Manning, J. E., Hershey, N. A., Broker, T. R., Pellegrini, M., Mitchell, H. K., and Davidson, N. (1975a), *Chromosoma 53,* 197.
Manning, J. E., Schmid, C. W., and Davidson, N. (1975b), *Cell 4,* 141.
Miller, W. L., Brenner, D. J., and Doctor, B. P. (1974), *Biochim. Biophys. Acta 366,* 188.
Orosz, J. M., and Wetmur, J. G. (1974), *Biochemistry 13,* 5467.
Pellegrini, M., Manning, J., and Davidson, N. (1977), *Cell* (in press).
Ritossa, F. M., Atwood, K. C., Lindsley, D. L., and Spiegelman, S. (1965), *Natl. Cancer Inst. Monogr. 25,* 449.
Ritossa, F. M., Atwood, K. C., and Spiegelman, S. (1966), *Genetics 54,* 663.
Serwer, P. (1975), *J. Mol. Biol. 92,* 433.
Sharp, P. A., Hsu, M., Ohtsubo, E., and Davidson, N. (1970), *J. Mol. Biol. 71,* 471.
Shih, T. Y., and Martin, M. A. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1976.
Tartof, K. D., and Perry, R. P. (1970), *J. Mol. Biol. 51,* 171.
Thomas, M., White, R. L., and Davis, R. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 2294.
Wallace, H., and Birnstiel, M. L. (1966), *Biochim. Biophys. Acta 114,* 296.
Wellauer, P. K., and Dawid, I. B. (1977), *Cell* (in press).
White, R. L., and Hogness, D. S. (1977), *Cell* (in press).

An Endonuclease from Calf Liver Specific for Apurinic Sites in DNA†

J. Philip Kuebler and David A. Goldthwait*

ABSTRACT: An endonuclease specific for apurinic sites in double-stranded DNA has been partially purified from calf liver extracts. The enzyme has a pH optimum of 9.5, is only slightly stimulated by low concentrations of Mg^{2+}, and has a molecular weight of 28 000. Inhibitors of the endonuclease include Ca^{2+}, EDTA, *p*-HOHgBzO, NaCl, and tRNA. The enzyme introduces single- and double-stranded breaks in depurinated DNA. High concentrations of the enzyme preparation degrade untreated single-stranded DNA, but not ultraviolet (UV) irradiated DNA or DNA treated with methylmethanesulfonate or 7-bromomethyl-12-methylbenz[*a*]anthracene. Enzymatic incisions produce 3′-hydroxyl and 5′-phosphate end groups. Some of the properties of the calf liver apurinic endonuclease differ from those of a similar endonuclease obtained from calf thymus by S. Ljungquist and T. Lindahl [(1974), *J. Biol. Chem. 249,* 1530] and in this laboratory. The data suggest that these are isozymes.

Deoxyribonucleic acid in cells may undergo depurination damage by various pathways. Base release by either chemical or enzymatic reactions can occur after the bases are altered by specific alkylating agents or γ irradiation. Lindahl and Nyberg (1972) demonstrated the slow spontaneous hydrolysis of purines under physiological conditions and calculated that up to 3% of the total purines could be lost during the lifetime of cells such as human neurones if there was no replacement. An endonuclease active only at depurinated sites was isolated from *Escherichia coli* by Paquette et al. (1972), while a preparation active on depurinated sites and alkylated DNA (Hadi and Goldthwait, 1971) has now been resolved into an enzyme active on depurinated sites and one active on alkylated DNA (endonuclease II, Kirtikar et al., 1976a). Evidence for the existence in mammalian tissues of endonucleases capable of recognizing depurinated sites was obtained by Verly and Paquette (1973). Ljungquist and Lindahl (1974) purified an endonuclease 830-fold from calf thymus and reported many of its properties. Subsequently, an enzyme active on apurinic sites in DNA was demonstrated in a number of human cell lines (Teebor and Duker, 1975) and an altered specific endonuclease for depurinated sites has been found in extracts of two cell lines from xeroderma pigmentosum patients (Kuhnlein et al., 1976). This paper reports the isolation and partial purification from calf liver of an endonuclease active on depurinated DNA which possesses some properties that differ from those observed with the enzyme obtained from calf thymus. The possibility of apurinic endonuclease isozymes is discussed.

Materials and Methods

Reagents. [^{3}H]Thymidine was obtained from the New England Nuclear Corp., Boston, Mass. Sodium borohydride (98% pure) was purchased from the Fisher Scientific Co., Fairlawn, N.J. Dithiothreitol and 2-mercaptoethanol were both from Sigma Chemical Co., St. Louis, Mo. Toluenesulfonyl fluoride, 97%, was obtained from Aldrich Chemical Corp., Milwaukee, Wis. 1,3-Bis[tris(hydroxymethyl)methylamino]propane (BTP buffer),[1] A grade (pK_{a_1} = 9.0, pK_{a_2} = 6.8), was purchased from Calbiochem, Los Angeles, Calif. DNase I was also obtained from this source. Bovine spleen and snake venom phosphodiesterases were obtained from the Worthington Co.

DNA. The procedure for preparing [^{3}H]thymine-labeled *Bacillus subtilis* or *Escherichia coli* DNA of Smith (1967) was employed. Specific activity of the DNA was approximately

† From the Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106. *Received October 13, 1976.* This work was supported by a grant from the National Institutes of Health (CA 11322) and a contract with ERDA (11-1) 2725. D.A.G. was supported by National Institutes of Health Research Career Award No. KO-6-GM-21444.

[1] Abbreviations used are: BTP buffer, 1,3-bis[tris(hydroxymethyl)-methylamino]propane-hydrochloric acid buffer; EDTA, ethylenediaminetetraacetic acid, sodium salt; *p*-HOHgBzO, *p*-hydroxymercuribenzoate; AP, alkaline phosphatase.

10^3 cpm/nmol. ^{3}H-Labeled T4 phage and DNA were prepared as described previously (Melgar and Goldthwait, 1968). ^{3}H-Labeled DNA from T7 bacteriophage was isolated from phage grown on *Escherichia coli* B3, Thy$^-$, in the presence of [^{3}H]thymidine (Richardson, 1965). ^{3}H-Labeled T7 DNA was prepared by the procedure of Summers and Szybalski (1968). The specific activity of the labeled T4 DNA ranged from 840 to 10 000 cpm/nmol of nucleotide, while that of the T7-DNA was 500 cpm/nmol. Salmon sperm DNA was obtained from Sigma Chemical Co., as was *E. coli* tRNA. ^{3}H-Labeled rat liver and *E. coli* rRNA were obtained from Dr. D. D. Anthony.

