A Mammalian Nicking Endonuclease†

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ABSTRACT: Purification and properties are described for an endonuclease isolated from calf thymus which attacks double-stranded, unmodified DNA, primarily by making single-strand breaks. No detectable acid-soluble products arise from the reaction. Double-strand breaks may occasionally be produced by the introduction of single-strand breaks on opposite strands in close proximity. The enzyme does not attack denatured DNA and is not inhibited by tRNA. Although added divalent cations are not required for activity, the enzyme is inhibited by EDTA, which suggests an essential role for bound cations; reaction is inhibited by Ca^{2+}. The endonuclease has a broad pH optimum and is inactivated by preincubation at temperatures of 45 °C and higher. The molecular weight as determined by gel chromatography is about 30 000. Analysis of the products of reaction on a defined substrate, bacteriophage T3 DNA, by sedimentation in alkaline sucrose density gradients indicates limit products with chain lengths of about 0.8×10^6 daltons. On electrophoresis in agarose gels these products were shown to be heterogeneous in size. The endonuclease appears to generate 3′-hydroxyl and 5′-phosphate ends. The ability of the endonuclease to utilize bovine DNA as substrate argues against a restriction role for this enzyme.

Thymus has been used as a source for the isolation of several enzymes that attack DNA. DNase II, the first such enzyme to be isolated from thymus (Fredericq and Oth, 1958), was shown to degrade DNA extensively. Subsequently, Strauss et al. (1968) located in crude calf thymus extracts an activity that attacks alkylated DNA. More recently, Ljungquist and Lindahl (1974) have described the isolation of an endonuclease that attacks double-stranded DNA at previously introduced apurinic sites.

In the course of an investigation into thymus nucleases that attack alkylated DNA (Lavin et al., 1972), we have isolated an enzyme that also makes a limited number of breaks in native double-stranded DNA. The purpose of the present paper is to describe the purification of this enzyme and to examine some of the molecular and kinetic properties of the endonuclease and its reaction with defined substrates. A preliminary account of some of these findings has been reported (Counsilman and Lavin, 1975).

Experimental Section

Materials

DEAE[1]-Cellulose (DE 52, preswollen) and CM-cellulose (CM 52, preswollen) were obtained from Whatman. Acrylamide and *N,N*-methylenebisacrylamide were supplied by Eastman Organic Chemicals. Methyl[^{3}H]thymidine (30 Ci/mmol) was obtained from New England Nuclear Corporation and carrier-free [^{32}P]phosphate from the Australian Atomic Energy Commission. Spleen phosphodiesterase and venom phosphodiesterase were Worthington products. Yeast tRNA was purchased from Boehringer.

Methods

Preparation of DNA Substrate. Mouse myeloma cell lines C1 and XC1 were grown in suspension culture as described by Byars and Kidson (1970). Methyl[^{3}H]thymidine (5 μCi) was added per 10-ml plate (2×10^5 cells/ml) and incubation carried out for 48 h (final cell density, 8×10^5 cells/ml). DNA was extracted as described by Kidson (1966) with certain modifications: (a) sodium triisopropylnaphthalenesulfonate (1%) was used as detergent; and (b) in lieu of ethanol precipitation, residual phenol was removed by ether extraction, followed by dialysis. Specific activities of DNA samples thus prepared were of the order of 10^4 cpm/μg DNA (3.3×10^3 cpm/nmol). [^{3}H]Thymidine-labeled DNA was also prepared from a bovine endothelial cell primary culture (a gift from Dr. S. Tzipori).

Bacteriophage. Bacteriophages T3 and T4 were gifts from Dr. R. B. Russell. The titers of these bacteriophages were increased according to the method of Thomas and Abelson (1966). Bacteriophage λ was induced by transferring a logarithmically growing culture of *Escherichia coli PB64* from 30 to 40 °C for 15 min. Lysis occurred after a further 45 min at 37 °C; bacteriophages were collected and DNA was isolated by the method of Thomas and Abelson (1966).

Endonuclease Assays. Throughout the purification procedure, endonuclease activity was measured by the method of Melgar and Goldthwait (1968). This procedure utilizes DNA trapped in a polyacrylamide gel and measures the release of fragments of less than 400 000 daltons.

