AN INVESTIGATION OF THE PROPERTIES OF DNase II ISOLATED FROM BOVINE INTESTINAL MUCOSA AND THE NATURE OF ITS REACTION WITH DNA

by

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ABSTRACT

Isolation of DNase II from bovine small intestine by chromatography on DEAE cellulose of a 105,000 xg supernatant solution prepared from an homogenate of the mucosa in Krebs Ringer phosphate buffer appeared to yield two activities, a major activity which was eluted from the column with 20 mM phosphate buffer and a minor activity which was eluted with a potassium chloride gradient. The two DNase II activities differed in their response to increasing ionic strength, pH, sulfate ion concentration and temperature for the hydrolysis of DNA. The major activity degraded native DNA more rapidly than denatured DNA whereas the minor activity degraded both at the same rate. Previous investigators have reported the presence of two DNases IIs with different properties in other tissues. In bovine intestinal DNase II, the minor activity, upon rechromatography on DEAE cellulose, eluted in the same position as the major DNase II and it was concluded that the appearance of the minor DNase II activity was an artifact of the chromatography. It is likely that a small quantity of DNase II was bound to endogenous DNA on the DEAE cellulose column in the 20 mM phosphate buffer and later eluted from the column along with some of the DNA with the potassium chloride gradient. DNA present in the minor DNase II preparation probably caused the apparent differences in properties of the two DNase IIs by interfering in the enzymic reactions.

Intestinal DNase II was partially purified by ion exchange chromatography and gel filtration and had properties similar to DNase IIs from other tissues. The enzyme hydrolysed calf thymus DNA endonucleolyti-

cally at acid pH in the absence of a divalent metal ion to oligonucleotides with 3'-phosphate and 5'-hydroxyl terminals. The activation energy for the reaction was 19 kcal/mole; that for denaturation of DNase II itself, 43 kcal/mole. Michaelis-Menton kinetics were observed for the reaction of DNase II with Escherichia coli DNA--the Michaelis constant was 2.42×10^{-7} M DNA-phosphate. The molecular weight of DNase II was estimated to be 41,000 by gel filtration on Sephadex G100.

The early stages of the digestion of DNA by DNase II were investigated by labelling the reaction products with ^{32}P at their 5'-terminals using polynucleotide kinase and $[\gamma\text{-}^{32}P]$ATP and at their 3'-terminals using terminal deoxynucleotidyl transferase and $[\alpha\text{-}^{32}P]$ATP.

The mode of cleavage of native DNA by DNase II was determined by comparing the polynucleotide-catalysed incorporation of ^{32}P from $[\gamma\text{-}^{32}P]$ATP into native and denatured DNase II reaction products. Since single-strand cleavage of DNA by DNase II released 5'-hydroxyl terminals that were inaccessible to polynucleotide kinase as long as the DNase II reaction products remained double-stranded, incorporation of ^{32}P into native products was proportional to the number of double-strand cleavages while incorporation of ^{32}P into denatured products was proportional to the number of double-strand cleavages plus single-strand cleavages. It was found that DNase II degraded native DNA primarily by a double-strand cleavage mechanism.

After DNase II catalysed hydrolysis of DNA each of the four bases present in DNA was found at the 5'- and 3'-terminals of the reaction products. Thus DNase II did not have an exclusive preference for one or two bases at either terminal, and likely cleaved a large number of

different base sequences in the DNA. The most susceptible internucleotide linkage was GpG; the most resistant, CpT. The base specificity at the 5'-terminal changed during the reaction, especially in the initial and terminal phases. In the initial phase the proportion of guanine was elevated and the order of cytosine and adenine was reversed compared to later stages in the reaction. These changes could reflect the presence of a preferred sequence that was selectively degraded and exhausted during the initial phase of the reaction. Different proportions of terminal bases in cleavage products of DNA from diverse species indicated that susceptible sequences occurred with different frequencies in the various DNAs.

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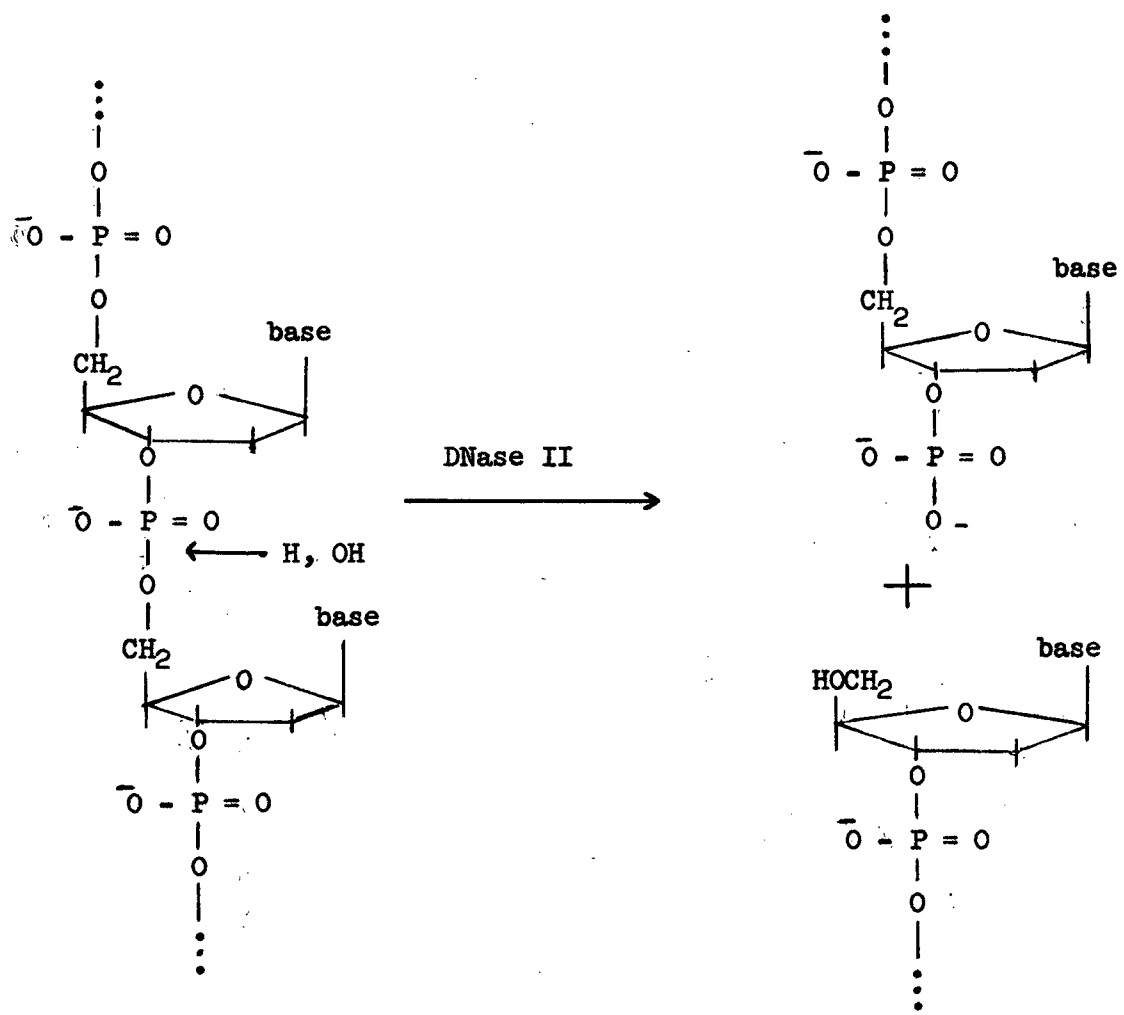
INTRODUCTION

According to international recommendations on Enzyme Nomenclature (1972), deoxyribonuclease II (EC 3.1.4.6), abbr., DNase II and also known as acid DNase, is defined as a phosphodiester hydrol ase that degrades deoxyribonucleic acid to 3'-phosphate terminated oligonucleotides:



The classification of animal DNases into DNase I- and DNase II-type enzymes was introduced by Cunningham and Laskowski (1953) in order to compare enzymes distributed in different tissues with their prototypes pancreatic DNase I (Kunitz, 1950) and spleen or thymic DNase II (Maver and Greco, 1948). Pancreatic DNase I has an optimum pH at about 7, requires magnesium ions for activity and is inhibited by citrate (Kunitz, 1950), whereas DNase II has a pH optimum at about 5 and is inhibited by magnesium ions (Maver and Greco, 1948).

Thus DNase II is an endonuclease which is specific for DNA and hydrolyses it to 3'-phosphate terminated oligonucleotides optimally at acid pH and without requiring a divalent metal ion. Cordonnier and Bernardi (1968) found that DNase II activity from a variety of different animal sources was associated with protein molecules similar in sedimentation coefficients, modes of action on native DNA, chromatographic behavior, and in enzymatic properties.

DNase II has been investigated with different goals in mind (Laskowski, 1961, 1967; Bernardi, 1968, 1971). DNase II has been studied with the purpose of elucidating its biological function. Since comparative DNase II activities of many fetal and adult tissue correspond in a general way with the capacity of those tissues for proliferation or regeneration (Allfrey and Mirsky, 1952), and since DNase II activity increases at that time in the cell cycle when DNA synthesis is taking place (Slor et al., 1973), DNase II may have an important biological function, the nature of which has not been firmly established. Physical, chemical and enzymatic studies of DNase II have dealt with the characterization of the enzyme as a protein and with the kinetics and specificity

of the enzymic reaction with DNA. DNase II has also been investigated in order to determine whether it could be used as a tool for probing the structure of DNA and chromatin.

The biological function of DNase II

Distribution in different tissues

A DNase II has been found in the cells of a number of animal tissues and species (Cordonnier and Bernardi, 1968; Bernardi, 1968; Laskowski, 1961, 1967; Private de Garilhe, 1967; Lehman, 1967). Cordonnier and Bernardi (1968) compared the chromatographic and enzymatic properties, the sedimentation coefficients, and the mechanism of action on native DNA exhibited by partially purified DNase II preparations obtained from 15 different animal sources. They concluded that the DNase II activity was associated with protein molecules endowed with very similar physical, chemical, and enzymatic properties. They also found that DNase II preparations from bull seminal plasma, hog serum, and liver and chicken erythrocytes, showed two peaks of activity upon chromatography on DEAE cellulose. A major component was not retained in low ionic strength buffer, and a minor one was eluted by a pH-molarity gradient. When Yamanaka et al. (1974) chromatographed DNase II activity from human gastric mucosa or cervix uteri on a phosphocellulose column, two peaks of DNase II activity appeared. The two activities had similar properties, but different isoelectric points. Zöllner et al. (1974) discovered two DNase II activities in extracts of human lymphocytes; one activity was similar to spleen DNase II, the other had a different electrophoretic

mobility, and showed no preference for native over denatured DNA.

Intracellular localization

DNase II is associated with the lysosomes in rat liver, with the related "droplets" from rat kidney, and with the related secretion granules in rabbit leucocytes (de Duve et al., 1962). The distribution of DNase II in mouse liver and pancreas, in rat brain, and in calf thymus suggest that DNase II is also lysosomal in these tissues (de Duve et al., 1962).

Lesca (1968) found a DNase II activity in the nuclear fraction of mouse liver cells and, since no cytochrome oxidase activity was found in the nuclear fraction, he concluded that the nuclear DNase II activity was not due to contamination of the nuclear preparation with lighter organelles. Shor and Lev (1971) found DNase II activity in purified calf thymus nuclei. During purification of the nuclei the activity ratio of DNase II to acid phosphatase, a lysosomal enzyme (de Duve et al., 1962), increased 30-40 fold indicating that the DNase II activity found in the nuclei was not due to lysosomal contamination. Slor (1973) purified ^{14}C-labelled DNase II from HeLa S3 lysosomes, and added it to cells during isolation of their nuclei. He found no specific binding of lysosomal DNase II to the nuclear fraction and concluded that the DNase II activity observed in isolated nuclei represented an intrinsic activity that might be involved in nuclear DNA metabolism.

Correlation of DNase II activity levels with rate of cell proliferation

Allfrey and Mirsky (1952) found that adult cells which do not divide, eg. heart, brain and red cells, had low DNase II levels whereas tissues capable of high mitotic activity, eg. spleen, intestinal mucosa, and liver, had high DNase II levels. Organs capable of partial regeneration like the kidney had intermediate DNase II concentrations. Fetal calf kidney was more than twice as active in DNase II as calf kidney; fetal liver had nearly 3 times the DNase II concentration of calf liver. Also the DNase II activity was higher in regenerating liver than in non-regenerating liver. Cordonnier and Bernardi (1968) confirmed that the highest DNase II levels were found in lymphatic and tumoral tissues which are capable of high rates of cell proliferation, and the lowest ones in cells like sperm cells and erythrocytes that do not reproduce themselves. The specific activity of DNase II in chicken brain (minus cerebellum) increases up to the 16th day of embryonic age and decreases after hatching (Shrivastaw et al., 1975). A similar pattern in DNase II activity has been reported in developing rat (Sung, 1968) and human (Rao-Subba, 1973) brains.

DNase II activity in malignant tissues

A four fold rise in DNase II activity levels in leukemia leukocytes relative to normal leukocytes was observed by Slor (1970a). Eschenbach (1971) found that acute bacterial and virus diseases caused an increase

in the DNase II activity in the cytoplasm of leukocytes in children. In children with acute leukemia the DNase II activity was decreased, but after therapeutic treatment, the number of leukocytes decreased and the DNase II activity increased.

Taper et al. (1971a) investigated the DNase II levels in benign and malignant tumors of the human central nervous system using a histochemical technique.* In the benign tumors, the DNase II activity was similar to that of those normal tissues from which the tumors originated; but in the malignant tumors no DNase II activity reappeared. Taper et al. (1971b) also measured DNase II activity histochemically in the rat liver parenchyma during N-nitrosomopholine-induced carcinogenesis. The DNase II activity was considerably decreased in focal areas and later in hyperplastic nodules, but was normal in the surrounding liver parenchyma. DNase II deficiency appeared at the 38th to 59th day of carcinogenesis, and preceded by approximately 56-75 days the morphological signs of cancer. In the necrobiotic cells of malignant hepatocellular tumors, a reappearance of DNase II activity was observed.

Bhattachaya et al. (1977) found a specific protein inhibitor of DNase II in mouse neuroblastoma cells. A marked decrease in the number of adhering viable cells, as evidenced by trypan blue staining, 24 hours after plating coincided with maximal DNase II activity. With the appearance of the DNase II inhibitor the DNase II activity diminished and, at the same time, the number of adhering viable cells increased.

The results suggest that although the amount of DNase II present in

*Similar results to those obtained for DNase II were found for DNase I and acid and alkaline RNases.

cancerous tissues may be high as expected in correlation with the rapid rate of proliferation of cancer cells, the DNase II activity in malignant tissues may be low *in vivo* due to the presence of a DNase II-specific inhibitor. A low level of DNase II activity may facilitate the incorporation or replication of abnormal nucleic acids that are able to induce malignant transformation. (Taper *et al.*, 1971b)

DNase II activity levels in relation to cell cycle

Slor *et al.* (1973) studied DNase II activity in relation to the cell cycle in synchronized HeLa S3 cells. A two to seven fold increase in DNase II activity occurred at those times when DNA synthesis was taking place, and the peaks of DNase II activity coincided with the peaks of DNA synthesis. The increase in DNase II activity was probably due to *de novo* enzyme synthesis since no increase in DNase II activity occurred when puromycin was added. Acid phosphatase, a marker for lysosomal enzyme (de Duve *et al.*, 1962), did not show an induction similar to that observed for DNase II in relation to the cell cycle. DNase II was assayed under conditions in which any DNase II-specific inhibitor present would not be detected. (Lesca, 1976)

Possible biological roles for DNase II

Since lysosomes appear to contain all the enzyme needed to degrade nucleic acids to nucleosides, and since DNase II activity has been found primarily in the lysosomes, a likely biological role for DNase II is a degradative one (Bernardi, 1971), probably performed in concert with

other lysosomal hydrolases, and associated with the many functions of lysosomes (Holtzman, 1976; Dingle and Fell, 1969, 1973).

DNase II might be involved in the protection of the genetic stability of normal cells against transforming nucleic acids (Taper et al., 1971ab). Spleen DNase II was found to be much more effective in inactivating transforming DNA from Haemophilus influenzae than DNase I, endonucleose I from Escherichia coli, or sonication (Bernardi and Bach, 1968).

DNase II may have a role in DNA metabolism in relation to cell division. Indirect support for this hypothesis comes from the correlation between DNase-levels and rate of DNA synthesis in vivo; the appearance, specifically in response to viral infection, of a large number and variety of DNases, and from the studies of DNA replication in vitro (Lehman, 1967; Lesca, 1971).

The enzymology of DNase II

The reaction of DNase II with DNA

Three different phases may be distinguished in the degradation of native DNA by DNase II (Bernardi, 1968).

The initial phase is defined as the phase in which the macromolecular and biological properties of DNA are modified, but in which no change occurs in the spectral properties of the DNA and no acid-soluble oligonucleotides are formed.

The middle phase is characterized by an increase in the absorbance of ultraviolet radiation, maximally at 260 nm , of the DNA solution being

digested with DNase II. Oligonucleotides which are soluble in an acidic solution such as 2% perchloric acid and which absorb ultraviolet radiation at 260 nm are also released from DNA in this phase.

The terminal phase shows a slow further increase in the absorbance of ultraviolet radiation at 260 nm of the DNA solution and a slow further release of acid-soluble oligonucleotides absorbing ultraviolet radiation at 260 nm.

The initial phase of the reaction

Studies done on the initial phase of the DNase II reaction with DNA have demonstrated that DNase II can cleave either one strand, or both strands of native DNA in each encounter. Oth et al. (1958) compared the rates of decrease of viscosity of DNA observed upon reaction of the DNA with DNase I and DNase II. The logarithm of the specific viscosity of DNA versus time was plotted during degradation by the DNases at pH 5.5 under conditions on similar activity. Under the action of DNase I the logarithm of the viscosity of the DNA decreased slowly and non-linearly in the initial stages of the reaction, but with DNase II the viscosity of the DNA decreased linearly and at the same rapid rate in the initial stages of the reaction as during the remainder of the reaction. Oth et al. (1958) suggested that since DNase I had been shown to cleave native DNA by random single strand scissions and since the DNase II reaction with DNA had a different viscosity curve than the DNase I reaction with DNA, DNase II might cleave both strands of the DNA simultaneously. Using electronmicroscopy MacHattie et al. (1963) observed a random distribution

of duplex fragments upon reaction of DNase II with DNA from bacteriophage T5. Using a light scattering technique Bernardi and Sadron (1964) followed the molecular weight decrease that occurred upon degradation of DNA with DNase II. They showed that DNase II could cleave either one or both strands of native DNA in each encounter, and estimated that the percent of total breaks which were double-strand breaks was 67%. Young and Sinsheimer (1975), using ultracentrifugation at neutral and alkaline pHs, demonstrated that DNase II degraded DNA from bacteriophage λ by double-strand cleavage, and also showed that one such cleavage per DNA-molecule destroyed the infectivity of the DNA. An average of four phosphodiester bonds could be hydrolysed by single-strand scissions by DNase I in a DNA molecule before the infectivity of the DNA was lost. Using the same ultracentrifugation technique in neutral and alkaline solutions, estimates of the number of double-strand cleavages as a percent of the total number of cleavages of DNA by DNase II ranged from 3% (Sicard et al., 1973) to 80% (Bernardi and Bach, 1968; Kopecka et al., 1973). Oshima and Price (1974) have suggested that a possible explanation for this range may be the presence or absence in the DNase II reaction mixture of divalent anions. They found that sulphate, malate, oxalate, citrate, and EDTA at low concentrations caused a 20% increase in DNase II activity. The percent of total breaks due to double-strand cleavage was found to decline from 80% in the absence of sulphate to 4% in the presence of concentrations of sulphate that activated DNase II.

Tsubota et al. (1974) examined the initial kinetics of DNase II from human gastric mucosa using as substrate twisted circular duplex DNA from bacteriophage λ.

The hydrolysis products were treated with ATP dependent DNase from Micrococcus luteus. This enzyme hydrolyses double-stranded DNA in the linear form to acid-soluble oligonucleotides, but does not attack circular duplex molecules (Takagi et al., 1972; Friedman et al., 1972). Sucrose density gradient sedimentation of the products revealed that gastric DNase II produced both open circular DNA and linear DNA at a very early stage of hydrolysis at 35° C. However, at 0° C, the gastric enzyme converted twisted circular duplex DNA to the open circular form by introducing single-strand scissions. These results suggest DNase II cleaves both strands of DNA at or near opposing nucleotides on adjacent strands not simultaneously, but by first making a scission in one of the strands, and then, in a secong reaction, making a second scission in the other strand of the DNA at or near the same level as for the first scission. These two reactions are coupled and occur in rapid succession at 37° C, but are uncoupled at 0° C (Tsubota et al., 1974).

The middle and terminal phases of the reaction

The kinetics of the middle and terminal phases of degradation of calf thymus DNA by hog spleen DNase II were investigated in detail by Soave and coworkers (1973). During the middle phase the ultraviolet absorbance, the acid solubility and the reciprocal average degree of polymerization, $\bar{P}n^{-1}$* of the oligonucleotides obtained by DNase II di-

* The average degree of polymerization, $\bar{P}n$ was taken as the absorbance at 271 nm of the nucleotides plus terminal nucleosides produced by digestion of oligonuceotides with spleen exonuclease divided by the absorbance at 271 nm of the terminal nucleosides alone.

gestion increased linearly with time.

When a DNA solution was digested with DNase II at 22° C, an increase in absorbance, a hyperchromicity, at 260 nm of the DNA solution and an absorbance at 260 nm due to acid-soluble oligonucleotide release first became apparent at a $\bar{P}n^{-1}$ value corresponding to a chain length of 100 nucleotides, thus defining in terms of average length of oligonucleotide, the limit between the initial and middle phases of the reaction. Since the $\bar{P}n^{-1}$ value of the initial DNA was very close to zero, it was concluded that initially a "preferred" class of nucleotide sequences, corresponding to about 8% of all susceptible sequences, was split, possibly by the double-strand cleavage mechanism, at a faster rate than the sequences split during the middle and terminal phases of the reaction (Soave et al., 1973). Melting curves were done on DNase II digests containing oligonucleotides of average length between 16 and 46 nucleotides. The melting temperatures of the oligonucleotides, and the resulting hyperchronicities at 260 nm, decreased with the decreasing average size of the fragments.

A comparison of the melting curves obtained for the starting DNA and for DNA degraded to a $\bar{P}n$ of 46 showed that the enzyme degradation caused a destabilization and a lowering of the Tm before any hyperchromicity was apparent. The total hyperchromic shift (enzymatic plus heating) of the oligonucleotides was found to be equal to a constant value for all samples, indicating that the hyperchromatic shift caused by the enzyme was essentially due to the melting of the fragments released, and that the contribution of phosphodiester bond cleavage was negligible in the range explored. The beginning of the terminal phase

is characterized by a sudden decrease in the reaction rate due to the progressive melting of double-stranded DNA fragments which takes place when the average size of the oligonucleotides reaches a threshold range of values. The single-stranded fragments so originated, though still containing a large number of susceptible sequences, are poor substrates for the enzyme which shows a marked preference for native over denatured DNA (Soave et al., 1973). The preceding argument may not be entirely correct, however, as the results that Soave et al. (1973) reported may be due more to the method of measurement than to an actual abrupt slowdown in reaction rate. Since hyperchromicity was found to be primarily due to melting of double-stranded DNA fragments, and since at the start of the terminal phase most of the DNA digest is single-stranded and acid-soluble, further cleavage by the enzyme would not be reflected by as much hyperchromicity or acid-soluble oligonucleotide liberation as was observed previously. That the reaction does slow down was shown by chromatography of DNase II digests of DNA on DEAE cellulose columns in the presence of urea. Even after prolonged digestion with large amounts of enzyme most of the digestion mixture was composed of oligonucleotides with chain lengths greater than 5 nucleotides long (Soave et al., 1973). It is likely that the decrease in the rate of single-strand cleavage by DNase II in the terminal phase is due to the production of progressively more resistant substrates as smaller and smaller DNA fragments are formed (Laskowski, 1967).

The structure of DNase II

Physical and Chemical Properties

DNase II activities have been isolated from many different tissues and appear to have similar properties to those of spleen DNase II (Cordonnier and Bernardi, 1968). Spleen DNase II is a basic globular protein having a molecular weight of about 38,000, and containing a carbohydrate moiety, and an even number of amino acid residues for all the amino acids which were present at low levels (Bernardi et al., 1965). Because of this latter finding and because what appeared to be monomer and dimer subunits were observed on sedimentation analysis of DNase II in naturing and denaturing solvents, Bernardi (1965) suggested that DNase II was composed of two probably identical subunits. This subunit structure was consistent with the double-strand cleavage mechanism for DNase II since one subunit could cleave one strand of the DNA at the same time as the other subunit cleaved the apposing strand (Bernardi, 1968).

However, Townend and Bernardi (1971) later found that the "monomers" that were previously observed in denaturing solvents (Bernardi, 1965) were of a molecular weight that was very close to that of the native enzyme molecule. Furthermore, the enzyme molecule could not be dissociated into subunits under a variety of conditions which are extremely effective with other proteins. Oshima and Price (1973) found that iodoacetate alkylated a single histidine residue in DNase II with complete destruction of enzymatic activity. This is more compatible with a mono-

mer structure for the enzyme, or at least with a single active site.

Lesca (1976) has found that beef liver DNase II is a complex of molecular weight 45,000 comprised of a catalytic subunit of molecular weight 26,500 and an inhibitor subunit of molecular weight 21,500. He found that the inhibitor subunit bound strongly and specifically to DNase II bound covalently to a Sepharose 4B column and could only be eluted by a solution of guanidine hydrochloride. Slor (1974) showed that the DNase II specific inhibitor in crude and partially purified extracts of mouse liver, spleen and kidney could be selectively denatured by heating for 30 minutes at 50° C, or by lowering the pH of the solution to 2.5. Such treatments did not denature the DNase II activity and highly purified DNase II contained no inhibitor (Slor, 1974). Since highly purified DNase IIs from different tissues appear to have molecular weights of from 38,000 to 45,000 (Bernardi, 1968, 1971; Dulaney and Touster, 1972; Oshima and Price, 1973), and have usually been prepared with procedures involving acidification to pH 2.5, it remains an open question whether these purified DNase IIs contain an inhibitor subunit complexed to a catalytic subunit, or whether the inhibitor protein has been inadvertently removed or inactivated during the purification procedures. A possible reason that a DNase II specific inhibitor has not been found by many laboratories is that, at the high DNA concentrations used for the DNase II assays based on hyperchromicity of a DNA solution or acid-soluble oligonucleotide release, the inhibitor protein is competitively displaced from the catalytic subunit by the DNA (Lesca, 1976).

In order to be able to follow the DNase II reaction at the low con-

centrations of DNA that are required to observe the effects of the inhibitor it is necessary to use radioactive DNA (Lesca, 1969, 1976; Slor, 1974).

Catalytic properties

DNase II activity is influenced by pH, ionic strength, mono- and divalent cations and anions, substrate concentration, and the presence of foreign proteins (Laskowski, 1961, 1967; Bernardi, 1968, 1971). The enzyme may be affected directly through an influence on its structure or catalytic groups, indirectly by stabilization or destabilization of the double-stranded structure of the DNA substrate, or not at all, the apparent effect being due to an artifact of the assay procedure used. For example, when DNase II activity is assayed by measuring the release of acid-soluble oligonucleotides the optimal DNA concentration is 0.4 mg/ml (Bernardi and Griffe, 1964) and higher subtrate concentrations appear to be inhibitory (Oth et al., 1958; Bernardi, 1965; Rosenbluth and Sung, 1969). This is so because an increased DNA concentration results in fewer breaks per unit length of DNA and decreases the efficiency of acid-soluble oligonucleotide release. If a more direct method of estimating enzyme activity is used, such as the determination of phosphatase-sensitive phosphate, it can be shown that "inhibition" by high substrate concentration is an artifact of the assay procedure measuring the release of acid-soluble oligonucleotides (Rosenbluth and Sung, 1969). Magnesium ion at a concentration of 1mM activates DNase II indirectly due to stabilization of the double-stranded structure of DNA, the pre-

ferred substrate for DNase II over denatured DNA. Higher concentrations of magnesium ion inhibit DNase II directly. Complications arise when DNA preparations used for assay of DNase II contain endogenous magnesium ion and when DNase II preparations of different purity are compared since the inhibitory effects of magnesium ion increase with the increasing purity of the DNase II preparation (Cordonnier and Bernardi, 1968).

Because their preparation of DNase II from hog spleen catalysed the slow hydrolysis of bis-(p-nitrophenyl) phosphate and the p-nitrophenylesters of deoxyribonucleoside 3'-phosphate, but not those of deoxyribonucleoside 5'-phosphate, Bernardi and Griffe (1964) concluded that this "phosphodiesterase" activity of DNase II was an intrinsic property of the enzyme molecule. Hodes et al. (1967) prepared DNase II from mouse liver and found the the purified enzyme was free of nonspecific phosphodiesterase activity. Swenson and Hodes (1969) separated the phosphodiesterase and DNase II activities of bovine spleen by heating the crude preparation prior to chromatographic purification. Although Sicard et al. (1970) could not separate the phosphodiesterase and DNase II activities of a purified preparation of hog spleen DNase II, Slor (1970b) found that DNA did not inhibit the nonspecific phosphodiesterase activity of hog spleen DNase II, and bis(p-nitrophenyl) phosphate did not inhibit the DNase II activity. He concluded that the nonspecific phosphodiesterase activity observed with the highly purified DNase II of Bernardi and Griffe (1964) was probably a contaminant.

Lesca (1976) was able to show that the nonspecific phosphodiesterase was a contaminant of beef liver DNase II by separation of the phosphodiesterase (MW 59,000) from DNase II using affinity chromatography on

DNase II-succinylaminooctyl-Sepharase 4B.

When purified DNase II (ie: the catalytic subunit) was assayed alone, Michaelis-Menten kinetics were observed (Lesca, 1976). A sigmoid-shaped curve of velocity versus substrate concentration was observed for the enzyme in the presence of inhibitor at pH 5.0. The enzyme-inhibitor interaction disappeared progressively with a small pH shift from 5.0 to 5.57.

Little work has been done on the active site of DNase II. Melzer (1969) suggested that the following amino acids might be involved in the enzyme's activity: tryptophan since N-bromosuccinimide inhibited the enzyme, methionine and/or histidine since iodoacetate and hydrogen peroxide were inhibitory whereas beta-butyrolacetone was not, ruling out the involvement of cysteine. Since diisopropylfluorophosphate was relatively ineffective as an inhibitor, it was concluded that serine residues were probably not very important to the enzymatic activity.

Oshima and Price (1973) found that DNase II could be inactivated completely by iodoacetate whereas iodoacetamide was without effect, and that the loss of enzyme activity paralleled the incorporation of one carboxymethyl group per enzyme molecule. The inactivated protein was separated from active enzyme by chromatography on phosphocellulose, and contained one residue of 3-carboxymethylhistidine as the sole product of the iodoacetate reaction. DNA at a concentration of 0.5 mg per ml protected the enzyme from inactivation by iodoacetate suggesting that the active site of the enzyme contains an essential histidine (Oshima and Price, 1974).

The use of DNase II for the study of the structure of DNA and chromatin

Investigation of the primary structure of DNA

DNase II enzymes split short nucleotide sequences in DNA, and can be used to assess the frequency of these sequences in a given DNA (Bernardi, 1973). The number of sequences split is very large, in the range of 20-50% of all sequences, and the susceptible sequences probably overlap to some extent (Bernardi, 1973). Since the frequency of nucleosides at the 5'- and 3'- terminal positions of fragments formed by the action of DNase II on DNA changes with the extent of degradation (Vaneko and Laskowski, Sr., 1962), there may be one or more "preferred" sequences that are split at faster rate than the remaining sequences, and progressively exhausted in the initial phase of the reaction (Soave et al., 1973).

The specificity of DNase II has been investigated primarily in the middle and terminal phases of the reaction. DNase II recognizes a large number of different sequences in these phases, and has not been used in sequencing DNA since the DNA fragments produced would be too small and too variable in sequence. Although DNase II may be more specific in the initial phases of the degradation of DNA, the base specificity of DNase II in the initial phase of the reaction has not been extensively investigated due to the technical difficulties of determining relatively small quantities of terminal nucleosides in DNA fragments of length greater than 100 base pairs (Vaneko and Laskowski, Sr., 1962). It has

recently become feasible to determine the terminal nucleotides of long oligonucleotides because of the development of methods of radioactively labelling the termini of long DNA fragments and of analysing the nucleotide sequences (see Methods in Enzymology 29, 1974).

Investigation of the structure of chromatin

Digestion with micrococcal nuclease (EC 3.1.4.7) of DNA in isolated nuclei has revealed the existence of a periodic structure in chromation, consisting of nucleosomes, each one containing about 200 base pairs of DNA (Noll, 1974a). A different type of periodicity, based on a 10 nucleotide repeating unit size, has been found in each strand of DNA in chromatin using DNase I (EC 3.1.4.5) (Noll, 1974b). Yaneva and Dessev (1977) studied the action of DNase II on DNA in chromatin, and found that the formation of acid-soluble products followed a two-phase kinetic curve. At the end of the more rapid phase about 25% of the DNA was degraded. Early in the degradation DNA was converted into double-stranded fragments, whose sizes were multiples of about 180 base pairs. As the degradation proceeded these fragments were reduced in size. The fragments contained single-strand nicks and, under denaturing conditions the DNA was resolved into discrete single-stranded fractions, which were exact multiples of a ten-nucleotide length and formed a pattern very similar to that observed with DNase I.

Altenburger et al. (1976) used DNase II as a probe into the conformational states of chromatin . Upon digestion of mouse liver chromatin

with spleen DNase II a shift in the products from a 200 to a 100 base pair repeat pattern occurred after prior condensation of the chromatin by divalent or monovalent cations.

Intestinal DNase II

In rat liver about 0.7% of the total number of cells is newly formed in one day, whereas in rat small intestine about 43% of the total cell population is newly formed per day. (Stevens et al., 1953). In intestine new epithelial cells continuously arise by mitosis in the crypts of Lieberkühn, move towards the lumen of the intestine along the walls of the villi and are finally extruded from the tips of the villi into the lumen about 24 hours after mitosis. (Wilson, 1962). Desquamated epithelial cells in the lumen of the intestine are responsible for many of the enzymes found free in the intestinal tract, and intestinal intracellular enzymes such as DNase II may, after autolysis, be involved in degrading dietary nucleic acids in the small intestine prior to absorption as the nucleoside and the free base components. (Wilson, 1962). Thus DNase II may have an additional digestive function in the small intestine.

DNase II may also have a role in maintaining the highly proliferative, but differentiated state of the normal intestinal mucosa since Allfrey and Mirsky (1952) found a correlation between the higher rate of desquamation and the higher DNase II activity in adult mucosa as opposed to lower rates for both in fetal intestinal mucosa. Lieberman

et al. (1971) separated crypt and villus cells of rat small intestine by a crude scraping method; found that DNase II activity was associated with the crypt cell preparation, and suggested that DNase II plays a role in the metabolism of the actively dividing crypt cells rather than a digestive one.

Stewart and Zbarsky (1963) reported the loss of nucleic acids, particularly DNA, from mucosal scrapings or slices from rat intestine during incubation of the preparations in vitro in Krebs Ringer phosphate buffer, pH 7.8. A crude cell-free extract of the mucosal tissue in Krebs-Ringer phosphate buffer was found to have high levels of DNase activity (Lee and Zbarsky, 1967). Two DNases were subsequently demonstrated to be present in cell-free extracts of rat intestinal mucosa (Lee, Lawrence and Zbarsky, 1972). One DNase had optimum activity at neutral pH and required magnesium ion for activity, and thus was a DNase I (Kunitz, 1950). The second enzyme had an acidic pH optimum and showed no requirement for magnesium ion and thus was a DNase II (Maver and Greco, 1948).

The present investigation

This research was begun in order to investigate the properties of intestinal DNase II and the nature of the enzymic reaction with DNA. DNase II was first isolated from extracts of rat intestinal mucosa. However, the amount of DNase II obtained after three purification steps was too little to be used for further purification and then for enzymatic

studies. In order to obtain larger amounts of enzyme, DNase II was isolated from bovine intestinal mucosa. Upon chromatography of a crude extract of intestinal mucosa on a DEAE cellulose column, two peaks of DNase II activity were observed. The first DNase II had properties similar to those of DNase II enzymes which have been isolated from many different tissues and purified and characterized to various degrees. (Laskowski, 1961; 1967; Bernardi, 1968, 1971). The second DNase II activity was similar to other DNase II activities which have been isolated from only a few tissues and studied only to a small extent. (Cordonnier and Bernardi, 1968; Yamanka et al., 1974; Zöllner et al., 1974). DNase II activity has been found associated with lysozomes (de Duve et al., 1962), and presumably with a degradative function (Bernardi, 1971), and with nuclei (Lesca, 1968; Slor and Lev, 1971; Slor, 1973) and a possible role in DNA metabolism in relation to cell division (Slor et al., 1973). The DNase II activities associated with separate organelles and with diverse biological functions could be due to distinct enzymes with different properties.

The properties of the two intestinal DNase II activities were compared in order to determine whether the activities were due to the same or different enzymes. Different amounts of inhibition of the two DNase II activities by ionic strength and by sulphate, an inhibitor of DNase II, were observed. The first DNase II degraded native DNA at a more rapid rate than denatured DNA whereas the second activity degraded both native and denatured DNA at the same rate. The activation energies for the hydrolysis of DNA by the two DNase II activities were also different.

Further investigation revealed, however, that the appearance of a second DNase II activity was probably due to the binding of a small amount of DNase II to DNA which had been bound to the DEAE cellulose column. The observation of different properties for the two DNase II activities was likely due to the presence of DNA in the second DNase II preparation.

DNase II enzymes from other tissues have been investigated in order to evaluate their usefulness as tools for sequencing DNA. Vaneko and Laskowski, Sr. (1962) have presented evidence that the base specificity of DNase II cleavage of DNA changes as the reaction progresses. Bernardi et al. (1973) analysed products from the middle and terminal phases of the DNase II reaction and found that the base specificity did not vary much in these phases. DNase II recognizes a large number of nucleotide sequences in DNA, but there may be a preferred class of sequences that is exhausted in the initial phase of the reaction (Soave et al., 1973). The reaction of intestinal DNase II with DNA was studied in order to determine whether the enzyme had a different base specificity than DNase IIs from other tissues, whether the base specificity changed as the reaction progressed, and whether there was any preferred class of sequences that were cleaved in the initial phase of the reaction. Intestinal DNase II was found to have a similar base specificity as spleen DNase II. The base specificity changed as the reaction progressed, primarily in the initial and terminal phases of the reaction. No evidence was obtained for a greatly increased specificity of cleavage of DNA by DNase II in the early stages of the reaction. The studies of the base specificity of the cleavage of DNA by DNase II suggest that the enzyme

recognizes a large number of nucleotide sequences in DNA and that some sequences are preferred over others, particularly GpG in the initial stages of the reaction.

Intestinal DNase II was investigated in order to compare its enzymic properties with those of DNase II enzymes from other tissues. The chromatographic behavior, pH optimum, activity in the presence of different ions, molecular weight, and mode of action on DNA of intestinal DNase II were similar to the respective properties of DNase II enzymes isolated from other tissues.

DNase II was also studied in vitro in order to provide a basis for future research into the biological role of DNase II in the metabolism of intestinal mucosa, a differentiated yet rapidly proliferating tissue, in vivo.

MATERIALS AND METHODS

Materials

Chemicals which were used in this investigation are listed in Table I; chromatographic media, in Table II; enzymes, and animals, in Table III.

TABLE I

CHEMICALS USED IN THE INVESTIGATION

Material	Abbreviation	Manufacturer/Supplier	Comments
diisopropylfluorophosfate	DFP	Aldrich Chemical Co., Milwaukee, Wis.	nerve poison since inhibits acetylcholinesterase (Aldridge, 1953) antidote: atropine sulfate, 2 mg i.m. q 15 min.
phenylmethylsulfonyl fluoride	PMS	Sigma Chemical Co., St. Louis, Mo.	does not inhibit acetylcholinesterase (Fahinez & Gold, 1973) but less effective in inhibiting proteases due to low solubility in aq. solution
acrylamide		Eastman Kodak Co., Rochester, N. Y.	acrylamide and bis were recrystallized from chloroform and aetone, respectively, as described by Loening (1967)
N, N' methylene bisacrylamide	bis		
riboflavin			
ammonium persulfate		E. C. Apparatus Co., Philadelphia, Pa.	
N, N, N', N' tetramethylethylene diamine	TEMED	Matheson, Coleman and Bell, Norwood, Ohio	
B-alanine		"	
Coomassie Brilliant Blue		Schwartz-Mann, Orangeburg, N. Y.	
protamine sulfate			
agar		Difco Laboratories, Detroit, Mich.	

TABLE I--Continued

Material	Abbreviation	Manufacturer/Supplier	Comments
2,5-diphenyloxazole	PPO	Kent Laboratories, Vancouver, B. C.	
p-bis[2-(4-methyl-5-phenyl oxazolyl)] benzene	dimethyl POPOP	Packard InstrumentsCo., La Grange, Ill.	
bovine serum albumin fraction V	BSA	Armour Pharmaceutical Co., Chicago, Ill.	
tris (hydroxymethyl) amino-methane	tris	Sigma Chemical Co., St. Louis, MO.	
dithiothreitol	DDT	"	
the 4 major 2'-deoxy-ribonucleoside-3'-phosphates	3'-dNMP's	"	The DNA's were dissolved in a 10mM sodium chloride solution by stirring at 4° C over a one to two day period. The calf thymus DNA was dissolved at a concentration of 4 mg per ml so that the same solution of DNA could be used for 3 different DNase II assays (see Methods). The other DNA's were dissolved at a concentration of o.4 mg per ml so that a ten-fold dilution would give a solution of about the optimal absorbance for measurement of DNase II activity by the hyperchromicity assay (see Methods)
calf thymus DNA type I		"	
salmon DNA type III		"	
Escherichia coli DNA type VIII		"	
bacteriophage λ DNA	λ DNA	"	

TABLE I--Continued

Material	Abbreviation	Manufacturer/Supplier	Comments
polydeoxyadenylate-(deoxy)thymidylate	polydAT	Miles Laboratories, Elkhart, Ind.	
yeast RNA		"	
adenosine-3'-phosphate	3'-AMP	"	
p-nitrophenyl thymidine-5'-phosphate	P-NO_2ØpT	Calbiochem, La Jolla, Calif.	
the 4 major 2'-deoxyribonucleoside-5'-phosphates	5'-dNMP's	"	
disodium p-nitrophenylphosphate	p-NO_2ØP	Raylo Chemicals Ltd., Edmonton, Alta.	
p-nitromethylthymidine-3'-phosphate	p-NO_2ØTp	"	
polydeoxyadenylate	polydA	P. L. Biochemicals Inc. Milwaukee, Wis.	
oligo(deoxythymidylate)$_{10}$	oligo(pT)$_{10}$	"	
Norit A		Fischer Scientific Co.	
Triethylammonium bicarbonnate	TEAB	"	Prepared by bubbling carbon dioxide through a 1 molar solution of redistilled triethylamine until the pH decreased to 7.5 (Porath, 1955)

TABLE I--Continued

Material	Abbreviation	Manufacturer/Supplier	Comments
Casein (Technical grade)		Nutritional Biochemical Corp., Cleveland, Ohio	
thymidine[methyl-^{3}H]		New England Nuclear, Boston, Mass.	Contained at least 85% thymidine-^{3}H as determined by thin layer chromatography on MN 300 cellulose plates run in distilled water according to Randerath and Randerath (1967) (thymine, Rf, o.65; thymidine Rf, 0.80)
sonicated ^{3}H DNA from Escherichia coli B	^{3}H DNA	Miles Laboratories Elknart, Ind.	Specific Activities: ^{3}H DNA 127 uCi/umole phosphate
[C_8-^{3}H] polydeoxyadenylic acid	polydA-^{3}H	"	polydA-^{3}H 31.4 "
methyl ^{3}H polythymidylate	polydT-^{3}H	"	polydT-^{3}H 55.7 "
polydeoxyadenylate-thymidylate (methyl-^{3}H)	polydAT-^{3}H	"	polydAT-^{3}H 19.8 "
α-^{32}P labelled adenosine triphosphate	[α-^{32}P] ATP	New England Nuclear or Amersham/Searle	Specific activities of from 2 to 20 Ci/umole
γ-^{32}P labelled adenosine triphosphate	[γ-^{32}P] ATP	"	
^{32}P labelled inorganic phosphate	^{32}Pi	"	

TABLE II

CHROMATOGRAPHIC MEDIA USED IN THE INVESTIGATION

Materials	Manufacturer/Supplier	Comments
MN 300 cellulose plates (20 x 20 cm)	Schleicher and Schwell Inc. Keene, N. H.	
polyethyleneimine impregnated MN 300 cellulose plates (20 x 20 cm x 0.1mm)	Brinkman Instruments (Canada) Ltd. Toronto, Ont.	When samples with Rf's greater than 0.7 were to be chromatographed, the plates were prerun with distilled water to remove a yellowish material which accumulated on the plates during storage and which ran near the solvent front.
Whatman Chromatography Products. DE 81 cellulose paper and ion exchange celluloses DEAE 22, 32, CM 22, 32, and phosphocellulose (P11)	Mandel Scientific Co. Ltd. Montreal, Quebec	DEAE and CM cellulose were precycled with 0.5N NaOH and 0.5N HCl according to the manufacturer's instructions and were equilibrated with the starting buffer (with respect to both pH and conductivity) before use.
Sephadex G25, G50 and G100 fine, sulfopropyl (SP) Sephadex G50 and G100 and sulfoethyl (SE) Sephadex C25	Pharmacia (Canada) Ltd. Dorval, Quebec	The Sephadex media were swollen in starting buffer for the recommended times and the ion exchange Sephadexes were equilibrated with starting buffer before use.
Molecular weight calibration kit: RNase A, MW 13,700 chymotripsinoglo A, MW 25,000 ovalbumen, MW 45,000 aldolase, MW 158,000	"	

TABLE II--Continued

Materials	Manufacturer/Supplier	Comments
blue dextran, MW 2 x 10^6	Pharmacia (Canada) Ltd. Dorval, Quebec	
hydroxyapatite	Bio-Rad Laboratories, Richmond, Calif.	
Munktell 410 cellulose	"	
DNA cellulose	"	DNA cellulose was prepared with native and with denatured calf thymus DNA and Munktell 410 cellulose by the method of Alberts and Herrick (1971).