Endonuclease Assays. DNA in Polyacrylamide Gel. The use of T4 DNA entrapped in acrylamide gel as a substrate for the assay of various endonucleases has been described (Friedberg et al., 1969; Melgar and Goldthwait, 1968). Native T4 DNA gel, prepared according to the method of Melgar and Goldthwait (1968), was depurinated by first suspending 1 vol of the packed DNA gel in 9 vol of 0.1 M sodium citrate (pH 3.5) containing 10^{-3} M EDTA. The suspension was heated for 30 min at 50 °C in a water bath, after which it was rapidly cooled and the pH was adjusted to 6.5 by the addition of 2 N NaOH. The pH was stabilized by the addition of $\frac{1}{3}$ vol of 2 M potassium phosphate buffer (pH 6.5). Sodium borohydride, 5 M, was then added to a final concentration of 0.25 M in three aliquots at 15-min intervals and the solution was allowed to stand an additional hour at room temperature. Finally, the DNA gel was washed repeatedly in 0.05 M BTP buffer (pH 9.5) and stored at 4 °C. Loss of DNA from the gel before reaction with enzyme amounted to less than 15%.

For assay of endonuclease activity, DNA-gel suspensions containing 5 to 15 nmol of DNA-phosphorus were added to incubation mixtures (1.5 mL) with a final concentration of 0.05 M BTP buffer (pH 9.5), 10^{-4} M dithiothreitol, 10^{-4} M 8-hydroxyquinoline (unless Mg was added), and 1 mg/mL of bovine serum albumin. Incubations with and without enzyme were for 30 min at 37 °C, and the reactions were stopped by the addition of 0.1 mL of 1% sodium dodecyl sulfate. After centrifugation, 1-mL aliquots of the supernatant were counted in a scintillation counter. One unit of activity for the calf liver endonuclease is that amount of enzyme which causes the release of 1 μmol of DNA nucleotide from the gel in 1 h. This DNA-gel is an excellent substrate for apurinic endonuclease purified from *E. coli* to homogeneity (Kirtikar et al., 1976a).

Initially, the enzyme was assayed on heavily alkylated DNA-gel. Preparations containing about 60 nmol of DNA-phosphorus per mL of packed gel were treated with methylmethanesulfonate at a ratio of methylmethanesulfonate:DNA nucleotide of greater than 6000:1. This degree of alkylation has been shown to produce a number of depurinated sites in the DNA which increase with time as the gel is stored.

DNA in Solution. Assays by sedimentation velocity were done with depurinated, depurinated-reduced, γ irradiated and UV irradiated, alkylated, and single-stranded DNA. ^{3}H-Labeled T4 DNA was depurinated at 37 °C in sodium citrate buffer at pH 3.5 and then reduced with sodium borohydride as noted above (Hadi and Goldthwait, 1971). An experiment was done to determine the number of alkali-labile sites produced by the incubation at pH 3.5 as a function of time (Figure 1). A sigmoid curve was obtained which will be discussed below. Reduction of the depurinated DNA with sodium borohydride stabilized most of the alkali-labile sites (Figure 1).

The preparation of γ-irradiated T7-DNA was as described by Kirtikar et al. (1975) and the details appear in the table

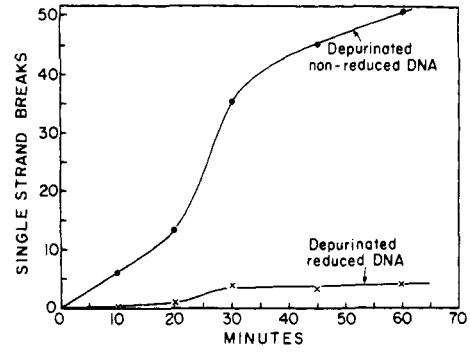

FIGURE 1: The number of depurinated sites as a function of the time of incubation at pH 3.5. Six 135-nmol samples of ^{3}H-labeled T4 DNA were depurinated under the conditions described under Materials and Methods. At the indicated times, a sample was removed from the 37 °C water bath and placed on ice, and the pH brought to 6.5 with 2 N NaOH. The pH was stabilized with potassium phosphate buffer and half of each sample was reduced with $NaBH_4$. The reduced and nonreduced portions were dialyzed overnight against 0.05 M BTP buffer (pH 9.5) and aliquots of each were added to the ingredients of the standard reaction mixture. After incubation, the samples were treated with 0.06 N NaOH for 20 min and then centrifuged through 5–20% alkaline sucrose gradients as described to determine single-strand breaks: (●) depurinated-nonreduced DNA; (×) depurinated-reduced DNA.

legends. Ultraviolet irradiation of T4 DNA was done in a sterile glass petri dish using a 15-W General Electric germicidal lamp at a fluence of 1 erg mm^{-2} s^{-1}. The dose rate was calibrated with a Fisher short-wave UV meter. The ^{3}H-labeled T4 DNA was irradiated in 0.05 M Tris-HCl (pH 8.0) at a final concentration of 5 μg/mL. The number of thymine dimers was determined by a modification (Shlaes et al., 1972) of the technique of Carrier and Setlow (1971) which involved two-dimensional paper chromatography. Approximately 0.06% of the thymine moieties in the DNA were converted to dimers or about 60 sites per T4 genome. After irradiation, the DNA was mixed 1:12 by weight with cold carrier T4 DNA, precipitated with ethanol, dried, and then dissolved in 0.05 M BTP buffer (pH 9.5). [^{3}H]Dimethyl sulfate treated T4 DNA and 7,12-dimethylbenz[*a*]anthracene treated ^{3}H-labeled T7 DNA were both gifts from Dr. Dollie Kirtikar. Calf-thymus DNA reacted with 7-[^{3}H]bromomethyl-12-methylbenz[*a*]anthracene was a gift from Dr. Anthony Dipple.

Single-stranded DNA was prepared by incubation of from 100 to 200 nmol of ^{3}H-labeled T4 or T7 DNA in a final concentration of 0.15 M NaOH for 10 min at 25 °C. The solution was partially neutralized by the addition of $\frac{1}{11}$ vol of 1.1 N HCl to which was added a final concentration of 0.2 M Tris. The final pH of the mixture was 8.5. The DNA was then dialyzed overnight against 1 L of 0.05 M BTP buffer (pH 9.5) and used the next day.

