclic-UMP and oligo-UMP were hydrolyzed with 80% acetic acid and subsequently treated in the same manner as the enzyme digest. The 120 minute digest which was not hydrolyzed with 80% acetic acid showed a predominate spot at the 2',3'-cyclic-UMP position as expected. In the corresponding sample which was subject to 80% acetic acid hydrolysis, the 2',3'-cyclic-UMP spot was essentially absent and the bulk of material now chromatographed in the same position as 2'- and 3'-UMP. The known 2',3'-cyclic-UMP standard which was hydrolyzed with 80% acetic acid had like-

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[4]F. M. Rottman, personal communication.

wise been converted to 2'- and 3'-UMP. The chromatographic pattern of oligo-uridylate which had been subjected to 80% acetic acid hydrolysis was essentially the same as the pattern of unhydrolyzed material. This indicates that the oligonucleotide material was not affected by 80% acetic acid hydrolysis.

DISCUSSION

The wheat DNase under investigation has been purified approximately 830-fold with a recovery of about 12% of the total DNase activity present in the crude homogenate. Some enzyme preparations have been obtained which were approximately 1000-fold purified with a recovery of about 20% of the total activity. The assays of the ammonium sulfate fraction were always quite low because of retention of ammonium sulfate which is inhibitory to the enzyme activities. Assays of the ethanol fraction were also poorly reproducible. This fraction routinely gave high values and it was found that the addition of a small amount of ethanol to the purified enzyme caused a slight but significant increase in activity. The crude homogenate contains an appreciable level of activity towards 5'-AMP and bis-p-nitrophenyl phosphate. Both of these activities are essentially completely removed by the heat treatment at 75°. Thus while the specific activity of the DNase activity decreased during this purification step, this step was necessary for the removal of at least two of the major contaminating enzyme activities from the enzyme preparation. Chromatography on the phosphocellulose column removed about 85% of the protein in the pass through and wash fractions. However, these fractions exhibited only slight enzyme activity. The recovery of DNase activity from the

phosphocellulose column was about 50% of that applied to the column. In some preparations recoveries as high as 70% have been obtained at this step while the recovery of protein was always about the same. The use of the combination column of phosphocellulose and P-30 was a very beneficial procedure. The enzyme activities were obtained in a volume only one twelfth of that applied to the column, an increase in the total units of all three activities was observed, and the material was completely free of salt. The latter eliminates the need for the dialysis of the phosphocellulose fraction, a procedure which resulted in a net loss in enzyme activity. Further concentration of the P-30 fraction was most rapidly achieved by flash evaporation of the pooled fractions at 28° and subsequent dialysis of the concentrate to bring the buffer concentration down to 0.05 M. Dialysis against carbowax and lyophilization were also attempted. Dialysis against 30% carbowax in Buffer B was an effective means of concentration, however, the procedure was slower and material from the carbowax which dialyzed into the enzyme solution could not be removed by subsequent dialysis against Buffer B alone. This material strongly interfered with the Lowry protein determination. Lyophilization repeatedly gave poor recoveries of enzyme activity.

It was necessary to carry the enzyme preparation through the heat step within about 12 hours after the initial homogenization. The activity in the cruder fractions was very instable, however, these fractions could be stabilized for

several days by making them 3 M in urea. This treatment appeared to prevent the aggregation and precipitation of the wheat proteins as is consistent with the findings of Wu et al. (43) on the effect of 3 M urea on wheat proteins. This procedure was not routinely employed, however, because of problems caused by the presence of urea in subsequent purification steps.

The point of sulfhydryl addition to the enzyme preparation was found to be quite critical. The sulfhydryl compound was routinely added to the enzyme preparation after the ethanol fractionation and before the heat treatment. Addition of sulfhydryl to any step prior to the ethanol fractionation resulted in a complete loss of enzyme activity within a few hours at 4°. This was presumably caused by the activation of some sulfhydryl requiring protease(s) in the cruder fractions which was finally removed by the ethanol fractionation. After the heat step the three enzyme activities showed an absolute requirement for the presence of a sulfhydryl compound at pH 4.5.