TABLE III

ENZYMES AND ANIMALS USED IN THE INVESTIGATION

Materials	Manufacturer/Supplier	Comments
trypsin	Schwartz-Mann Orangeburg, N. Y.	Specific Activity
DNase I (EC 3.1.4.5) from bovine pancreas	P. L. Biochemicals Milwaukee, Wis.	52,000 units/mg by the method of Kunitz (1950)
DNase II (EC 3.1.4.6) from hog spleen	"	52,800 units/mg " "
alkaline phosphatase (EC 3.1.3.1) from Escherichia coli	"	47 units/mg " "
acid phosphatase (EC 3.1.3.2) from potato	"	67 units/mg " "
phosphodiesterase I (EC 3.1.4.1) from Crotalus adamentus venom	Worthington product purchased from ICN Canada Ltd. Montreal, Quebec	1.06 units/mg Inactivation of contaminating 5'nucleotides was done by the method of Sulkowski and Laskowski (1971). After treatment 96% of the original phosphodiesterase activity and 3.3% of the 5' nucleotidase activity remained.
phosphodiesterase II (EC 3.1.4.18) from spleen	"	0.32 units/mg with the assay using p-NO_2ØTp 22 units/mgwith the assay measuring the conversion of 3'-phosphate oligonucleotides to 3'-phosphate nucleotides.

TABLE III--Continued

Materials	Manufacturer/Supplier	Comments
RNase A (EC 2.7.7.16) from bovine pancreas	Sigma Chemical Co. St. Louis, Mo.	76.5 units/mg
polynucleotide kinase (EC 2.7.1.78) from T4 x α-1 infected E. coli B	P. L. Biochemicals Milwaukee, Wis.	8,700 units/mg where one unit catalyses the transfer of 1 mole of phosphate from ATP to polynucleotide in 30 min. at 37^{o}C
terminal transferase (EC 2.7.7.31) from calf thymus	Miles Laboratories Elkhart, Ind.	2,500 units/mg where one unit catalyses the incorporation of 1 mole of dAMP into acid insoluble material in 1 hour at 37^{o}C
male Wistar rats	Animal unit of the University of British Columbia.	The rats' weight was 180-200 g after 24 hours of starvation.
cattle	Intercontinental Packers 8950 Shaunessy, Vancouver	Small intestines were removed from cattle about 10-15 minutes after slaughter and were tied off, placed in an ice-water bath and transported to the university

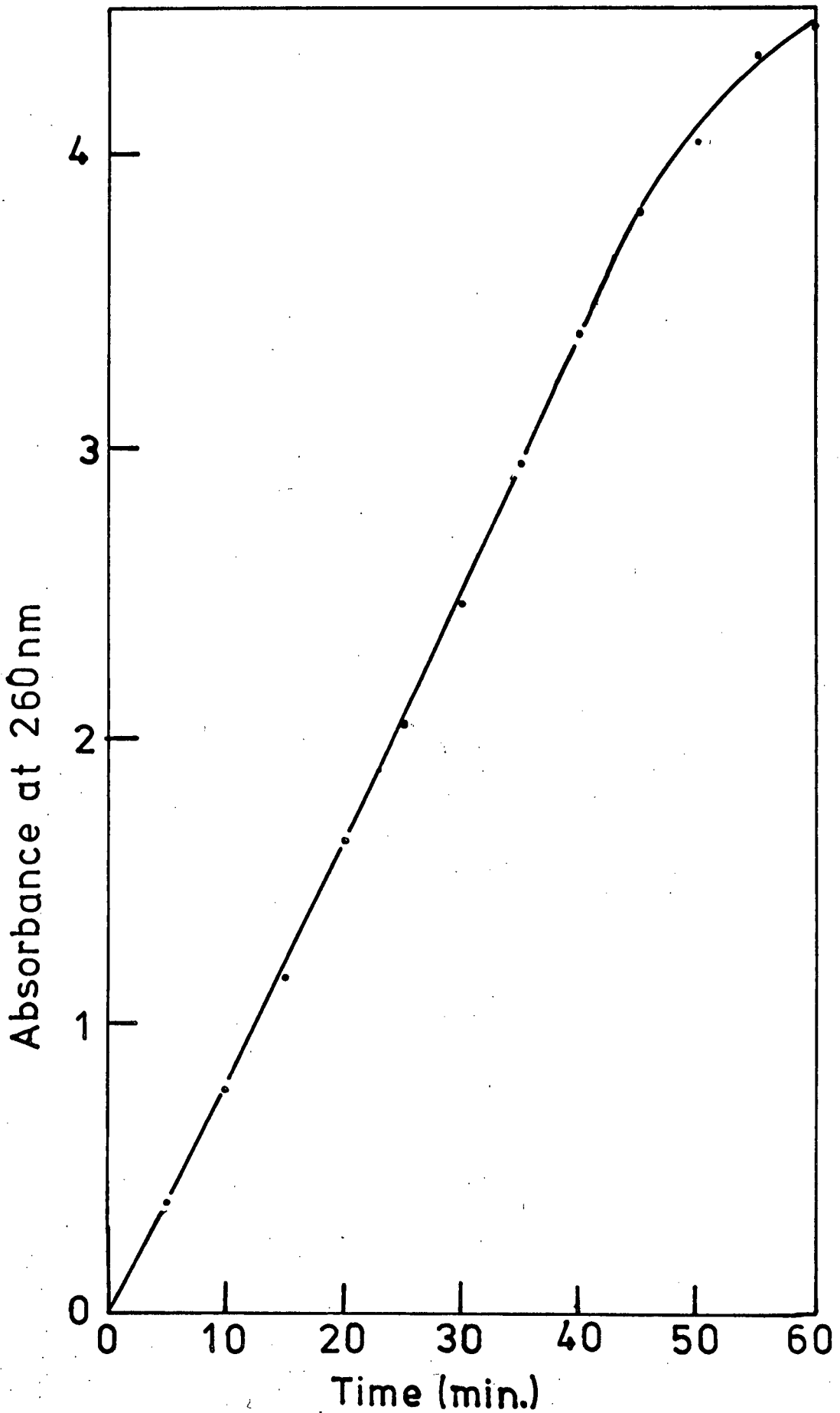
Methods

Measurement of DNase II activity

Acid Soluble oligonucleotide assay

This assay was used to determine DNase II activity by measuring the absorbance at 260 nm of the acid-soluble oligonucleotides liberated from DNA upon digestion with the enzyme. (Bernardi, 1964). DNase II was incubated at 37°C with 400 ugm of calf thymus DNA in 120 mM sodium acetate buffer pH 5.0 containing 8 mM ethylenediamininetetracetate (EDTA) in a total volume of 1.0 ml. After periods of time which ranged from 5 to 30 minutes, depending on the activity of the enzyme, 0.5 ml of cold 6% perchloric acid was added to stop the reaction. The reaction mixture was chilled in an ice-water bath for 10 minutes and then centrifuged at 12,000 xg for 10 minutes in a Sorvall RC-2B refrigerated centrifuge. The absorbance at 260 nm of the supernatant solution was measured with respect to a substrate blank in which the DNA had been incubated without enzyme, the perchloric acid added, and then the enzyme added. The readings were also corrected for dilution with perchloric acid. As illustrated in Fig. 1, the absorbance at 260 nm of the acid-soluble oligonucleotides released increased linearly with time between the absorbance values of 0.8 and 3.5. When lower concentrations of enzyme were used, there was an initial lag phase in which the increase in absorbance was not linear with time. This lag phase was due to the

Fig. 1 Release of acid-soluble oligonucleotides from calf thymus DNA by DNase II. DNase II was incubated at 37°C with 400 ugm DNA in 1.0 ml of 120 mM sodium acetate buffer, pH 5.0, containing 8 mM EDTA. The absorbance at 260 nm of the oligonucleotides which were soluble in 2% perchloric acid was measured with respect to a substrate blank solution in which the enzyme had been added after the perchloric acid.

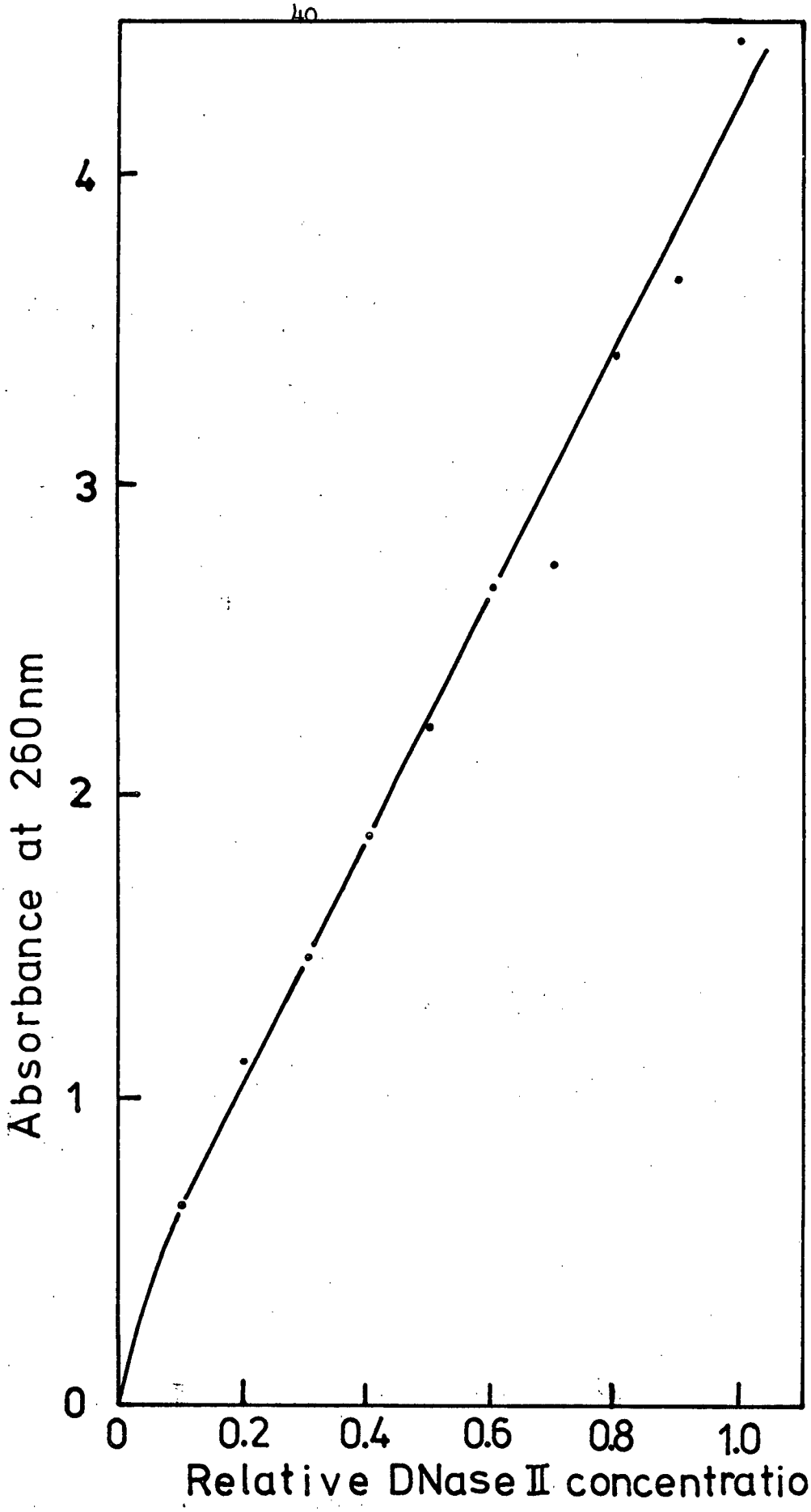

Absorbance at 260nm
0
1
2
3
4
0
10
20
30
40
50
60
Time (min.)

hydrolysis of DNA by DNase II initially to fragments which were not acid-soluble (Rosenblüth and Sung, 1969).

The effect of enzyme concentration upon the release of acid-soluble oligonucleotides from DNA is shown in Fig. 2. The absorbance at 260 nm of the oligonucleotides released increased linearly between the absorbance values of 0.8 and 3.5 as the enzyme concentration was increased. In this linear range the acid-soluble oligonucleotide assay can be used to measure DNase II activity in both crude and purified preparations of DNase II because the acid precipitation procedure clarifies murky solutions so that the absorbance can be read without interference. The assay has therefore been used to measure DNase II activity at different stages of the purification procedure in order to estimate the extent of purification and to compare the properties of different DNase II preparations.

One unit of DNase II activity is defined as the amount of enzyme that liberates acid-soluble oligonucleotides with an absorbance of 1.0 at 260 nm in 1 minute under the conditions of this assay. The specific activity was obtained by dividing the enzymic activity by the milligrams of protein present in the same volume of solution.

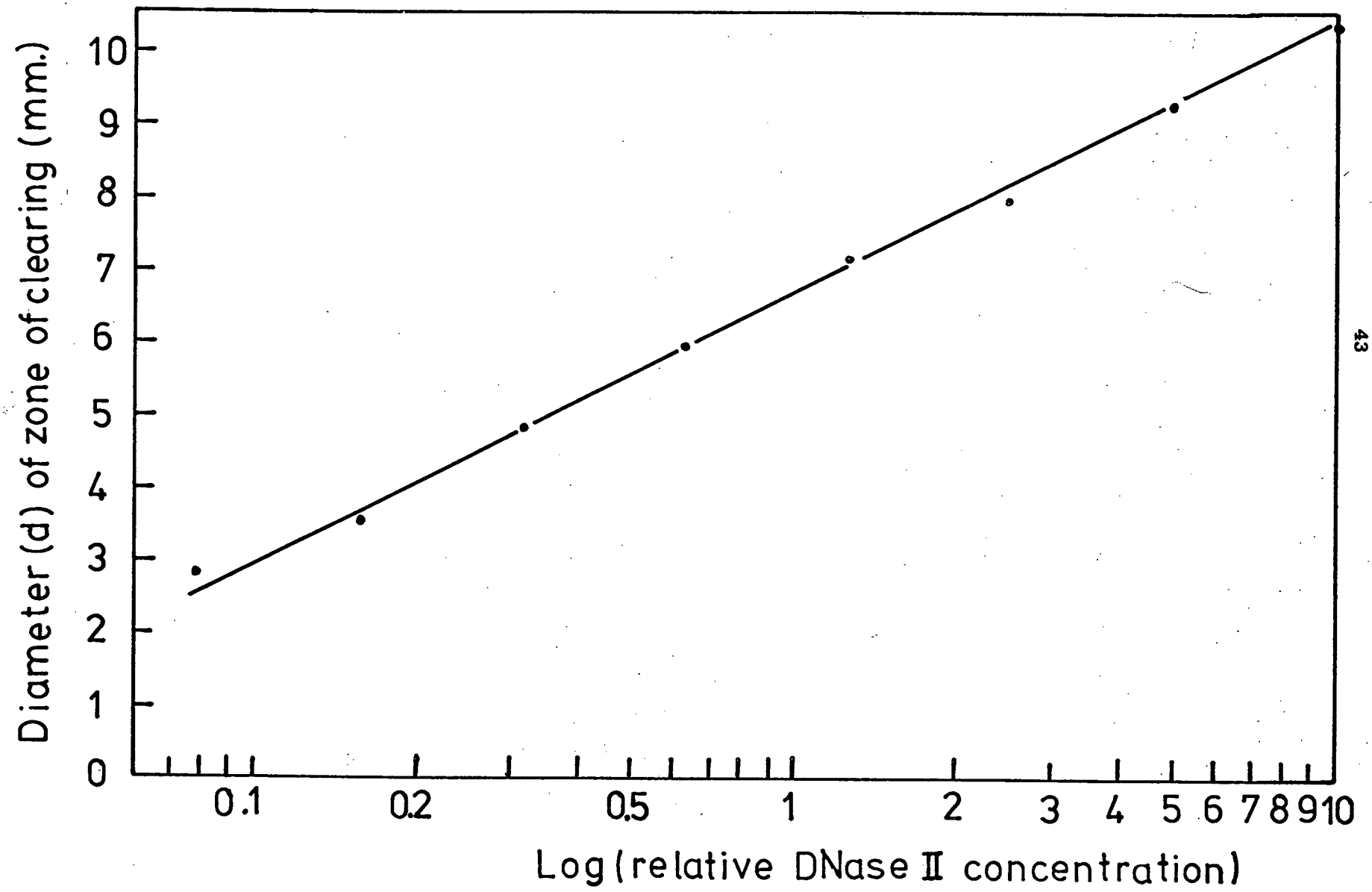
Fig. 2 Effect of different concentrations of DNase II on the release of acid-soluble oligonucleotides from calf thymus DNA. DNase II was incubated for 20 minutes at 37°C with 400 ugm DNA in 1.0 ml of 120 mM sodium acetate buffer, pH 5.0, containing 8 mM EDTA and the absorbance at 260 nm of the acid-soluble oligonucleotides released was measured.


Absorbance at 260nm
0
1
2
3
4
0
0.2
0.4
0.6
0.8
1.0
Relative DNase II concentration

DNA-agar gel assay

This assay is a modification of the assay used by Jarvis and Lawrence (1969) to measure DNase I activity. The diameter of the zone of clearing produced by the action of DNase II on DNA embedded in agar gel was measured after the unhydrolysed DNA had been precipitated with hydrochloric acid. Agar, 200 mg, was dissolved in 15 ml of 150 mM sodium acetate buffer, pH 5.0, containing 10 mM EDTA by heating the mixture until the boiling temperature was just reached. The solution was allowed to cool to about 60°C, and 5 ml of a solution containing 20 mg calf thymus DNA in 10 mM NaCl were added. Three millilitre portions of the resulting solution were pipetted onto microscope slides over areas of 2 by 3 inches bordered by cellulose tape. After the gels had set, holes 2 mm in diameter were punched (with a gel puncher from Biorad), and 4 ul portions of the DNase II containing solutions were added to the wells. The slides were placed in a covered plastic box containing a moist paper towel, incubated at 37°C overnight, and then immersed in 1 N HCl for a few minutes. In Fig. 3 it may be seen that the diameters of the zones of clearing, (minus the diameters of the holes) were proportional to the logarithms of the enzyme concentrations. Since a large number of solutions could be assayed for DNase II simultaneously, this method was used to measure DNase II in fractions from column chromatography. Because the diameter of the zone of clearing varied as the logarithm of the DNase II concentration, a small error in the determination of a diameter resulted in a larger relative error in the calculation of the corresponding DNase II concentration. Therefore, the

Fig. 3 The linear relationship between the logarithm of the DNase II concentration and the diameter of the zone of clearing determined by the DNA agar gel assay. Different dilutions of the same solution containing DNase II activity were incubated at 37°C overnight with 0.1% calf thymus DNA embedded in 1% agar gel in sodium acetate buffer, pH 5.0 containing EDTA. The unhydrolysed DNA was precipitated with 1 N HCl and the diameters of the zones of clearing (minus the diameters of the wells in which the enzyme solutions had been placed) were found to be linear with the logarithms of the DNase II concentrations. This assay is a modification of the assay used by Jarvis and Lawrence (1969) to measure DNase I activity.

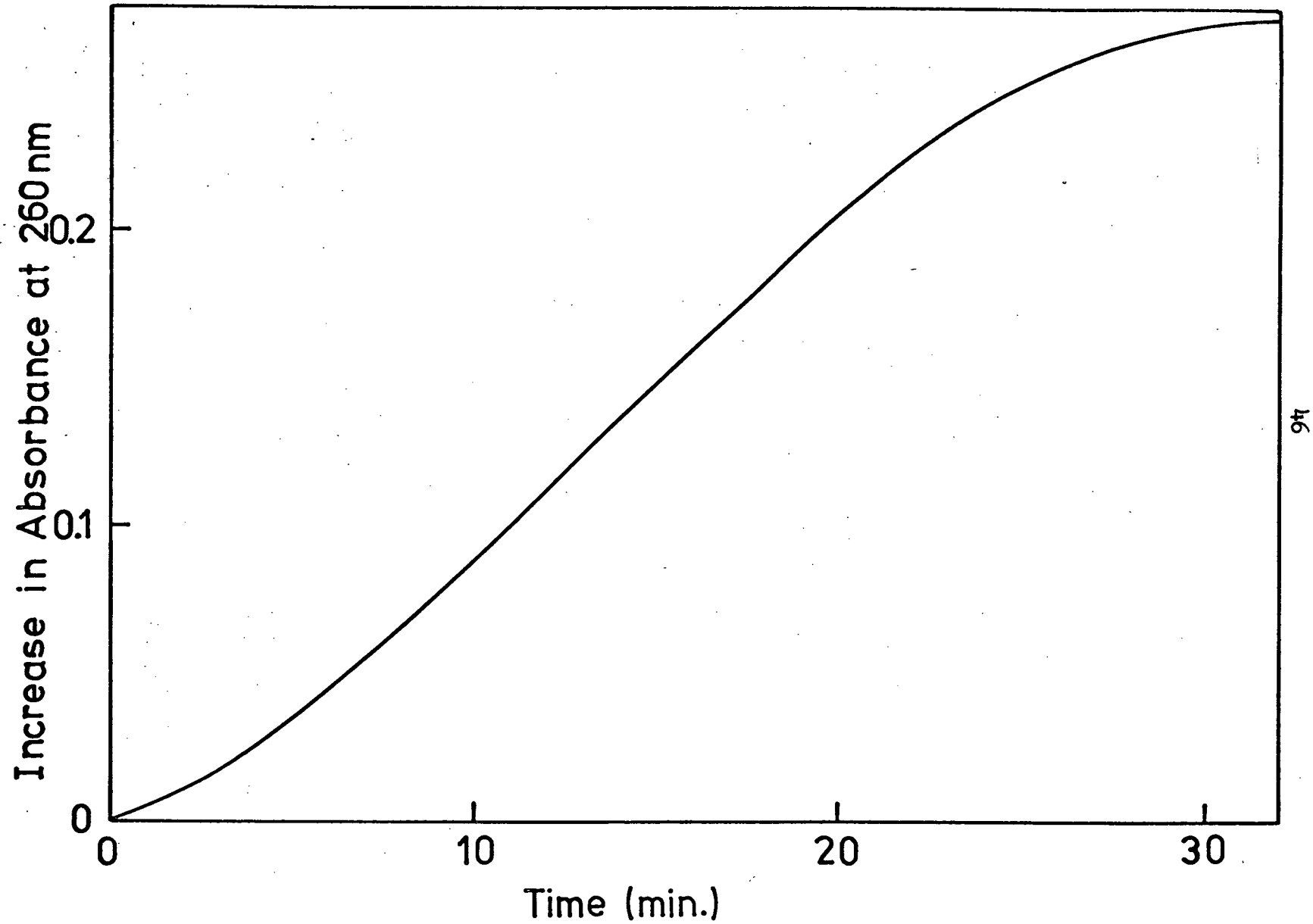

Diameter (d) of zone of clearing (mm.)
0
1
2
3
4
5
6
7
8
9
10
0.1
0.2
0.5
1
2
3
4
5
6
7
8
9
10
Log (relative DNase II concentration)

DNase II activity could not be measured as accurately with this assay as it could be with the acid-soluble oligonucleotide assay in which there was a linear relationship between the absorbance of the acid-soluble oligonucleotides liberated and the DNase II concentration.

Hyperchromicity assay

The increase in absorbance, the hyperchromicity, of a DNA solution upon digestion with DNase II was measured by a modification of the method used by Kunitz (1950) to determine DNase I activity. The enzyme was incubated with 40 ugm calf thymus DNA in 1.0 ml of 100 or 150 mM sodium acetate buffer containing 10 mM EDTA. The solutions were maintained at 37°C in cuvettes placed in the thermostated compartment of a Gilford model 2000 or a Cary model 15 spectrophotometer. Fig. 4 is a tracing of a plot of the increase in absorbance at 260 nm versus time obtained from the Gilford spectrophotometer, with respect to the absorbance of a solution containing only buffer and substrate. The same initial lag phase observed using low enzyme concentrations in the acid-soluble oligonucleotide assay was also seen here, presumably for the same reason. In Fig. 5 a linear plot was obtained when the slopes from the linear portions of the curves in Fig. 4 were plotted against the different amounts of enzyme that were used. The advantage of this assay was that each determination of DNase II activity resulted in a curve of hyperchromicity versus time instead of the single values that were obtained for the two previous assays. The disadvantage was

Fig. 4 Increase in absorbance of a DNA solution upon treatment with DNase II. DNase II was reacted at 37°C with 40 ugm DNA, in 1.0 ml of 150 mM sodium acetate buffer pH 5.0, containing 10 mM EDTA. The reaction was continuously monitored at 260 nm with respect to a control solution which contained no enzyme and which was set to read zero absorbance at 260 nm.

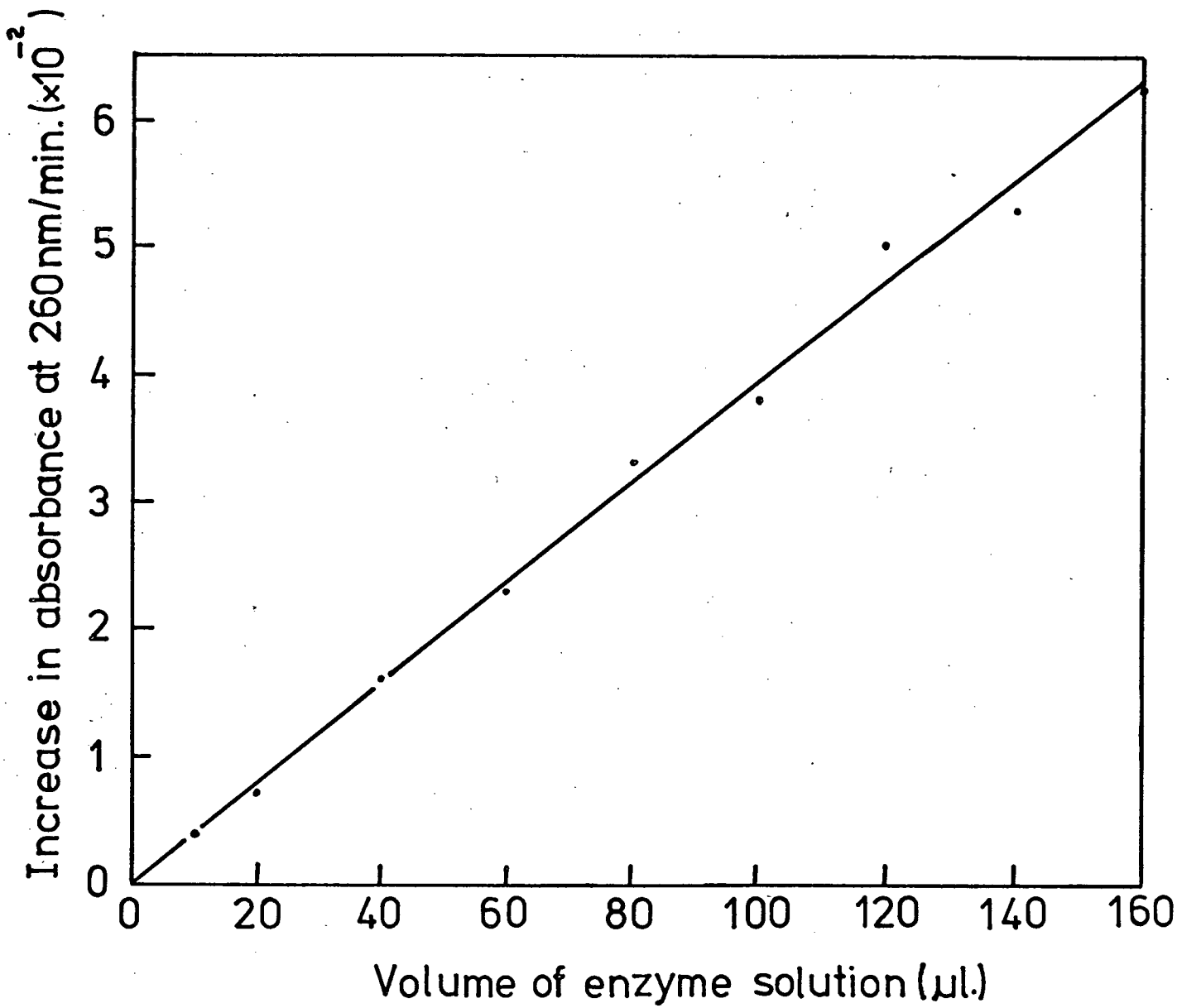

Increase in Absorbance at 260nm
0.2
0.1
0
0
10
20
30
Time (min.)

that the assay could not be used with crude enzyme preparations due to the formation of a precipitate which interfered with the measurement of the absorbance of the DNA solution.

^{3}H-DNA assay

The acid-soluble radioactive oligonucleotides released from sonicated ^{3}H-DNA from Escherichia coli by digestion with DNase II were measured by a modification of the method of Lesca (1976). DNase II was incubated with ^{3}H-DNA at 37°C in 1.0 ml of 150 mM sodium acetate buffer, pH 5.0, containing 10 mM EDTA. At various times 0.1 ml aliquots were removed and added to a mixture of 0.2 ml of 6% $HClO_4$ and 10 ul of bovine serum albumin solution (50 mg/ml). The mixtures were allowed to stand for 10 minutes at 0°C and then centrifuged at 12,100 xg for 10 minutes. Aliquots (200 ul) of the supernatant solutions were added to 10 ml portions of a napthalene fluor (see: Measurement of radioactive compounds), and the solutions were counted in the ^{3}H channel of a Packard Model 2543 liquid scintillation counter. In Fig. 6 the counts per minute of acid-soluble radioactivity released from ^{3}H-DNA by DNase II action is plotted as a function of time. Although the curve was linear for less than five minutes, there was no initial lag phase present when either sonicated ^{3}H-DNA from E. coli or polydAdT-methyl-^{3}H was used. This simplified the determination of initial velocities.

Fig. 5 Effect of different concentrations of DNase II on the hyperchromicity of a DNA solution. Points on this curve correspond to slopes taken from the linear portions of curves similar to that shown in Fig. 4. The assays were done under the conditions described in Fig. 4 for different amounts of enzyme.


Increase in absorbance at 260nm/min.(×10⁻²)
0
1
2
3
4
5
6
0
20
40
60
80
100
120
140
160
Volume of enzyme solution (µl.)

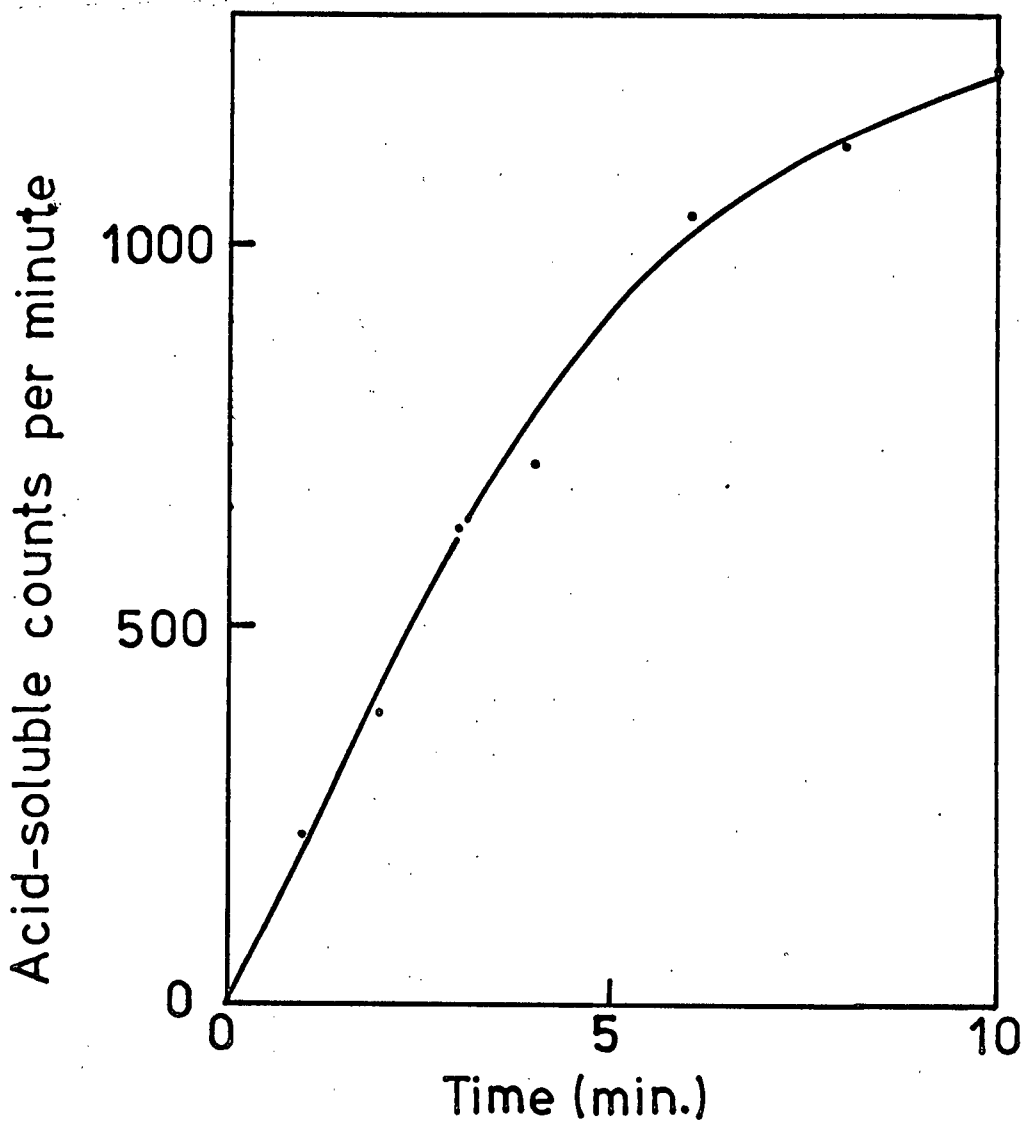


Fig. 6 Rate of release of tritiated acid-soluble oligonucleotides from sonicated ^{3}H-DNA from Escherichia coli DNA. The reaction was done at 37°C in 150 mM sodium acetate buffer, pH 5.0, containing 10 mM EDTA. The oligonucleotides that were soluble in 4% perchloric acid were counted in a napthalene based liquid scintillation fluid.

Measurement of the activities of other nucleases

DNase I

DNase I activity was measured by a modification of the method of Kunitz (1950). The increase in absorbance at 260 nm of a solution containing 40 ug calf thymus DNA in 1.0 ml of 0.1 M TrisHCl, pH 7.2, 10 mM $MnCl_2$ upon treatment with enzyme solution was measured at 37°C using a Gilford model 2000 spectrophotometer. One unit of DNase I activity was defined as the amount of enzyme that coused an increase in absorbance of 1.0×10^{-3} per minute at 260 nm.

Alkaline phosphatase

Solutions containing alkaline phosphatase were incubated at 37°C with 1 mM p-nitrophenylphosphate in 0.6 M TrisHCl, pH 8.0, and the absorbance at 400 nm of the p-nitrophenol released was recorded as a function of time. (Torriani, 1966). One unit of enzyme activity was defined as the amount of enzyme that released 1 umole of p-nitrophenol per minute under the above assay conditions. The molar absorbance (ε) for p-nitrophenol was taken to be 17×10^3 (Hynie and Zbarsky, 1970). A similar definition for units of enzyme activity was used for phosphodiesterase I and II and acid phosphatase.

Phosphodiesterase I

Incubation was carried out at 37°C with 0.4 mM p-nitrophenyl-thymidine-5'-phosphate in 33 mM TrisHCl, pH 8.9, containing 0.5 mM $CaCl_2$. The absorbance at 400 nm of the p-nitrophenol released was recorded as a function of time (Hynie and Zbarsky, 1970).

5' nucleotidase

Contaminating 5' nucleotidase present in venom phosphodiesterase from Worthington was measured before and after inactivation by the method of Sulkowski and Laskowski (1971). Solutions containing 5' nucleotidase were incubated for 24 hours at 37°C with 3 mM adenosine-5'-phosphate in a 200 mM glycine-NaOH, pH 9.0, containing 10 mM $MgCl_2$. The reaction was stopped by the addition of an equal volume of 20% trichloroacetic acid and the phosphate that had been released was measured by the method of Ames (1966).

Acid Phosphatase

Solutions containing acid phosphatase were incubated at 37°C with 1 mM p-nitrophenylphosphate in 150 mM sodium acetate buffer, pH 5.0, containing 10 mM EDTA. After 10 minutes the reaction was stopped by the addition of 200 ul of 2 N NH_4OH and the absorbance of the solution was measured at 400 nm. A substrate blank solution was prepared by incubation

of the substrate, addition of the NH_4OH, and then addition of the enzyme. The absorbance at 400 nm of this solution was subtracted from that of the assay solution.

Phosphodiesterase II

Assay #1. Solutions containing phosphodiesterase II were incubated with 1 mM p-nitrophenylthymidine-3'-phosphate in 0.15 M sodium acetate buffer, pH 5.0, containing 10 mM EDTA. After 10 minutes at 37°C the reaction was stopped by the addition of 200 ul of 2 N NH_4OH and the absorbance of the solution was read at 400 nm. The absorbance at 400 nm of a substrate blank solution which was prepared in a similar manner as the blank solution for acid phosphatase was prepared, was subtracted from the absorbance reading taken for the enzymic reaction.

Assay #2. Deoxyribooligonucleotide-phosphate, 0.8 umoles, the product of exhaustive digestion of calf thymus DNA with hog spleen DNase II (P.L. Biochemicals), was incubated with solutions containing phosphodiesterase II in 0.15 M sodium acetate buffer, pH 5.0, containing 10 mM EDTA. After 10 minutes at 37°C, an equal volume (1.0 ml) of 2.5% perchloric acid containing 0.25% uranyl acetate was added. The mixture was cooled at 0°C for 10 minutes and then centrifuged at 12,000 xg for 10 minutes at 4°C. The absorbance of the supernatant solution was read at 260 nm, and the absorbance at 260 nm of a substrate blank was subtracted. (Bernardi and Bernardi, 1966). One activity unit is the amount of enzyme that liberates mononucleotides having an absorbance of 1.0 at 260 nm per minute under the conditions of the assay.

Acid RNase

The solution containing acid RNase was incubated with 0.4 mg yeast RNA in 1.0 ml of 100 mM sodium acetate buffer, pH 5.0, containing 10 mM EDTA. After 30 minutes at 37°C the reaction was stopped by the addition of 0.5 ml of 6% perchloric acid. The mixture was cooled at 0°C for 10 minutes, and then centrifuged at 12,100 xg for 10 minutes at 4°C. The absorbance of the supernatant solution was read at 260 nm and the absorbance at 260 nm of a substrate blank was subtracted. One activity unit is the amount of enzyme that liberates acid-soluble oligonucleotides having an absorbance of 1.0 at 260 nm under the conditions of the assay.

Alkaline RNase

The assay procedure was the same as that described for acid RNase except that the buffer used was 20 mM TrisHCl pH 7.5.

Measurement of Protein

Protein content was determined by the method of Lowry et al. (1951) using standard curves which were constructed by measuring different quantities of bovine serum albumin. Protein content was also estimated by absorbance at 280 nm; 1 unit of absorbance at 280 nm was taken to indicate a protein concentration of 1.2 mg per ml. (Kunitz, 1950) This latter method was useful in estimating the protein content of solutions containing high concentrations of Tris buffer since Tris has been found to interfere with the determination of protein by the method of Lowry et al. (1951) (Rej and Richards, 1974)

Measurement of DNA

DNA was measured by determining deoxyribose content with diphenylamine by a modification of the method of Dische (Burton, 1968). Calf thymus DNA (Sigma, type 1) was used as a standard.

Measurement of Phosphate

Inorganic and total phosphate were determined by the method of Ames (1966) using sodium phosphate solutions as standards.

Measurement of Proteolytic Activity

Proleolytic activity was measured by the method of Kunitz (1947) as described by Laskowski (1955). A solution containing proteolytic enzymes was incubated with 0.5% casein in 100 mM phosphate buffer, pH 7.6, at 37°C. The reaction was stopped by the addition of trichloroacetic acid (TCA) to 1% final concentration, and the mixture was cooled at 0°C for 10 minutes and then centrifuged at 12,100 xg for 10 minutes. The extent of proteolysis was determined by reading the absorbance at 280 nm. An enzyme blank solution was prepared by incubation of the enzymic solution in the absence of substrate, addition of the trichloroacetic acid, and then addition of the casein substrate. The absorbance at 280 nm of the enzyme blank was subtracted from that of the reaction mixture. This served as a correction for proteolysis of any endogenous protein present in the solution containing the proteolytic enzymes. In order to obtain an estimate of the amount of proteolytic enzyme present in a given solution, the solution was assayed for proteolytic activity by the casein digestion assay and the resulting absorbance at 280 nm was compared with absorbances at 280 nm resulting from the digestion of casein with known amounts of trypsin. In Fig. 7 the absorbance at 280 nm of acid-soluble fragments of casein released by trypsin digestion is plotted against the quantity of trypsin used in the assay.

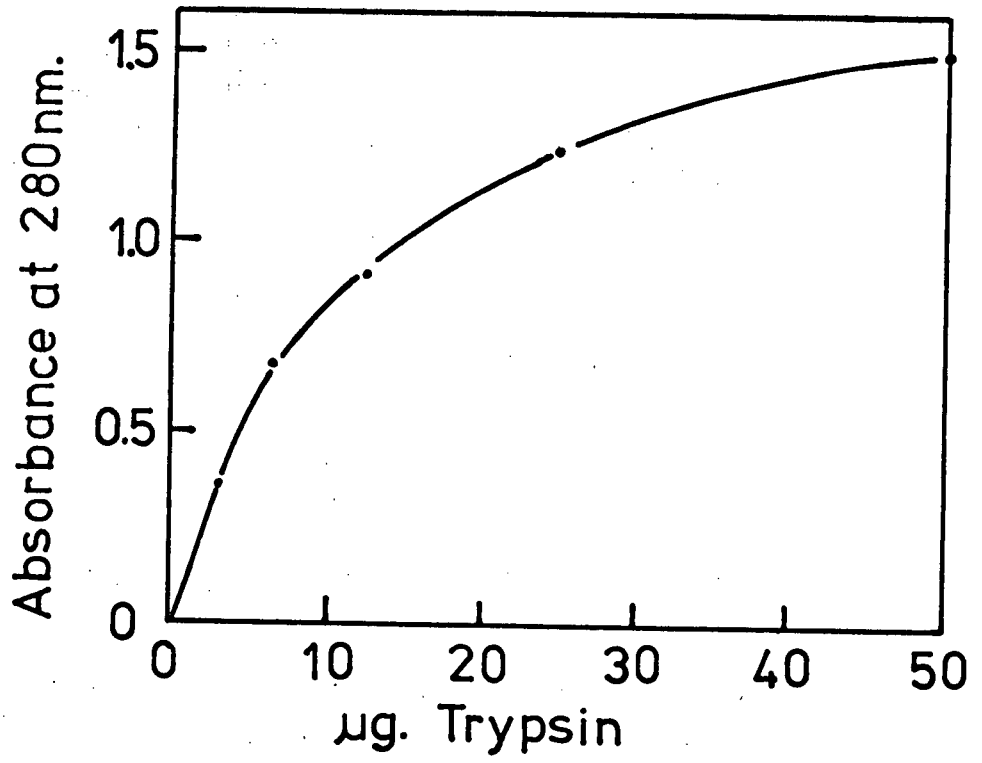


Fig. 7 Effect of different amounts of trypsin on the release of acid-soluble fragments from casein. Trypsin was reacted at 37°C with 0.5% casein in 100 mM phosphate buffer, pH 7.6, and the absorbance at 280 nm of casein fragments that were soluble in 1% trichloroacetic acid was measured.

Measurement of radioactive compounds

^{32}P-labelled compounds were counted in 10 ml of aqueous solution by Čerěnkov radiation in the tritium channel of a Packard 2425 liquid scintillation counter. Čerěnkov radiation is the electromagnetic radiation emitted when a charged particle passes through a dielectric medium at a greater velocity than the velocity of light in that medium. (Parker and Elrick, 1970). Electronically polarized molecules, which are produced along the path of the charged particle, return to their ground state with the emission of electromagnetic radiation. When the velocity of the charged particle is greater than that of light in the medium, there is a certain direction in which the emitted electromagnetic radiation interferes constructively. Although 90% of the ^{32}P β-spectrum is above the Čerěnkov threshold, most of the continuous spectrum of radiation is directionally emitted in the ultraviolet and counting in the ^{3}H channel of a liquid scintillation counter is consequently of relatively low efficiency. An efficiency of 35% for Čerěnkov radiation was estimated by comparing the observed counts per minute with the manufacturers' data accompanying the ^{32}P compounds. The counts per minute were corrected for the decay of the ^{32}P.

Tritiated compounds were counted in the tritium channel of the Packard 2425 liquid scintillation counter using a napthalene fluor that was composed of 15 gm 2,5-diphenyloxazole (PPO), 150 mg p-bis[2-(4-methyl-5-phenyl-oxazolyl)] benzene (dimethyl POPOP), 240 gm napthalene, and 1 litre each of toluene, dioxane and absolute ethanol (Chiu and Sung, 1972).

The counts per minute were not corrected for quenching, but all had similar external channel ratios.

Preparation of ^{3}H thymidine labelled DNA from the mucosa of rat small intestine

A male Wistar rat weighing 180-200 gm was starved for 24 hours and then anesthetized with ether. ^{3}H-methyl thymidine (1.3×10^{9} cpm in 200 ul of water) was injected into the tail vein and the rat was allowed to recover from the effects of the anesthesia. Thirty minutes after the injection the rat was stunned by a blow to the head and decapitated. Intestinal DNA was prepared by the method of Colter et al. (1962) as modified by Meyer (1964). From 5 gm of fresh intestinal mucosa 31.5 mg DNA were recovered with a total radioactivity of 18.7×10^{6} (1.4% of the injected counts) and with a specific radioactivity of 592 cpm per mg DNA (219 cpm/umole DNA-phosphate). Unlabelled rat intestinal DNA was also prepared: 22.3 mg DNA were recovered from 4 gm of wet mucosa.

Characterization of the DNA preparations from rat intestine and calf thymus

Thermal denaturation curves for DNA from rat intestine and calf thymus were obtained by the method of Mandal and Marmur (1968). DNA at a concentration of 20 ug/ml was heated in 150 mM sodium chloride-15 mM sodium citrate pH 6.5 (standard saline citrate) solution in the thermostated chamber of a Gilford model 2000 spectrophotometer. The temperature rise in ^{o} C and increase in absorbance at 260 nm of the DNA solution with respect to a blank solution containing standard saline citrate buffer were continuously recorded versus time. The absorbance values were corrected for the thermal expansion of the solutions, and the ratio of the absorbance at 260 nm at particular temperature t to the absorbance

at 25° was plotted as a function of the temperature t. Fig. 8 shows the thermal denaturation curve obtained for DNA isolated from rat intestine. The midpoint of the transition (Tm) was at 85.7°. This corresponded to a GC content of 40% as calculated from the equation GC = (Tm - 69.3) x 2.44 (Mandel and Marmur, 1968). The increase of the relative absorbance from 1.0 at 50° (not shown) to about 1.05 at 70° indicated that there was a low molecular weight species in the DNA preparation that melted in this low temperature range. Since there was a large transition at a relatively high temperature, most of the preparation consisted of native DNA molecules.

Fig. 9 depicts the thermal denaturation curve obtained for DNA from calf thymus. The midpoint of the transition was 84.9° and corresponded to a GC content of 38%.