For sedimentation velocity experiments, incubation mixtures contained in 0.25 mL 5–50 nmol of ^{3}H-labeled T4 or T7 DNA nucleotide, 1×10^{-4} M β-mercaptoethanol, 1×10^{-4} M 8-hydroxyquinoline, 0.01 M Tris-HCl (pH 8.0), and 0.035 M BTP buffer (pH 9.5) plus enzyme as stated. The final pH of the reaction mixture was 9.3. After 60 min at 37 °C, the enzyme reaction was terminated by the addition of sodium dodecyl sulfate and sodium ethylenediaminetetraacetate (NaEDTA) at final concentrations of 0.33% and 1.3×10^{-2} M, respectively. Sodium hydroxide was added to the DNA samples to a final concentration of 0.06 N and the samples were held at 37 °C for an additional 20 min in order to break phosphodiester bonds at depurinated sites in the DNA (Kirtikar et al., 1975). Aliquots (0.25 mL) were centrifuged

TABLE I: Partial Purification of Calf-Liver Apurinic Endonuclease.[a]

Fraction	Total Act.	Sp Act.	Purification
II. 40–80% ammonium sulfate	68	0.002	0
III. phosphocellulose	329	0.23	123
IV. DEAE-cellulose	282	0.29	152
V. DNA–agarose	228	1.12	590
VI. Concentration by vacuum dialysis	71	0.83	435
VII. Bio-Gel A-1.5m	33	0.89	460
VIII. Concentration by DNA–agarose and vacuum dialysis	18	1.38	727

[a] Assays were done as described under Materials and Methods. Enzyme units were calculated from the linear portion of a plot of activity vs. protein concentration. Values obtained for the 40–80% $(NH_4)_2SO_4$ fraction are only approximate. The crude extract was not assayed due to interference from contaminating nucleases and heme pigments (see text). The degree of purification is subject to qualifications noted in the text.

through 5–20% alkaline sucrose gradients, fractionated, and counted and the number average molecular weight and single-strand breaks were then calculated using a computer program. Molecular weights for single-stranded T4 and T7 DNA were 52.5×10^6 (Smith, 1967) and 12.5×10^6 (Freifelder, 1970), respectively.

Other Enzyme Assays. The exonuclease assay, in which single- or double-stranded T4, T7, *Bacillus subtilis,* or *E. coli* DNA was used, has been described (Friedberg and Goldthwait, 1969). For the alkaline phosphatase assay, 0.001 M *p*-nitrophenyl phosphate in 1.0 M Tris-HCl (pH 8.0) was used. To 1.0 mL in a cuvette, 0.01 mL of the enzyme dilution was added and the optical density at 410 nm was followed as a function of time on a Zeiss spectrophotometer. Details for the assays involving the bovine spleen and snake venom phosphodiesterases are given in the appropriate figure legends.

Isolation of a Calf-Thymus Apurinic Endonuclease. The procedure for the partial purification of the apurinic acid endonuclease from calf thymus was modified from that reported by Ljungquist and Lindahl (1974). It was as follows: fresh calf-thymus glands, obtained from a local slaughterhouse, were freed of fat and connective tissue. Four-tenths kilogram of tissue was washed in buffer containing 0.05 M Tris-HCl (pH 8.0), 10^{-3} M EDTA, and 3 mM toluenesulfonyl fluoride, cut into small pieces, and homogenized in portions in a Waring blender for 1 min at high speed and 4 min at low speed with an equal amount of the buffer noted above. The mixture was sonicated for a total of 4 min at 100 W with a Branson sonifier. Cellular debris was removed by centrifugation at 50 000*g* for 2 h at 4 °C. An aliquot of the supernatant fraction was saved and later assayed for activity on the various gel substrates. Half of the sonicated sample was applied to a 2 × 32.5 cm phosphocellulose column previously equilibrated in 0.02 M potassium phosphate (pH 6.5), containing 10% glycerol and 10^{-4} M dithiothreitol. After washing the column with 0.5 L of this buffer, a linear NaCl gradient (2 × 400 mL) between 0 and 0.4 M in the same buffer was applied. Assay on depurinated-reduced DNA–gel revealed that the enzyme which acted on apurinic acid sites began to elute from the column soon after the gradient was started, and its peak was observed at 0.26 M NaCl. Activity on gel containing native DNA or DNA treated with methylmethanesulfonate at a methylmethanesulfonate to nucleotide ratio of 100:1 was absent both in the crude preparation and throughout the entire column. The DNA treated with this ratio and examined immediately has been shown by two different methods to contain no observable depurinated sites. This DNA is an excellent substrate for a homogeneous preparation of endonuclease II (Kirtikar et al., 1976a).

The symmetrical peak of apurinic acid endonuclease activity from the phosphocellulose column was pooled, dialyzed against 0.05 M Tris-HCl (pH 8), containing 10^{-4} M dithiothreitol and 10% glycerol, concentrated by vacuum dialysis, and stored at −15 °C.

Electrophoresis, Miscellaneous. Sodium dodecyl sulfate gel electrophoresis was performed according to the method of Weber and Osborn (1969) and non-sodium dodecyl sulfate gel electrophoresis by the procedure of Davis (1964). The gels were sliced into 2-mm sections with a gel slicer and incubated overnight in 0.05 M Tris-HCl (pH 8.0) with 10^{-4} M dithiothreitol and 10% glycerol to elute the enzyme from the gel. Aliquots were then assayed with a gel containing depurinated-reduced DNA under standard conditions. Protein concentrations were determined by direct optical density measurements at 260 and 280 nm during chromatography or by the method of Lowry et al. (1951).

Results

A summary of the purification procedure is given in Table I. All operations were at 4 °C. Calf liver, 1.4 kg, was obtained from a local slaughterhouse and chilled immediately in ice. The tissue was cut into pieces and washed in 0.3 M sucrose, buffered with 0.05 M Tris-HCl (pH 8.0). It was then ground in a meat grinder and mixed in the proportion 1:1.5 with the sucrose buffer containing 3×10^{-3} M toluenesulfonyl fluoride and 10^{-3} M EDTA. The liver mixture was then homogenized in portions by stirring for 1 min at low speed and for 15 s at high speed in a Waring blender, after which cellular debris was removed by centrifugation (fraction I).

To the supernatant (1500 mL), 364 g of $(NH_4)_2SO_4$ was added and slowly dissolved by stirring to achieve 40% saturation. After an additional 30-min period of slow stirring, the precipitate was removed by centrifugation and discarded. To the supernatant solution (1400 mL), an additional 394 g of $(NH_4)_2SO_4$ was added, to 80% saturation. After centrifugation, the precipitate was suspended in 365 mL of buffer A which was 0.04 M potassium phosphate (pH 6.5), containing 10^{-4} toluenesulfonyl fluoride, 10^{-3} M dithiothreitol, and 10% glycerol (fraction II). Approximately half of fraction II was applied to a phosphocellulose column (6.6 cm diameter × 15 cm), equilibrated with the same buffer. The column was washed first with 0.05 M NaCl dissolved in buffer A and then with this same buffer containing 0.5 M NaCl. Determination of enzyme activity using the gel assay revealed that most of the activity on heavily alkylated DNA was eluted at the higher salt concentration (fraction III). Very little activity on native DNA was detected throughout the enzyme peak.