The enzyme preparation exhibits no detectable hydrolysis of 5'-AMP, bis-p-nitrophenyl phosphate, 5'-p-nitrophenyl thymidylate, or 5'-p-nitrophenyl adenylate. A very low level of hydrolysis of p-nitrophenyl phosphate and of 3'-p-nitrophenyl thymidylate was observed. These activities were assayed with a 10-fold greater level of enzyme and for 24 hours at 37° which is a 144-fold excess of time over the normal DNase assay conditions. Thus it would appear that

the purified wheat enzyme preparation contains little, if any, contaminating enzyme activities.

The lanthanum nitrate-acid soluble product assay was found to be a relatively rapid and reproducible assay for the hydrolysis of both denatured and native DNA and of rRNA. The fact that the rates of hydrolysis of both denatured DNA and RNA were not proportional to enzyme concentration at high and low concentrations was somewhat of a problem. This is similar to the results obtained by Curtiss, Burdon, and Smellie (71) with the rat liver DNase and to the results obtained with other nucleases. This problem could be effectively overcome, however, by always choosing an aliquot of enzyme which gave values in the range of 1.0 to 2.0 A_{260} units which was the most nearly linear region of the curve. Values in this region were relatively proportional to each other and were found to be quite reproducible. The DEAE-cellulose assay is rather advantageous because it can distinguish between exo- and endonuclease activities. The assay is fairly rapid and quite reproducible. However, because this assay is dependent on the binding of oligonucleotides to the resin, any appreciable change in the pH or ionic strength of the sample will greatly effect the results. For this reason the assay can only be used for a given, well defined set of assay conditions.

The ability of the enzyme preparation to hydrolyze both denatured DNA and RNA is not surprising by itself, since many purified nucleases exhibit no specificity towards the

sugar moiety of the substrate and thus hydrolyze both DNA and RNA. The associated 3'-nucleotidase activity could also simply be an additional activity of a non-specific nuclease. However, experiments on the mode of action of the wheat enzyme on denatured DNA and rRNA indicate that the hydrolysis proceeds by different mechanisms for these two substrates. The mononucleotides produced on hydrolysis of denatured DNA are exclusively 5'-mononucleotides, while those produced on hydrolysis of rRNA appear to be mainly the 2',3'-cyclic compounds. The mononucleotides isolated from a digest of denatured DNA were subjected to treatment with alkaline phosphatase, snake venom 5'-nucleotidase, and rye grass 3'-nucleotidase. The 5'-nucleotidase released essentially the same amount, if not more, of P_i from the mononucleotides as did alkaline phosphatase. The 3'-nucleotidase released only a small amount of P_i from the same mononucleotides. Thus the mononucleotides produced on digestion of denatured DNA by wheat DNase are predominately, if not exclusively, 5'-mononucleotides. The mononucleotides produced from rRNA digestion were found to be resistant to both 3'- and 5'-nucleotidase. Chromatography on DEAE-cellulose paper in 0.1 M ammonium formate of a digest of oligo-uridylate showed that 2',3'-cyclic-UMP was produced by the wheat RNase. This was further substantiated by conversion of the cyclic-UMP to a mixture of 2'- and 3'-UMP on hydrolysis with 80% acetic acid at room temperature. Under the hydrolysis conditions employed, the oligo-uridylate showed no detectable hydrolysis.

Thus the wheat RNase forms 2',3'-cyclic-UMP on hydrolysis of oligo-uridylate and it is tacitly assumed that the mononucleotides produced from rRNA are also the 2',3'-cyclic compounds. The observation that alkaline phosphatase, which does not hydrolyze 2',3'-cyclic mononucleotides, caused conversion of the purine nucleotides to the corresponding nucleosides is of interest. It is possible that the wheat RNase forms 2',3'-cyclic mononucleotides initially and then in a second reaction converts the purine nucleotides to the curresponding 2'-mononucleotides. These would then be hydrolyzed by alkaline phosphatase as was observed. If, indeed, this were the case the 3'-nucleotidase activity of wheat may simply represent the second stage of this reaction in which the 3'-phosphoester bond is cleaved. Since in the 3'-mononucleotide the phosphoryl group is not attached to the 2' position the phosphoryl group would be removed instead of transfered to the 2' position as would occur with the 2',3'-cyclic mononucleotide. This type of activity has been reported by Mcleod and Huang (72) for a ribonuclease from mouse lymphosarcoma.