Table IV gives the results of ultraviolet and phosphate analyses of the DNA which were carried out by the methods of Felsenfeld (1968) and Ames (1966) respectively. The values obtained for umoles nucleotide per mg DNA and umoles phosphate per mg DNA agreed fairly well considering that two disparate methods of analysis were used. The extinction coefficient at 260 nm with respect to phosphate (ε(P) 260) was close to 6,600, a value characteristic of many native DNA molecules, and was not over 7,200, a value indicating denaturation (Chargaff, 1955). The maximal thermal hyperchromicity at 260 nm (h 260) is equal to

$$\left[\frac{\text{maximum absorbance at 260 nm of a DNA solution after heating}}{\text{absorbance at 260 nm of a DNA solution at room temperature}} - 1 \right]$$

(Mahler and Cordes, 1971). The values obtained were representative of native DNA (Mahler and Cordes, 1971). The fraction of the DNA originally

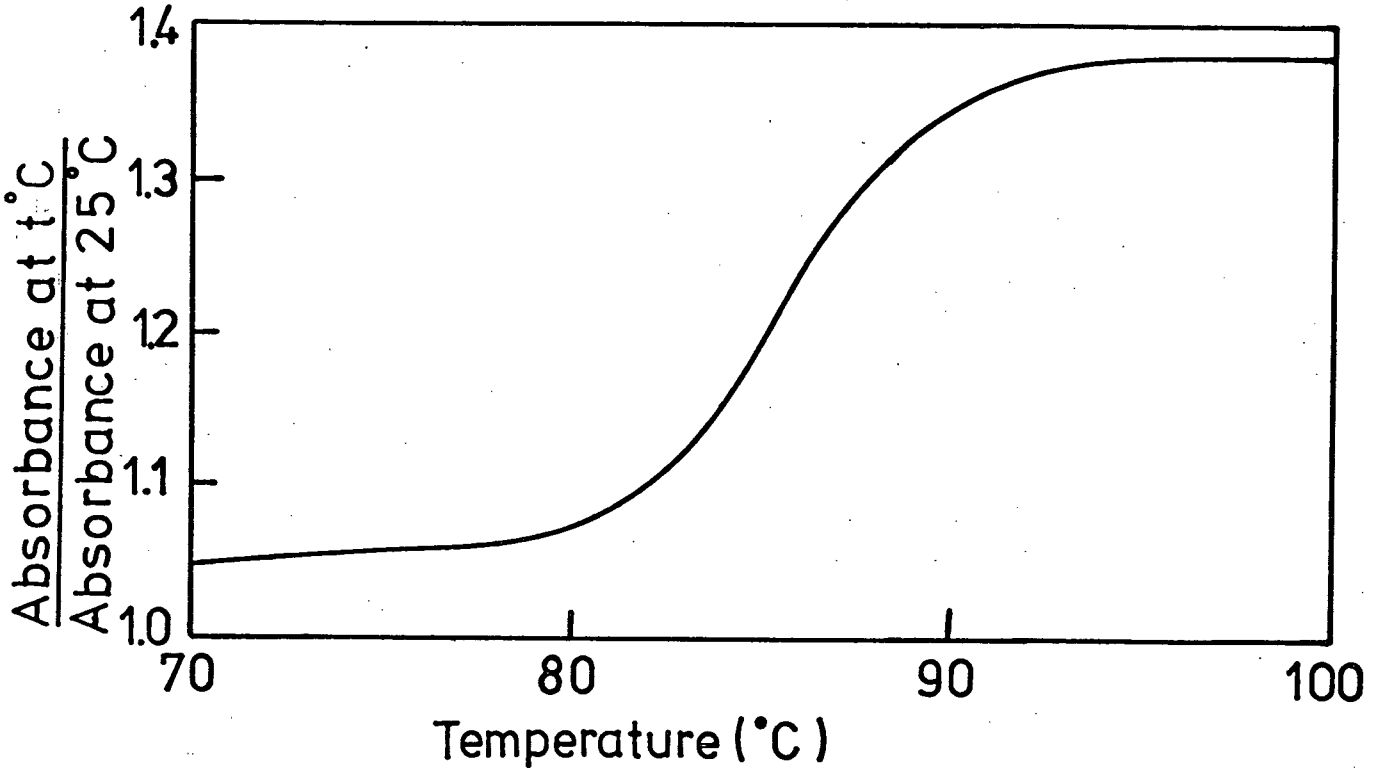


Fig. 8 Thermal denaturation curve for DNA isolated from rat intestinal mucosa. The DNA at a concentration of 20 ugm/ml was heated in standard saline citrate solution and the relative absorbance of the DNA solution was corrected for thermal expansion and plotted as a function of the temperature.

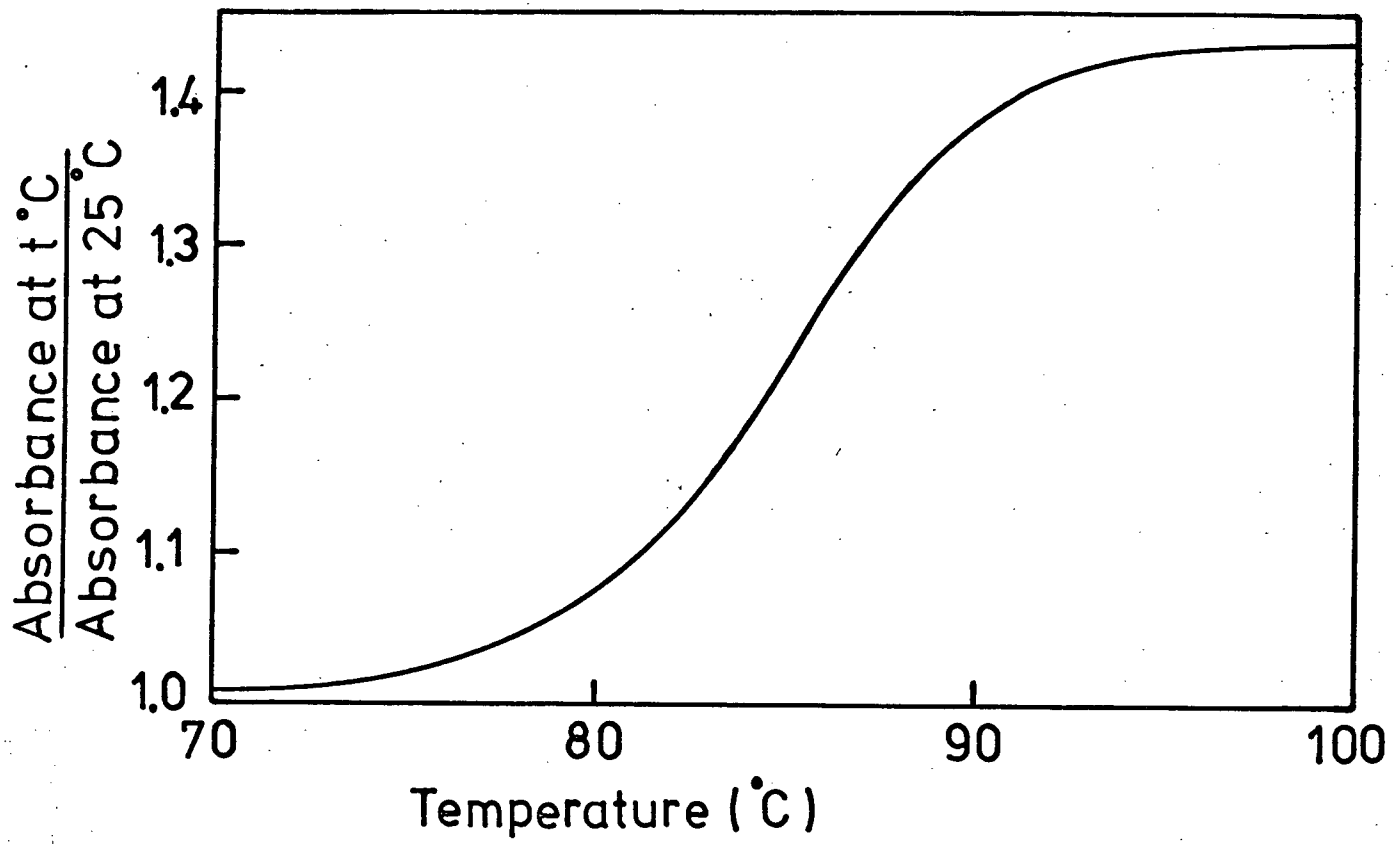


Fig. 9 Thermal denaturation curve for calf thymus DNA (Sigma type I). The DNA was heated in a similar manner as for Fig. 8.

TABLE IV

ULTRAVIOLET AND PHOSPHATE ANALYSES OF DNA ISOLATED FROM RAT SMALL INTESTINE AND OF CALF THYMUS DNA (SIGMA TYPE I)

DNA	umoles nucleotide per mg DNA	umoles phosphate per mg DNA	Extinction coefficient at 260mm with respect to phosphate $\epsilon(P)$ 260	Maximal thermal hyperchromicity at 260 mm h_{260}	Fraction of DNA originally involved in helix
Rat DNA (unlabelled)	2.29	2.45	6,755	0.38	0.93
Rat ^{3}H-DNA	2.54	2.70	6,685	0.38	0.94
Calf thymus DNA (Sigma type I)	2.14	2.00	6,800	0.43	1.0

involved in helix was calculated by dividing the concentration of DNA that underwent denaturation by the concentration of DNA that was present in denatured form at 100°C (Felsenfeld, 1968). These values also indicated that the DNA was present in the double-stranded form.

Preparation of [γ - ^{32}P]ATP from ^{32}P labelled inorganic phosphate

The prodedure of Glynn and Chappell (1964) as modified by Schendel and Wells (1973) involves the substrate level phosphorylation of ADP during the conversion of glyceraldehyde-3-phosphate to 3-phosphoglycerate by enzymes of the glycolytic pathway as illustrated in Fig. 10. The glyceraldehyde-3-phosphate reaction is made irreversible by oxidizing the NADH to NAD^+ with thiazoylblue and phenazine methosulfate. Because the nucleotide content of commercially available glyceraldehyde-3-phosphate dehydrogerase can vary, NAD^+ should be added to the reaction mixture to ensure that a high yield of [γ - ^{32}P]ATP is consistently obtained (Martin and Voorheis, 1977). The [γ-^{32}P]ATP was separated from reactants and byproducts by chromatography on a DEAE-cellulose column, 0.8 x 16 cm, equilibrated with 0.05 M triethylammonium bicarbonate (TEAB), pH 7.5. The column was eluted 4°C with a linear gradient of 0.05 to 0.5 M TEAB, pH 7.5. One microlitre of each 2 ml fraction collected was added to 10 ml of water and counted by Čerěnkov radiation. The elution profile of radioactivity from the column is illustrated in Fig. 11. The identity of the eluted radioactive compounds was determined by subjecting aliquots from peak fractions to thin layer chromatography on polyethylenemine cellulose plates run in 1.25 M LiCl by the method of Randerath and Randerath (1967). The Rf values were: 5' AMP, 0.66; Pi, 0.63; ADP, 0.53; 5' ATP, 0.22. Since 5'-AMP and Pi were not resolved, ^{32}P labelled inorganic phosphate was determined by measuring the amount of radioactivity that would not bind to Norit A under the conditions described by Weiss *et al.* (1968). The fractions containing the [γ- ^{32}P]ATP were

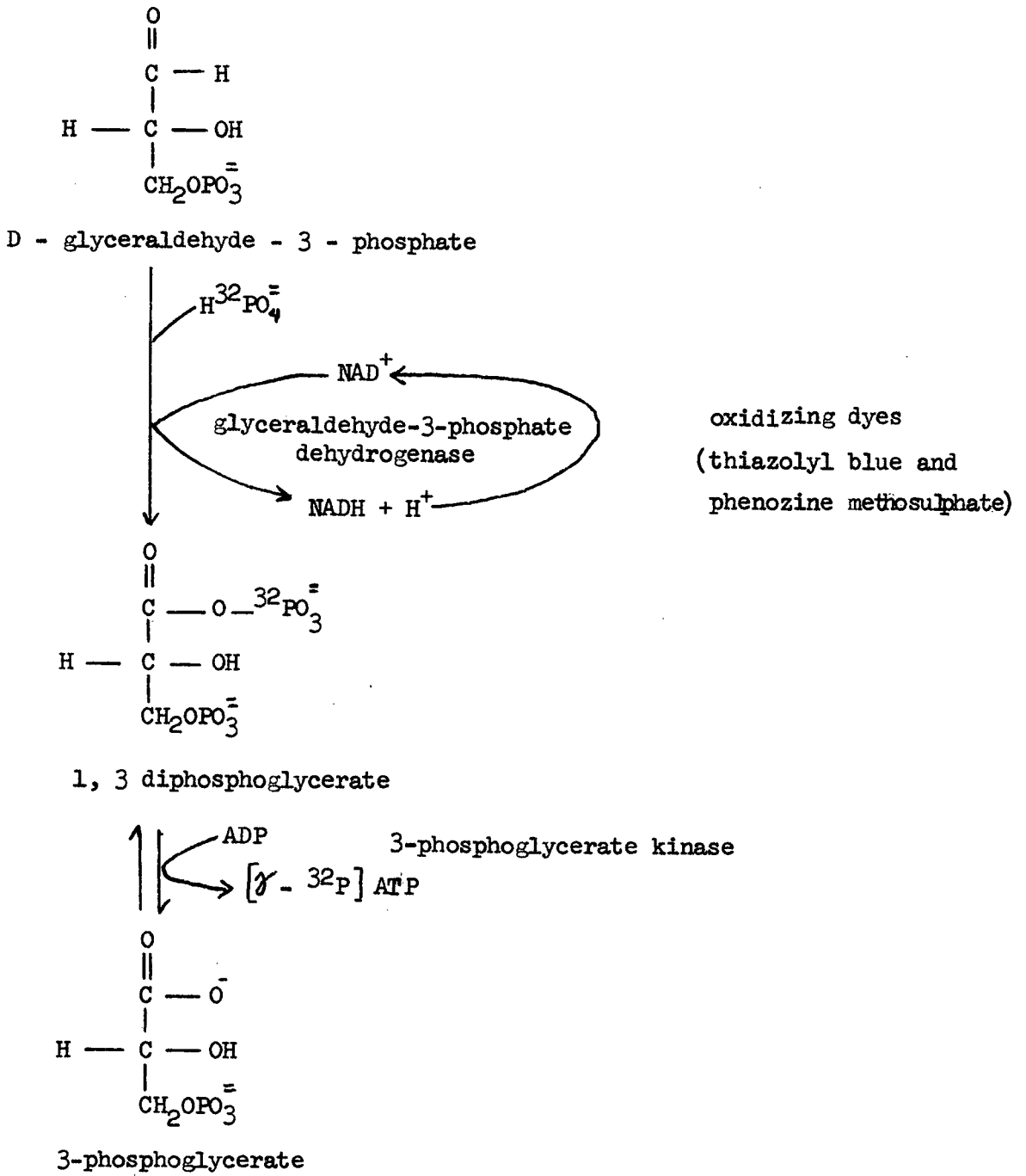


Fig. 10 Schematic representation of the reactions involved in the synthesis of $[\gamma - {}^{32}P]$ATP

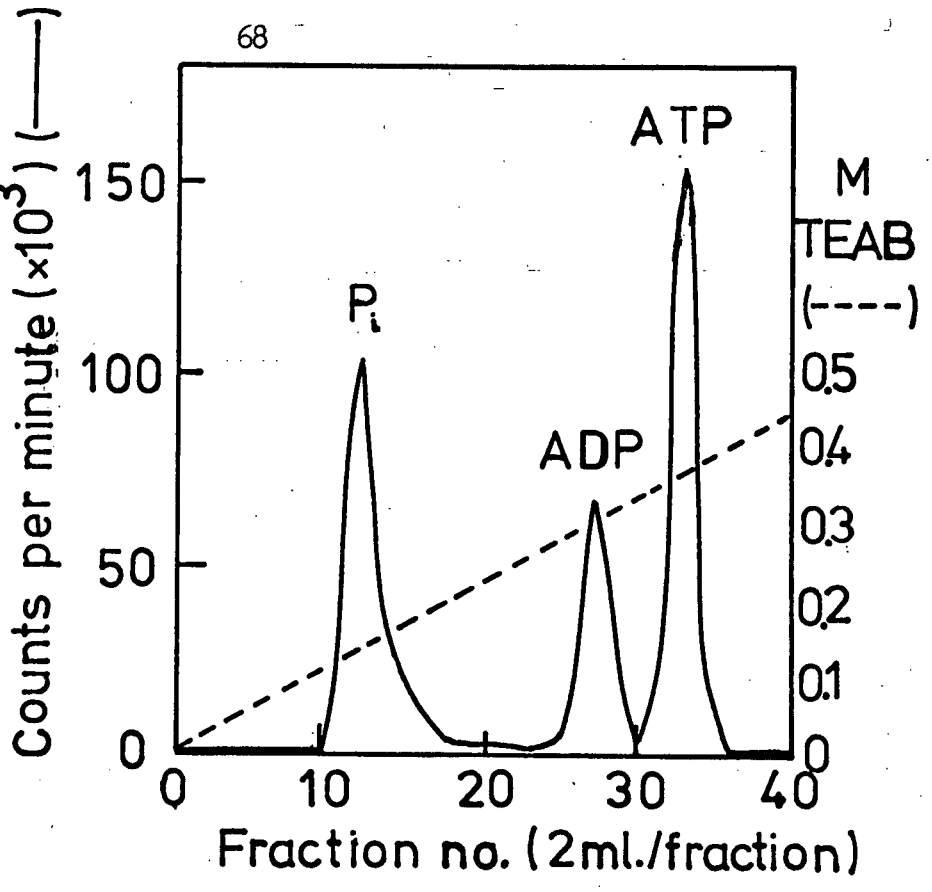


Fig. 11 Chromatography of the [γ - ^{32}P]ATP preparation on DEAE cellulose. The column, 0.8 x 16 cm, was eluted at 4°C with a linear gradient of 0.05 to 0.5 M triehylammonium bicarbonate (TEAB), pH 7.5. One microlitre of each 2 ml fraction collected was added to 10 ml of water and the radioactivity was measured by the Čerenkov radiation emitted.

pooled; the solution contained 0.54% ^{32}P - ADP and 1.2% ^{32}P - inorganic phosphate. The triethylammonium bicarbonate was removed by lyophilization of the solution.

Determination of the specific radioactivity of [γ - ^{32}P]ATP

Known amounts of oligodT(pT)9 that were determined by ultraviolet absorbance were phosphorylated using [γ - ^{32}P]ATP and polynucleotide kinase by the method of Chaconas et al. (1975). OligodT(pT)9 was prepared by dephosphorylation of oligo(pT)10 with alkaline phosphatase from Escherichia coli (Worthington). Because Weiss et al. (1968) found that an endonuclease contaminant was present in the alkaline phosphatase preparation from Worthington, the alkaline phosphatase was preincubated at 65°C and the reaction was carried out at 65°C in 0.6 M TrisHCl, pH 8.0, containing 10 mM $MgCl_2$ in an attempt to inactivate the contaminating enzyme. In accord with the observation of Torriani (1966), the alkaline phosphatase activity was stable under these conditions. After the reaction with alkaline phosphatase the solution containing the oligodT(pT)9 was cooled to 0°C and chromatographed on a Sephadex 650 column, 0.9 x 50 cm. The oligodT(pT)9 was eluted with 50 mM triethylammonium bicarbonate, pH 7.5; the peak fractions absorbing at 266 nm were pooled and freeze-dried. The oligodT(pT)9 was dissolved in water; the solution was made 0.5 N in NaOH and incubated at 37°C for 15 minutes in order to inactivate the alkaline phosphatase (Ho and Gilham, 1973; Delaney and Spencer, 1976). The solution was then neutralized with HCl

and the concentration of oligodT(pT)9 was determined using a nucleotide extinction coefficient of 8.7×10^3 at 226 nm (Cassani and Bollum, 1969). Known amounts of oligodT(pT)9 were phosphorylated using [γ- ^{32}P]ATP and polynucleotide kinase (Chaconas et al., 1975). The reaction mixture contained 6.28 or 12.56 pmoles oligodT(pT)9 and [γ- ^{32}P]ATP (486,000 cpm) in 50 mM Tris HCl, pH 7.6, with 10 mM dithiothreitol, 5 mM $MgCl_2$ and 2 units of polynucleotide kinase from bacteriophage T4 in a volume of 20 ul. One microlitre aliquots were removed at various times and spotted along one side of a 23 by 28 cm sheet of DE81 cellulose paper which had been prespotted twice with 50 ul of 1 mM ATP, 50 mM EDTA. The prespotting was required to prevent irreversible binding of radioactive ATP to the origin. One microlitre of the reaction mixture was taken before addition of the polynucleotide kinase and was spotted as a control. Descending paper chromatography was carried out for 2 hours at room temperature in 0.35 M ammonium formate buffer, pH 5.5 (van de Sande et al., 1973). Under these conditions the Rfs of ATP, AMP and inorganic phosphate (Pi) were 0.46, 0.48 and 0.71 respectively, and oligonucleotides of at least 10 nucleotides in length remained at the origin (Sgaramella and Khorana, 1972). The DE81 paper was dried and the origins were cut out, placed in 10 ml of water in glass scintillation vials and counted by Čerěnkov radiation. After subtraction of control values of about 100 cpm for the reaction mixture without the addition of enzyme, the counts per minute for ^{32}P incorporation at various times were multiplied by 20 to relate the 1 ul aliquots back to the original 20 ul reaction mixture. In Fig. 12 a time curve of the incorporation of ^{32}P into oligodT(pT)9 is shown.

For 6.28 and 12.56 pmoles, 64,000 and 126,000 cpm were incorporated respectively. Two curves of incorporation were done as a check on the reproducibility of the determination. The specific radioactivity of the [γ- ^{32}P]ATP was taken as the cpm incorporated over the pmoles of oligodT(pT)p present. That is, $128{,}000/_{12.56} = 64{,}000/_{6.28} = 10{,}191$ cpm/pmole. Assuming 35% efficiency for measuring ^{32}P by Čerěnkov radiation, this is equivalent to 27 Ci/umole.

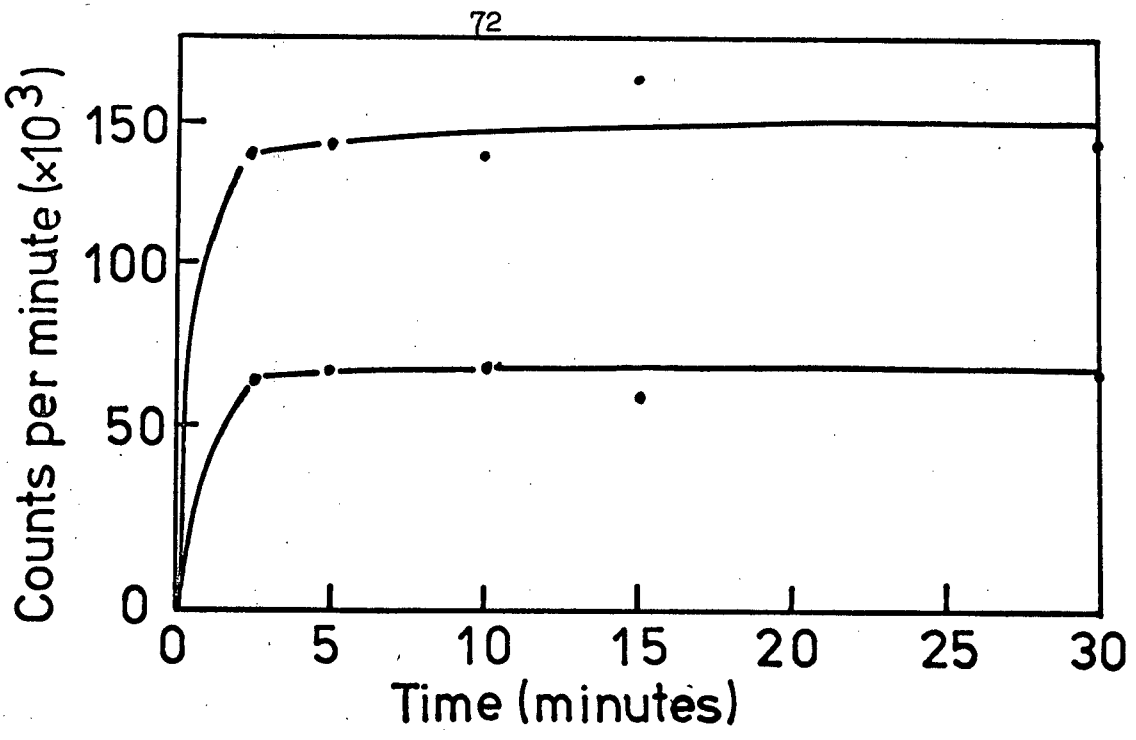


Fig. 12 Incorporation of ^{32}P into oligodT(pT)9 using polynucleotide kinase and [γ-^{32}P]ATP. Aliquots of the reaction mixture were spotted on DE81 cellulose paper. Descending paper chromatography was carried out in 0.35 M ammonium formate buffer, pH 5.5. The paper was dried, the origins cut out, and the ^{32}P present was determined by measuring the Čerěnkov radiation emitted.

RESULTS AND DISCUSSION

A Isolation of DNase II activity from bovine intestinal mucosa

Preparation of a crude extract containing DNase II activity from the mucosa of bovine small intestine

A bovine small intestine obtained from a freshly slaughtered animal and kept at 0°C, was cut into five foot lengths. Each length was thoroughly flushed with tap water and the fat and other tissues were cut off with scissors. The section of intestine was cut longitudinally and placed mucosal side up on a metal tray inverted over crushed ice. The mucosa was scraped off with a dull meat cleaver and suspended in an equal volume of Krebs Ringer phosphate buffer, pH 7.8, to give a total volume of 5 litres. The mixture was homogenized 2.5 litres at a time for 3 minutes at the "high" setting in a Waring Commercial Blender, cooled to 0°C in an ice-water bath, and rehomogenized as before. After the first homogenization, clumps of cells still remained in the mucosal mixture as could be seen by phase contrast light microscopy. After the second homogenization, many nuclei, but no intact cells, were observed. The homogenate was centrifuged at 16,300 xg for 30 minutes at 4°C in a Sorvall RC 2B refrigerated centrifuge. The precipitate was resuspended in a minimum volume of Krebs Ringer phosphate buffer, pH 7.8, and recentrifuged as before in order to recover as much DNase II activity as possible. The two supernatant solutions were filtered through 8-12 layers of cheese cloth to remove lipid material, combined, and frozen in

300 ml lots at -80°C.

This relatively low speed centrifugation was done to remove some cellular debris and organelles in order to improve the subsequent centrifugation at 105,000 xg.

Table V indicates the volumes of solution and the amounts of protein and DNase II activity that were involved in the preparation of a crude extract of bovine intestinal mucosa. The large volume of solution and the large amount of protein present made it difficult to process the entire solution by ultracentrifugation or column chromatography, but attempts to concentrate the solution and remove a substantial amount of protein by acidification and/or ammonium sulfate precipitation procedures similar to those described by Bernardi et al. (1966) resulted in a large loss of DNase II activity. This may have been due to the different properties of intestinal DNase II, or to the different number and kinds of proteins present in the intestinal extract. All of the DNase II activity present in the homogenate was recovered in the combined supernatant solution with a modest increase in specific activity. That the DNase II activity was higher in the combined supernatant solution than it was in the homogenate may have been due to the release of "latent" DNase II from lysozomes during the procedure.

TABLE V

PREPARATION OF A CRUDE EXTRACT CONTAINING DNase II ACTIVITY FROM THE MUCOSA OF BOVINE SMALL INTESTINE

Preparation	Volume (ml)	Protein (gm)	DNase II Units	Specific Activity (Units/mg protein)
Homogenate	4484	159	9681	0.0606
First 16,300xg Supernatant	3560	85.0	8259	0.0967
Resuspended Precipitate	1730	42.4	1285	0.0303
Second 16,300xg Supernatant	925	15.3	1073	0.0703
Resuspended Precipitate	975	18.5	263	0.0142
Combined 16,300xg Supernatants	4480	103	10,125	0.0983

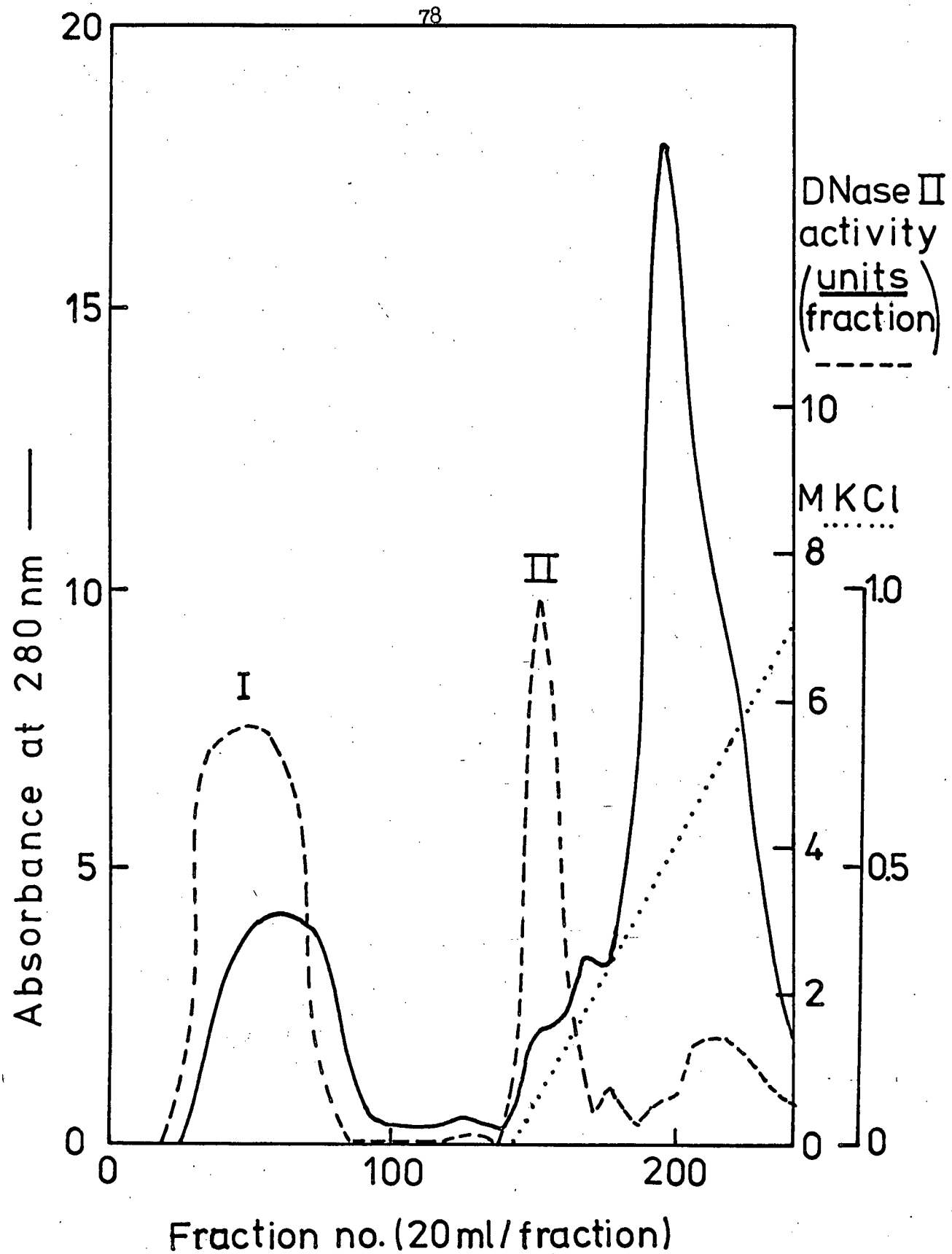
Chromatography of the crude DNase II preparation on DEAE cellulose

A 300 ml lot of the 16,300 xg supernatant solution was thawed, adjusted to pH 7.8 with 1 N NaOH, and centrifuged at 105,000 xg for 1 hour at 4°C in a Beckman model L centrifuge. A DEAE 22 cellulose column, 5 x 40 cm, was prepared and equilibrated with 20 mM phosphate buffer, pH 7.8. The 105,000 xg supernatant solution was diluted $2\frac{1}{2}$ times with distilled water, so that the conductivity of the sample solution was the same as the buffer with which the DEAE cellulose column was equilibrated. The sample was applied to the column, and the column was eluted at a flow rate of 2.5 ml per minute first with 20 mM phosphate buffer, pH 7.8, and later with a linear 0 to 1 M potassium chloride gradient in the same buffer. Fractions of 20 ml were collected and were assayed for protein by absorbance at 280 nm, for DNase II by the acid-soluble oligonucleotide assay, and for concentration of potassium chloride by conductivity measurements using known amounts of potassium chloride in the same buffer as standards. The elution profile from the DEAE cellulose column is illustrated in Fig. 13.

There were two large peaks of absorbance at 280 nm, but the second peak was not due entirely to protein. Although the absorbance at 280 nm of the pooled fractions indicated a protein concentration of 8.5 mg per ml of solution, the protein concentration as determined by the method of Lowry et al. (1951) was only 0.37 mg/ml. Since the 280 nm to 260 nm absorbance ratio was 0.60, it is suggested that nucleic acids present in the solution could have accounted for much of the absorbance seen at 280 nm.

DNase II activity was determined by the acid-soluble oligonucleotide

Fig. 13 Chromatography on DEAE cellulose of a crude extract prepared from bovine intestinal mucosa. The column, 5 x 40 cm, was eluted with 20 mM phosphate buffer, pH 7.8, and with a linear 0 to 1 M potassium chloride gradient in the same buffer. Protein concentration was estimated by absorbance at 280 nm, and DNase II activity, by the acid-soluble oligonucleotide assay.


20
15
10
5
0
Absorbance at 280 nm
I
II
0
100
200
Fraction no. (20 ml / fraction)
DNase II activity (units / fraction)
10
8
6
4
2
0
M KCl
1.0
0.5
0

assay (see methods). Two major peaks of DNase II activity were observed; the second peak was sometimes eluted throughout the potassium chloride gradient. The first peak of DNase II activity contained 35% and the second peak, 10% of the DNase II units which had been applied to the column.

An aliquot of the first DNase II activity was rechromatographed on a DEAE cellulose column, 2 x 18 cm. The column was eluted with 20 mM phosphate buffer, pH 7.8, and then with 0.5 M potassium chloride in the same buffer. The elution profile from the column is illustrated in Fig. 14. Of the 8.34 units of DNase II activity applied to the column 6.66 units (80%) were eluted with the 20 mM phosphate buffer.

The second DNase II activity, 5.87 units, was dialyzed against 20 mM phosphate buffer, pH 7.8, for 30 minutes using a Biorad "Biofibre beaker-50" to yield 1.62 DNase II units. After standing overnight at 4°C the solution, now containing only 0.771 units of DNase II activity, was applied to a DEAE cellulose column, 2 x 18 cm. The column was eluted with 20 mM phosphate buffer, pH 7.8, and then with 0.5 M potassium chloride in the same buffer. Fig. 15 depicts the elution profile from the column. The DNase II activity, 0.590 units, was eluted with the potassium chloride step. This corresponded to a yield of 77% of the DNase II activity actually placed on the column, and to an overall yield of 10% of the aliquot of DNase II activity taken from the second peak of the original DEAE cellulose column.

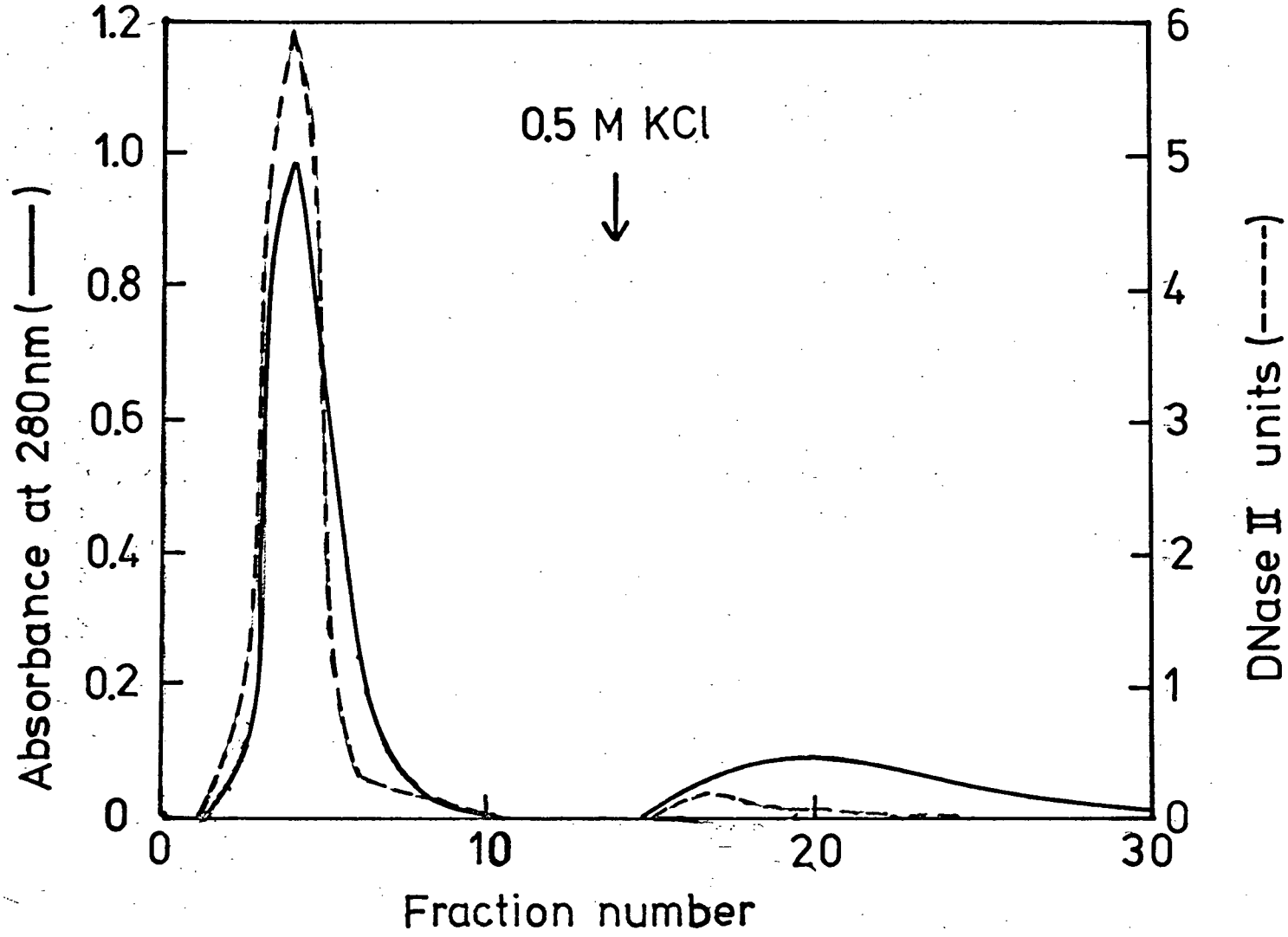


Fig. 14 Rechromatography of the first peak of DNase II activity from Fig. 13 on DEAE cellulose. The column, 2 x 18 cm, was eluted firstly with 20 mM phosphate buffer, pH 7.8, and secondly with 0.5 M potassium chloride in the same buffer. Protein concentration was estimated by absorbance at 260 nm and DNase II activity by the acid-soluble oligonucleotide assay.

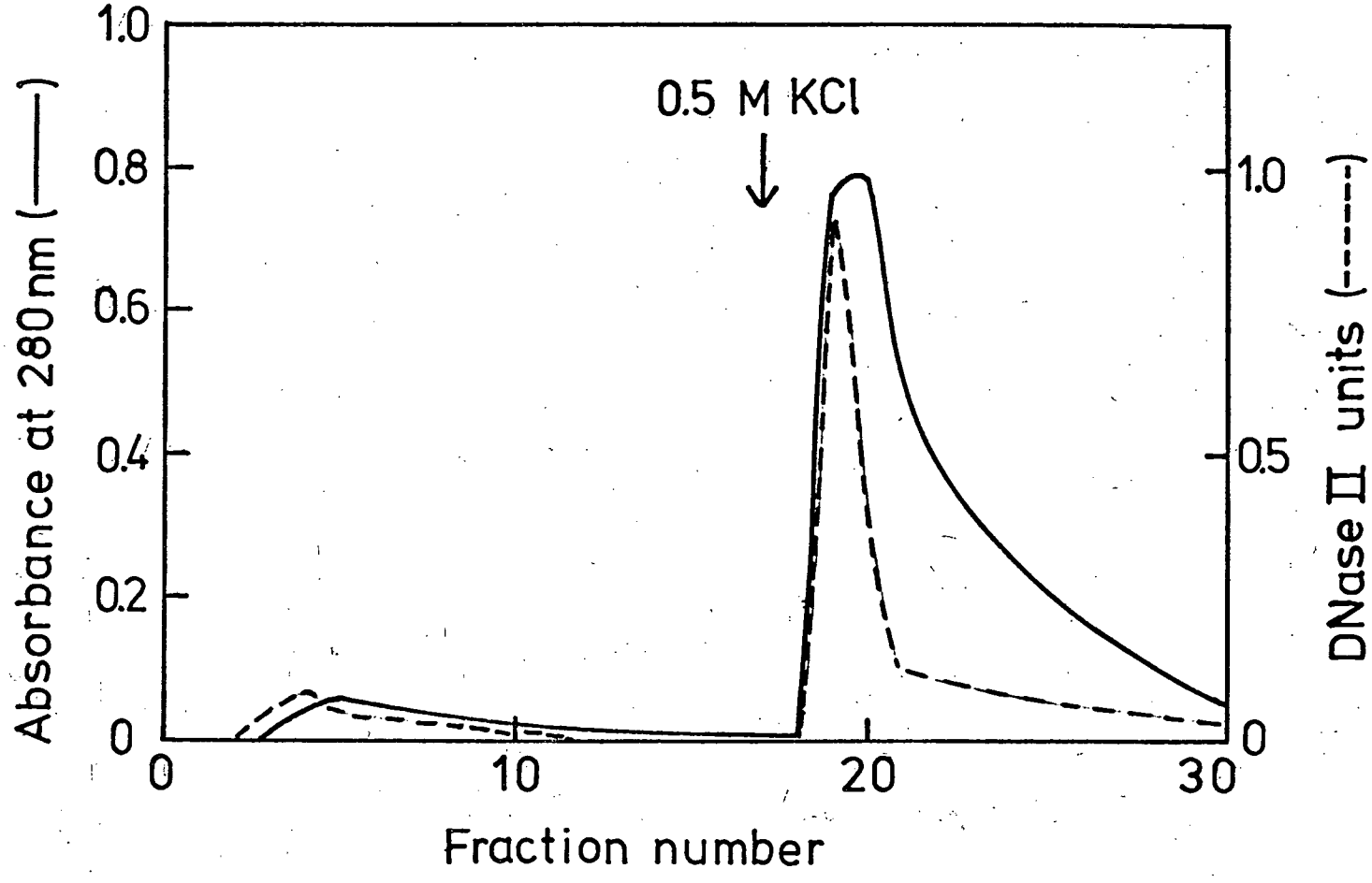


Fig. 15 Rechromatography of the second peak of DNase II activity from Fig. 13 DEAE cellulose. The column 2 x 18 cm was eluted with 20 mM phosphate buffer, pH 7.8, and then with 0.5 M potassium chloride in the same buffer. The protein concentration was estimated by the absorbance at 280 nm and the DNase II activity in each fraction by the acid-soluble oligonucleotide assay.

Some factors affecting the stability of DNase II

A possible reason for the low yields of DNase II activity obtained after chromatography, dialysis, and storage at 4°C may have been the instability of DNase II activity in the 20 mM phosphate buffer used. Separate solutions containing the first and second DNase II activities respectively in 20 mM phosphate buffer, pH 7.8, and in the same buffer containing either 10 mM EDTA or 10 mM $MgCl_2$, were stored at 4°C for 9 days. Aliquots of these solutions were taken at different times and assayed for DNase II activity by the acid-soluble oligonucleotide assay. The curves in Fig. 16a and b demonstrate the decrease in activity observed for the first and second DNase II activities respectively. The addition of 10 mM $MgCl_2$ to the phosphate buffer destabilized the DNase II activity whereas the addition of 10 mM EDTA stabilized DNase II. The second DNase II activity did not decrease as rapidly or as much in the phosphate buffer with or without $MgCl_2$ as did the first DNase II activity. This may have been due to protection of the second DNase II activity by endogenous substrate. Evidence for the presence of DNA in preparations of the second DNase II activity will be presented later.

The loss of DNase II activity may have been due to proteolysis of the enzyme. The solutions that contained the first and second DNase II activities also had proteolytic activity equivalent to 2.6 and 18 ugm of trypsin per ml, respectively, as determined by the casein digestion assay. Furthermore, each ml of undiluted 105,000 xg supernatant contained proteolytic activity equivalent to 60 ugm of trypsin.