Preparation of DNA Gel. Radiolabeled DNA was incorporated in a polyacrylamide gel as described (Melgar and Goldthwait, 1968). For assay of endonucleases with a preference for alkylated DNA, alkylation (methylation) of DNA was carried out by adding methylmethanesulfonate (0.08 M) to a 20-ml suspension of the gel (140 μg of DNA) in 0.05 M Tris buffer, pH 8.0, then shaking for 3 h at ambient temperature. The gel was washed with buffer until no further release of radioactivity occurred. Alkylation by methylmethanesulfonate occurs primarily at N-7 of guanine moieties (Brookes and Lawley, 1961).

Enzyme Units. The amount of DNA retained in the gel was monitored for each set of assays, to permit calculation of DNA concentration. On the basis of the known specific activity of the DNA, the radioactivity released from the gel during in-

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[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; CM, carboxymethyl; Tris, tris(hydroxymethyl)aminomethane.

cubation with enzyme was converted to nanomoles of DNA nucleotide released. For the purposes of assessing purification, one unit of endonuclease was arbitrarily defined as that amount catalyzing the release of 1 nmol of nucleotide in 1 h from a gel under the conditions of the assay.

Endonuclease Recognizing Alkylated DNA. In addition to an aliquot of the enzyme to be assayed, the reaction mixture contained, in a total volume of 1.5 ml, 4 μg of gel-trapped, alkylated DNA, 5 mM $MgCl_2$, 150 mM KCl, 0.1 mM dithiothreitol, and 50 mM Tris buffer, pH 8.0. Incubation was carried out for 60 min at 37 °C with shaking, the reaction was stopped by addition of 0.5 ml of 100 mM EDTA, pH 6.5, and the mixture was centrifuged for 5 min at 10 000g at 4 °C. The DNA released was precipitated from 0.5 ml of the supernatant by the addition of 0.1 ml of 25% trichloroacetic acid and collected on glass fiber discs (GF/C, Whatman), and radioactivity was assayed in a toluene phosphor by liquid scintillation spectrometry.

Endonuclease Recognizing Unmodified DNA. In addition to an aliquot of the enzyme fraction to be assayed, the reaction mixture contained, in a total volume of 1.5 ml, 4 μg of gel-trapped DNA, 5 mM $MgCl_2$, 150 mM KCl, 0.1 mM dithiothreitol, and 50 mM sodium acetate, pH 5.5. Activity was determined as for methylated DNA.

Exonuclease. In all enzyme fractions during the isolation steps, exonuclease activity was assayed as follows. In addition to an aliquot of the enzyme fraction to be assayed, the reaction mixture contained, in a final volume of 0.5 ml, 2 μg of DNA, 8 mM $MgCl_2$, 0.1 mM dithiothreitol, and 50 mM sodium acetate, pH 5.5. After incubation for 30 min at 37 °C, the reaction was terminated by adding 0.3 ml of 0.5% bovine serum albumin followed by 0.2 ml of 25% trichloroacetic acid. After cooling to 0 °C, centrifugation was carried out at 10 000g for 5 min. An aliquot of the supernatant was neutralized with 4 M NH_4OH and counted in a dioxane phosphor by liquid scintillation spectrometry.

Assay by Zonal Sedimentation. Further characterization of enzyme activity involved zonal sedimentation of DNA digestion products on linear sucrose gradients (5–20%) using intact bacteriophage DNA markers. A stock solution of enzyme was usually diluted 1 in 3 with an incubation mixture containing 6 mM $MgCl_2$, 28 mM Tris buffer (pH 7.8), and 5–10 μg/ml of radiolabeled DNA in a total volume of 180 μl. After incubation of reaction mixtures at 37 °C for specified time intervals, the reaction was stopped by cooling on ice, prior to loading on gradients (100 μl). Density gradient centrifugation of native and enzyme-treated DNAs was performed at 50 000 rpm in a Spinco Model L2-65B ultracentrifuge fitted with an SW56 rotor. Fractions were collected, precipitated with 5% Cl_3CCOOH, and counted in a toluene phosphor. Sedimentation coefficients were estimated by the method of Martin and Ames (1961). Because of the heavy reliance that this method places on the linearity and extent of the gradient, sucrose concentrations were monitored routinely by Abbé refractometry. Sedimentation coefficients ($s_{20,w}^0$) of the nucleic acids used as markers in these experiments were determined by conventional velocity centrifugation in a Spinco Model E ultracentrifuge. Conversion of sedimentation coefficients to molecular weights was based on the empirical calibration plots of Studier (1965).

In order to take into account possible variations in the skewness of the centrifuged zones, number-average zone positions were used in the estimation of sedimentation coefficients to provide a more direct index of the average number of strand scissions. Interpretation of the sedimentation coefficients so obtained in terms of number-average molecular weights requires the corresponding calibration plot. Although that of Studier (1965) refers to weight-average values, it was used here because it is based on essentially homogeneous DNA samples, for which assumed identity of weight-average and number-average quantities should be a good approximation.

