ABSTRACT

A STUDY OF WHEAT SEEDLING NUCLEASE AND ITS PROPERTIES RELATING TO THE CATALYTIC MECHANISMS INVOLVED IN THE HYDROLYSIS OF DNA, RNA, AND THE 3'-NUCLEOSIDE MONOPHOSPHATES

By

Warren Dean Kroeker

Wheat seedling nuclease has been purified over 10,000 fold with a 5% yield of activity. This procedure produced 0.5 mg of protein from 7 kilograms of wheat. The isolated preparation was judged to be nearly homogenous in regards to size and shape as indicated by the following results: a) sedimentation in the analytical ultracentrifuge revealed a single, symmetrical boundary, b) sedimentation through a sucrose gradient resulted in a single, coincident peak of protein and enzyme activity, c) filtration in Sephadex G-100 yielded a single protein-activity peak, throughout which the activity remained constant, and d) polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol and SDS revealed the presence of only one major protein band comprising greater than 90-95% of the total protein stained on the gel.

However, when electrophoresis of this preparation of wheat seedling nuclease was performed at pH 8 in the absence of SDS and 2-mercaptoethanol, 6-12 protein bands were detected. Furthermore, the 3 activities associated with wheat seedling nuclease were associated with only one of these bands. The band responsible for the enzyme activities was one of the least negatively charged bands and contained less than 10% of the total protein stained on

the gel. All of the multiple-ionic forms of wheat seedling nuclease were determined to be approximately equal in size. This conclusion is based on the fact that when the percent acrylamide contained in the gel was plotted as a logarithmic function of the relative mobility, similar slopes were obtained for all of the bands.

The molecular weight of wheat seedling nuclease was determined to be approximately 43,000 as determined by the following methods: a) sedimentation velocity experiments on the analytical ultracentrifuge, b) sucrose gradient sedimentation with proteins of known molecular weights, c) SDS gel electrophoresis with a system calibrated with standard proteins. Since the SDS electrophoresis procedure yielded a molecular weight which was similar to the other two methods, it is likely that wheat seedling nuclease exists as a single polypeptide chain.

Kinetic analysis of the rates of hydrolysis of 3'-AMP and pApA by wheat seedling nuclease was performed by using a continuous, spectrophotometric assay developed for this purpose. The assay was based on the decrease in absorbance produced when either adenosine or 5'-AMP (products of the reaction catalyzed by wheat seedling nuclease) was deaminated by a specific amino hydrolase coupling enzyme. The Km and Vmax values obtained for the dephosphorylation of 3'-AMP were 1.5×10^{-5} M and 1.3×10^{2} μmoles/min/mg respectively. The hydrolysis of pApA yielded Km and Vmax values of 9.1×10^{-5} M and 1.67×10^{3} μmoles/min/mg.

Kinetic inhibition studies were conducted using

3'-AMP as the substrate. 5'-AMP, a molecule containing a single phosphomonoester linkage, was found to be a competitive inhibitor while the phosphodiester compounds, 2',3'-cyclic AMP and ApA, were found to inhibit the reaction in a noncompetitive manner.

A general salt inhibition was noted for wheat seedling nuclease. The 3'-AMPase reaction was inhibited competitively by NaCl. However, when NaCl was included in the pApA hydrolysis reactions, noncompetitive inhibition resulted.

A consistent order of base preference for all three of the activities associated with wheat seedling nuclease was observed at pH 5.0. This preference was found to be $A > T(U) > C > G$. Phosphodiester bonds adjacent to cytidylic and guanylic acid residues were found to be quite resistant to cleavage by wheat seedling nuclease.

When high molecular weight, native E. coli DNA or intact, linear-duplex viral DNA molecules are hydrolyzed by wheat seedling nuclease, only a few, specific double-stranded cleavages occur. The resulting "limit polymers", still of high molecular weight, are resistant to further hydrolysis by wheat seedling nuclease.

The nature of the sites on the duplex DNA recognized by the enzyme was investigated. The production of limit polymers (MW = 3×10^6) from gh-1 DNA (MW = 23×10^6) was accompanied by the release of 0.04-0.08% acid-soluble material. This corresponds to less than 8 nucleotide equivalents released as acid-soluble material for each limit

polymer produced by the action of wheat seedling nuclease. The 5' termini of limit polymers labeled with ^{32}P by the action of polynucleotide kinase contained either cytidylic or guanylic acid, but no adenylic or thymidylic residues.

Wheat seedling nuclease exhibits a very large preference for denatured DNA as compared to native, double-stranded DNA. However, the fact that wheat seedling nuclease can hydrolyze the double-stranded, synthetic polymer, poly d(A-T) at rates comparable to the hydrolysis of denatured DNA, indicates that wheat seedling nuclease may recognize A-T rich areas in native DNA. This contention is further supported by the following evidence: a) λ DNA, which contains an A-T rich region near its center, is rapidly cleaved to produce λ halves, b) wheat seedling nuclease possesses a considerable preference for bonds adjacent to A and T residues while showing a marked resistance to the hydrolysis of bonds adjacent to C and G residues, c) the nucleotide composition of an area within 118 nucleotides of the 3' end of the gh-1 DNA limit polymers showed a slight increase in A-T content as compared to the whole gh-1 DNA molecule and d) the hydrolysis rate of native λ DNA increased 15 fold between 20° and 30° C indicating a major structural effect of the increased temperature on the substrate.

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INTRODUCTION

The ubiquitous presence of nucleolytic activities in the cells of organisms in all life's phylogenetic levels has been recognized for some time. Nucleic acids, the target molecules for these activities, serve the important function of maintaining and perpetuating the biological information necessary for life. It is not surprising, then, that nucleases have been the subject of a great deal of interest to the biochemist. During the past two decades, which saw numerous attempts by enzymologists to purify and characterize many enzymes of diverse nature, a large number and variety of nuclease activities has been reported. These nucleolytic activities have been characterized and cataloged according to their hydrolytic and degradative properties (1). Until very recently, however, little attempt has been made to ascribe these activities to particular cellular functions. The molecular mechanisms and enzymatic involvement in such subcellular phenomena as genetic recombination, DNA replication and repair, and restriction of foreign DNA molecules has led to a renaissance of interest in nucleolytic enzymes.

Of these cellular processes, perhaps the one with the most information concerning nuclease involvement is that of DNA replication and repair. The early evidence indicating

the involvement of nucleolytic enzymes was based on the results from three experimental approaches (2). The first was the correlation between high levels of DNase activities with the period of growth corresponding to the fastest rates of DNA synthesis. Secondly, it was pointed out that the infection of a host bacterial cell with a phage usually resulted in the appearance of a variety of nuclease activities even in the case where the host DNA was not degraded. And, finally, the appearance of nucleolytic activity associated with a DNA polymerase purified to a single protein species indicates an intimate relationship between the nucleolytic and polymerase activities.

Goulian (3) has recently reviewed the properties of the nuclease activities associated with E. coli DNA polymerase. There appear to be two separate DNase activities; one acting in the 5' to 3' direction and the other in the 3' to 5' direction. The 5' to 3' activity requires a double helical structure for activity and a 5' OH or 5' phosphate terminus provides an equally acceptable substrate. In contrast, the 3' to 5' activity is greater with single-stranded DNA and requires the presence of a 3' OH group. The 3' to 5' degradation produces some di- and oligonucleotides. Recently Kornberg et al. (4) have succeeded in physically separating 5' to 3' nuclease activity from E. coli DNA polymerase molecule following proteolytic digestion. A hypothetical scheme has been proposed for the concerted hydrolysis and polymerization of DNA by E. coli DNA

polymerase and is reviewed by Goulian (3). This scheme postulates a spatial orientation for the catalytic sites on the enzyme which is compatable with the overall polymerization reaction. The ability of the 5' to 3' nuclease to remove segments of DNA in which the bases are not paired suggests a repair role for E. coli DNA polymerase and, indeed this may be the exclusive role for the enzyme.

The subject of DNA repair and its relationship to nucleases has been reviewed by Howard-Flanders (5). The excision of mismatched base pairs as well as pyrimidine dimers from UV irradiated DNA suggests the presence of endonucleases with the ability to recognize these particular sites on the DNA molecule.

In its broadest sense the biological process of recombination has been defined as "any of a set of pathways in which elements of nucleic acid interact with a resultant change of linkage of genes or parts of genes" (6). Recombination has been most thoroughly studied and best understood as a genetic phenomenon rather than a biochemical process involving particular enzymes and pathways. Nevertheless, certain authors have proposed various models or schemes for recombination which postulate the involvement of exonucleases, endonucleases, ligases and polymerases (6,7). In its original genetic perception, recombination occurred through a process of synapsis occurring at the same loci on homologous regions of DNA followed by crossing over to form the recombinant molecules. The phenomenon of

"site-specific" recombination, which involves the integration and excision of temperate phage with the host genome, has recently received considerable attention (8,9). The proposed model for this process is based on evidence from genetic studies with bacterial mutants and postulates the recombination between specific but nonidentical regions (phage and host attachment regions).

The final area of nuclease involvement to be reviewed is that of the bacterial modification-restriction systems. These systems are responsible for degrading foreign unmodified DNA molecules which might enter a bacterial cell. The modification and restriction activities appear to be genetically linked and separate systems have been identified in five strains of E. coli as well as in several other bacteria including Hemophilus influenza. The latter example contains a restriction-like endonuclease but as yet no detectable modifying activity. A common feature of all the restriction endonucleases is their ability to produce a few, specific double-stranded scisions in unmodified DNA molecules, yielding limit fragments of different lengths.

A considerable amount of information about the restriction endonucleases has recently been obtained (10). All of the restriction endonucleases investigated to date require Mg^{++} and in the case of at least three of the enzymes, ATP and S-adenosylmethionine are either required or provide activating effects. The enzymes are generally large proteins (MW 80-300,000) and subunits have been identified

in some cases. All of the restriction endonucleases investigated thus far exhibit a pH optimum between 7-8. Furthermore they apparently lack the ability to catalyze hydrolysis of single-stranded DNA.

Since restriction endonucleases produce such a limited number of double strand scisions (0.01-0.1% of the phosphodiester bonds available) the question naturally arises as to the nature of the substrate site recognized by the enzyme. The cleavage site for the restriction-like endonuclease from H. influenza has been studied in detail (11, 12). The site is uniquely defined by a hexanucleotide sequence of the following sort:

5'---pGpTpPy↓pPupApCp---3'

3'---pCpApPup↑PypTpGp---5'

where the arrows indicate the bonds cleaved. The striking feature of this sequence is the two fold rotational axis of symmetry perpendicular to the DNA helix. It was postulated (11) that symmetry elements in the protein (possibly at the subunit level) are able to recognize the base symmetry in the DNA sequence.

Similarly, Hedgpath et al. (13) have established the base sequence recognized by the E. coli. RI restriction endonuclease:

5'---A/TpG↓pApApTpTpCpT/A---3'

3'---T/ApCpTpTpApAp↑GpA/T---5'

Again a two fold rotational symmetry is noted. While the H. influenza enzyme attacks diester bonds directly across

from each other, the RI endonuclease produces a staggered cleavage resulting in cohesive ends. In addition it is noted that although the RI enzyme is specific for the bond adjacent to the 3' side of guanine, twelve of the sixteen bases in the recognition site are either A or T.[1]

While the aforementioned cellular processes are certainly not the only events involving nucleolytic activity, they clearly are vital to the life of the cell. Other possible functions for nuclease activities in the cells may involve processing of both nuclear and cytoplasmic RNA molecules as well as providing a general pool of nucleic acid components. The omnipresence and diverse functions of nucleases coupled with the obvious need for a cell to maintain its genetic integrity would seem to indicate the importance of control and regulation mechanisms for the expression of various nucleolytic activities. Apart from the known examples of induction of nuclease activities following viral infection little is known concerning the control of nuclease activities already present in a normal cell. It has been suggested (14) that control of intracellular RNase activity could arise from a) spacial segregation, b) complexing of the RNA to form a resistant structure, c) complexing of the enzyme with an inhibitor, and d) specificity for one form of macromolecular structure of RNA. Most of the evidence centers on b and c. RNA in ribosomes has been shown to be resistant to RNase attack. Finally, a number of nuclease inhibitors have been found, some of which

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[1] A list of abbreviations may be found in the appendix.

are proteins.

Wheat seedling nuclease, originally isolated in this laboratory by D. Hanson (15), can be classified among other nucleolytic enzymes by a number of catalytic properties. The enzyme hydrolyzes the phosphodiester bond in RNA and DNA producing 5' phosphoryl terminated oligonucleotides and mononucleotides. In addition, the enzyme possesses the ability to cleave the phosphate moiety from 3'-nucleoside monophosphates. Wheat seedling nuclease appears to cleave dDNA in an endonucleolytic manner while degrading rRNA exonucleolytically (16). Denatured or single stranded DNA is hydrolyzed at a much faster rate than native helical DNA.

Several other nucleases also demonstrate a definite preference for single-stranded substrates and have been isolated from lamb brain (17), sheep kidney (18), yeast cells (19), Neurospora crassa conidia (20), Staphylococcus aureus (21), Aspergillus oryzae (22), and mung beans (23). These enzymes show several common properties in regards to substrate specificity: a) they are all capable of hydrolyzing internal phosphodiester bonds (endonucleases), b) the the reaction products are 5' phosphoryl terminated oligo- and mononucleotides except in the case of S. aureus which produces 3'-P terminated products, and c) there is no specificity for the sugar moiety (ie. DNA and RNA are both substrates).

The single-stranded specificity of these enzymes

has found utility in several cases for use in hybridization experiments where selective removal of nonhybridized areas is desirable (24,20). In addition, the ability of these enzymes to recognize differences in the ordered structure of the substrate makes them potential tools for probing nucleic acid structure. For example, sheep kidney enzyme has been used to study the looped regions in tRNA (18). Furthermore, it has been demonstrated that the enzyme from S. aureus can recognize A-T rich areas in native DNA (21). This preference was ascribed to the fact that the dynamic equilibrium (single-strand⇌double-strand) for areas rich in A and T is shifted to the left.

One of the single strand specific nucleases most thoroughly studied is that isolated from mung beans (23, 25-29). This enzyme appears quite similar to the wheat seedling nuclease and will be reviewed here in some detail.

Like the wheat seedling nuclease, the mung bean enzyme is a) a 5' phosphate former, b) sugar unspecific, c) an endodeoxyribonuclease and d) is capable of catalyzing the hydrolysis of the phosphomonoester bond of 3'-AMP. In addition, Johnson and Laskowski (27) have demonstrated a high degree of enzymatic preference for single-stranded substrates as opposed to the native or double helical structures. Their most striking comparison is that for native and heat denatured T_4 DNA. The initial velocities of hydrolysis of these two substrates differ by 30,000 fold when measured by a hyperchromic assay. The authors tentatively suggest

that the small amount of hydrolysis of native T_4 DNA may be due to single-stranded contamination. This is consistant with the kinetics observed as well as the lack of decrease in $s_{20,w}$ for the native substrate. Somewhat smaller differences are noted for the initial rates of native and heat denatured forms of E. coli. and calf thymus DNA. Using the differences in buoyant density between native and denatured DNA, it was demonstrated that T_4 DNA corresponding to the denatured DNA peak on a CsCl gradient was selectively and completely removed from a mixture of native and denatured forms by the mung bean enzyme without changing the native DNA peak. The relative degree of susceptibility of the substrate's secondary structure was tested by comparing rates of hydrolysis of a number of denatured substrates which had been renatured to varying extents. These results were also consistant with a very high level of specificity of the enzyme for the single-stranded form of nucleic acid polymer. The only exception noted by the authors to the resistance of double stranded substrates was the synthetic copolymeric duplex, poly d(AT) which was hydrolyzed at rates comparable to denatured DNA. In a rather dramatic experiment using high levels of enzyme at 17° C, it was shown that mung bean nuclease could selectively cleave the linear duplex, λ DNA, near its center producing two undamaged halves which were separable due to their difference in G plus C content, hence buoyant density. At temperatures above 17° C this center-recognizing specificity was lost. The

authors point out that λ DNA has a well characterized A-T rich region near its center (spanning 0.1 of the total genome). This fact taken together with the susceptibility of poly d(A-T) to mung bean nuclease led the authors to coin the phrase "region specific nuclease".

The mung bean nuclease exhibits a chemical preference for the rate of hydrolysis of the diester bonds adjacent to the five naturally occurring bases in single stranded nucleic acid polymers (28). The order of preference in denatured DNA and riboheteropolymers was found to be A > T(U) > C > G. This is the same order as that found for the monophosphatase activity of mung bean nuclease on 3'-nucleotide substrates. Loring et al. (26) working at a higher pH found a different relative order - namely, A>G > U >C.

The work to be described in this thesis represents an attempt by the investigator to provide a general picture of the nature of the molecular events occurring during the enzymatic catalysis by wheat seedling nuclease. Theoretically, a true definitive description of the molecular mechanism of any chemical reaction as complex as the hydrolysis of a polynucleotide would require an exceedingly large number of assignments of the coordinates of all involved atoms in space and time. Therefore, any"mechanistic study" represents an investigation yielding a qualitative description, the detail of which is limited by the amount of experimental data. The portion of this thesis directed toward elucidation of the biochemical "mechanism

of action" of the wheat seedling nuclease, then is given in this general descriptive sense.

It has been widely observed in the biochemical literature that protein structure plays an important part in enzymatic function. With this structure-function relationship in mind, wheat seedling nuclease was isolated in a highly purified form and some of its physical and chemical properties are reported. Furthermore, some of this data has led to the construction of a possible model for the enzymatic control of wheat seedling nuclease in the cell.

A third section of this thesis reports in a somewhat detailed manner on the mode of enzymatic hydrolysis of double stranded, native DNA by wheat seedling nuclease. The special attention to this aspect of the reaction mechanism seems warrented for several reasons. The overall biological implications of the manner in which native DNA is hydrolyzed by the wheat enzyme may relate to some of the cellular functions for nucleases reviewed earlier in this introduction. Furthermore, these studies indicate a potentially important role for wheat seedling nucleases as a probe of DNA structure.

EXPERIMENTAL PROCEDURE

Materials

Resins

Carboxymethyl cellulose cation exchange resin (0.07 meq/gm capacity, coarse mesh) was purchased from Sigma. It was prepared before use by washing with 2 cycles of 0.5 M NaCl - 0.5 N NaOH and water, then with 0.1 N HCl and water, with another cycle of 0.5 M NaCl - 0.5 N NaOH and finally by extensive washing with water.

Cellulose phosphate cation exchange resin (0.8 meq/gm capacity, coarse mesh) was purchased from Sigma and treated prior to use with alternate washings of 0.1 N KOH and water until it was colorless.

Dowex 1-X4 was purchased from Bio Rad Laboratories as analytical grade Ag 1-X4, 100-200 mesh, chloride form. Before use it was subjected to 3 cycles of 1 N NaOH and 1 N HCl and then washed extensively with water. DEAE Sephadex A-25 and Sephadex G-100 were products of Pharmacia and were used without additional treatment. Bio-Gel P-60 (50-150 mesh) was obtained from Bio Rad Laboratories and DEAE cellulose (DE-52) was a product of Whatman.

Proteins

The proteins used as standards in sucrose gradient sedimentation and SDS gel electrophoresis were egg

ovalbumin, Grade III from Sigma; cytochrome C, Type III from Sigma; trypsin, Type III from Sigma; and hemoglobin which was a gift from Dr. A. Morris.

Enzymes

The following enzymes were obtained commercially and used without further purification: adenosine deaminase, Type I from Sigma; DNase I and DNase II; DPFF and HDAC respectively from Worthington; and micrococcal nuclease, Grade IV from Sigma.

Alkaline phosphatase was purchased from Sigma as Type III and was carried through an additional purification by chromatography on a DEAE cellulose column (30). Venom phosphodiesterase was purchased from Worthington (VPH) and trace amounts of 5'-nucleotidease were removed by the method of Laskowski (31). 5'-AMP amino hydrolase was either a gift of Dr. C. Suelter or was purified from rabbit muscle by the method of Suelter et al. (32). Muskmelon nuclease was prepared in this laboratory by Dr. L. Muschek. The RNA dependent DNA polymerase induced by infecting Pseudomonas putida with the gh-1 phage was a gift of H. Towle of this department. Polynucleotide kinase was purified from T_4 infected E. coli B through the phosphocellulose step after the method of Richardson (33).

Nucleic Acids

The DNA routinely used as a substrate for DNase activity was highly polymerized salmon sperm DNA (Sigma Type III - sodium salt). Ribosomal RNA was prepared in this lab by

the method of Cresfield (34). High molecular weight *E. coli* DNA was made by Dr. D. Hanson[2] by a modification of the method of Berns and Thomas (35). The polyribohomopolymers, poly d(A-T), and poly dG - poly dC were purchased from Miles Laboratories. Poly rA,U was a gift from Dr. J. Boezi. The 5' ribo- and deoxyribonucleoside monophosphates were purchased from Sigma as was 3', 5' - cyclic AMP. The 3'- and 2'-ribonucleoside monophosphates were products of PL Labs. The 3'-deoxyribonucleoside monophosphates were a gift from Dr. D. Hanson.

DNA from the bacteriophages T_4, T_7, λ and gh-1 was prepared by the following procedure. SDS was added to a phage suspension (concentration of phage was adjusted to give 10-25 optical density units at 260 nm per ml) to give a final concentration of 0.25%. The suspension was then heated to 60° C for 3 minutes. The DNA was extracted 3 times with freshly distilled, water-saturated phenol. The aqueous phase was then extensively dialyzed against 50 mM NaCl, 10 mM tris-HCl (pH 8) until all of the phenol had been removed. Φ-X 174 RF DNA was the generous gift of Dr. R. Wells, Department of Biochemistry, University of Wisconsin.

The di- and trinucleotides, pApA and pApApA were made by digesting poly A with muskmelon nuclease. The reaction mixture contained 20 ml 0.05 M succinate buffer, pH 6.0, 5 ml poly A (212 optical density units at 260 nm)

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[2] Research Division, Veterans Administration Hospital, Bedford, Massachusetts.

and 0.11 ml muskmelon nuclease. After incubating at 37° C for two hours, the reaction was stopped by heating at 95° C for 3 minutes. The mixture was then applied to a DEAE Sephadex A-25 column (2.5 x 60 cm) which had been equilibrated with 0.14 M triethylammonium bicarbonate (TEAB), pH 8.0. The column was washed with 1 liter of 0.14 TEAB and then an 8 liter gradient of 0.14-0.46 M TEAB was applied to the column. A flow rate of 1.0 ml/min was maintained and 20 minute fractions were collected. Three peaks of absorbance at 260 nm were observed. They were identified as pA, pApA and pApApA by comparing the inorganic phosphate to nucleotide ratio after treatment with alkaline phosphatase. The pApA and pApApA peaks were pooled and flash evaporated to dryness three times. The residue from the last evaporation was dissolved in water and passed through a phosphocellulose column at pH 7.0 to remove traces of triethylamine.

ATP labeled in the γ position with ^{32}P was made by a modification (33) of the method of Glynn and Chappell (36).

Bacteria and Bacteriophages

E. coli B, Pseudomonas putida and gh-1 bacteriophage were supplied by Dr. J. Boezi. The temperature sensitive mutant of E. coli ($C_{600}\lambda CI_{ts}857$) containing λ prophage and T_4 bacteriophage were gifts from Dr. L. Snyder, Department of Microbiology, Michigan State University. T_7 bacteriophage was supplied by Dr. D. Hanson.

Chemicals

Cesium chloride (optical grade) was purchased from Schwarz/Mann. Ethidium bromide was a product of Calbiochem. Guanidine-HCl (ultra pure) was a product of Schwarz/Mann. Agarose used in gel electrophoresis was obtained from Sigma as "electrophoresis grade." Acrylamide was a product of the Canalco Company.

Wheat

The wheat used as a source of wheat seedling nuclease was Genesee soft white wheat (either certified or one year removed from certification) which had been treated with a mercurial fungicide.

Methods

Assays for Activity of Wheat Seedling Nuclease

Standard Acid-Soluble DNase and RNase Assay Unless otherwise stated, all assays for RNase and DNase activities of the wheat enzyme were performed with the acid-soluble technique of D. Hanson (15). The precipitating reagent is $La(NO_3)_3$ - HCl (0.02 M $La(NO_3)_3$, 0.2 m HCl) which is mixed with an equal volume of reaction mixture. A unit of activity is defined as that amount which catalyzes in 10 minutes the formation of lanthanum acid-soluble material with an A_{280} of 1.0 per ml of incubation mixture under standard conditions.

Nucleotidase Assay In order to study the nucleotidase activity of wheat seedling nuclease, two different assay

techniques were used. A fixed time phosphate assay procedure was used to evaluate various substrate analogs as well as determine pH optima for certain substrates. The procedure measures the formation of a colored molybdate complex and is a modification (15) of the method of Dreisbach (37). One unit of activity is defined as the amount of enzyme which liberates 1 μmole of Pi per ml of incubation mixture in 15 minutes under standard conditions.

3'-AMPase Assay The second method was developed as a continuous spectrophotometric method and used to study the kinetics of the release of phosphate from 3'-AMP. The coupling enzyme used in the assay was adenosine deaminase. This enzyme catalyzes the hydrolysis of the amino group at the 6 position of adenosine converting it to inosine which has a considerably smaller molar absorbtivity at 265 nm. Adenosine deaminase is specific for adenosine and will not deaminate 3'-AMP (38). The enzyme's Km, broad pH optimum and high catalytic turnover number (38) make it an efficient coupling enzyme in this reaction. The difference in the extinction coefficients for adenosine and inosine at 265 nm is such that a difference of 1.0 A_{265} unit equals the conversion of 0.14 μmoles adenosine per ml to inosine. The kinetic assays on 3' AMP were performed at 30° C, pH 6.0 in 0.01 M cacodylate buffer. Under these conditions 1 unit of 3'-AMPase activity is defined as the hydrolysis of 1 μmole of 3'-AMP per minute. In all cases the amount of wheat seedling nuclease used was such that the rate of

hydrolysis fell on the linear portion of the enzyme vs. rate curve. Furthermore, the amount of adenosine deaminase used was always such that additional deaminase produced no change in the reaction rate.

Hyperchromic Assay In certain instances the increase in optical density at 260 nm due to the hyperchromic phenomenon was used to measure the rate of hydrolysis of nucleic acid polymers by wheat seedling nuclease. These measurements were made in a Beckman DB spectrophotometer with the aid of a Sargent model SRL recorder. Rates were observed in the 0.5 to 1.5 OD range and except when noted, were linear for at least the first minute. Initial rates are reported as the increase in A_{260} units per minute.

Viscosity Assay A loss of viscosity was used to measure the reduction in molecular weight of *E. coli* DNA during the time course of hydrolysis by wheat seedling nuclease. Viscosities were determined with a Contraves Low-Shear Rheometer type LS100 (Contraves AG, Zurich) with a minimum shear rate of 0.07 per second. At the times specified 0.35 ml of reaction mixture were placed in the viscometer. Viscometer values at various shear rates were recorded and essentially no shear dependence was noted at the lower shear rates. Molecular weights were calculated from the empirical equations of Eigner (39) where k, the concentration dependency constant was 0.6.

Other Enzyme Assays

RNA Polymerase Assay Phage-induced, DNA-dependent

RNA polymerase from Pseudomonas putida was assayed according to the procedure of H. Towle. The assay procedure consists of measuring the amount of ^{3}H- labeled CTP incorporated into acid-insoluble form in 15 minutes at 30° C. The reaction mixture contained 25 μl of prereaction mix (200 μl 1 M tris-HCl, pH 8, 50 μl 1 M $MgCl_2$, 50 μl 0.1 M dithiothreitol, 200 μl 10 mg/ml BSA, 100 μl of nucleoside triphosphates -0.02 M in each nucleotide, 100 μl ^{3}H - CTP 0.5 Ci/ml, 300 μl H_2O), 10 μl DNA substrate and 10 μl polymerase. The reaction was terminated by the addition of 0.1 ml 0.1% SDS, 2 drops of salmon sperm carrier DNA and 5.0 ml cold 10% TCA - 1% PPi. After standing for 5 minutes in ice, the material was filtered and rinsed 3 times with 10% TCA - 1% PPi on a millipore filter. The filters were dried, covered with scintillation fluid and counted in a liquid scintillation counter. One unit of activity equals 1 nmole of ^{3}H-CTP made acid-insoluble in 10 minutes.

Polynucleotide Kinase Assay Polynucleotide kinase was assayed by the method of Richardson (33). The assay measures the conversion of radioactivity from γ - ^{32}P-ATP into acid-insoluble form. The substrate used was made by digesting salmon sperm DNA with micrococcal nuclease. A unit of polynucleotide kinase activity is defined as the amount catalyzing the production of a mμmole of acid-insoluble ^{32}P in 30 minutes at 37° C.

Alkaline Phosphatase Assay The method used to assay alkaline phosphatase was that of Garen and Levinthal (30)

where the change in absorbancy at 410 nm corresponds to the release of p-nitrophenol from p-nitrophenyl phosphate. One unit liberates one μmole of p-nitrophenol per minute at 25° C.

Venom Phosphodiesterase Assay Venom phosphodiesterase was assayed using the procedure of Laskowski (31). One unit equals the amount of enzyme needed to liberate one μmole of p-nitrophenol per minute from p-nitrophenyl phosphate at 25° C.

Protein Assays

Protein determinations were performed after the method of Lowry et al. (40) or by the turbidometric method using tannic acid as a precipitating reagent (41). The latter method has several advantages - namely, tris buffer and cysteine do not interfere, the assay is linear from 20-100 μgms/ml of protein, and finally, determinations can be made in ten minutes. BSA was used as a standard protein with both methods.

Gel Electrophoresis

Polyacrylamide Disc Gel Electrophoresis Polyacrylamide disc gel electrophoresis was performed by the method of Ornstein (42) and Davis (43). Except where otherwise noted, 5 cm of 7 percent running gel with 2 cm of stacking gel on top was polymerized in a 0.5 cm (inside diameter) glass tube. Protein (50-100 μgms) was layered on top of the gels in 20% sucrose in a volume less than 0.1 ml. Bromophenol blue was used as a tracking dye and electrophoresis was stopped when the dye band was just off the gel,

(about 90 minutes). Gels were stained for protein with Coomassie Blue, destained and stored in 10% acetic acid.

SDS Gel Electrophoresis SDS gel electrophoresis was performed following the procedure of Weber and Osborn (44). Protein solutions were prepared in 1% SDS and 1% 2-mercaptoethanol and heated at 100° C for 10 minutes prior to electrophoresis. Ten-cm gels containing 5% acrylamide (w/v) were used. Electrophoresis was carried out at room temperature at 8 ma/tube for 3 hours. Under these conditions the dye band had migrated through approximately one half of the gel. The smallest polypeptide moved about 10% farther than the dye band. Protein was stained and destained as described previously.

Agarose Gel Electrophoresis Electrophoresis of DNA on agarose gels in the presence of 0.5 μg/ml of ethidium bromide was accomplished following the procedure of J. Sambrook et al. (45). The DNA appeared as fluorescent bands under short-wave UV illumination. The gels were photographed with Polaroid type 55 P/N film after removing the illuminating short-wave UV with a Kodak No. 23A red filter.

Isoelectric Focusing

Two different procedures were employed for isoelectric focusing experiments. The first procedure used to determine the isoelectric point of the wheat seedling nuclease activities was carried out in an LKB Model 8101 electrofocusing column (total capacity 106 ml) which contained a pH gradient of ampholyte from 3.0-10.0. A

potential of 300 volts was applied to the column for 48 hours using a Krohn-Hite Model U4R-240 constant voltage power supply.