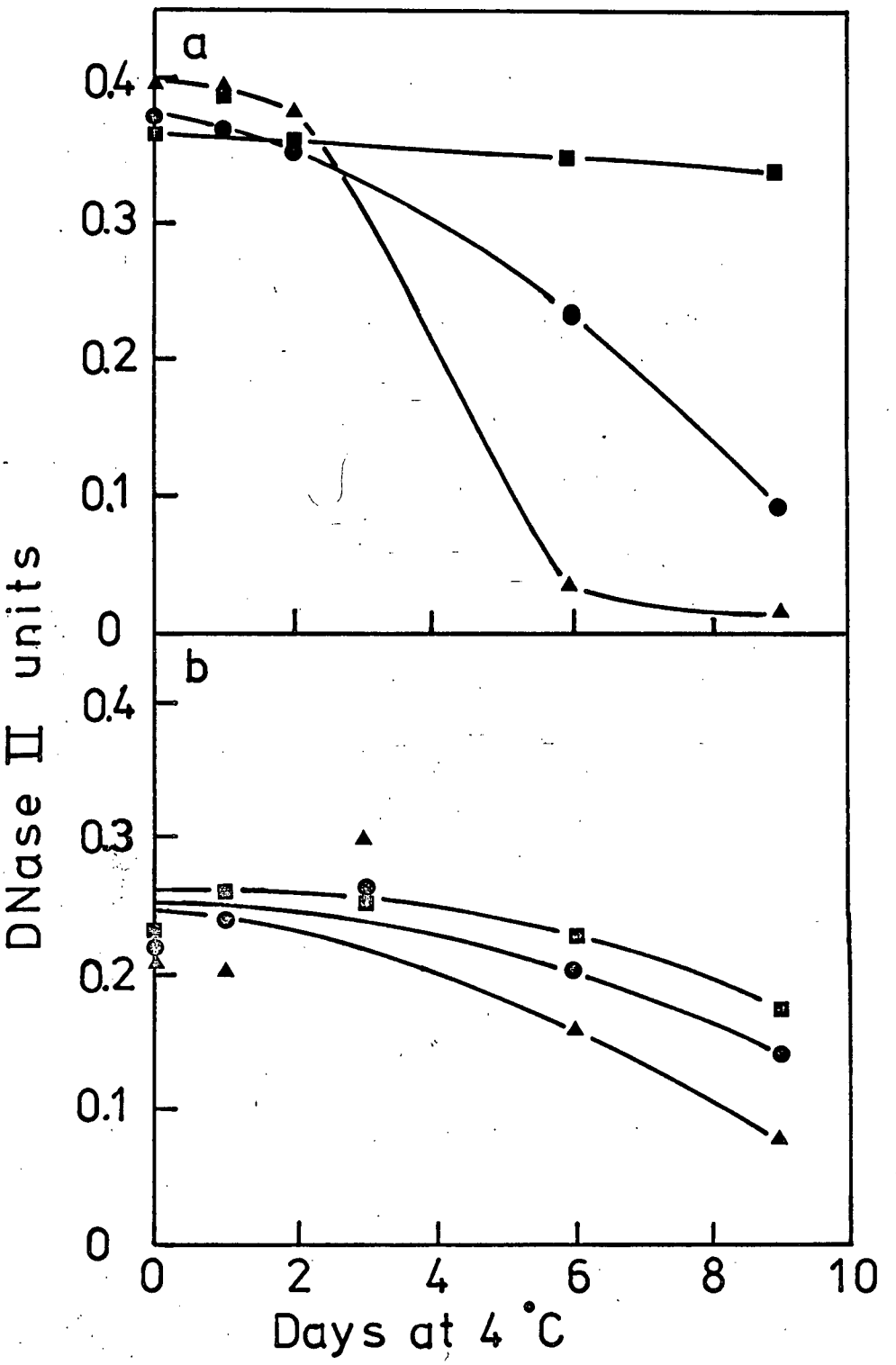
In order to determine whether proteolysis of DNase II was causing

Fig. 16 Stability of intestinal DNase II. DNase II activity was measured by the acid-soluble oligonucleotide assay and the units of DNase II activity per ml of enzyme solution were plotted as a function of the time that the enzyme solutions had remained at 4°C. (a) and (b) refer respectively, to the first and second DNase II activities for Fig. 13.

—●— 20 mM phosphate buffer, pH 7.8

—▲— same buffer containing 10 mM $MgCl_2$

—■— same buffer containing 10 mM EDTA


a
b
0.4
0.3
0.2
0.1
0
DNase II units
0
2
4
6
8
10
Days at 4 °C

the decrease in DNase II activity, 1 ml of diisopropyl fluorophosphate (DFP), a protease inhibitor which irreversibly inactivates proteolytic enzymes containing serine at their active sites by alkylating that serine, was added to 260 ml of 105,000 xg supernatant solution. The proteolytic activity was reduced to 5% of that originally present. DNase II was not affected by the addition of the DFP and remained stable at 4°C in the solution containing DFP over a period of at least 14 days.

When a 105,000 xg supernatant solution which had been treated with DFP, was chromatographed on a DEAE cellulose column under conditions described previously, 80-90% of the applied DNase II units were eluted with the 20 mM phosphate buffer, pH 7.8, and about 10%, with the potassium chloride gradient. A similar result was obtained with untreated 105,000 xg supernatant when 10 mM EDTA was added to the buffer solutions that were used for elution of the column. The EDTA may chelate the divalent metal ions that are required for optimal activity of some proteases. It was decided to include 10 mM EDTA in all buffers used in elutions of DNase II from columns and in concentration and dialysis of solutions containing DNase II. This was done both because of the toxicity of DFP and because DFP does not inactivate the zygomen precursors of proteolytic enzymes which may later be converted to active proteases. Otsuka and Price (1974) found that even after treatment of the enzyme preparation with DFP, DNase I activity was lost in a solution that did not contain divalent metal ions. They suggested that the loss of DNase I activity was due to activation of zygomen precursors of proteolytic enzymes.

Discussion

A procedure was described for the preparation of large amounts of mucosal extract from bovine small intestine with very little loss of DNase II activity. The extract was stored at -80°C for at least two years without any further loss of DNase II activity.

Two DNase II activities were separated on DEAE cellulose, and on rechromatography on DEAE cellulose, each activity was eluted in the same position as observed originally. Although there was a problem of the low yields of DNase II activity, the results appeared to indicate that there were two different DNase II activities in accord with the findings of previous investigators that two DNase II activities were present in some tissues (Cordonnier and Bernardi, 1968; Yamanaka et al., 1974; Zöllner et al., 1974).

EDTA probably stabilized DNase II by chelating metal ions which would otherwise have caused a loss of DNase II activity either directly by denaturation of the enzyme molecule, or indirectly through enhancement of the activity of proteases present in the DNase II preparation. The latter hypothesis is supported by experiments demonstrating the presence of proteolytic activity in solutions containing DNase II and the stabilizing effect of diisopropyl fluorophosphate, a protease inhibitor, on DNase II activity.

B Investigation of the properties of two DNase II activities isolated from bovine intestinal mucosa

pH optima

The two DNase II activities were observed in the presence of EDTA and had acidic pH optima as shown in Fig. 17. The absorbance at 260 nm of the acid-soluble oligonucleotides released from calf thymus DNA, 400 ugm/ml, upon incubation with enzyme for 10 minutes in 120 mM sodium acetate buffer containing 8 mM EDTA, was taken as a measure of the enzymatic activity at the various pH values. The first DNase II activity had a broad acidic pH optimum centered at about pH 4.8 (Fig. 17a), whereas the second DNase II activity had a narrower pH optimum also centred at pH 4.8 (Fig. 17b).

Degradation of native and denatured DNA

Calf thymus DNA was denatured by placing the DNA solution, 4 mg DNA per ml of 10 mM sodium chloride, into a stoppered test tube in a boiling water bath for 15 minutes and then cooling the DNA solution in an ice-water bath. DNase II activity was determined by the acid-soluble oligonucleotide assay using 400 ugm/ml of either native or denatured DNA. Table VI indicates that the first DNase II activity degraded native DNA at a rate nearly three times faster than it degraded denatured DNA, whereas the second DNase II activity degraded both native and denatured DNA at about the same rate.

Fig. 17 pH optima of the DNase II activities. The absorbance at 260 nm of the acid-soluble oligonucleotides released from calf thymus DNA (400 ugm/ml) upon digestion with enzyme for 10 minutes in 120 mM sodium acetate buffer containing 8 mM EDTA was taken as a measure of the enzymatic activity at different pH values.

(a) pH optimum of the first DNase II activity

(b) pH optimum of the second DNase II activity

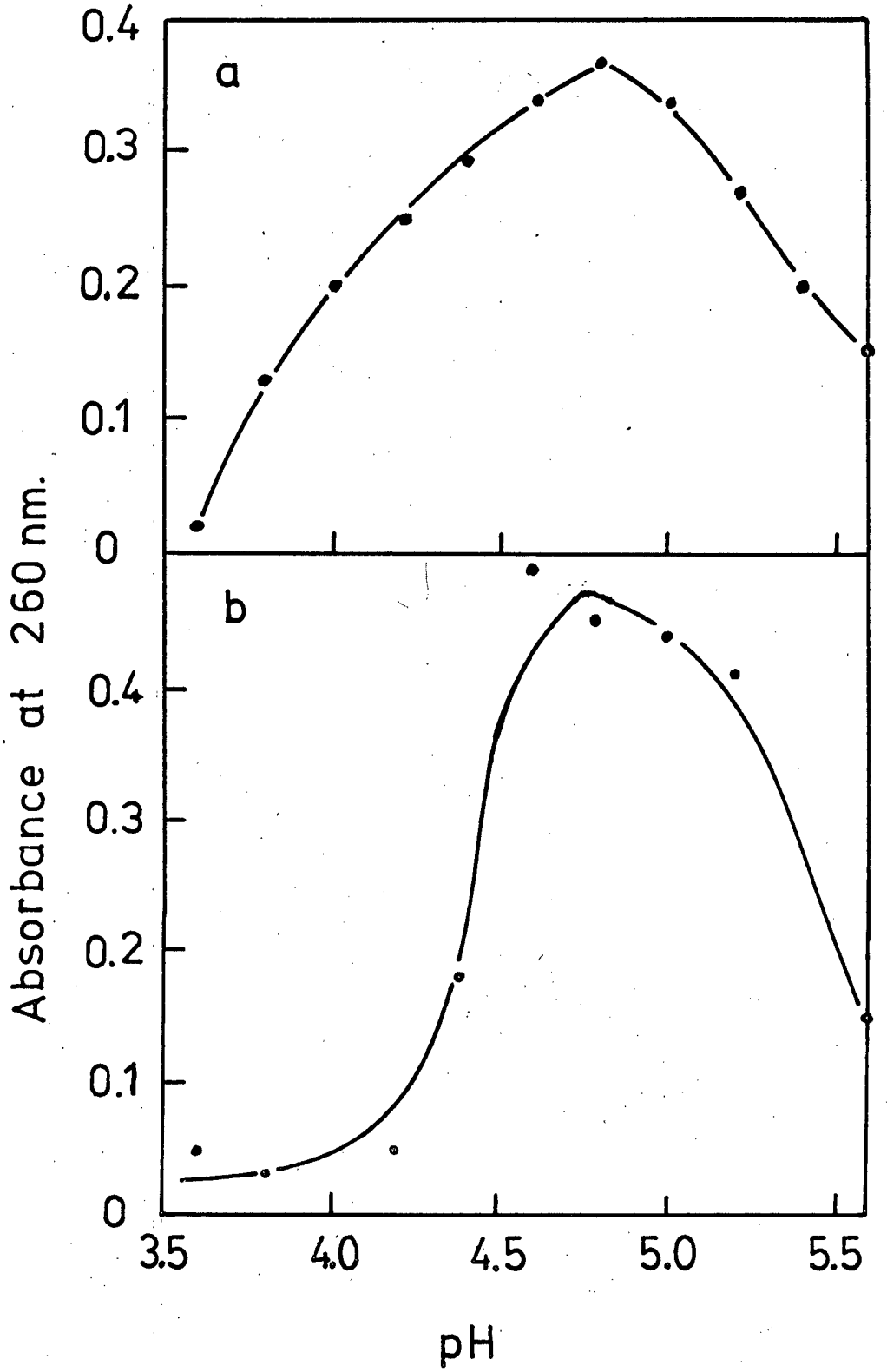

a
b
0.4
0.3
0.2
0.1
0
0.4
0.3
0.2
0.1
0
Absorbance at 260 nm.
3.5
4.0
4.5
5.0
5.5
pH

TABLE VI

RATE OF RELEASE OF ACID-SOLUBLE OLIGONUCLEOTIDES FROM NATIVE AND DENATURED DNA BY DNase II

	Increase in absorbance at 260nm per minute per ml of enzymic solution	
	Native DNA	Denatured DNA
First DNase II Activity	0.56	0.20
Second DNase II Activity	0.13	0.13

Effects of ionic strength

In order to determine whether an increase in ionic strength affected the two DNase II activities differently the enzymes were assayed in 20 mM sodium acetate buffer, pH 5.0, containing 10 mM EDTA and different concentrations of sodium chloride. Fig. 18 compares the two DNase II activities in solutions of different ionic strength. DNase II activity was measured by the acid-soluble oligonucleotide assay and expressed as a percent of the activity observed in the buffer containing no sodium chloride. Slight increases in activity were seen for the first DNase II at 0.1 M NaCl, and for the second DNase II at 0.05 M NaCl. The second DNase II was less active than the first DNase II in solutions of high ionic strength.

Effects of sulfate, an inhibitor of DNase II

Since sulfate is an inhibitor of DNase II from other tissues (Bernardi and Griffe, 1964; Koener and Sinsheimer, 1957; Rosenblüth and Sung, 1969), the effect of different concentrations of sulfate on the two intestinal DNase II activities was measured using the acid-soluble oligonucleotide assay. As may be seen from Fig. 19, both DNase II activities were inhibited by low concentrations of sulfate. The second DNase II was inhibited more than the first DNase II activity at high concentrations of sulfate. The shapes of the curves for the two DNase II activities were different: a slight increase in activity was observed

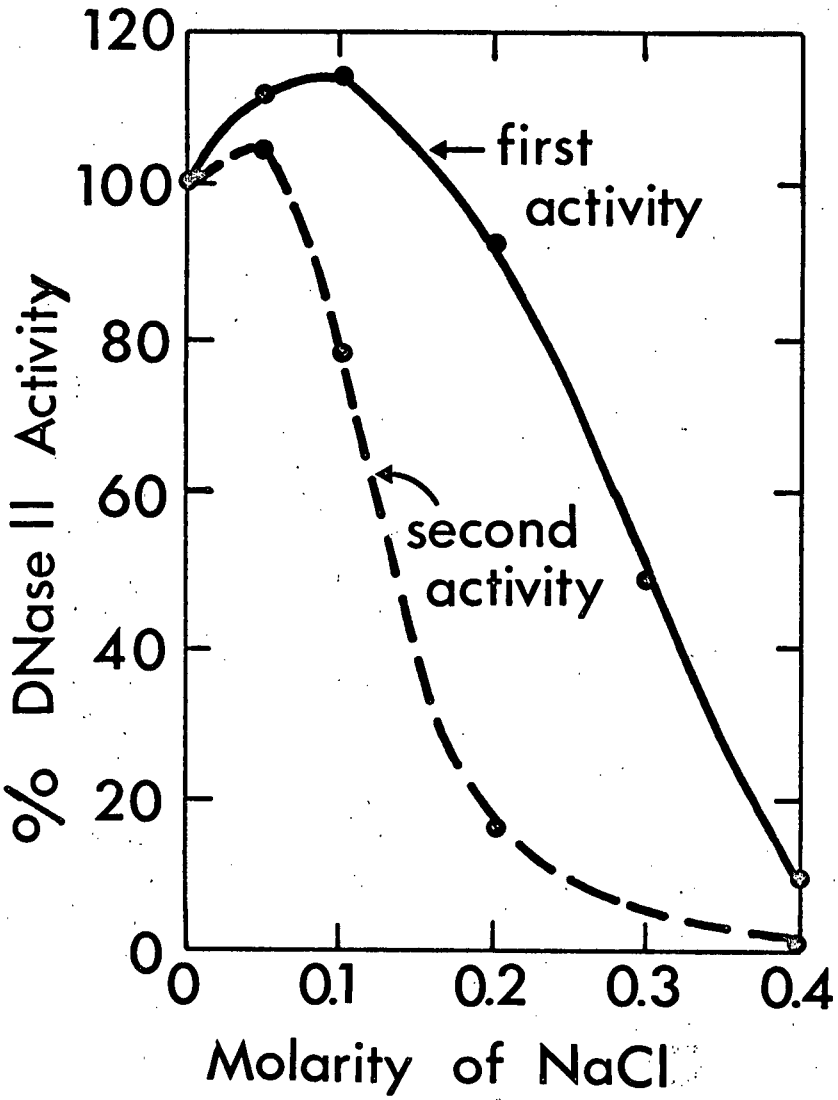


Fig. 18 Comparison of the first and second DNase II activities at different ionic strengths. DNase II activity was measured by the acid-soluble oligonucleotide assay in 20 mM sodium acetate buffer, pH 5.0, containing 10 mM EDTA and sodium chloride to the indicated molarity. The DNase II activity was expressed as a percent of the activity observed in the buffer containing no sodium chloride.

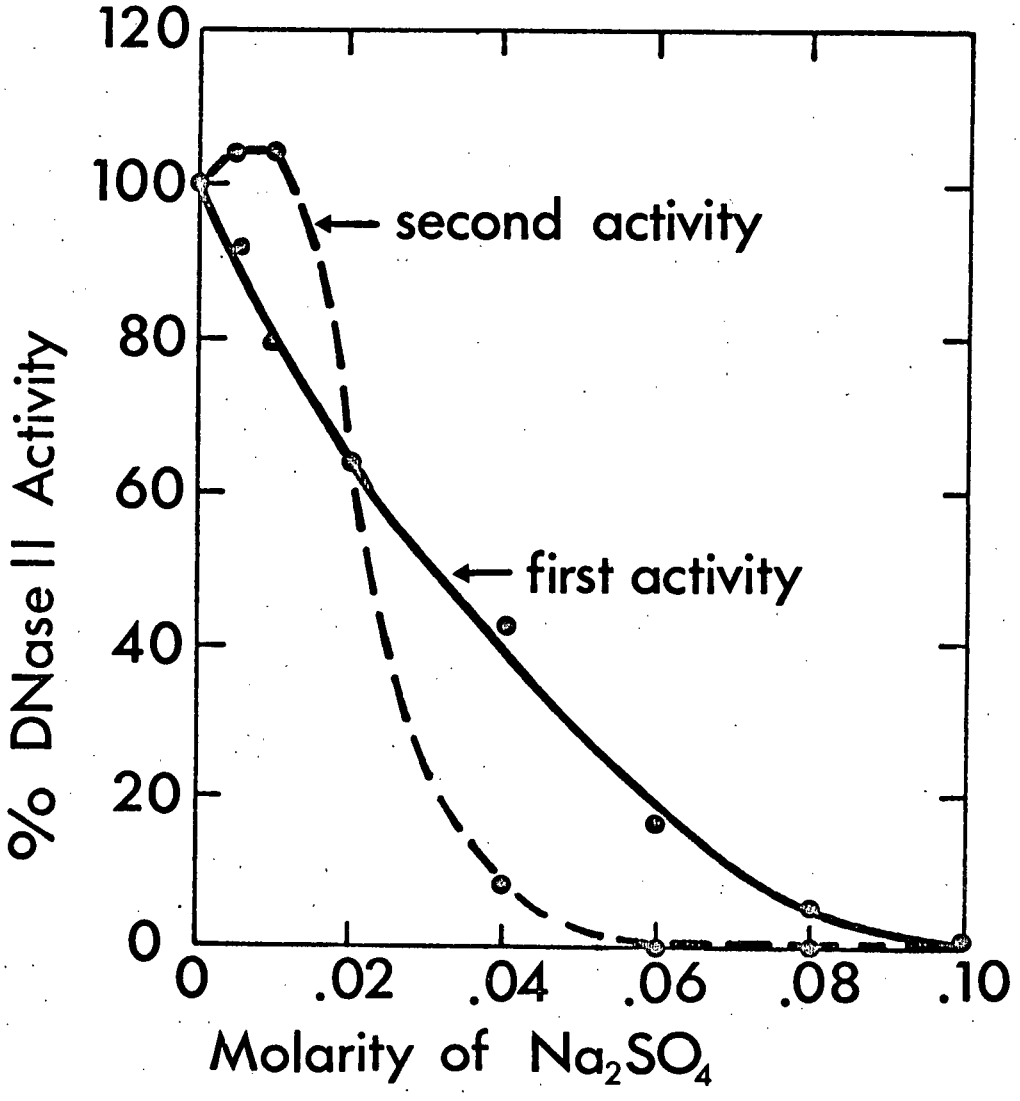


Fig. 19 Effect of sulfate on the first and second DNase II activities. DNase II activity was measured by the acid-soluble oligonucleotide assay in 20 mM sodium acetate buffer, pH 5.0, containing 10 mM EDTA and sodium sulfate to the indicated molarity. The DNase II activity was expressed as a percent of the activity observed in the buffer containing no sodium sulfate.

for the second DNase II activity at 0.01 M sulfate concentration whereas no such increase in activity was seen for the first DNase II.

Activation energies for the hydrolysis of DNA

Different activation energies for the DNase II catalysed hydrolysis of DNA could indicate that the two DNase II activities were due to different enzyme molecules. Hydrolysis of calf thymus DNA by DNase II was measured as a function of time at different temperatures by the acid-soluble oligonucleotide assay. The slopes of the linear portions of the absorbance at 260 nm versus time curves were taken as indicative of the rate of hydrolysis of the DNA at the various temperatures. Fig. 20 demonstrates that, when the rates of hydrolysis were plotted on a logarithmic scale as a function of the reciprocal of the absolute temperature, straight lines of different slopes could be drawn through the points for the first and second DNase II activities. The activation energies for the hydrolysis of native calf thymus DNA were calculated from the Arrhenius equation (Mahler and Cordes, 1971) to be 19 kcal/mole for the first DNase II and 8.1 kcal/mole for the second DNase II. Oth et al. (1958) found an activation energy of 23 kcal/mole for the hydrolysis of calf thymus DNA by DNase II from thymus.

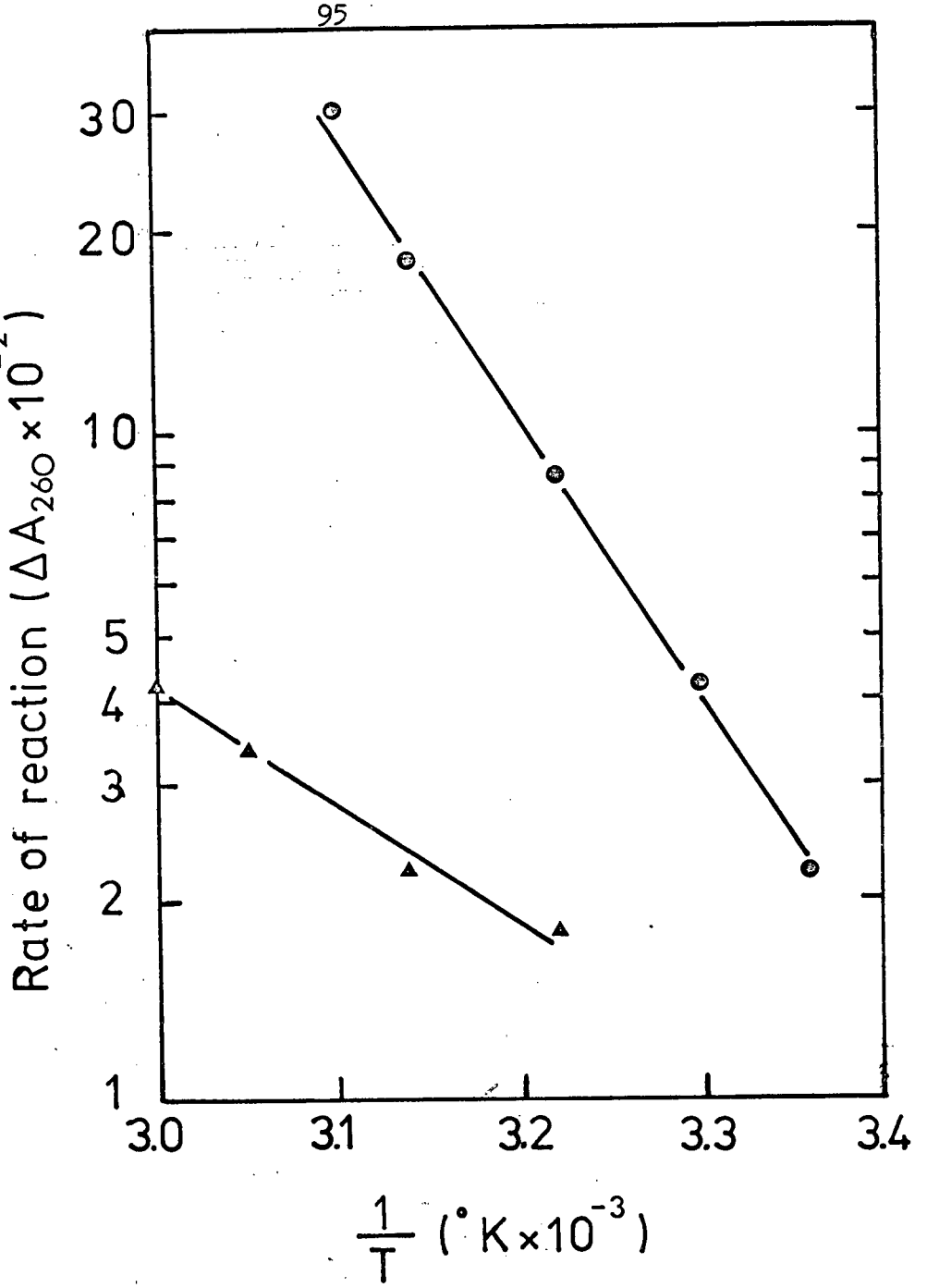


Fig. 20 Arrhenius plot showing the temperature dependence of the rate of hydrolysis of calf thymus DNA by DNase II. The logarithm of the reaction rate was plotted as a function of the reciprocal of the absolute temperature.

—●— plot using the first DNase II activity

—▲— plot using the second DNase II activity

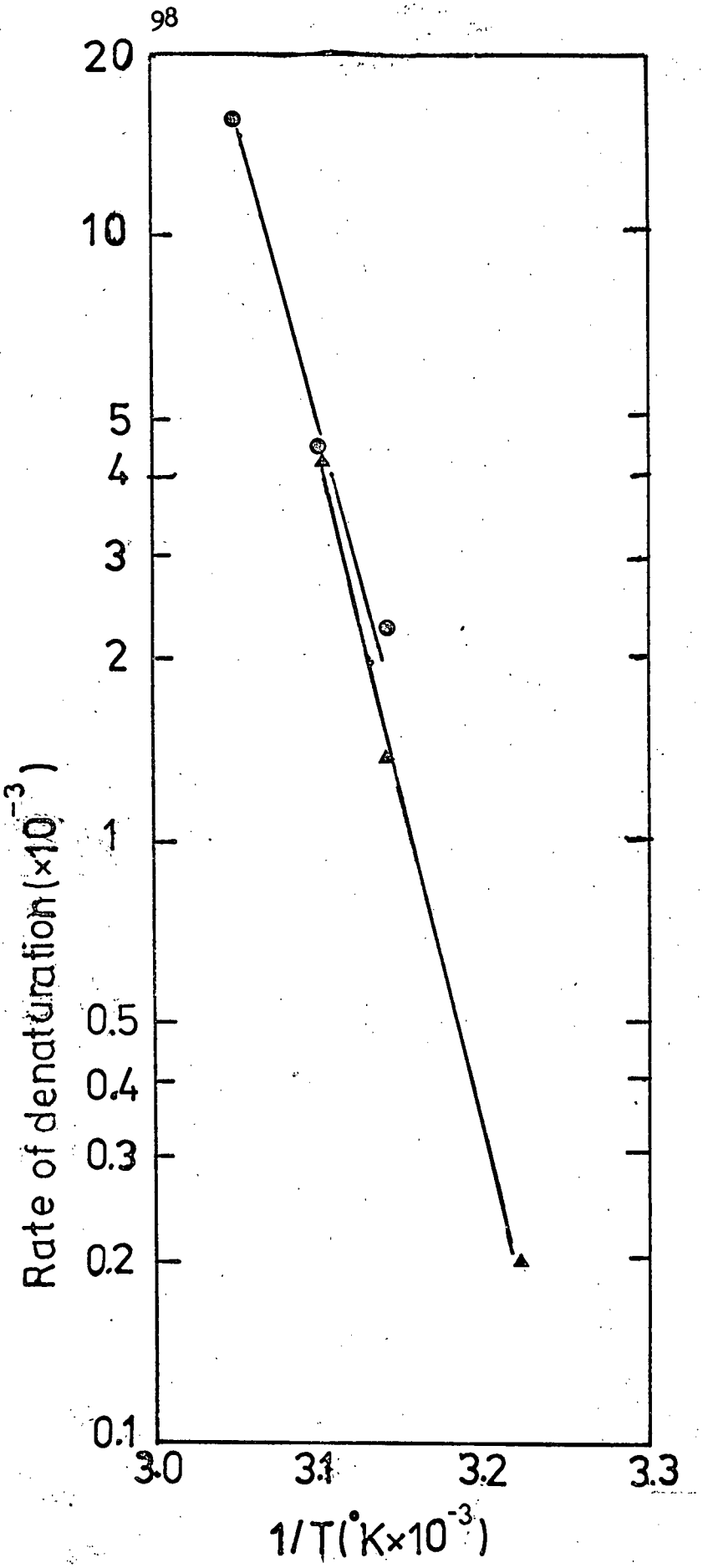
Activation energy for the denaturation of DNase II

In order to compare the enzyme molecules more directly, the decrease in DNase II activity which occurred after heating at various temperatures was measured. Aliquots of the first or second DNase II activity were heated at 37, 45, or 50°C for times up to 30 minutes. The enzymic solutions were then cooled rapidly to 0°C to stop further denaturation, and the DNase II activity remaining was determined at 37°C by the acid-soluble oligonucleotide assay. The DNase II activity remaining was plotted as a function of the time that the enzyme solution had been heated at a particular temperature. Slopes of tangents drawn to these curves at zero time were taken as indicative of the initial rates of denaturation of the enzyme molecules. Fig. 21 exhibits a plot of the logarithm of the rate of denaturation as a function of the reciprocal of the absolute temperature. The "activation energy" for denaturation was calculated from the Arrhenius equation (Mahler and Cordes, 1971) to be 43 kcal/mole for the first DNase II and 51 kcal/mole for the second DNase II; these values fall within the range observed for other enzymes (Mahler and Cordes, 1971). Although the values are fairly close, especially considering the different purity of the two preparations, this does not necessarily imply that the two DNase II activities are due to the same enzyme molecule since different enzymes can denature at the same rate. However, should the activation energies for denaturation have been very different this would have indicated the presence of different enzyme molecules.

Fig. 21 Temperature dependence of the rate of denaturation of DNase II. The DNase II activity remaining was plotted as a function of the time that the enzyme solution had been heated at a particular temperature. Slopes of tangents drawn to these curves at zero time were taken as indicative of the initial rates of denaturation and were plotted on a logarithmic scale as a function of the reciprocal of the absolute temperature.

denaturation of the first DNase II activity

denaturation of the second DNase II activity


20
10
5
4
3
2
1
0.5
0.4
0.3
0.2
0.1
Rate of denaturation (×10⁻³)
3.0
3.1
3.2
3.3
1/T(°K×10⁻³)

Presence of endogenous DNA in the second DNase II preparation

The properties of the first and second DNase II activities described above were determined using the acid-soluble oligonucleotide assay because the second activity preparation contained a large amount of material which precipitated in the hyperchromicity assay and obscured the results. The usual blank or control for the acid-soluble oligonucleotide assay was a solution in which DNA had been incubated for the same time and at the same temperature as for the enzymatic reaction. The perchloric acid was added to the control solution at the same time as the enzymatic reaction was stopped. An amount of enzyme solution equal to that used in the enzymatic reaction was then added to the control solution. This solution, which was called a "substrate blank" because it was the substrate that was incubated, now contained all the components that were present in the reaction mixture except that the enzyme had not acted on the substrate. The absorbance at 260 nm found for the substrate blank solution was subtracted from the absorbance at 260 nm found for the reaction mixture.

In order to determine whether there was endogenous substrate in the enzyme preparations "enzyme blank" solutions were prepared. That is, the enzyme solutions were incubated in buffer for the same time and at the same temperature as for the reaction, perchloric acid was added, and then substrate (DNA) was added. Thus, the enzyme blank contained all the components of the reaction mixture except that it was the enzyme and not the substrate that had been incubated alone.

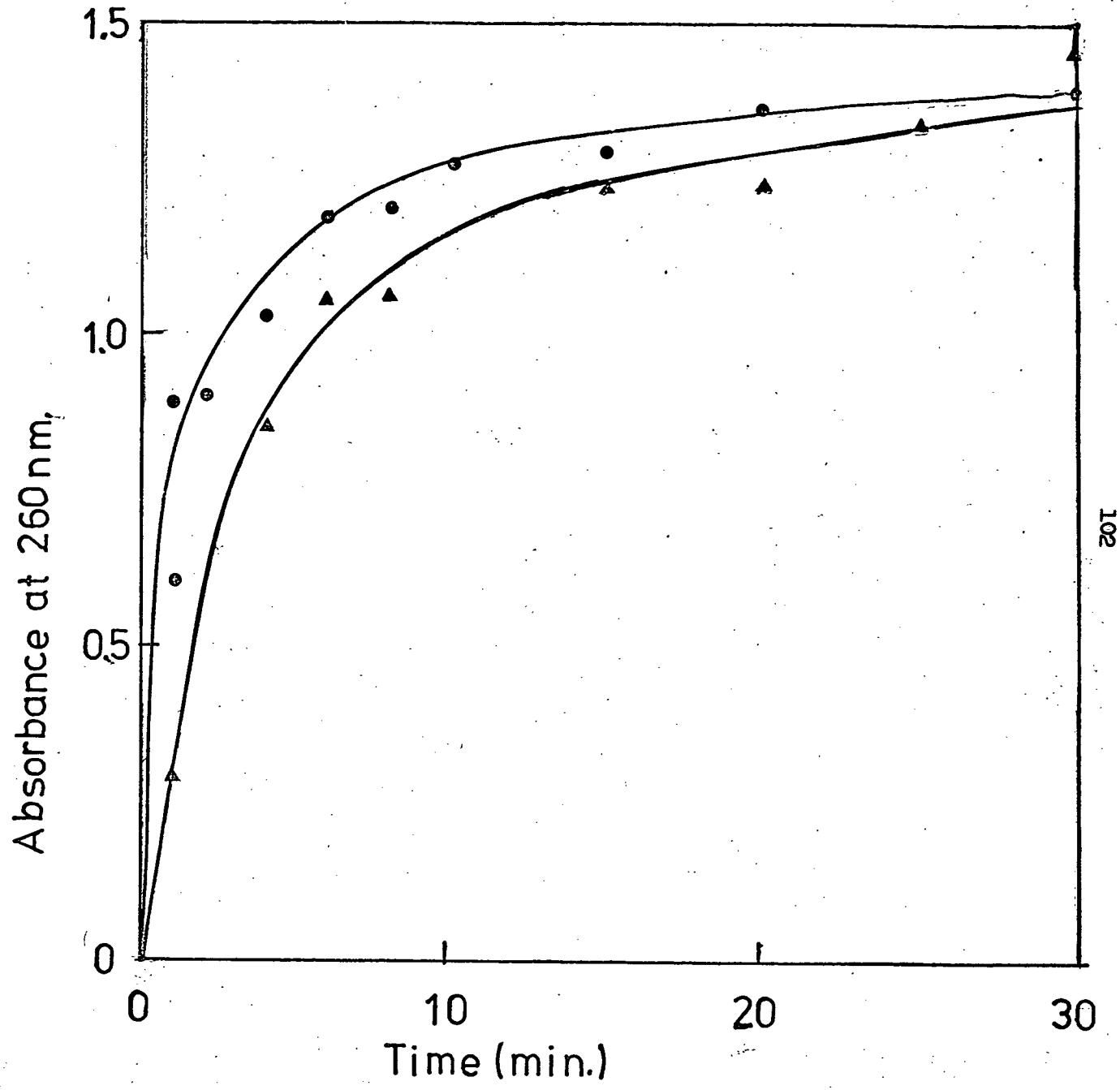
For the first DNase II about the same low absorbance at 260 nm was obtained for the substrate and enzyme blank solutions. Fig. 22 demonstrates that for the second DNase II almost identical curves of absorbance at 260 nm against time were observed for the release of acid-soluble oligonucleotides in the presence and absence of added substrate. This indicates that the second DNase II preparation contained an amount of endogenous substrate almost equal to that added in the acid-soluble oligonucleotide assay. The second DNase II was prepared by pooling fractions from the DEAE cellulose column, and concentrating the solution by ultrafiltration through an Amicon PM 10 membrane using nitrogen at 5.0 p.s.i. pressure. Salt was removed from the solution by "diafiltration": aliquots of 20 mM phosphate buffer, pH 7.8, containing 10 mM EDTA were added sequentially to the concentrated solution containing the second DNase II and the solution was subjected to ultrafiltration until the conductivity of the solution was similar to that of the buffer which had been added. The solution containing the second DNase II was then lyophilized.

In order to determine whether the endogenous substrate in the second DNase II preparation was nucleic acid, protamine sulfate was added to the preparation. Protamine, a basic protein, binds to the negatively charged phosphate groups of nucleic acids. The resulting nucleoprotein complex is insoluble at salt concentrations equivalent to about 0.14 M sodium chloride (Davidson, 1972). The lyophilized powder containing the second DNase II was dissolved in water at a concentration of 25 mg/ml. To 2.0 ml of this preparation was added 0.6 ml of a 1% solution of protamine sulfate. The mixture was allowed to stand in an ice-water bath for

Fig. 22 Release of acid-soluble oligonucleotides by the second DNase II in the presence and absence of added substrate. The reaction was carried out in 0.15 M sodium acetate buffer, pH 5.0, containing 10 mM EDTA.

—●— 40 ugm/ml calf thymus DNA present in the reaction mixture

—▲— no exogenous DNA present in the reaction mixture


1.5
1.0
0.5
0
Absorbance at 260nm,
0
10
20
30
Time (min.)

10 minutes and then was centrifuged in the cold at 12,100 xg for 10 minutes. The supernatant solution was decanted and the precipitate resuspended in 2 ml of water. Table VII demonstrates that the absorbances at 280 nm and 260 nm of the solution containing DNase II were reduced 3 and 5 fold respectively by protamine sulfate treatment. The A280/A260 ratio was increased from 0.65 to 0.89. Pure nucleic acids have an A280/A260 ratio of 0.5; mucoproteins, 0.8, and proteins containing only amino acids, 1.75 (White, Handler and Smith, 1974). Since protamine forms an insoluble complex with nucleic acids, the formation of a precipitate as well as the decrease in the absorbances at 280 nm and 260 nm and the increase in the A280/A260 ratio upon treatment of the second DNase II preparation with protamine sulfate indicate the presence of nucleic acids in the second DNase II preparation. In Table VII, it may be seen that for the untreated DNase II, the enzyme blank was much higher than the substrate blank, whereas after treatment with protamine sulfate the enzyme blank was only slightly higher than the substrate blank. This indicates that it was likely the endogenous nucleic acids in the untreated second DNase II preparation that were responsible for the high enzyme blank.

Although the second DNase II preparation contained nucleic acids, these could have been DNA and/or RNA. In order to determine whether the second DNase II activity contained DNA specifically, the deoxyribose content was determined by the method of Dische as modified by Burton (1968). The lyophilized powder containing the second DNase II activity was found to contain 14% (w/w) DNA by this method. Thus, the 25 mg/ml solution of

TABLE VII

EFFECT OF TREATMENT OF THE SECOND DNase II PREPARATION WITH PROTAMINE SULFATE

Preparation	Volume (ml)	Absorbances of Solutions			DNase II Activity (Acid-soluble oligonucleotide assay) (0.1 ml x 15')				
					assay value	1 substrate blank	2 enzyme blank	Total DNase II units with respect to	
		A_{280}	A_{260}	A_{280}/A_{260}	A_{260}	A_{260}	A_{260}	1	2
second DNase II	2.0	82.8	126.5	0.65	3.99	1.06	3.63	5.82	0.72
second DNase II supernatant	2.2	24.6	27.5	0.89	3.14	1.10	1.36	4.49	3.92
protamine sulfate resuspended precipitate	2.4	--	--	--	0.46	0.27	0.42	1.09	0.096

second activity used for the protamine sulfate experiment, contained 3.5 mg DNA per ml. Since the 0.1 ml of enzyme solution added to each assay tube contained 0.35 mg DNA and since the amount of calf thymus DNA added to each assay was 0.4 mg, the assay value for the enzyme blank for the second DNase II activity was of a comparable magnitude to the assay value for the reaction with calf thymus.

Rechromatography of the two DNase II activities on DEAE cellulose

Since it had been found that 10 mM EDTA stabilized the two DNase II activities, and since the presence of DNA in the second activity preparation could have caused apparent differences in the properties of the two activities, each of the two DNase II activities were again rechromatographed separately on DEAE cellulose columns in order to determine whether the two activities were due to chromatographically distinct species or to an artifact of the chromatographic procedure.

The first DNase II was eluted from the DEAE cellulose column with 20 mM phosphate buffer, pH 7.8, containing 10 mM DETA as in the first rechromatography experiment (see Fig. 14) except that the yield of DNase II units was now 99%.

The second DNase II activity was rechromatographed on DEAE cellulose. The column, 2 x 18 cm, was eluted with 20 mM phosphate buffer, pH 7.8, containing 10 mM EDTA, and with 1 M potassium chloride in the same buffer. Fig. 23 shows the elution profile from the column. Ninety percent of the applied DNase II units were eluted with the 20 mM phosphate buffer,

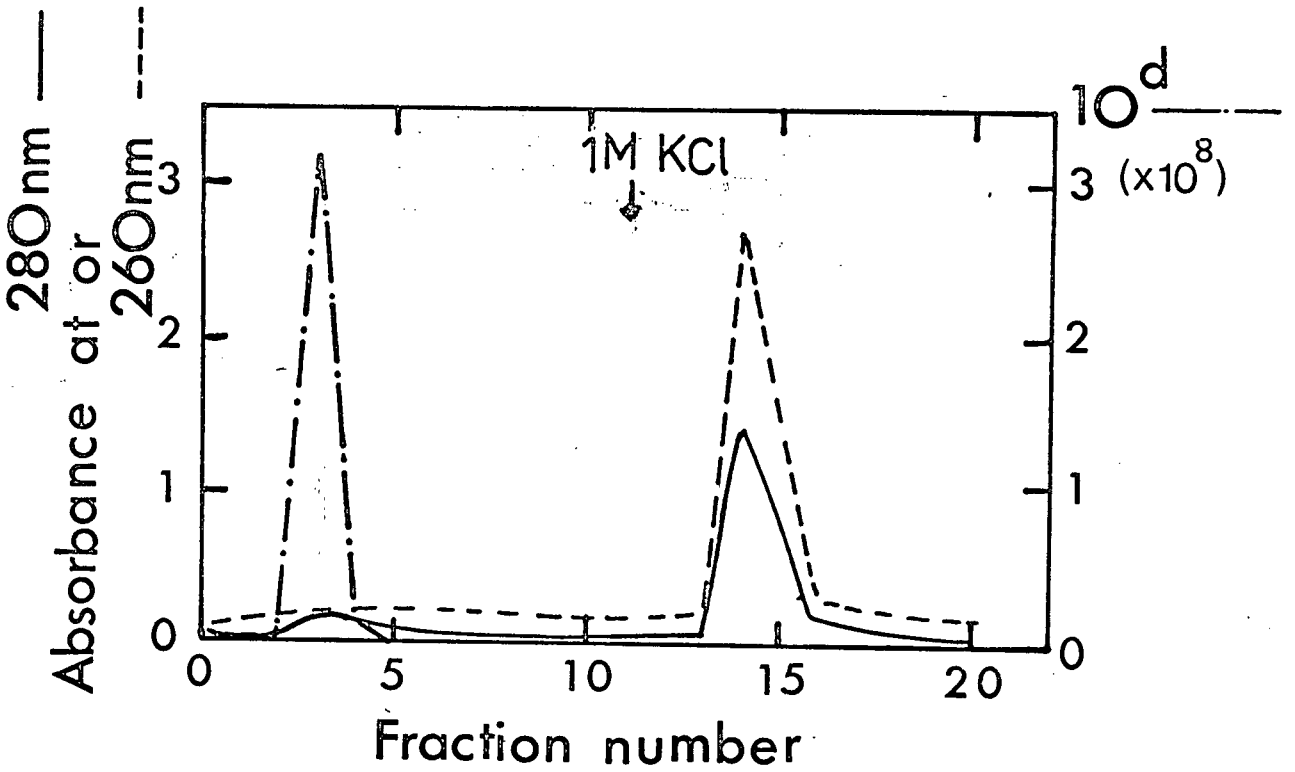


Fig. 23 Rechromatography of the second DNase II activity on DEAE cellulose. The column, 2 x 18 cm, was eluted with 20 mM phosphate buffer, pH 7.8, containing 10 mM EDTA, and then with 1 M potassium chloride in the same buffer. Protein was estimated by absorbance at 280 nm, nucleic acid, by absorbance at 260 nm and DNase II activity by the DNA-agar gel assay. The antilogarithm of the diameter of the zone of clearing produced by the action of DNase II on DNA (10^d) gives the relative enzyme concentration in the different fractions.

and 5-10% with 1 M potassium chloride in the same buffer. It may be recalled that in a previous rechromatography experiment (Fig. 15) no DNase II activity had been eluted with the 20 mM phosphate buffer, and 77% of the applied DNase II activity had been eluted with the 1 M potassium chloride step. A possible explanation for the different results obtained in the two experiments may be deduced from the observation that, in the first experiment, due to the absence of the stabilizing agent EDTA in the enzyme solution, the overall yield of DNase II activity from the fractions of the original chromatography to the rechromatography fractions was 10%--the DNase II activity that eluted with the 1 M potassium chloride step. In the second experiment with the inclusion of 10 mM EDTA in all solutions, the overall yield of DNase II activity was 95-100% and of this 5-10%, about the same overall percent as before, was eluted with the 1 M potassium chloride step. It may be that in the first experiment all of the DNase II enzyme was denatured or degraded except for 10% which was stabilized by binding to endogenous DNA. Most of the 10% of the DNase II activity was then eluted with the 1 M potassium chloride step.

Chromatography of the first DNase II activity on DNA cellulose

In order to determine whether the first DNase II activity could be bound to DNA that was bound to cellulose and in this way give rise to a "second" DNase II activity, a DNA cellulose column, 2 x 18 cm, was prepared by the method of Alberts and Herrick (1971) and the first DNase II activity was chromatographed on it. The column was eluted with 20 mM

Tris HCl, pH 7.8, containing 1 mM EDTA and with a linear gradient of 0 to 1 M potassium chloride in the same buffer. Fig. 24 demonstrates that the DNase II activity was bound to the DNA cellulose column in the 20 mM Tris buffer and was eluted at the beginning of the potassium chloride gradient.