Diesterase Assays. Spleen and venom diesterase digestions of DNA and DNA fragments were carried out as described by Laval and Paoletti (1972). Alkaline phosphatase treatment of T3 DNA was carried out as follows. Untreated DNA and limit T3 DNA degradation product were heated to 100 °C for 7 min to denature DNA. Incubation mixtures containing radiolabeled denatured DNA (10 μg), 28 mM Tris buffer (pH 7.8), and 0.5 unit of alkaline phosphatase were incubated at 37 °C for 60 min. At the end of reaction, the pH of the incubation mixture was adjusted to pH 4.5 with dilute acetic acid prior to reaction with spleen phosphodiesterase.

Agarose Gel Electrophoresis. Gels were prepared according to Hayward and Smith (1972). Electrophoresis was carried out at 4 °C and an applied potential gradient of 60 V per tube. Prior to analysis DNA samples were made 10% (w/v) with sucrose and 50-μl aliquots were layered onto the gels with a wide bore Hamilton syringe to avoid DNA shearing. After electrophoresis for 6 h, the gels were cut into 1-mm slices. In experiments with ^3H-labeled DNA, slices were placed in 0.2 ml of soluene (Packard) and counted by liquid scintillation spectrometry using a toluene phosphor. ^{32}P-Labeled DNA was estimated as Cerenkov radiation by counting slices in 1 ml of distilled water.

Results

Enzyme Purification. Frozen calf thymus (Miles) was homogenized in 0.05 M Tris buffer, pH 8.0, in a Sorvall omnimixer for 30 s (speed 10), using 1.3 ml of buffer per g of tissue. The homogenate was then centrifuged at 2 °C for 3 h at 80 000g in a Spinco Model L2-65B ultracentrifuge using a number 30 rotor. RNA was removed from the supernatant fraction by precipitation with streptomycin sulfate (Friedberg and Goldthwait, 1969). The supernatant from this step was fractionated with ammonium sulfate and the 45–75% precipitate resuspended in 0.05 M Tris (pH 8.0), 0.1 mM Na_2EDTA, and 0.1 mM dithiothreitol (0.5 ml/g of tissue originally extracted).

DEAE-Cellulose Chromatography. After dialysis against more of the Tris buffer used for its dissolution, the sample was added to a DEAE-cellulose column (16 × 1.8 cm) equilibrated with the same Tris buffer. The column was washed with an additional 500 ml of buffer, followed by elution with a linear gradient of 0–0.3 M NaCl in the same buffer (volume 600 ml). Some activity against alkylated DNA was eluted by the 500-ml wash, and some by the gradient (as measured by release of DNA from the gel). Fractions corresponding to the salt range 0.15–0.24 M were pooled for further purification. This particular cut was selected on the basis of minimal overlap both with endonuclease activity against unmodified DNA and with exonuclease activity; it represented about 30% of the observed activity against alkylated DNA. Ammonium sulfate, 80% saturation, was used to concentrate the active fraction from DEAE-cellulose chromatography, and the precipitate dissolved in 50 ml of 0.02 M potassium phosphate (pH 6.5), 0.1 mM dithiothreitol (buffer A). The ammonium sulfate was removed by dialysis against the same buffer in readiness for chromatography on phosphocellulose.

Phosphocellulose Chromatography. Enzyme from the previous step was applied to a phosphocellulose column (14 × 1.2

TABLE I: Purification of Endonuclease Bound to DEAE-Cellulose, Using Gel-Entrapped, Alkylated DNA Substrate.

Fraction	Volume (ml)	Total Enzyme Units	Total Protein[a] (mg)	Spec Act.[b]	Recovery (%)	Purification
Crude extract	363	7549	15 792	0.48	100	1.0
Streptomycin sulfate	540	9653	14 429	0.67	128	1.4
Ammonium sulfate	300	5776	5 719	1.01	77	2.1
DEAE-Cellulose	100	1782	569	3.13	24	6.5
Phosphocellulose	25	1268	14.6	86.85	17	181
Hydroxylapatite	5	538	1.5	358.7	7	747

[a] Protein was determined by the method of Lowry et al. (1951). [b] In units/mg protein.

cm) equilibrated with buffer A and elution was carried out with a linear gradient of 0–0.3 M NaCl in buffer A in a total volume of 600 ml. Fractions with activity against alkylated DNA (0.06–0.19 M NaCl) were pooled, concentrated in a Diaflo cell (PM-10 membrane), and dialyzed against buffer A prior to chromatography on hydroxylapatite.