The following procedure (46) was used for the isoelectric focusing experiments conducted on the purified Type III wheat seedling nuclease. A column (154 mm x 10 mm) containing ampholites giving a final pH gradient of 3-6 was used. A 1 cm plug was formed in the bottom of the column with a mixture containing 1 percent agar and 3 percent ethanolamine. To the upper part of the column 3 percent phosphoric acid was carefully layered and the complete upper reservoir was then filled with 3 percent phosphoric acid. The lower reservoir was filled with 3 percent ethanolamine. In a cold room a 4° C and with the use of a Heathkit constant voltage power supply, 200 volts were applied to the column with the upper reservoir being positive and the lower reservoir being negative. After the current had dropped to less than 10 percent of the original value (about 4 hours), the column was fractionated by removing 0.2 ml fractions off the top with a spring-loaded syringe fitted with a piece of polyethylene tubing.

Ultracentrifugation Techniques

Sedimentation of DNA in Alkaline Sucrose Gradients

Alkaline sucrose gradients were prepared by forming a 4.5 ml linear gradient of 5-20% sucrose which was 0.9 M NaCl and 0.1 M NaOH. DNA samples (0.1 ml) were allowed to set 30 minutes in 0.9 M NaCl, 0.1 M NaOH and then

layered on top of the gradient with polyethylene tubing (1.0 mm I.D.) and a syringe microburet. The tubes were placed in a SW-39 rotor and spun in a Beckman Model L-2 centrifuge for 3 hours at 35,000 RPM at 20° C. In the case of ^{32}P labeled DNA, 1-5 μgms DNA was applied to the gradient and 30 fractions collected. The fractions were either counted directly as Cerenkov radiation or were TCA precipitated, filtered and counted in scintillation fluid. When unlabeled DNA was sedimented, 15-30 μgms DNA was layered on top of the gradient and 25 fractions collected. Optical density at 260 nm of the individual fractions was measured in 0.2 ml cuvettes with a Gilford spectrophotometer. The following formula was used to correlate S values with molecular weight - $S=0.0528 \times MW^{.400}$.

Sedimentation of DNA in Neutral Sucrose Gradients

Sedimentation of DNA in neutral sucrose gradients was performed in an analogous manner except that the DNA was not treated with NaOH and NaCl prior to centrifugation. The 5-20% sucrose gradient contained 1.0 M NaCl and 10 mM tris-HCl, pH 8.0. The gradients were spun at 50,000 RPM for 3 or 3.5 hours at 5° C. Under these conditions $S=0.0882 \times MW^{.346}$.

CsCl Buoyant Density Banding To form a gradient of CsCl, 4.5 ml of CeCl with a refractive index of 1.4000 were placed in a centrifuge tube. After layering the DNA sample (0.1 ml) on top of the gradient, the tubes were spun at 32,000 RPM for 63 hours at 10° C in a fixed-angle rotor,

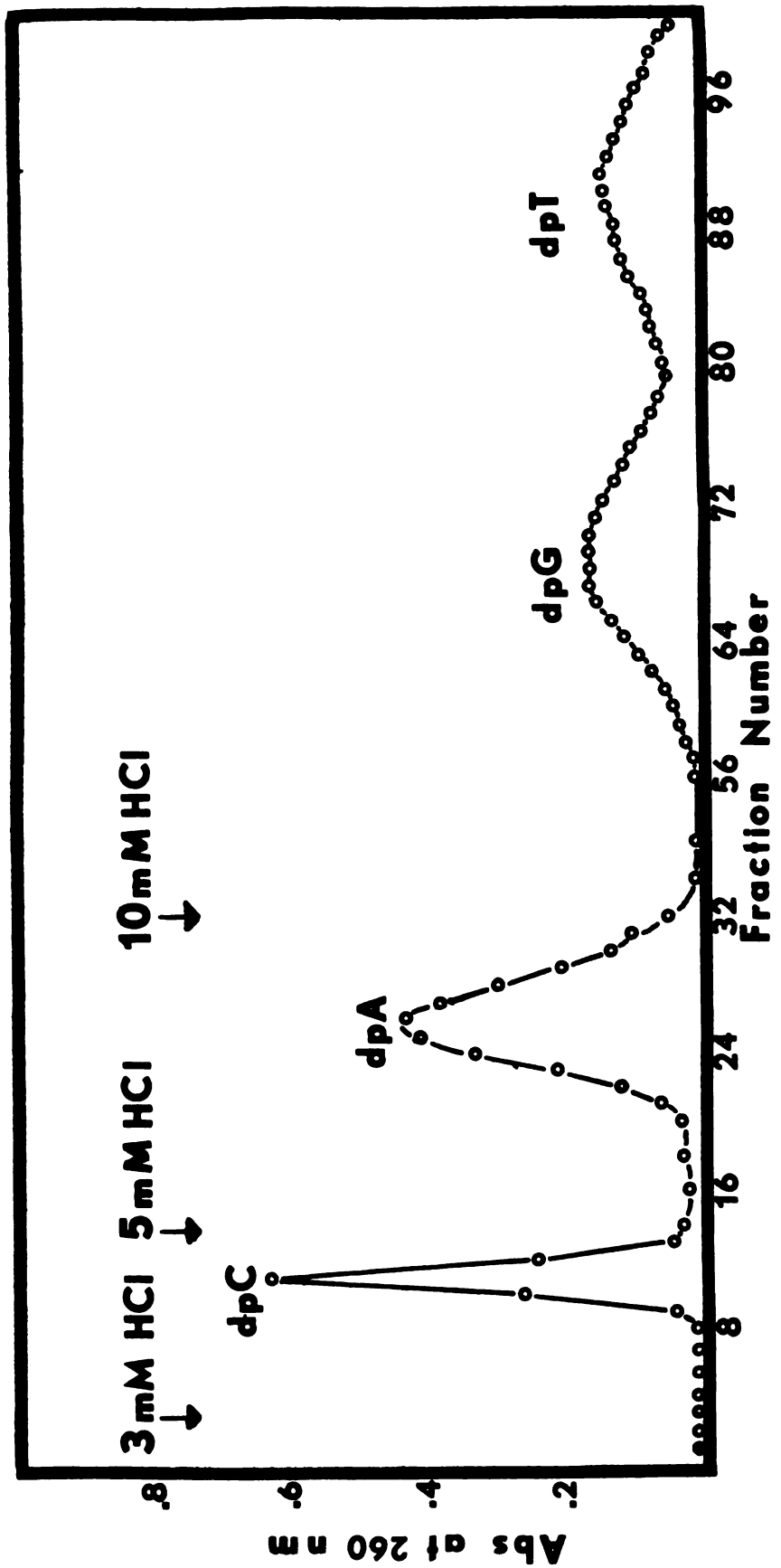
number 40. Forty fractions were collected and the refractive index was determined on every fourth fraction.

Model E Analytical Ultracentrifugation The sedimentation coefficient for wheat seedling nuclease was determined from sedimentation velocity analysis in a Spinco Model E analytical ultracentrifuge. Protein and solvent, respectively, were placed in the two chambers of a 30 mm double sectored cell. An AN-E rotor number 414-V was spun at 50,714 RPM at a temperature of 9° C. Photographs of the boundary pattern were taken at either 8 or 16 minute intervals.

Chromatographic Separation of 5'-Deoxyribonucleotides

Separation of the 5'-dNMP's was accomplished by chromatography using Bio Rad AG1 x 4 (100-200 mesh, Cl^- form) resin. The resin was washed with 3 cycles of 1 N acid, water and base and then washed extensively with water prior to use. A column (1.1 x 12 cm) was then packed and washed with 200 ml of 2 mM HCl. After the sample was applied to the column, a 1.0 ml/min flow rate was maintained with a Polystaltic pump and 5 minute fractions were collected. Figure 1 shows a typical separation. The sample applied to this column was comprised of 0.02 ml 1 M tris-HCl, pH 7.8, 0.05 ml of each of the 5'-NMP's (10 mg/ml), 0.10 ml 0.1 M $MgCl_2$ and 0.1 ml 0.7 M glycine buffer, pH 9.2. This mixture was diluted to 20 ml with water before application to the column. Elution of the 4 nucleotides was then accomplished with 3 mM HCl until pdC was off the column, then with 5 mM HCl until pdA had been eluted and finally with

Figure 1: Chromatography of 5'-deoxyribonucleoside monophosphates on a Dowex-1 column. A column (1.1 x 12 cm) was equilibrated with 2 mM HCl and the four nucleotides were applied as described in Methods. A step gradient of increasing HCl concentration was applied to the column as indicated by the arrows. The flow rate was maintained at 1.0 ml/min and 5 minute fractions were collected. The peaks were identified by comparing the spectra with those of known compounds.


Abs at 260 nm
.8
.6
.4
.2
3mM HCl
5mM HCl
10mM HCl
dpC
dpA
dpG
dpT
8
16
24
32
56
64
72
80
88
96
Fraction Number

10 mM HCl which eluted pdG and pdT from the column. The peaks were identified by comparing the absorbancy at 250, 260 and 290 nm with known spectra for the nucleotides in acid.

Growth of Bacteria and Bacteriophages

T_4r^+ phages were grown[3] at 37° C by inoculating a culture of E. coli B cells (10^8 cells/ml in the logarithmic phase of growth) with T_4r^+ phages at a multiplicity of infection of 0.01. After shaking the infected cells at high speed for 2 hours, lysis was completed with an additional incubation for 4 hours at low shaker speed. Lysis was followed by monitoring the absorbance at 650 nm. Phages were purified by 2 series of differential centrifugations (DNase I was added before the last low speed spin) and then by banding in a step gradient of 20-70% saturated CeCl. The purified phages were dialyzed against decreasing NaCl concentration starting with 2 M NaCl and ending with 0.5 M NaCl.

Lambda phages were made by growing a culture of temperature sensitive mutants of E. coli, $C_{600}\lambda CI_{ts}875$ to 0.5 A_{660} units at 30° C. The growth flask was placed in a 42° C water bath for 15 minutes. It was then shaken vigorously at 42° C for 1-2 hours until the A_{660} was 0.2. DNase was added and the phages were purified by differential centrifugation at 17,000 x g for 10 minutes after which the supernatant fluid was spun an additional 5 hours to

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[3]See appendix for the composition of the various growth media.

pellet the phages. The resuspended phages were subjected to 10 minute and 5 hour spins and the resuspended phages were then further purified by banding in a step gradient of 20-70% saturated CeCl. The phage suspension was dialyzed against 0.5 M NaCl, 0.01 M tris-HCl pH 8.0 before the DNA was extracted.

^{32}P uniformly labeled gh-1 DNA was made by growing Pseudomonas putida in a low phosphate media at 32° C to 0.2 A_{660}. ^{32}P was then added (3 mCi per 500 ml growth medium) and 30 minutes later gh-1 phages were added at a multiplicity of infection of 0.2. After lysis was complete, a few drops of chloroform were added and the lysate stored overnight at 4° C. The lysate was first spun at 16,000 x g for 10 minutes. Then the supernatant fluid was spun at 16,000 x g for 3 hours. The pellet was resuspended overnight in 10 mM tris-HCl (pH 8) plus 0.2 M NaCl. After another series of spins, the resuspended phages were passed through a DEAE cellulose column at room temperature. The column (2.5 x 10 cm) had been previously equilibrated with 10 mM tris-HCl (pH 8) plus 0.2 M NaCl. The fractions containing the phage particles were collected and concentrated by centrifugation before extracting the DNA.

RESULTS

Wheat Seedling Nuclease Purification Procedure

In order to conduct most of the experiments reported in this thesis, wheat seedling nuclease was required in relatively pure form with moderate yields of enzyme. In addition, since the enzyme may possess utility as a tool for research in other laboratories, expediency and ease of preparation were also considerations. In order to meet these requirements, several purification procedures were developed during the course of this investigation. As a starting point, the original isolation procedure of D. Hanson (15) was used. This procedure consisted of six steps. In the first step, the germinated wheat seedlings were homogenized through cheese cloth. In the next step the liquid portion of the crude homogenate was subjected to precipitation by a 57% saturation with $(NH_4)_2SO_4$. A second treatment with 75% $(NH_4)_2SO_4$ precipitated most of the enzyme activity. In a likewise manner, the third step consisted of a double precipitation with ethanol. The fourth step, the heat stable step, involved heating the material from the ethanol step to 75° C for 10 minutes and discarding the resulting

precipitate. The next step was chromatography on a phosphocellulose column (PC step). The sixth and last step in the original procedure was chromatography on a Bio-Gel P-60 column.

Type I Purification

Initial efforts were centered on minor modifications of the original isolation procedure outlined above and resulted in the Type I purification procedure. Centrifugation of the crude homogenate at 10,000 x g for 15 minutes instead of squeezing through cheese cloth was found to result in larger yields of enzyme. Typically about 100,000 units of DNase activity and 100 grams of protein were present in the crude homogenate which represents a three fold increase in activity and 50% increase in protein.

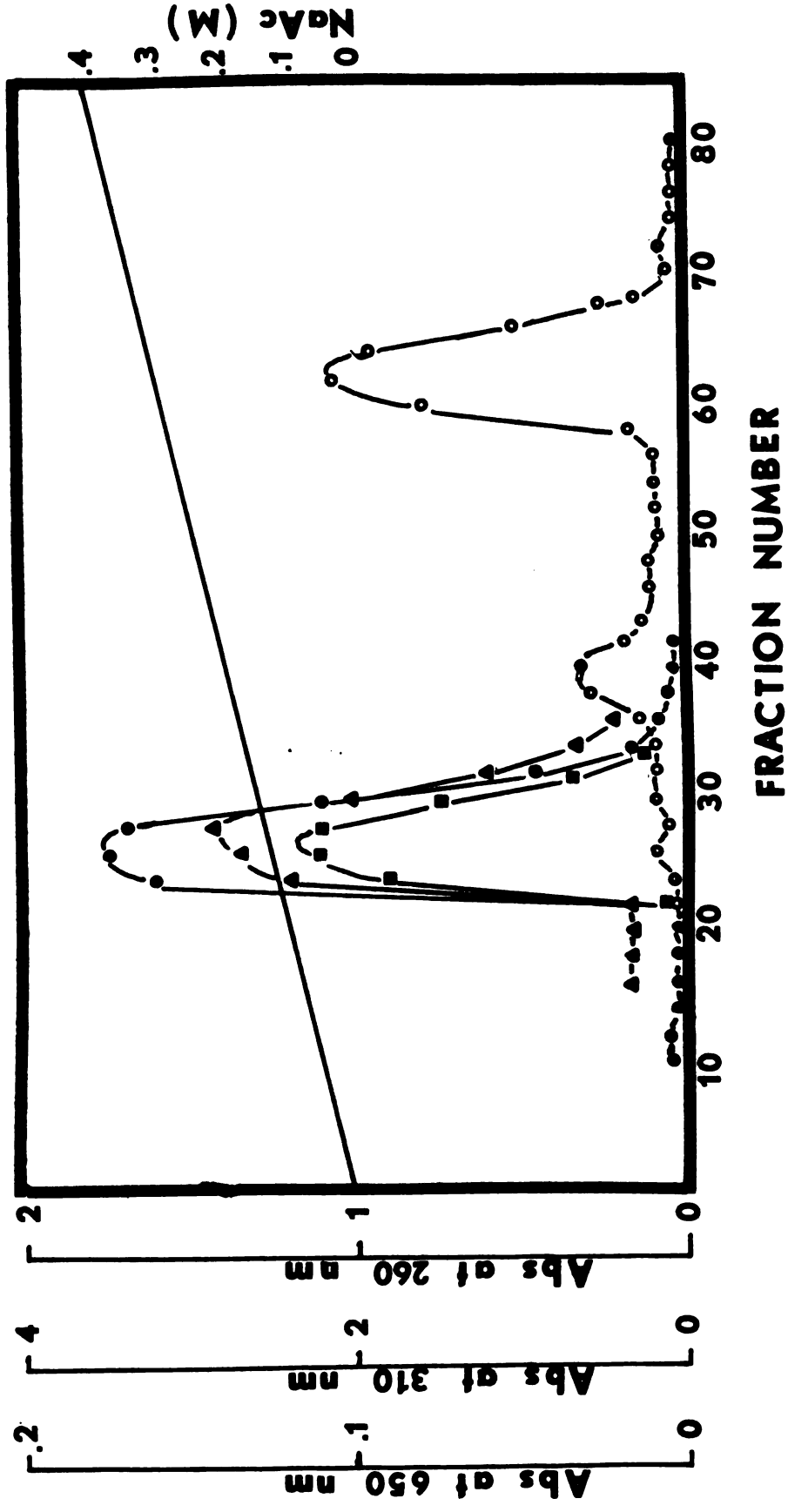
Ammonium sulfate was added by an automatic continuous shaking process during the $(NH_4)_2SO_4$ precipitations. The pellet following the second $(NH_4)_2SO_4$ precipitation was dissolved in 0.05 M NaAc, pH 4.5, 2 mM cysteine and 1.0 mM zinc acetate and stored overnight at 4° C with no loss of DNase activity. This modification is a convenience since the preparation can be stopped after about 8 hours instead of the more than 12 hours previously required to reach the heat stable step. The protein collected and pooled from the final P-60 column step was concentrated by pressure dialysis on an Amicon ultrafiltrator (UM-10 filter) which resulted in no loss of

activity. This material was dialyzed against 0.05 M NaAc, pH 5.0, 0.1 mM $Zn(Ac)_2$ and 0.05% 2-mercaptoethanol and was stable for months at 4° C. This purification procedure (Type I) involved the same six steps as the original procedure - namely, crude homogenate, $(NH_4)_2SO_4$ step, ethanol step, 75° C heat treatment, phosphocellulose chromatography and chromatography on a P-60 column and required 5 days. This procedure was used through the heat stable step as a starting point for the Type II purification procedure and through the phosphocellulose step for the Type III procedure.

Type III Purification

It was noted in batch studies with various ion exchangers that the wheat seedling nuclease had a very low binding affinity for carboxymethyl cellulose at pH 5.0. This property was utilized in the Type III purification procedure. The pooled fractions from the phosphocellulose step of Type I purification were concentrated to 10-20 ml by ultrafiltration on a UM-10 filter and dialyzed overnight against two changes of 0.01 M NaAc, pH 5.0, 0.1 mM $Zn(Ac)_2$ and 1.0 mM cysteine (CMC buffer). This material (about 11,500 DNase units and 70 mg protein) was applied to a carboxymethyl cellulose column and eluted at room temperature with a 600 ml linear gradient of increasing NaAc concentration as shown in Figure 2. As can be seen from the figure, the three activities

Figure 2: Chromatography of wheat seedling nuclease on a carboxymethyl cellulose column following the phosphocellulose purification step. Carboxymethyl cellulose was treated as described in Materials and packed into a column 2.5 x 23 cm. After equilibrating the column with 0.1 mM $Zn(Ac)_2$, 1.0 mM cysteine and 0.01 M NaAc, pH 5.0, the protein was applied as described in the text. A 600 ml linear gradient of increasing sodium acetate concentration (0.1 mM $Zn(Ac)_2$, 1.0 mM cysteine and 0.01 M NaAc, pH 5.0 to 0.1 mM $Zn(Ac)_2$, 1.0 mM cysteine and 0.4 M NaAc, pH 5.0) was applied to the column at a flow rate of 1.5 ml/min. Five minute fractions were collected. Protein was assayed on every other fraction by the tannic acid method and is indicated by absorbance at 650 nm. DNase and RNase assays were acid soluble assays as described in Methods and reported as absorbance at 260 nm. The 3'-AMPase activity was assayed by the fixed time phosphate assay described in Methods and is reported as absorbance at 310 nm. DNase activity, ●———● ; RNase activity ■———■ ; 3' AMPase activity, ▲———▲ ; protein o———o .


Abs at 650 nm
Abs at 310 nm
Abs at 260 nm
NaAc (M)
FRACTION NUMBER

associated with wheat seedling nuclease eluted in the early fractions while the bulk of the protein eluted in two later peaks. The fractions containing the enzyme activity were pooled and concentrated to approximately 1 ml by ultrafiltration on an Amicon UM-2 filter and dialyzed against 0.05 M NaAc, pH 5.0, 0.1 mM $Zn(Ac)_2$ and 0.05% 2-mercaptoethanol. Over 40% of the enzymatic activity and less than 0.8% of the protein applied to the column was collected in the pooled fractions representing an increase in the specific activity of over 62 fold. The activity of this preparation remained stable for several months when stored at 4° C. This procedure yielded wheat seedling nuclease in its purest form and was used for the studies in the "protein properties" section except where noted.

Type II Purification

A slightly less pure preparation (Type II) of wheat seedling nuclease requiring 3 days to prepare was used for most of the "mechanism of action" studies. In this procedure, material from the heat stable step of the Type I procedure was concentrated by ultrafiltration to 10-20 ml on an Amicon UM-10 filter and dialyzed overnight against two changes of CMC buffer. This dialyzed solution (about 16,000 DNase units and 120 mg protein) was applied to a carboxymethyl cellulose column and eluted at room temperature with a 600 ml linear gradient of increasing

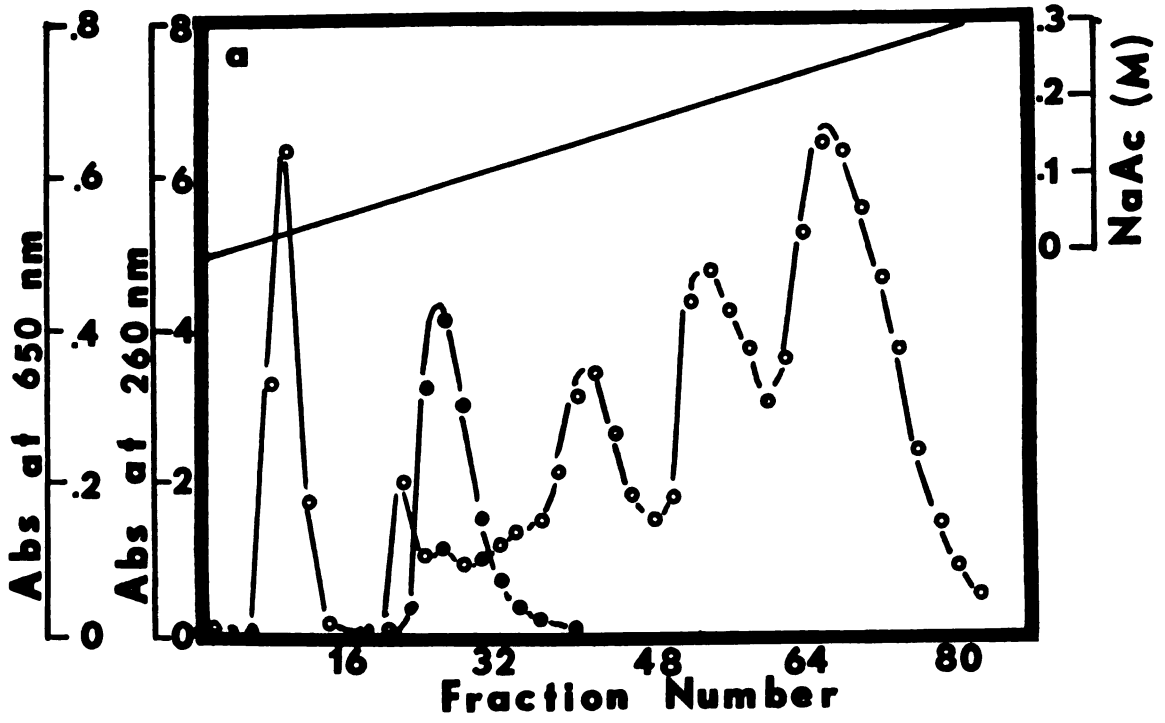
NaAc concentration as shown in Figure 3a. The pooled fractions of enzyme activity were concentrated and dialyzed as described for the Type III procedure and were also stable at 4° C for several months. Greater than 55% of the DNase activity and less than 2% of the protein applied to the column was recovered in the pooled fractions. As a result, this purification step represents a 31 fold increase in the specific activity of the enzyme. The Type II purified wheat seedling nuclease contained no detectable 3'-nucleotidase or 5'-nucleotidase activity at pH 5 or pH 7. In addition no hydrolysis of p-nitrophenyl phosphate or bis-p-nitrophenyl phosphate was noted at pH 5 or pH 8. In an experiment to be described later measuring acid-soluble material released from ^{32}P labeled native gh-1 DNA, a small amount of a nonspecific exodeoxyribonuclease was detected. In this case all of the contaminating activity was removed by passing the enzyme through a Bio Rad P-60 gel column as shown in Figure 3b. The figure also shows a roughly uniform specific activity across the elution peak indicative of a relatively high degree of purity of the enzyme.

Summary of Purification Procedures

Table I compares the properties of the various purification procedures. The numbers listed are typical values based on at least three separate preparations. Normally 3.5 kilograms of wheat were used as the starting

Figure 3a: Chromatography of wheat seedling nuclease on a carboxymethylcellulose column following the heat stable purification step. Carboxymethylcellulose was prepared as described in Materials. A column (2.5 x 25 cm) was equilibrated with 0.1 mM $Zn(Ac)_2$, 1.0 mM cysteine and .01 M NaAc, pH 5.0. The protein was prepared and applied as described in the text. A 600 ml linear gradient of increasing NaAc concentration (0.1 mM $Zn(Ac)_2$, 1.0 mM cysteine, .01 M NaAc, pH 5.0 to 0.1 mM ZnAc, 1.0 mM cysteine, 0.3 M NaAc, pH 5.0) was used to elute the enzyme. Fractions of 7 ml were collected and every second fraction was assayed for protein (as absorbance at 650 nm), o———o and DNase activity (as absorbance at 260 nm), ●———● .

Figure 3b Gel filtration of wheat seedling nuclease on a Bio Gel P-60 column following the carboxymethylcellulose step in Figure 3a. A column (2.3 x 46 cm) was equilibrated with 0.1 mM $Zn(Ac)_2$, 0.1% 2-mercaptoethanol, and .025 M NaAc, pH 5.0. This same buffer was used to elute the enzyme at a flow rate of 0.8 ml/min. Fractions of 5 minutes were collected and each fraction was assayed for protein (absorbance at 650 nm), o———o and DNase activity (absorbance at 260 nm), ●———●. The specific activity of the eluted enzyme is plotted as DNase activity (absorbance at 260 nm) per mg/ml of protein.


a
Abs at 650 nm
Abs at 260 nm
NaAc (M)
Fraction Number
0
.2
.4
.6
.8
2
4
6
8
.1
.2
.3
16
32
48
64
80

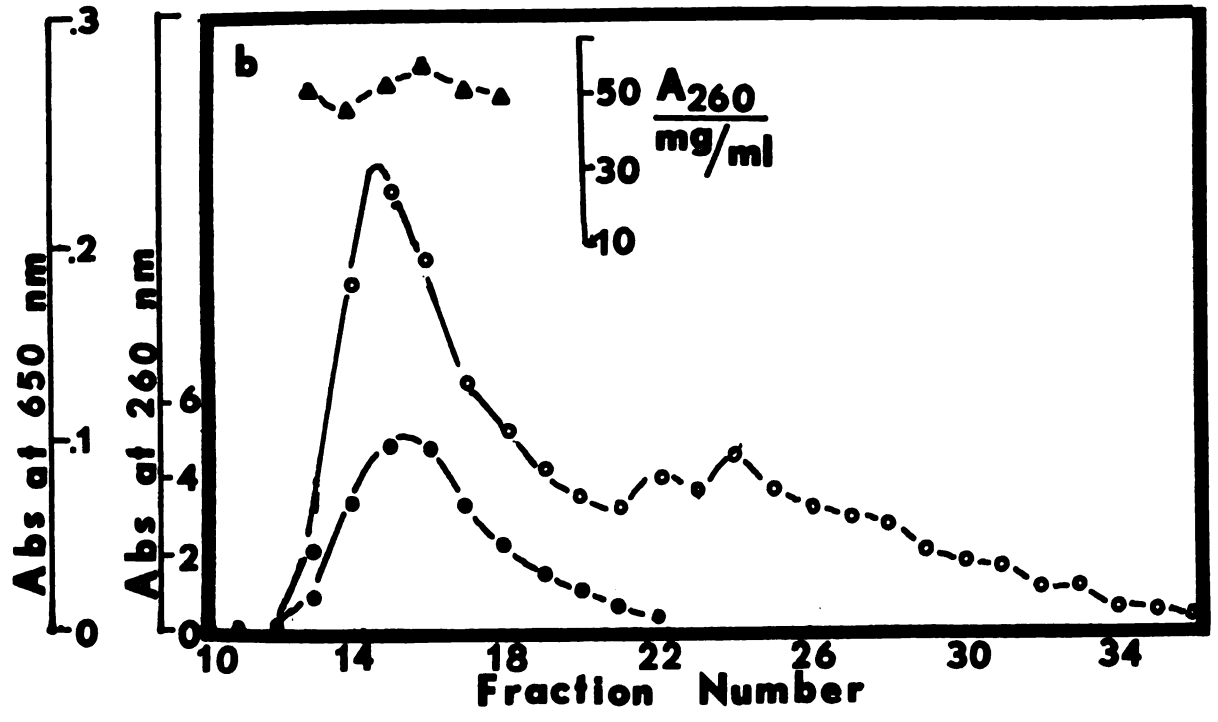

b
Abs at 650 nm
Abs at 260 nm
A260
mg/ml
Fraction Number
0
.1
.2
.3
2
4
6
10
30
50
10
14
18
22
26
30
34

Table I

Comparison of various procedures for the isolation of wheat seedling nuclease[a]

Procedure	Total protein	Total activity	Specific activity	Fold purified[c]	Yield[c]
	mg	units	units/mg		%
Original[b]	10.5	4,400	418	839	12.2
Type I	13.0	15,000	1,150	1,150	15.0
Type II	2.2	9,000	4,090	4,090	9
Type III	0.5	5,000	10,000	10,000	5

[a]These data are based on assays of the final purification step of the procedure where the starting material was 7 kilograms of wheat seedlings. The standard DNase assay was used to determine enzyme activity and, in the case of procedures I-III, the data represent typical values of 3 or more purifications.

[b]The original procedure is that of D. Hanson (15).

[c]Fold purified and percent yield are calculated by comparing the final purification steps with the crude homogenate.

material and two preparations were combined after the heat stable step. The original isolation procedure requires 5 days which included 12-18 hours at the beginning before the enzyme is stable enough to store overnight. The Type I procedure also requires 5 days but can be stored overnight after about 8 hours. The Type III procedure produced the purest enzyme and was judged to be greater than 90-95% pure (see "Protein Properties" section). This procedure requires 4 days for completion. The Type II procedure was judged to give protein which was greater than 80% pure (see the "Protein Properties" section) and took 3 days to complete.

Protein Properties

In order to study some of the physical and chemical properties of wheat seedling nuclease, it was necessary to establish the degree of homogeneity to which the enzyme had been purified. The Type III preparation was used for these studies and several criterion were used to estimate its purity.

Sedimentation in the Model E Analytical Ultracentrifuge

Wheat seedling nuclease Type III was sedimented in the analytical ultracentrifuge as described in Methods. A single symmetrical boundary was observed during the sedimentation. The $S_{20,w}$ value which was calculated

from the sedimentation velocity data is 3.59. Assuming that the enzyme is roughly spherical in shape, this $S_{20,w}$ value corresponds to a protein of approximately 43,000 daltons.