Chromatography of the two DNase II activities on Sephadex G100

In order to estimate the molecular weights of the two DNase II enzymes each was chromatographed separately on a Sephadex G100 column, 2.5 x 78 cm. The column was eluted at a flow rate of 17.4 ml per hour with 0.25 M Tris HCl, pH 7.5, containing 10 mM EDTA and 4 ml fractions were collected. The column was calibrated by the application, separately, of blue dextran, bovine serum albumin (MW 67,000) and chymotrypsinogen A (MW 25,000), ovalbumin (MW 45,000) and ribonuclease A (MW 13.700). Each sample was applied in 1.0 ml of the elution buffer. Upward elution was used and 2 ml of a solution of 10% sucrose in the elution buffer was added after the sample in order to ensure even application of the sample to the column. The positions of elution of the above samples were determined by absorbance at 280 nm. DNase II activity in column fractions was determined by the acid-soluble oligonucleotide assay. The elution profile for the second DNase II activity is illustrated in Fig. 25. The positions of the maxima of the symmetrical elution peaks for the other samples and for the first DNase II activity are indicated by arrows. Multiple peaks of activity were observed for the second DNase II; the

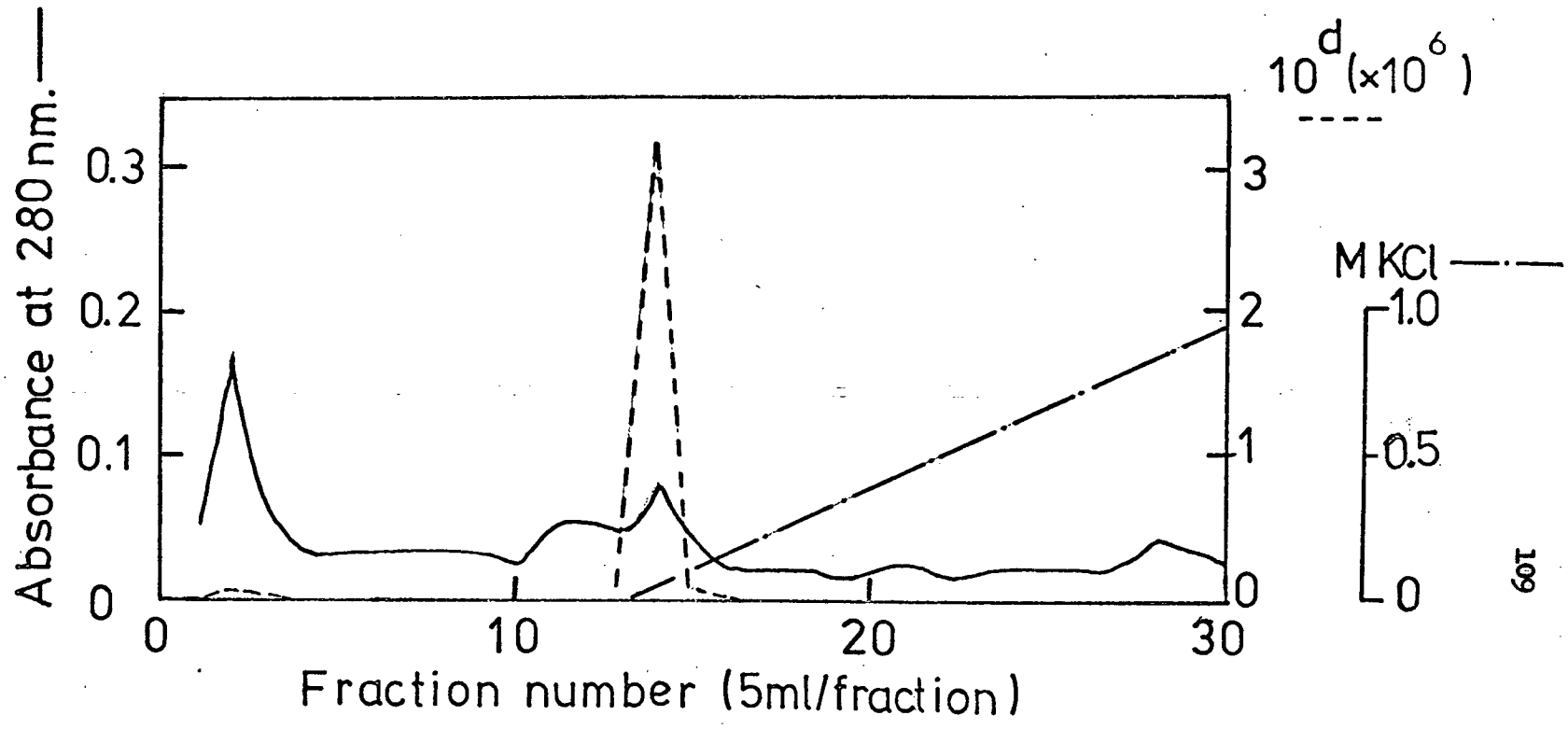


Fig. 24 **Chromatography of the first DNase II activity on DNA cellulose. The column, 2 x 18 cm, was eluted with 20 mM tris HCl, pH 6.8, containing 1 mM EDTA and then with 1 M potassium chloride in the same buffer.**

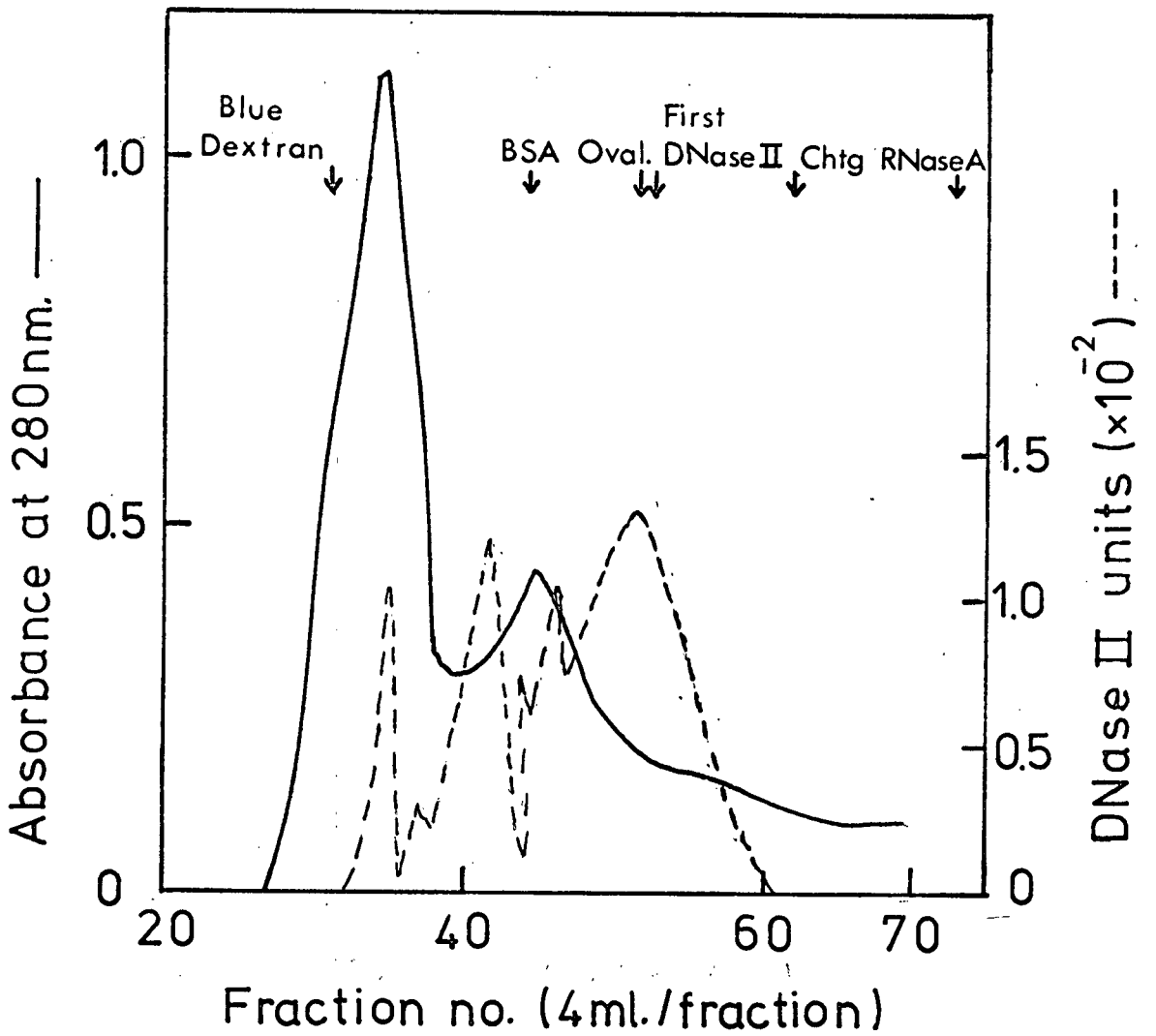


Fig. 25 Chromatography of the second DNase II activity on Sephadex G100. The column, 2.5 x 78 cm, was eluted upwards with 0.25 M Tris HCl, pH 7.5, containing 10 mM EDTA. Protein was estimated by absorbance at 280 nm; the second DNase II activity, by the acid-soluble oligonucleotide assay. The arrows indicate the elution positions of blue dextran, bovine serum albumin (BSA), ovalbumin (oval), chymotrypsinogen A and RNase A as determined by maximal absorbance at 280 nm. In a separate experiment the first DNase II was found to elute in a single symmetrical peak with a maximum at the position indicated by the arrow.

peak associated with the species of lowest molecular weight coincided with the elution position of the first DNase II. This is consistant with the second DNase II being the same molecular weight as the first DNase II and binding with progressively larger fragments of DNA to yield peaks of enzymatic activity eluting from the column in different molecular weight ranges.

From elution positions shown in Fig. 25 the partition coefficients (Kav values) of proteins of known molecular weight between the mobile phase, the buffer solution moving down the Sephadex column, and the entire gel phase, the liquid imbibed in the gel phase plus the gel matix itself, were calculated by the method described by Fischer (1969) and Reiland (1971). These partition coefficients were plotted in Fig. 26 against the logarithms of the molecular weights of the proteins. The partition coefficients for the first DNase II was determined and by interpolation on Fig. 26 the molecular weight of the first DNase II was estimated to be 41,000. As suggested previously the molecular weight of the second DNase II is likely the same as the first DNase II.

Discussion

Previous investigators found two DNase II activities in some tissues (Cordonnier and Bernardi, 1968; Yamanaka et al., 1974; Zöllner et al., 1974). Since DNase II activity had been found in calf thymus nuclei (Slor and Lev, 1971) as well as in lysosomes (de Duve et al., 1962), it was considered possible that the two DNase II activities isolated from

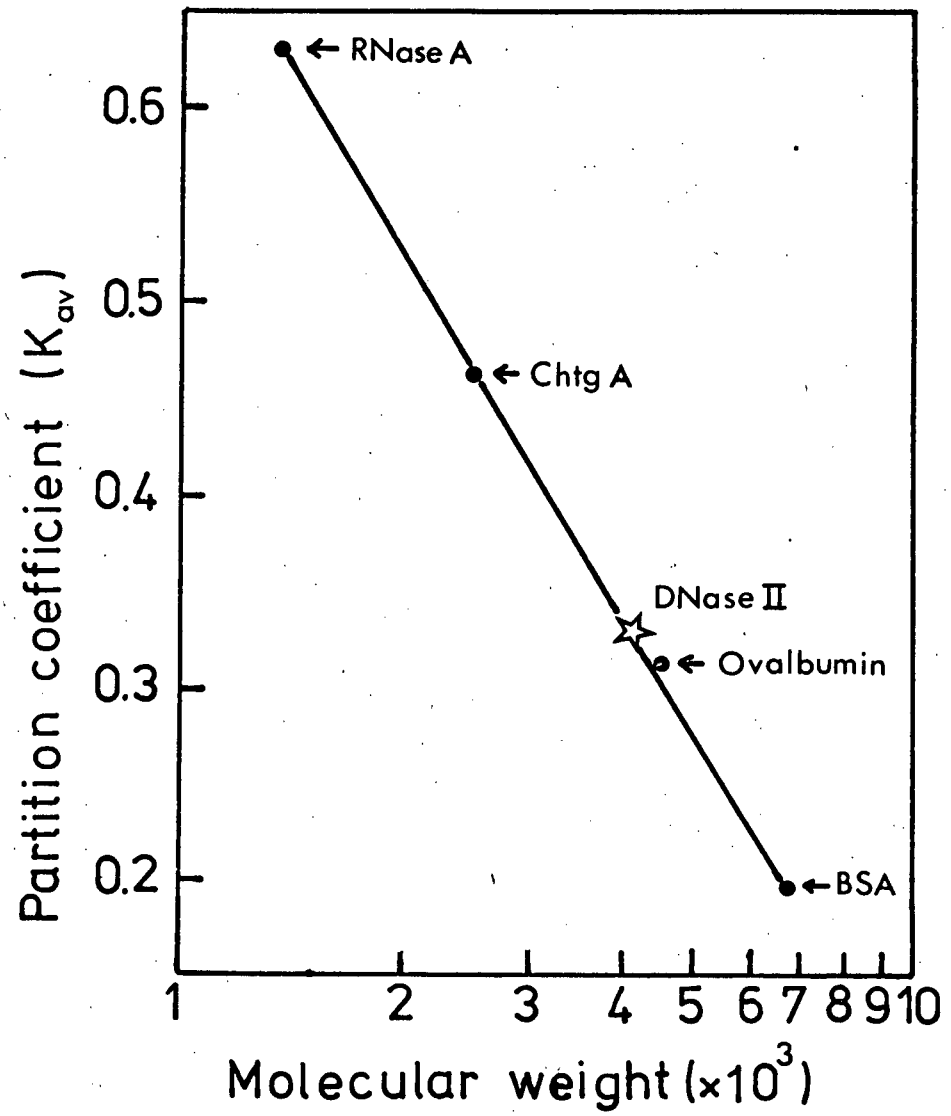


Fig. 26 Estimation of the molecular weight of the first DNase II by gel filtration on Sephadex G100. The partition coefficients (Kav values) of the protein whose elution positions are shown in Fig. 25 were plotted against the logarithms of the molecular weights of the proteins. The molecular weight of the first DNase II was estimated by interpolation.

intestine and other sources might have different intracellular locations and biological functions.

We found, however, that the appearance of a second DNase II activity was an artifact of the DEAE cellulose chromatography. Negatively charged DNA present in the 105,000 xg supernatant solution was bound electrostatically to the positively charged DEAE cellulose column and about 10 percent of the DNase II activity was bound to the DNA. When the column was eluted with a potassium chloride gradient, the bound DNase II activity was eluted along with some, but not all of the DNA. The DNA may also have been degraded somewhat due to DNase II action.

Evidence that the first and second DNase II activities are not due to distinct enzyme molecules comes from the rechromatography experiment in which 90% of the second activity was eluted with 20 mM phosphate buffer in a similar manner to the first activity. The earlier rechromatography experiment appeared to give contradictory results because in the absence of EDTA only 10% of the DNase II that was associated with DNA was stable. This DNase II was bound to the column with the DNA; the 90% of the DNase II activity that would have been eluted with the phosphate buffer had been denatured or digested by proteases and was not observed. That DNase II can bind to and be eluted from DNA which is bound to a cellulose column was shown by the DNA cellulose chromatography experiment. The second DNase II preparation contained DNA, and several peaks of DNase II activity were seen when the second DNase II was chromatographed on a Sephadex G100 column. This suggests that the DNase II activity may have been bound to DNA fragments of different lengths. These fragments could

have been liberated by the action of DNase II itself.

The presence of DNA in the second DNase II preparation could account for some of the apparently different properties of the first and second DNase II activities. Some of the variance in properties may also have resulted from the different purity of the two preparations--the first and second DNase II preparations had specific activities of about 0.5 and 0.05 DNase II units per mg of protein, respectively.

The second DNase II apparently degraded denatured DNA at the same rate as native DNA. However, in the assay with denatured DNA, endogenous native DNA was being added along with the enzyme. If the enzyme did degrade denatured DNA at a lower rate than native DNA, this would not be apparent because of the presence of endogenous native DNA in the reaction mixture.

The second DNase II had lower activity in solutions of high ionic strength than the first DNase II. A precipitate formed in the pH 5 assay solution containing the second DNase II activity, but not in the solution containing the first DNase II activity. Due to the presence of excess DNA and/or protein in the second DNase II preparation, precipitation of a DNA-protein complex may have occurred, perhaps more readily at higher ionic strengths, and caused an apparently lower activity for the second DNase II by removal of substrate from the assay solution. This effect would have been superimposed upon the effect of inhibition of the enzyme itself by high ionic strength which was also seen for the first DNase II activity.

The second DNase II had lower activity than the first DNase II

in solutions containing 0.03 to 0.1 M sulfate. This may have been due to the precipitation of a DNA-protein complex from the assay solution in a similar manner as described previously at high ionic strength. The similar shape of the curves of the second DNase II activity as a function of increasing ionic strength and sulfate concentration indicates that a similar process might have occurred in both experiments.

The apparently lower activation energy for the DNA hydrolysis reaction with the second DNase II as opposed to the first DNase II may have been due to the presence of larger amounts of DNA in the assay tubes for the second DNase II activity. The assay tubes for the first DNase II contained 400 ugm DNA per ml; those for the second DNase II contained 400 ugm exogenous DNA plus 350 ugm endogenous DNA per ml. High DNA concentrations cause an apparent inhibition of DNase II because not all cleavages of the DNA result in acid-soluble oligonucleotide release (Rosenbluth and Sung, 1969).

Since the activation energies for the denaturation reactions were fairly close, the first and second DNase II activities were due to enzymes that denatured initially at about the same rate. Although the initial rates of denaturation for both activities were approximately similar, after a period of time the second activity was not denatured to the same extent as the frist activity. This implies that either other proteins or the DNA in the second DNase II preparation had a stabilizing effect on a portion of the second activity.

We found that the appearance of a second intestinal DNase II activity was an artifact. It is possible that the appearances of

second DNase II activities in other tissues were also artifactual. Cordonnier and Bernardi (1968) used DEAE cellulose chromatography to separate the two DNase II activities existing in some tissues and suggested that the appearance of a second DNase II activity may have been due to the presence of other proteins which may have interfered with the chromatographic behavior of the enzyme. When Zöllner et al. (1974) subjected extracts from human lymphocytes to electrophoresis on 13.4% polyacrylamide gels containing 0.3 mg herring sperm DNA per ml, he found five bands of DNase II activity. It is possible that these bands could be due to a single DNase II enzyme which formed complexes with other proteins or DNA during the electrophoresis. Yamanaka et al. (1974) separated two DNase II activities by phosphocellulose column chromatography, by isolectric focusing and by polyacrylamide gel electrophoresis of extracts from human gastric mucosa and cervix ueteri. Since all other properties of the two DNase II activities were identical, and since the activities were not separated completely from each other, one of them may have been an artifact.

C Partial purification of bovine intestinal DNase II

The following procedure was routinely used to prepare intestinal DNase II. The first step of the procedure, DEAE cellulose column chromatography, was done as described previously except that the buffers contained 10 mM EDTA. The first DNase II activity was purified further; the second DNase II activity was discarded.

A 105,000 xg supernatant was prepared from a crude extract of bovine intestinal mucosa and applied to a DEAE cellulose column as described previously. The column, 5 x 40 cm, was eluted at a flow rate of 2.5 ml per minute with 20 mM phosphate buffer, pH 7.8, containing 10 mM EDTA, and then with a linear 0 to 1 M potassium chloride gradient in the same buffer. Fractions of 20 ml were collected and were assayed for protein by absorbance at 280 nm, for DNase II by the DNA-agar gel assay, and for concentration of potassium chloride by conductivity measurements. Fig. 27 shows the elution profile obtained for the DEAE cellulose column. Three peaks of absorbance at 280 nm were seen. The concentration of DNase II was proportional to the antilogarithm (10^d) of the diameter (d) of the zone of clearing produced in the DNA-agar gel assay. Most of the DNase II activity, 80% of the applied units, was eluted with the 20 mM phosphate buffer. The fractions containing the DNase II activity were pooled and the enzyme solution was concentrated by ultrafiltration through an Amicon PM 10 membrane with a molecular weight cut off of 10,000. During ultrafiltration aliquots of water were added until the conductivity of the enzyme solution was the same as that of the buffer used in the next

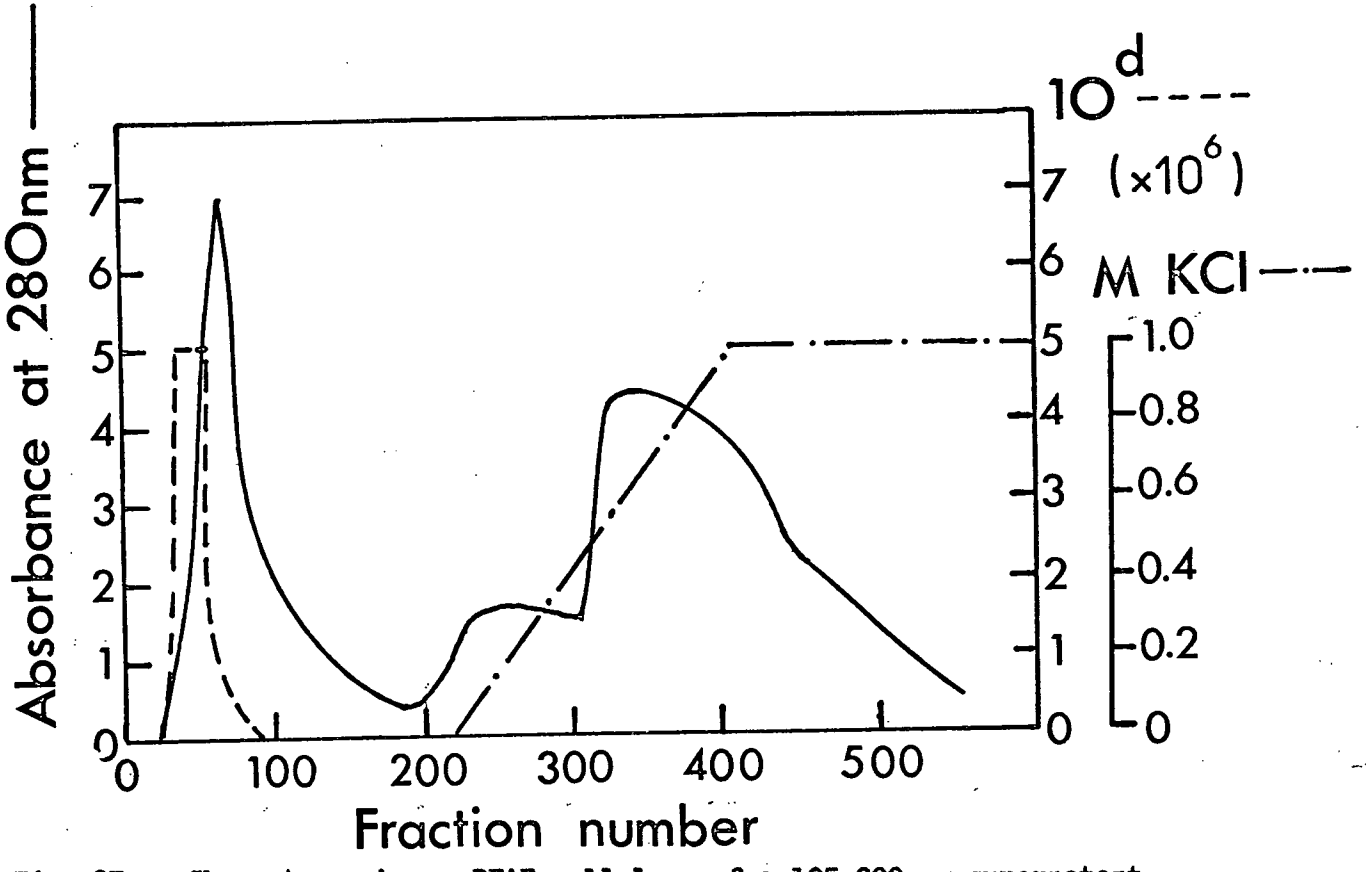


Fig. 27 Chromatography on DEAE cellulose of a 105,000 xg supernatant solution prepared from bovine intestinal mucosa. The column, 5 x 40 cm, was eluted with 20 mM phosphate buffer, pH 7.8, containing 10 mM EDTA, and then with a linear 0 to 1 M potassium chloride gradient in the same buffer. Fractions of 20 ml were collected. The protein concentration was estimated by the absorbance at 280 nm and DNase II concentration by the antilogarithm (10^d) of the diameter (d) of the zone of clearing produced in the DNA-agar gel assay.

purification step, 10 mM phosphate buffer, pH 6.0, containing 10 mM EDTA. The pH of the enzyme solution was adjusted to 6.0 with 1N HCl.

The DNase II solution was applied to a CM 22 cellulose column, 5 x 40 cm. The column was eluted at a flow of 5 ml per minute with 10 mM phosphate buffer, pH 6.0, containing 10 mM EDTA, and then with a linear 0 to 1 M potassium chloride gradient in the same buffer. Fractions of 20 ml were assayed for protein, DNase II and potassium chloride concentration as described previously. The elution profile obtained for the CM cellulose column is illustrated in Fig. 28.

Two peaks of absorbance at 280 nm were seen. The DNase II activity was eluted at a concentration of about 0.25 M potassium chloride. The fractions containing the enzyme activity were pooled and the solution was concentrated to about 50 ml by ultrafiltration.

The DNase II solution was applied to a Sephadex G100 column, 5 x 90 cm. The column was eluted at a flow rate of 1.0 ml per minute with 0.25 M Tris HCl buffer, pH 7.5, containing 10 mM EDTA, and 20 ml fractions were collected. Fig. 29 illustrates the elution profile from the column. Protein was estimated by absorbance at 280 nm and DNase II activity, by the DNA-agar gel assay. The fractions containing the DNase II activity were pooled and the enzyme solution was concentrated by ultrafiltration through an Amicon PM 10 membrane. The solution was desalted by sequentially adding aliquots of 20 mM Tris HCl, pH 7.5, containing 10 mM EDTA to a concentrated solution containing the DNase II activity and subjecting the solution to ultrafiltration. As this was repeated the buffer composition of the solution approached that of the added buffer asymptotically. The con-

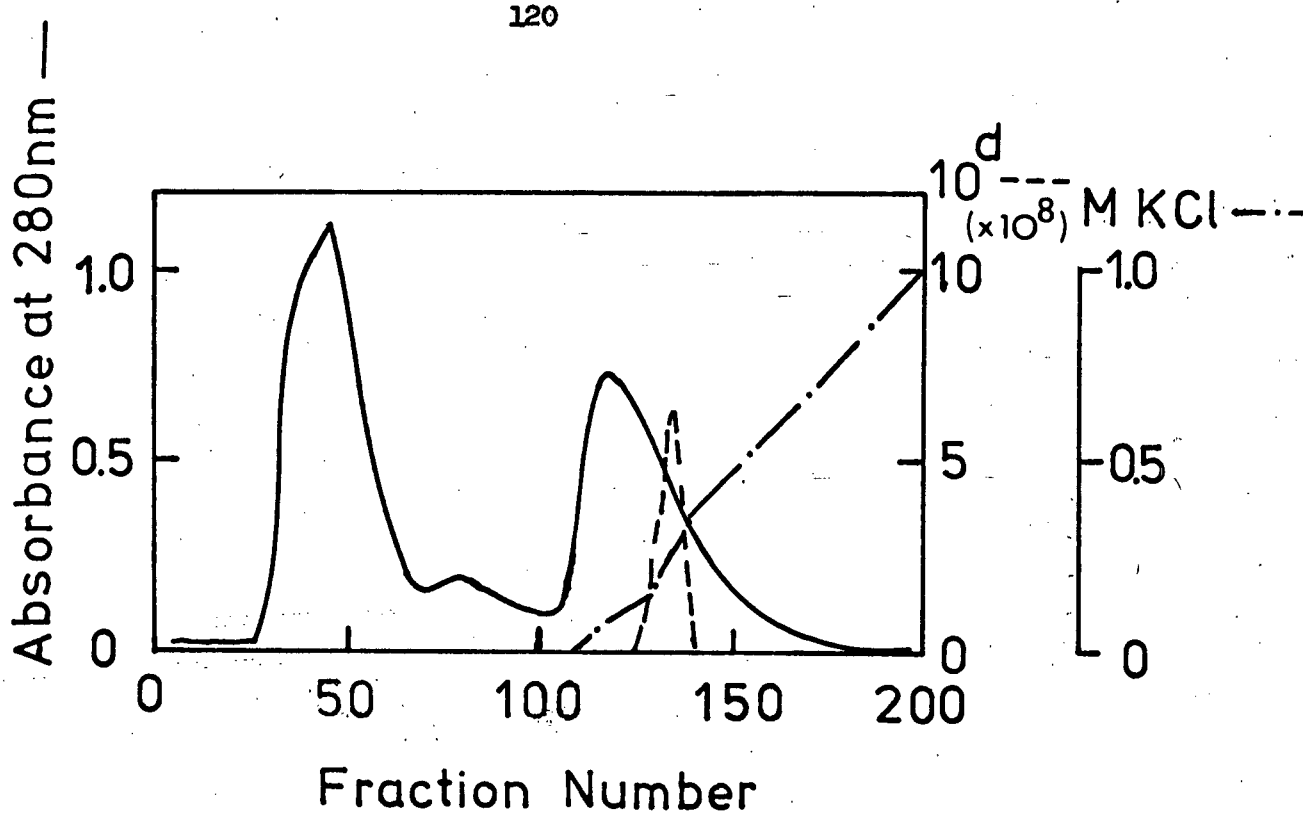


Fig. 28 Chromatography on CM cellulose of the pooled DNase II activity from the DEAE cellulose column. The column, 5 x 40 cm, was eluted with 10 mM phosphate buffer, pH 6.0, containing 10 mM EDTA, and then with a linear gradient of 0 to 1 M potassium chloride. Fractions of 20 ml were collected. The protein and DNase II concentrations were measured as described in Fig. 27.

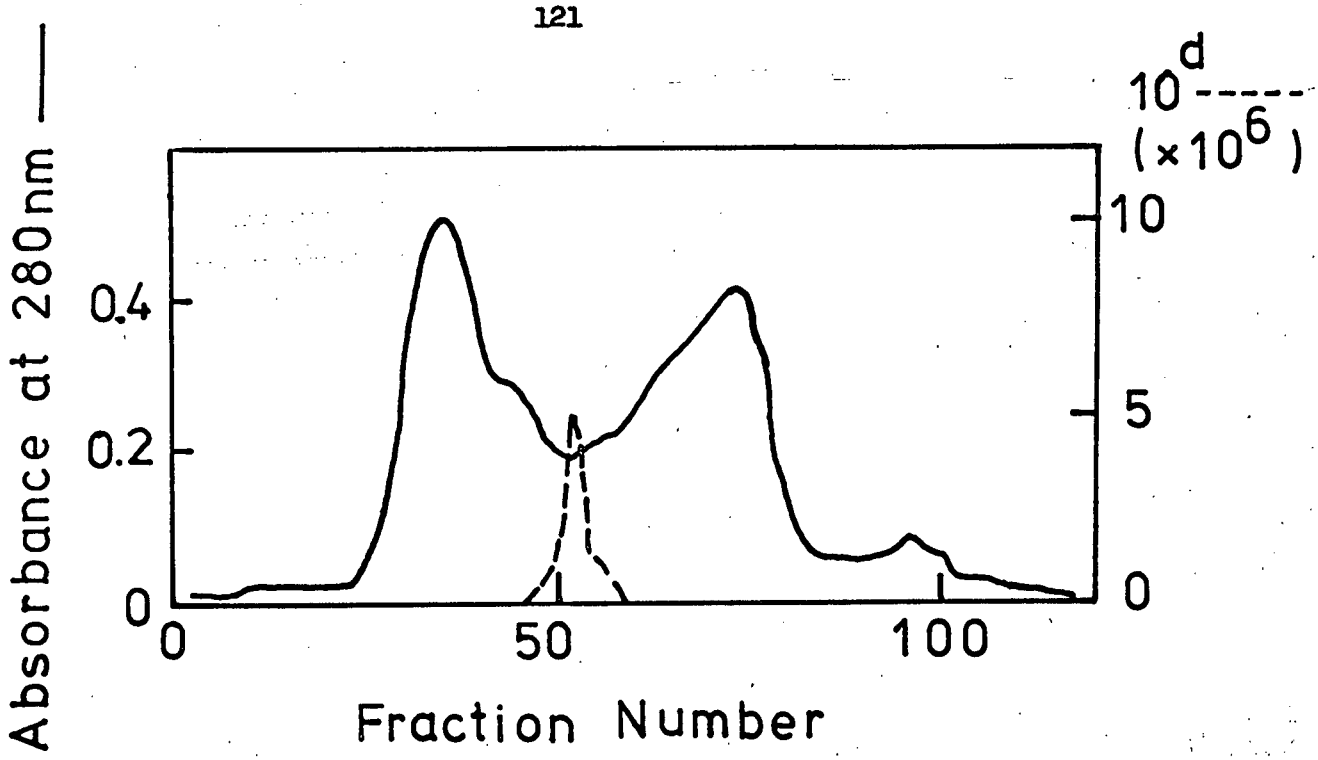


Fig. 29 Chromatography on Sephadex G100 of the pooled DNase II activity from the CM cellulose column. The column, 5x90 cm, was eluted with 0.25 M Tris HCl, pH 7.5, containing 10 mM EDTA and 20 ml fractions were collected. Protein and DNase II concentration were measured as described in Fig. 27.

centrated, desalted solution containing DNase II activity was stored in 1 ml aliquots at -80° C.

Estimation of the yield and specific activity of DNase II after each step in the purification procedure are given in Table VIII. Centrifugation of the crude extract at 105,000 xg resulted in the loss of about 12% of the DNase II activity; more activity was lost if the pH was less than 7.8. The first DEAE cellulose effluent contained the "first DNase II activity" referred to previously and was processed further on CM cellulose and Sephadex columns. The second DEAE cellulose effluent contained the "second DNase II activity", an artifact of the DEAE cellulose chromatography, and was discarded. The greatest loss of DNase II activity occurred upon chromatography on the Sephadex G100 column. However, this loss may have been more apparent than real since intestinal phosphodiesterase II of molecular weight 150,000 - 170,000 (Flanagan and Zbarsky, 1977) was likely partially separated from DNase II, of molecular weight 41,000, on the column. Since phosphodiesterase II is active under the same conditions as DNase II, it is likely that there would be less contribution made to the DNase II assay values by the phosphodiesterase II enzyme after the Sephadex chromatography. Overall, DNase II was purified 32 fold with respect to proteins present in the solution, and the yield of DNase II activity was 41%.

Electrophoresis of the partially purified DNase II preparation in polyacrylamide gels

In order to obtain an estimate of the number and amounts of

TABLE VIII

PARTIAL PURIFICATION OF INTESTINAL DNase II

Preparation	DNase II Units	Protein (mg)	Specific Activity (Units/mg protein)	% Yield Units	Purification
Combined Supernatants	487	5310	0.0917	100	1
105,000xg Supernatant	427	4240	0.101	88	1.1
105,000xg Precipitate	24.3	1094	0.0222	5	0.24
First DEAE Cellulose Effluent	348	666	0.522	71	5.7
Second DEAE Cellulose Effluent	75	1678	0.0447	15	0.49
CM Cellulose Effluent	329	307	1.07	68	12
Sephadex G100 Effluent	198	67	2.96	41	32

other proteins present as impurities, the DNase II preparation was subjected to polyacrylamide gel electrophoresis using methods described by Gabriel (1971a). The separating gel was composed of 7.5% acrylamide and 0.2% bis in a 0.37 M potassium acetate buffer pH 4.3, and the stacking gel, of 2.5% acrylimide and 0.625% bis in a 0.062 M potassium acetate buffer, pH 6.7. The electrophoresis buffer was 0.035 M β-alanine - 0.014 M acetic acid, pH 4.5. About 50 ul of the DNase II solution obtained from the Sephadex G100 column containing 10% sucrose and a drop of 0.1% methyl green as a marker was applied to the gels which were contained in glass tubes, 5 x 75 mm. Electrophoresis was carried out at a current of 5 milliamperes per tube for 2 hours at 4°C. After electrophoresis, some of the gels were removed, stained for protein with Coomassie blue by the method of Gabriel (1971b), and scanned at 550 nm in a Gilford spectrophotometer equipped with a linear scanning device. Fig. 30 is a densitometer tracing of a polyacrylamide gel showing the electrophorectic separation of proteins present in the DNase II preparation. The position of the DNase II activity was estimated by placing unstained gels in contact with a DNA-agar gel plate which was prepared similarly as for the DNA-agar gel assay described in the methods section except that no holes were punched in the gel. After incubation overnight at 37°C, the unreacted DNA was precipitated with 1 N HCl and an oval zone of clearing was observed with the long axis being along the long axis of the gel. The centre of this zone of clearing was judged to be adjacent to the original position of the DNase II in the polyacrylamide gel. This position is shown in Fig. 30 by an arrow.

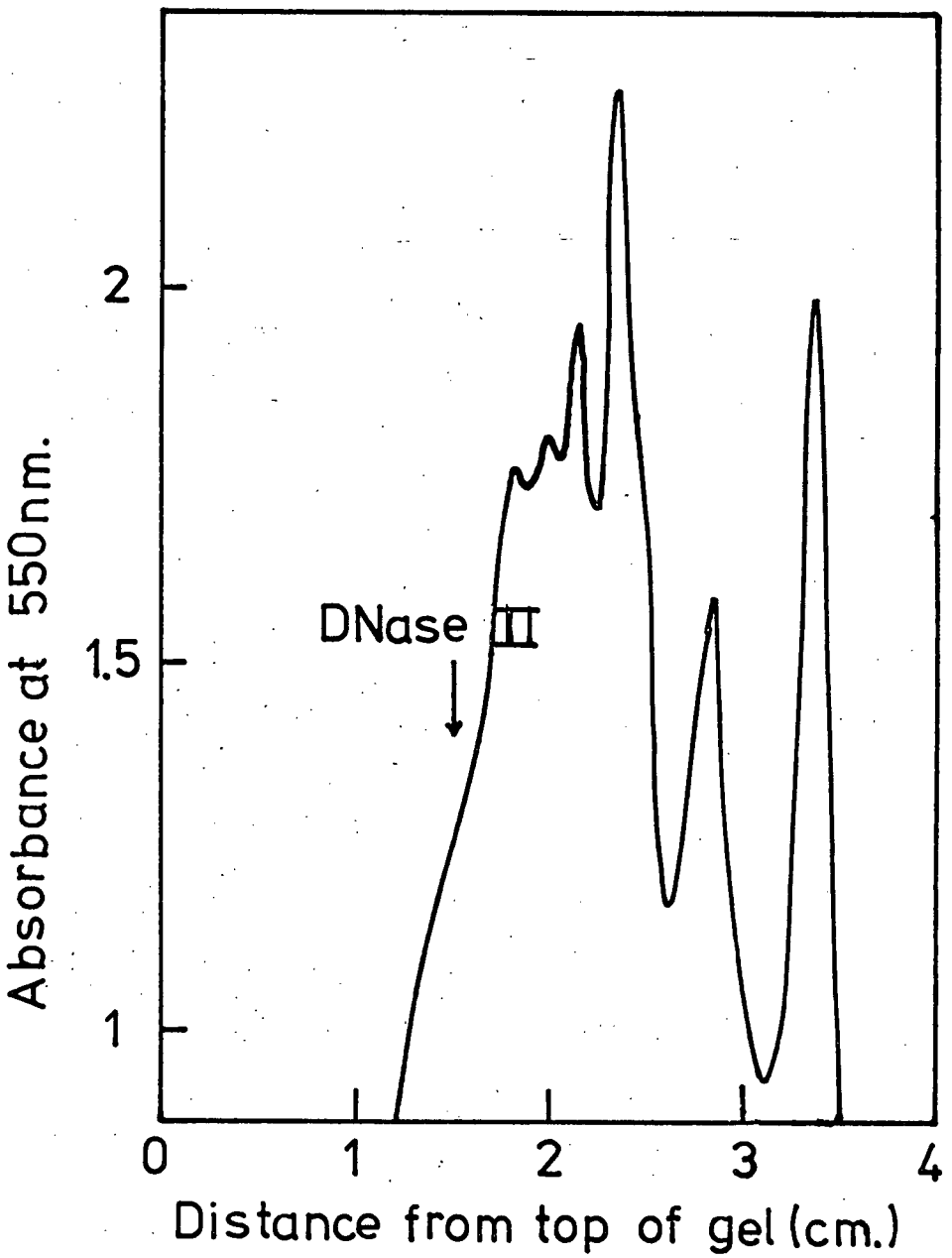


Fig. 30 Densitometer tracing of a polyacrylamide gel showing the electrophoretic separation of proteins present in the DNase II preparation. The gel was stained with Coomassie blue by the method of Gabriel (1971a) and the densitometer tracing was done at 550 nm using a Gifford spectrophotometer equipped with a gel scanning device. DNase II was assayed by placing an unstained gel in contact with a DNA-agar gel plate prepared similarly as for the DNA-agar gel assay. After incubation overnight at 37°C, the unreacted DNA was precipitated with 1 N HCl and an oval zone of clearing was deemed to be adjacent to the original position of the DNase II on the gel. This position is shown by an arrow in Fig. 30.

Measurement of contaminating nucleases in the DNase II preparation

Aliquots of the partially purified DNase II preparation were assayed for different nucleases by procedures described in the Methods section. Table IX demonstrates that there was no DNase I activity present and that the amounts of alkaline and acid phosphatase, and phosphodiesterase I present were low. However, larger amounts of acid and alkaline RNase and phosphodiesterase II activities were evident in the DNase II preparation. The phosphodiesterase II activity may be lower than indicated. Assay #1 measures the hydrolysis of thymidine 3-p-nitrophenylphosphate; DNase II also degrades this substrate at a slow rate (Bernardi, 1964). Assay #2 measures the hydrolysis of 3-phosphate oligonucleotides prepared with hog spleen DNase II. The limit digest of DNA prepared with hog spleen DNase II may have contained oligonucleotides susceptible to the action of bovine intestinal DNase II because of differences in the two enzymes with regard to base specificity and/or extent of degradation of DNA.

Of the contaminating nucleases present phosphodiesterase II would have interfered the most with studies of degradation of DNA by DNase II since phosphodiesterase II acts on the same substrate as DNase II and is active at acid pH in the presence of EDTA (Bernardi and Bernardi, 1966), the same conditions which are optimal for DNase II activity.

TABLE IX

OTHER NUCLEASES PRESENT IN THE DNase II PREPARATION*

Enzyme Activity		Units / mg Protein
DNase II		2.19
DNase I		0.00
Alkaline phosphatase		3.5×10^{-4}
Phosphodiesterase I		9.4×10^{-3}
Acid phosphatase		2.0×10^{-2}
Phosphodiesterase II:	Assay #1	0.28
	Assay #2	5.91
Acid RNase		3.77
Alkaline RNase		2.77

* Aliquots of the Dnase II preparation obtained from the Sephadex G100 column (Fig. 29) were assayed for nucleolytic activities by procedures described in the Methods section.

Further purification of DNase II

In order to reduce the phosphodiesterase II contamination of DNase II several different chromatographic purification methods were attempted. Hydroxyapatite chromatography by the method of Bernardi (1971b) resulted in the absorption of the DNase II activity to the column in 20 mM phosphate buffer, pH 6.8, but less than 4% of the applied DNase II units could be eluted either with a linear gradient of 0.02 to 0.5 M phosphate buffer, pH 6.8, or, in a separate experiment, with a linear gradient of 0 to 0.5 M potassium chloride in 20 mM phosphate buffer, pH 6.8. That a loss of DNase II activity occurs in phosphate buffer especially in the presence of magnesium, a divalent metal ion, has been shown previously (Fig. 16). It is possible that the low yield of DNase II activity upon chromatography on hydroxyapatite was due to loss of DNase II activity in the phosphate buffer perhaps exacerbated by the presence of calcium, an integral part of hydroxyapatite (Bernardi, 1971b). It is not possible with hydroxyapatite chromatography to use EDTA in the eluting buffers to stabilize the DNase II activity since the presence in the solvent of substances such as EDTA and citrate having a stronger affinity for calcium than phosphate can reduce the binding capacity of hydroxyapatite to zero (Bernardi, 1971b). Oshima and Price (1973) purified porcine spleen DNase II by chromatography on sulfoethyl-Sephadex and phosphocellulose. However, their methods were not found to be effective for purifying intestinal DNase II since the enzyme was not retained by the columns at ionic strengths sufficient to remove any appreciable amounts of contaminating protein. The difference in chromatographic behavior of spleen and intestinal DNase II

may be due to the different tissue origins or the different methods of preparation of the two enzymes.

DNase II was eventually purified further by repeating the procedure described previously using DEAE and CM cellulose and Sephadex columns of smaller size and the purified DNase II from the Sephadex G100 column (Fig. 29) as the starting material. Since intestinal phosphodiesterase II has a molecular weight of 150,000 to 170,000 for the rat enzyme (Flanagan and Zbarsky, 1977) and intestinal DNase II, a molecular weight of 41,000, the two enzymes should have been separated in the original Sephadex G100 column. In order to overcome any electrostatic interactions which may have hindered the complete separation of the two activities, the Sephadex column used in this second purification procedure was eluted with 20 mM Tris HCl, pH 7.5, containing 10 mM EDTA and 1 M potassium chloride instead of with 0.25 M Tris HCl, pH 7.5, containing 10 mM EDTA. Table X compares DNase II, phosphodiesterase II and acid and alkaline RNase activities before and after further purification. After purification the specific activities of DNase II and acid RNase were increased and those of phosphodiesterase II and alkaline RNase were decreased. The ratio of phosphodiesterase II, acid RNase and alkaline RNase to DNase II had decreased 11, 3, and 4 fold respectively. This indicates that the phosphodiesterase II contaminant had been substantially reduced with respect to the DNase II activity.

TABLE X

EFFECT OF FURTHER PURIFICATION ON THE ACTIVITIES OF OTHER NUCLEASES PRESENT IN DNase II PREPARATION

	Enzyme Activity	Units / mg Protein	Ratio of Nuclease to DNase II
Before Further Purification	DNase II	2.19	1.0
	Phosphodiesterase II	5.91	2.7
	Acid RNase	3.77	1.7
	Alkaline RNase	2.77	1.3
After Further Purification	DNase II	7.12	1
	Phosphodiesterase II	1.73	0.24
	Acid RNase	4.58	0.64
	Alkaline RNase	2.20	0.31

Discussion

During the purification procedure 10 mM EDTA was included in all buffers in order to prevent loss of DNase II activity. When EDTA was omitted from the buffers used for elution of the columns very little DNase II activity was recovered after the first three chromatographic purification steps. EDTA probably stabilized DNase II by chelating metal ions which would otherwise have caused a loss of DNase II activity either directly by denaturation of the enzyme molecule, or indirectly through enhancement of the activity of proteases present in the DNase II preparation.

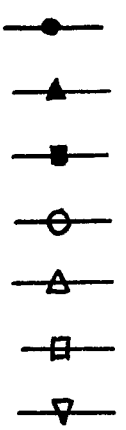
Intestinal DNase II was partially purified by ion exchange column chromatography and gel filtration. Several different proteins other than DNase II were demonstrated in the DNase II preparation by polyacrylamide gel electrophoresis. The major contaminating nucleases were acid and alkaline RNase which do not act on DNA, and phosphodiesterase II. The phosphodiesterase II activity present in the DNase II preparation was reduced substantially by further purification. DNase II had a specific activity of 7.12 units per mg protein in the most purified preparation. This represented a 78 fold purification of DNase II with respect to the original crude extract of bovine intestinal mucosa. The overall yield of DNase II units was 13%.