It also appears that while the wheat enzyme attacks denatured DNA primarily, if not exclusively, in an endonucleolytic manner, the mode of attack on rRNA is primarily exonucleolytic. The results of the gel filtration elution patterns on P-100 indicate a predominately endonucleolytic attack on denatured DNA. The pattern obtained was that of an endonuclease which produces a variety of oligonucleotides

of various sizes. This was further substantiated by the results of the DEAE assay which distinguishes between endo- and exonuclease type activities. The curve for the 0.3 M NaCl wash (oligonucleotides) was much higher than that of the 0.1 M NaCl wash (mononucleotides) at all periods of incubation. When snake venom phosphodiesterase, a known exonuclease, was assayed by this procedure the two curves coincided throughout the incubation as would be expected since only mononucleotides were being produced by this enzyme. The distribution of oliognucleotides on DEAE-Sephadex chromatography of the digestion mixtures also indicates that a wide variety of oligonucleotides are produced from denatured DNA by the wheat DNase. This plus the fact that at earlier periods of incubation a larger proportion of the material was present in the higher oligonucleotide peaks is taken as further proof of the endonucleolytic action of wheat DNase on denatured DNA.

The gel filtration elution patterns of digests of rRNA indicate that RNA hydrolysis in the presence of Zn^{++} proceeds primarily in an exonucleolytic manner. With this gel filtration procedure even very low levels of endonuclease activity would cause a detectable spreading of the RNA peak before the mononucleotide peak became very evident. Even after considerable hydrolysis of the RNA had occurred there was still a good separation between the RNA and mononucleotide peaks. This indicates that few, if any, small oligonucleotides were produced. The dialysis experiment

also substantiates the exonucleolytic action of the wheat RNase. In the presence of 1 mM Zn^{++} about 80% of the lanthanum nitrate-acid soluble products from rRNA hydrolysis were dialyzable in 5 hours at 4°. When this same experiment was performed in the absence of Zn^{++} only 50% of the lanthanum nitrate-acid soluble material was dialyzable in the same period of time. Since Zn^{++} inhibits the RNase to a certain extent (about 15%) the digestion in the presence of zinc had only proceeded to about 85% of that observed in the absence of zinc, however, 30% more of the products were found to be dialyzable in the zinc digest. Thus it seems likely that Zn^{++} may play a role in determining the mode of attack of wheat RNase on rRNA. A similar effect of Zn^{++} was observed by Walters and Loring (23) with the mung bean RNase. It is also possible that the purified RNase contains a low level of a contaminating endo-ribonuclease activity which is inhibited by Zn^{++}. Thus in the presence of Zn^{++} only the exonuclease activity is observed while in the absence of Zn^{++} both endo- and exonuclease activities are observed.

The difference in the mode of action alone might suggest that at least two distinct enzymes are present in the preparation. But the fact that all attempts to separate the DNase and RNase activities and the associated 3'-nucleotidase activity have been uniformly unsuccessful, together with the extreme similarity in the properties of the three activities, as described below, lead us to suspect that the three enzyme

activities reside in a single entity. This close association of DNase, RNase and 3'-nucleotidase activities appears to be similar to that observed with several other plant nucleases. The three associated activities of mung bean sprouts have been extensively studied (20-24), as have the activities of germinating barley (17, 29), ryegrass (18), and rice bran (25). This association may also exist for the enzymes from soybean (19) and the fraction III nuclease of germinating garlic (26). Walters and Loring (23) have concluded that the mung bean DNase and RNase-3'-nucleotidase activities reside in different proteins, since the DNase activity alone was lost on standing in the cold. It is also possible that this represents the differential alteration of the structure of a protein with two different active sites. Work in this laboratory on muskmelon seed extracts[5] has produced an enzyme fraction, purified several hundred fold, which also possesses DNase, RNase and 3'-nucleotidase activities.

The three wheat enzyme activities cochromatographed on phosphocellulose at pH 4.5 regardless of whether a salt or pH gradient was employed for the elution of the protein. Thus the three activities remained inseparable when two different elution parameters were applied to the same column. The three wheat enzyme activities also cochromatographed on DEAE-cellulose at pH 8, on ECTEOLA-cellulose at pH 6.5, and on passage through either P-60 or P-30 gel filtration columns.

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[5]A. B. Adams, L. D. Muschek, and J. L. Fairley, unpublished observations.