Sedimentation through a Sucrose Gradient

Type III enzyme (0.14 mg) was sedimented through a 5-20% sucrose gradient in the swinging bucket rotor of an L-2 Beckman ultracentrifuge following the procedure of Martin and Ames (47). Centrifugation was done at 4° C and stopped after 24 hours. Fractions were collected and assayed for protein and DNase activity. A single,coincident peak of protein and activity was found. The specific activity of the peak remained roughly constant in all the fractions. Wheat seedling nuclease sedimented to the same fraction number as egg ovalbumin (MW= 45,000) which was included in another centrifuge tube in the same experiment.

SDS Gel Electrophoresis

Acrylamide gel electrophoresis in the presence of SDS was performed on protein samples from the last three steps in the Type II purification procedure and protein samples from the last two steps in the Type III procedure as outlined in Methods. Typical gels are shown in Figure 7a and 7b. The slowest moving band represents the wheat seedling nuclease. As can be seen in the figure,

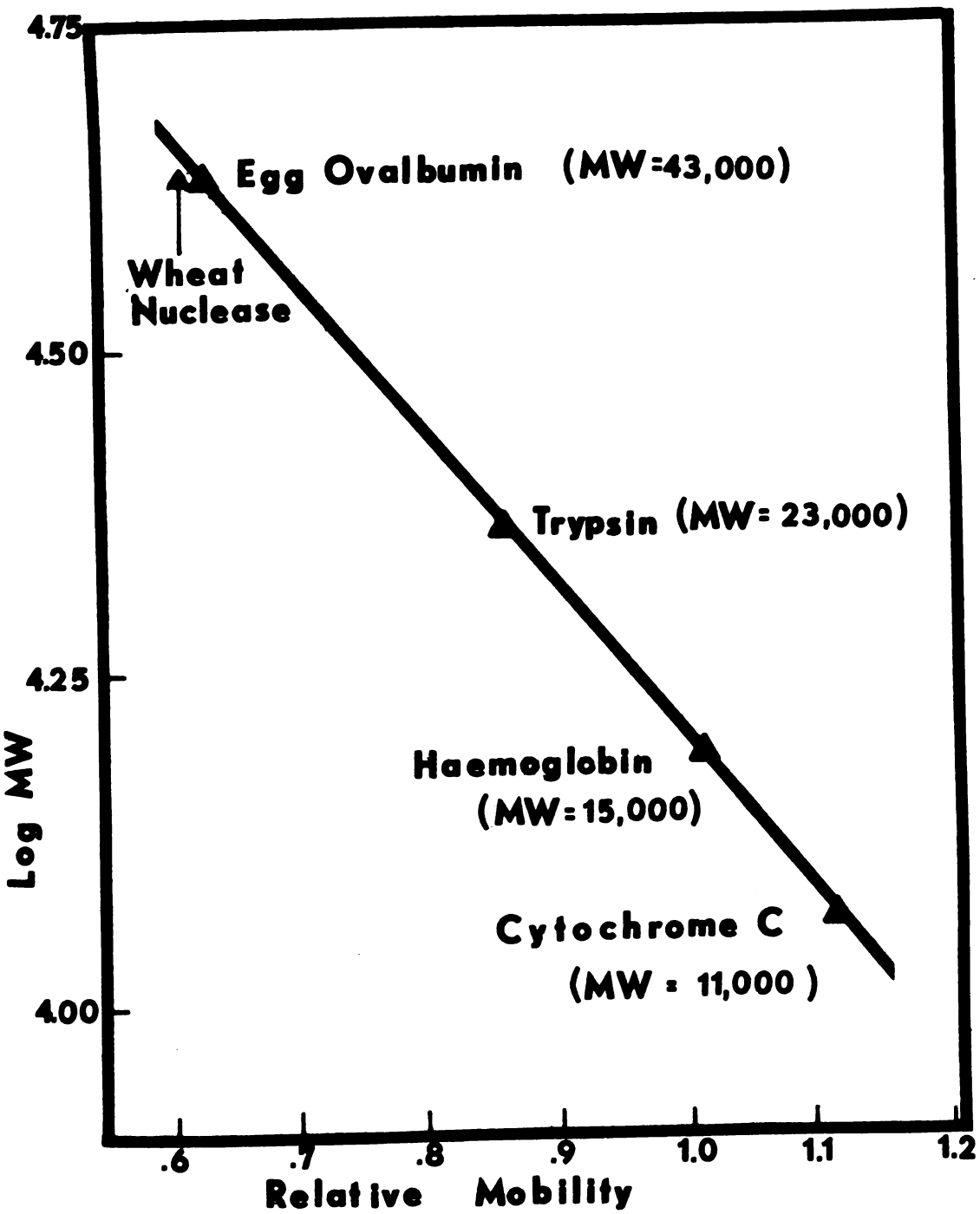
there are two other major protein bands after the heat stable step. The CMC step removes most of these contaminating proteins and the band corresponding to the wheat seedling nuclease is judged to comprise better than 80% of the protein on the gel. The last step in the Type II procedure, P-60 chromatography, increases the estimated purity to better than 90%. The PC step of Type III purification removes a significant amount of one of the two contaminating bands present after the heat stable step. The last step in the Type III procedure, CMC chromatography, results in a purification of wheat seedling nuclease judged to be greater than 90-95% pure.

Figure 4 shows the relative mobility of several standard proteins on SDS gels plotted against the logarithm of their molecular weights. When the relative mobility of the single major band of the purified Type III enzyme is compared to this standard curve, its molecular weight is 43,500. Since this value is similar to that derived from other methods (where conditions are not expected to disrupt the subunit structure of proteins like the conditions during SDS electrophoresis), wheat seedling nuclease appears to be a protein comprised of a single polypeptide chain.

Sephadex G-100 Gel Filtration

As a final criterion for estimating the homogeneity of the Type III purified enzyme, wheat seedling

Figure 4: Relative mobility of standard proteins after SDS gel electrophoresis. Relative mobility refers to the distance moved by the protein divided by the distance moved by the dye band. Wheat seedling nuclease relative mobility was 0.62 which corresponds to a molecular weight of 43,500.


4.75
4.50
4.25
4.00
Log MW
Egg Ovalbumin (MW=43,000)
Wheat
Nuclease
Trypsin (MW= 23,000)
Haemoglobin
(MW=15,000)
Cytochrome C
(MW = 11,000)
.6
.7
.8
.9
1.0
1.1
1.2
Relative Mobility

nuclease was chromatographed on a Sephadex G-100 column. The result is shown in Figure 5. All of the protein eluted in a single peak with the enzyme activity between 1 and 2 times the column void volume. The specific activity remained constant throughout the peak. The various techniques used to estimate the size and purity of wheat seedling nuclease Type III are summarized in Table II.

Isoelectric Point Determination

The isoelectric point for the 3'-AMPase and DNase activities of Type I wheat seedling nuclease was determined on an LKB 8101 electrofocusing column as described in Methods. The result is displayed in Figure 6. As expected, the two activities focused together and the isoelectric point was determined to be 5.2.

Spectral Properties

The ultraviolet absorption spectrum for the purified wheat seedling nuclease indicated an abnormal ratio for the extinction indexes at 280 nm and 260 nm and was therefore examined in some detail. The A_{280}/A_{260} ratio of the Type III purified enzyme after dialysis against tris-HCl buffer at pH 8.0 varied between 1.09 and 1.30 depending on the individual preparation. This ratio compares with 1.6-1.7 found for most proteins.

An abnormal distribution of aromatic amino acids in the protein could account for the low A_{280}/A_{260}

Figure 5: Gel filtration of purified Type III wheat seedling nuclease. Sephadex G-100 was packed in a column (1.9 x 80 cm) and equilibrated with 0.1 mM $Zn(Ac)_2$, 1.0 mM cysteine, and 0.05 M NaAc, pH 5.0. Wheat seedling nuclease Type III was applied as described in the text and 5 minute fractions were collected at a flow rate of 0.4 ml/min. Every third tube was assayed for protein by the tannic acid method (absorbance at 650 nm), o——o. Every second tube was assayed for DNase activity (absorbance at 260 nm), •———•. Specific activity is plotted as DNase activity (A_{260}) per microgram of protein. The arrows indicate positions where one and two times the void volume of eluting buffer had passed through the column.

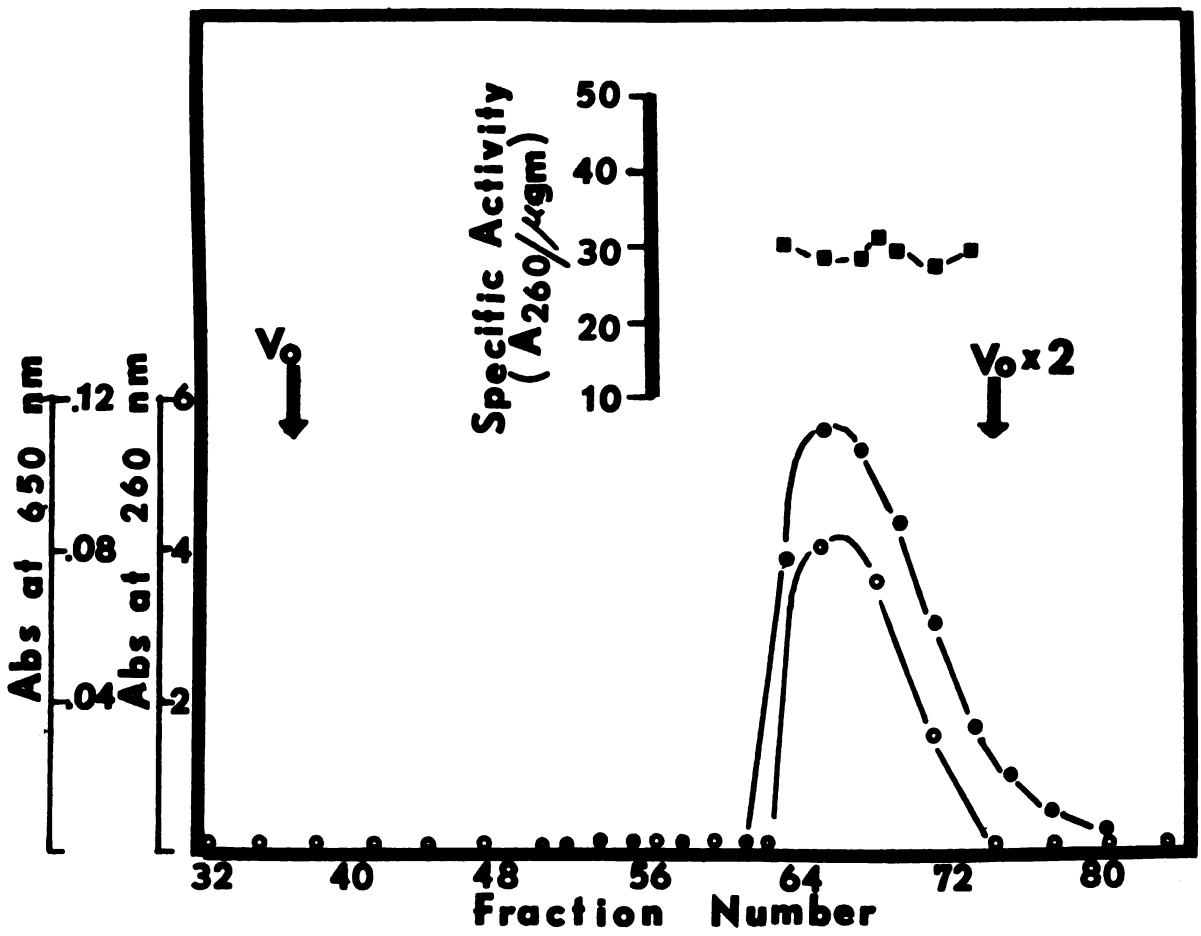

Specific Activity
(A260/μgm)
50
40
30
20
10
Vo
Vo x 2
Abs at 650 nm
.12
.08
.04
Abs at 260 nm
6
4
2
32
40
48
56
64
72
80
Fraction Number

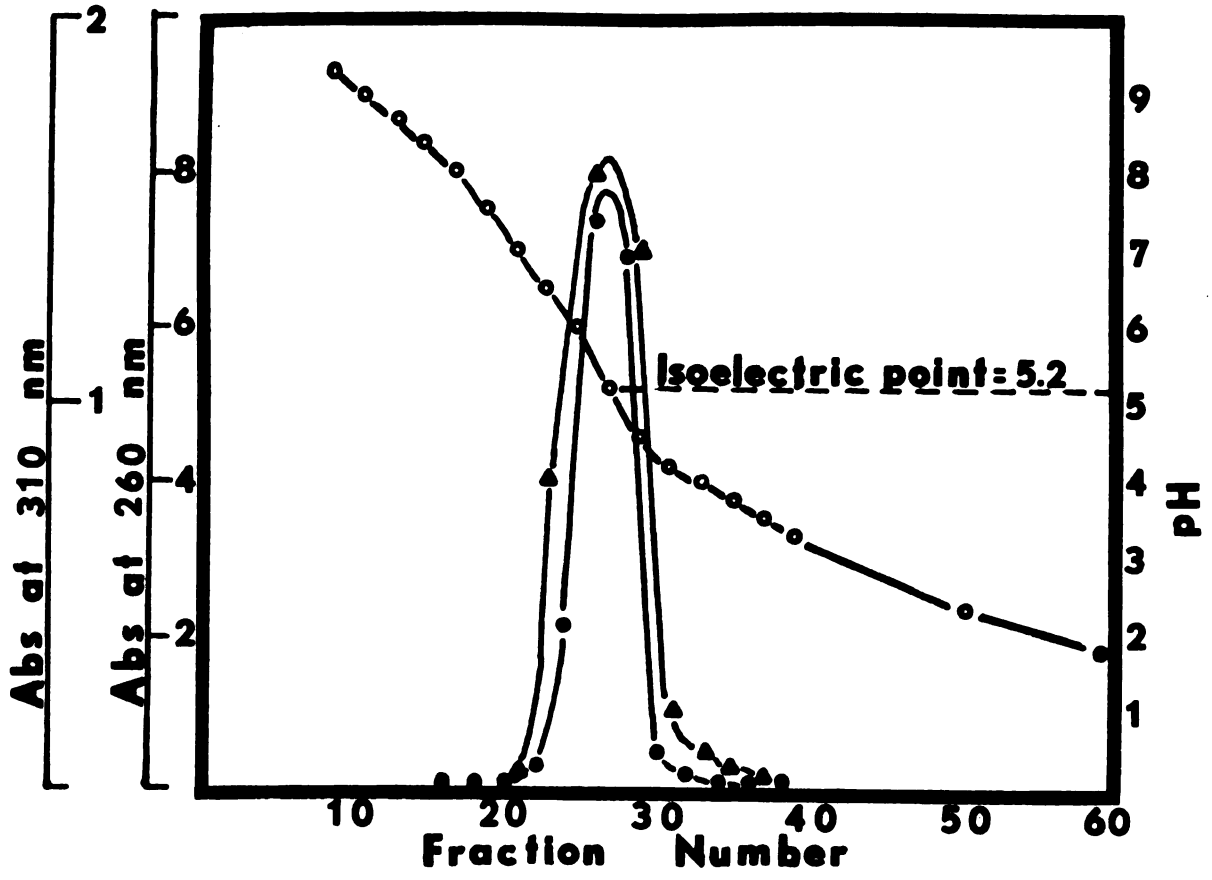
Table II

Molecular weight values and evidence of purity for Type III enzyme

Method	Molecular weight	Evidence of purity
SDS gel electrophoresis	43,500	single major protein band
Sucrose gradient sedimentation	45,000	single symmetrical peak
Sephadex G-100 gel filtration		constant specific activity across elution peak[a]
Analytical ultracentrification	43,000	single symmetrical boundary

[a]See figure 5.

Figure 6: Isoelectric focusing of DNase and 3'-AMPase activities. Wheat seedling nuclease Type I (800 units, 1 mg) was focused on a LKB Model 8101 electrofocusing column as described in Methods. Fractions of 1.5 ml were collected and enzyme assays and pH were determined on every other fraction. 3'-AMPase activity (absorbance at 310 nm), ▲———▲; DNase activity (absorbance at 260 nm), ●———●; pH, ●——o.


Abs at 310 nm
Abs at 260 nm
pH
Isoelectric point = 5.2
Fraction Number
10
20
30
40
50
60

ratio observed for wheat seedling nuclease. To test this possibility trytophan and tyrosine content in the Type III enzyme was determined using the spectrophotometric method of Edelhoch (48). A 0.18 mg/ml solution of Type III enzyme was dissolved in 6 M guanidine hydrochloride and the absorbance (measured on a Guilford spectrophotometer in a 1 cm light path, 0.2 ml cuvette) at 280 nm and 288 nm was 0.130 and 0.095 respectively. Since the molar extinctions for tyrosine and tryptophan are known and the concentration of the protein in the cuvette can be calculated (assuming a homogeneous protein), the two unknowns, moles of tryptophan and moles of tyrosine per mole of protein can be calculated[4] by solving two simultaneous equations. The result is 4.31 and 5.07 moles of tryptophan and tyrosine per mole of wheat seedling nuclease respectively.

Phenylalanine has an extinction maximum at 260 nm. The molar extinction coefficient at this wavelength, however, is only about one tenth as large as tryptophan and about one third as large as that for tyrosine at 280 nm. Therefore there would have to be a very large number of phenylalanine residues per molecule of wheat seedling nuclease to cause a low A_{280}/A_{260} ratio. The spectra of phenylalanine has a characteristic fine structure region around 260 nm which produces multiple peaks. For proteins with an appreciable amount of phenylalanine this

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[4] See appendix for calculations..

fine structure causes irregularities in the protein spectra around this wavelength. Wheat seedling nuclease Type III with an A_{280}/A_{260} ratio of 1.26 was placed in a Cary spectrophotometer and the spectra from 300 nm to 240 nm was recorded. No obvious irregularities occurred in the region around 260 nm indicating a relatively low content of phenylalanine. It therefore seems likely that the abnormal A_{280}/A_{260} ratio for wheat seedling nuclease is not due to an abnormal distribution of aromatic amino acid residues.

The final spectral properties studied were fluorescense excitation and emission spectra. These spectra, along with UV absorption spectra, were obtained with the aid of a computer-centered spectrophotometer-fluorimeter constructed by Dr. J. Holland of this department. After the appropriate spectra were obtained, the computer was asked to construct a UV absorption spectra which would be produced if all the absorption at 285 nm,which had produced fluorescence,were subtracted from the original UV absorption spectra. For a normal protein containing only aromatic amino acid chromophores, this constructed curve should be flat. The computer-constructed UV absorption spectrum for wheat seedling nuclease, however, contained a small peak at 255 nm. This result indicates the possible presence of a chromophore present in wheat seedling nuclease which absorbs light at 255 nm but does not produce any

fluorescence due to absorption at this wavelength.

Multiple Ionic Forms of Wheat Seedling Nuclease

During the initial attempts to develop a homogeneous purification of wheat seedling nuclease, polyacrylamide disc gel electrophoresis was used as a criterion for estimating purity. It was noted that a number of bands (6-12) persisted even when the specific activity of the enzyme preparation had been considerably increased.

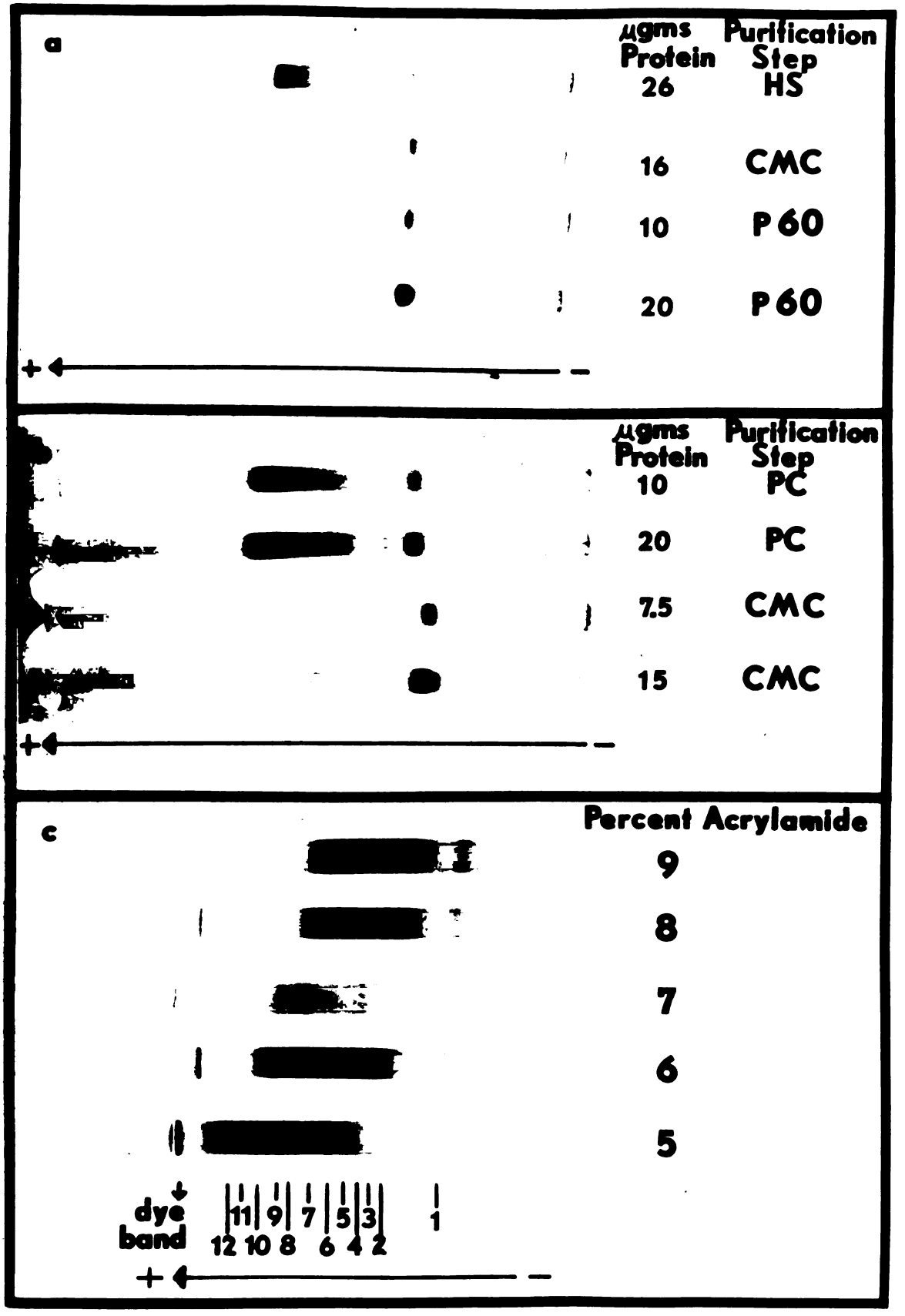
When wheat seedling nuclease Type III purified enzyme (considered to be of high purity) is electrophoresed on polyacrylamide gels, 6-12 bands are easily discernible. Figure 7c shows photographs of the multiple bands produced by staining gels (for protein) containing different percentages of acrylamide. The 7% acrylamide gel was sliced lengthwise. One half was stained for protein while the other half was sliced into 2 mm slices and the DNase activity was eluted and assayed. The DNase activity was associated with the third band. The first band was independently determined to be a contaminating protein from the PC purification step.

When proteins are electrophoresed on gels containing different percentages of acrylamide and the logarithm of their relative mobilities on each gel are plotted versus the percent acrylamide, the slope of the resulting plot is a function only of the size of the protein and the ordinate intercept is a function of the total

Figure 7a: SDS gel electrophoresis of the last three steps in the Type II purification procedure. The amount of protein indicated in the figure was layered on top of SDS acrylamide gels and electrophoresed as described in Methods. The arrow indicates the length of the gels and the direction of protein migration. The + and − signs indicate the polarity of the electrodes during electrophoresis. HS refers to the heat stable fraction, CMC to the carboxymethyl cellulose fraction and P-60 to the P-60 column step of purification.

Figure 7b: SDS gel electrophoresis of the last two steps in the Type III purification procedure. Notations are the same as in figure 7a except PC refers to protein from the phosphocellulose step and CMC refers to the final carboxymethyl cellulose column of the Type III purification.

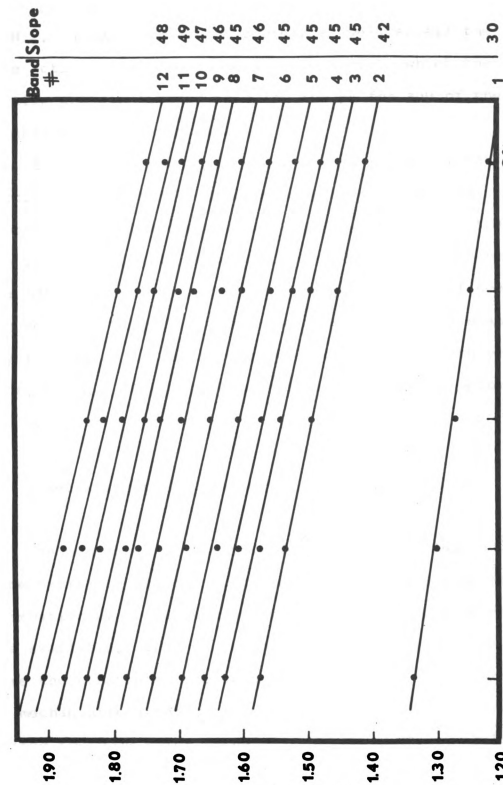
Figure 7c: Polyacrylamide gel electrophoresis of the purified wheat seedling nuclease Type III on gels containing different percentages of acrylamide. Electrophoresis was performed as described in Methods except that it was terminated before the tracking dye band was off the gels. The 7% gel was sliced in half lenghtwise and one half was stained for protein while the other half was sliced in 2 mm segments from which enzyme activity was eluted. The arrow indicates the direction of migration of the protein bands which are numbered chronologically from the top of the gel.


a
µgms Protein
Purification Step
26
HS
16
CMC
10
P60
20
P60
+
−
µgms Protein
Purification Step
10
PC
20
PC
7.5
CMC
15
CMC
+
−
c
Percent Acrylamide
9
8
7
6
5
dye band
12 11 10 9 8 7 6 5 4 3 2 1
+
−

charge carried by the protein at the pH of electrophoresis (49). Using this procedure, wheat seedling nuclease was electrophoresed on gels containing 5-9% acrylamide (w/v). A plot of the logarithm of relative mobilities for each band versus the percentage of acrylamide in the gel is shown in Figure 8. Band number one has a slope of 30 and was determined independently to be a contaminating protein carried through from the PC purification step. Using the same system, Hendrick and Smith (49) showed that a protein with a slope of 49 corresponds to a molecular weight of 47,000. The plots in Figure 8 are all approximately parallel, indicating that the differently charged protein bands are all about the same size. A slight increase in the slope, 42-49, is noted as the bands moved farther to the positive electrode. This corresponds roughly to a difference of about 2,000 daltons between bands 2 and 12. DNase activity was associated with only one band (number three) which represents one of the least negatively charged proteins. Another striking feature of the graph is that the vertical displacement of the various plots is roughly the same between any two bands indicating similar differences in net protein charge between adjacent bands.

Isoelectric focusing of the purified Type III enzyme was performed in the hope of providing a technique for studying the differently charged proteins. Figure 9 shows the results of focusing the purified enzyme on

Figure 8: Plot of relative mobilities of protein bands during electrophoresis on gels containing different percent of acrylamide. Wheat seedling nuclease Type III was electrophoresed on gels containing different percentages of acrylamide (w/v) as shown in Figure 7c. The relative mobility (Rm) was calculated by dividing the distance moved by the protein band by the distance moved by the dye band. Slopes of the individual plots were calculated by dividing the log (100 x Rm) at 5% minus the log (100 x Rm) at 9% by 4 and multiplying by a factor of 1000.


Band Slope
#
12 48
11 49
10 47
9 46
8 45
7 46
6 45
5 45
4 45
3 45
2 42
1 30
1.90
1.80
1.70
1.60
1.50
1.40
1.30
1.20
Log (100 x Rm)
5%
6%
7%
8%
9%
Percent Acrylamide in Gel

a small, custom-made isoelectric focusing column. Most of the protein formed a precipitant band which focused at pH 2. A small amount of protein as well as all the DNase activity focused at pH 5.2. The pH to which the inactive protein focused is below the pK for any of the commonly occuring amino acids.

In order to ascertain whether some or all of the inactive protein bands found on acrylamide gels were responsible for the overall low A_{280}/A_{260} ratio for the Type III enzyme, gels were scanned at 260 nm and 280 nm on a Gilford spectrophotometer equipped with a gel scanning device. Figure 10 shows the result of such a scan. Only four major bands are easily discernible. Of these bands, the A_{280}/A_{260} ratio decreases as the bands become more negatively charged.

Mechanism of Action During Enzymatic Catalysis

As mentioned in the introduction to this thesis a "mechanism of action" study is limited in descriptive information by the amount and detail of the experimental data available. This is especially true for nucleases where the substrates represent complex biomacromolecules. The mechanistic areas of investigation reported here are limited to those which illustrate unique features of the enzymatic hydrolysis by wheat seedling nuclease as compared with other nucleases appearing in the literature.

Figure 9: Isoelectric focusing of Type III enzyme. The enzyme was focused in a small (154 mm x 10 mm) custom made column as described in Methods. Fractions of 0.2 ml were removed and analyzed for DNase activity (absorbance at 260 nm), ▲——▲, protein (absorbance at 650 nm), ●——●, and pH, o——o.