Fraction III was dialyzed against 0.05 M Tris-HCl (pH 8.0), containing 10^{-3} M dithiothreitol, and 10% glycerol (buffer B), and then applied to a column of DEAE-cellulose (2.5 cm diameter × 11 cm) equilibrated with the same buffer. The enzyme washed through the column without binding to the DEAE-cellulose. Since a significant amount of protein which contained most of the activity on native DNA remained on the column, this step provided a reproducible purification of the enzyme. The fractions active on depurinated-reduced DNA which did not adhere to the column were pooled (fraction IV). Fraction IV was concentrated by affinity chromatography

employing a small column (4.75 cm diameter × 5 cm) of single-stranded DNA–agarose. The enzyme bound to the column and was removed with buffer B containing 0.25 M NaCl. The salt was removed from the pooled fractions by dialysis, and the sample was further concentrated to 7.25 mL by vacuum dialysis (fraction V).

Fraction V was then applied to a column of Bio-Gel A-1.5m (5 cm diameter × 122 cm), equilibrated with buffer B. The column was washed with this same buffer and the fractions were assayed for activity. The most active fractions were pooled (fractions VI) and immediately applied to a column containing single-stranded DNA–agarose (4.75 cm diameter × 5 cm) equilibrated in buffer B. The enzyme was eluted with 0.25 M NaCl (fraction VII), dialyzed overnight against buffer B, and then concentrated by vacuum dialysis against 0.05 M Tris-HCl (pH 8.0) containing 10^{-3} M β-mercaptoethanol and 20% glycerol. The final enzyme, 1.38 units/mg and 2.5 units/mL, was frozen in several small aliquots.

When stored at −20 °C, activity was retained unchanged for 6 months. Repeated thawing and refreezing caused small losses of activity. If the protease inhibitor toluenesulfonyl fluoride was excluded from the initial homogenization buffer, recovery of enzyme was markedly reduced. The crude extract, fraction I, could not be assayed accurately for enzyme activity, as the heme pigments present in liver interfered with the determination of radioactive DNA released in the gel assay. In addition, the prevalence of enzymes active on native DNA in this early fraction obscured the activity associated with apurinic endonucleases. Table I shows that an inhibitor of enzyme activity present in the ammonium sulfate fraction was also removed during purification. This resulted in a higher yield of total apurinic endonuclease activity in subsequent steps. Because of the difficulty in determining the activity in the starting material and because of the probable removal of an inhibitor, it is not possible to assign an exact figure for purification. Sodium dodecyl sulfate gel electrophoresis of the endonuclease preparation revealed several bands. Non-sodium dodecyl sulfate gel electrophoresis using 7.5% gels was also done, and the gel was sliced into 2-mm sections. Enzyme activity was found very close to the top of the gel. This same behavior was observed with 5% acrylamide gels, and, together with the absence of binding to DEAE-cellulose, implied that the enzyme may have very little negative charge. On further attempts at purification, using Sephadex or phosphocellulose, enzyme activity was separated from most of the protein in fraction VIII, and the pooled fractions contained only one band on sodium dodecyl sulfate electrophoresis. However, attempts to concentrate the more purified preparation resulted in almost total loss of activity. This loss after the purification step suggests instability rather than the separation of two enzyme activities.

Properties of the Enzyme. A pH optimum at 9.5 was demonstrated using BTP buffer. The activity of the enzyme increased from pH 7.5 through 9.0 when Tris-HCl was used as the reaction buffer. In addition, activity began to decrease at concentrations of BTP buffer above 0.05 M; at 0.1 M approximately 80% of the maximal activity was observed. In subsequent experiments, 0.05 M BTP buffer at pH 9.5 was used. Enzyme activity was only slightly stimulated by low concentrations of Mg^{2+} or Mn^{2+} (optimal at 0.01 mM), in the reaction mixture, while Ca^{2+} inhibited the enzyme at concentrations above 0.002 mM. The endonuclease was inhibited 50% by 3×10^{-6} M EDTA and also 50% by 4.6×10^{-6} M *p*-hydroxymercuribenzoate (*p*-HOHgBzO) as shown in Table II. Reactivation by Mg^{2+} or dithiothreitol, respectively, was observed. The enzyme reaction was sensitive to ionic strength; 50% inhibition was observed with 0.025 M NaCl. Although caffeine had no effect on the enzyme activity, *E. coli* tRNA was observed to inhibit the endonuclease 50% at a concentration of 0.2 mg/mL either in the presence or absence of 0.01 mM Mg^{2+}. This effect was reversible since no inhibition of activity was observed when the enzyme was preincubated with 0.2 mg/mL of tRNA which was then diluted to a final concentration of 0.007 mg/mL and incubated in the standard reaction mixture. The enzyme was also heat labile; 75% of the activity was lost after heating at 45 °C for 10 min in 0.05 M Tris-HCl (pH 8.0), 10^{-3} M β-mercaptoethanol, and 20% glycerol.

TABLE II: Inhibition of Enzyme Activity by *p*-Hydroxymercuribenzoate, EDTA, NaCl, and tRNA.[a]

Inhibitor	Concn Yielding 90% Inhibition (M)	Concn Yielding 50% Inhibition (M)
p-HOHgBzO	3.2×10^{-5}	4.6×10^{-6}
EDTA	1.9×10^{-5}	3.0×10^{-6}
NaCl	1.9×10^{-1}	2.3×10^{-2}
tRNA	1.3×10^{-4}	5.0×10^{-6}

[a] All of the values were taken from inhibition vs. concentration curves for each compound. Assays were performed with the gel assay as described in Materials and Methods, except that 8-hydroxyquinoline was not used. Bovine serum albumin and dithiothreitol were left out of the assays when *p*-hydroxymercuribenzoate (*p*-HOHgBzO) was tested and $MgCl_2$ (0.01 mM) was included. *p*-HOHgBzO was preincubated with the enzyme for 10 min prior to the start of the reaction. Bovine serum albumin and dithiothreitol were used when the other inhibitors were assayed. Magnesium was not added to the tubes containing EDTA, but was used when NaCl was tested. The inhibition by tRNA was studied both with and without Mg^{2+}. No significant difference was observed and the results given here were obtained without Mg^{2+}. The amount of enzyme used was 0.003 unit.

Gel filtration through Sephadex G-100 revealed that the endonuclease had a mol wt of 28 000.

Substrate Specificity. To determine the specificity of the enzyme, various types of substrate DNAs were treated with varying amounts of the enzyme preparation, and the products were subjected to centrifugation through alkaline sucrose gradients. The number of breaks introduced into the DNA was then calculated. On incubation of depurinated–reduced T4 DNA with the calf-liver endonuclease, single-strand cleavage was observed at 2.8×10^{-4} units of enzyme (Figure 2), a concentration approximately 1000-fold less than that needed to make breaks in native DNA. In this experiment, 0.14 unit of enzyme was observed to cleave about 68% of the total number of depurinated sites. Higher concentrations of enzyme made only a few breaks in native T4 DNA and even less in native T7 DNA. The linear relationship between the log of the enzyme concentration and activity observed in Figure 2 may reflect limiting substrate conditions for the apurinic enzyme, as there were only about 61 depurinated sites present per single strand of T4 DNA.