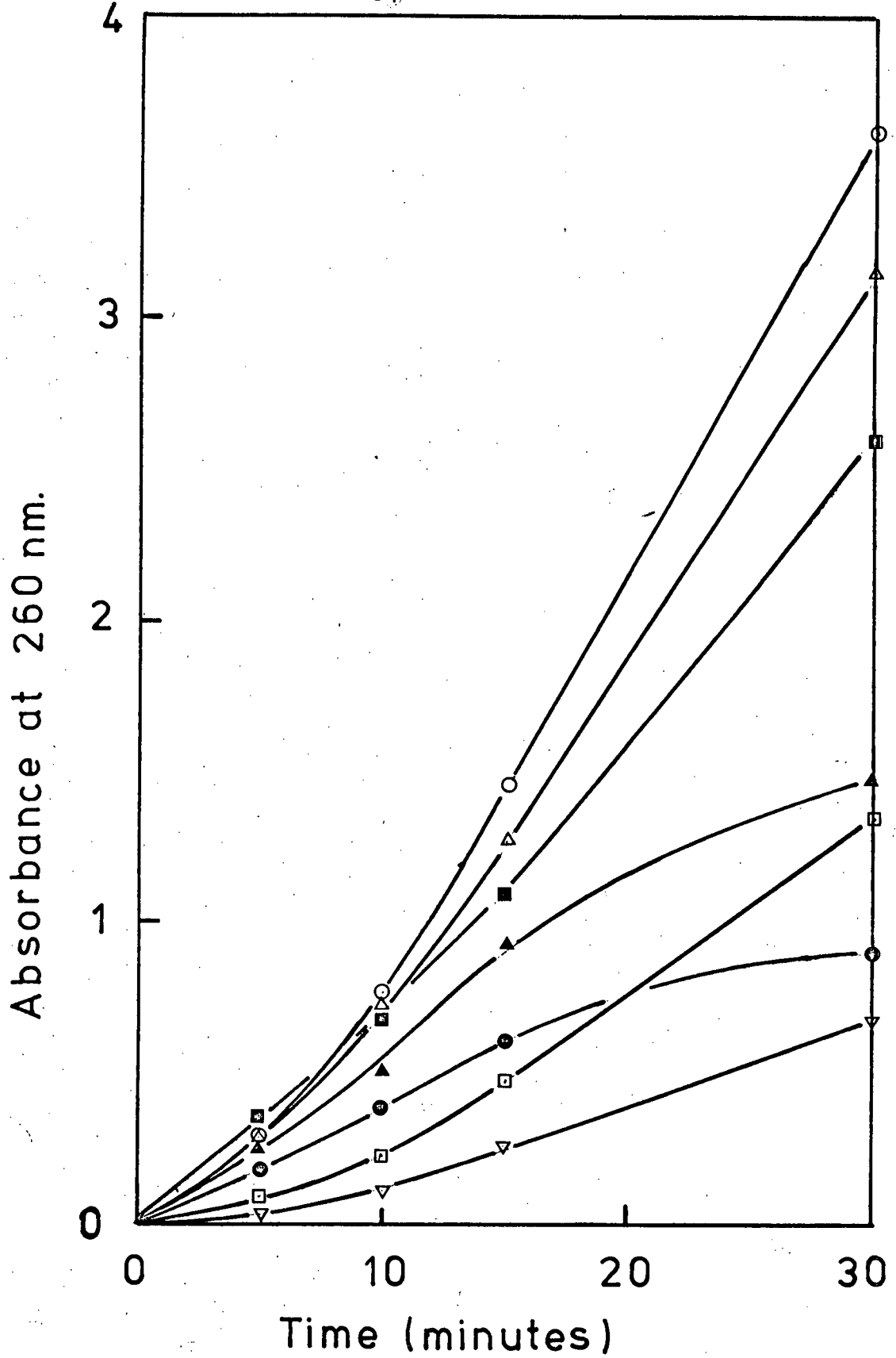
D Some properties of intestinal DNase II

pH optimum

The pH optimum of the partially purified intestinal DNase II was determined by measuring the DNase II activity as a function of time by the acid-soluble oligonucleotide assay. All buffers were made up to a constant conductivity of 6.5 mmhos by the addition of sodium chloride and the pH was measured in each assay tube. Fig. 31 illustrates the effect of pH on the rate of release of acid-soluble oligonucleotides from DNA by DNase II. In all the curves an initial lag phase in the release of acid-soluble oligonucleotide was observed. This was likely due to additional cleavages of DNA by DNase II that did not produce DNA fragments small enough to be acid-soluble. Cleaver and Boyer (1972) found that oligomucleotides, 17 nucleotides in length, were 50% precipitated by 5% perchloric acid. Since the final concentration of perchloric acid in the DNase II assay mix was 2%, it is likely that only small oligonucleotides less than 20 nucleotides in length, were acid-soluble to an appreciable extent and contributed to the absorbance at 260 nm in the assay. Another feature of the reaction which is shown in Fig. 31 is that the extent of degradation of DNA was lower at lower pHs. The reaction at pH 3.8 slowed down at 30 minutes, whereas the reaction at pH 5.7 showed no sign of leveling off at 30 minutes. Lowering the pH of a solution causes denaturation of calf thymus DNA; the pH at which denaturation occurs depends on the amounts and kinds of salts present and the

Fig. 31 Effect of pH on the rate of release of acid-soluble oligonucleotides from DNA by DNase II. The reaction was carried out with 400 ugm DNA per ml in 120 mM sodium acetate buffer containing 8 mM EDTA.




4
3
2
1
0
Absorbance at 260 nm.
0
10
20
30
Time (minutes)

length of the DNA fragments present (Beaven et al., 1955; Reisner and Romen, 1973). At low pH DNA fragments were probably denatured earlier in the reaction than at high pH. Since DNase II degrades denatured DNA at a slower rate than native DNA (Table VI), the denaturation of the DNA fragments likely resulted in a decrease in the rate of acid-soluble oligonucleotide formation. Fig. 32 demonstrates that DNase II had a broad pH optimum centered about pH 4.8. Each point on the graph represents the slope of the linear portion of the curve for that pH from Fig. 31. By taking the pH values corresponding to half the maximum velocity, two apparent pKa values for DNase II were estimated to be 3.9 and 5.6. The values are within the ranges that would be expected for a β- or γ-carboxyl group of aspartic or glutamic acid and an imidizolium group of histidine respectively in the active site of the enzyme (Mahler and Cordes, 1971; Segel, 1975).

Effect of magnesium ion

DNase II activity was measured in the presence of different concentrations of magnesium chloride by the hyperchromicity assay using 40 ugm calf thymus DNA in 1.0 ml of 120 mM solium acetate buffer, pH 5.0. The slopes of the hyperchromicity at 260 nm versus time curves were plotted against the magnesium chloride concentration. Fig. 33 shows that the DNase II activity was activated slightly by low $MgCl_2$ concentrations, and inhibited by $MgCl_2$ concentrations greater than 10 mM.

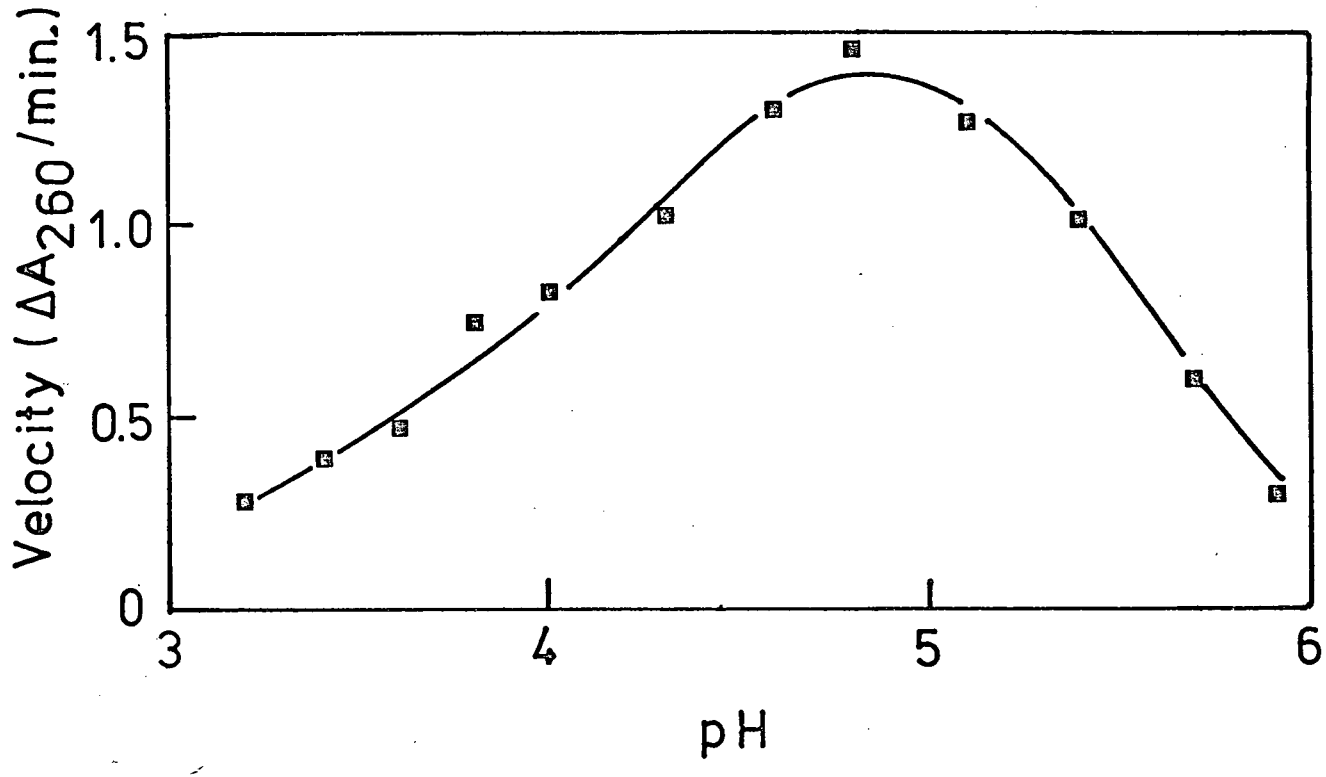


Fig. 32 pH optimum of intestinal DNase II. Each point on this graph represents the slope of the linear portion of the curve for that pH from Fig. 31.

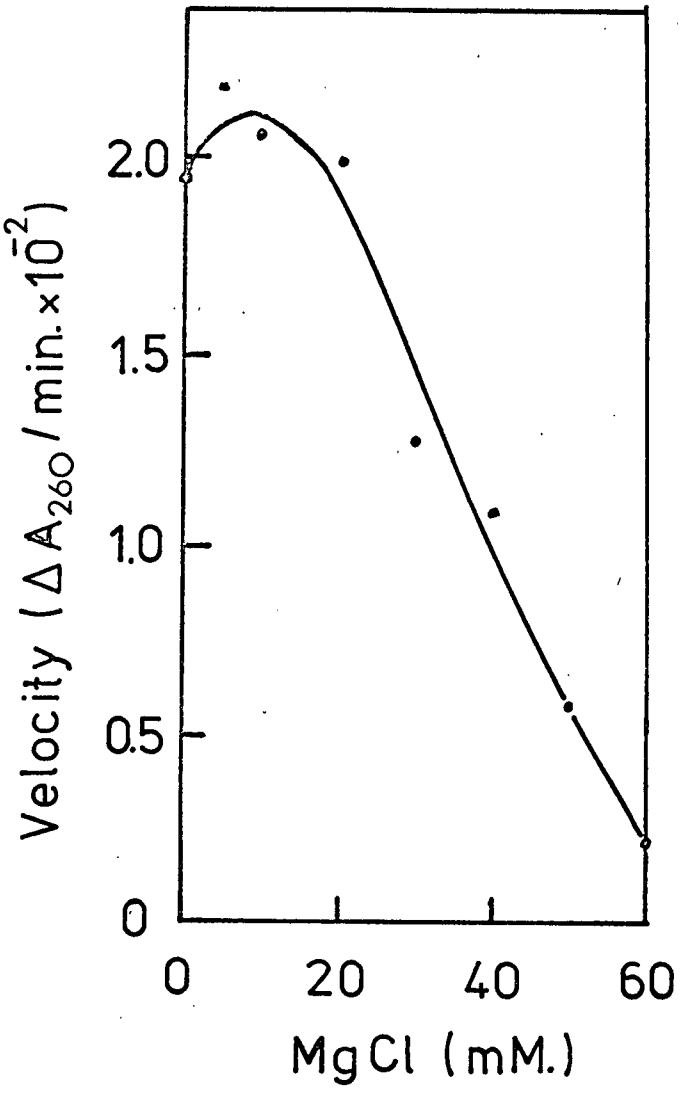


Fig. 33 Effect of magnesium ions on DNase II activity. DNase II activity was measured by the hyperchromicity assay in 120 mM sodium acetate buffer, pH 5.0, containing 40 ugm calf thymus DNA/ml. Velocities were taken as the slopes of the linear portions of hyperchromicity curves obtained for various $MgCl_2$ concentrations.

Effect of ionic strength

Fig. 18 demonstrates that DNase II ("first activity") was activated on addition of NaCl to 0.1 M and inhibited by further additions of NaCl.

Effect of sulfate, an inhibitor of DNase II

Fig. 19 illustrates the inhibition of DNase II by sulfate concentrations from 0.01 to 0.1 M.

The effect of very low sulfate concentrations on DNase II activity may be seen in Fig. 34. DNase II activity was measured by the hyperchromicity assay in 120 mM sodium acetate buffer, pH 5.0, containing 40 ugm DNA per ml. Velocities were taken as the slopes of the linear portions of hyperchromicity curves obtained for different concentrations of sodium sulfate. DNase II was activated maximally at a 1 mM concentration of sulfate. The addition of 10 mM EDTA also resulted in about a 20% increase in DNase II activity. Oshima and Price (1974) found that 0.5 mM sulfate increased the activity of hog spleen DNase II by about 20% corresponding to a 15 fold increase in the number of single strand cleavages of the DNA substrate.

Activation energy for hydrolysis of DNA

The activation energy for the hydrolysis of calf thymus DNA by DNase II first activity was previously estimated to be 19 kcal/mole (Fig. 20)

Fig. 34 Effect of millimolar concentrations of sulfate ion on DNase II activity. DNase II activity was measured by the hyperchromicity assay in 120 mM sodium acetate buffer, pH 5.0, containing 40 ugm calf thymus DNA/ml. Velocities were taken as the slopes of the linear portions of hyperchromicity curves obtained for different concentrations of sodium sulfate. The DNase II activity in the presence of 10^{-5} M sulfate ion was the same as that found when no sulfate ion was present.

Velocity (ΔA_{260} / min. $\times 10^{-2}$)
0
1
2
3
5
4
3
2
1
$-\log[SO_4^{=}]$

Activation energy for denaturation of DNase II

The activation energy for denaturation of the DNase II molecule was estimated to be 43 kcal/mole (Fig. 21).

Molecular weight of DNase II

The molecular weight of DNase II was estimated to be 41,000 by gel filtration on Sephadex G100 (Fig. 26). DNase II is probably a positively charged molecule at neutral pH since it was not retained on a positively charged DEAE cellulose column in 20 mM phosphate buffer, pH 7.8, but was bound to a negatively charged CM cellulose column in 20 mM phosphate buffer, pH 6.0.

As will be discussed in the next section, the products of the reaction of DNase II with DNA can be labelled with ^{32}P using polynucleotide kinase and [γ- ^{32}P]ATP, separated from mononucleotides, and degraded to 5'-^{32}P nucleotides. This implies that DNase II cleaves DNA endonucleolytically to form oligonucleotides with 5' hydroxyl and 3' phosphate-terminals.

Further evidence for endonucleolytic cleavage of DNA by DNase II comes from the observation that DNase II was active under conditions used to measure endonucleolytic activity, but showed no activity under exonuclease assay conditions. Hog spleen DNase II, an endonuclease, can be assayed by measuring the degradation of DNA into products soluble in 2% perchloric acid (Bernardi, 1968). Spleen exonuclease can be assayed by

measuring the products of DNA digestion that are soluble in 1.3% perchloric acid containing 0.13% uranyl acetate (Bernardi and Bernardi, 1966).

When DNA is degraded by intestinal DNase II, absorbances of 0.6 at 260 nm in 2% perchloric acid and zero in 1.3% perchloric acid containing 0.13% uranylacetate were observed. This indicates that DNase is active under conditions used to measure endonucleases, not exonucleases.

Discussion

The enzyme isolated from bovine intestinal mucosa has an acidic pH optimum, is active in the presence of EDTA and is inhibited by magnesium. This is the original definition of a DNase II that was proposed by Cunningham and Laskowski (1953).

As will be demonstrated in a latter section the enzyme also degrades DNA endonucleolytically to 3'-phosphate terminated oligonucleotides, and thus is a DNase II by the current definition (Enzyme Nomenclature, 1972). Other properties of intestinal DNase II such as chromatographic behavior, molecular weight, inhibition by high ionic strength and by high sulfate ion concentration, are similar to those of DNase II enzymes isolated from other sources (Cordonnier and Bernardi, 1968; Laskowski, 1961, 1967; Bernardi, 1968, 1971).

E The reaction of DNase II with DNA and other substrates

The Michaelis constants and the maximum velocities for the reaction with DNA and polydAT

A first attempt to determine the Michaelis constant, Km,for the reaction of DNase II with DNA was made by using the hyperchromicity assay to follow the DNase II reaction with different concentrations of calf thymus DNA in 120 mM sodium acetate buffer containing 8 mM EDTA. Fig. 35 shows that the extent of degradation as measured by the total increase of the absorbance at 260 nm over the course of the reaction was proportional to the concentrations of calf thymus DNA used: 40, 20, and 10 ugm per ml in Fig. 35 a, b, and c, respectively. However, the rate of degradation of DNA was the same in all three reactions. This implies that the substrate was present in a saturating concentration with respect to the enzyme.

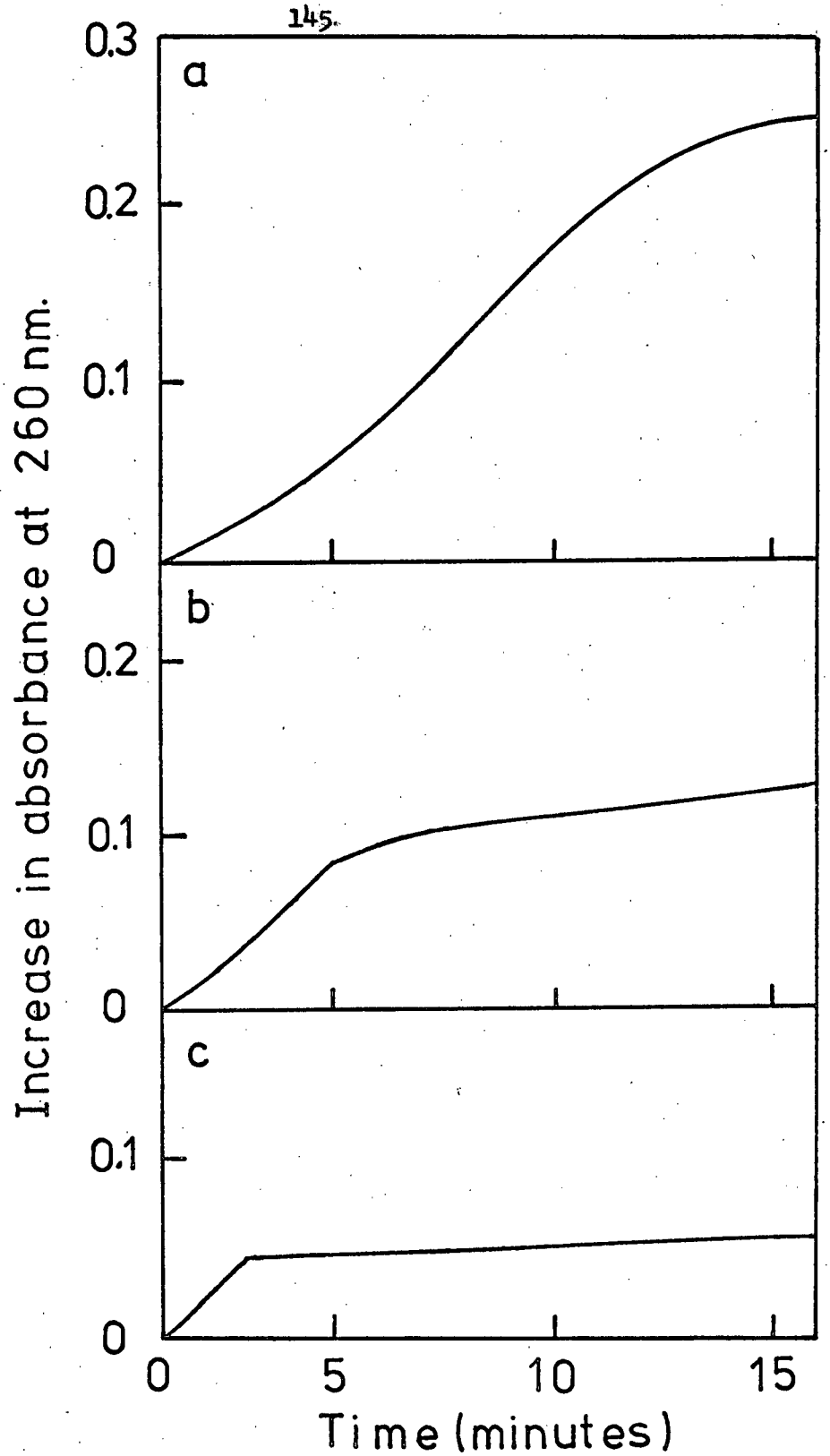
In order to have lower concentrations of DNA in the reaction it was necessary to use radioactively labelled DNA so that the acid-soluble oligonucleotides released could be detected. ^{3}H-DNA, sonicated, from Escherichia coli was a good substrate with which to determine the Km for DNase II because there was no initial lag phase in the release of acid-soluble oligonucleotides and thus initial velocities could be determined.

^{3}H-DNA was digested with DNase II and the acid-soluble radioactivity released was measured and plotted as a function of time, as described in the Methods section.

The initial slopes of these curves were taken as indicative of the

Fig. 35 The rate and extent of the reaction of DNase II with different concentrations of calf thymus DNA. The increase in absorbance, the hyperchromicity, at 260 nm, of a DNA solution upon digestion with DNase II in 120 mM sodium acetate buffer, pH 5.0, containing 8 mM EDTA was plotted as a function of time. The rate of the reaction was taken as the slope of the linear portion of the curve, and the extent of degradation, as the total increase in absorbance at 260 nm.

a 40 ugm DNA/ml
b 20 ugm DNA/ml
c 10 ugm DNA/ml

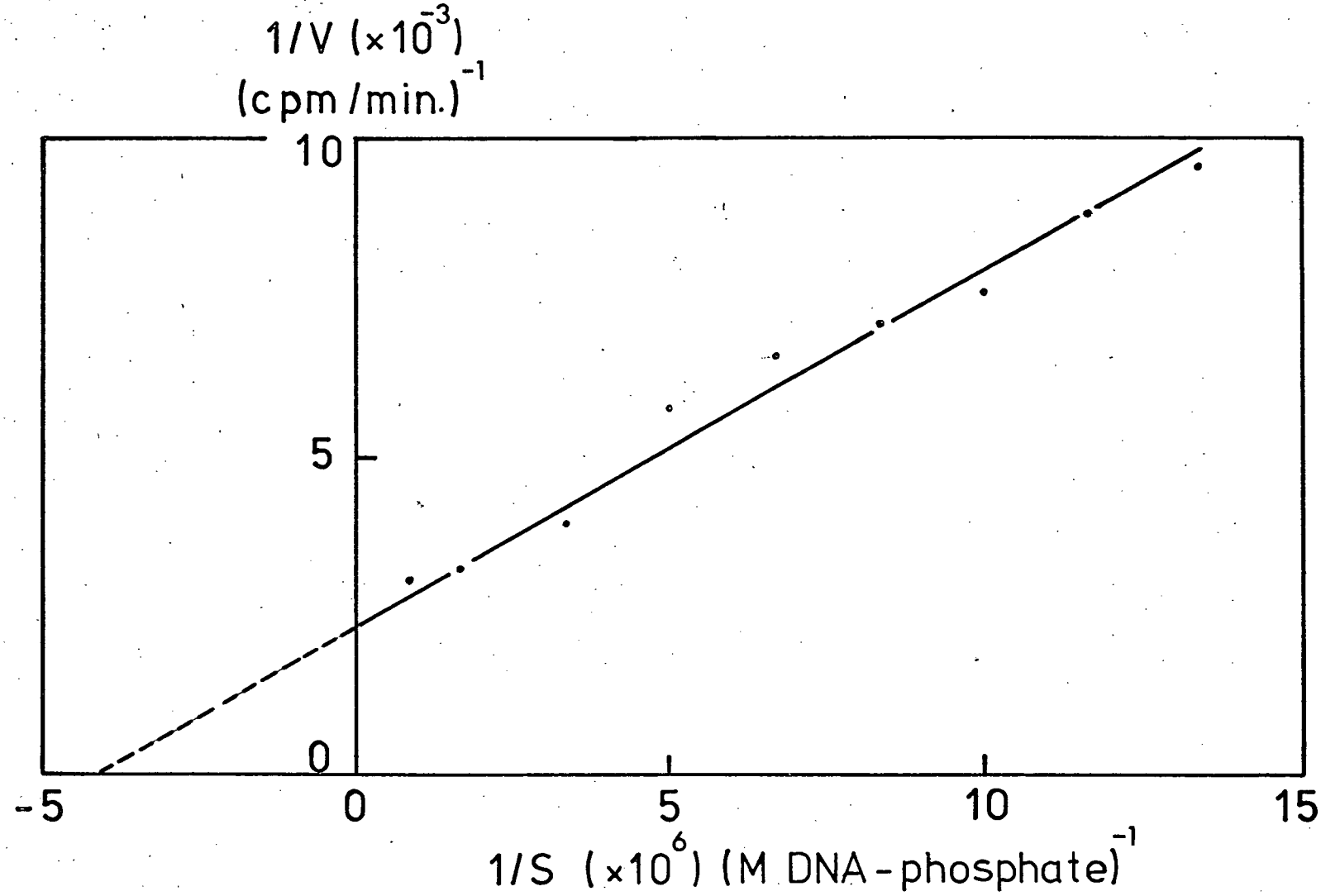

a
b
c
0.3
0.2
0.1
0
0.2
0.1
0
0.1
0
0
5
10
15
Increase in absorbance at 260 nm.
Time (minutes)

initial velocities of the reaction at different DNA concentrations. Fig. 36 exhibits a Lineweaver-Burk plot for the reaction of DNase II with sonicated ^{3}H DNA from Escherichia coli. The Michaelis constant, Km, was estimated to be 2.42×10^{-7} M DNA-phosphate, and the maximum velocity, 435 cpm of acid-soluble radioactivity released per minute. In reactions that were allowed to go to completion, an average of 33.4 cpm of acid-soluble radioactivity was released for each pmole of DNA-phosphate present. Thus the maximum amount of DNA digested per minute was 435/33.4=13.0 pmoles DNA-phosphate. Because the DNase II preparation was not pure, the maximum velocity could not be expressed in terms of the pure enzyme, but was, instead, expressed in terms of the protein present in the DNase II preparation. The maximum velocity was 441 pmoles DNA-phosphate/min/ugm protein.

Fig. 37 illustrates the Lineweaver-Burk plot obtained for the reaction of DNase II with polydAT-methyl-^{3}H. The Michaelis constant, Km, was estimated to be 2.63×10^{-7} M polydAT-phosphate, and the maximum velocity, 556 cpm of acid-soluble radioactivity per minute, or 326 pmoles polydAT-phosphate/min/ugm protein.

For the reaction of DNase II with ^{3}H DNA the Km was 2.42×10^{-7} M DNA-phosphate. DNase II was, therefore, saturated with substrate in the hyperchromicity and acid-soluble oligonucleotide assays which contained 8.0×10^{-5} M and 8.0×10^{-4} M concentrations of DNA-phosphate, respectively.

Fig. 36 Lineweaver-Burk plot for the reaction of DNase II with sonicated ^{3}H-DNA from Escherichia coli. Reactions were carried out at 37°C in 150 mM sodium acetate buffer, pH 5.0, containing 10 mM EDTA and various concentrations of ^{3}H-DNA. The initial slopes of curves of acid-soluble radioactivity released as a function of time were taken as indicative of the initial velocities of the reaction at different DNA concentrations.


1/V (×10⁻³)
(cpm/min.)⁻¹
10
5
0
-5
0
5
10
15
1/S (×10⁶) (M DNA-phosphate)⁻¹

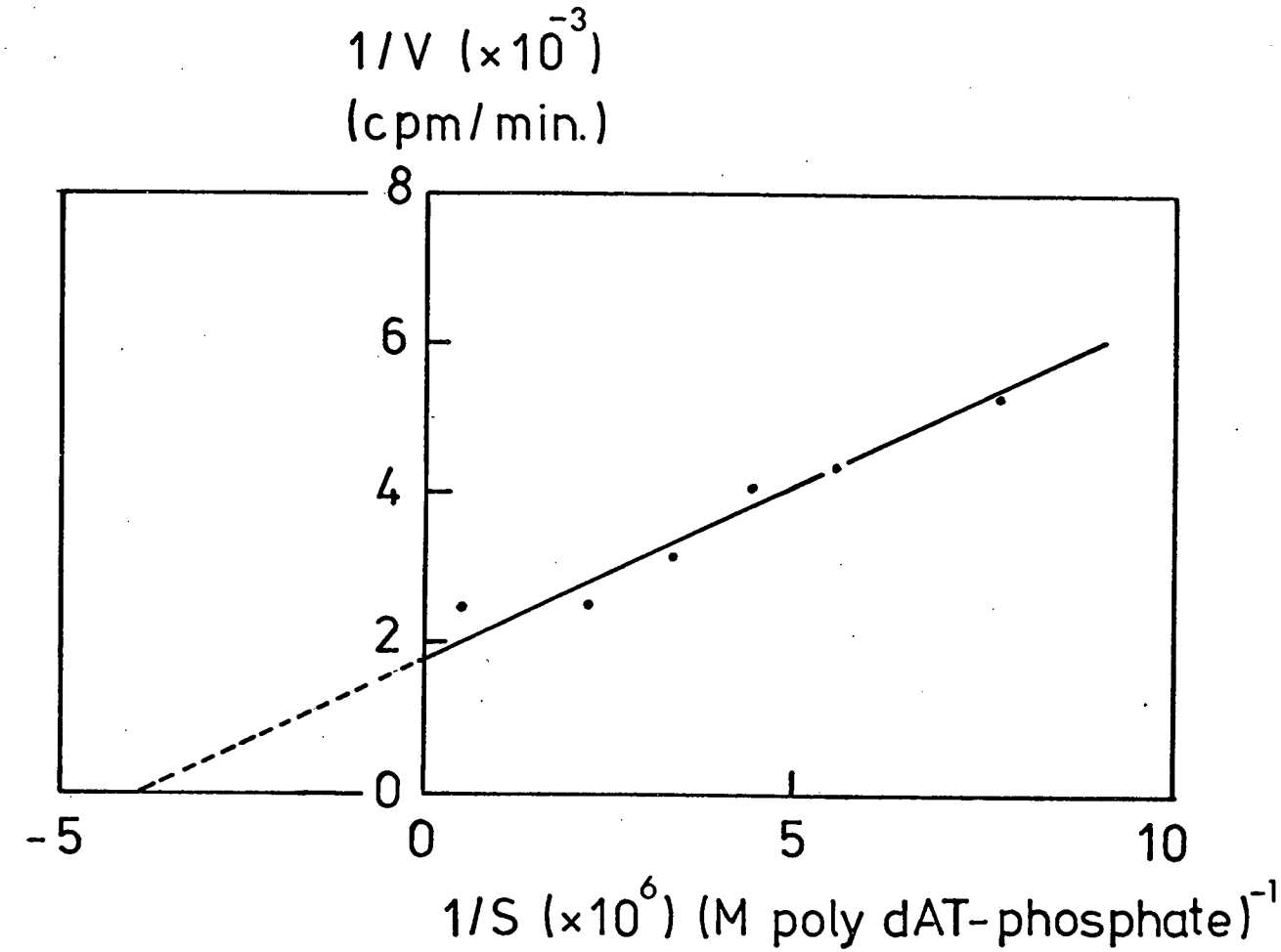


Fig. 37 **Lineweaver-Burk plot for the reaction of DNase II with polydAdT-methyl-^{3}H. The experiment was carried out and the values calculated as for ^{3}H-DNA (Fig. 36).**

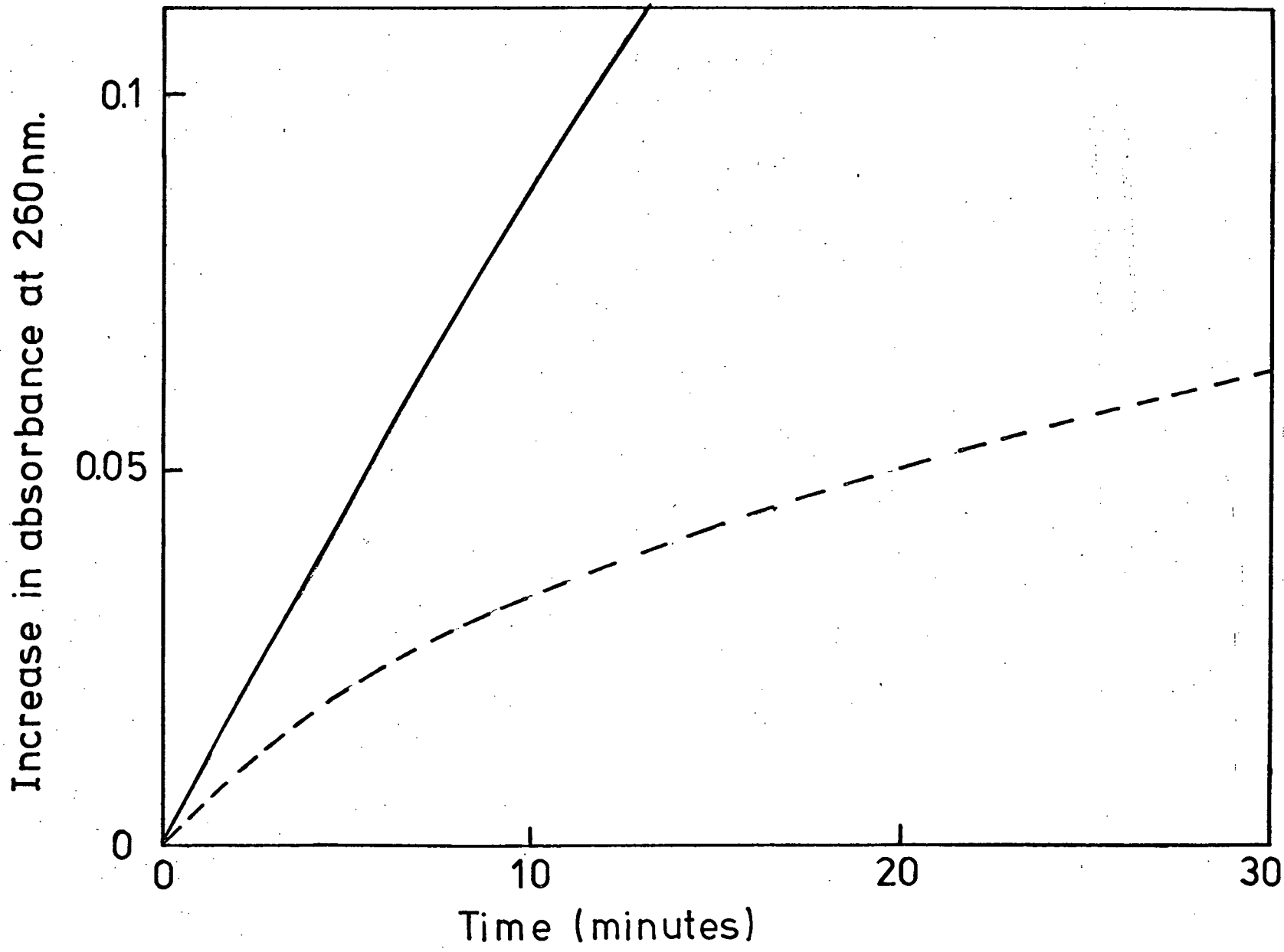
Degradation of native and denatured DNA

The rates of hydrolysis of native and denatured DNA by DNase II were compared. Calf thymus DNA at a concentration of 0.4 mg per ml in 10 mM sodium chloride was denatured by heating in a boiling water bath for 15 minutes, and then cooling in an ice-water bath. The reaction of DNase II with 40 ugm/ml of either native, or denatured DNA was carried out in 100 mM sodium acetate, pH 5.0, containing 10 mM EDTA. The hyperchromicity of each solution was plotted as a function of time; the curves obtained are shown in Fig. 38. With native DNA absorbance at 260 nm increased linearly with time at first and then gradually decreased; at this high enzyme to DNA ratio the initial lag phase was not apparent. With denatured DNA the absorbance increased non-linearly at first and then increased linearly with time. Some double-stranded regions of the DNA were probably reformed by aggregation upon cooling of the denatrued DNA since a relatively high concentration, 0.4 mg/ml, of DNA was used. (Marmur et al., 1963). It is likely that the rate of degradation observed initially with the denatured DNA was due to digestion of the small number of double-stranded regions that may have reformed in the DNA upon cooling. The linear region of the curve probably represented the actual rate of degradation of denatured DNA. Since the slopes of the linear portions of the curves for native and denatured DNA were 0.009 and 0.0014 absorbance units at 260 nm per minute respectively, DNase II degraded native calf thymus DNA 6.4 times faster than denatured calf thymus DNA. An earlier estimate of the relative rates of degradation of native and denatured DNA by DNase II (Table VI) probably included the degradation of

Fig. 38 Comparison of the reaction of DNase II with native and denatured calf thymus DNA. DNase II was reacted with 40 ugm/ml of either native, or denatured DNA in 100 mM sodium acetate buffer, pH 5.0, containing 10 mM EDTA. The hyperchromicity of each solution was plotted as a function of time.

———— with native DNA

- - - - - - with denatured DNA


Increase in absorbance at 260nm.
0.1
0.05
0
0
10
20
30
Time (minutes)

double-stranded regions in the calculation of the reaction rate for denatured DNA.

Digestion of polydAT, polydA, and polydT

The rates of degradation of polydAT, polydA and polydT by DNase II were compared by measuring the rate of release of acid-soluble radioactivity from the tritiated compounds upon reaction with DNase II in 150 mM sodium acetate buffer, pH 5.0, containing 10 mM EDTA by the procedure used for the ^{3}H DNA assay, described in the Methods section. Fig. 39 illustrates the acid-soluble radioactivity released as a function of time for each polynucleotide. The rates of reaction of DNase II with 0.23 uM polydAT-methyl-^{3}H, 0.29 uM polydA-8-^{3}H, and 0.22 uM polydT-methyl-^{3}H were 245, 26, and 15 cpm of acid-soluble radioactivity released per minute, respectively. That the rate of reaction of DNase II with polydAT was at least 10 times that with polydA or polydT is probably due to the double helical regions such as "hairpin loops" which can occur in polydAT (Scheffler et al., 1968) and to the preference of DNase II for the double-stranded over the single-stranded configuration.

Mode of cleavage of native DNA

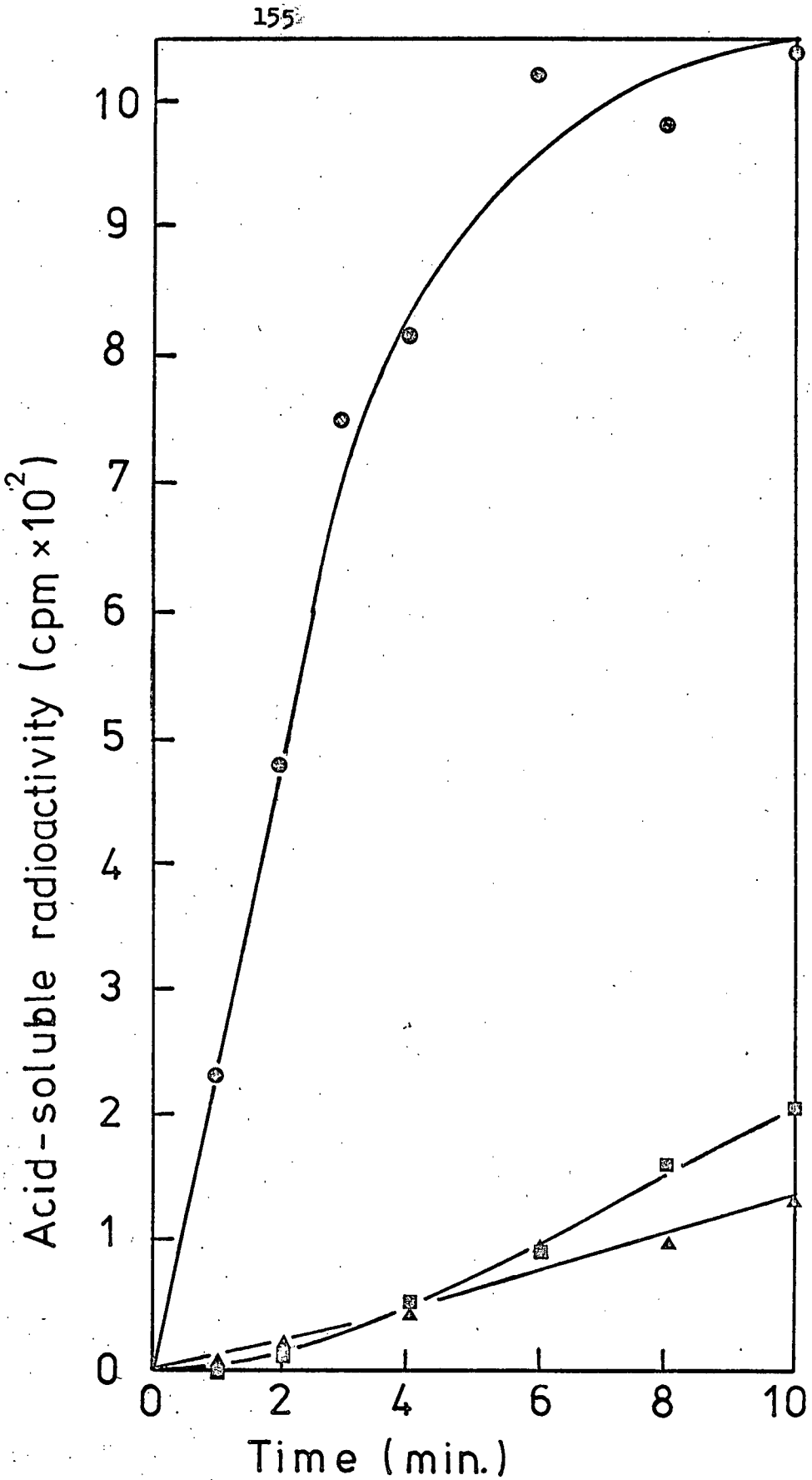
DNase II can cleave either one or both strands of native DNA. Different values for the relative numbers of single-strand and double-strand cleavages of DNA by DNase II from other tissues have been found

Fig. 39 Reaction of DNase II with polydAT, polydA and polydT. The acid-soluble radioactivity released from the tritiated compounds upon reaction with DNase II at 37°C in 150 mM sodium acetate buffer, pH 5.0, containing 10 mM EDTA, was plotted as a function of time.

—●— polydAT-methyl-^{3}H

—■— polydA-8-^{3}H

—▲— polydT-methyl-^{3}H

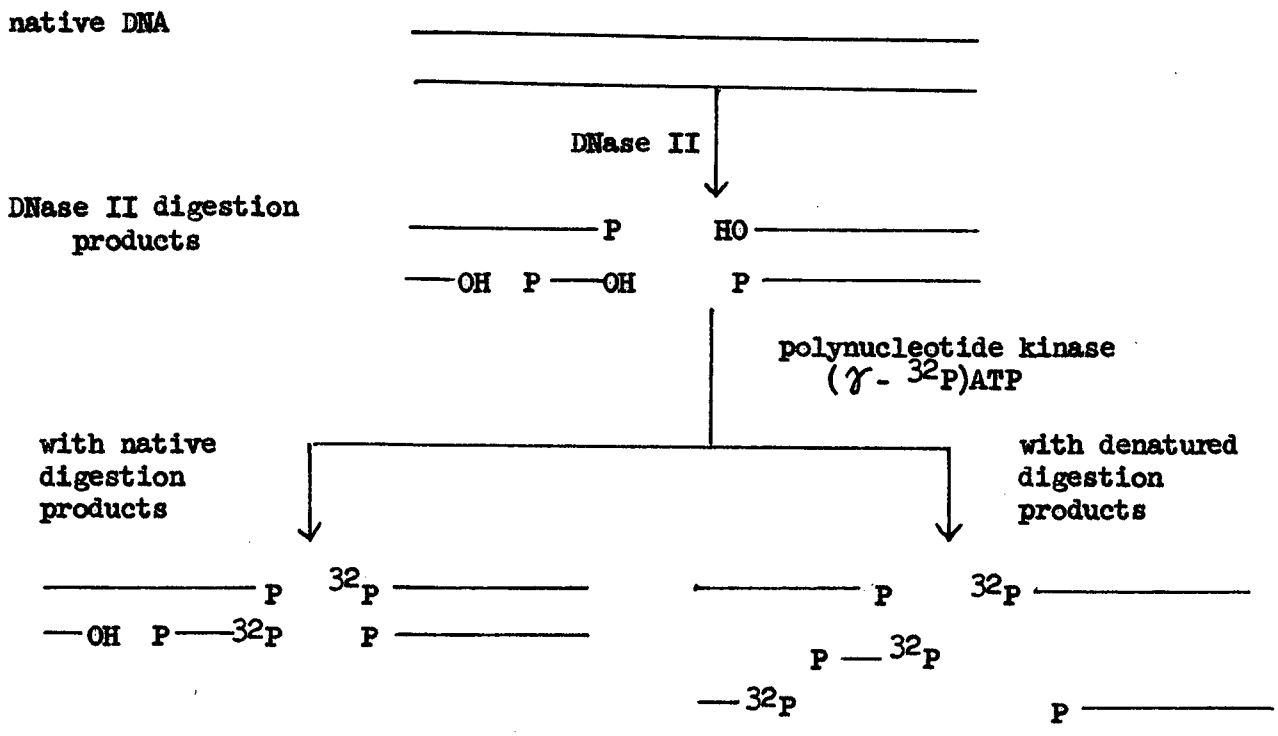

Acid-soluble radioactivity (cpm ×10²)
0
1
2
3
4
5
6
7
8
9
10
0
2
4
6
8
10
Time (min.)

by previous investigators using ultracentrifugation in neutral and alkaline media (Young and Sinsheimer, 1965; Bernardi and Bach, 1968; Kopecka et al., 1973; Oshima and Price, 1974).

Radioactive labelling of DNase II reaction products using polynucleotide kinase and [γ-^{32}P]ATP is a rapid and direct way of determining the mode of cleavage of DNA by DNase II.

Fig. 40 indicates how the relative amounts of single-strand and double-strand cleavages of DNA by DNase II were determined. In a certain fragment of native DNA, DNase II made one single-strand and one double-strand cleavage. When the native digestion products from the DNase II reaction were incubated with polynucleotide kinase and [γ-^{32}P]ATP, only the 5'-hydroxyl groups at the double-stranded ends were labelled since incorporation of ^{32}P into an internal 5'-hydroxyl group occurred at a much slower rate than into an external 5'-hydroxyl group (Weiss et al., 1968; Oshima and Price, 1974). Conditions used for the polynucleotide kinase reaction were not favourable for the exchange reaction between 5'-phosphate terminated oligonucleotides and [γ-^{32}P]ATP noted by van de Sande et al. (1973) and by Beckner and Folk (1977). Since two 5'-hydroxyl groups were liberated for each double-strand cleavage, half the counts per minute or pmoles incorporated was proportional to the number of double-strand cleavages.