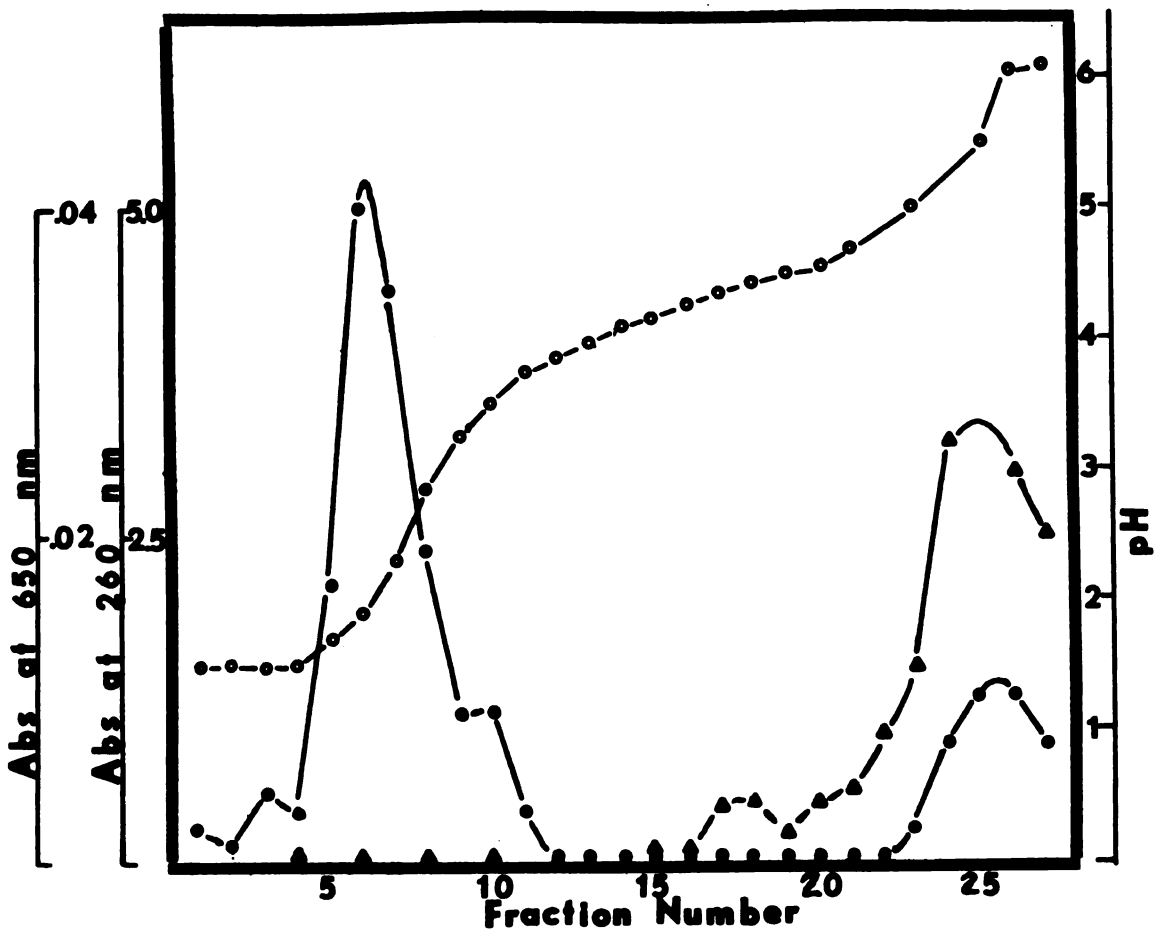
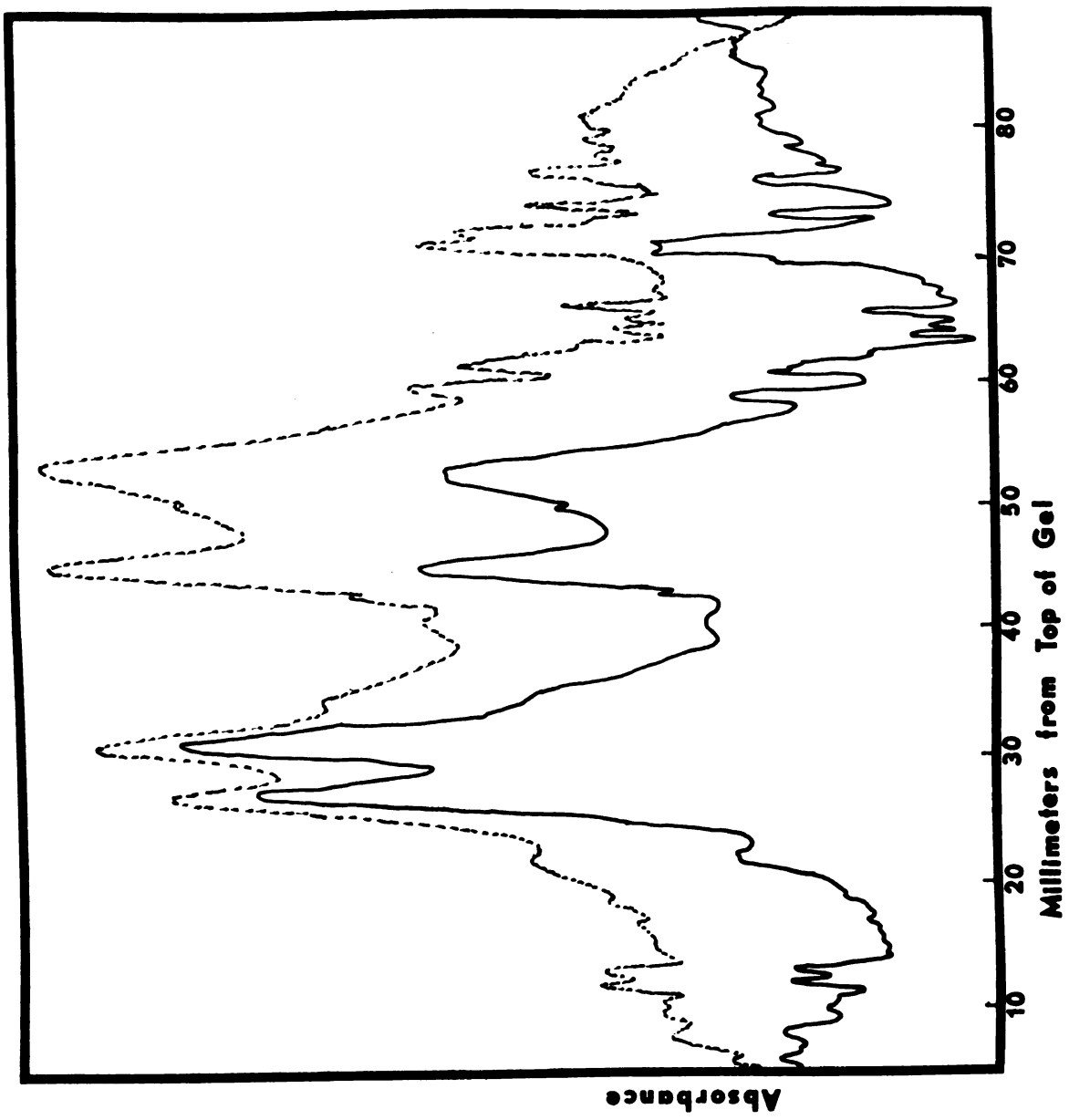

.04
.02
Abs at 650 nm
5.0
2.5
Abs at 260 nm
6
5
4
3
2
1
pH
5
10
15
20
25
Fraction Number

Figure 10: Ultraviolet absorbance scans of a polyacrylamide gel following electrophoresis of Type III enzyme. Polyacrylamide gel electrophoresis was conducted as described in Methods except 750 μgms of protein was layered on the gel after the gel had been pre-electrophoresed for 1.5 hours at 250 V. Scans were performed on a Gilford spectrophotometer equipped with a gel scanner. An absorbance of 0. 89 and 1.307 was dialed into the spectrophotometer to blank the gel at 280 nm and 260 nm respectively and the scans were made with the recorder set to 0-0.1 OD full scale.——— , absorbance at 280 nm;- - - -, absorbance at 260 nm.


Absorbance
10
20
30
40
50
60
70
80
Millimeters from Top of Gel

The mechanisms involved in the hydrolysis of native, double-stranded DNA molecules appear to have particular significance and are described in a separate section.

Exo-and Endonucleolytic Cleavage

Early studies done in this laboratory by D. Hanson (16) indicated that there may be a difference in the mode of cleavage between dDNA and rRNA. Using the gel filtration of reaction products after the method of Birnboim (50) it was shown that dDNA is degraded by wheat seedling nuclease primarily in an endonucleolytic fashion and rRNA hydrolysis more nearly resembled the exonucleolytic mode of cleavage. It was also observed (16) that the presence of Zn^{++} in the reaction mixture was slightly inhibitory but increased the production of mononucleotides during the hydrolysis of rRNA.

Early work in this investigation confirmed the above results. When Zn^{++} was included in the reaction with rRNA as the substrate, the reaction products were even more characteristic of an exoribonuclease when chromatographed on Birnboim columns.

The Birnboim technique is a rough qualitative method for these purposes. In order to define the mode of cleavage in a more quantitative manner the following experiment was done. Polyadenylic acid was incubated at 37° C with wheat seedling nuclease. The reaction contained 0.9 ml poly A (200 OD_{260} units per ml), 8.0 ml 0.05 M

succinate buffer, pH 6 and 0.09 ml wheat seedling nuclease (30 DNase units). At intervals of 10 minutes 0.9 ml aliquots were removed and combined with an equal volume of $La(NO_3)_3$-HCl reagent. Acid-soluble material was determined as described in Methods. Also at ten minute intervals 0.05 ml aliquots were removed and heated at 95° C for 3 minutes. The amount of 5'-AMP in these samples was evaluated by diluting the samples to 1.0 ml with 0.05 M succinate buffer, pH 6 in a 1 cm light path cuvette. After the addition of 5'-AMP amino hydrolase, the decrease in absorbance at 265 nm was measured in a Beckman DB spectrophotometer. 5'-AMP amino hydrolase deaminates only 5'-AMP (no deamination of oligonucleotides) converting it to 5'-IMP (38). The difference in the molar extinctions between adenylic acid and inosinic acid under these conditions is such that a change of 1 OD at 265 nm equals the deamination of 0.125 μmoles per ml of 5'-AMP.

Figure 11 shows the results of the experiment described above. The left hand ordinate indicates the amount of 5'-AMP released and the amount of material (A_{260}) converted to acid-soluble form. The right hand ordinate is calibrated as percent of 5'-AMP and acid-soluble material produced during the reaction with 100% representing complete degradation of poly A to mononucleotides. As can be seen in the case of wheat seedling nuclease digestion of poly A, the production of 5'-AMP and acid-soluble material increases in a parallel fashion as would be expected

Figure 11: Time course for the hydrolysis of polyadenylic acid by wheat seedling nuclease Type II and muskmelon nuclease. The reaction mixture contained 0.9 ml poly A (200 OD units at 260 nm per ml), 8.0 ml 0.05 succinate buffer, pH 6.0, and either 0.09 ml wheat nuclease Type II (30 DNase units of activity) or 0.018 ml muskmelon nuclease. The reaction was carried out at 37° C and 0.9 ml aliquots were removed at the times indicated and combined with 0.9 ml of $La(NO_3)_3$-HCl reagent for the acid-soluble assay. Also at the times indicated 0.05 ml aliquots were removed and heated at 95° C for 3 minutes and then assayed for 5'-AMP as described in the text. Reactions lacking any enzyme were run as a control. ▲———▲ 5'-AMP produced by the action of wheat seedling nuclease; ●———●, acid-soluble material produced by the action of wheat seedling nuclease; o———o, acid-soluble material produced by the action of muskmelon nuclease; Δ———Δ, 5'-AMP produced by the action of muskmelon nuclease.

[5'AMP] μmoles/ml
0.2
1.0
2.0
Abs at 260 nm
0
10
20
0
10
20
30
40
50
60
70
80
Minutes of Incubation
Percent of Products as 5'AMP
20
40
60
80
100
Percent of Products Acid Soluble
20
40
60
80
100

for an exoribonuclease. Theoretically the percentages of 5'-AMP and acid-soluble production should be equal during exonucleolytic degradation until the latter stages of the reaction. During the final stages of the reaction, 5'-AMP production should continue after acid-soluble material has ceased to increase since small oligonucleotides are acid-soluble. As can be noted from the figure, the point in the reaction which has produced 50% acid-soluble products results in 43% production of 5'-AMP and similarly 20% acid-soluble products compares with 19% 5'-AMP. The slight lag in 5'-AMP production can be interpreted as a slight amount of endonucleolytic activity.

Figure 11 also shows the same experiment done with muskmelon nuclease instead of wheat seedling nuclease. Muskmelon nuclease is known to be largely endonucleolytic in its mode of cleavage of RNA (51). As expected for a known endonuclease the mononucleotide production lags substantially behind the formation of acid-soluble products.

The experiment in Figure 11 was repeated for wheat seedling nuclease with 1.0 mM Zn^{++} in the reaction mixture. It was found that Zn^{++} had no effect on the shape of the curves or on the rate of the reaction.

3' Nucleotidase Activity

A number of different nucleotides were tested as substrates for the wheat seedling nuclease's monophosphatase activity and are compared in Table III. The reactions

Table III

Dephosphorylation rates on various nucleotide substrates

Rates were determined with the fixed-time phosphate assay described in Methods. The reaction mixture contained 0.05 ml of 50 mM dithiothreitol, 1 ml of 2 mM nucleotide (dissolved in 0.05 M sodium acetate, pH 5.0), 0.1 ml of wheat seedling nuclease - Type III (diluted to contain 8 DNase units) and 1.35 ml of sodium acetate buffer (0.05 M, pH 5.0).

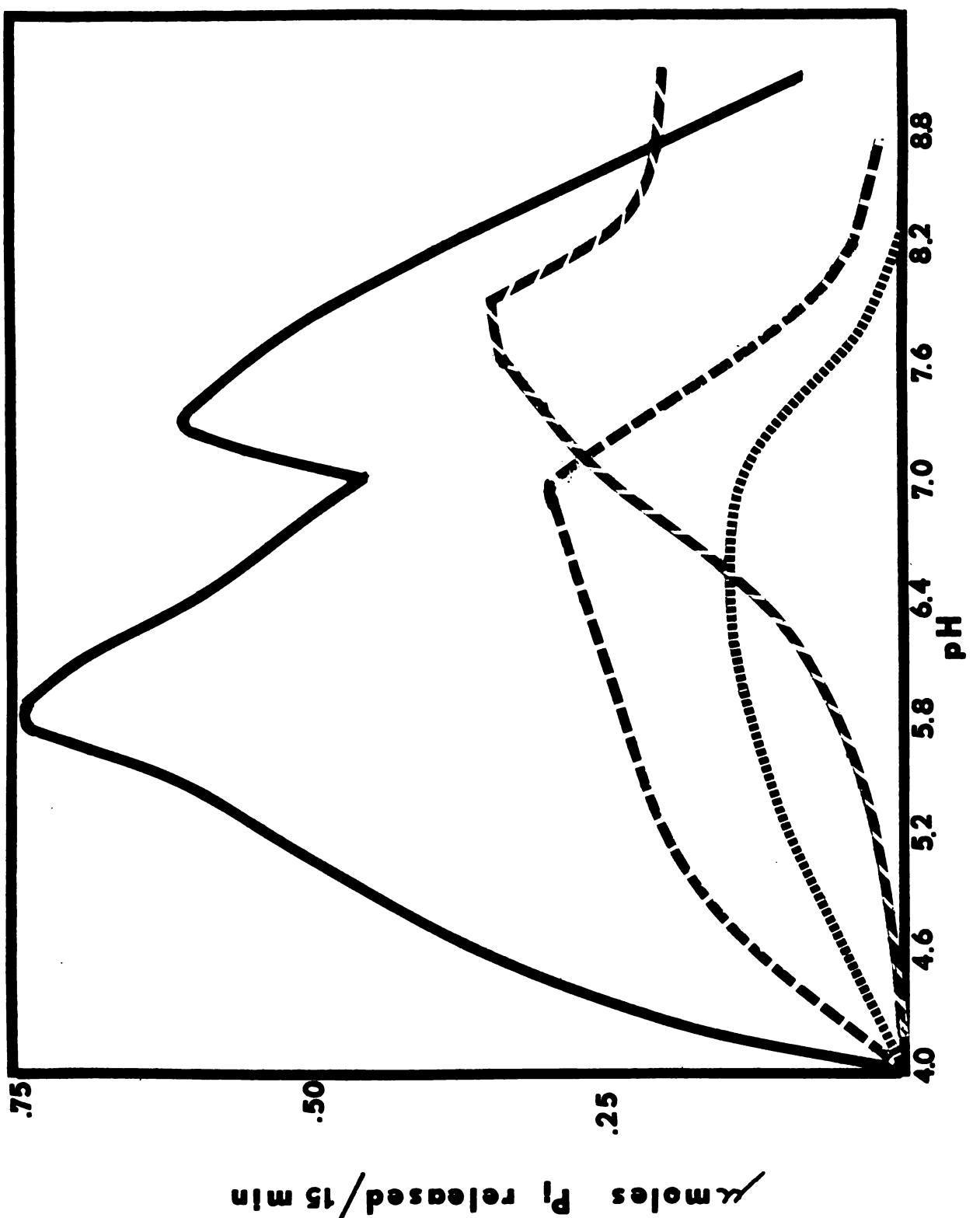
Substrate	Rate[a]	Percent of 3'-AMP rate
	moles Pi/15 min	%
3'-AMP	7.40	100
3'-UMP	3.60	46
3'-CMP	2.10	28
3'-GMP	1.68	23
2'-AMP	0.04	0
2'-UMP	1.94	27
2'-CMP	0.03	0
2'-GMP	0.04	0
3'-dAMP	3.04	41
3'-dTMP	0.51	7
3'-dCMP	0.03	0
3'-dGMP	0.05	1
5'-dXMP	0.05	1
5' -XMP	0.05	1

[a]Rates listed are the average values of 2 determinations. The background subtracted for the minus enzyme control was 0.05.

were carried out at pH 5.0 which is near the pH optimum of the DNase activity. The 3'-ribonucleoside monophosphates were the best substrates and were dephosphorylated in the following order: 3'-AMP > 3'-UMP > 3'-CMP > 3'-GMP. Of the 3'-deoxyribonucleoside monophosphates only 3'-dAMP and 3'-dTMP were dephosphorylated to any extent. These two substrates were hydrolyzed at 41 and 7 percent of the rate of 3'-AMP respectively. The only 2'-ribonucleoside monophosphate which was dephosphorylated was 2'-UMP at a rate 27% that of 3'-AMP. This observed rate could be due to the presence of some 3'-UMP in the commercial preparation of 2'-UMP. None of the 5'-ribo- or deoxyribonucleoside monophosphates served as substrates for the monophosphatase activity. Thus, except for 2'-UMP, 3'-nucleotidase is an appropriate classification for the monophosphatase activity of wheat seedling nuclease.

Figure 12 shows the pH profiles for the nucleotidase activity on the four 3'-ribonucleoside monophosphates. The fixed time phosphate assay described in Methods was performed at 0.3 pH intervals with the following buffers: acetate-pH 4.0-5.2, maleate-pH 5.5-6.7 and tris-HCl-pH 7.0-9.1. When 3'-AMP is used as a substrate, a rather broad pH profile is noted. It is noted that the relative rates of dephosphorylation for the various nucleotide substrates changes with pH. The pH profile of nucleotidase activity when 3'-GMP is used as a substrate shows a fairly sharp optimum at pH 7.9.

Figure 12: pH profiles for the hydrolysis of the 3'-ribonucleoside monophosphates by wheat seedling nuclease. The fixed time phosphate assay described in Methods was used to determine the rate of hydrolysis of the various nucleotide substrates at different pH values by the Type II enzyme. Acetate was used to buffer the reaction between pH 4.0 and 5.2; Maleate was used between pH 5.5 and 6.7; tris-HCl was used between pH 7.0 and 9.1. 3'-AMP, ▬▬▬▬ ; 3'-UMP, ▬ ▬ ▬ ▬ ; 3'-CMP, ▮▮▮▮▮▮▮▮▮▮▮▮▮ ; 3'-GMP, ▰▰▰▰


μmoles Pi released/15 min
.75
.50
.25
4.0
4.6
5.2
5.8
6.4
7.0
7.6
8.2
8.8
pH

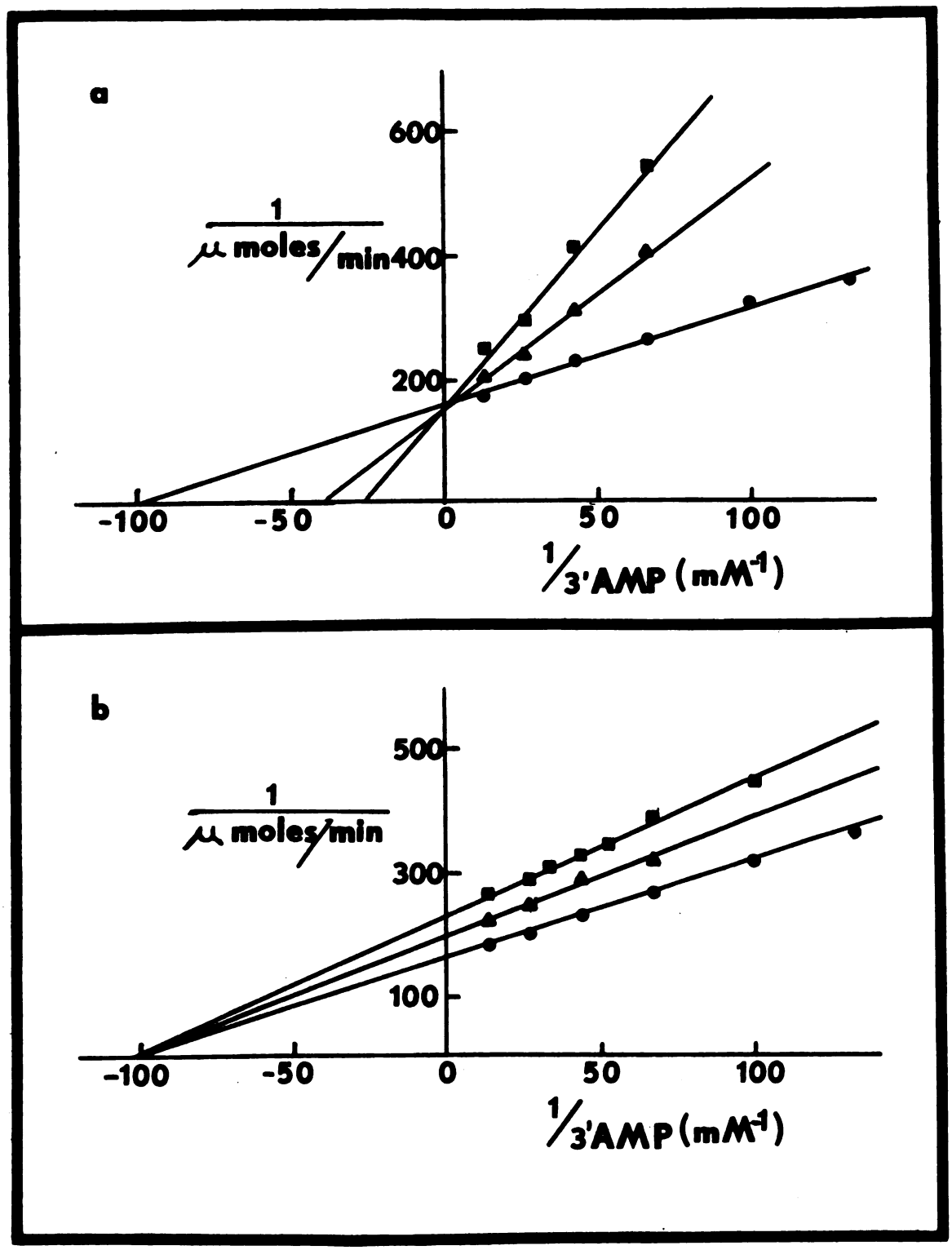
Kinetic Analysis of the Hydrolysis of 3'-AMP and pApA

Kinetic analysis of the 3'-nucleotidase and RNase activities was undertaken in order to study the relationship of these activities in wheat seedling nuclease. A continuous spectrophotometric assay was developed in order to measure the initial rate of dephosphorylation of 3'-AMP. This assay, which is described in Methods, measures the decrease in absorbance at 265 nm produced when 3'-adenylic acid is dephosphorylated to adenosine which is converted to inosine by the coupling enzyme, adenosine deaminase. Reaction mixtures contained 0.05 ml 0.2 M cacodylate buffer, pH 6, 0.01-0.12 ml 3'-AMP (0.75 mM), 0-0.05 ml inhibitor, 0.03 ml adenosine deaminase (1:500 dilution in 0.025 M NH_4Ac, pH 8.0) and enough H_2O to make 1.0 ml. The reaction mixture was placed in a 1 cm light path cuvette and equilibrated at 30° C in a water bath. It was then placed in a Beckman DB spectrophotometer which had 30° C water circulating through the cuvette chamber. The reaction was initiated with the addition of 2.1 DNase units of wheat seedling nuclease and the reaction rate monitored by measuring the decrease in absorbance at 265 nm. Reaction rates were linear for at least the first two minutes and were proportional to enzyme concentration over a 10 fold range.

Figure 13a shows double reciprocal plots of substrate concentration versus rate for the hydrolysis of 3'-AMP with either no 5'-AMP present or 0.016 mM or 0.032 mM

Figure 13a: Lineweaver-Burk plots for the hydrolysis of 3'-AMP by wheat seedling nuclease Type II showing the inhibition by 5'-AMP. Reaction rates were determined at 30° C using the continuous spectrophotometric assay for 3'-AMPase activity described in Methods. Each reaction contained 0.05 ml 0.2 M cacodylate buffer, pH 6.0, 0.01-0.12 ml 3' AMP (0.75 mM), none, 0.025 or 0.05 ml 5' AMP (0.64 mM), 0.03 ml adenosine deaminase (diluted 1:500 with NH_4Ac, 0.025 M pH 8.0) and enough water to make 1.0 ml. The reaction was initiated with 0.00215 ml of wheat seedling nuclease Type II (2.2 DNase units). ●——●, no 5'-AMP; ▲——▲, 0.016 mM 5'-AMP; ■——■, 0.032 mM 5'-AMP.

Figure 13b: Lineweaver-Burk plots for the hydrolysis of 3'-AMP by wheat seedling nuclease Type II showing the inhibition by 2', 3'-cAMP and ApA. The reaction conditions were those described in the caption to Figure 13a except 0.05 ml of 2',3'-cAMP (0.82 mM) and ApA (0.75 mM) were used instead of 5'-AMP. ●——●, no inhibitor; ▲——▲, 0.041 mM 2', 3'-cAMP; ■——■ 0.038 mM ApA.


a
600
400
200
1
μ moles/min
-100
-50
0
50
100
1/3'AMP (mM-1)
b
500
300
100
1
μ moles/min
-100
-50
0
50
100
1/3'AMP (mM-1)

5'-AMP present in the reaction mixture. 5'-AMP is not a substrate for wheat seedling nuclease or adenosine deaminase. The inhibition produced by 5'-AMP is seen to be competitive in nature. The Km and Vmax values for the nucleotidase activity are 1.5×10^{-5} M and 1.3×10^{2} μmoles/min/mg. The K_I value for 5'-AMP inhibition is calculated to be 1.1×10^{-5} M.

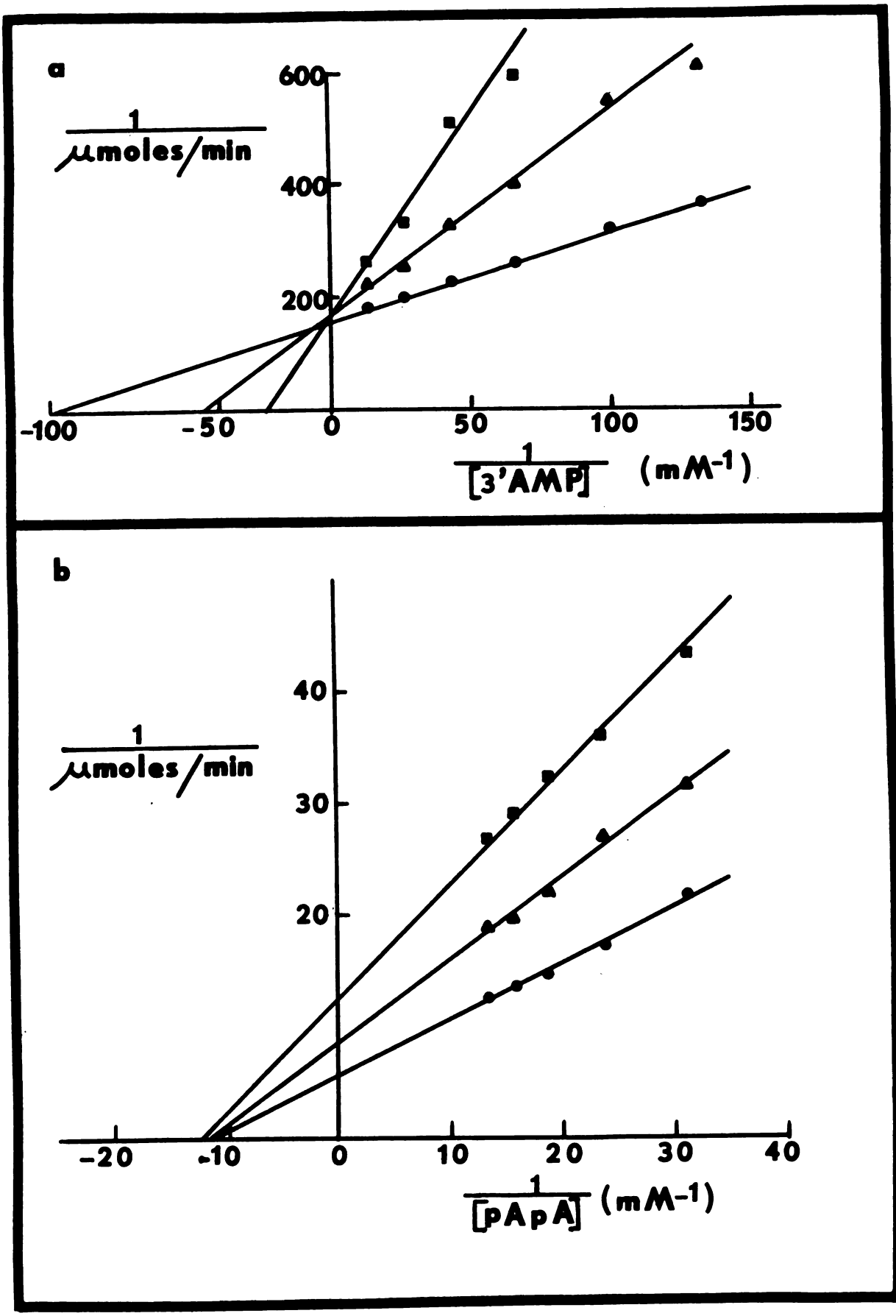
Figure 13b is a double reciprocal plot for hydrolysis of 3'-AMP in the presence of no inhibitor, 0.82 mM 2',3'-cyclic AMP, or 0.75 mM ApA. The inhibition produced by these compounds results in no change in the Km for the reaction which is consistent with a noncompetitive type of inhibition. Both of the inhibitory compounds of Figure 13b contain phosphodiester linkages. The K_I values derived from the inhibition by 2',3'-cAMP and ApA were calculated to be 2.15×10^{-4} M and 1.2×10^{-4} M respectively.

When various salts were included in the reaction mixture, inhibition of the rate of 3'-AMP hydrolysis was observed. Figure 14a shows the result of making the reaction mixture 0.05 M or 0.1 M in NaCl. The Lineweaver-Burk plots reveal a competitive type of inhibition with a K_I for the salt-enzyme complex of 4.5×10^{-2} M.

In order to make kinetic measurements for the RNase activity of wheat seedling nuclease an appropriate substrate had to be found. When complex substrates such as nucleic acid polymers are used, kinetic measurements are difficult to obtain as well as to interpret. Therefore, a simple, well

Figure 14a: Lineweaver-Burk plots for the hydrolysis of 3'-AMP by wheat seedling nuclease Type II showing the inhibition by NaCl. The reaction conditions were the same as those described in the caption to Figure 13a except either 0.05 ml or 0.10 ml NaCl (1 M) was used instead of 5'-AMP. ●——● , no NaCl; ▲——▲, 0.05 M NaCl; ■——■ , 0.1 M NaCl.