The elution pattern from the DEAE-cellulose column at pH 8 showed a rather long tail of enzyme activity on the higher salt side of the peak. The ratios of the three activities were essentially constant throughout this tail as well as throughout the main body of the peak itself. The behavior of the three activities subjected to a batch treatment with a total of 8 different ion-exchange resins and 2 adsorbents at pH 4.5, 6.5, and 8 was also identical. The three activities either did not bind to the resin or bound to relatively the same degree at each pH employed. Disc electrophoresis of the purified nuclease on 10% polyacrylamide gel at both pH 9.5 and 8.3 produced a number of protein bands, however, the three enzyme activities were located only in one of these. The ratios of the three activities throughout this band were constant. Similarly the ratio of the DNase to RNase and DNase to 3'-nucleotidase activity remained essentially constant from the ethanol fractionation step throughout the remainder of the purification procedure. These ratios were also constant throughout the various chromatographic and electrophoresis procedures described above. The fact that these ratios remain so constant throughout so many different procedures is rather strong evidence that the three enzyme activities reside in a single unit of some sort.

Further evidence for the assignment of the three enzyme activities to a single entity may be summarized as follows: (a) All three enzyme activities have similar pH optima (within about 0.5 pH units). (b) The three activities

are inactivated at approximately the same rate by sulfhydryl compounds at pH 8. (c) The presence of sulfhydryl compounds is essential for maintaining the three activities at pH 4.5. (d) At pH 4.5, Zn^{++} is essential for stabilization of the three activities. (e) Each of the three activities exhibits similar stability on storage at 4^{o} and at -20^{o} in the presence of Zn^{++} and sulfhydryl compounds. (f) All three activities show similar rates of heat inactivation. (g) The three activities all showed an increase in activity in the range of 65^{o} to 70^{o}. (h) Treatment with N-bromosuccinimide completely destroyed all three activities, while several other common amino acid modifying reagents caused no inactivation of any of the activities. (i) The three activities were effected, at least qualitatively, to the same extent by the addition of divalent metal cations, although the quantitative effects were different in some cases. Thus MN^{++}, Co^{++}, Ni^{++}, and Cd^{++} were inhibitory to all activities to about the same degree. (j) The DNase and RNase activities were inhibited in a similar manner by sodium fluoride and P_i. (k) Both the DNase and RNase activities showed the same unusual type of response to EDTA.

Evidence for the presence of more than one protein includes the apparent activation only of the RNase activity by Mg^{++}, Ca^{++}, alkylating agents and arsenite. Since the degree of binding of metal cations by the three substrates is undoubtedly different it is felt that the Ca^{++} and Mg^{++} activation of the RNase activity, as well as the quantitative

differences observed with other metal cations may be due to effects of the metal ions on the substrate and not on the enzyme itself. These differences may accordingly not be significant. The activation of wheat RNase by alkylating agents and arsenite suggest at least two possibilities: (a) Two separate enzymes are responsible for RNase and DNase-3'-nucleotidase activity. (b) One enzyme was responsible for all three activities, but the alkylating agents affected only a site absolutely essential for RNase activity. This latter possibility may also explain the difference in the extent of inhibition of the DNase and RNase activities by EDTA, assuming that EDTA affects the RNase site to a greater extent. A similar difference in the degree of EDTA inhibition was observed with the mung bean RNase and 3'-nucleotidase activities (24). The unusual shape of the EDTA inhibition curve suggests that the EDTA may be doing more than simple metal ion removal from the enzyme. Below 10^{-6} M EDTA little or no inhibition of the activities was observed. In the range from about 10^{-5} to 10^{-2} M EDTA the degree of inhibition remained constant for both the DNase and RNase activities. This represented approximately a thousand fold increase in the EDTA concentration before the second phase of inhibition occurred. While this may simply be titration of the metal attached to the enzyme protein the biphasic nature of the curve suggests that EDTA may be reacting with at least two different groups in the protein or with metals bound to different degrees. What the exact nature of the

EDTA effect(s) might be is not clear at present.

The three enzyme activities exhibited very similar heat stabilities. Thus the temperature values at which the activities were reduced to 50% of the unheated control after 8 minutes were 76°, 75° and 75° for the DNase, RNase, and 3'-nucleotidase activities respectively. Above 85° all three enzyme activities were completely destroyed. The three activities also showed rather similar stability to heating at 70° for various periods of time. The activity was reduced to 50% of the control after heating at 70° for 25, 26, and 31 minutes for the DNase, RNase, and 3'-nucleotidase activities respectively. The Q_{10} values in the temperature range from 27° to 47° were also determined. The RNase exhibited the highest Q_{10} values of 4.2 to 4.5 while the DNase and 3'-nucleotidase exhibited Q_{10} that were somewhat lower, near 3.1 and 2.1 respectively. The high value for the RNase activity may reflect an effect of temperature on the secondary structure of rRNA and may not be an effect on the enzyme itself. This may also be true for the Q_{10} value for the DNase activity on denatured DNA.