The endonuclease also degraded single-stranded DNA, but this activity was not observed until concentrations of enzyme exceeding 6×10^{-2} units were added, approximately 500-fold greater than that needed for depurinated–reduced DNA (Figure 2). Above this concentration, the number of single-stranded breaks introduced into the DNA increased rapidly and reached a plateau at approximately 2×10^{-1} units. Addition of large amounts of enzyme to DNA, single stranded by

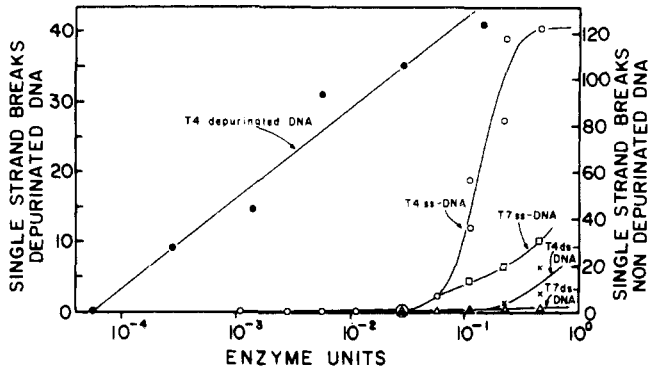

FIGURE 2: Activity of the calf-liver preparation on various DNA substrates. Sedimentation velocity analysis was done on various ^{3}H-labeled DNA substrates before and after reaction with varying concentrations of enzyme as described under Materials and Methods under endonuclease assays, DNA in solution. The depurinated T4 DNA which was not reduced contained 61 alkali-labile sites. Single-strand T4 and T7 DNA were obtained by treatment with alkali: (●) depurinated-reduced T4 DNA; (○) single-stranded T4 DNA; (□) single-stranded T7 DNA; (×) double-stranded T4 DNA; (△) double-stranded T7 DNA.

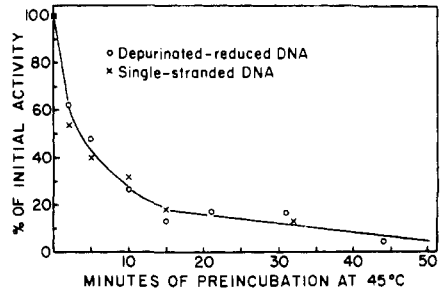

FIGURE 3: Heat inactivation of enzyme activity with depurinated-reduced DNA and with single-stranded DNA. The enzyme was preincubated for varying times at 45 °C in 0.05 M Tris-HCl (pH 8.0), containing 20% glycerol and 10^{-3} M dithiothreitol. Incubations were under standard conditions with equal amounts of depurinated-reduced or single-stranded DNA in the gel. The final concentration of enzyme in assays on depurinated-reduced DNA was 0.0018 unit; that on single-stranded DNA was 0.14 unit: (○) depurinated-reduced DNA; (×) single-stranded DNA.

either heat treatment or alkali incubation, resulted in each case in a limit digest with molecules of approximately 11 S.

To determine whether the activity on depurinated-reduced DNA and that on single-stranded DNA were due to the same enzyme, heat inactivation studies were done employing the gel assay. After preincubation of the enzyme at 45 °C in buffer containing 0.05 M Tris-HCl (pH 8.0), 10^{-4} M dithiothreitol, and 20% glycerol, for varying periods of time, aliquots were incubated with gel in which either single-stranded or depurinated-reduced double-stranded DNA was entrapped. Figure 3 shows that the decrease in activity on these two substrates was identical. Furthermore, it was noted that tRNA inhibited the degradation of single-stranded DNA to the same degree as the breakdown of depurinated-reduced DNA. For example, a tRNA concentration of 5 mg/mL inhibited enzyme activity by 90% on gels which contained depurinated-reduced DNA, and inhibited activity on single-stranded DNA by 93%. This evidence supports the hypotheses that the degradation of single-stranded DNA noted at high concentrations of enzyme is due to the same protein molecule responsible for the introduction of breaks in depurinated DNA. It was not possible to measure the ratio of these activities in fractions eluted from a column because of the requirement for large amounts of enzyme for activity on single-stranded DNA.

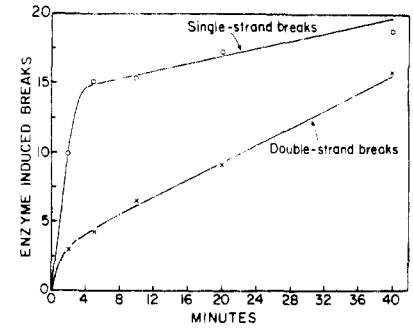

FIGURE 4: Production of single- and double-strand breaks in depurinated-reduced DNA by the calf-liver enzyme. ^{3}H-Labeled T4 DNA was depurinated and reduced as described. Standard reaction mixtures were set up with and without 0.12 unit of enzyme so that aliquots could be removed at the indicated times for centrifugation. Single-strand breaks were determined on alkaline sucrose gradients and double-strand breaks on neutral sucrose gradients. An aliquot of depurinated DNA which was not reduced had 21 alkali labile sites per single strand: (○) single-strand breaks; (×) double-strand breaks.

Since double-stranded DNA is normally used in making the depurinated-reduced DNA gel, it was assumed that the apurinic endonuclease in order to release DNA into the supernatant was introducing some double-stranded breaks into the substrate. This was confirmed by following with time the production of either single-strand breaks in alkaline or double-strand breaks in neutral sucrose gradients. Figure 4 shows that the enzyme produced in 5 min 15 single-strand breaks in each strand of a DNA which had 21 depurinated-reduced sites per strand. At 5 min, only about one-third of the breaks were due to double-strand breaks. After 5–6 min, the rate of single-strand breakage decreased to about half the rate of formation of double-strand breaks. This result suggests that few if any double-strand breaks occur as single events. The data are consistent with the hypothesis that the enzyme must make sequential single-strand breaks which then result in double-strand breaks. The second single-strand break could occur directly across from or within about eight nucleotides of a break previously made at a depurinated site.