When the DNase II digestion products were denatured before incubation with polynucleotide kinase, ^{32}P was incorporated into the 5'-hydroxyl ends resulting from single-strand cleavages as well as those resulting from double-strand cleavages. The difference between the ^{32}P incorporated into the denatured DNase II digestion products and the ^{32}P incorporated



let n = pmoles ^{32}P incorporated into native digestion products

let d = pmoles ^{32}P incorporated into denatured digestion products

no. of double-strand cleavages $\propto \frac{n}{2}$

no. of single-strand cleavages $\propto (d - n)$

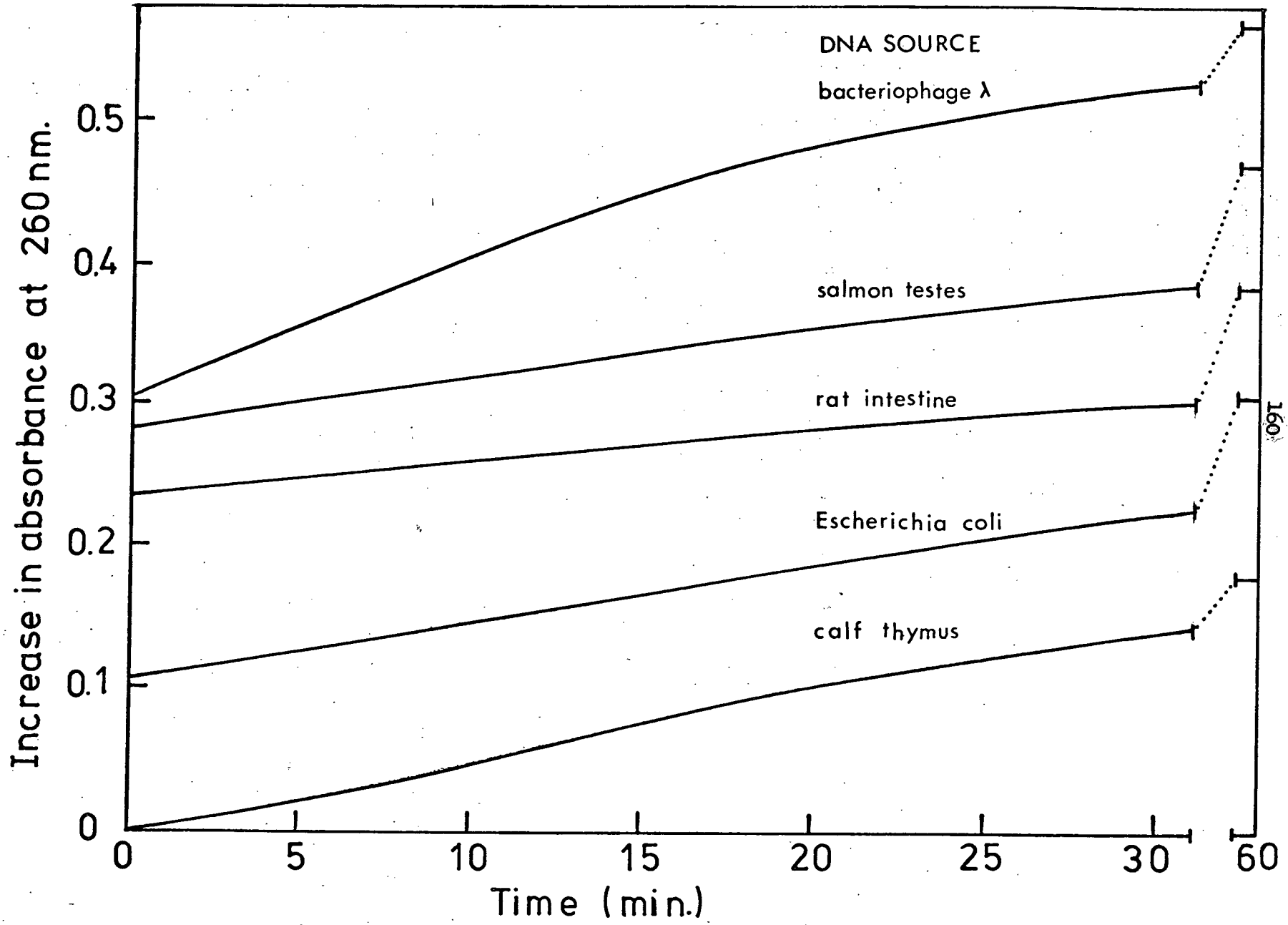
Fig. 40 Determination of mode of cleavage of DNA by DNase II.

into the native digestion products was proportional to the number of single-strand cleavages.

The DNase II reaction

DNase II was incubated at 37°C with 40 ugm DNA in 1.0 ml of 100 mM sodium acetate buffer, pH 5.0, containing 10 mM EDTA. The reaction was followed by measuring the increase in absorbance at 260 nm of the DNA solution versus time with respect to a control solution to which no enzyme had been added. Fig. 41 illustrates the hyperchromicity plots obtained for the digestion of DNA from different sources by DNase II. Duplicate DNA solutions were incubated with DNase II for 5 minutes at 37°C. The reactions were stopped by the addition of 50 ul of 1.0 M tris HCl, pH 8.0, 20 ul of 1.0 M $MgCl_2$, and 45 ul of 1 N NaOH; the final pH was 8.0. This was done in order to ensure that the solutions had an optimal pH and divalent metal ion concentration for the polynucleotide kinase reaction. The extent of degradation of the DNA was estimated from Fig. 41, by expressing the increase in absorbance at 260 nm after 5 minutes of reaction as a percent of the total increase in absorbance at 260 nm. The reaction of DNase II with calf thymus DNA was also carried out for 1, 10, 20, and 30 minutes at 37° C. The reaction mixtures, as well as control solutions containing DNA, were each divided into two equal portions. One portion of the solution was heated in a boiling water bath for 15 minutes, and then quickly cooled in an ice-water bath in order to denature the double-stranded oligonucleotides or DNA; the other remained at 0° C and con-

Fig. 41 Digestion of DNA from various sources by DNase II. The reaction was carried out with 40 ugm/ml of DNA in 100 mM sodium acetate buffer, pH 5.0, containing 10 mM EDTA. The hyperchromicity of the reaction solution was plotted as a function of time, and the extents of degradation of a particular DNA at various times were estimated by expressing the absorbances of the DNA solution at those times as percents of the maximum absorbance attained.


DNA SOURCE
bacteriophage λ
salmon testes
rat intestine
Escherichia coli
calf thymus
Increase in absorbance at 260 nm.
0
0.1
0.2
0.3
0.4
0.5
0
5
10
15
20
25
30
60
Time (min.)

tained native oligonucleotides, or DNA.

The polynucleotide kinase reaction

The reaction conditions were worked out with consideration of the properties of polynucleotide kinase from bacteriophage T4 (Richardson, 1971; van de Sande et al., 1973; Lillehaug and Kleppe, 1975a,b; Okazaki et al., 1975; Lillehaug et al., 1976). A reducing agent, 10mM dithiothreitol was added to stabilize the polynucleotide kinase (Richardson, 1971). The reaction was carried out at a pH of 8.0 which is within the optimum pH range for the enzyme for the phosphorylation of 5' hydroxyl end groups in DNA and which is high enough so that the exchange reaction of 5'-phosphate end groups with $[\gamma\text{-}^{32}P]$ ATP is minimized (van de Sande et al., 1973). The $[\gamma\text{-}^{32}P]$ ATP was of sufficient radioactivity, at least 200 cpm per pmole, so that the extent of ^{32}P incorporation could be reliably determined. The concentration of $[\gamma\text{-}^{32}P]$ ATP was 20 mM in order to ensure quantitative incorporation of ^{32}P into the 5'-hydroxyl groups of double-stranded DNA (Lillehaug et al., 1976). Although this concentration is much lower than the Km for the enzyme (Richardson, 1971), a higher concentration was not used because of the possibility of increasing the rate of the exchange reaction (van de Sande et al., 1973). Only 2 units of polynucleotide kinase were used per assay because of the possibility of phosphorylation of single-strand nicks in native DNA using larger amounts of enzyme (Lillehaug et al., 1976).

To 100 ul of a solution containing DNase II digestion products

or DNA, were added 2 ul of 0.98 mM ATP, enough $[\gamma\text{-}^{32}P]$ ATP to give a specific radioactivity of at least 200 cpm per pmole ATP and 2 units of T4 polynucleotide kinase. The incubation was carried out at 37° C and at various times 5 ul aliquots were removed from the reaction mixture and placed on sheets of DE 81 cellulose paper, 23 x 28 cm which had been prespotted twice with 50 ul aliquots of 1 mM ATP, 50 mM EDTA. A 5 ul aliquot of the reaction mixture was taken before the addition of the polynucleotide kinase and was spotted as a control for the possible absorption of $[\gamma\text{-}^{32}P]$ ATP to the origin. Descending chromatography was run for two hours at room temperature with 0.35 M ammonium formate buffer, pH 5.5, (van de Sande et al., 1973). Under these conditions the Rfs of ATP, AMP and Pi were 0.46, 0.48 and 0.71 respectively. Oligonucleotides of at least 10 nucleotides in length remained at the origin (Sgaramella and Khorana, 1972). The DE 81 cellulose paper was dried, and the origins were cut out and placed in 10 ml of water in liquid scintillation vials. The radioactivity retained at the origin was estimated by measuring the Čerěnkov radiation produced in the ^{3}H channel of a liquid scintillation counter. The counts per minute were converted into pmoles of ^{32}P incorporated into the original amount of digestion product present in the reaction mixture. This was done by calculating the specific radioactivity of the $[\gamma\text{-}^{32}P]$ ATP used, either from the manufacturer's data, or by using oligodT (pT) 9 as described in the Methods section.

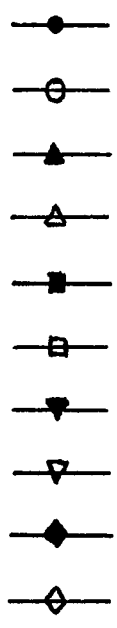
Cleavage of calf thymus DNA

Fig. 42 depicts the ^{32}P radioactivity incorporated by polynucleotide kinase into the native and denatured products of the digestion of native calf thymus DNA by DNase II as well as into native and denatured calf thymus DNA. A significant incorporation of ^{32}P into native DNA indicated that there was a substantial number of 5'-hydroxyl groups present in the DNA before digestion with DNase II. The higher incorporation of ^{32}P into denatured DNA than into native DNA demonstrated that there were single-stranded 5'-hydroxyl nicks in the DNA prior to digestion with DNase II and that this technique of ^{32}P incorporation using polynucleotide kinase was able to differentiate between external and internal 5'-hydroxyl groups.

The incorporation of ^{32}P by polynucleotide kinase into denatured products of digestion of native DNA by DNase II was greater than for the control DNA and increased with thr duration of the DNase II reaction. This indicates that the products of the reaction of DNase II with DNA had 5'-hydroxyl terminals. Because the ^{32}P radioactivity incorporated remained at the origin in the DE 81 paper chromatography system in which AMP migrated with an Rf of 0.48, DNase II cleaved the DNA endonucleolytically to liberate oligonucleotides.

Table XI illustrates the method used to calculate the percent of cleavages of DNA by DNase II that were double-strand cleavages. In order to determine the amount of ^{32}P incorporated into 5'-hydroxyl groups released from DNA by DNase II action, the pmoles of ^{32}P incorporated into denatured and native DNA were subtracted from the

Fig. 42 Polynucleotide kinase catalysed incorporation of ^{32}P into products of the reaction of DNase II with native calf thymus DNA. The DNase II reaction with 40 ugm/ml DNA was carried out for various times in 100 mM sodium acetate buffer, pH 5.0, containing 10 mM EDTA. The reaction mixtures, as well as control solutions containing DNA, were each divided into two equal portions. One part of the solution was denatured; the other was allowed to remain native. The ^{32}P radioactivity incorporated into these products was plotted as a function of the time of reaction with polynucleotide kinase.



native DNA

denatured DNA

native 5 minute reaction products

denatured 5 minute reaction products

native 10 minute reaction products

denatured 10 minute reaction products

native 20 minute reaction products

denatured 20 minute reaction products

native 30 minute reaction products

denatured 30 minute reaction products

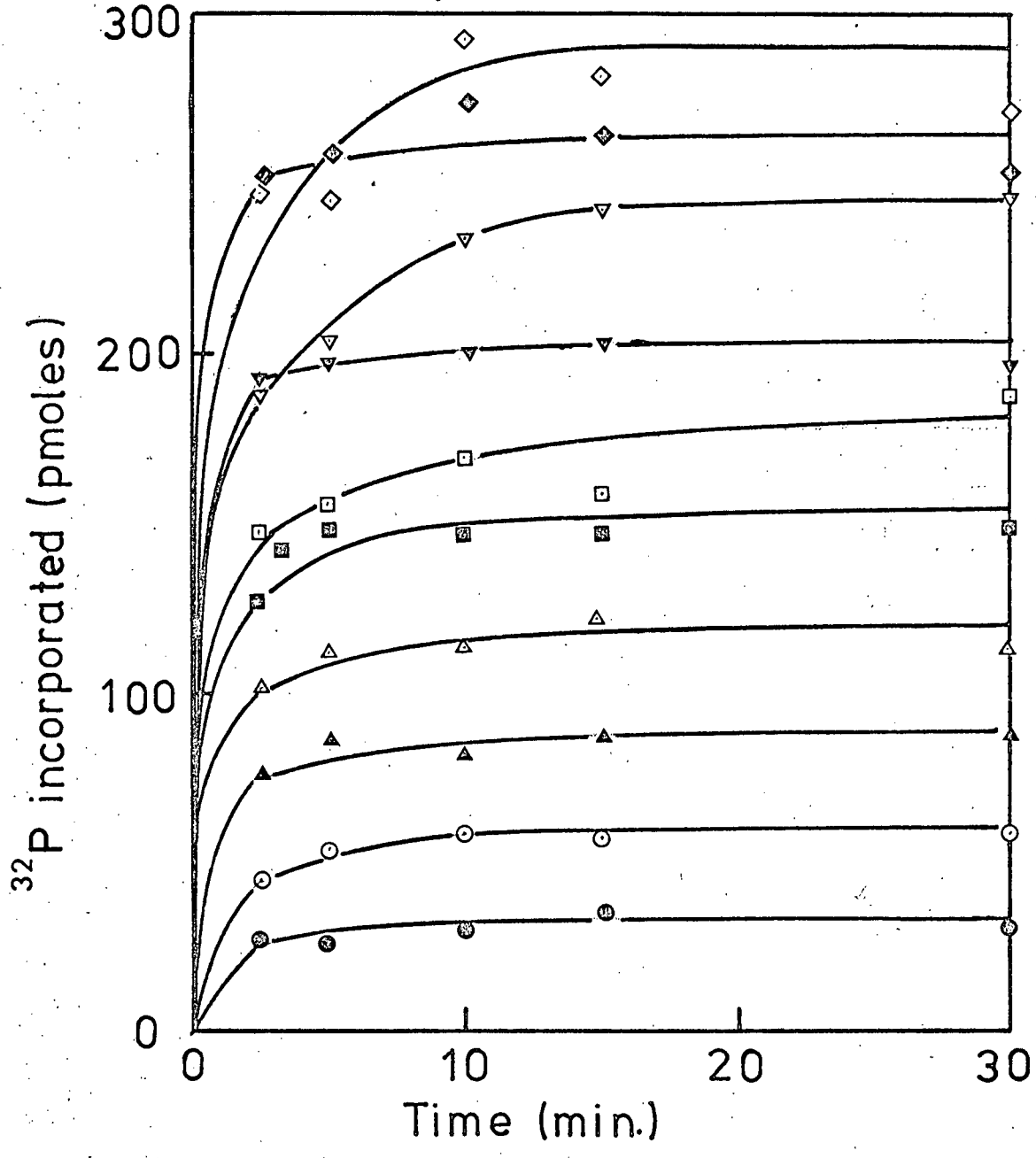

300
200
100
0
0
10
20
30
Time (min.)
^{32}P incorporated (pmoles)

TABLE XI

PROPORTION OF CLEAVAGES OF DNA BY DNase II
THAT WERE DOUBLE STRAND CLEAVAGES

Substrate	Total ^{32}P incorporated (pmoles)	^{32}P incorporated due to DNase II action (pmoles)	^{32}P incorporated proportional to no. of double-strand cleavages	^{32}P incorporated proportional to no. of single-strand cleavages	double-strand cleavages % of total
denatured 5 minute reaction products	120	120 - 60 = 60	$\frac{53.4}{2} = 26.7$	60 - 53.4 = 6.6	$\frac{26.7}{26.7-6.6} \times 100$ = 80%
denatured calf thymus DNA	60				
native 5 minute reaction products	86.7	86.7 - 33.3 = 53.4			
native calf thymus DNA	33.3				

pmoles of ^{32}P incorporated into denatured and native products of digestion of DNA by DNase II. Because two 5'-hydroxyl groups originated from each double-strand cleavage, the number of double-strand cleavages was proportional to half the amount of ^{32}P incorporated into native digestion products. Since ^{32}P was incorporated into 5'-hydroxyls from single-strand cleavages only after denaturation of the reaction products, the number of single-strand cleavages was proportional to the pmoles of ^{32}P incorporated into denatured products minus that incorporated into native products. The relative number of double-strand cleavages was calculated as a percent of the total number of cleavages. Table XII gives values for percent double-strand cleavage calculated in this way for 5 minute reactions of DNase II with 40 ugm/ml of calf thymus DNA in 100 mM sodium acetate buffer, pH 5.0, containing 10 mM EDTA in 10 separate experiments. The mean value obtained was 86.1 $\pm$12.6.

The relatively large background incorporation of ^{32}P due to nicks and ends present in calf thymus DNA before digestion with DNase II made it more difficult to measure accurately the incorporation of ^{32}P into 5'-hydroxyl groups released by DNase II action and probably accounted for some of the variability of the results.

Degradation of DNA from other sources.

In order to determine the mode of cleavage of other DNA by DNase II, and the background incorporation, with DNA from other species, DNA from salmon testes, rat intestinal mucosa, bacteriophage λ, and

TABLE XII

MODE OF CLEAVAGE OF CALF THYMUS DNA BY DNase II

Experiment Number	Double strand cleavages[a] (% of total cleavages)	
1	90	Mean = 86.1
2	71	Standard Deviation = ± 12.6
3	86	
4	81	Coefficient of Variation = 14.6%
5	90	Standard Deviation of the Mean:
6	100	$S\bar{x} = \pm 3.98$
7	63	[b]95% Confidence Limits
8	80	$\bar{x} = 86.1 \pm 9.0$
9	100	99% Confidence Limits
10	100	$\bar{x} = 86.1 \pm 11.2$

(a) Values for % double strand cleavages that were greater than 100, were assumed to be 100. If this was not not done, these values were rejected by Chauvenet's criterion.

(b) Using Student's t-test (Brewer et al., 1974).

Escherichia coli was digested with DNase II under the same conditions used previously for the digestion of calf thymus DNA. Polynucleotide kinase catalysed incorporation of ^{32}P into salmon, rat, and λ DNA and their respective reaction products formed on digestion with DNase II is illustrated in Fig. 43. The lower incorporation of ^{32}P into salmon and λ DNA than into calf thymus and rat DNA indicated that there were fewer 5'-hydroxyl groups present in salmon and λ DNA. For all three DNA species in Fig. 44 the incorporation of ^{32}P into digestion products resulting from DNase II action was substantially greater than into the corresponding DNA control.

Effect of 1 mM sulfate on the mode of cleavage of DNA from Escherichia coli

Fig. 44 illustrates the effect of 1 mM sulfate on the mode of cleavage of DNA from Escherichia coli by DNase II. The reaction was carried out for 5 minutes and ^{32}P was incorporated as previously described for the reactions of DNase II with DNA.

In the presence of sulfate ^{32}P incorporation increased slightly for both native and denatured digestion products, but no large increase was seen in the incorporation of ^{32}P into 5'-hydroxyl groups resulting from single-strand cleavages.

Effect of 10 mM EDTA on the mode of cleavage of calf thymus DNA

The effect of 10 mM EDTA on the mode of cleavage of calf thymus

Fig. 43 Incorporation of ^{32}P by polynucleotide kinase into products of the reaction of DNase II with DNA from salmon testes, rat intestinal mucosa, and bacteriophage λ. The DNase II reaction was carried out for 5 minutes and ^{32}P was incorporated as described in Fig. 42.

(a) salmon DNA
(b) rat DNA
(c) λ DNA

—●— native DNA

—○— denatured DNA

—▲— native 5 minute reaction products

—△— denatured 5 minute reaction products

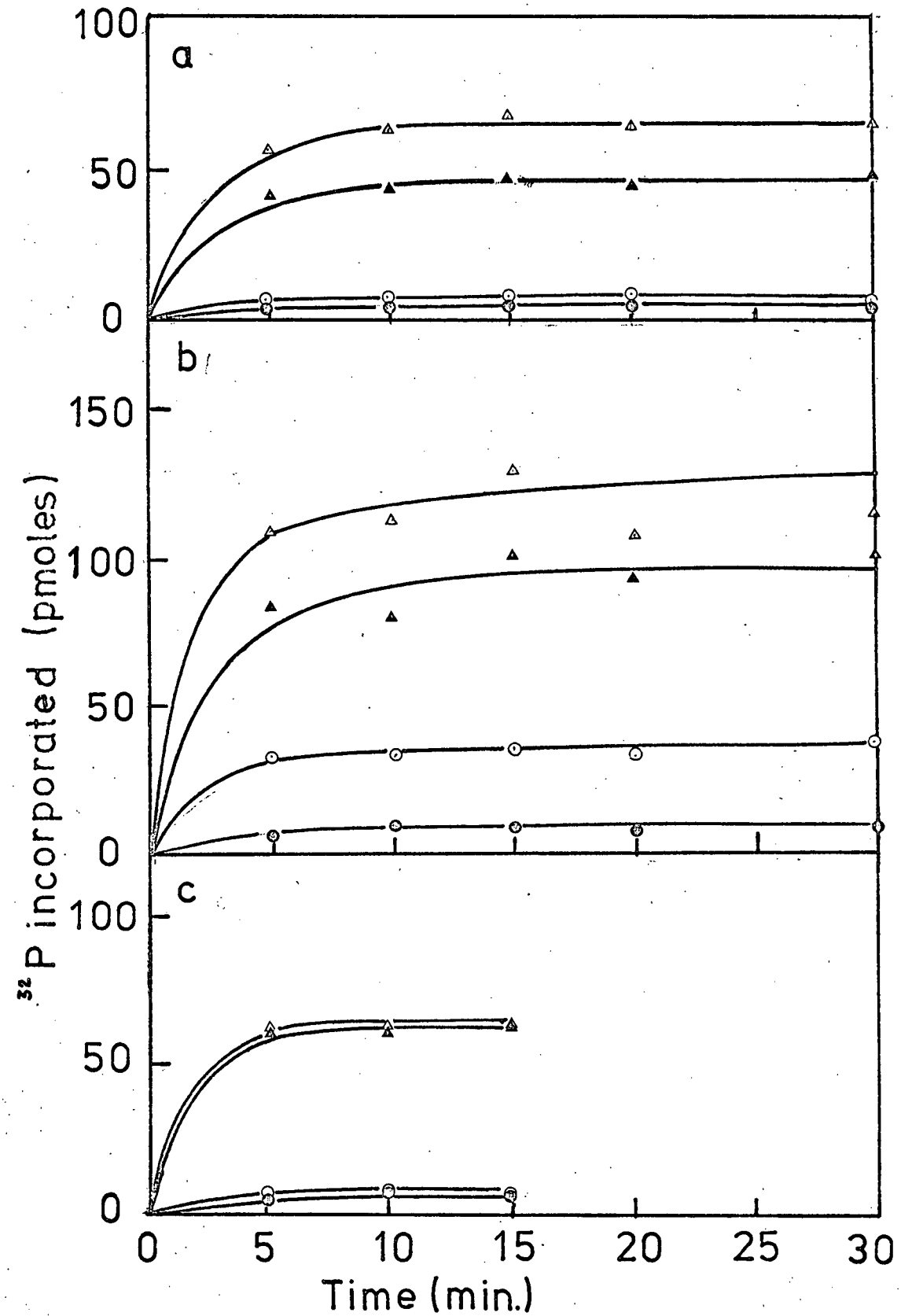

a
b
c
100
50
0
150
100
50
0
100
50
0
^{32}P incorporated (pmoles)
0
5
10
15
20
25
30
Time (min.)

Fig. 44 Effect of 1 mM sulfate on the mode of cleavage of DNA from Escherichia coli by DNase II. The DNase II reaction was carried out for 5 minutes and ^{32}P incorporated as described in Fig. 42.

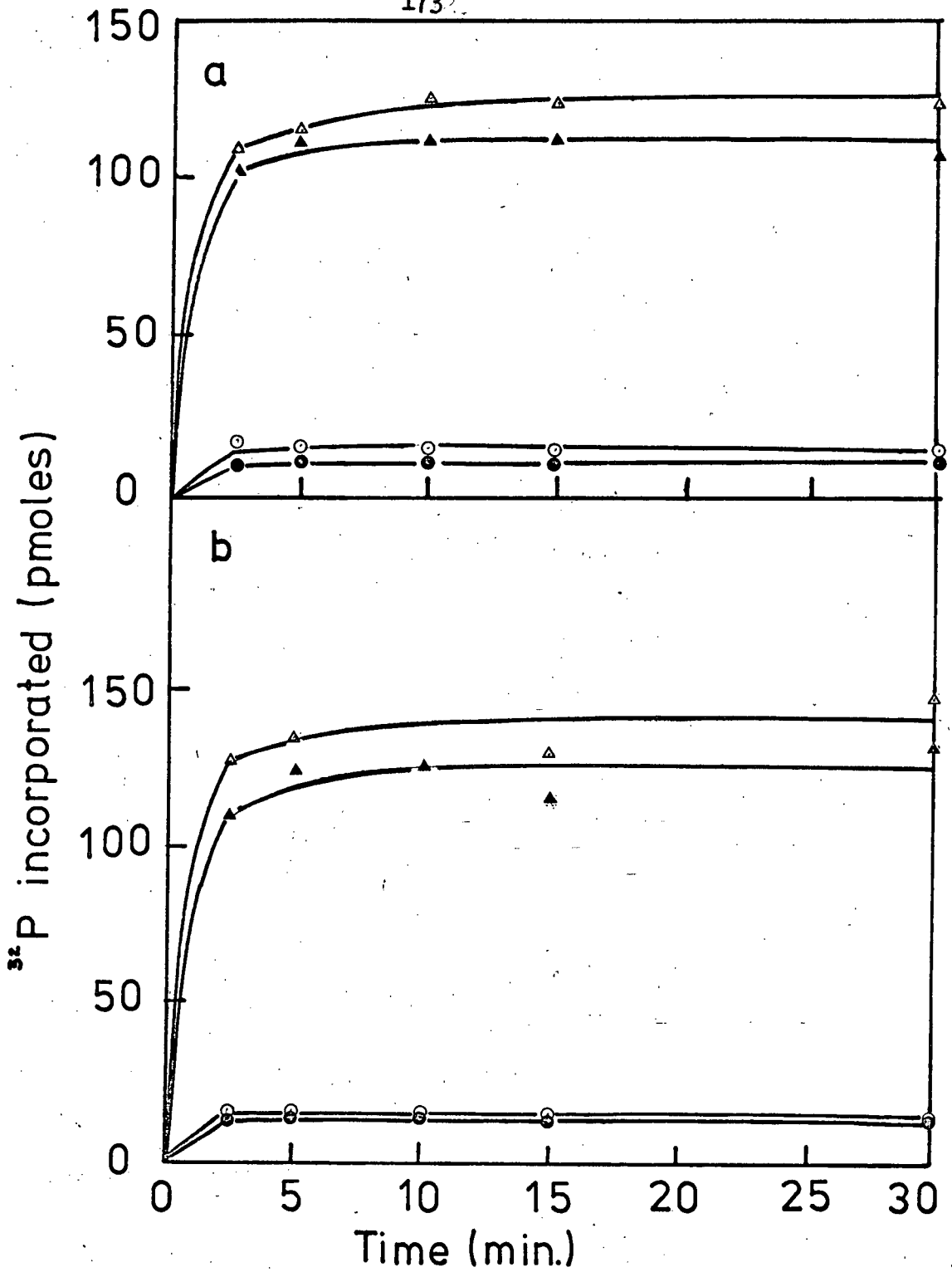
(a) no sulfate ion present
(b) with 1 mM sodium sulfate present

—●— native DNA

—○— denatured DNA

—▲— native reaction products

—△— denatured reaction products


a
b
150
100
50
0
150
100
50
0
0
5
10
15
20
25
30
Time (min.)
^{32}P incorporated (pmoles)

DNA by DNase II is illustrated in Fig. 45. In the presence of EDTA the ^{32}P incorporation increased slightly for both native and denatured reaction products. No evidence was seen for a large increase in the number of single-strand cleavages by DNase II in the presence of EDTA.

In Table XIII are given the values for percent double-strand cleavage of DNA from different species by DNase II calculated from the ^{32}P incorporation curves displayed in Figs. 43, 44, and 45. From the results shown in Table XIII as well as from those found in Table XII, it appears that intestinal DNase II cleaved native DNA primarily by a double-strand cleavage mechanism. Neither the addition of 1 mM sulfate to the reaction mixture, nor the removal of the EDTA from it seemed to have any substantial effect on the mode of cleavage of DNA by DNase II.

Characterization of the products and substrates of the DNase II reaction by electrophoresis on polyacrylamide gels

Although the size distribution of the products of the degradation of DNA by DNase II from other tissues has been investigated by chromatography of reaction mixtures on DEAE cellulose columns (Bernardi, 1968; Soave et al., 1973), even after very prolonged incubation of DNA with DNase II, 50% or more of the products were unresolved by this technique. (Soave et al., 1973).

Polyacrylamide gel electrophoresis of the products of the reaction of DNase II with DNA and other substrates was carried out in order to estimate the size ranges of the digestion products as well as to determine

Fig. 45 Effect of 10 mM EDTA on the mode of cleavage of calf thymus DNA by DNase II. The DNase II reaction was carried out for 5 minutes and ^{32}P was incorporated as described in Fig. 42.

(a) complete DNase II reaction mixture including 10 mM EDTA
(b) with omission of EDTA

—●— native DNA

—○— denatured DNA

—▲— native reaction products

—△— denatured reaction products

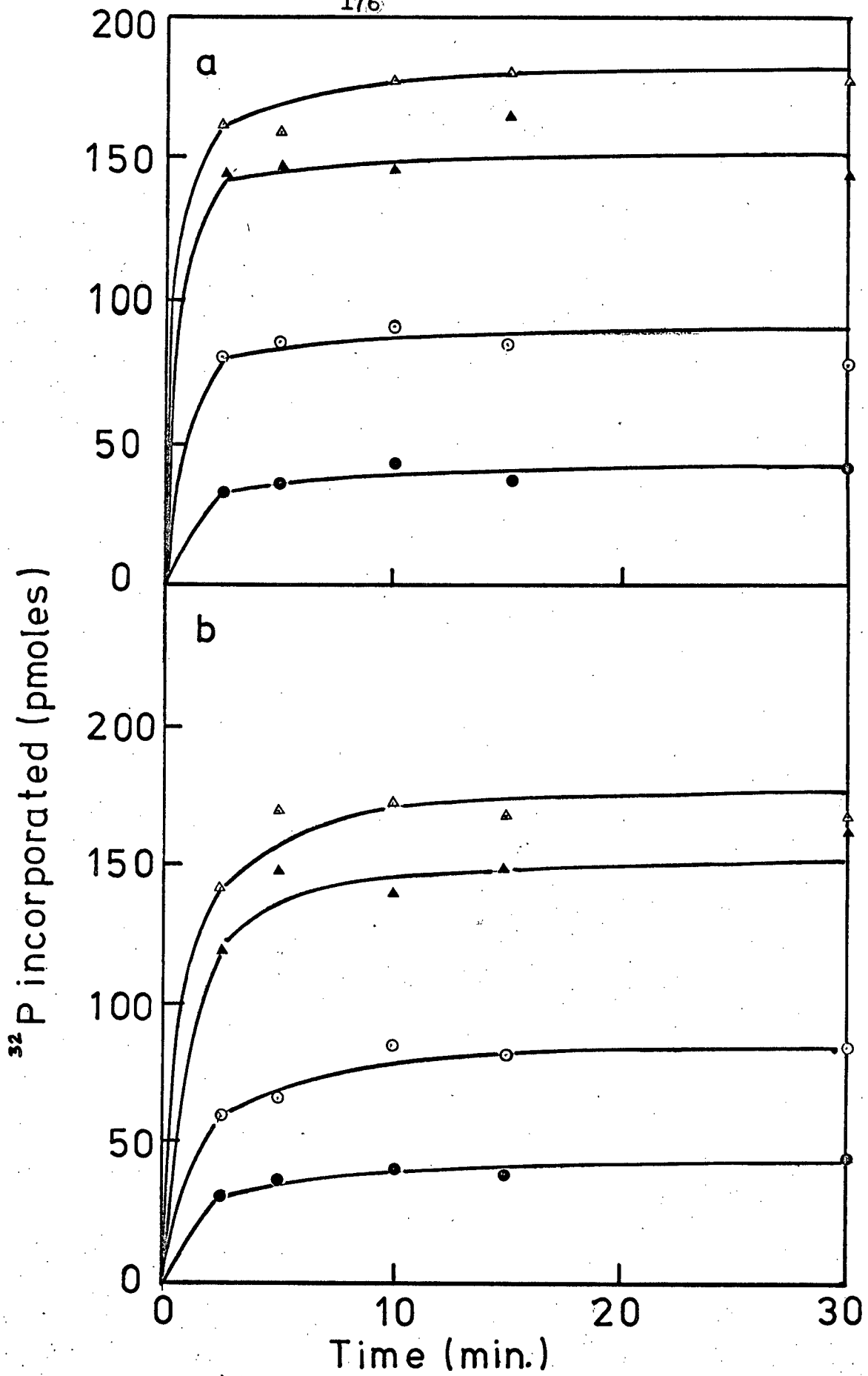

a
b
200
150
100
50
0
200
150
100
50
0
0
10
20
30
Time (min.)
32P incorporated (pmoles)

TABLE XIII

MODE OF CLEAVAGE OF DNA FROM DIFFERENT SPECIES BY DNase II*

Preparation Used		Double-strand Cleavages (percent of total cleavages)
DNA from salmon testes		82
DNA from rat intestinal mucosa		84
DNA from bacteriophage λ		100
DNA from Echerichia coli	- sulfate	80
	+ 1 mM sulfate	79
DNA from calf thymus	+ EDTA	100
	- EDTA	100

* DNase II was reacted with 40 ugm/ml DNA in 100 mM sodium acetate buffer, pH 5.0, containing 10 mM EDTA except when the EDTA was omitted.

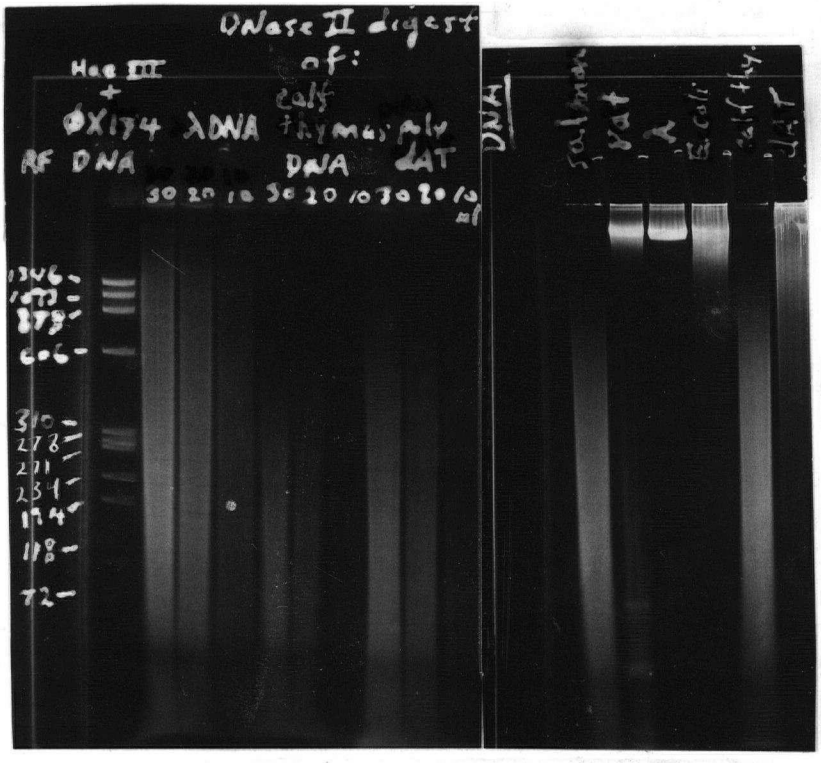
whether discrete sizes of products were formed in the early stages of the reaction.

DNA from bacteriophage λ and calf thymus, and polydAT were digested with DNase II under the same conditions used to determine the mode of cleavage of DNA by DNase II. The reactions were stopped by extraction of the solutions with equal volumes of water-saturated phenol. Phenol dissolved in the aqueous layer was removed by three extractions with ether; traces of ether were removed by bubbling nitrogen through the solution. Substrate solutions which had not been digested with DNase II were treated similarly.

The samples were electrophoresed on 5% polyacrylamide gels at 5 volts/cm in 90 mM Tris-90 mM boric acid-2.5 mM EDTA by the method described by Maniatis et al. (1975). Since intercalation of ethidium bromide between base pairs in double-stranded DNA causes a large increase in the ethidium bromide fluoresence emission spectrum (LePecq and Paoletti, 1967), treatment of the gels with ethidium bromide allowed the DNA fragments to be visualized.* The lengths of the DNA fragments were estimated by comparison of their distances of migration on the gels with those of DNA fragments resulting from digestion of replicative form DNA from bacteriophage ϕX 174 by a restriction endonuclease from Haemophilis aegyptius, Hae III. The lengths of the ϕX 174 DNA fragments have been determined by sequence analysis (Sanger et al., 1977). As illustrated in Fig. 46, continuous fluorescent patterns resulting from oligonucleo-

*Thanks are due to Anne Lui who performed this experiment.

Fig. 46 Electrophoresis of products and substrates of the DNase II reaction on polyacrylamide gels. The sample applied at the extreme left was a digest of replicative form DNA from bacteriophage ØX 174 by a restriction endonuclease from Haemophilis aegyptius, Hae III. From top to bottom the bands shown represented double-stranded DNA fragments of 1346, 1073, 873, 606, 310, 278, 271, 234, 194, 118 and 72 base pairs. (Sanger et al., 1977). The other samples applied were 10, 20, and 30 ul portions of 5 minute DNase II digests of polydAT and DNA from bacteriophage λ and calf thymus as well as 30 ul portions of the unreacted substrates.


DNase II digest of:
Hae III
+
ΦX174 λDNA
RF DNA
calf thymus poly DNA
DAT
50 20 10 50 20 10 50 20 10 ng
1346
1078
872
606
310
278
271
234
194
118
72
DNA
salmon
yeast
E. coli
calf thy.
DAT

tides ranging from less than 100 to more than 1,300 base pairs in length were seen for 5 minute DNase II digests of polydAT and DNA from bacteriophage λ and calf thymus. Fluorescent bands with positions on the gel comparable to those of species of high molecular weight were seen for polydAT and DNA from bacteriophage λ and Escherichia coli. Electrophoresis of DNA from calf thymus and salmon testes resulted in fluorescent patterns indicating the presence of DNA fragments of high and low molecular weights.

Base specificity of cleavage of DNA by DNase II

Thiery et al. (1973) have investigated the base specificity of the cleavage of DNA by DNase II from hog spleen in the middle and terminal phases of the reaction, that is, with oligonucleotide products of average length from 15 to 40 nucleotides. They demonstrated that DNase II cleaved specific sets of short nucleotide sequences at least four nucleotides long and presented evidence that indicated that DNase II might be more specific, or at least have a different base specificity in the initial phase of the reaction which had not been studied.

Accordingly the base specificity of the cleavage of DNA by intestinal DNase II was investigated in the early stages and throughout the reaction in order to determine whether the base specificity of cleavage changed as the reaction progressed, and whether DNase II cleaved DNA more specifically in the initial stages of the reaction.

The base specificity at the 5' terminals

Although radioactive labelling using polynucleotide kinase has been a useful technique for determination of the base sequence of cleavage of DNA by restriction enzymes (Smith et al., 1974), the method has not been used for DNase II.

Fig. 47 presents a scheme outlining the procedure used to determine the 5'-terminal base specificity of cleavage of DNA by DNase II. DNA was digested with DNase II and the products of the reaction were labelled with ^{32}P at the 5'-hydroxyl groups in a reaction with polynucleotide kinase and [γ - ^{32}P]ATP. The labelled oligonucleotides were separated from unreacted [γ - ^{32}P]ATP by chromatography on Sephadex G50 and then degraded to 5'-phosphate terminated nucleotides with DNase I and phosphodiesterase I. The nucleotides were separated by thin layer chromatography and the amount of radioactivity incorporated into each terminal nucleotide was determined.

The DNase II and polynucleotide kinase reactions

DNase II and polynucleotide kinase reactions were carried out as described previously for determination of the mode of cleavage of DNA by DNase II. After 32 minutes of incubation with polynucleotide kinase, 20 ul of 0.2 M EDTA was added, the reaction mixture was heated at 100° C for 5 minutes and then cooled to 0°C.

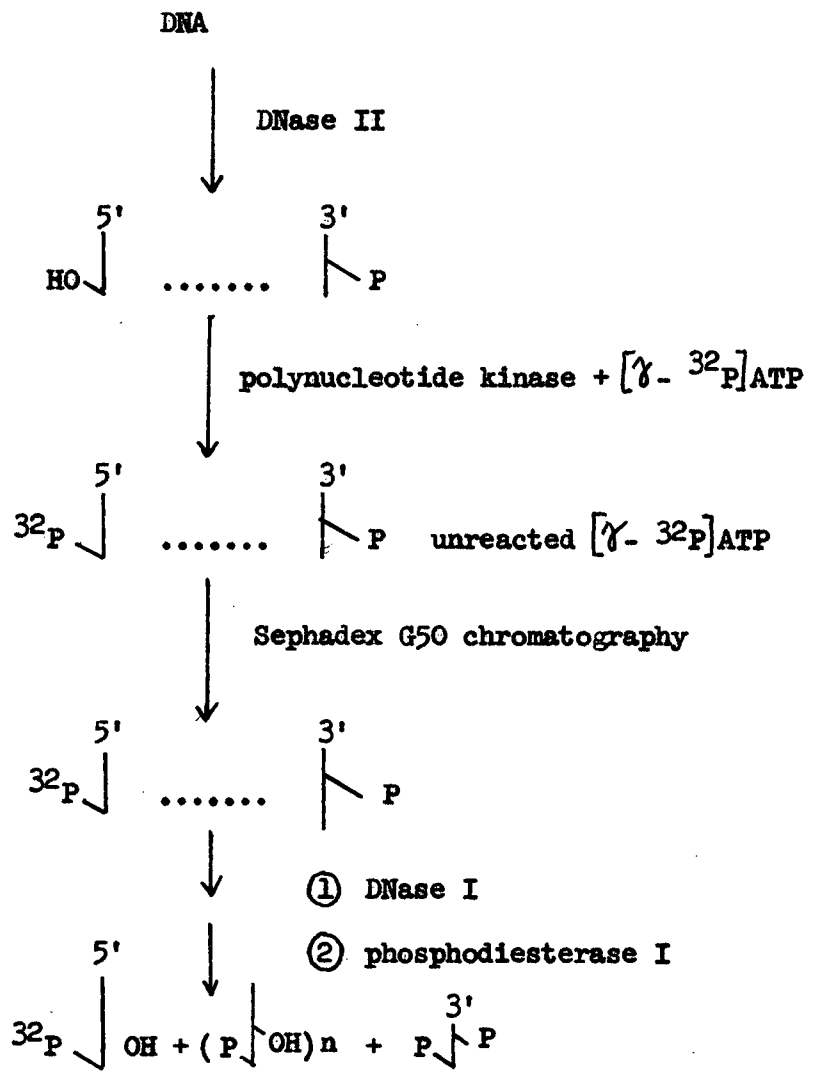


Fig. 47 Scheme for determination of the base specificity at the 5'-terminals of products of cleavage of DNA by DNase II. The oligonucleotide products form the DNase II reaction were labelled at the 5'-terminals with ^{32}P using polynucleotide kinase and then were enzymatically degraded to 5'-^{32}P nucleotides. The 5'-^{32}P nucleotides were separated by thin layer chromatography and the amount of radioactivity in each nucleotide was determined by measuring the Čerěnkov radiation emitted.

Separation of the 5'-^{32}P oligonucleotide products of the polynucleotide kinase reaction from unreacted [γ-^{32}P]ATP

The reaction mixture was chromatographed on Sephadex G50. The column, 0.9 x 50 cm, was eluted at 4°C with 50 mM triethylammonium bicarbonate buffer, pH 7.5. Fractions of 0.5 ml were collected in 4 ml glass vials, and after the addition of 3.5 ml of water to each, these vials were placed in larger scintillation vials and the radioactivity counted by Čerénkov radiation. Fig. 48 shows the elution profile from the column. The first small peak of radioactivity eluted from the column was due to the 5'-^{32}P-oligonucleotides, and the second large peak of radioactivity was due to unreacted [γ-^{32}P]ATP. The fractions containing the 5'-^{32}P-oligonucleotides were pooled and the solution was lyophilized in order to remove the triethylammonium bicarbonate.

Degradation of the 5'-^{32}P-oligonucleotides to 5'-^{32}P nucleotides

This was done by a method similar to that used by Richardson (1966). The reaction mixture contained 80 ul of a solution containing the 5'-^{32}P oligonucleotides dissolved in water, 10 ul of 0.1 M Tris HCl pH 7.2, 10 ul of 0.1 M $MgCl_2$, and 5 ugm of pancreatic DNase I. The reaction was incubated at 37° C for 30 minutes; 3 ul of 1 N NH_4OH were added to change the pH to 9.0; 5.3 ugm phosphodiesterase I was added, and the reaction was allowed to continue at 37° C for a further 30 minutes.