The observation that a higher percentage of the three activities remained after heating at 70° then remained after heating at 65° for the same period of time is of interest. This may be similar to the heat reactivation effect observed with the associated DNase and RNase activities of chicken pancreas (45). Again this is strong circumstantial evidence that the three activities reside in a single entity.

The effect of Zn^{++} and sulfhydryl compounds are also interesting aspects of this enzyme preparation. Although Zn^{++} was somewhat inhibitory to the purified enzyme activities, the presence of Zn^{++} at a concentration of at least 10^{-4} M was an absolute requirement for the stabilization of the activities at pH 4.5. This suggests that Zn^{++} may be essential for stabilization of the proper tertiary or quaternary structure of the protein. Such a role for Zn^{++} has been demonstrated in several enzymes such as horse liver alcohol dehydrogenase (46), B. subtillis α-amylase (47), and E. coli alkaline phosphatase (48, 49). In these enzymes the Zn^{++} is essential for either the proper association of subunits to form the active enzyme or for the maintenancy of the proper conformation of the active enzyme. The observation that Zn^{++} has an effect on the distribution of products produced during the hydrolysis of RNA also suggests that the metal may play a role in determining the specificity of, at least, the RNase activity. The absolute requirement for a sulfhydryl compound for maintenance of activity at pH 4.5 and the complete destruction of all enzyme activity by the same sulfhydryl compounds at pH 8 appears at first to be contradictory. This appears to be a general sulfhydryl effect since essentially the results were obtained regardless of whether the sulfhydryl source was dithiothreitol, cysteine, or mercaptoethanol, provided that the total sulfhydryl concentration was the same. A similar effect of sulfhydryl compounds at pH 4.5 and 7.5 was observed by Schuster

(18) with the associated DNase, RNase, and 3'-nucleotidase activities of rye grass. Since disulfide bonds are not reduced by sulfhydryl compounds to any appreciable extent at pH 4.5, the possibility exists that the enzyme contains a sulfhydryl group and a disulfide bond, both of which are essential for normal enzyme function. This would be similar to the enzyme papain, which has been shown to contain one sulfhydryl group which is essential and three disulfide bonds (50). The possibility cannot be ruled out that at pH 8 a significant proportion of the sulfhydryl compound is auto-oxidized and that the observed inactivation of the three activities is due to reaction of the oxidized material with the essential sulfhydryl group of the protein. In this case one would not need to postulate the presence of an essential disulfide bond. This seems unlikely in that treatment of the enzyme at pH 8 with alkylating agents caused no loss of enzyme activity. If the sulfhydryl was being oxidized by the media at pH 8 it should also be alkylated by the alkylating agents at that pH. One possible explaination might be that a conformational change occurs in the protein between pH 4.5 and 8 which causes the sulfhydryl group to become "shielded" and inaccessible to the alkylating agents, but somehow, still accessible for reaction with the oxidized compounds in the media.

The similarity in the rates of inactivation of the three enzyme activities by sulfhydryl compounds at pH 8 is another good circumstantial indication that the three activ-

ities are associated in some manner.

At pH 8 the amino acid modifying reagent, N-bromosuccinimide, is capable of reacting with a variety of different amino acid residues in a protein (51). It is, therefore, not possible to assess which amino acid residues were modified by this reagent. However, it seems important that all three enzyme activities were destroyed to the same extent in this process.

The wheat DNase activity appears to be highly preferential for denatured DNA. While native DNA was hydrolyzed by the enzyme, the initial rate was only about 10% of that observed with denatured DNA. As the digestion of native DNA proceeded the rate fell to only 1.3% of that observed with denatured DNA in the same period of incubation. It is not felt that the enzyme activity itself decreased during this time period since the rate of hydrolysis of denatured DNA did not decrease. Since this experiment was performed on commercial salmon sperm DNA it is quite possible that the low level of activity observed on native DNA simply represented the hydrolysis of single-stranded regions in the native DNA preparation or of contaminating RNA. If this were the case then one would expect that the rate of hydrolysis should decrease with time as this material is removed from the native DNA preparation. This was, indeed, what was observed. This is similar to the results obtained by Linn and Lehman (32) with the endonuclease from N. crassa which also is highly preferential for denatured DNA.