Two types of sites are possible which the endonuclease might recognize in order to make the second break across from or near a cleaved apurinic sugar residue. First, it is possible that the unaltered DNA strand opposite a cleaved depurinated site is the substrate and is attacked by the endonuclease. To ascertain whether the endonuclease can cleave native DNA opposite a single-strand break, T4 DNA was treated with DNase I to produce a small number of single-strand breaks; in this case 4 were introduced into each strand of the DNA. The apurinic acid endonuclease was unable to introduce any additional breaks into this DNA. However, this substrate is not quite the same as one with a nick at a depurinated site. Second, it is possible that the depurinated sites occur in clusters on both strands and that cleavage of depurinated sites on both strands but in close proximity results in double-stranded breaks. Although a nonrandom occurrence of alkali-sensitive sites has not been reported by other investigators, a sigmoid curve has been observed in this laboratory for the formation of depurinated sites as a function of time (Figure 1). One explanation for this curve could be that there is cooperativity in the formation of depurinated sites and therefore they would not be random. With the apurinic acid endonuclease from *E. coli* we have also observed double-strand breaks. The precise reason for the double-strand breaks has not been established, and explanations other than those above are possible.

TABLE III: Activity of Calf-Liver Endonuclease on γ-Irradiated T7 DNA.[a]

Expt	DNA	Enzyme Source	Enzyme Units	Enzyme-Induced Breaks
1	Reduced	Calf liver	0.03	3
	Reduced	Calf liver	0.06	5
	Reduced	Calf liver	0.11	6
	Reduced	Calf liver	0.22	13
	Reduced	Calf liver	0.44	18
2	Nonreduced	Calf liver	0.05	3
	Nonreduced	Calf liver	0.10	3
	Nonreduced	Calf liver	0.28	6
	Nonreduced	*E. coli*	0.05	9
	Nonreduced	*E. coli*	0.10	18

[a] Irradiation of the DNA was done according to a standardized procedure (Kirtikar et al., 1975). In experiment 1, 0.1 mL of T7 DNA, 209 nmol (388 cpm/nmol), in 0.05 M Tris-HCl (pH 8.0) was diluted with 0.9 mL of potassium phosphate buffer (pH 7.0) in a vial with a screw top cap. Nitrogen was bubbled through this mixture at 0 °C for 15 min. The DNA was then irradiated for 3 min at 0 °C with a ^{60}Co source which delivered 7.5 krads/min (total dose = 22.5 krads). The vial was then incubated for 4 h at 37 °C to increase enzyme-sensitive sites (Kirtikar et al., 1975). In experiment 2, 0.15 mL of the same T7 DNA, 314 nmol, was diluted to 2 mL with Tris-HCl (pH 8.0) and irradiated as in experiment 1 above. The pH was then stabilized by the addition of sodium phosphate buffer (pH 6.5) to a final concentration of 0.25 M. Reduction was carried out by the addition of 0.06 mL of 5 M $NaBH_4$ in aliquots of ⅙, ⅓, and ½ of the total amount. The pH at this point was 8.5. The DNA was then preincubated for 4 h at 37 °C as in experiment 1. The reaction and analysis by sedimentation were done as described under Materials and Methods. Sedimentation was at 40 000 rpm in experiment 1 and 50 000 rpm in experiment 2. *E. coli* endonuclease II was obtained from Dr. Dollie Kirtikar.

Previous studies have shown that γ-irradiated DNA contains sites recognized by endonuclease II of *E. coli* which are presumably due to base damage (Kirtikar et al., 1975). Other sites are also present which are alkali labile and which can be stabilized by reduction with $NaBH_4$. At least some of those sites may be due to depurination or depyrimidination. Data in Table III, experiment 1, show that when γ-irradiated DNA was reduced with $NaBH_4$ and then treated with the calf-liver endonuclease increasing numbers of sites were recognized by increasing amounts of the enzyme. In experiment 2, the DNA was not treated with $NaBH_4$ prior to incubation with the calf-liver enzyme. In both experiments, at the end of the reaction, the mixture was incubated in 0.066 N NaOH at 37 °C for 10 min to degrade all alkali-labile phosphodiester bonds in both the control and enzyme-treated samples (Kirtikar et al., 1975). The enzyme-induced breaks in the reduced DNA could represent both depurinated (depyrimidinated) sites and sites due to base damage, while the sites in the nonreduced DNA could not include depurinated (depyrimidinated) sites. The calf-liver enzyme made twice as many single-strand breaks in the reduced DNA as in the nonreduced DNA and the tentative conclusion is that the difference represents depurinated (depyrimidinated) reduced sites. The nature of the sites recognized by the calf-liver enzyme in the nonreduced DNA is not clear. Some sites in this DNA are recognized more efficiently by endonuclease II of *E. coli* but whether they are the same as those recognized by the calf-liver enzyme is not clear. Further work is required to resolve this problem.

When T4 DNA was exposed to UV irradiation to produce 60 thymine dimers per single strand (Shlaes et al., 1972), treated with the calf-liver enzyme, and then examined as above by alkaline sucrose gradient sedimentation, there was no change in the sedimentation pattern. Therefore, base damage produced by UV was not recognized by the enzyme.

The ability of the enzyme to react with DNA treated with methylmethanesulfonate was then tested using the DNA-gel assay. This was done by treating the DNA-gel with methylmethanesulfonate at a ratio of 1000:1 methylmethanesulfonate to DNA nucleotide and using this preparation immediately under the conditions used for the analysis of endonuclease II of *E. coli* (Kirtikar et al., 1976a). Although *E. coli* endonuclease II readily released DNA from this type of gel, the calf-liver enzyme was unable to utilize this substrate. In addition, the enzyme was allowed to react with DNA previously treated with 7-bromomethyl-12-methylbenz[*a*]anthracene. This substrate remained unaltered when centrifuged through alkaline gradients, even when high concentrations of enzyme were used. These experiments indicate that the endonuclease does not cleave alklated or aralkylated DNA. Experiments were done to rule out any *N*-glycosidase activity. No activity of the enzyme on DNA treated with ^3H-labeled dimethyl sulfate was detected when base release was assayed either by alcohol-soluble counts or by the isolation of 3-methyladenine and 7-methylguanine on thin-layer chromatography. Furthermore, there was no release of ^3H from DNA treated with 7-[^3H]bromomethyl-12-methylbenz[*a*]anthracene. The endonuclease was also found to lack exonuclease activity toward either single- or double-stranded T4, T7, or *E. coli* DNA.