Figure 14b: Lineweaver-Burk plots for the hydrolysis of pApA by wheat seedling nuclease Type II showing the inhibition by NaCl. The reaction rates at 30° C were measured as described in the text. Each reaction contained 0.05 ml succinate buffer (0.5 M, pH 6.5) 0.1-0.4 ml pApA (0.21 mM), 0-0.1 ml NaCl (1 M), 0.005 ml 5'-AMP aminohydrolase and water to give a total volume of 1.0 ml. Reactions were initiated with 0.005 ml wheat seedling nuclease Type II (1.2 DNase units) ●——● , no NaCl; ▲——▲, 0.05 M NaCl; ■——■, 0.1 M NaCl.


a
1/μmoles/min
600
400
200
-100
-50
0
50
100
150
1/[3'AMP] (mM-1)
b
1/μmoles/min
40
30
20
-20
-10
0
10
20
30
40
1/[pApA] (mM-1)

defined substrate was sought. The dinucleoside monophosphate ApA was not hydrolyzed by wheat seedling nuclease. The dinucleoside diphosphate, pApA is a good substrate and was made as described in Materials. Rate measurements were obtained by monitoring the decrease in absorbance at 275 nm produced by the cleavage of pApA yielding two molecules of 5'-AMP which were converted to 5'-IMP by the action of the coupling enzyme, 5'-AMP amino hydrolase. This coupling enzyme, like adenosine deaminase, has a high catalytic turnover number and is specific for 5'-AMP (adenine, adenosine, ApA and pApA are not substrates), (38).

Saturating levels of substrate for pApA hydrolysis by wheat seedling nuclease are relatively high. Therefore, in order to measure rates at substrate concentrations near the Km and still remain under an absorbancy of 2 in the spectrophotometer, a wavelength was chosen where the extinction of pApA would be lower than at 265 nm. At 275 nm the molar absorptivity was considerably lower and the difference in extinctions between 5'-AMP and 5'-IMP at this wavelength was such that a difference of 1 OD corresponded to the conversion of 0.43 μmoles/ml of 5'-AMP to 5'-IMP.

Figure 14b shows the Lineweaver-Burk plots for pApA hydrolysis with and without NaCl present. In the absence of NaCl the Km and Vmax are 9.1×10^{-5} M and 1.67×10^{3} μmoles/min/mg respectively. In the presence of NaCl inhibition is seen to be noncompetitive in nature with the

K_I for the NaCl enzyme complex equal to 1.43×10^{-1} M.

To summarize these kinetic results, then, the non-substrate, 5'-AMP, inhibits the nucleotidase activity competitively while the phosphodiester-containing non-substrates, 2',3'-cyclic AMP and ApA, inhibit in a non-competitive manner. Furthermore, NaCl acts as a noncompetitive inhibitor towards the phosphodiesterase activity of wheat seedling nuclease using the RNA substrate, pApA, while acting as a competitive inhibitor towards the 3'-nucleotidase activity. These results are consistent with the supposition that wheat seedling nuclease contains separate active sites (subsites); one site catalyzing the hydrolysis of the phosphomonoester bond of 3'-AMP and the other site catalyzing the hydrolysis of the phosphodiester bond in pApA.

Base Preference at the Cleavage Site

Wheat seedling nuclease does not appear to possess absolute base specificity towards single stranded substrates; i.e., phosphodiester bonds adjacent to the four bases are all hydrolyzed to some extent. Wheat seedling nuclease does, however, have a preference for the base at the cleavage site. In other words, rates at the different sites appear to vary considerably depending on the base present next to the bond being cleaved. For example, if the mononucleotides produced during a digestion of dDNA by wheat seedling nuclease are separated from the other reaction products, the relative content of 5'-dAMP, 5'-dTMP, 5'-CMP

and 5'-dGMP is 100:38:16:8 (16).

As has already been noted from the results in Table III, the rate of dephosphorylation of the various 3'-nucleotides varies in the following order 3'-AMP > 3'-UMP > (3'-TMP) > 3'-CMP > 3'-GMP. In this case the specificity of the 3'-deoxyribonucleoside monophosphates is even more pronounced since 3'-dCMP and 3'-dGMP are not even hydrolyzed at detectable rates.

The same relative order of base preference is observed when the ribohomopolymers are digested with wheat seedling nuclease. Rates of hydrolysis of the various ribohomopolymers were measured at 30° C using the hyperchromic assay described in Methods. The reaction mixture was 0.05 M in acetate, pH 5.0 and contained 0.7 OD_{260} units of each of the polymers. The initial rates of hydrolysis of poly U, poly C and poly G were 53%, 10% and 4% respectively as fast as the rate of poly A hydrolysis by wheat seedling nuclease. It should be pointed out, however, that the amount of secondary structure of the polymers varies considerably under the reaction conditions. Poly G, for example, possesses considerable secondary structure.

The results of the base preference experiments are summarized in Table IV. As can be noted, the same relative order of base preference is present in all of the experiments.

The interaction of poly G with wheat seedling nuclease

Table IV

Base preference at cleavage site

Base	Relative production of mononucleotides from d DNA[a]	Relative rate of 3'-NMP hydrolysis[b]		Relative rate of hydrolysis of ribohomopolymers[c]
		ribo	deoxyribo	
Adenine	1.00	1.00	1.00	1.00
Thymine (Uracil)	.38	.46	.16	.53
Cytosine	.16	.28	.00	.10
Guanine	.08	.23	.00	.04

[a]Data taken from thesis by D. Hanson (16). The hydrolysis of denatured DNA was terminated when 54.5% of the product was in acid soluble form. At this point 5.8% of the products were mononucleotides. The relative distribution of the four nucleotides were normalized so that dpA equals 1.0.

[b]Data taken from Table III. Rates are compared to 3'-AMP equals 1.00 in the "ribo" column and to 3'-dAMP equals 1.00 in the deoxyribo column. 3'-dAMP was hydrolyzed at a rate 41% that of 3'-AMP.

[c]Rates of ribohomopolymers were measured at 30° C. using the hyperchromic assay described in Methods. The reaction mixture was .05 M in acetate, pH 5.0 and contained 0.7 optical density units at 260 nm of each of the polymers. Initial rates were measured after the addition of 2.0 DNase units of Type-II enzyme and normalized to equal 1.0 for polyadenylic acid.

was investigated in more detail. Acid-soluble assays were carried out as described for the DNase assay except poly G was used instead of dDNA. No detectable formation of acid-soluble material occurred even after 30 minutes with 16 DNase units of wheat seedling nuclease. This represents ten fold more enzyme than would be needed to degrade dDNA completely to acid-soluble material in 30 minutes. Likewise no formation of acid-soluble material occurred when the reaction was performed at pH 7.5.

Poly G was found to be an excellent inhibitor of the DNase activity. When 0.03 mg/ml of poly G was included in the standard DNase assay, 50% inhibition resulted.

Poly G was also studied as an inhibitor of the 3'-nucleotidase activity. Using the kinetic system outlined in the previous section, Lineweaver-Burk plots were obtained for 3'-AMP hydrolysis with and without poly G present in the reaction mixture. The rates of the reactions with poly G present produced non linear, concave upward double reciprocal plots indicating a complex inhibitor-enzyme interaction.

Mechanism of Action on Native DNA

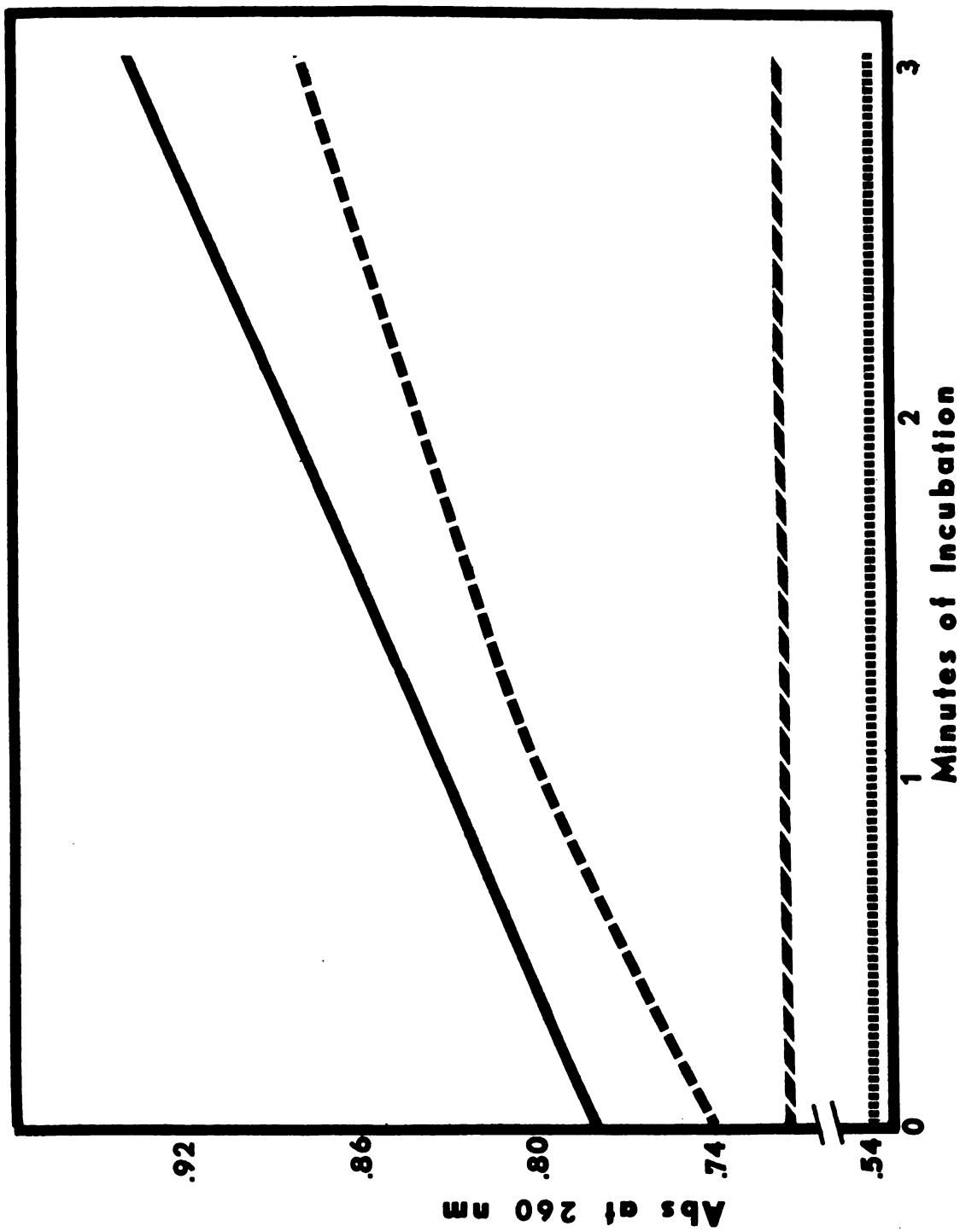
It had been noted early in the investigation of wheat seedling nuclease that single-stranded, denatured DNA was a much better substrate than native, double-stranded DNA. In fact, the rate of formation of acid-soluble

products from native DNA (nDNA) was only a few percent that released from denatured DNA (dDNA) using commercial preparations of salmon sperm and calf thymus DNA. There were several possibilities which might explain the low rate with nDNA. First, the small amount of acid-soluble material released from native DNA could arise from the hydrolysis of small amounts of dDNA or RNA which were endogenous to the commercial DNA preparation. Secondly, a contamination of another nuclease in the wheat seedling nuclease preparation might be responsible for the action on nDNA. A third possibility remained that wheat seedling nuclease possessed the ability to hydrolyze nDNA to a limited extent. In order to investigate these possibilities, a study was conducted on the hydrolysis of native DNA with a highly purified preparation of wheat seedling nuclease.

Hydrolysis of Double-Strand Synthetic Polymers

The alternating copolymeric duplex, poly d(A-T), was compared as a substrate to native and denatured calf thymus DNA using the hyperchromic assay described in Methods. Reactions were carried out at 30° C in 0.04 M cacodylate, pH 5.0. Under these conditions poly d(A-T) was hydrolyzed at a rate comparable to dDNA as can be seen in Figure 15. Under these same conditions nDNA and the synthetic duplex, poly dG-poly dC, showed only a very slight increase in absorbance at 260 nm.

Figure 15: Time course for the hydrolysis of various double-stranded DNA substrates by wheat seedling nuclease Type II. Rates are measured as the increase in absorbance at 260 nm at 30° C as described under "hyperchromic assay' in Methods. Reaction volume was one ml containing 0.04 M cacodylate, pH 5.0, 0.5-0.8 optical density units at 260 nm nucleic acid polymer. Reactions were initiated with the addition of 2.4 DNase units of wheat seedling nuclease Type II. ▬▬▬▬, poly d(A-T); ▬▬▬▮, denatured calf thymus DNA; ▰▰▰▰, native calf thymus DNA; ▮▮▮▮▮▮▮▮▮▮▮▮, poly dG - poly dC.


Abs at 260 nm
.92
.86
.80
.74
.54
0
1
2
3
Minutes of Incubation

The effects of ionic strength and pH on the hydrolysis of poly d(A-T) were looked at in some detail. It was found that the rate of hydrolysis of poly d(A-T) by wheat seedling nuclease increased sharply as the pH was lowered from 7 to 4.5, especially at low salt concentrations. For example, at pH 4.8 in 0.04 M cacodylate buffer, poly d(A-T) was hydrolyzed about 10% faster than dDNA as estimated by the hyperchromic assay. Figure 16 shows the effect of pH and ionic strength on the hydrolysis of poly d(A-T). At pH 5.0 in 0.2 M cacodylate, the rate for poly d(A-T) was only 30% that of dDNA. When the reaction was carried out at pH 6.2 the rates for poly d(A-T) at 0.04 M, 0.1 M and 0.2 M cacodylate were 18%, 8% and 0% respectively when compared to the rate of dDNA hydrolysis. The melting transition temperatures, Tm's, for poly d(A-T) were measured under the various assay conditions and found to be at least 20° C above the 30° C temperature used in the assays.

Figure 17 shows the time course for the hydrolysis of poly rA,U, native gh-1 DNA and denatured gh-1 DNA. In this case the DNA substrate was uniformly labeled with ^{32}P and the RNA synthetic polymer was ^{3}H labeled. Acid-soluble assays were performed as described in the figure caption. As can be seen from the figure, poly rA,U and denatured gh-1 DNA were rapidly converted to acid-soluble form whereas native gh-1 DNA showed no decrease in acid-insoluble material over a period of one hour.

Figure 16: Ionic strength and pH effects on the initial velocities of hydrolysis of poly d(A-T) and dDNA. Poly d(A-T) and denatured calf thymus DNA (0.5 OD_{260} units) were dissolved in 1 ml cacodylate buffer and initial rates were measured at 37° C using the hyperchromic assay described in Methods. Cacodylate buffers were prepared by dissolving the indicated concentration of cacodylate in water and titrating to the indicated pH with NaOH. ●——● , dDNA in .04 M buffer; ▲——▲ dDNA in 0.1 M buffer; ■——■ dDNA in 0.2 M buffer; o- - -o , poly d(A-T) in 0.04 M buffer; Δ- - -Δ , poly d(A-T) in 0.1 M buffer; □- - -□ , poly d(A-T) in 0.2 M buffer.

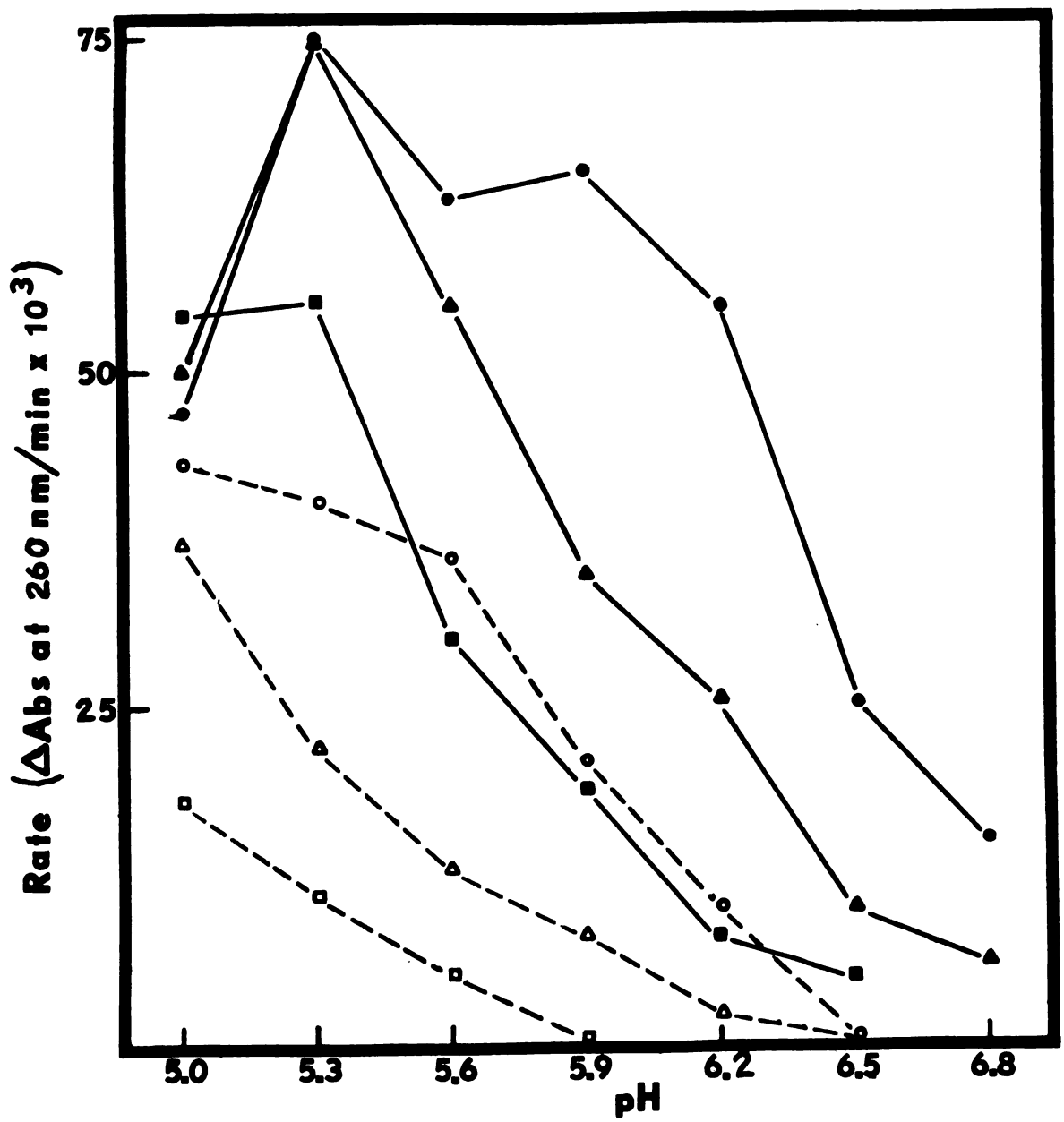
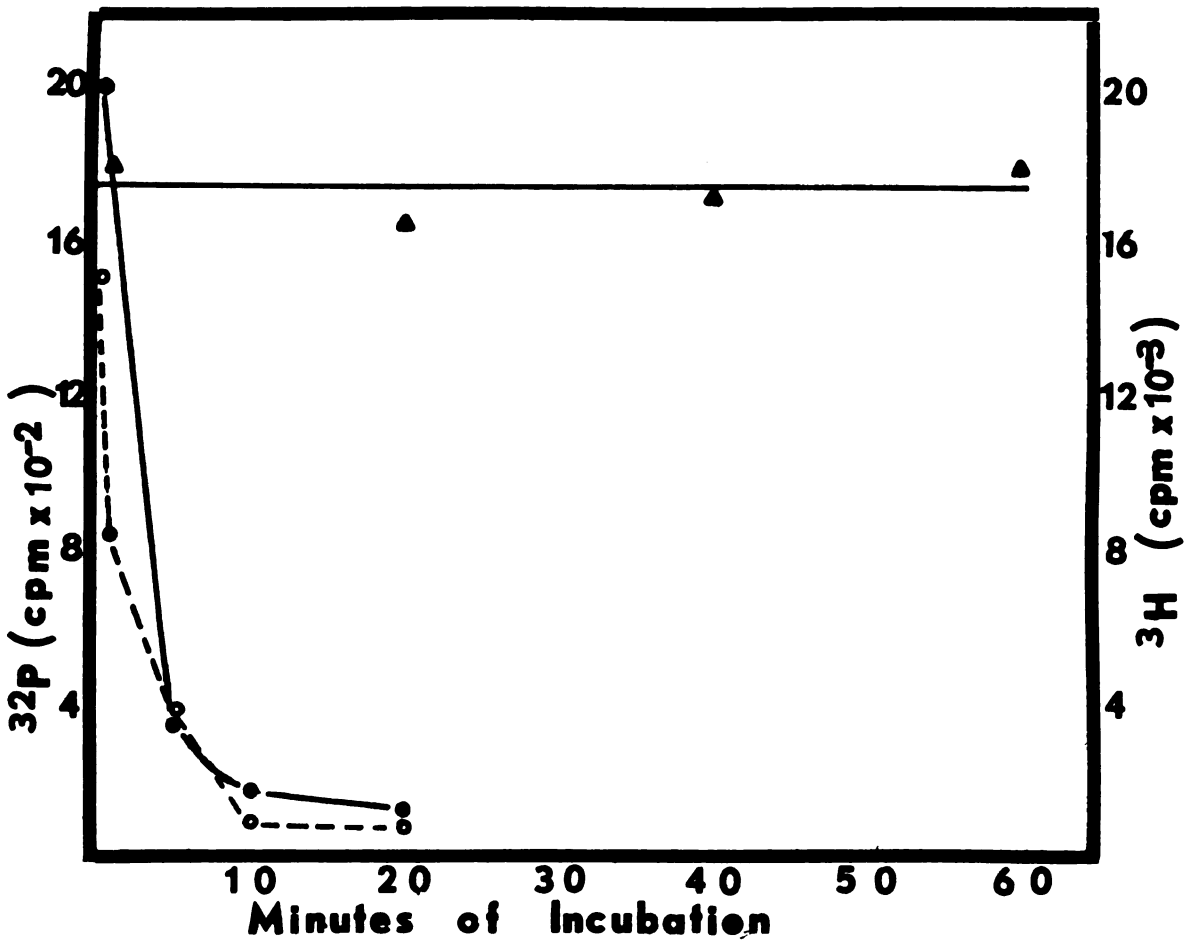

75
50
25
Rate (ΔAbs at 260 nm/min x 10³)
5.0
5.3
5.6
5.9
6.2
6.5
6.8
pH

Figure 17: Time course for the loss of acid insoluble substrate during the hydrolysis of native gh-1 DNA, denatured gh-1 DNA and poly rA,U. The reaction with gh-1 DNA contained 0.150 ml ^{32}P gh-1 DNA (550 μgms/ml, 131 cpm/μgms), 0.06 ml 0.5 M acetate buffer, 0.06 ml 10 mM 2-mercaptoethanol, 0.24 ml H_2O and 0.09 ml Type II enzyme (3 DNase units). The reaction was carried out at 30° C and 0.09 ml aliquots were removed at the times indicated and combined with 5 ml cold TCA. Salmon sperm carrier DNA was added and the precipitate was collected and washed on a filter, dried and counted in scintillation fluid. The denatured DNA was made by heating the gh-1 DNA at 100° C for 10 minutes followed by quickly cooling in ice. The reaction with rA,U was done the same way except that it contained 0.025 ml poly r(A,U) (2 mg/ml and ^{3}H labeled at 3820 cpm per μl). ▲———▲, native gh-1 DNA; ●———● , denatured gh-1 DNA; o---o, poly rA,U.


20
16
12
8
4
32P (cpm x10-2)
3H (cpm x10-3)
10
20
30
40
50
60
Minutes of Incubation

Limit Digests of Native DNA

The action of wheat seedling nuclease on naturally occurring native DNA molecules was investigated next. Commercially available preparations of DNA were not satisfactory for this purpose due to a) possible contamination with denatured DNA, RNA or enzymes and b) the heterogeneity produced by random shearing of the DNA during preparation, shipment and storage. Either high molecular weight E. coli DNA or intact viral DNA molecules were used in the following studies.

The first substrate to be tested was E. coli DNA. A high molecular weight preparation was made as described in Materials. The action of wheat seedling nuclease on native E. coli DNA was monitored by measuring viscosity changes during the time course of the reaction. In order to correlate viscosity with molecular weight of the DNA, an ultra low shear viscometer capable of measuring small volumes of DNA was used.[5] The reaction (2.0 ml) was conducted at 22° C at pH 5.0, and contained 39.4 μgms E. coli DNA, 0.04 mmoles acetate buffer, 0.002 mmoles 2-mercaptoethanol, 0.38 mmoles NaCl. The reaction was initiated by adding 10.5 DNase units of wheat seedling nuclease Type II and 0.35 ml aliquots were placed in the viscometer at the times indicated. Viscosity measurements were obtained and molecular weights calculated as described in Methods.

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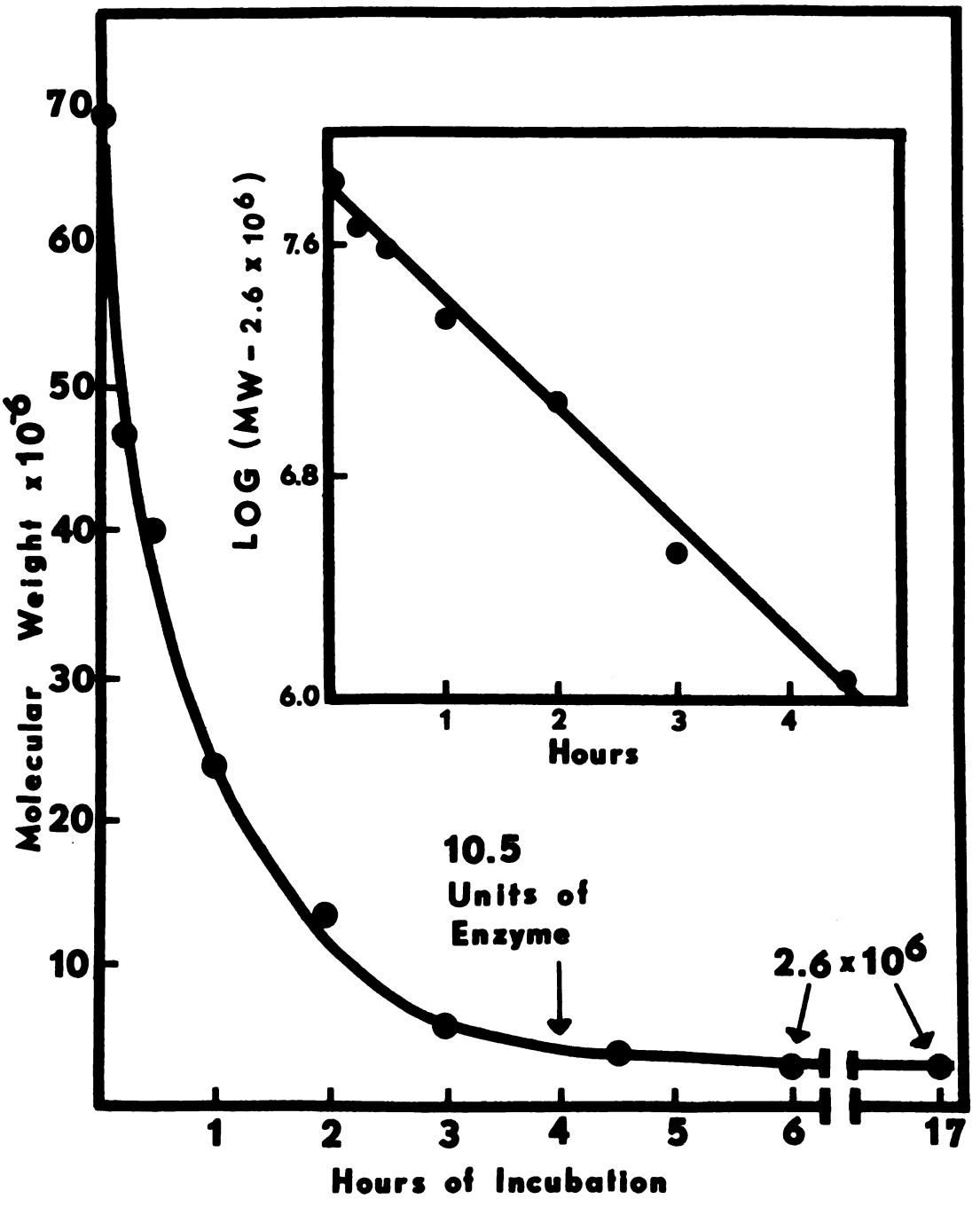
[5]These experiments were conducted at the Veterans Administration Hospital, Research Division, Bedford, Massachusetts.

The time course for this reaction is plotted in Figure 18. The striking feature of this plot is the appearance of a limit DNA polymer (MW = 2.6 x 10^6) which is resistant to further hydrolysis for extended periods of time even after additional enzyme is added. The decrease in molecular weight with time appeared to be an exponential function. When the limit molecular weight was subtracted from the molecular weight of the reaction products during the first 4.5 hours and the logarithms of these molecular weights were plotted versus the time of incubation, a straight line resulted (inset to the figure). The first order kinetics observed suggest that all the cleavage sites are hydrolyzed at approximately equal rates by wheat seedling nuclease.

When the reaction was conducted at pH 6.4 a decrease in molecular weight of about 10 x 10^6 was observed during the first hour. After that the molecular weight remained at 6.0 x 10^6 even after additional enzyme (10.5 units) was added at 4 hours. At pH 8.0 no reduction in molecular weight was observed during the first two hours of incubation.