A good separation of the 4 deoxymononucleotides was effected on DEAE-Sephadex employing a gradient of triethylammonium bicarbonate (TEAB). The separation of dAMP and dGMP was complete. The dTMP and dCMP appeared in the same peak but the relative amounts of these two nucleotides can be determined either by spectrophotometric analysis of the binary mixture or by subsequent chromatography of this material on Dowex-I-Chloride by the method of Volkin et al. (53) which uses dilute HCl as solvent and effects a good separation of the pyrimidine nucleotides. The same separation of mononucleotides could be effected in a batch-wise treatment of the DEAE-Sephadex with 0.18 M TEAB. This system has the added advantage that the solvent is volatile and can be completely removed by repeated flash evaporation. The final product can thus be obtained in an essentially salt free form. This separation system was used to study the production of mononucleotides at various stages of the digestion of denatured DNA. At all periods of hydrolysis investigated dAMP was the predominate mononucleotide and was present in at least a 2-fold greater amount than any of the other 3 deoxymononucleotides. The production of dTMP remained relatively constant during the hydrolysis. The rate of release of dCMP was constant at least through the first 60 minutes of incubation but had almost tripled by the end of 300 minutes. After 15 minutes incubation dGMP accounted for about 24% of the total mononucleotides, however, at the end of 60 minutes the dGMP fraction was only

5% of the total mononucleotides and after 300 minutes it was only about 2% of the total. Thus it would appear that the rate of release of dGMP from denatured DNA decreases appreciably as the hydrolysis proceeds. The recovery of the 4 known deoxymononucleotides is essentially quantitative from the DEAE-Sephadex column. Therefore, it is felt that the low level of deoxyguanylate observed is real and is not an artifact of the separation procedure. Furthermore, by total analysis of all the oligonucleotide peaks from the DEAE-Sephadex column, it was possible to account for 100% of the deoxyguanylate residues that were present in the original DNA. Thus the oligonucleotides which are present at later stages of digestion appear to be rich in deoxyguanylate residues. The formation of high levels of dAMP and low levels of dGMP suggests that the wheat DNase possess some degree of base specificity. It would appear that bonds involving deoxyadenylate residues are preferentially attacked, while those involving deoxyguanylate residues are relatively resistant. It is not possible at present to say anything further about the base specificity of this enzyme. In light of the apparent effect of Zn^{++} on the products formed on hydrolysis of rRNA, it should be mentioned that these digestions of denatured DNA were also performed in the presence of 1 mM Zn^{++}. What effect the presence or absence of Zn^{++} has on the distribution of mononucleotides that are produced is not known. The production of mononucleotides by the wheat nuclease is similar to that observed with the N. crassa

endonuclease (32) except that the fungal enzyme produces large amounts of dGMP and very little dCMP. The possibility exists that these two enzymes could be used to complement each other in base sequence determinations on deoxyoligonucleotides and on denatured DNA. The DNase activity from soybean sprouts (19), rice bran (25), and from the digestive juice of larvae of the silkworm, Bombyx mori (52) also have been reported to produce low levels of dGMP relative to the levels of the other three deoxymononucleotides.

The exact nature of the association of DNase, RNase, and 3'-nucleotidase activities cannot be ascertained at present. The following possibilities seem most likely: (a) The three enzyme activities are the property of a single protein, with one or more active sites. (b) The protein is a very stable complex of two or more distinct enzymes. (c) Three distinct, separate enzyme proteins are present in the purified preparation. Several lines of evidence can be interpreted as indicating the presence of subunits in the enzyme. These include the Zn^{++} stabilization of the enzyme activity, the disappearance of the active protein band on electrophoresis of enzyme treated with sulfhydryl compounds and 8 M urea, and the lack of proportionality of the rate of hydrolysis to enzyme concentration in the lanthanum-acid soluble product assay. It seems likely that the Zn^{++} effect on the enzyme is at least in part due to some stabilization of the proper tertiary and quaternary structure of the enzyme. The lack of proportionality of the rate of hydrolysis to