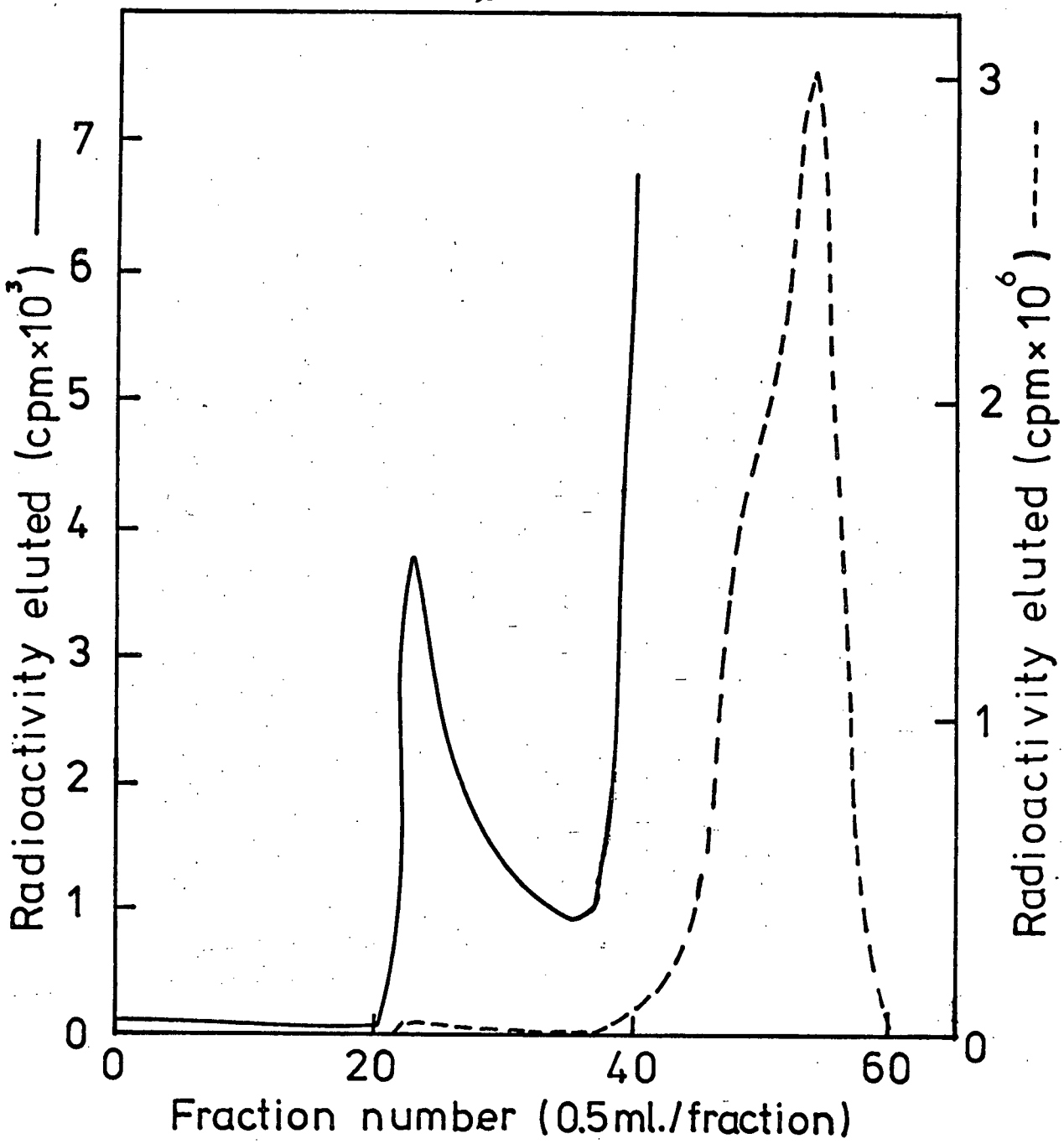


Fig. 48 Separation of 5'-^{32}P oligonucleotides from unreacted [γ-^{32}P]ATP by chromatography of the polynucleotide kinase reaction mixture on Sephadex G50. The polynucleotide kinase reaction mixture which contained radioactively phosphorylated products of the digestion of DNA with DNase II was placed on a column of Sephadex G50, 0.9 x 50 cm. The column was eluted with 50 mM triethylammonium bicarbonate buffer, pH 7.5; each fraction was made up to 4 ml with water and the amount of radioactivity was measured by Čerenkov radiation.

Separation of the 5'-^{32}P nucleotides

Twenty-five microlitres of the reaction mixture was spotted on a polyethyleneimine (PEI) cellulose plate 1 ul at a time. The plate had been previously spotted in the same way with 5 ul of a mixture of 1 mg each of 5'dAMP, 5' dCMP, 5' TMP, 5' dGMP in 1 ml of water.

The thin layer chromatograph was run upwards for 5.0 cm in 1 N acetic acid, transferred while still wet, and run a further 10-11 cm in the same direction in 0.3 M lithium chloride (Randerath and Randerath, 1967). The plate was dried in a stream of warm air, and the nucleotides except for GMP, which fluoresced, appeared as dark spots under ultraviolet irradiation at 254 nm. The Rfs for dGMP, TMP, dAMP, and dCMP were 0.16, 0.34, 0.46 amd 0.69, respectively. The areas of the chromatogram containing the nucleotides were cut out and counted by Čerěnkov radiation as described in the Methods section.

Table XIV shows the radioactivity incorporated into each 5'-terminal nucleotide and the method of calculation of the ^{32}P incorporation. The percent of ^{32}P incorporation into a particular nucleotide was taken as indicative of the prevalence of the corresponding base at the 5'-terminals of products formed after DNase II cleavage of DNA. For each nucleotide the ^{32}P incorporation in the 5' terminal nucleotide was taken to be equal to the ^{32}P incorporated into the nucleotide for the native or denatured product of the DNase II reaction minus the ^{32}P incorporated into the native or denatured DNA, respectively. The incorporation of radioactivity into each nucleotide was expressed as a percent of the total amount of

TABLE XIV

CALCULATION OF THE ^{32}P INCORPORATED INTO EACH NUCLEOTIDE AT 5' TERMINAL AS A PERCENT OF THE TOTAL ^{32}P INCORPORATION

Material Analysed		^{32}P Incorporated CPM	^{32}P Incorporated Percent of Total Incorporation
(1) Native calf thymus DNA	Origin	25	
	GMP	154	33
	TMP	82	18
	AMP	94	20
	CMP	131	28
(2) Denatured calf thymus DNA	Origin	44	
	GMP	324	38
	TMP	132	15
	AMP	146	17
	CMP	260	30
(3) Native products of the 5 minute reaction of DNase II with native DNA	Origin	108	
	GMP	572	
	TMP	230	
	AMP	356	
	CMP	396	
(4) Denatured products of the 5 minute reaction of DNase II with native DNA.	Origin	105	
	GMP	765	
	TMP	258	
	AMP	368	
	CMP	629	
(5) 5' terminal nucleotides produced by double strand cleavage of DNA by DNase II (3) - (1)	GMP	418	38
	TMP	148	14
	AMP	262	24
	CMP	265	24
(6) 5' terminal nucleotides produced by both double-strand and single strand cleavage of DNA by DNase II (4) - (2)	GMP	441	38
	TMP	126	11
	AMP	222	19
	CMP	369	32

radioactivity incorporated into all four nucleotides. The low amount of radioactivity observed at the origins of the thin layer chromatograms indicated that the degradation of 5'-^{32}P oligonucleotides to 5'-^{32}P nucleotides had been essentially complete.

Fig. 49 illustrates the base specificity at the 5'-terminals for cleavage of native calf thymus DNA by DNase II. For the native digestion products the base that was present in the highest percentage at the 5'-terminals of the cleavage sites was guanine, which ranged from 30-40% of the total. The base which was present in the lowest percentage was thymine, at about 11-17%. Adenine and cytosine has intermediate values. For the native products the order of the bases did not change during the middle and terminal phases of the reaction, but in the terminal phase there was a greater variation in the percentages of the bases at the 5'-terminus than during the middle phase of the reaction. These results are in accord with those of Soave et al.(1973) for digestion of calf thymus DNA with hog spleen DNase II.

In the early stages of the reaction the percent of guanine was elevated and the order of cytosine and adenine was reversed. These changes may reflect the presence of a preferred sequence that is selectively degraded and exhausted during the initial phase of the reaction.

The situation for the denatured digestion products is more complex because the percentage obtained for each base represents the percentage of the base prrsent at the 5'-terminals of single-strand cleavages as well as double-strand cleavages of DNA by DNase II. In the early stages of the reaction less than 15% of the cleavages of calf thymus DNA by

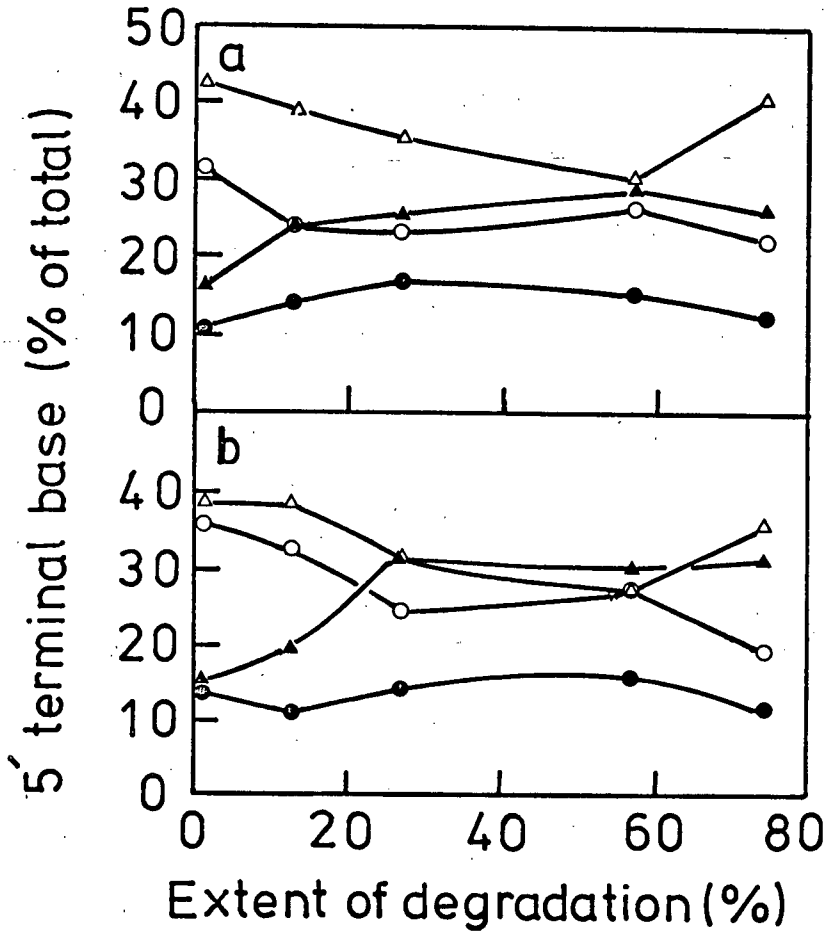


Fig. 49 Base specificity at the 5'-terminals for cleavage of native calf thymus DNA by DNase II. The percent of the 5'-terminal base was calculated as in Table XIII. The extent of degradation was determined from Fig. 42.
(a) native products of digestion of DNA by DNase II
(b) denatured products of digestion of DNA by DNase II

—●— thymine

—○— cytosine

—▲— adenine

—△— guanine

DNase II were single-strand cleavages (Table XII). These may have had a prevalence of cytosine at their 5'-terminals since the percent of cytosine was higher for the denatured digestion products (Fig. 49b) than for the native digestion products (Fig. 49a). The percentage of cleavages that were single-strand cleavages probably increased as the reaction progressed because, due to denaturation of short DNA fragments, more single-stranded substrates were likely present at later stages in the reaction. In the later stages of the reaction higher percentages of adenine at the 5'-terminals were observed for denatured digestion products (Fig. 49b) than for native digestion products (Fig. 49a). This suggests either that DNase II cleaved small single-stranded DNA fragments with a preference for adenine at the 5'-terminals, or that the small fragments that the enzyme was degrading had already been depleted of the preferred sequences with guanine or cytosine at the 5'-terminals.

Table XV compares the base specificities at the 5'-terminals for DNase II cleavage of DNA from several different sources. Although the order of the bases was similar for all the DNAs with guanine present in the highest percent and thymine, in the lowest, a different pattern was observed for each DNA. For the eukaryotic DNAs the 5'-terminals of the native digestion products had high percentages for guanine, low percentages for thymine and intermediate approximately equal percentages for adenine and cytosine. For DNA from Escherichia coli and bacteriophage λ the percentages of adenine and cytosine at the 5'-terminals of the native digestion products were not equal. The salmon DNA had a higher percent

TABLE XV

BASE SPECIFICITY AT THE 5' TERMINALS FOR CLEAVAGE OF DNA FROM DIFFERENT SOURCES BY DNase II

DNA Source	Extent of Degradation		% 5' Terminal Base: Native Products	% 5' Terminal Base: Denatured Products
Calf thymus	13%	Guanine	38	38
		Thymine	14	11
		Adenine	24	19
		Cytosine	24	32
Salmon	13%	Guanine	46	39
		Thymine	10	13
		Adenine	22	22
		Cytosine	21	26
Rat	9%	Guanine	36	44
		Thymine	13	4
		Adenine	26	26
		Cytosine	25	27
E. coli	12%	Guanine	37	42
		Thymine	9	7
		Adenine	22	19
		Cytosine	33	32
Bacteriophage λ	17%	Guanine	46	53
		Thymine	11	10
		Ademine	16	9
		Cytosine	27	28

of guanine at the 5'-terminals of the native digestion products than did the two mammalian DNAs. The patterns for the bases at the 5'-terminals of the native digestion products for Escherichia coli DNA and λ DNA also differred from each other as illustrated in Table XV.

The patterns for the bases at the 5'-terminals of denatured digestion products resulted from the combined processes of double-strand and single-strand cleavage reactions and were too complex to be compared for DNA from different species, but were compared with the patterns obtained for native digestion products from DNA of the same species. The increase in the percent of cytosine for the denatured digestion products from calf thymus and salmon DNA with respect to the native digestion products suggested that for these DNAs DNase II preferred cytosine at the 5'-terminals of single-strand cleavages early in the reaction. For rat, E. coli and λ DNA, the percent of guanine was elevated in the denatured digestion products with respect to the native digestion products, suggesting that guanine was preferred at the 5'-terminals of early single-strand cleavages.

The base specificity at the 3'-terminals

The terminal addition of riboadenylic acid to deoxyoligonucleotides by terminal deoxynucleotidyl transferase has been used to specifically label deoxyoligonucleotides at the 3' ends (Kössel and Roychoudhury, 1971; Kössel et al., 1974). Terminal transferase catalysed radioactive labelling of the 3'-terminals of products of the reaction of DNA with spleen DNase II has allowed the analysis of the 3'-terminal nucleotides for cleavage of DNA by spleen DNase II (Bertazzoni et al., 1973; ibid,

1974). Since both mono- and diaddition of riboadenylic acid to the deoxyoligonucleotides occur, it is necessary to remove the second riboadenylic acid either by treatment with base and phosphatase (Kössel et al., 1974), or by separation of the ribonucleotide from the deoxyribonucleotides after degradation to nucleotides (Bertazzoni et al., 1974). Terminal deoxynucleotidyl transferase catalyses the polymerization of deoxynucleotide triphosphates, elongating polydeoxynucleotide chains (Bollum, 1974). Perhaps because a ribonucleotide triphosphate and not the preferred substrate was used, large concentration of enzyme and substrate were required for appreciable labelling of 3'-terminals (Bertazzoni, 1973).

Products of the reaction of DNA with DNase II were labelled with ^{32}P using terminal transferase and [α-^{32}P]ATP by the method of Kössel et al., (1974) as outlined in Fig. 50. DNA was reacted with DNase II to yield 3'-phosphate oligonucleotides. The 3' phosphate group was removed by incubation with acid phosphatase. ^{32}P labelled 5'-AMP was added to the resulting 3'-hydroxyl group on the oligonucleotide by using terminal transferase and [α-^{32}P]ATP. Since both mono- and diaddition reactions occurred, the second adenosine was hydrolysed with base and the second ^{32}P labelled phosphate was removed with alkaline phosphatase. The radioactivity labelled oligonucleotide was then separated from ^{32}P labelled phosphate by chromatography on Sephadex G50 and was digested with spleen DNase II and phosphodiesterase II to yield 3'-^{32}P labelled nucleotides from the 3'-terminals of the sites of cleavage of DNA by DNase II. The labelled nucleotides were then separated by thin layer chromatography on PEI cellulose plates and the percent of radioactivity incorporated

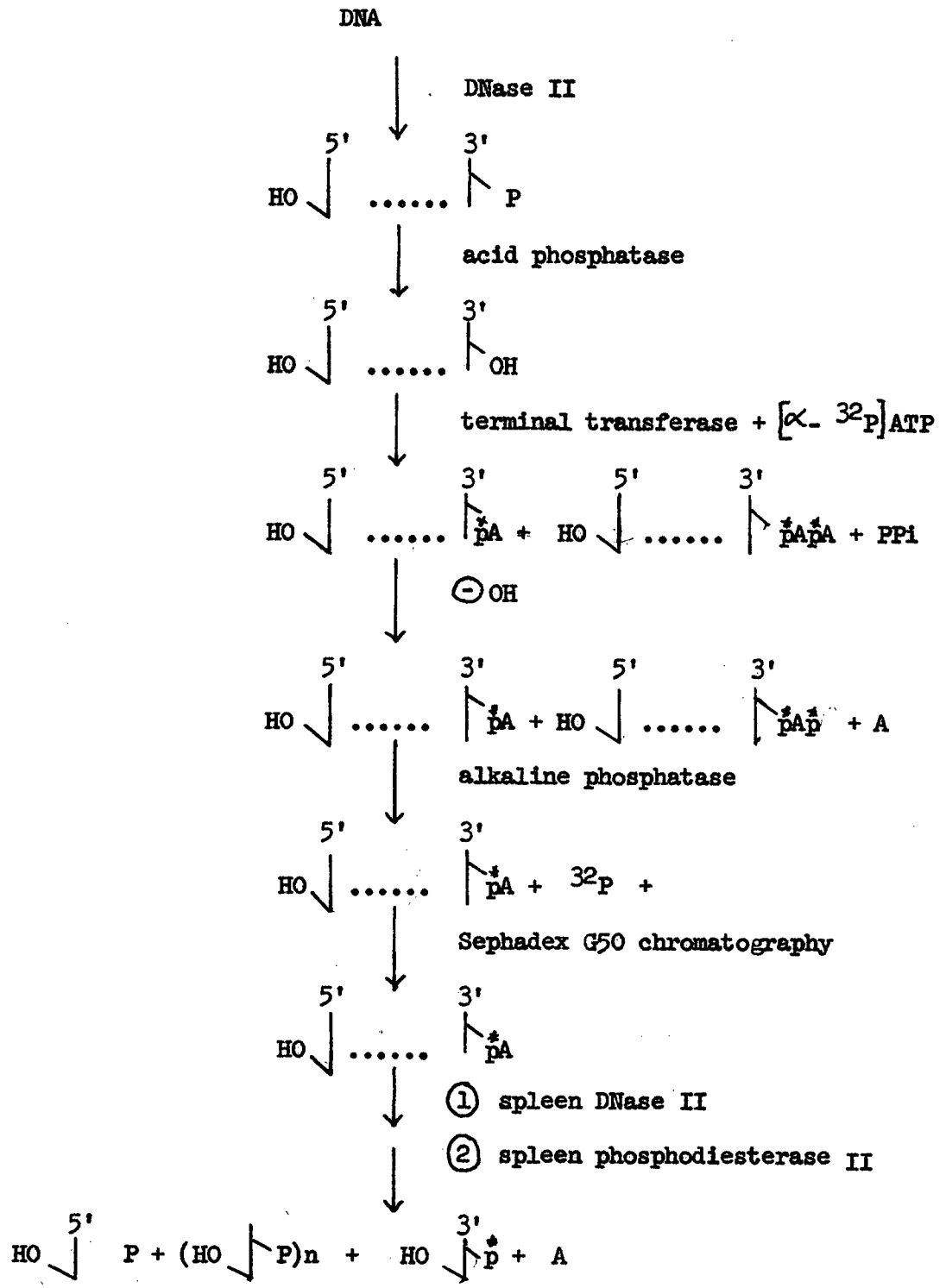


Fig. 50 Scheme for determination of the base specificity at the 3'-terminals of products of cleavage of DNA by DNase II. Dephosphorylated digestion products were labelled at the 3'-terminals with ^{32}P using terminal transferase and, after removal of excess label, were enzymatically degraded to 3'-^{32}P nucleotides. The 3'-^{32}P nucleotides were separated by thin layer chromatography and the amount of radioactivity in each nucleotide was determined from the Čerěnhov radiation emitted.

into each nucleotide was determined by measuring the Čerěnkov radiation emitted.

The DNase II reaction

As for the determination of the base specificity at the 5'-terminal, DNase II was incubated at 37° C with 40 ugm DNA in 1.0 ml of 100 mM sodium acetate buffer containing 10 mM EDTA and the reaction was followed by the hyperchromicity assay. The reaction was stopped and the enzyme and DNA denatured by placing the reaction mixtures, in rubber stoppered test tubes, into a boiling water bath for 15 minutes and then into an ice-water bath.

The acid phosphatase reaction

Potato acid phosphatase, 5 ugm, was added to the reaction mixture containing the denatured products of the DNase II reaction with DNA. After a 30 minute incubation at 37° C, the solution was heated to 100° C for 5 minutes in order to inactivate the enzyme

The terminal transferase reaction.

The method of Kossel et al. (1974) was followed with some modifications. To 1.0 ml of the acid phosphatase treated reaction mixture

were added 50 ul of 1 M Tris HCl*, pH 7.5, 10 ul of 1 M $ZnCl_2$**, 8 ul of 1 M $MgCl_2$, and 42 ul of 1 N NaOH to give a final pH of 7.5. To 100 ul of this solution were added 1 ul of 0.1 M dithiothreitol, 149 pmoles of [α-^{32}P]ATP at 1.72 x 10^4 cpm/pmole and 24 units of terminal deoxyribonucleotidyl transferase. The reaction was followed by removing 5 ul aliquots from the reaction mixture before the addition of the terminal transferase and at various times during the reaction and spotting the aliquots on DE81 paper which had previously been prespotted with 50 ul of a solution of 1 mM ATP, 50 mM EDTA. Descending paper chromatography was carried out for two hours with 0.35 M ammonium formate buffer, pH 5.5. The DE81 paper was dried, the origins were cut out, and the radioactivity that was retained at the origin was determined by measuring the Čerenkov radiation emitted.

Fig. 51 depicts the terminal deoxynucleotidyl transferase catalysed incorporation of ^{32}P into products of the five minute reaction of DNase II with DNA from various sources and into the indigested DNA molecules. A substantial amount of ^{32}P was incorporated into Escherichia coli, bacteriophage λ, and rat intestinal DNAs and the respective reaction products

*Little or no radioactive incorporation was observed with the buffer commonly used for terminal transferase reactions: 40 or 200 mM potassium cacodylate pH 6.8 or 7.2 (Bollum, 1974). Tris buffer was used instead of cacodylate buffer because more incorporation of radioactivity into 3'-hydroxyl oligonucleotides was observed using the former buffer.

**The $ZnCl_2$ was added in order to complex the EDTA present in the solution from the DNase II reaction and prevent the inhibition of the enzyme which occurred in the presence of small quantities of EDTA and was probably due to chelation of the Zn from the active site of the enzyme (Chang and Bollum, 1970).

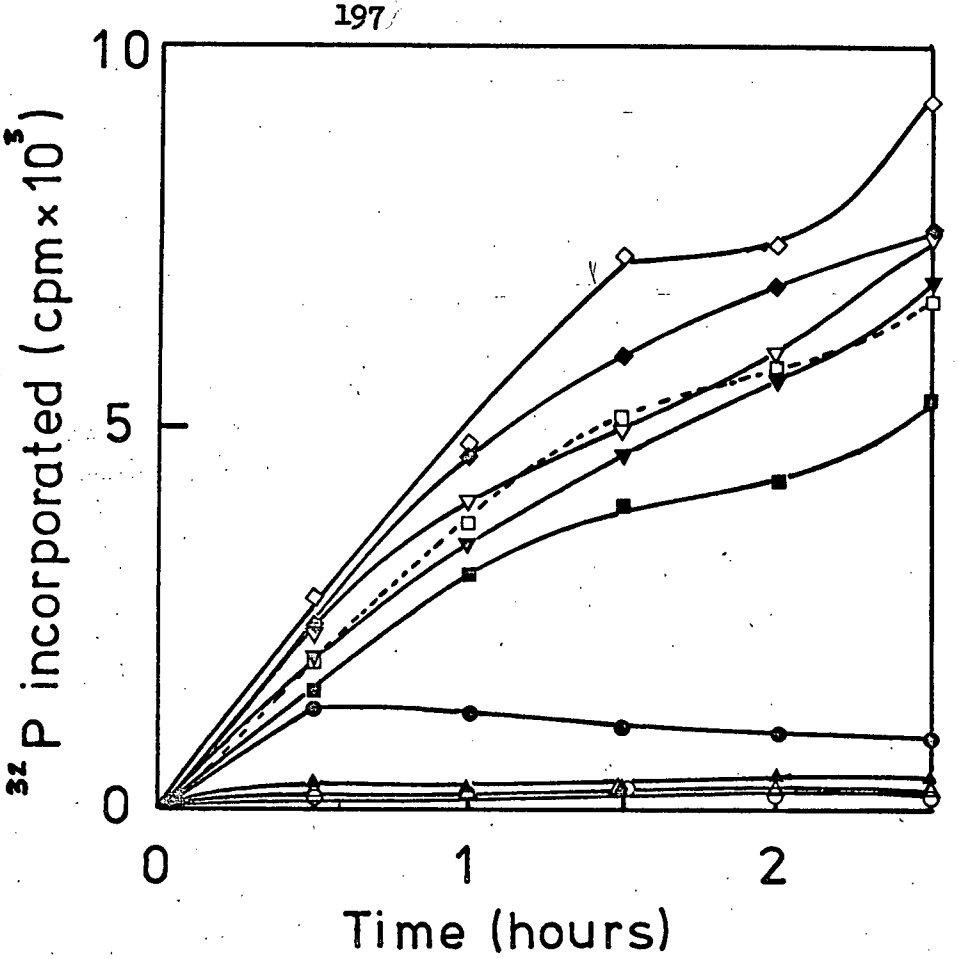


Fig. 51 Terminal deoxynucleotidyl transferase catalysed incorporation of ^{32}P into products of the reaction of DNase II with DNA from various sources. Incorporation of ^{32}P into products of a five minute DNase II reaction with DNA is indicated by open symbols, and the incorporation of ^{32}P into undigested DNA, by closed symbols. The sources of the DNA were:

●, ○ calf thymus,

▲, △ salmon testes,

■, □ bacteriophage λ,

▼, ▽ rat intestine,

◆, ◇ Escherichia coli.

liberated after digestion with DNase II, but little ^{32}P was incorporated into DNA from salmon testes and calf thymus and the respective DNase II reaction products. For most of the samples a biphasic curve was observed as was expected for a diaddition reaction. However, a large amount of ^{32}P was incorporated into DNA from E. coli, rat intestine, bacteriophage λ and calf thymus. The amount of incorporation of ^{32}P into calf thymus DNA was greater than into the respective DNase II reaction products. The ^{32}P compounds may have absorbed non-specifically to DNA which was itself absorbed at the origin of the DE81 cellulose paper.

Separation of the 3'-^{32}P oligonucleotide products of the terminal transferase reaction from ^{32}P-labelled inorganic phosphate

The terminal transferase reaction was stopped by the addition of 3 ul of 10 N KOH. Upon incubation at 37° C overnight, the base hydrolysed the terminal ribonucleotide residue resulting from two additions of ^{32}P-AMP (Bock, 1967). The next day 40 ul of water, 10 ul of 1.0 M Tris HCl, pH 8.0, and 2 ul of 60% perchloric acid were added at 0° C to the 60 ul of the terminal transferase reaction mixture. The mixture was cooled at 0° C for 10 minutes, and then centrifuged at 12,100 xg for 10 minutes at 4° C.

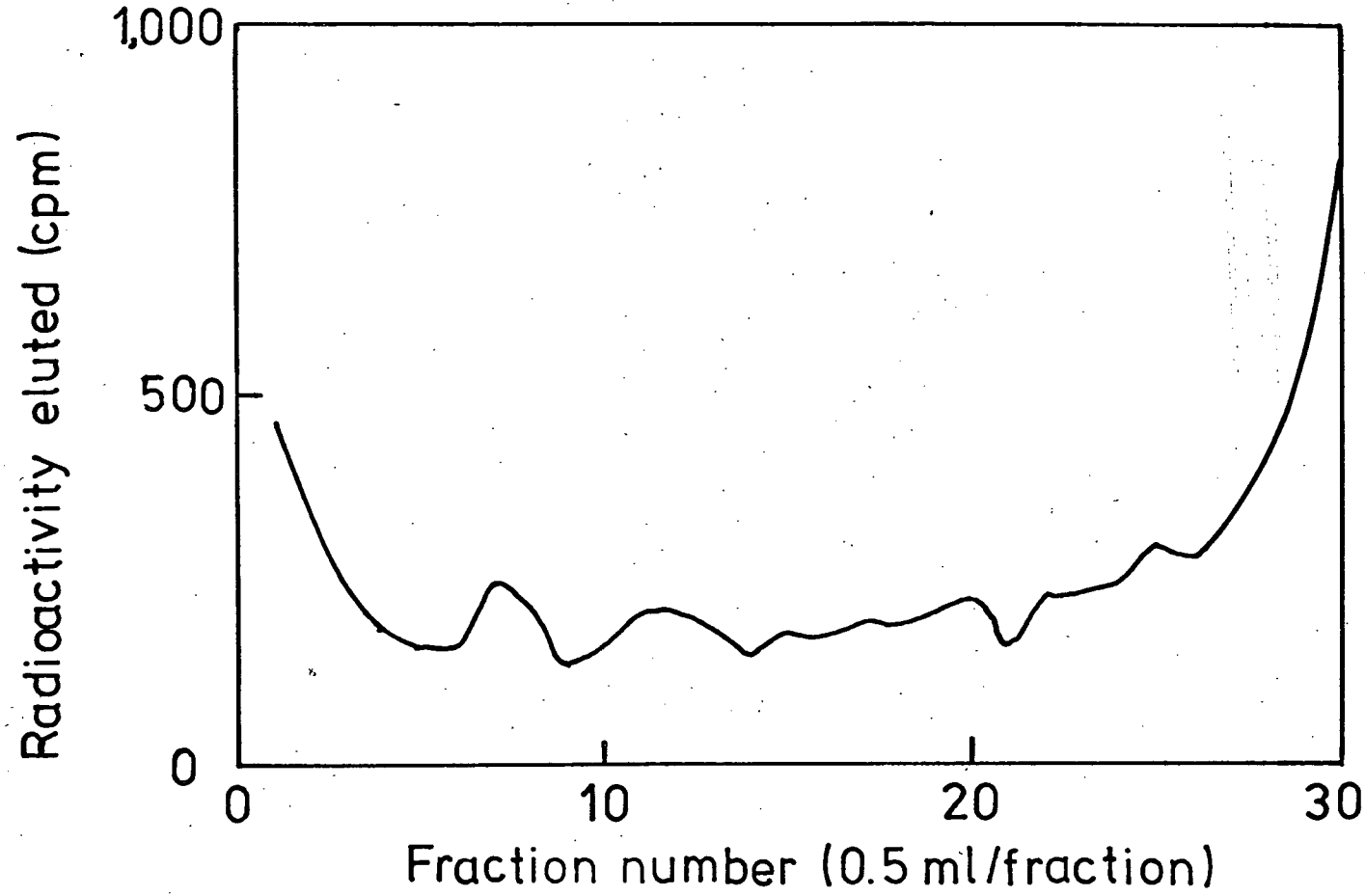
Alkaline phosphatase, 5 ugm, was incubated with the supernatant solution at pH 8 and 37° C for 15 minutes; 5 ul of 10 N KOH were then added and the solution was incubated for a further 15 minutes at 37° C in order to inactivate the alkaline phosphatase (Ho and Gilham, 1973; Delaney and Spencer, 1976). The solution was neutralized with 4 ul of

concentrated hydrochloric acid, and was applied to a Sephadex G50 column, 0.9 x 50 cm. The column was eluted at 4° C with 50 mM triethylammonium bicarbonate, pH 7.5 and 0.5 ml fractions were collected in 4 ml glass vials. The radioactivity present in each fraction was determined by adding 3.5 ml of water to the small vials, placing the small vials in larger vials, and measuring the Čerěnkov radiation emitted. The elution profile of radioactivity from the Sephadex G50 column is illustrated in Fig. 52. ^{32}P labelled inorganic phosphate was eluted starting at fraction number 30; the elution profile is not shown in Fig. 52. Although judging from Fig. 51, about 7,000 counts per minute of radioactivity were incorporated into products of the digestion of DNA from Escherichia coli with DNase II, very little radioactivity was eluted from the Sephadex column in the position expected for oligonucleotides, fractions number 19-25. These fractions were pooled, nevertheless, and the solution was lyophilized to remove the triethylammonium bicarbonate. Similar elution profiles were obtained for the other DNAs and digestion products.

Degradation of the 3'-^{32}P-oligonucleotides to 3'-^{32}P nucleotides

A method similar to that of Bertazzoni et al. (1973) was used. To the 3'-^{32}P oligonucleotides in 35 ul of water were added 5 ul of 1 M ammonium acetate, pH 5.6, and 42 ugm of hog spleen DNase II. After one hour of incubation at 37° C, 10 ugm of spleen phosphodiesterase II was added and the solution was incubated for a further hour at 37° C.

Fig. 52 Separation of 3'-^{32}P oligomucleotides from ^{32}P-labelled inorganic phosphate by chromatography of the base and alkaline phosphatase treated terminal transferase reaction mixture in Sephadex G50. Products of a five minute reaction of DNase II with DNA from Escherichia coli were radioactively phosphorylated with terminal transferase and [α-^{32}P]ATP, and placed on a Sephadex G50 column, 0.9 x 50 cm. The column was eluted with 50 mM triethylammonium bicarbonate, pH 7.5. Radiactivity in each fraction was determined by measuring the Čerěnkov radiation emitted. A large peak of ^{32}P-labelled inorganic phosphate, which eluted after fraction number 30, is not shown in Fig. 52.


1,000
500
0
Radioactivity eluted (cpm)
0
10
20
30
Fraction number (0.5 ml/fraction)

Separation of the 3'-^{32}P nucleotides

A PEI cellulose thin layer chromatography plate was spotted and run in the same manner as for the separation of the 5'-^{32}P nucleotides except that 3'-P deoxyribonucleotides were used as markers, and interfering salts were removed, after spotting but before chromatography, by immersing the TLC sheet flat in 500 ml methanol for 10 minutes, and then drying it (Randerath and Randerath, 1964). The Rfs of 3'd GMP, 3' TMP, 3'd AMP and 3'd CMP were 0.19, 0.30, 0.42, 0.58, respectively. The areas containing the nucleotides were cut out and the radioactivity incorporated into each nucleotide was measured by the Čerěnkov radiation emitted. The amount of radioactivity incorporated into each nucleotide was calculated as a percent of the total incorporation.

Table XVI shows the percent of radioactivity incorporated into each nucleotide for the 3'-terminals of products of the reaction of DNase II with different DNAs. The highest percent incorporation was found for guanine, and the lowest, for cytosine. Thymine and adenine were also labelled at fairly low levels. DNase II had a strong preference for guanine at the 3'-terminals of products of DNase II catalysed hydrolysis of DNA from several different sources. The patterns of incorporation observed for the different DNAs differed primarily in the percent incorporation for guanine. This may indicate different frequencies in the various DNAs of the preferred sequences containing guanine at the 3'-terminals of products released from calf thymus DNA by spleen DNase II (Thiery et al., 1973).

TABLE XVI

BASE SPECIFICITY AT THE 3' TERMINALS FOR CLEAVAGE OF DNA FROM DIFFERENT SOURCES BY DNase II

DNA Source	Extent of Degradation		% 3' Terminal Base Denatured Products
Calf thymus	13%	Guanine	49
		Thymine	22
		Adenine	23
		Cytosine	6
Salmon testes	13%	Guanine	58
		Thymine	17
		Adenine	15
		Cytosine	11
Rat intestine	9%	Guanine	72
		Thymine	10
		Ademine	14
		Cytosine	6
Escherichia coli	12%	Guanine	69
		Thymine	11
		Adenine	14
		Cytosine	6
Bacteriophage	17%	Guanine	79
		Thymine	7
		Adenine	6
		Cytosine	7

Discussion

The Michaelis constant, Km, for the hydrolysis by DNase II of ^{3}H-DNA from Escherichia coli was 2.42 x 10^{-7} M DNA-phosphate. This provides a basis for future experiments to test for the presence of a possible DNase II-specific inhibitor in crude and purified preparations of DNase II. A decreased DNase II activity in a crude preparation at a saturating concentration of DNA would indicate the presence of an inhibitor. It is unlikely that the highly purified DNase II preparation contains a DNase II-specific inhibitor because the sigmoidal kinetics characteristic of interaction of the inhibitor with DNase II (Lesca, 1976) were not observed. Also, although enzymes from different tissues are being compared, Lesca (1976) found that the beef liver DNase II-specific inhibitor was evident at a DNA concentration of 8.9 x 10^{-7} M DNA-phosphate.

That DNase II degrades double-stranded substrates such as native DNA and polydAT hairpin loops at a greater rate than single-stranded substrates such as native DNA, polydA and polydT is in accord with results reported for spleen DNase II (Bernardi, 1968).

DNase II degraded native DNA primarily by a double-strand cleavage mechanism in the presence and absence of 10 mM EDTA. Addition of 1 mM sodium sulfate to a DNase II assay solution containing 10 mM EDTA had little or no effect on the mode of cleavage of DNA. These results are in agreement with those of Bernardi and Sadron (1964), Young and Sinsheimer (1965), Bernardi and Bach (1968) and Kopecka et al. (1973) who found that DNase II degraded DNA primarily by a double-strand

cleavage mechanism.

When products of the reaction of DNase II with DNA were subjected to electrophoresis on polyacrylamide gels and visualized by fluorescence after interchelation with ethidium bromide, continuous fluoresecent patterns resulting from oligonucleotides ranging from less than 100 to more than 1,300 base pairs were observed. This indicated that DNase II cleaved a large number of different base sequences in DNA to yield a wide distribution of fragment sizes. The base specificity values together with the electrophoresis results indicated that intestinal DNase II cleaved a large number of different base sequences in DNA because the percentage values for the bases did not indicate a preference for one or two bases to the exclusion of the others. The most susceptible internucleotide linkage for intestinal DNase II was GpG and the most resistant was CpT. These results are in accord with those of Vanecko and Laskowski (1962) and Bernardi et al. (1973) for the cleavage of calf thymus DNA by spleen DNase II. In addition the most susceptible linkages for single-strand cleavages were probably GpC and GpG in the initial phase of the reaction and perhaps GpA with small single-stranded fragments in the later stages of the reaction. The finding that the base specificity at the 5'-terminal changed during the course of the reaction, especially in the initial and terminal phases of the reaction, is in accord with the results of Vanecko and Laskowski (1962) for the reaction of spleen DNase II with calf thymus DNA. Intestinal DNase II was not much more specific early in the reaction with DNA than it was at later stages of the reaction. Different patterns for percentages of terminal bases were observed in cleavage products of DNA from various species

likely because of the different frequencies with which sequences susceptible to cleavage by DNase II occurred in the DNAs. These results are in accord with those of Bernardi et al. (1973) who found similar patterns for the base specificities of cleavage of different mammalian DNAs by DNase II, but different patterns when the base specificities for DNase II cleavage of mammalian, yeast nuclear, and bacterial DNA were compared.

SUMMARY

1. DNase II activity was isolated from bovine small intestine by homogenization of the mucosa in an equal volume of Krebs Ringer phosphate buffer, pH 7.8, and centrifugation of the mixture first at 16,300xg, and then at 105,000xg. Buffer solutions containing DNase II were made 10 mM in ethylenediaminetetraacetate (EDTA) in order to stabilize the enzyme. Since addition of diisopropylflurophosphate, a protease inhibitor, also prevented a loss of DNase II activity, it is likely that the decline in DNase II activity observed in the absence of both reagents was due to proteolysis of the enzyme. When the 105,000xg supernatant solution was chromatographed on DEAE cellulose, two peaks of DNase II activity were eluted, a major peak with 20 mM phosphate buffer, pH 7.8, and a minor one with a 0-1M potassium chloride gradient in the same buffer. Similar results indicating the presence of two DNase II activities in several other tissues have been reported by previous investigators (Cordonnier and Bernardi, 1968; Yamanaka et al., 1974; Zöllner et al., 1974). The two DNase II activities seemed to have different properties. The major DNase II activity degraded native DNA more rapidly than denatured DNA, whereas the minor one digested both at the same rate. The activation energies for hydrolysis of native DNA by the two DNase IIs were different, although the activation energies for denaturation of the two enzyme molecules were similar. The two activities also differed in their response to increasing ionic strength, pH and sulfate ion concentration. Upon rechromatography on DEAE cellulose of the minor DNase II under

conditions in which the DNase II activity remained stable, most of the enzyme was eluted with 20mM phosphate buffer, pH 7.8, containing 10mM EDTA in the same position as that in which the major DNase II activity had been eluted. The major DNase II activity could be bound in low ionic strength buffer to DNA that was physically absorbed to a cellulose column, and could be eluted from the column along with fragments of DNA by a potassium chloride gradient in the same buffer. The minor DNase II preparation contained a substantial quantity of endogenous DNA and upon chromatography in Sephadex G100 split into several peaks of activity that were associated with species of molecular weight greater than or equal to that of the major DNase II. Thus it was concluded that the appearance of a minor DNase II activity was an artifact of the DEAE cellulose chromatography, likely due to the binding of a small amount of DNase II to DNA which was electrostatically bound to the positively charged DEAE groups of the column. Apparent differences in the properties of the two DNase II activities were probably due to the presence in the minor DNase II preparation of a substantial amount of endogenous DNA which interfered in the DNase II reactions.

2. Intestinal DNase II was partially purified by ion exchange chromatography and gel filtration. A 105,000xg supernatant prepared from bovine intestinal mucosa was applied to DEAE cellulose and the DNase II activity was eluted with 20mM phosphate buffer, pH 7.8, containing 10mM EDTA. The pooled DNase II activity from the DEAE cellulose column was bound to CM cellulose in 10mM phosphate buffer, pH 6.0, containing 10mM EDTA and later eluted with a 0-1M potassium chloride

gradient in the same buffer. The enzyme was then subjected to gel filtration on Sephadex G100 in 0.25M TrisHCl, pH 7.5, containing 10mM EDTA. Electrophoresis of the DNase II preparation from the Sephadex column on polyacrylamide gels revealed that only a few other protein bands were present. The only nuclease contaminants present in significant amounts were acid and alkaline RNase, which do not degrade DNA, and phosphodiesterase II, an exonuclease. The amounts of these enzymes relative to DNase II were reduced substantially by a repetition of the purification procedure using the partially purified DNase II preparation from the Sephadex column as the starting material. The enzyme was purified 78 fold with a yield of 13% compared to the 16,300xg supernatant.

3. The enzyme isolated from intestinal mucosa was a DNase II according to the criteria of Maver and Greco (1948) and the international recommendations on enzyme nomenclature (1972) because it hydrolysed DNA to oligonucleotides with 3'-phosphate and 5'-hydroxyl terminals endonucleolytically at acid pH and showed no requirement for a divalent metal ion. DNase II had a broad pH optimum centred at pH 4.8, and was inhibited by magnesium ions, high ionic strength and sulfate ions. The activation energy for hydrolysis of native calf thymus DNA was 19 Kcal/mole. The activation energy for denaturation of the DNase II molecule was 43 Kcal/mole. The molecular weight of DNase II was estimated to be 41,000 by gel filtration on Sephadex G100.

4. The reaction of DNase II with DNA and other subtrates was studied.

The Michaelis constants for the reactions of DNA with sonicated ^{3}H-DNA from Escherichia coli and polydAT-methyl-^{3}H were 2.42 x 10^{-7} M DNA-phosphate and 2.63 x 10^{-7}M polydAT-phosphate, respectively.

DNase II degraded native calf thymus DNA 6.4 times faster than denatured calf thymus DNA. The rate of reaction of DNase II with polydAT was at least 10 times that with polydA or polydT, probably because of the presence of double-stranded "hairpin loops" in the polydAT (Scheffer et al., 1968).

DNase II can cleave one or both strands of native DNA. Double-strand cleavage released "external" 5'-hydroxyl groups; single-strand cleavage resulted in "nicks" in which access to the "internal" 5'-hydroxyl groups formed was restricted by the surrounding DNA. Upon denaturation of the reaction products, both external and internal 5'-hydroxyls became equally accessible. Under specific conditions polynucleotide kinase catalyses the incorporation of ^{32}P from [γ-^{3}P] ATP into external 5'-hydroxyl groups only. DNase II was reacted with native DNA. ^{32}P incorporation into the native DNase II digestion products was into the external 5'-hydroxyls produced by the double-strand cleavage mechanism, whereas ^{32}P incorporation into the denatured DNase II digestion products was into the external plus the internal 5'-hydroxyl groups resulting from double-strand plus single-strand cleavages. Intestinal DNase II degraded native DNA from various sources, at different stages in the reaction and under different reaction conditions, primarily by a double-strand cleavage mechanism.

5. Products of the reaction of DNase II with DNA were subjected to electrophoresis on polyacrylamide gels and visualized by

fluorescence after interchelation with ethidium bromide. Early in the reaction continuous fluorescent patterns resulting from oligonucleotides ranging from less than 100 to more than 1,300 base pairs in length were observed. This indicated that DNase II had cleaved a large number of different base sequences in the DNA to yield a wide distribution of fragment sizes.

6. The base specificity of cleavages of DNA by DNase II was investigated using radioactive labelling techniques.

The 5'-hydroxyl terminals of DNase II digestion products were labelled with ^{32}P using polynucleotide kinase and [$\gamma^{32}P$] ATP. The 5'-^{32}P oligonucleotides were degraded to 5'-^{32}P nucleotides. The amount of ^{32}P incorporated into each nucleotide as a percentage of the total ^{32}P incorporation indicated the percent of the corresponding base at the 5'-terminals. Guanine was present in the highest percentage; thymine, in the lowest. Adenine and cytosine had intermediate values. The percentage for each base changed during the reaction, especially in the initial and terminal phases. Although the order of the bases was similar with guanine present in the highest percent, and thymine in the lowest, different patterns for percentages of terminal bases were observed for the cleavage by DNase II of DNA from various species. This indicated that sequences susceptible to cleavage by DNase II were present in different frequencies in the various DNAs.

The 3'-phosphate terminals of DNase II digestion products were dephosphorylated and then labelled with ^{32}P-riboadenylic acid using terminal deoxynucleotide transferase and [α-^{32}P] ATP. The 3'-^{32}P oligonucleotides were degraded to 3'-^{32}P nucleotides. The amount

of ^{32}P incorporated into each nucleotide as a percent of the total ^{32}P incorporated indicated the percent of the corresponding base at the 3'-terminals. Guanine was present at the 3'-terminals in the highest percentage; cytosine, in the lowest. Thymine and adenine were also present at low levels. DNase II had a strong preference for guanine at the 3'-terminals of products of the DNase II catalysed hydrolysis of DNA from several sources. The percent of guanine at the 3'-terminals varied with DNA from different species. This indicated that the preferred sequences containing guanine at their 3'-terminals occurred with different frequencies in the various DNAs.

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