Hydroxylapatite Chromatography. Enzyme was next applied to a hydroxylapatite column (8 × 1.5 cm) equilibrated with buffer A. After washing the column with 50 ml of buffer A, elution was carried out with a linear gradient of 0.02–0.4 M potassium phosphate, pH 6.5, containing 0.1 mM dithiothreitol (total volume 300 ml). Most of the activity against alkylated DNA was eluted in a single peak (Figure 1). No exonuclease activity was detected in the active fraction. Fractions (0.20–0.32 M phosphate) were pooled, concentrated using a Diaflo cell, removed therefrom by washing into 0.05 M Tris buffer, pH 8.0, containing 0.1 mM dithiothreitol, and stored at −20 °C. This concentrated preparation is the endonuclease referred to in the remainder of the text.

Purification and Yield. Selection of fractions in the chromatographic steps has been based on elimination as far as possible of nuclease activities other than those against alkylated DNA. Table I describes a typical purification for the endonuclease against alkylated DNA as measured by release of radioactivity from a gel: an overall purification of some 750-fold was obtained routinely. Since in the earlier stages of purification other endonuclease activities were present, it is difficult to assess the significance of the apparent specific activities against alkylated DNA in the more crude fractions. This difficulty is compounded by the presence of several enzyme activities against this substrate. It seems likely, therefore, that the degree of purification calculated above falls short of the value actually obtained.

Despite the selection procedure used for isolation of the endonuclease which suggested a requirement for alkylated substrate, evidence of activity against unmodified DNA was obtained by density gradient centrifugation. This is a more precise means of determining endonuclease activity since it allows the use of well-defined native DNA substrates.

When sedimentation analysis was employed using alkylated T3 DNA as substrate, the number of breaks introduced by the endonuclease was not increased over that using unmodified DNA. The ability of the endonuclease to release DNA fragments from alkylated DNA gels but not from unmodified DNA gels reflects the presence of preexisting breaks in the former resulting from the high level of alkylation employed. In unmodified DNA gels the number of breaks resulting from endonucleolytic cleavage alone was too few to lead to release of DNA from the gels.

General Properties of the Endonuclease. Molecular Weight.

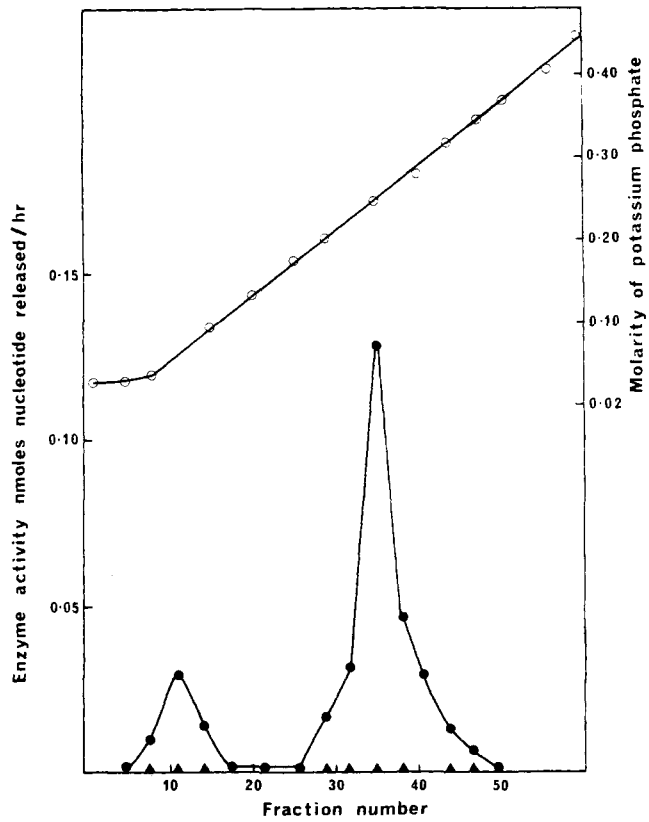

FIGURE 1: Chromatography of endonuclease eluted from phosphocellulose on hydroxylapatite. (▲) Absorbance at 280 nm; (●) endonuclease activity against alkylated DNA; (○) elution molarity.