enzyme concentration may indicate the presence of some type of association-disassociation phenomenom.. At very low enzyme concentrations the activity may be low because of disassociation of the active enzyme, while at high enzyme concentrations the activity is enhanced because of association of active enzyme molecules. The addition of bovine serum albumin did not effect the non-linearity of the assay. Thus this does not appear to be just a general protein effect and may represent the specific disassociation of the enzyme protein. The simplest explaination of the electrophoresis patterns is that in the presence of a sulfhydryl compound and 8 M urea at pH 8 the protein is dissociated into subunits which have an electrophoretic mobility quite different from that of the intact native protein. Since after this treatment the enzyme is completely inactive it is not possible to tell whether the subunits are part of a single enzyme protein (a) or whether they are separate enzyme proteins which are only active when associated in a complex (b). Several lines of evidence also indicate that more than one active site may be present in the enzyme. The activation of only the RNase activity by Ca^{++}, Mg^{++}, and alkylating agents, and the greater degree of inhibition of RNase by EDTA are suggestive of more than one active site, however, other explainations of these results are also possible. The fact that denatured DNA inhibits the RNase activity to a greater extent (about 65%) than rRNA inhibits the DNase activity (about 42%) is interesting. Since these

two values are both near 50% inhibition it could be postulated that a single site is involved in the hydrolysis of both denatured DNA and rRNA and that the two substrates are competing for that site. The possibility of two sites cannot be ruled out, however, since it is known that many specific nucleases bind and are inhibited by nucleic acids which they cannot hydrolyze. Thus two sites could be present in the protein with one site responsible for the hydrolysis of each substrate but both capable of binding DNA and RNA. The difference in the percent inhibition observed with the two substrates might only represent a difference in the relative degree of binding of each nucleic acid by the individual sites. Finally, the apparent difference in the mode of action of the DNase and RNase activities is strongly suggestive, although certainly not definite, evidence for the presence of more than one active site.

If three separate enzymes are responsible for the three enzyme activities (c) then these three enzyme proteins would have to be extremely similar in amino acid composition and sequence in order to display the similarity of properties exhibited by these three activities. If, indeed, this were the case then these proteins might represent an example of a series of duplications of a single gene at some time in the evolution of plants and the subsequent independent course of random mutation of these genes. At present it is impossible to conclude definitely which of the three possible models

for the associated wheat activities is correct. A final decision on the nature of the association of the three activities must await large-scale preparations making possible a variety of physical chemical experiments.

The apparent association of DNase, RNase, and 3'-nucleotidase activities that has been observed in a variety of plants may simply represent an artifact peculiar to plant tissues. One might expect that a similar phenomenom would occur in closely related plant species such as wheat, barley, and rye grass, all of which are monocots. The presence of the same association of enzyme activities in dicots such as mung bean, muskmelon, and soybean as well, suggest that this association may be a general one throughout the plant kingdom. Many of the nucleases which have been isolated from bacterial, mold, and animal sources have been shown to possess both DNase and RNase activities. Furthermore, many of these purified enzymes still contain some phosphomonoesterase activity. Others have only been tested for nucleotidase activity using α-glycerol phosphate or p-nitrophenyl phosphate, substrates which may not be hydrolyzed at an appreciable rate by specific nucleotidases. A careful study of these enzymes might reveal that the association of DNase, RNase, and 3'-nucleotidase activities is more widespread and not simply peculiar to plant tissues.

The possible biological significance of such a complex of related enzyme activities is intriguing. The relative abundance of these three associated enzymes in a variety of

germinating, rapidly growing seedlings might suggest a possible role for these enzymes in DNA replication or repair. It is possible to postulate a role for a single-strand endonuclease in DNA repair and related processes such as recombination and replication. As Lehman (15) has suggested, such enzymes might be capable of "seeking out" and attacking unordered regions in the DNA double-helix where damage to the DNA had occurred. They might also be capable of cleaving single-stranded fragments of DNA which might occur in the overlap regions during recombination. The DNA joining enzymes (polynucleotide ligase) which have been reported to date from E. coli (54, 55) and from T_4-infected E. coli (56) all have at least one requirement in common. This requirement is for the presence of a free 3'-hydroxyl group on the adjacent nucleotide residue at the site of phosphodiester bond formation. The presence of a 3'-nucleotidase activity closely associated with a repair enzyme would be a means of insuring that the 3'-hydroxyl position would be free and capable of participating in the formation of a phosphodiester bond. This might explain the role of such a 3'-nucleotidase activity in DNA repair. At present it is not known whether the wheat 3'-nucleotidase activity is capable of removing the phosphate group from 3'-deoxymononucleotides for from the 3'-terminus of oligonucleotides or DNA. Two enzymes which remove 3'-phosphoryl groups in DNA have been reported by Becker and Hurwitz (57). One of these enzymes, 3'-deoxynucleotidase, is specific for the 3'-phosphate ends