The interaction of intact linear duplex viral DNA molecules with wheat seedling nuclease was studied next. Figure 19 shows the result of incubating λDNA with wheat seedling nuclease followed by sedimenting the reaction products on an alkaline sucrose gradient. Reaction mixtures of 0.1 ml containing 0.03 mg λ DNA, 5 μmoles acetate

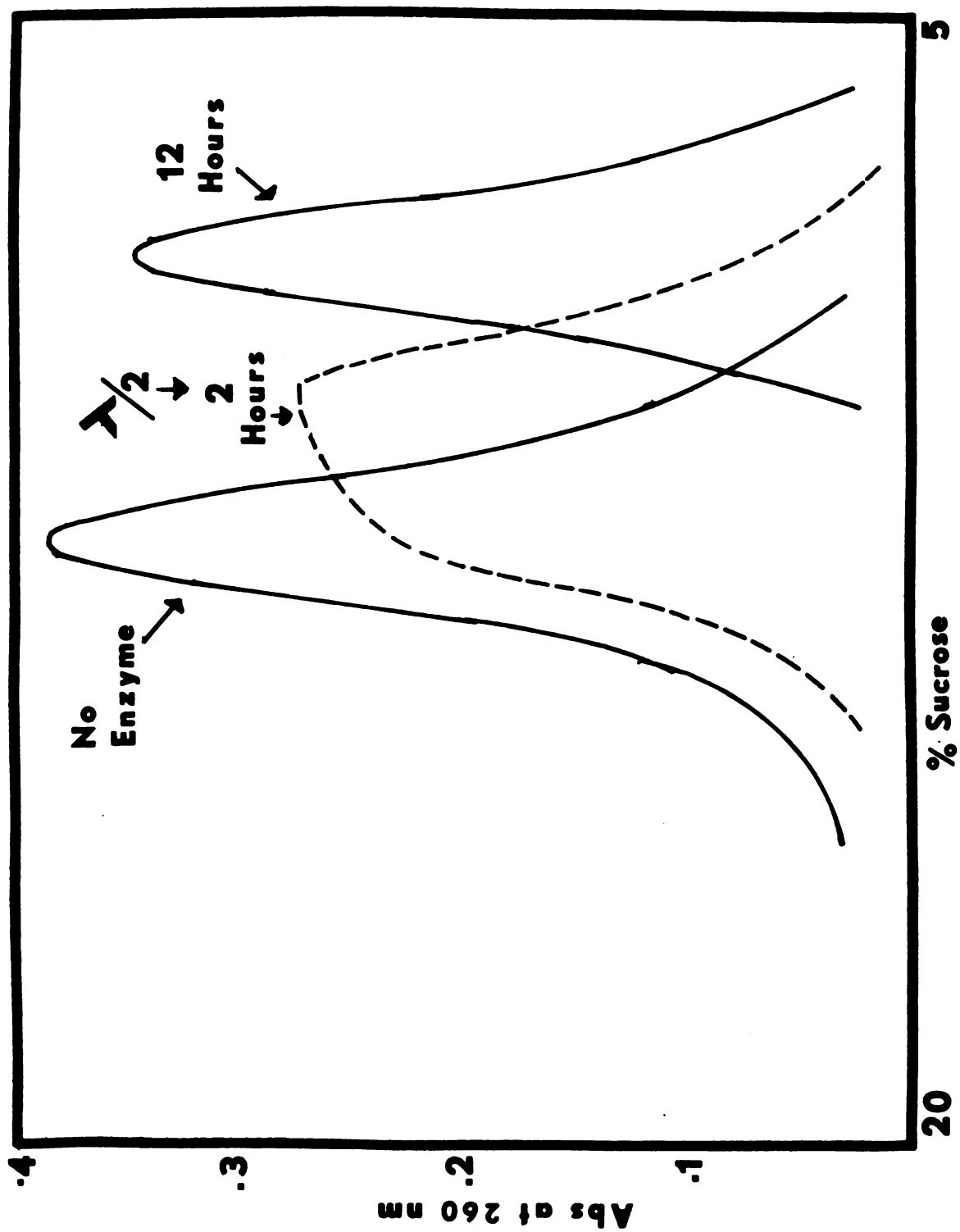
Figure 18: Time course for the decrease in molecular weight during the hydrolysis of native *E. coli* DNA by wheat seedling nuclease Type II. Reaction mixture contained 0.08 ml 0.5 M sodium acetate buffer (pH 5.0), 0.02 ml 0.1 M 2-mercaptoethanol and 1.92 ml *E. coli* DNA (20.5 μgms/ml in 0.2 M NaCl) plus 10.5 DNase units of enzyme. The reaction was carried out at 22° C and an additional 10.5 units of enzyme were added at 4 hours. At the times indicated, 0.35 ml aliquots were removed and placed in the viscometer and viscosities and molecular weights were determined as described in Methods. The inset in the figure is a replot of the same data after subtracting the "limit" molecular weight of the reaction products and taking the logarithm at the various stages of hydrolysis.


70
60
50
40
30
20
10
Molecular Weight x10^6
1
2
3
4
5
6
17
Hours of Incubation
10.5
Units of
Enzyme
2.6 x 10^6
LOG (MW - 2.6 x 10^6)
7.6
6.8
6.0
1
2
3
4
Hours

buffer, 0.1 μmole 2-mercaptoethanol and 1.0 DNase unit wheat seedling nuclease Type II were incubated at 22° C at pH 5.0. The reactions were terminated at the times indicated by the addition of 0.015 ml 1 N NaOH, 0.03 ml 4.5 M NaCl and 0.005 ml H_2O. After standing for half an hour at room temperature, 0.1 ml samples were layered on 5-20% alkaline sucrose gradients and centrifuged as described in Methods. The molecular weight of the DNA from the 12 hour digest was calculated to be 1.05×10^6. This value corresponds to 2.09×10^6 for the weight of the equivalent duplex form. After 2 hours of incubation a large percentage of the reaction products were about half the original size of λDNA, suggesting many early cleavages near the middle of the λDNA molecule. It is interesting to note that λDNA possesses a well characterized A-T rich region near its center.

When λDNA was incubated under conditions described above for 24 hours with an additional 1.0 DNase unit of enzyme added at 18 hours, the molecular weight of the reaction products were still approximately 1×10^6. When a 24 hour digest of λDNA was sedimented in a neutral sucrose gradient, the resulting molecular weight of the digestion products was calculated to be about 2×10^6. Since DNA exists entirely as single-stranded molecules during alkaline sucrose gradient sediemntation, the above result indicates that wheat seedling nuclease produces exclusively double-strand scissions in λDNA.

Figure 19: Alkaline sucrose gradient sedimentation of λ DNA and its reaction products. Reaction mixtures contained 0.06 ml λ DNA (30 μgms), 0.01 ml 0.5 M acetate buffer (pH 5.0), .01 ml 10 mM 2-mercaptoethanol. 0.01 ml H_2O and 0.01 ml wheat seedling nuclease Type II (1.0 unit). Reactions were carried out at 22° C and terminated at the times indicated by the addition of 0.015 ml 1 N NaOH, 0.03 ml 4.5 M NaCl and 0.005 ml H_2O. Sedimentation in alkaline sucrose gradients was performed as described in Methods. The position which half λ molecules would be expected to sediment is indicated on the figure and is calculated by comparing the calculated S value for λ/2 with the S value for whole λ molecules.


Abs at 260 nm
.4
.3
.2
.1
20
5
% Sucrose
No Enzyme
T/2 → 2 Hours
12 Hours

Gh-1 DNA is a linear duplex DNA (MW = 23 x 10^6) containing 57% G + C and 43% A + T (52). A limit digest averaging 3.0 x 10^6 daltons is obtained as indicated by the results of sedimentation in neutral sucrose gradients which are summarized in Figure 20. Additional incubation for 24 hours with an extra 1.0 DNase unit of enzyme added at 18 hours results in no further reduction in molecule size.

Electrophoresis of the limit polymers produced from gh-1 DNA was performed with agarose gels as described in Methods, using ϕ-X174RF DNA as a 3 x 10^6 dalton marker. The limit polymers migrated as a single wide band, the center of which corresponded to the distance moved by the marker. Greater than 90% of the material in the limit polymer band was judged to be within $\pm$ 0.5 x 10^6 daltons of the marker (MW = 3.0 x 10^6).

Table V compares the molecular weights found for various DNA's after hydrolysis with wheat seedling nuclease for 24 hours as described above for λ and gh-1 DNA. T_4 DNA produced the smallest limit polymers (1.8 x 10^6 daltons - duplex equivalent molecule weight). The polymers produced by extensive treatment of gh-1 DNA with wheat seedling nuclease yielded the largest products (3.0 x 10^6 daltons).

Mechanism of gh-1 DNA Hydrolysis

DNA from the bacteriophage gh-1 was chosen as a

Figure 20: Neutral sucrose gradient sedimentation of gh-1 DNA and its reaction products. The reaction mixture contained .05 ml ^{32}P gh-1 DNA (445 μgms/ml, 117 cpm/μgms), 0.015 ml 10 mM 2-mercaptoethanol, 0.045 ml H_2O and 0.025 ml Type II enzyme (0.5 DNase units). The reaction was conducted at 22° C and 0.1 ml aliquots were removed at the times indicated and combined with 0.025 ml 1.5 M tris base. Neutral sucrose gradient sedimentation was accomplished as described in Methods. The S value and molecular weight calibration shown on the figure is based on the sedimentation of gh-1 DNA as a marker (31 S, 23 x 10^6 daltons).

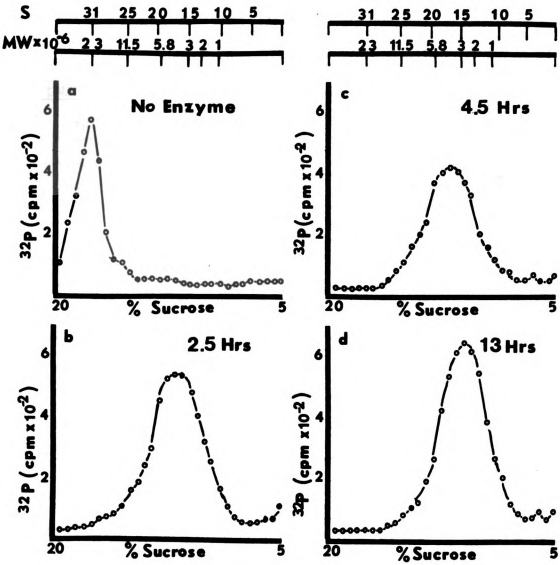

S
31 25 20 15 10 5
MW x10⁻³
2.3 11.5 5.8 3 2 1
a
No Enzyme
c
4.5 Hrs
b
2.5 Hrs
d
13 Hrs
32P (cpm x10⁻²)
% Sucrose
20
5

Table V

Summary of molecular weights of limit digest polymers from various DNA sources

DNA		Molecular weight of limit polymer			
source	molecular weight	single strand	method	double strand	method
E. coli	75×10^6			2.6×10^6	c
T_7	24×10^6	1.2×10^6	a		
T_4	130×10^6	0.9×10^6	a		
λ	31×10^6	1.05×10^6	a	2.0×10^6	d
gh-1	23×10^6	1.7×10^6	a	3.0×10^6	d
		1.49×10^6	b	3.1×10^6	e

a Alkaline sucrose gradient sedimentation

b. ^{32}P end labeling (see Methods and Table VII)

c Viscometric analysis

d Neutral sucrose gradient sedimentation

e Agarose gel electrophoresis

substrate for a more detailed study of the action of wheat seedling nuclease on native DNA.

The production of limit polymers from various intact DNA molecules suggested that only a few specific sites in the DNA molecule were being cleaved. Earlier studies with commercial preparations of DNA indicated that a small amount of acid soluble material (up to several percent as much as that liberated from dDNA) was produced during hydrolysis. This acid-soluble production from commercial DNA's however could arise from a) dDNA, RNA, or enzyme contamination or b) heterogeneous ends of sheared DNA as was pointed out earlier. The viral DNA's used were essentially free of these complications. RNA is not present in the phages and the DNA was shown to have molecular weights equal to the known values for intact DNA in alkaline or neutral sucrose gradients. Therefore this DNA is native, intact and unnicked.

Acid Soluble Assays Acid soluble assays were performed on uniformly labeled ^{32}P gh-1 DNA. Initial studies with Type II enzyme indicated that several percent of the total ^{32}P was converted to acid-soluble material during extensive hydrolysis of gh-1 DNA. This activity was eliminated, however, by adding the P-60 chromatography step to the Type II purification (see "Purification" section of Results). Table VI lists the percentage of ^{32}P distributed between acid-soluble and acid-insoluble products after extensive hydrolysis of ^{32}P gh-1 DNA at

Table VI

Acid soluble material produced during digestion of ^{32}P uniformly labeled gh-1 DNA by wheat seedling nuclease at 22° and 37° C.

Reaction mixtures contained 0.1 ml ^{32}P gh-1 DNA (15 µgms, 3 x 10^3 cpm/µgm) 0.03 ml sodium acetate buffer (0.5 M, pH 5), 0.003 ml 100 mM 2-mercaptoethanol, 0.159 ml H_2O and 0.008 ml Type-II enzyme containing 0.5 DNase units. Reactions were terminated by adding 0.2 ml salmon sperm DNA (1 mg/ml) and 0.5 ml cold $La(NO_3)_3$ - HCl reagent. After standing for 20 minutes at 4° C the samples were centrifuged at 10,000 x g for 20 minutes. Cerenkov radiation of the acid soluble fraction was counted directly and the acid precipitate was dissolved in 0.9 ml H_2O plus 0.1 ml 1 N NaOH. After counting, 0.1 ml of the redissolved acid precipitated fraction was sedimented in an alkaline sucrose gradient for evaluation of the molecular weight as described in Methods.

Temp.	Time	Acid soluble material		Acid precipated material		Molecular weight of digestion products[a]
°C	hours	cpm	%	cpm	%	
22°	18	14	0.04	43,512	99.96	3.2 x 10^6
37°	3	29	0.07	41,822	99.93	2.7 x 10^6

[a]molecular weights determined on single strand polymers, but have been multiplied by 2 and reported as duplex equivalent values

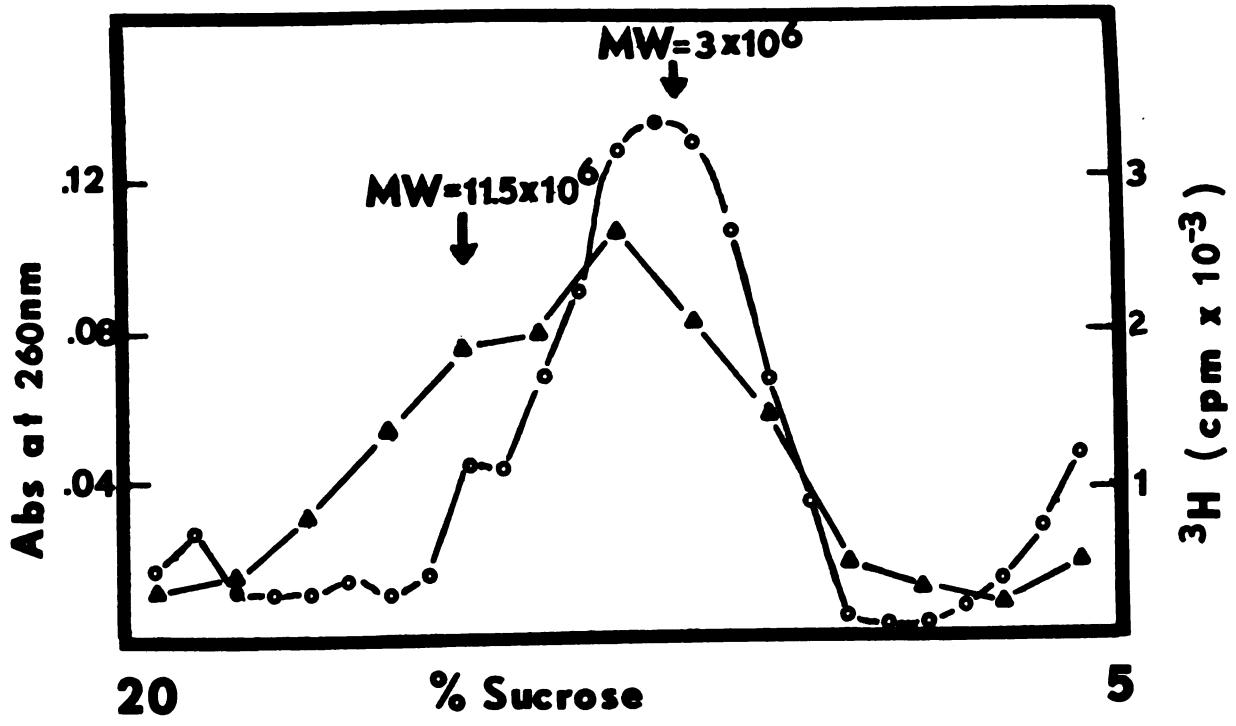
22° C and 37° C. Sucrose gradient analysis indicated that the products had been reduced to "limit" size during the incubation with wheat seedling nuclease. All of the ^{32}P radioactivity in the starting material was recovered as acid-soluble or acid-insoluble material. Only 14 and 29 counts per minute of ^{32}P radioactivity were present in the acid-soluble fraction. These values were based on ten minute counts after subtracting 30 cpm due to background radiation. The background radiation was found to vary between 24 and 40 cpm. Thus the ^{32}P in the acid-soluble fraction represents a negligible amount of radioactivity.

Transcription of gh-1 DNA Limit Polymers Since the size of the average transcriptional unit in gh-1 DNA is thought to be about 3×10^6 daltons[6], the possibility existed that the limit polymers produced by extensive hydrolysis with wheat seedling nuclease were separate transcriptional units. Accordingly, they were examined as templates for the transcribing activity of gh-1 phage-induced DNA-dependent RNA polymerase. Limit polymers of gh-1 DNA hydrolysis were sedimented on a neutral sucrose gradient and the fractions from the gradient were assayed for DNA and polymerase template activity. As can be seen from Figure 21, DNA, throughout the peak served as an acceptable template for transcription.

Material from the three peak fractions (OD_{260}) of the experiment shown in Figure 21 was pooled and dialyzed against 10 mM tris-HCl, pH 8 and 0.05 M NaCl. These

[6]Personal communication from Dr. J. Boezi.

Figure 21: RNA polymerase activity on a limit digest of gh-1 DNA after sedimentation through a neutral sucrose gradient. The limit gh-1 DNA digest was produced by the following reaction: 0.5 ml gh-1 DNA (680 μgms/ml), 1.0 ml 0.1 M acetate buffer, pH 5.0, 0.02 ml 100 mM 2-mercaptoethanol, 0.38 ml H_2O and 10 DNase units of Type II enzyme. The reaction was carried out at 22° C for 20 hours with an additional 10 units of enzyme added at 16 hours. The reaction was terminated with the addition of 0.4 ml of 1.0 M tris base. A 0.1 ml aliquot was sedimented through a neutral sucrose gradient as described in Methods. Aliquots of 5 μl from every second fraction were used as substrate for the gh-1 phage-induced DNA dependent RNA polymerase reaction described in Methods. Molecular weights shown in the figure were calculated using gh-1 DNA as a marker (31 S, 23 x 10^6 daltons). o——o, gh-1 DNA limit polymer (absorbance at 260 nm); ▲——▲, RNA polymerase activity (^{3}H-CTP incorporated).


MW=3x10^6
MW=11.5x10^6
.12
.08
.04
Abs at 260nm
3
2
1
3H (cpm x 10^-3)
20
% Sucrose
5

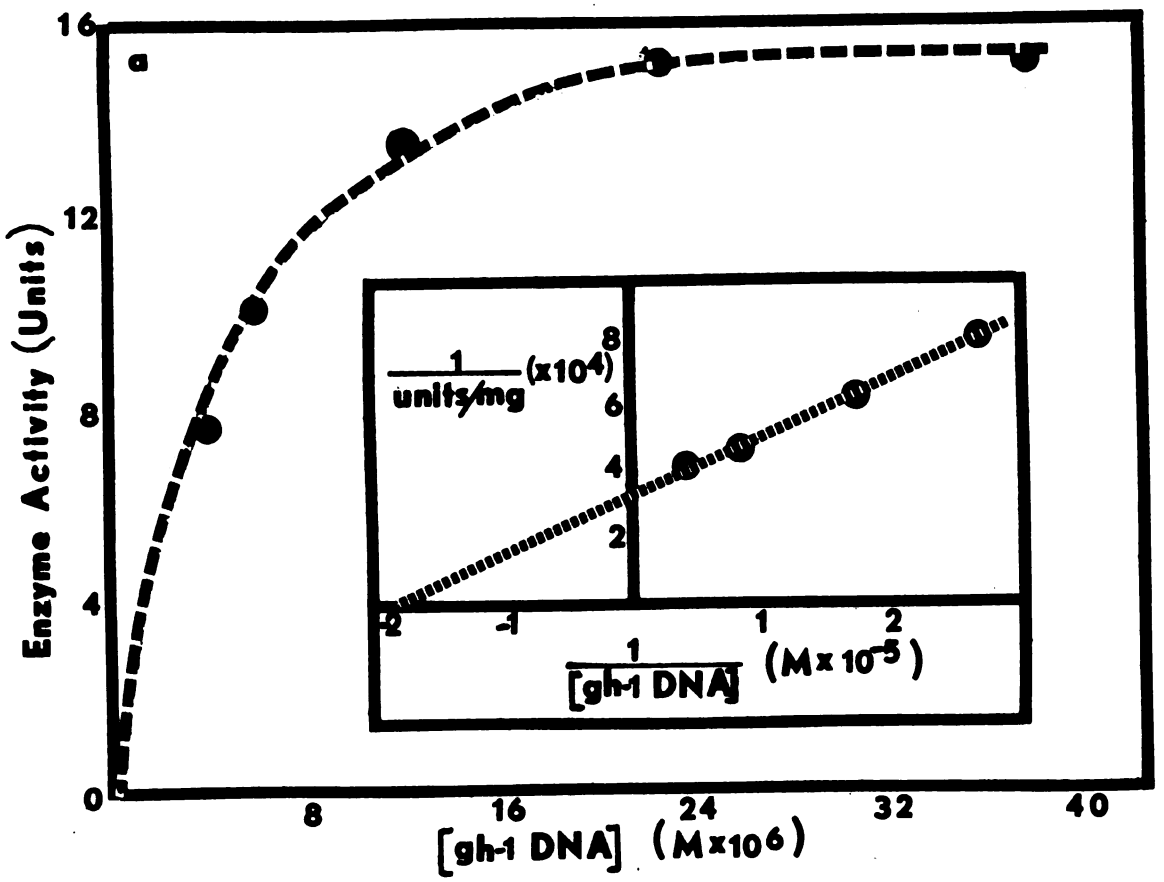
polymers (gh-1 DNA pieces), as well as whole gh-1 DNA molecules, were used as substrates to study the kinetics of the transcription reaction. Figure 22 shows the substrate-saturation curves as well as the double reciprocal substrate versus rate plots when these two templates were used. When the gh-1 DNA pieces were used as a template the Km = 2.78×10^{-6} M and the Vmax = 1×10^{3} units/mg. On the other band, using whole gh-1 DNA molecules as templates, the Km = 5.56×10^{-6} M and the Vmax = 3.17×10^{3} units/mg. Thus the maximum rate of transcription for the gh-1 DNA pieces is almost one third that for whole gh-1 DNA molecules.

Buoyant Density Banding of gh-1 DNA Limit Polymers

Given the fact that gh-1 DNA is 23×10^{6} daltons and the limit polymers are all approximately 3×10^{6} daltons, an average of about 7-8 DNA pieces are produced from each gh-1 DNA molecule by the action of wheat seedling nuclease. In order to ascertain whether any of these DNA pieces contained abnormal guanylic plus cytidylic acid content, CsCl gradients were prepared and the DNA was banded during centrifugation as described in Methods. The results are shown in Figure 23. The DNA pieces all banded at fractions with the same refractive index (and therefore at the same buoyant density) as the whole gh-1 DNA molecules. Thus the average G + C content of the limit polymers were all about the same as that of the whole gh-1 DNA molecule (57% G + C).

Figure 22a: Substrate saturation and Lineweaver-Burk plots for the transcription of gh-1 DNA by gh-1 phage-induced DNA dependent RNA polymerase. Assays for polymerase activity were conducted as described in Methods except that reaction volumes were 0.5 ml and 0.125 ml aliquots were withdrawn and assayed at 5 minute intervals. In all cases reaction rates were linear for the first 15 minutes. One unit of polymerase activity equals 1 nmole ^{3}H-CTP made acid insoluble per 10 minutes. The concentration of DNA is reported as the molarity of nucleotide equivalents and determined by measuring absorbance at 260 nm (1 mg/ml = 20 OD_{260}).

Figure 22b: Substrate saturation and Lineweaver-Burk plots for the transcription of gh-1 DNA limit polymers by gh-1 phage induced DNA dependent RNA polymerase. Limit polymers of gh-1 DNA produced by the action of wheat seedling nuclease (see Figure 21) were sedimented on neutral sucrose gradients and the fractions corresponding to material with a molecular weight of 3 x 10^{6} were pooled and dialyzed against 10 mM tris-HCl, pH 8.0 and 0.05 M NaCl. Using this material as substrate, kinetic measurements were made as described in Figure 22a.


a
Enzyme Activity (Units)
16
12
8
4
0
8
16
24
32
40
[gh-1 DNA] (Mx10⁶)
1/units/mg (x10⁴)
8
6
4
2
-2
-1
1
2
1/[gh-1 DNA] (Mx10⁻⁵)

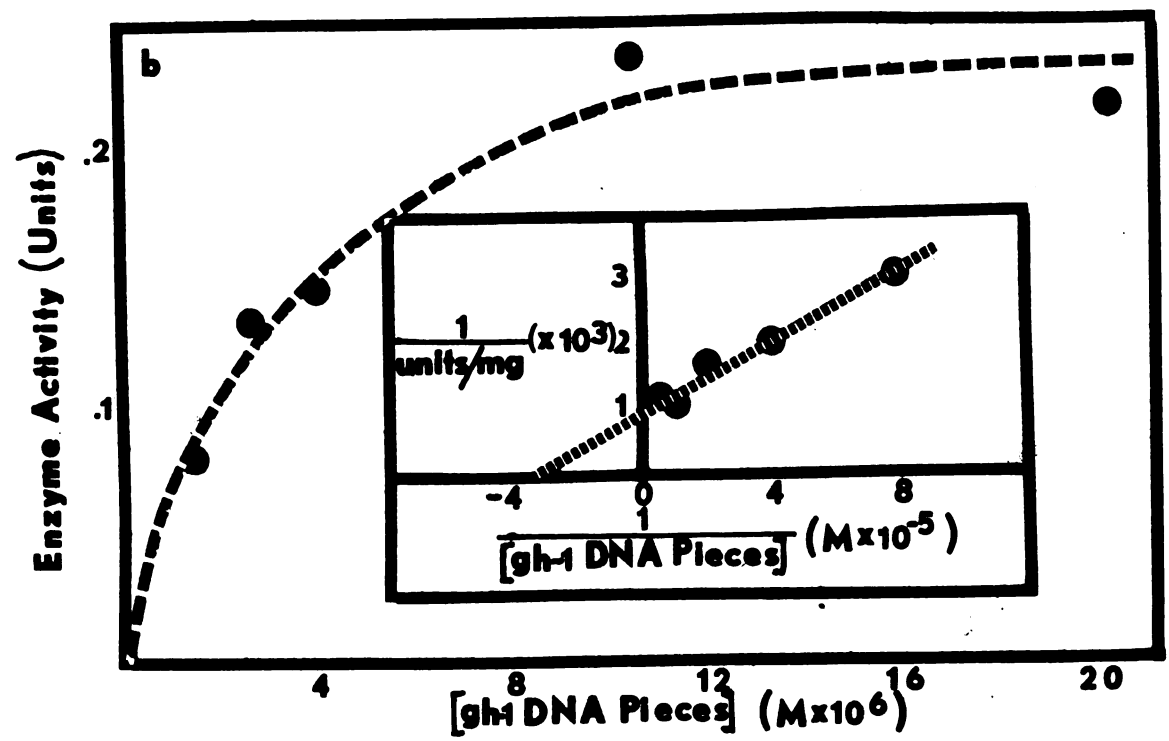
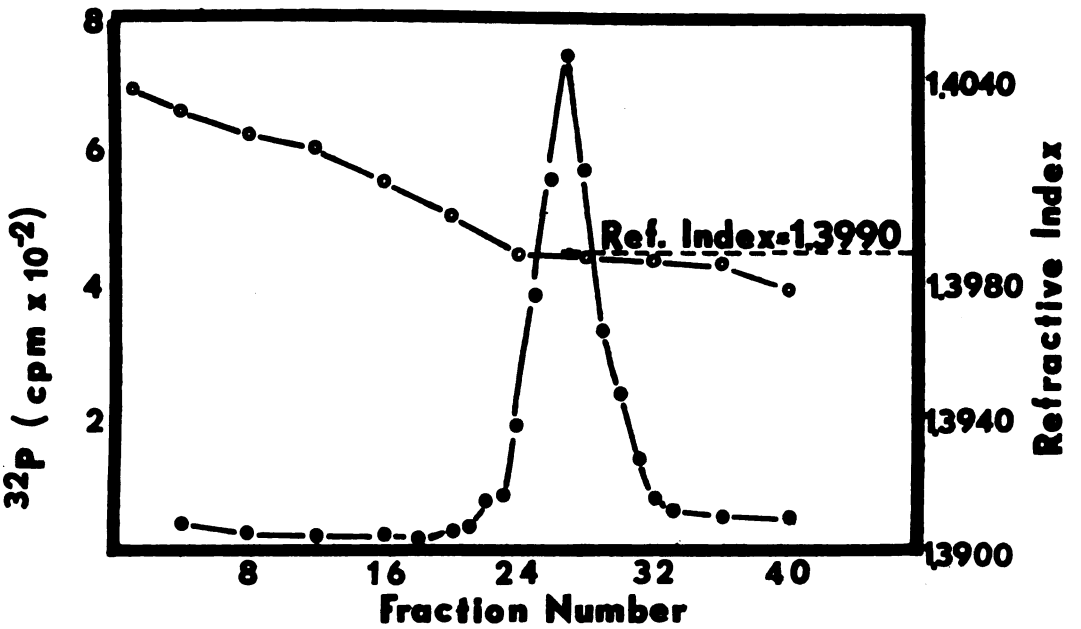
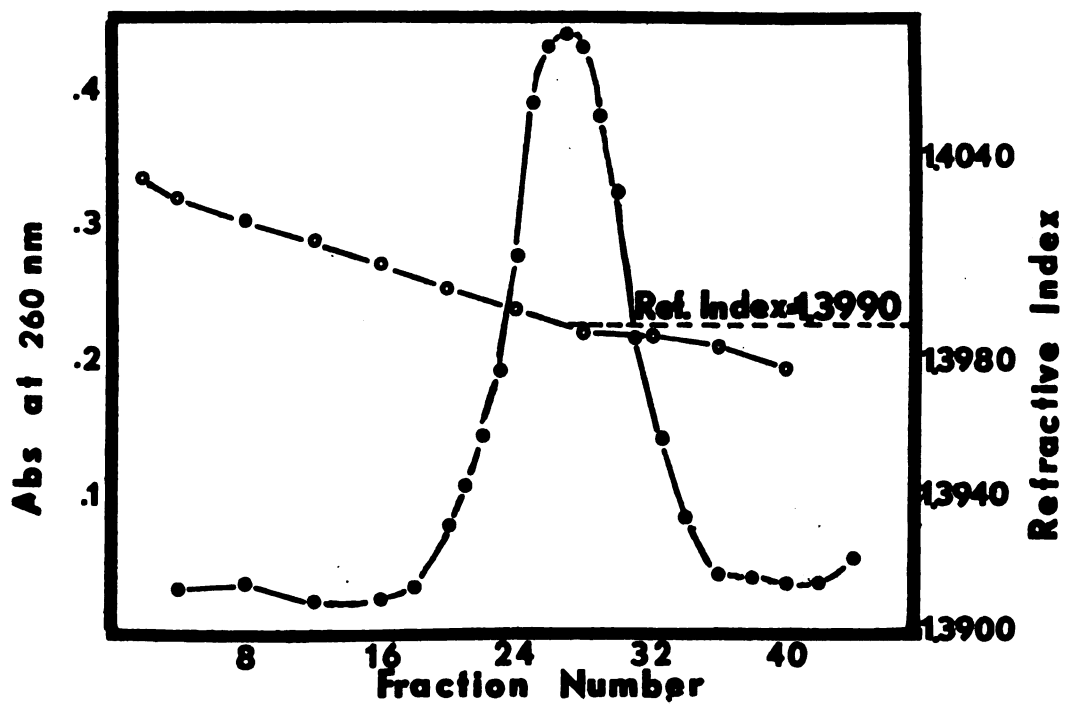

b
Enzyme Activity (Units)
.2
.1
4
8
12
16
20
[gh-1 DNA Pieces] (Mx10⁶)
1/units/mg (x10⁻³)₂
3
1
-4
0
4
8
1/[gh-1 DNA Pieces] (Mx10⁻⁵)

Figure 23a: Buoyant density banding of gh-1 DNA in a CsCl gradient. ^{32}P labeled gh-1 DNA (30 μgms) was layered on top of a CsCl solution and banded as described in Methods.

Figure 23b: Buoyant density banding of gh-1 DNA limit polymers in a CsCl gradient. The limit polymers were made as described in Figure 21. After layering 30 μgms on top of a CsCl solution, banding was accomplished as described in Methods.