Production of 3′-Hydroxyl and 5′-Phosphate Groups. End group analysis to determine the type of strand breakage by the calf-liver endonuclease was performed, taking advantage of the specificity of spleen and venom exonucleases. Bovine spleen exonuclease initiates attack at free 5′-hydroxyl groups and liberates mononucleotides with a 3′-phosphate. Its action is blocked by a 5′-phosphate. The snake venom exonuclease initiates hydrolysis at the 3′-hydroxyl end and yields mononucleotides with 5′-phosphates. Its action is blocked by a 3′-phosphate. *E. coli* DNA treated with DNase I, which produces 3′-hydroxyl and 5′-phosphate end groups, was found to be a good substrate for the snake venom exonuclease, but was attacked by the spleen enzyme only after pretreatment with alkaline phosphatase. The opposite results were obtained after treatment of the DNA with micrococcal nuclease, which produces 3′-phosphate and 5′-hydroxyl end groups. When *E. coli* DNA was first depurinated and then treated with the calf-liver endonuclease, it was shown to be a good substrate for the venom exonuclease without (or with) alkaline phosphatase (AP) (Figure 5, + enzyme, AP). The spleen exonuclease, however, released alcohol-soluble counts only if the DNA was pretreated with alkaline phosphatase (+ enzyme, + AP). The slow and delayed appearance of mononucleotides on incubation of the DNA with venom exonuclease alone (− enzyme, ± AP) may reflect contamination of the enzyme preparation by an endonuclease as described by Laskowski (1967). These results therefore demonstrate that the apurinic acid endonuclease from calf-liver produces 3′-hydroxyls and 5′-phosphate end groups. At the present time, it is not known whether the enzyme hydrolyzes the phosphodiester bond on the 3′ or 5′ side of the sugar residue which lacks the purine base.

A Comparison of Calf-Liver Apurinic Acid Endonuclease with Calf-Thymus Apurinic Acid Endonuclease. Ljungquist and Lindahl (1974) have isolated and purified an enzyme from calf thymus which is specific for depurinated sites. A crude preparation of this enzyme was made to compare its properties with the calf-liver enzyme. In Table IV, data are presented

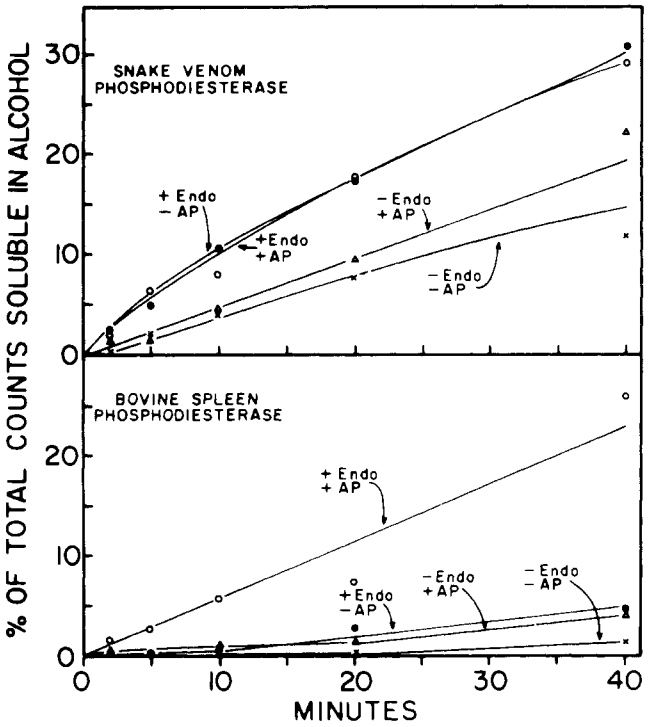

FIGURE 5: Action of specific exonucleases on depurinated-reduced DNA treated with the calf-liver enzyme. ^{3}H-Labeled *E. coli* DNA (537 nmol/mL, 947 cpm/nmol) was depurinated and reduced as described under Materials and Methods. The DNA was precipitated by the addition of 0.1 vol of 2.5 M sodium acetate and 2 vol of 95% EtOH. It was dissolved in 3.0 mL of 0.05 M Tris-HCl (pH 8.0) and dialyzed overnight against this same buffer before reaction with calf-liver apurinic acid enzyme. Half the DNA sample (555 nmol) was treated with 0.50 unit of enzyme in a total reaction volume of 1.9 mL containing final concentrations of 0.05 M Tris-HCl (pH 8.0), 10^{-4} M β-mercaptoethanol, and 1% glycerol. After 60 min at 37 °C, the reaction was terminated by the addition of 750 μg of cold carrier calf-thymus DNA and the DNA was precipitated with EtOH as above, dissolved in 1.0 mL of 0.1 N NaOH, neutralized with 0.1 mL of 2 M Tris-HCl (pH 6.45), and dialyzed overnight against 0.05 M Tris-HCl (pH 8). The other half of the DNA was treated as a control. The sample volumes were brought to 1.8 mL with buffer. Recovery of DNA was 52%. For assays with 10 mg of snake venom phosphodiesterase, reaction volumes of 0.15 mL contained 8 nmol of substrate DNA, 0.025 nmol of β-mercaptoethanol, 7.5 μmol of Tris buffer, and 7.5 μmol of $MgCl_2$. At various times, the reactions were stopped by the addition of 250 μg of cold calf-thymus DNA and precipitated with 2 vol of ethanol alone. Aliquots of the supernatant and precipitate dissolved in base and neutralized were counted and the percentage of the DNA substrate made ethanol soluble by the exonuclease was determined. Similar reactions were run with 0.050 unit of bovine spleen phosphodiesterase except that 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer (pH 6.5) was used instead of Tris-HCl and no $MgCl_2$ was added. When alkaline phosphatase was employed, 0.2 unit of this enzyme was allowed to react with substrate DNA in the absence of buffer and exonuclease. After 10 min at 37 °C, all constituents were added and the reaction was run as above. Specificity of the exonucleases was checked using a substrate DNA previously treated with DNase I or micrococcal nuclease: (●) + endonuclease (endo), + alkaline phosphatase (AP); (○) + endonuclease, − alkaline phosphatase; (△) − endonuclease, + alkaline phosphatase; (×) − endonuclease, − alkaline phosphatase.

which were obtained with the liver apurinic acid endonuclease purified in this laboratory and with the calf-thymus apurinic acid endonuclease purified extensively by Ljungquist and Lindahl (1974) and with the same enzyme only partially purified in this laboratory. Assays done in this laboratory were with the DNA-gel method while assays done by Ljungquist and Lindahl (1974) were with transforming DNA. The pH optima for the two enzymes differed. The stimulation by Mg^{2+} was small with the liver endonuclease but pronounced with the

TABLE IV: Comparison of Calf Apurinic Endonucleases.[a]

Property	Liver[b]	Thymus[b]	Thymus[c]
Purification	727-900	10-30	830
pH optimum	9.5		8.5
Optimum Mg^{2+} concn (mM)	0.01-0.05	0.5-2.0	3
Max stimulation by Mg^{2+} (fold)	1.5	9	50-1000
NaCl, 50% inhibition (M)[d]	0.025	0.12	0.2
NaCl stimulation at low concn	None	4× at 0.01 M	
3′-Hydroxyl, 5′-phosphate end groups produced	+	+	+
Mol wt	28 000		32 000