in DNA and 3'-mononucleotides. The other activity, E. coli C phosphatase, is non-specific at the mononucleotide level and will dephosphorylate both 3'- and 5'-mononucleotides but reacts with only 3'-phosphate ends in DNA and RNA. What role the associated wheat RNase might play in the repair process, if any, is not clear at present. Stable DNA-RNA complexes have been isolated from plant cells (58-60), animal cells (61-63), and from some bacteria (64). These complexes do not appear to be artifacts of the DNA extraction procedures employed. The RNA in these complexes incorporates labeled uracil at a rate several times faster than the total cellular RNA. The role of this DNA-RNA complex in the cell is not known. The possibility exists that the associated RNase and DNase activities may participate in reactions involving such DNA-RNA complexes.

SUMMARY

A nuclease has been purified from germinating wheat seedlings to an extent of over 830-fold with a recovery of about 12% of the total DNase activity present in the crude homogenate. The purified preparation hydrolyzes denatured DNA, rRNA and the 3'-phosphoester linkage of 3'-AMP at similar rates. The preparation exhibits no appreciable 5'-nucleotidase, phosphodiesterase, or phosphomonoesterase activity.

The DNase, RNase, and 3'-nucleotidase activities have remained associated throughout a variety of purification procedures, including chromatography on several ion-exchange resins, gel filtration, and polyacrylamide disc electrophoresis at pH 9.5 and 8.3. The ratios of the three activities remain essentially constant throughout the last four steps in the purification procedure as well as throughout all of the various procedures mentioned above. The three activities exhibit a great degree of similarity with respect to the following properties: (a) pH optima, (b) requirement for Zn^{++} and sulfhydryl compounds at pH 4.5, (c) rate of destruction of activity by sulfhydryl compounds at pH 8, (d) stability to storage at both 4^{o} and -20^{o}, (e) effect of temperature and duration of heating, (f) reactivation on heating between 65^{o} and 70^{o}, (g) effect of N-

bromosuccinimide, (h) effect of metal cations, (i) effect of inhibitors and EDTA. It is tentatively concluded that the three associated enzyme activities are either the properties of a single protein or of a very stable complex of two or more proteins. The possibility that three separate enzymes are responsible for the three activities seems unlikely but cannot be definitely ruled out at present. A final decision on the nature of the association of the three activities must await large-scale preparations making possible a variety of physical chemical experiments.

The DNase activity is highly preferential for denatured DNA. Although native DNA was hydrolyzed by the enzyme, the rate was only about 1.3% of that observed on denatured DNA. The possibility exists that this low level of hydrolysis of native DNA represents simply the hydrolysis of denatured regions in the native DNA preparation or of contaminating RNA. The hydrolysis of denatured DNA by the wheat DNase was found to be endonucleolytic in manner by a number of criteria. The mononucleotides and presumably the oligonucleotides produced bear 5'-phosphoryl groups. At all stages of digestion investigated, dAMP was the predominate mononucleotide component. The level of dAMP was always about 2-fold greater than any of the other three deoxymononucleotides. The dAMP level also increased as the digestion proceeded. The level of dTMP and dCMP were relatively constant throughout the early stages of digestion. A very low level of dGMP was observed throughout the digestion and this

level decreased as a percentage of the mononucleotide fraction as the digestion proceeded. Essentially all of the deoxyguanylate residues could be accounted for in the oligonucleotide fractions. Thus it would appear that the wheat DNase exhibits a relative degree of base specificity with bonds involving deoxyadenylate residues being preferentially cleaved and bonds involving deoxyguanylate residues being relatively resistant.

The mode of action of the wheat RNase on rRNA was found to be exonucleolytic and the mononucleotides produced appear to be the 2',3'-cyclic compounds.

The association of DNase, RNase, and 3'-nucleotidase activities seems common in plants and may also be common in other biological sources as well. The biological significance of this association is not clear but the observation that these activities exist at relatively high levels in germinating, rapidly growing seedlings suggests that these enzymes may play some role in DNA repair and replication.

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