8
6
4
2
^{32}P (cpm x 10^{-2})
Ref. Index=1.3990
1.4040
1.3980
1.3940
1.3900
Refractive Index
8
16
24
32
40
Fraction Number


.4
.3
.2
.1
Abs at 260 nm
Ref. Index=1.3990
1.4040
1.3980
1.3940
1.3900
Refractive Index
8
16
24
32
40
Fraction Number

Nucleotide composition at 3' end of gh-1 DNA limit polymers In order to determine the nucleotide composition near the cleavage site in gh-1 DNA, a small number of nucleotides were cleaved from the 3' termini of the gh-1 DNA limit polymers by the action of venom phosphodiesterase. The reaction contained 0.1 ml ^{32}P gh-1 limit polymers (20 μgms/ml, 1.05 x 10^4 cpm/μgms), 0.06 ml 0.7 M glycine buffer, pH 9.2, 0.03 ml 0.3 M $MgCl_2$ and 0.11 ml H_2O. Venom phosphodiesterase was added (0.01 ml which had previously been treated to remove trace amounts of 5'-AMPase activity as described in Methods) and the mixture was incubated at 22° C for 15 minutes. The reaction was terminated by adding 0.2 ml salmon sperm DNA (1 mg/ml) and 0.5 ml cold 10% TCA. After standing in ice for 20 minutes the precipitate was collected by centrifugation at 20,000 x g for 20 minutes. The supernatant was counted directly as Cerenkov radiation and the precipitate was redissolved in 0.01 N NaOH and counted. The acid-soluble portion contained 584 cpm of ^{32}P and the acid-insoluble portion contained 20,752 cpm. This represents the conversion of 2.6% of the gh-1 DNA limit polymer to acid-soluble material. The limit gh-1 DNA polymers have an average molecular weight of 3 x 10^6. This corresponds to 9.1 x 10^3 nucleotides per limit polymer or a chain length of 4.55 x 10^3 nucleotide pairs. Venom phosphodiesterase removes nucleotides progressively from the 3' terminus of DNA. Therefore 2.6% conversion of the gh-1 DNA limit polymers to acid-soluble material represents the cleavage of 118 nucleo-

tides on the average from both 3' termini. To these nucleotides contained in the acid soluble supernatant was added 0.2 ml 0.7 M glycine buffer, pH 9.2 and the mixture was titrated to pH 9 with 1 N NaOH. The four deoxynucleotides (0.05 ml each 10 mg/ml pdC, pdA, pdG, pdC) were added and the nucleotides were separated on a Dowex column as described in Methods. Table VII compares the ^{32}P radioactivity eluting in each nucleotide peak on a percentage basis. The nucleotides released from the exonuclease action are 46% A + T and 54% G + C. For comparison the same experiment was done on whole gh-1 DNA. In this case the G + C mole percent equals 57% and A + T equals 43% which agrees with that determined by other methods for gh-1 DNA (52). Thus the area representing an average of 118 nucleotides from the cleavage site is not greatly different in nucleotide composition as compared to the entire gh-1 DNA molecule although a slight enrichment of A + T was found.

^{32}P 5' End Labeling of gh-1 DNA Limit Polymers The production of limit polymers from native DNA by wheat seedling nuclease yielding less than 0.1% acid soluble material suggests a specific, well defined site of cleavage on the DNA molecule. In order to learn more about this specificity, polynucleotide kinase was used to label the 5' termini of the limit gh-1 DNA polymers with ^{32}P. Polynucleotide kinase catalyzes the reaction which transfers the γ phosphate of ATP to the 5' OH end of a polynucleotide chain. Thus by using ATP labeled in the γ

Table VII

Nucleotide composition at the 3' end of uniformly ^{32}P labeled gh-1 DNA limit polymers

Acid soluble material representing 2.6% of the total ^{32}P counts in the limit polymers was produced by the limited action of venom phosphodiesterase (see text for details). This material containing the nucleotides released by the venom exonuclease was applied to a dowex column and the nucleotides were separated as described in Methods. For comparison, uniformly ^{32}P labeled gh-1 DNA was reduced completely to nucleotides by the combined action of DNase I and venom phosphodiesterase and the individual nucleotides separated as described in Methods.

Nucleotide	Acid soluble fraction		gh-1 DNA	
	cpm	%	cpm	%
pdC	123	27	835	27
pdA	123	26	614	20
pdG	127	27	941	30
pdT	98	20	751	23
pdA = pdT		46		43
pdG = pdC		54		57

position with ^{32}P it is possible to label the 5' terminus of a polynucleotide chain, providing there is a 5' OH group present at the terminus.

The limit gh-1 DNA polymers were made in the following manner. A reaction mixture of 1.0 ml at pH 5 containing gh-1 DNA (354 μgms), 1 μmole 2-mercaptoethanol, 0.05 mmoles acetate buffer and 10 DNase units of wheat seedling nuclease was incubated at 22° C for 18 hours. An additional 10 units of enzyme were added and the incubation was continued for 2 more hours. SDS (0.05 ml, 10%) was added and the solution was incubated at 60° C for 5 minutes. After two phenol extractions, the aqueous phase was dialyzed for 3 days against 3 changes of 50 mM KCl and 0.01 M tris-HCl, pH 8.0.

The limit polymers as well as whole gh-1 DNA molecules were labeled with ^{32}P by the following procedure. DNA (0.1 ml 295 μgms/ml gh-1 DNA or 0.1 ml 280 μgms/ml gh-1 DNA limit polymer) was denatured by adding 0.11 ml 1.0 M NaOH and the solutions were kept at room temperature for 5 minutes. Next, 0.11 ml of 1.0 M tris-HCl, pH 7.8 was added. After adding 0.005 ml alkaline phosphatase (containing 0.037 units and prepared as described in Methods) the reaction was allowed to proceed at 37° C for 30 minutes. An additional 0.005 ml of alkaline phosphatase was added and the incubation continued for 30 minutes. This procedure was necessary to remove the phosphate group at the 5' terminus of the limit polymer leaving a

5' OH group to react with ^{32}P-ATP. After adding 0.28 ml H_2O the reaction mixtures were phenol-extracted twice with equal volumes of water-saturated, freshly distilled phenol. The aqueous phases were then dialyzed for 48 hours against 4 changes of 0.01 M $MgCl_2$ and 0.07 M tris-HCl, pH 7.6. To each sample was then added 0.05 ml 50 mM dithiothreitol and 0.01 ml ^{32}P-ATP (1.27 μmoles/ml, 2.38 x 10^8 cpm/μmole). The reaction was started by adding 0.02 ml of polynucleotide kinase (5 units). The reaction mixtures were incubated at 37° C for 90 minutes with an additional 0.02 ml of polynucleotide kinase added at 30 and 60 minutes. The samples were then dialyzed against 0.5 M NaCl and 0.03 M tris-HCl, pH 7.6 for 48 hours with 3 changes of buffer. They were then dialyzed against 2 changes of 0.01 M $MgCl_2$ and 0.02 M tris-HCl, pH 7.6, over a 24 hour period. At this point all of the ^{32}P had been dialyzed away from the samples which had no polynucleotide kinase treatment.

The samples were removed from the dialysis tubing and counted directly by measuring the Cerenkov radiation. The results are shown in Table VIII. The controls lacking phosphatase treatment indicate that the gh-1 DNA contained a significant number of free 5'-OH groups, a number not increased by prior treatment with alkaline phosphatase. However, treatment of the limit polymers with phosphatese markedly increased the number of 5'-OH groups capable of undergoing the polynucleotide kinase reaction. The high value encountered in the case of the minus-phosphatase control may reflect the presence of some phosphatase activity during the kinase reaction.

Table VIII

^{32}P incorporation into gh-1 DNA and gh-1 DNA limit polymers by polynucleotide kinase

The method used for end labeling with polynucleotide kinase is described in detail in the text. All samples were carried through the alkaline phosphatase and polynucleotide kinase treatments except the specified enzyme was left out where noted. The final reaction mixture was dialyzed until only incorporated counts remained inside the dialysis bag. This material was counted directly using Cerenkov radiation.

DNA	Alkaline phosphatase treatment	Polynucleotide kinase treatment	^{32}P incorporated (cpm)
gh-1 DNA	x	-	18
gh-1 DNA	-	x	610
gh-1 DNA	x	x	595
limit polymer	x	-	17
limit polymer	-	x	1989
limit polymer	x	x	4421

Since each labeled DNA molecule contains only one ^{32}P atom and the amount of DNA in each reaction is known as well as the specific activity of the ^{32}P-ATP, the number of nucleotides in each DNA molecule and hence molecular weight of the DNA can be calculated. This number-average molecular weight calculation (see appendix for actual calculations) gave a value of 1.49×10^6 for the gh-1 DNA limit polymers and 1.2×10^7 for the whole gh-1 DNA molecules. These values are for denatured single stranded molecules and therefore must be multiplied by 2 to compare with duplex equivalent molecular weights. The known molecular weight for denatured gh-1 DNA is 1.25×10^7. The value for the limit polymers also agrees closely with the values obtained by other methods (see Table V).

The identity of the 5' terminally labeled nucleotides in gh-1 DNA and gh-1 DNA limit polymers was determined as follows. The DNA was reduced to 5' mononucleotides by the successive action of DNase I and venom phosphodiesterase. To the samples in Table VIII which had been treated with phosphatase and kinase, was added 0.1 mg of pancreatic DNase (DNase I). They were incubated at 37° C for 30 minutes. Then, 0.1 ml 0.7 M glycine buffer, pH 9.2 was added followed by 0.4 units of venom phosphodiesterase (treated as described in Materials to eliminate trace amounts of 5' AMPase activity). The reaction was carried out at 37° C for 30 minutes after which 0.05 ml of each of the deoxyribonucleotides (10 mg/ml) was added. The mixture was diluted to 20 ml with H_2O and chromatographed

on a Dowex column as described in Methods.

Figure 24 shows the distribution of ^{32}P in the nucleotide peaks eluting from the column. The radioactivity was distributed among the unlabeled nucleotide markers with the percentages listed in Table IX. The 4% dpA and 5% dpT values are high since some A and T residues were found to be present at the termini of whole gh-1 DNA molecules. After correcting for the presence of these nucleotides the composition at the 5' ends of gh-1 DNA limit polymers produced by the action of wheat seedling nuclease is 48% dpC and 52% dpG.

Effect of Temperature on λDNA Hydrolysis

A large increase in the rate of hydrolysis of nDNA with moderate increases in temperature had been observed in earlier studies. In order to quantitate this effect, the extent of hydrolysis of λDNA by wheat seedling nuclease at 20° C and 30° C was investigated. Reactions containing 0.05 ml λDNA (30 μgms), 0.01 ml 0.5 M NaAc, pH 5.0, 0.035 ml H_2O and 1 DNase unit of wheat seedling nuclease Type II were incubated at either 20° or 30° C for one hour. Reactions were terminated by adding 0.015 ml 1 N NaOH and 0.03 ml 4.5 NaCl. The samples were then sedimented on alkaline sucrose gradients as described in Methods. The average duplex equivalent molecular weight of the products from the 20° C incubation was 15 x 10^6. The 30° C reaction produced products with an average duplex equivalent mole-

Figure 24: Dowex-1 separation of nucleotides produced by degrading gh-1 DNA limit polymers, which had been labeled with ^{32}P at the 5' termini, to the mononucleotide level. Limit polymers of gh-1 DNA were labeled with ^{32}P at their 5' termini by polynucleotide kinase and degraded completely to mononucleotides by the successive action of DNase I and venom phosphodiesterase as described in the text. Unlabeled 5' dNMP's (0.05 ml of each nucleotide, 10 mg/ml) were added and the entire mixture was diluted to 20 ml and separated on a Dowex-1 column as described in Methods. Approximately 100% of the applied ^{32}P radioactivity was recovered from the column and the percentages of radioactivity associated with the unlabeled nucleotide peaks are displayed in Table IX. o——o , absorbance at 260 nm of unlabeled nucleotides; ——— radioactivity from ^{32}P labeled nucleotides.

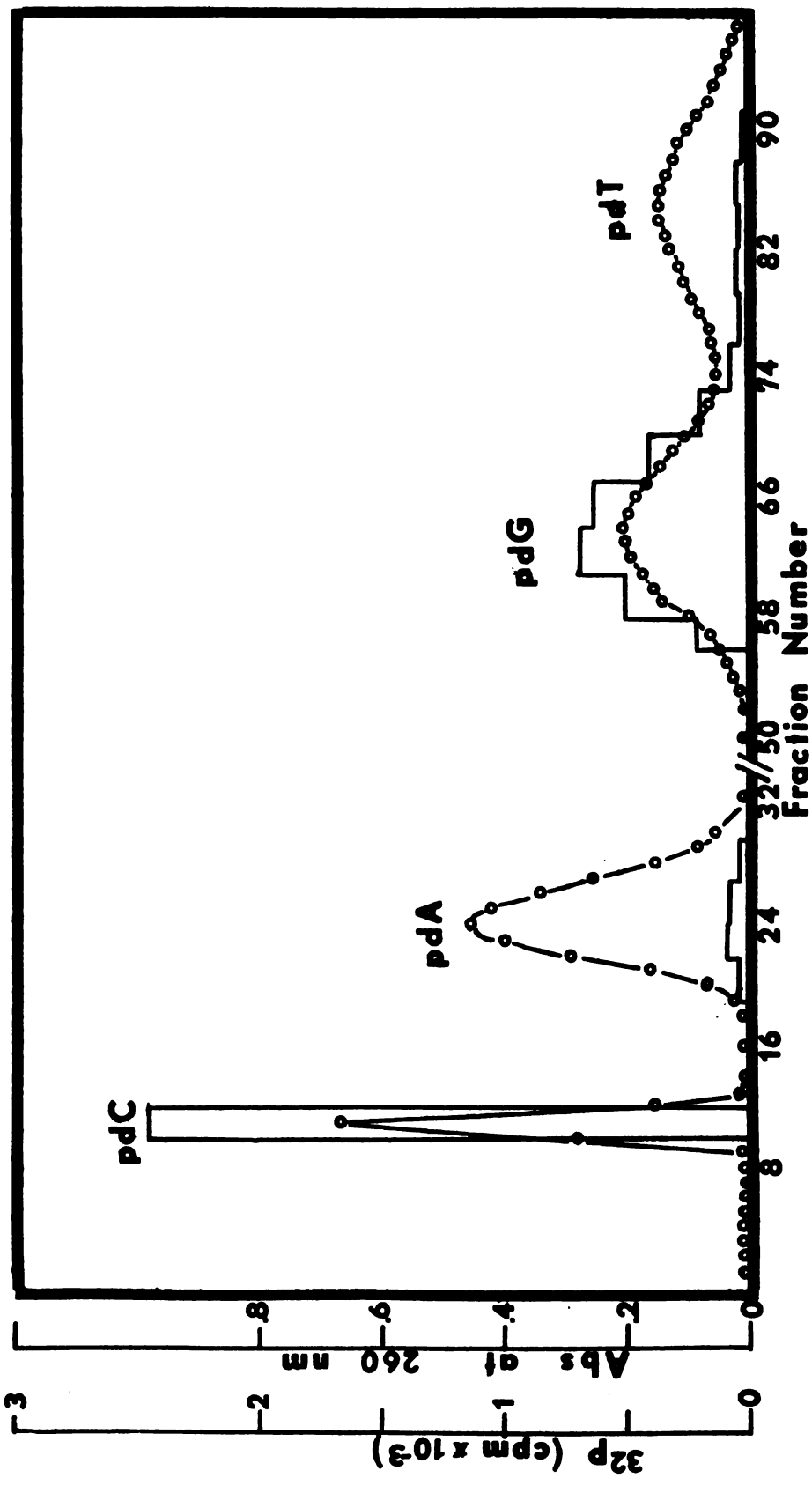

pdC
pdA
pdG
pdT
Fraction Number
Abs at 260 nm
32P (cpm x 10-3)

Table IX

5' terminal nucleotide composition of gh-1 DNA limit polymers

Limit polymers which had been labeled with ^{32}P at the terminal nucleotide position were completely degraded to mononucleotides by the successive action of DNase I and venom phosphodiesterase and were then separated by dowex chromatography as described in Methods.

Nucleotide	^{32}P (cpm)	Percent of total counts
dpC	2392	43
dpA	228	4
dpG	2647	48
dpT	297	5
dpC + dpG	5039	91
dpA + dpT	522	9

cular weight of 2×10^6. Since λDNA is 30×10^6 daltons, the number of bonds broken during the 20° C and 30° C incubations is 1 and 15 respectively. Since a 15 fold increase in activity is much larger than that expected from the thermal activation of the enzymatic catalysis (Q_{10} effect), the increase is attributed to changes in substrate structure.

DISCUSSION

The purification procedures reported for wheat seedling nuclease were developed in order to achieve a highly purified form of the enzyme requiring a minimum number of days to prepare. Chromatography on a carboxymethyl cellulose (CMC) column was found to be a very useful step in the latter stages of purification. Since wheat seedling nuclease has a much lower binding affinity to this material at pH 5.0 compared to the other proteins present, most of the contaminating protein was removed during this step. When the CMC column step is used following the heat step (Type II procedure), a large increase in the specific activity of wheat seedling nuclease results. The enzyme resulting from this procedure represents an increase in specific activity of over 4,000 compared to the crude homogenate and is better than 80% pure as judged by the protein bands on SDS gels. This enzyme preparation , which requires three days and was devoid of any phosphatase or phosphodiesterase contaminating activities, provided a convenient source of enzyme for the studies on the "mechanism of action" of wheat seedling nuclease.

The Type III purification procedure also utilized the CMC column step (following the phosphocellulose step) and yielded wheat seedling nuclease in a highly purified

form which could be used for the investigation of protein properties.

Various methods based on the determination of the size or the size and shape of protein molecules seemed to indicate that the Type III enzyme preparation contained predominantly a single protein species. These methods yielded the following results: 1) a single symmetrical boundary observed during the sedimentation velocity experiment in the analytical ultracentrifuge, 2) a single, coincident peak of protein and enzyme activity found after sedimenting wheat seedling nuclease Type III through a sucrose gradient and 3) a single peak of protein and wheat seedling nuclease activity eluted from a Sephadex G-100 column. The fact that the specific activity remained constant throughout this peak indicates a close correlation between total protein and enzyme activity. This protein-activity peak eluted between one and two times the column void volume where maximum separation of protein occurs. In addition, electrophoresis of the wheat seedling nuclease in polyacrylamide gels in the presence of SDS gave one major band comprising 90-95% of the total protein stained on the gel.

The molecular weights determined from the sedimentation velocity experiments in the analytical ultracentrifuge and the sucrose gradient sedimentation analysis (43,000 and 45,000 respectively) are estimations based on assumptions regarding the shape of the protein molecule.

The molecular weight of wheat seedling nuclease determined by SDS gel electrophoresis was 43,500. The standard proteins used to calibrate the gel system had molecular weights from 11,000-43,000. Thus, the estimation of size for wheat seedling nuclease requires a slight extrapolation of the standard curve. Weber and Osborn (44) have studied the migration of 40 proteins in SDS gels and found that all the proteins studied fell on the standard curve, regardless of the shape of the protein in the native state. The conditions used during the SDS electrophoresis method are expected to produce single polypeptide chains. Therefore, since this method yields a molecular weight which corresponds to that determined by other methods for the intact protein molecule, wheat seedling nuclease probably contains one polypeptide chain.

After these indications that the preparation was rather homogeneous, the existence of multiple ionic forms of purified wheat seedling nuclease was a somewhat surprising discovery. Isozymes bearing different net protein charges have been observed for a number of enzymes (53). Use of the term "isozymes" to describe the multiple ionic forms of wheat seedling nuclease is not appropriate, however, since, only one of the 6-12 differently charged proteins, possesses any enzymatic activity.

Multiple protein species produced by proteolytic activities during purification of several enzymes have been noted (54,55). In these cases, all of the protein species possessed enzymatic activity. However, when the esterase inhibitor, phenylmethylsulfonyl fluoride (PMSF) was added during the purification, the multiple forms of the enzyme were eliminated yielding one active protein. When wheat seedling nuclease was prepared in the presence of PMSF, the same pattern of multiple protein bands remained after polyacrylamide gel electrophoresis of the purified enzyme. Furthermore, the specific activity of the purified enzyme was essentially the same with or without PMSF present during purification. The possibility remains, however, that wheat seedling nuclease is modified by a proteolytic enzyme(s) which is not inhibited by the general protease inhibitor PMSF. The fact that the inactive components of the wheat seedling nuclease preparation appear to be at least as large, if not larger, than the active protein, would indicate that the enzyme has not undergone extensive proteolytic digestion.

Another consideration which might explain the presence of the multiple, inactive forms of wheat seedling nuclease is chemical alteration of the enzyme during purification. This possibility does not seem too likely, since

no greater than 50% of the total DNase activity is lost during any individual step in the purification procedure, yet the active protein band on polyacrylamide gels comprises less than 10% of the total protein present in all of the bands.

The electrophoresis experiments with purified wheat seedling nuclease on gels containing various percentages of acrylamide indicate that all of the protein species are approximately the same size. A slight increase in molecular weight (about 2,000) was observed for the more negatively charged proteins, although this value is certainly not much larger than the experimental accuracy for this procedure. The active band represents one of the least negatively charged proteins with only one protein band(comprising just a few percent of the total protein on the gel) being more positively charged. Negatively charged, non-protein moities associated with wheat seedling nuclease could account for these observations assuming that binding to these moieties caused complete inactivation of the enzyme.

The low A_{280}/A_{260} ratio for wheat seedling nuclease may be due to a non-protein group associated with the enzyme. Analysis of the aromatic amino acid groups present in wheat seedling nuclease indicates that the low A_{280}/A_{260} ratio is probably not due to an abnormal distribution of aromatic amino acids. Furthermore, the fluorescence data indicate the possible presence of a

chromophore in wheat seedling nuclease which absorbs light energy at 255 nm but which does not fluoresce.

The possible association of nucleic acid components with the wheat seedling nuclease is consistent with the spectral data. Nucleic acids absorb light energy in the ultraviolet region of the spectrum but do not produce any fluorescence at neutral pH (56). Assuming that nucleotides and/or oligonucleotides are bound to wheat seedling nuclease, the observed A_{280}/A_{260} ratios of 1.09 to 1.30 would correspond to two to four moles of nucleotide per mole of enzyme based on average extinctions for proteins and nucleic acids at 280 nm and 260 nm.

The isoelectric focusing experiment on purified wheat seedling nuclease resulted in the focusing of only a small amount of protein, along with all of the activity, at pH 5.2. The remainder of the protein focused at pH 2.0. This pH is well below that of any ionizable group in any of the common amino acids. The phosphate ester group in nucleotides and/or oligonucleotides, however, has an ionizable hydrogen with a pK near this value.

Other evidence consistent with the presence of a negatively charged ligand which absorbs light energy at 260 nm is seen from the A_{280} and A_{260} scans of multiple protein bands on polyacrylamide gels. As the four major protein bands become more negatively charged, their A_{280}/A_{260} ratio decreases. In addition, it can be seen from the plot of relative mobility versus percent

acrylamide in the gel that the various protein bands appear to be separated by roughly equal charge increments (vertical displacement on the ordinate scale). It is interesting, then, to speculate that the various ionic forms of wheat seedling nuclease arise from the binding of integral multiples of negatively charged ligands.

The following model system is offered as one explanation for the existence of multiple, inactive forms of wheat seedling nuclease. Nucleotides and/or oligonucleotides might be bound to the enzyme yielding a variety of inactive proteins containing different net charges. If this occurs in the cell, it might represent a control mechanism by which the cell is able to limit nuclease activity in certain situations.

If negatively charged ligands are indeed bound to wheat seedling nuclease, they are either very tightly bound or are covalently linked, since extensive dialysis produces no change in the band pattern on polyacrylamide gels. Polyguanylic acid is not hydrolyzed by wheat seedling nuclease but, never the less, binds tightly to the enzyme as demonstrated by inhibition studies. Furthermore, it has a λmax at 255 nm which is the same wavelength as the peak produced by subtracting the enzyme's absorbance (which produces fluorescence) from the absorption spectrum of wheat seedling nuclease. It might therefore be possible that oligomers of guanylic acid are bound tightly by the enzyme - perhaps buried in the protein's interior

by a conformational change in the protein's structure during binding. Another explanation for the presence of a non-dialyzable ligand would be a covalent enzyme-ligand bond produced by another enzyme activity in the cell. Glutamine synthetase is an example of an enzyme containing adenylic acid covalently linked by a separate enzyme activity (57).

As pointed out earlier in this thesis, the mechanistic studies conducted on wheat seedling nuclease were limited to those areas which established an unusual or unique facet of enzymatic catalysis compared to mechanisms of other nucleases reported in the literature. For example, wheat seedling nuclease appeared to have the ability to hydrolyze dDNA by an endonucleolytic mode of cleavage while degrading rRNA exonucleolytically.

The conclusion that the DNase activity is endonucleolytic is based on the observations that gel filtration of early reaction products showed the presence of large oligonucleotides, while extensive hydrolysis yielded a mixture of small oligonucleotides and mononucleotides (16). However, these observations do not rule out the possibility of an additional exonucleolytic component to the DNase activity.

Gel filtration of early stages of rRNA digests revealed primarily the presence of small products (presumably mononucleotides) although some large products were also observed. Since rRNA has a very complicated secondary structure, these results are difficult to interpret. For example, perhaps small products were being produced from

small single-stranded, looped areas in the rRNA with areas of secondary structure remaining intact. The experiment in which polyadenylic acid was hydrolyzed by wheat seedling nuclease and assayed concurrently for 5'-AMP and acid-soluble material, provided a more quantitative evaluation for the mode of cleavage on a better defined RNA substrate. These results indicate that primarily mononucleotides are released during hydrolysis of poly A, although the presence of a small amount of endonucleolytic activity cannot be ruled out. These results tend to indicate that DNA is cleaved primarily endonucleolytically by wheat seedling nuclease, while RNA is, for the most part degraded by an exonucleolytic mode of cleavage.

The monophosphatase activity associated with wheat seedling nuclease has been referred to as a 3'-nucleotidase activity. This is possibly a slight error in nomenclature since 2'-UMP is also dephosphorylated to a small extent. However, none of the other 2'-ribonucleoside monophosphates, nor any of the 5' isomers of the ribo- or deoxyribonucleoside monophosphates are hydrolyzed by wheat seedling nuclease. The small amount of activity associated with 2'-UMP could be due to the presence of 3'-UMP in the commercial preparation. Therefore, it is still felt that "3'-nucleotidase" is the most descriptive term.

The pH profiles for the 3'-nucleotidase activity are considerably different for the four 3'-ribonucleoside monophosphate substrates (Ap, Cp, Up, Gp).

This probably reflects the different extent of ionization of the various charged groups in the heterocyclic bases of the nucleotides at the various pH values. The pH profiles are rather broad for 3'-AMP, 3'-CMP and 3'-UMP, whereas 3'-GMP hydrolysis shows a pH optimum at pH 7.9. Thus at pH 5-6.4 the relative order of hydrolysis is 3'-AMP > 3'-UMP > 3'-CMP > 3'-GMP. On the other hand, at pH 7.3-8.5, the relative order is 3'-AMP > 3'-GMP > 3'-UMP > 3'-CMP. This observation probably explains the discrepancy observed in the relative order of hydrolysis of the 3'-ribonucleoside monophosphates by a similar enzyme, mung bean nuclease. For this enzyme, Walters and Loring (26) published the following order which was determined at pH 8: 3'-AMP > 3'-GMP > 3'-UMP > 3'-CMP. Laskowski (28), at pH 5, observed the same order as that for wheat seedling nuclease at the same pH - namely, 3'-AMP > 3'-UMP > 3'-CMP > 3'-GMP.

Because of their complex substrates, kinetic analysis of nucleases has largely been avoided. When nucleic acid polymers are degraded during the course of nuclease-catalyzed hydrolysis, the substrates continually change and inhibitor products may form, producing autoacceleration or autoretardation (58). Furthermore, in most cases an initial collection of homogeneous polymeric molecules is impossible to obtain. For these reasons, the kinetic analysis of initial rates is difficult to interpret.

Wheat seedling nuclease possesses a 3'-nucleotidase

activity which is amenable to study by classical kinetic methods. The assay methods available for this type of activity, however, are fixed-time assays which are difficult and tedious methods for obtaining kinetic data. Therefore, a continuous spectrophotometric assay was developed which could be used to measure initial rates of 3'-AMP dephosphorylation. This assay uses adenosine deaminase to convert the adenosine produced from 3'-AMP by the action of wheat seedling nuclease to inosine, which has a much lower molar absorptivity at 265 nm. This assay technique could have general utility for a number of nucleotidase activities which dephosphorylate any of the isomers of adenylic acid.

The Km and Vmax values obtained for the hydrolysis of 3'-AMP indicate that it could be a natural physiological substrate for wheat seedling nuclease. The mononucleotide, 5'-AMP, which is not hydrolyzed by wheat seedling nuclease, was seen to act as a competitive inhibitor towards the 3'-AMPase activity. When the phosphodiester-containing non-substrates, 2',3'-cyclic AMP and ApA were included in the reaction mixture, non-competitive inhibition of the 3'-AMPase activity was observed. This is an interesting result in light of the fact that wheat seedling nuclease has the ability to hydrolyze two types of substrates - namely, the phosphodiester molecules, RNA and DNA and the phosphomonoester-containing 3'-nucleoside monophosphates.

The inhibition of the 3'-AMPase activity by NaCl appears to be a general salt effect since other chloride salts, as well as inorganic phosphate, are also inhibitory. The inhibition probably represents the binding of the salt ions to charged groups on the enzyme at or near the active site. This type of inhibition has been observed for several other enzymes (58,59). Since all the chloride salts were approximately equally effective inhibitors, the inhibition may be explained by the binding of Cl^- ions to a positively charged center(s) which also binds the substrate (s). It is again interesting that two different types of inhibition were observed: one for the 3'-nucleotidase activity and the other for the RNase activity. NaCl inhibits the hydrolysis of 3'-AMP competitively while producing a noncompetitive inhibition for the pApA hydrolysis.

While kinetic studies are seldom able to establish a particular mechanism unequivocally, they can often aid in suggesting mechanistic possibilities. Wheat seedling nuclease contains three distinct catalytic activities: DNase; RNase; and 3'-nucleotidase activities. The kinetic data suggest some interesting possibilities for the interrelationship of these activities. One possibility consistent with the data, is that the 3'-AMPase (3'-nucleotidase) activity and the pApA hydrolysis (RNase activity) occupy separate and distinct active sites on the wheat seedling nuclease molecule. This would explain the

noncompetitive and competitive inhibition by phosphodiester and phosphomonoester inhibitors,respectively, towards the 3'-AMPase activity. This 2-site model is also consistant with the fact that NaCl inhibits the 3'-AMPase activity competitively while acting as a noncompetitive inhibitor towards the hydrolysis of pApA. Another possibility is that the phosphodiester-containing inhibitors, 2',3'-cyclic AMP and ApA, are not binding to the pApA site, but are binding to another site - possibly the DNase site or to a separate allosteric or effector site. Finally, the possibility exists that the three activities are all contained in subsites of the same active center. In this case, for example, the Cl^- ion inhibition could be explained if Cl^- competed for the positively charged center(s) which binds 3'-AMP, but bound very tightly to the center(s) or configuration responsible for binding pApA. If this were the case, pApA may not be able to "compete out" the Cl^- ion, until very high concentrations of pApA are obtained, and thus demonstrate a type of non-competitive inhibition. To test this type of noncompetitive inhibition, substrate concentrations well above the Km need to be tested. Unfortunately, this data could not be obtained since the pApA reaction has a high Km and the pApA concentration in the assay is limited to that amount which produces an A_{275} below 2. This fact also explains why the ultraviolet absorbing inhibitors could not be tested in the pApA hydrolysis reaction.