[a] Data for pH optima, magnesium concentration, and NaCl sensitivity of the calf liver and crude calf-thymus enzyme were obtained in this laboratory from experiments where the gel assay was employed. [b] Purified in this laboratory. [c] Data on the more highly purified thymus enzyme were obtained by Ljungquist and Lindahl (1975) who used a transformation assay. The nature of the end groups investigated by Ljungquist and Lindahl (1975) was determined by the use of polynucleotide kinase. [d] Compared to activity without NaCl.

thymus enzyme, particularly the more purified material as noted by Ljungquist and Lindahl (1974). Inhibition by NaCl of the liver enzyme occurred at a lower molarity than inhibition of the thymus enzyme. The activity of the thymus enzyme with 0.01 M NaCl was fourfold greater than in the absence of NaCl. The liver enzyme showed no such stimulation. When compared in the absence of added Mg^{2+}, the liver enzyme was much more sensitive to inhibition by tRNA. A concentration of 0.05 mg/mL of tRNA was necessary to observe a 40% decrease in activity on gels for the calf-liver enzyme, while a similar inhibition for the thymus enzyme required 5 mg/mL of tRNA. Both enzymes produced 3′-hydroxyls and 5′-phosphates, and the molecular weights differed only slightly. All of the differences suggest that these endonucleases are isozymes.

Discussion

The endonuclease from calf liver described in this paper recognizes depurinated sites and depurinated-reduced sites in DNA. The enzyme was purified 700- to 900-fold although some of this increase may be due to removal of an inhibitor. The enzyme was stable at this stage, but it became unstable on further purification steps. Associated with the activity on depurinated sites was an activity on single-stranded DNA. The minimal amount of enzyme required to observe this activity was 500 times that needed to observe activity on depurinated-reduced DNA. The heat inactivation of the single-strand activity paralleled that of the depurination activity and both activities were inhibited in a comparable fashion by tRNA. These results suggest that the two activities are associated with one protein, but this remains to be proven. The preparation has no ability to remove thymine dimers from DNA and does not recognize DNA treated with methylmethanesulfonate or with 7-bromomethyl-12-methylbenz-[*a*]anthracene.

With depurinated-reduced DNA, the enzyme makes both single- and double-strand breaks in what appears to be a sequential manner. Since one double-strand break, depending on assumptions, requires up to 200 random single-strand breaks (Thomas, 1956), it is apparent that some nonrandom event is occurring. Depurination may occur nonrandomly since

single-stranded DNA is depurinated more easily than double-stranded DNA (Greer and Zamenhof, 1962; Lindahl and Nyberg, 1972) and a plot of the number of depurinated sites produced as a function of time (Figure 1) has a sigmoid shape. It is also possible that some property of the enzyme preparation is responsible. A similar observation was made with an enzyme preparation from *E. coli* in which one double-strand break was made for every four single-strand breaks in depurinated–reduced DNA (Hadi and Goldthwait, 1971). In both cases, the number of enzyme-sensitive sites was equal to the number of alkali-labile sites. Thus, although the precise reason for the double-strand breaks in depurinated–reduced DNA made by the liver and *E. coli* enzymes is not known, there is some suggestive evidence that the depurinated sites do not occur randomly in the DNA.

A comparison of some of the properties of the calf-liver enzyme with the calf-thymus enzyme leads to the proposal that these enzymes are isozymes (Kirtikar et al., 1976). Distinct differences in pH optima, concentration of Mg^{2+} for maximum stimulation, degree of stimulation by Mg^{2+}, stimulation at low and inhibition at high ionic strength, and inhibition by tRNA all point to the suggestion of isozymes. Evidence for isozymes has been obtained in *E. coli* where two different proteins with activity toward apurinic sites have been separated chromatographically (Kirtikar et al., 1976, 1977). Ljungquist et al. (1976) have also presented evidence for two forms of apurinic endonuclease in *E. coli*, one of which is more stable to heat and is not inhibited by EDTA. Finally, data on apurinic acid endonuclease from lines of normal and xeroderma pigmentosa fibroblasts suggest that there may be isozymes in these cells (Kuhnlein et al., 1976).

Acknowledgment

The authors wish to thank Dr. Dollie Kirtikar for many helpful suggestions during the course of this work.

References

Carrier, W. L., and Setlow, R. B. (1971), *Methods Enzymol. 21,* 230.
Davis, B. J. (1964), *Ann. N.Y. Acad. Sci. 121,* 404.
Freifelder, D. (1970), *J. Mol. Biol. 54,* 867.
Friedberg, E. C., and Goldthwait, D. A. (1969), *Proc. Natl. Acad. Sci. U.S.A. 62,* 934.
Friedberg, E. C., Hadi, S. M., and Goldthwait, D. A. (1969), *J. Biol. Chem. 244,* 5879.
Greer, S., and Zamenhof, S. (1962), *J. Mol. Biol. 4,* 123.
Hadi, S. M., and Goldthwait, D. A. (1971), *Biochemistry 10,* 4986.
Kirtikar, D. M., Cathcart, R. C., and Goldthwait, D. A., (1976a), *Proc. Natl. Acad. Sci. U.S.A.,* 4324–4328.
Kirtikar, D. M., Kuebler, J. P., Dipple, A., and Goldthwait, D. A. (1976b), *Cancer Enzymology,* Schultz, J., and Ahmed, F., Ed., 8th Miami Winter Symposium, New York, N.Y., Academic Press, pp 139–155.
Kirtikar, D., Slaughter, J., and Goldthwait, D. A. (1975), *Biochemistry 14,* 1235.
Kuhnlein, V., Penhaet, E. E., and Linn, S. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 1169.
Laskowski, M. S. (1967), *Adv. Enzymol. 29,* 165.
Lindahl, T., and Nyberg, B. (1972), *Biochemistry 11,* 3610.
Ljungquist, S., and Lindahl, T. (1974), *J. Biol. Chem. 249,* 1530.
Ljungquist, S., Lindahl, T., and Howard-Flanders, P. (1976), *J. Bacteriol. 126,* 646.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Melgar, E., and Goldthwait, D. A. (1968), *J. Biol. Chem. 243,* 4401.
Paquette, Y., Crine, P., and Verly, W. G. (1972), *Can. J. Biochem. 50,* 199.
Richardson, C. C. (1965), *Proc. Natl. Acad. Sci. U.S.A. 54,* 158.
Shlaes, D. M., Anderson, J. A., and Barbour, S. D. (1972), *J. Bacteriol. 111,* 723.
Smith, M. G. (1967), *Methods Enzymol. 12,* 545.
Summers, W. C., and Szybalski, W. (1968), *Virology 34,* 9.
Teebor, G. W., and Duker, N. J. (1975), *Nature (London) 258,* 544.
Thomas, C. A. (1956), *J. Am. Chem. Soc. 78,* 1861.
Verly, W. G., and Paquette, Y. (1973), *Can. J. Biochem. 51,* 1003.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.