The fact that the relative order of base preference expressed by the enzyme is the same for all three activities is, perhaps, not surprising if one makes the assumption that all three activities are catalyzed by the same general active center. The data from experiments on the production of the four different mononucleotides during the hydrolysis of dDNA, as well as the hydrolysis of the four different ribohomopolymers indicate a reluctance of wheat seedling nuclease to hydrolyze phosphodiester bonds adjacent to guanylic and cytidylic acid residues. The ribohomopolymer data should be interpreted with some caution, however, since the polymers exhibit different degrees of ordered structure with the given conditions of the assay. At a value near the pH optimum for the DNase and RNase activities (pH 5), the relative order of base preference for the 3'-nucleotidase activity is the same as that for the RNase and DNase activities. In fact, when the 3'-deoxyribonucleoside monophosphates are used as substrates, 3'-dGMP and 3'-dCMP are not hydrolyzed at all. As was pointed out, however, the base preference for the 3'-nucleotidase activity changes at higher pH - at least in the case of the 3'-ribonucleoside monophosphates.

Perhaps the most exciting area of investigation was the hydrolysis of native DNA molecules by wheat seedling nuclease. Early work with wheat seedling nuclease demonstrated a large difference in the rate of acid-soluble products produced when native and denatured DNA were

hydrolyzed by the enzyme. When the limited hydrolysis of native DNA was examined by measuring the size of the products produced during the course of the reaction, it was found that wheat seedling nuclease catalyzed the hydrolysis of only a limited number of phosphodiester bonds in large DNA molecules, producing "limit polymers" still of high molecular weight, which were resistant to further hydrolysis by additional enzyme over extended periods of time. This somewhat surprising result indicated that there may be a few, highly specific sites or areas in the native DNA molecules which are recognized and cleaved by wheat seedling nuclease.

A number of methods were used to investigate the extent and nature of this apparent site specificity. High molecular weight E. coli DNA or intact, linear-duplex viral DNA of known molecular weight was used for these studies. Since the average molecular weight of the limit polymers produced by wheat seedling nuclease is also known, the number of double-strand scissions made in the original DNA can be estimated. When this estimation is made for all of the DNA's tested, the result is one double-strand cleavage for every 6,100 to 9,090 phosphodiester bonds present in the original DNA, or one double-strand scission per 3,500-4,545 nucleotide pairs. In other words, for example, gh-1 DNA ($MW = 23 \times 10^6$) is cleaved about seven times to yield limit polymers of 3×10^6 daltons.

An analysis of the acid soluble products produced

during the limit digestion of ^{32}P gh-1 DNA, revealed that the acid-soluble material was near the borderline of detectability. The sensitivity of the method was such that 0.1% conversion to acid-soluble products would be just detectable. Since this value would correspond to only seven nucleotides per cleavage, little, if any, acid-soluble material is released during the limit digestion of native gh-1 DNA. This fact obviously contradicts the early results obtained with commercial calf thymus and salmon sperm DNA. A possible explanation for the small amount of acid-soluble production observed with these substrates is the contamination with single-stranded DNA, RNA, or loose, single-stranded ends produced from physical shearing of the DNA in preparation, shipment and storage. Also, the contamination of the commercial DNA with other nucleolytic enzymes could explain the appearance of acid-soluble products.

Wheat seedling nuclease was judged to produce only double-stranded cleavages in native DNA for the following reason. After gh-1 DNA or λ DNA was digested to limit polymers, it yielded the same duplex-equivalent molecular weight in both neutral and alkaline sucrose gradients. The alkaline sucrose gradient technique sediments DNA as single,randomly-coiled strands, whereas the neutral sucrose gradient technique sediments the DNA as hydrogen-bonded double-strands. Therefore, the equivalence in molecular weight indicates the absence of any "nickase"

activity or single-strand interruption in the limit polymers.

The data collected by labeling the 5' termini of the gh-1 DNA limit polymers can be used to evaluate several parameters of the wheat seedling nuclease activity. First of all, since only one nucleotide is labeled per single-stranded DNA polymer, it is possible to calculate the average number of nucleotides in the limit polymers and hence the number-average molecular weight. Furthermore, this data also indicates the number of new termini produced during the limit digestion and therefore the number of cleavages catalyzed by the enzyme. Finally, by separating the labeled nucleotides, it is possible to identify the nucleotide present on the 5' ends of the limit polymers which were produced by the hydrolysis of wheat seedling nuclease.

Treatment of the data in this manner for the experiments with the limit digestion products of gh-1 DNA gave the following results (see the appendix for the calculations). An average of 6.7 cleavages were produced in gh-1 DNA (MW 23×10^6) yielding limit polymers containing 8.54×10^3 nucleotides which corresponds to a number-average molecular weight of 2.98×10^6. The nucleotide composition at the 5' ends of the limit polymers was 52% G and 48% C after subtracting the contribution of the nucleotides present initially at the 5' end of gh-1 DNA. The possible significance of these results is discussed later.

There existed the possibility that wheat seedling nuclease could recognize a large area of DNA, perhaps several hundred nucleotides in length, which was structurally unique because of an abnormal nucleotide sequence. In order to test this possibility, venom diesterase was allowed to cleave an average of 118 nucleotides from the 3' end of ^{32}P-labeled gh-1 DNA limit polymers. An analysis of these nucleotides revealed that there was an enrichment of several mole-percent in the A-T content, compared with that of the whole gh-1 DNA molecule. This enrichment in A-T does not seem sufficiently great to cause a change in the DNA structural conformation. However, the possibility remains that this value reflects a much higher enrichment in A-T in a much smaller area near the cleavage site.

Another interesting aspect of the hydrolysis of native DNA by wheat seedling nuclease is the separation of the specific cleavage sites along the DNA molecule. The limit polymers produced by treatment of gh-1 DNA with wheat seedling nuclease appear to be roughly the same size, indicating a regular or periodic spacing of the cleavage sites in the DNA. The homogeneity of the size of the gh-1 DNA limit polymers is suggested by the similar molecular weight values obtained by sucrose gradient analysis (weight-average molecular weight) and by the 5' end labeling experiment (number-average molecular weight). Furthermore, the agarose gel electrophoresis

of the limit polymers reveals that about 90% of the polymers are within roughly $\pm 0.5 \times 10^6$ daltons of the ϕ-X174 marker DNA ($MW = 3 \times 10^6$).

The fact that the rate of hydrolysis of E. coli DNA is first order throughout the reaction suggests that all of the cleavage sites are hydrolyzed at approximately the same rate. This result would be expected if all of the cleavage sites were identical in nature.

The supposition that an A-T rich area may be involved at the cleavage site in native DNA is suggested by several lines of experimental evidence. Although native DNA is hydrolyzed at a much slower rate by wheat seedling nuclease than is single-stranded or denatured DNA, the synthetic duplexes, poly d(A-T) and poly rA,U, are hydrolyzed at rates comparable to denatured DNA. On the other hand, the duplex poly dG-poly dC is not hydrolyzed by wheat seedling nuclease. The rapid hydrolysis of poly d(A-T) may reflect the relatively weak bonding of the A-T base pairs which holds the two self-complementary strands together (60).

Poly d(A-T) existed primarily in the duplex form in all the assay conditions, since they were performed well below the Tm in all cases. As the conditions were changed to those favoring denaturation (low ionic strength and low pH), a large increase in the rate of hydrolysis was noted, although the synthetic polymer was still mainly in the

duplex form. These observations probably indicate that for poly d(A-T), at least, the enzyme acts on single-strand areas produced by disruption of the secondary structure.

Other experimental data which seems to implicate the involvement of A-T rich areas at the cleavage site in native DNA is the early and rapid production of λ halves during the hydrolysis of λ DNA. The A-T rich region near the center of λ DNA has been well characterized (61). Inman and Schnos (61) have demonstrated with electron microscopic pictures that when λ DNA is partially denatured at high pH, the A-T rich region in the center tends to loop out, appearing as two single-stranded chains for a short distance. Wheat seedling nuclease may be recognizing this area as being single-stranded in nature.

As previously mentioned the 3' end analysis revealed only a slight increase in the mole-percent of A + T over an area representing an average of 118 nucleotides from the limit polymer terminus. If all of this enrichment were due to an area of only 6-12 nucleotides, say, from the end of the limit polymer, then this area would be enriched 20-40% in A + T, compared to the whole gh-1 DNA molecule. Thus, while there does not appear to be a significantly large abberration in nucleotide content in the general area of the cleavage site, the possibility of a high A-T area in the immediate vicinity of the cleavage

site cannot be ruled out.

As pointed out earlier, the fact that the rate of hydrolysis for λDNA is increased by 15 fold between 20° and 30° C, suggests a structural effect of the increased temperature on the substrate. Such an effect might be expected if an A-T rich region were involved at the cleavage site. Such an area would be expected to undergo partial denaturation, thus shifting the dynamic equilibrium (single-strand configuration ⇌ double-strand configuration) to the left, thereby providing a much better substrate for wheat seedling nuclease. This would be analogous to the large increase in rate noted for poly d(A-T) as the ionic strength and pH were lowered.

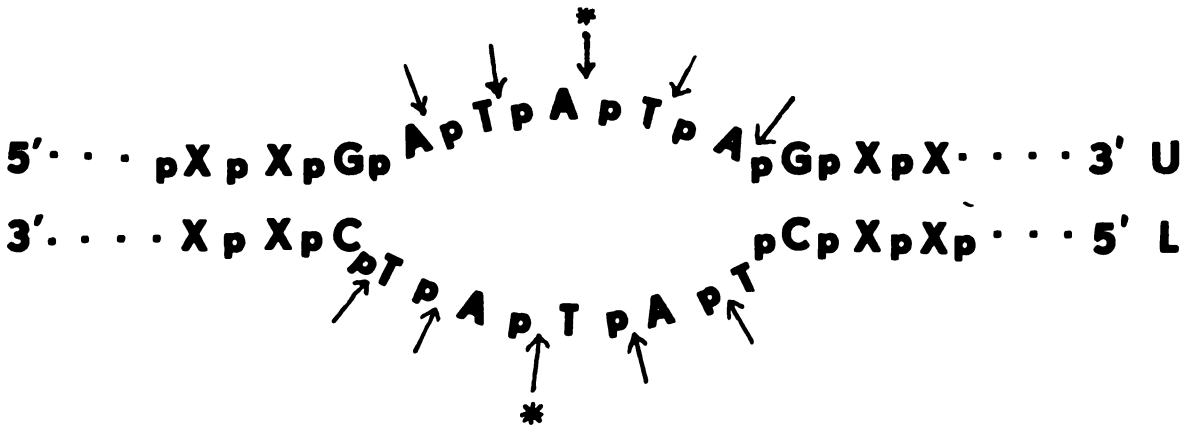
The conceptual idea of "structural breathing" has been noted by von Hippel and Felsenfeld (62). These authors postulated that at temperatures below the Tm, regions rich in A-T undergo strand separation to a greater extent than areas rich in G-C. Therefore, it is possible that the single-strand specificity of wheat seedling nuclease endows the enzyme with the ability to recognize A-T rich areas in native DNA.

It is interesting to compare the base preference exhibited by wheat seedling nuclease with the possibility of the involvement of A - T rich areas near the cleavage site. The hydrolysis of the phosphate bond adjacent to A and T was seen to be preferred to a considerable extent compared to bonds adjacent to C and G

residues in dDNA, ribohomopolymers and 3'-nucleoside monophosphates. Thus it may be that this base preference is acting in tandem with the enzyme's ability to recognize structural difference in the nucleotide composition of a given region of DNA in order to produce a specific site on the native DNA molecule which is recognized by wheat seedling nuclease.

It is once again tempting to construct a model, for the sake of discussion, which attempts to coalesce the variety of experimental information into a hypothetical explanation which is consistent with a maximum number of the observed experimental results. The following model, then, is offered for the production of limit polymers from native DNA by wheat seedling nuclease.

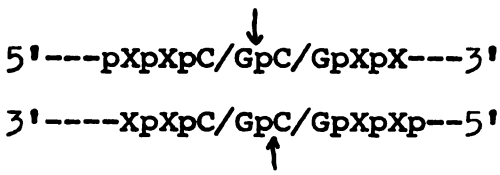
In the model illustrated below, there is a small A-T area which is diagramatically drawn to illustrate the "structural breathing" phenomenon. Wheat seedling nuclease catalyzes the hydrolysis of one of the A-T bonds (arrows with asterisk) on both the upper(U) and lower (L) strand where the single-strand character of the DNA is the greatest. The loose A-T ends are then hydrolyzed (arrows) producing 5' phosphoryl terminated products, until a G-C pair is encountered. At this point the enzyme is unable to bind to the double stranded DNA molecule and is released by the substrate.



This model predicts that the 5' termini of the limit polymers should contain 50% G and 50% C residues which agrees with the experimental evidence. It also predicts that the 3' terminus on both the L and U strands should be either A or T. This would be consistant with the observation that the bonds adjacent to G and C residues are resistant to attack by wheat seedling nuclease. This model also predicts that a small amount of acid-soluble material should be released in the form of 5'-AMP and 5'-TMP. The A-T area drawn in the model would only release 8 mononucleotides in the limit polymer. This value was about the limit of detectability in the ^{32}P-gh-1 acid-soluble experiment. The experimental values of 0.04% and 0.07% acid-soluble products could easily be in error by as much as $\pm$ 0.04%.

Other possibilities exist, of course, to explain the specificity indicated for the cleavage of native DNA by wheat seedling nuclease. If no acid-soluble material

is released during hydrolysis, the cleavage configuration would have to be of the following sort:



where C/G indicates the presence of either C or G. Therefore, this would necessitate the cleavage of a CpG or GpC bond in order to explain the 50% G and 50% C 5' terminal data if the double-strand scission was made directly across the duplex chain (i.e. if the cleavage was not staggered). This possibility seems less likely since the base preference data suggest a considerable reluctance by wheat seedling nuclease to hydrolyze bonds adjacent to C and G residues.

Other areas in the DNA molecule besides A-T rich regions may play a role in the site recognition. For example, symmetry elements in the DNA molecule or unique base sequences may be responsible for the recognition of certain areas by wheat seedling nuclease. In addition, the possibility remains that the binding of wheat seedling nuclease to native DNA may contribute to the opening of the duplex to permit hydrolysis of the single stranded areas.

The data pertaining to the transcription by the phage-induced, DNA-dependent RNA polymerase from the limit polymer templates is interesting for several reasons. First, the fact that the Km and Vmax values are similar

to those obtained when whole gh-1 DNA is used for a template, demonstrates a high catalytic efficiency for the limit polymer templates. This would seem to indicate that either the promotor and terminator regions were pretty much intact (at least functionally) in the limit polymers or that they are not required to affect efficient transcription. Also it is known[7] that the phage induced polymerase transcribes guanylic acid as the first residue incorporated into RNA chains. This fact may be significant since the limit polymers end in 50% C and 50% G.

It is particularly intriguing to speculate about the significance of the manner in which wheat seedling nuclease degrades native DNA molecules. Although the mechanism of native DNA hydrolysis may be purely happenstance,and therefore of no significance to the cell, this seems unlikely due to the unusual specificity involved. Since wheat seedling nuclease produces only a limited number of double-strand cleavages which are spaced along the DNA molecule at average intervals, comparable to individual cistrons in size, a mechanism for site-specific recombination could be envisaged. Such a mechanism has been demonstrated <u>in vitro</u> for the restriction endonuclease, R I (63). In this case, the specific cleavage produced by the endonuclease results in a number of fragments which can be recombined by a separate ligase activity to produce a new population of recombinant molecules. H. Sobell has also proposed a molecular mechanism for genetic recombination

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[7]Personal communication from Dr. J. Boezi.

which postulates a specific double-strand cleavage by an endonuclease (64). In these respects, it is interesting to note that, in the case of gh-1 DNA, wheat seedling nuclease produces fragments which correspond in size to the average transcriptional unit of the phage DNA and which retain a good portion of their template activity.

Although no modification activity has been demonstrated to be associated with wheat seedling nuclease, restriction of foreign DNA molecules might also be a function of the action of wheat seedling nuclease on native DNA. As noted in the introduction to this thesis, the bacterial restriction endonucleases also demonstrate the same high degree of specificity towards the hydrolysis of native DNA.

The fact that wheat seedling nuclease appears to possess the ability to recognize certain structural and/or chemical sites in a double-stranded DNA molecule, suggests a role for the enzyme as a probe of DNA structure. R. D. Wells (65) has studied the physical and chemical properties of a number of synthetic DNA molecules of known base sequence. He goes on to postulate that areas of abnormal base sequence in naturally-occurring DNA's may be involved in structure-function relationships. For example, areas rich in A-T have been demonstrated to be involved at the insertion site for λ prophage in *E. coli* DNA (66) and at the sites in T_5 and T_7 where *E. coli* RNA polymerase binds (promotor sites) (67). The operator region in λ DNA, to

which the lactose repressor binds,has recently been demonstrated to have a unique base sequence (68). Therefore, enzymes such as wheat seedling nuclease, which are able to recognize certain specific areas in DNA may have potential use as probes of these structure-function relationships.

SUMMARY

Wheat seedling nuclease has been purified over 10,000 fold with a 5% yield of activity. This procedure produced 0.5 mg of protein from 7 kilograms of wheat. The isolated preparation was judged to be nearly homogeneous in regards to size and shape as indicated by the following results: a) sedimentation in the analytical ultracentrifuge revealed a single, symmetrical boundary, b) sedimentation through a sucrose gradient resulted in a single, coincident peak of protein and enzyme activity, c) filtration in Sephadex G-100 yielded a single protein-activity peak, throughout which the activity remained constant, and d) polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol and SDS revealed the presence of only one major protein band comprising greater than 90-95% of the total protein stained on the gel.

However, when electrophoresis of this preparation of wheat seedling nuclease was performed at pH 8 in the absence of SDS and 2-mercaptoethanol, 6-12 protein bands were detected. Furthermore, the 3 activities associated with wheat seedling nuclease were associated with only one of these bands. The band responsible for the enzyme activities was one of the least negatively charged bands and

contained less than 10% of the total protein stained on the gel. All of the multiple-ionic forms of wheat seedling nuclease were determined to be approximately equal in size. This conclusion is based on the fact that when the percent acrylamide contained in the gel was plotted as a logarithmic function of the relative mobility, similar slopes were obtained for all of the bands.

The molecular weight of wheat seedling nuclease was determined to be approximately 43,000 as determined by the following methods: a) sedimentation velocity experiments on the analytical ultracentrifuge, g) sucrose gradient sedimentation with proteins of known molecular weights, c) SDS gel electrophoresis with a system calibrated with standard proteins. Since the SDS electrophoresis procedure yielded a molecular weight which was similar to the other two methods, it is likely that wheat seedling nuclease exists as a single polypeptide chain.

Wheat seedling nuclease has an A_{280}/A_{260} ratio of 1.1-1.3 which is lower than that observed for most proteins. This ratio may represent the presence of a non-protein group bound to the enzyme since the aromatic amino acid content cannot explain this low A_{280}/A_{260} ratio.

Wheat seedling nuclease has been demonstrated to possess the ability to degrade denatured DNA by an endonucleolytic mode of cleavage yielding 5'-phosphoryl terminated mono- and oligonucleotides (16). When polyriboadenylic acid is degraded by wheat seedling nuclease, nearly

all of the products released throughout the reaction are 5'-AMP, indicative of an exonucleolytic mode of hydrolysis.

Kinetic analysis of the rates of hydrolysis of 3'-AMP and pApA by wheat seedling nuclease was performed by using a continuous, spectrophotometric assay developed for this purpose. The assay was based on the decrease in absorbance produced when either adenosine or 5'-AMP (products of the reaction catalyzed by wheat seedling nuclease) was deaminated by a specific amino hydrolase coupling enzyme. The Km and Vmax values obtained for the dephosphorylation of 3'-AMP were 1.5×10^{-5} M and 1.3×10^{2} μmoles/min/mg respectively. The hydrolysis of pApA yielded Km and Vmax values of 9.1×10^{-5} M and 1.67×10^{3} μmoles/min/mg.

Kinetic inhibition studies were conducted using 3'-AMP as the substrate. 5'-AMP, a molecule containing a single phosphomonoester linkage, was found to be a competitive inhibitor while the phosphodiester compounds, 2',3'-cyclic AMP and ApA, were found to inhibit the reaction in a noncompetitive manner.

A general salt inhibition was noted for wheat seedling nuclease. The 3'-AMPase reaction was inhibited competitively by NaCl. However, when NaCl was included in the pApA hydrolysis reactions, noncompetitive inhibition resulted.

A consistent order of base preference for all three of the activities associated with wheat seedling nuclease was observed at pH 5.0. This preference was found to be A > T(U) > C > G. Phosphodiester bonds adjacent to cytidylic and guanylic acid residues were found to be quite resistant

to cleavage by wheat seedling nuclease.

When high molecular weight, native E. coli DNA or intact, linear-duplex viral DNA molecules are hydrolyzed by wheat seedling nuclease, only a few, specific double-stranded cleavages occur. The resulting "limit polymers," -still of high molecular weight - are resistant to further hydrolysis by wheat seedling nuclease.

The nature of the sites on the duplex DNA recognized by the enzyme was investigated. The production of limit polymers (MW = 3×10^6) from gh-1 DNA (MW = 23×10^6) was accompanied by the release of 0.04-0.08% acid-soluble material. This corresponds to less than 8 nucleotide equivalents released as acid-soluble material for each limit polymer produced by the action of wheat seedling nuclease. The 5' termini of limit polymers labeled with ^{32}P by the action of polynucleotide kinase contained either cytidylic or guanylic acid but no adenylic or thymidylic residues.

Wheat seedling nuclease exhibits a very large preference for denatured DNA as compared to native, double-stranded DNA. The fact that wheat seedling nuclease can hydrolyze the double-stranded, synthetic polymer, poly d(A-T) at rates comparable to the hydrolysis of denatured DNA, indicates that wheat seedling nuclease may recognize A-T rich areas in native DNA. This contention is further supported by the following evidence: a) λ DNA, which contains an A-T rich region near its center, is rapidly cleaved to produce λ halves, b) wheat seedling nuclease possesses a considerable preference for bonds adjacent to A and T residues while showing

a marked resistance to the hydrolysis of bonds adjacent to C and G residues, c) the nucleotide composition of an area within 118 nucleotides of the 3' end of the gh-1 DNA limit polymers showed a slight increase in A-T content as compared to the whole gh-1 DNA molecule and d) the hydrolysis rate of native λ DNA increased 15 fold between 20° and 30° C indicating a major structural effect of the increased temperature on the substrate.

The biological significance of the wheat seedling nuclease remains to be established; however, the enzyme's catalytic properties are compatible with functions such as restriction, that is, the recognition and cleavage of foreign DNA's - or genetic recombination. Finally, wheat seedling nuclease may be of current importance as a probe of native DNA structure.

APPENDIX

APPENDIX A

Abbreviations

A,G,U,T,C,X - nucleosides (adenosine, guanosine, uridine, thymine, cytidine, x any of these nucleotides)

dA, dG, dX - deoxyribonucleosides

pApA - adenylyl-(5',3')-adenosine 5'-phosphate

2'-NMP, 3'-NMP, 5'-NMP - isomers of the nucleoside monophosphates

2'-dNMP, 3'-dNMP, 5'-dNMP - isomers of the deoxyribonucleoside monophosphates.

poly d(A-T) - alternating copolymeric duplex of A and T

poly dG-poly dC - homopolymeric duplex of poly dG and poly dC

poly rA,U - random copolymer of equal amounts of A and U

rRNA - ribosomal RNA

dDNA - denatured DNA

nDNA - native (double-stranded) DNA

DEAE- - diethylaminoethyl-

TEAB - triethylammonium bicarbonate

SDS - sodium dodecalsulfate

BSA - bovine serum albumin

TCA - trichloracetic acid

tris-HCl - tris(hydroxymethyl) aminoethane titrated to the given pH with HCl

NaAc, $Zn(Ac)_2$ - sodium and zinc acetate

CMC - carboxymethyl cellulose

PC - phosphocellulose

Pi, PPi - orthophosphate, pyrophosphate

A_λ, OD_λ unit - amount of material dissolved in 1.0 ml to give an absorbancy of 1.0 at the wavelength, λ, in a cuvette with a 1 cm lightpath

Q_{10} - the increase in an enzymatically catalyzed reaction for a given 10^o C increase in temperature

APPENDIX B

Growth Media

E. coli $C_{600}\lambda CI_{ts}875$ medium:

Per liter of 6.8 buffer:

5.0 gm casamino acids (0.5% final conc.) - autoclave for 15 minutes, then add aseptically
Add 0.25 ml of thiamine (B_1) 4 mg/ml H_2O - this has to be filter sterilized, cannot be autoclaved.
Add 20 ml of 25% glucose in H_2O - sterilize this by itself, then add it aseptically

6.8 buffer: per liter distilled H_2O

K_2HPO_4	8.7 gm
KH_2PO_4	6.8 gm
$(NH_4)_2Cl$	1.0 gm
$MgSO_4$-H_2O	0.1 gm
$CaCl_2$ (20 mg/ml stock)	0.25 gm
$FeCl_3$-$7H_2O$ (1 mg/ml stock)	0.25 ml

Adjust pH to 6.8 with KOH or KCl

Add $CaCl_2$ solution drop wise as the last addition (i.e. after everything else is in solution) with stirring to prevent precipitate of $CaPO_4$

E.coli infected with T_4 phages

Per liter:

NH_4Cl	1 gm
Na_2HPO_4	5.54 gm
KH_2PO_4	3 gm
NaCl	5 gm
$MgSO_4$	0.5 gm
Casamino acids	10 gm
glucose	4 gm

Pseudomonas putida - low phosphate medium

C medium phosphate*	600 ml
KH_2PO_4 (0.01 M)	50 ml
tris-HCl (1.0 M, pH 7.5)	80 ml
Casamino acids	5 gm
glucose (20%)	100 ml
H_2O	170 ml

*C medium minus phosphate

NH_4Cl	2 gm
KCl and NaCl (each)	4 gm
$MgCl_2$ (1 M)	0.4 ml
Na_2SO_4 (0.2 M)	10 m ml
H_2O	600 ml
$FeCl_3$, $Ca(NO_3)_2$, $MnSO_4$, $NaMoO_4$	trace amounts

APPENDIX C

Calculations

Tyrosine and tryptophan content in wheat seedling nuclease

The two unknowns, N_{trp} (the number of moles of tryptophan per mole of protein) and M_{tyr} (the number of moles of tyrosine per mole of protein), are found by solving the two simultaneous equations:

$$\varepsilon_{288} = N_{trp}4815 + M_{tyr}385$$

$$\varepsilon_{280} = N_{trp}5690 + M_{tyr}1280$$

where ε_{288} and ε_{280} are the extinctions of the unknown protein solution at 288 nm and 280 nm. 5690 and 1280 are the known extinctions of tryptophan and tyrosine at 280 nm. 4815 and 385 are the known extinctions at 288 nm

Since $A = \varepsilon \times$ conc.(for a 1 cm cuvette)

$$\varepsilon_{280} = \frac{A_{280}}{[\text{Protein}]} = \frac{1.3 \times 10^{-1}}{4.19 \times 10^{-5} M} = 3.10 \times 10^{4}\ M^{-1}$$

$$\varepsilon_{288} = \frac{A_{288}}{[\text{Protein}]} = \frac{9.5 \times 10^{-2}}{4.19 \times 10^{-5} M} = 2.27 \times 10^{4}\ M^{-1}$$

$$N_{trp} = (\varepsilon_{288}/3.103 \times 10^{3}) - (\varepsilon_{280}/1.032 \times 10^{4})$$

$$= (2.23 \times 10^{4}/3.103 \times 10^{3}) - (3.10 \times 10^{4}/1.032 \times 10^{4})$$

$$= \underline{4.31}$$

$$\varepsilon_{288} = N_{trp}(4.815 \times 10^{3}) + M_{tyr}(3.85 \times 10^{2})$$

$$2.27 \times 10^{4} - 2.075 \times 10^{4} = M_{tyr}(3.85 \times 10^{2})$$

$$1.95 \times 10^{3} = M_{tyr}(3.85 \times 10^{2})$$

$$M_{tyr} = \underline{5.07}$$

Number average molecular weights for denatured gh-1 DNA and denatured gh-1 DNA limit polymers

Specific activity of ^{32}P-ATP = 2.38×10^8 cpm/μmole

A. denatured gh-1 DNA

$$\mu\text{moles } ^{32}\text{P incorporated} = \frac{^{32}\text{P (cpm) incorporated}}{^{32}\text{P-ATP specific activity}}$$

$$= \frac{6 \times 10^2 \text{ cpm}}{2.38 \times 10^8 \text{ cpm}/\mu\text{mole}}$$

$$2.52 \times 10^{-6} \mu\text{mole } ^{32}\text{P incorp.}$$

$$\text{moles of gh-1 DNA*} = \frac{\text{weight of gh-1DNA (gms)}}{\text{MW of nucleotide (gm/mole)}}$$

$$= \frac{30 \times 10^{-6} \text{gms}}{3.5 \times 10^2 \text{ gms/mole}}$$

$$= 8.6 \times 10^{-2} \mu\text{moles}$$

$$\frac{\mu\text{moles of gh-1 DNA}}{\mu\text{moles } ^{32}\text{P incorporated}} = \frac{8.6 \times 10^{-2} \text{ moles gh-1 DNA}}{2.52 \times 10^{-6} \text{ moles } ^{32}\text{P incorp.}}$$

$$3.4 \times 10^4$$

Therefore one ^{32}P atom was incorporated at the 5' end of polymers containing an average of 3.4×10^4 nucleotides.

MW of denatured gh-1 DNA = MW of nucleotide x no. of nucleotides

$$= 3.5 \times 10^2 \times 3.4 \times 10^4$$

$$= \underline{1.2 \times 10^7}$$

* moles of DNA are expressed as moles of nucleotide equivalents

B. denatured gh-1 DNA limit polymers

$$\mu\text{moles}^{32}\text{P incorporated} = \frac{4.45 \times 10^3 \text{ cpm}}{2.38 \times 10^8 \text{cpm}/\mu\text{mole}}$$

$$= 1.87 \times 10^{-5} \mu\text{moles } ^{32}\text{P incorp.}$$

$$\text{moles of gh-1 DNA limit polymers} = \frac{28 \times 10^{-6} \text{ gms}}{3.5 \times 10^2 \text{ gms/mole}}$$

$$= 8 \times 10^{-2} \ \mu\text{moles}$$

$$\frac{\mu\text{moles of gh-1 DNA limit polymers}}{\mu\text{moles } ^{32}\text{P incorporated}} = \frac{8 \times 10^{-2}}{1.87 \times 10^{-5}}$$

$$= 4.27 \times 10^3$$

Therefore one ^{32}P atom was incorporated at the 5' end of the polymers containing an average of 3.4×10^4 nucleotides.

MW of denatured limit polymer = MW of nucleotide x no. of nucleotides

$$= 3.5 \times 10^2 \times 4.27 \times 10^3$$

$$= \underline{1.49 \times 10^6}$$

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