The molecular weight of the endonuclease was estimated by Sephadex G-100 gel filtration according to the method of Andrews (1964). The Sephadex G-100 column was successively calibrated with cytochrome *c*, myoglobin, ovalbumin, and bovine serum albumin by measuring the elution volume (V_e) of each protein in a separate run. The void volume (V_0) was measured with dextran blue. Endonuclease activity, as measured by sucrose gradient analysis of the degradation of T3 DNA, was eluted as a single symmetric peak in 0.05 M Tris, pH 8. Assuming a globular structure, the molecular weight of the enzyme was about 30 000.

pH Dependence of Enzyme Activity. The enzyme was found to be active over the pH range 4.0–9.0, with no obvious peak optimum.

Heat Inactivation. The effect of temperature was investigated by preincubation of the enzyme for 30 min at temperatures between 37 and 50 °C prior to incubation under normal

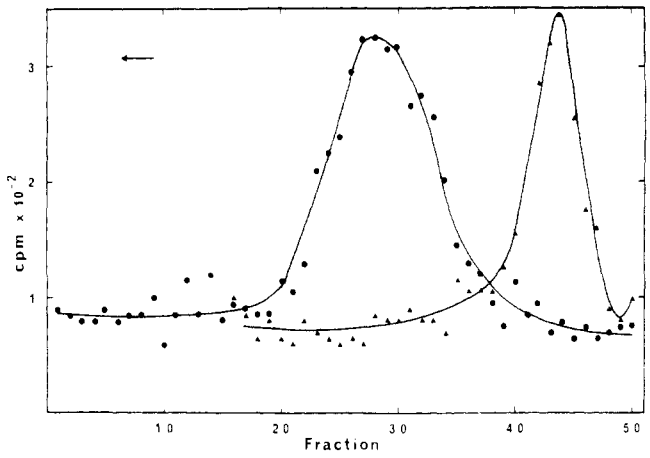

FIGURE 2: Density gradient centrifugation of DNA from bovine cells degraded by endonuclease (10 μg/ml). After 60 min at 37 °C, a 100-μl sample was layered on an alkaline sucrose gradient. Centrifugation was for 2 h at 50 000 rpm in an SW50 head of a Beckman ultracentrifuge. Untreated DNA (●); DNA incubated with enzyme for 60 min (▲).

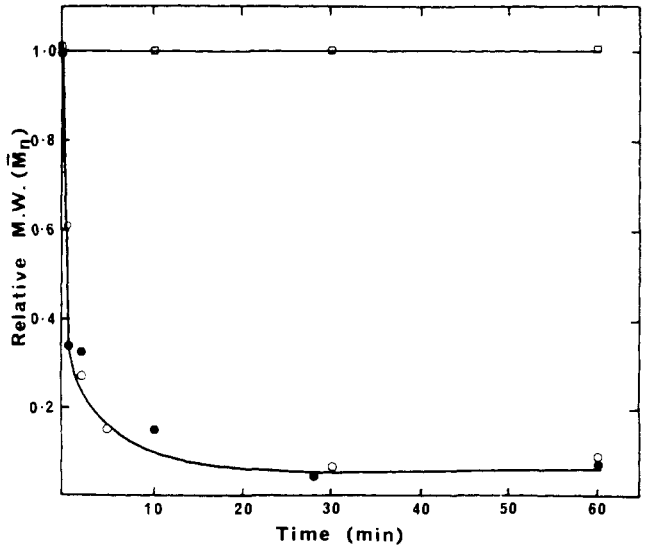

FIGURE 3: Effect of enzyme concentration on the rate and extent of degradation of T3 DNA. Enzyme (15 μg/ml) (●); 150 μg/ml enzyme (○)—analysis using alkaline sucrose gradients. Enzyme (15 μg/ml) (□)—analysis using neutral sucrose gradients.

assay conditions at 37 °C. Preincubation at 37 and 40 °C had no effect on enzymic activity but preincubation at 45 and 50 °C abolished activity completely.

Cation Dependence. Although the standard incubation mixture contained Mg^{2+} ions, omission of Mg^{2+} did not reduce enzyme activity significantly. However, increasing the Mg^{2+} concentration above 25 mM led to a decrease in enzyme activity. Replacement of Mg^{2+} by Mn^{2+} or NH_4^+ did not affect activity, but the addition of Ca^{2+} (2 mM) completely inhibited the enzyme reaction. EDTA (50 mM) inhibited activity completely.

Effect of tRNA. At concentrations up to 0.1 mg/ml, tRNA did not alter the rate of hydrolysis of DNA.

Substrate Specificity. Density gradient centrifugation showed that the enzyme attacked unmodified DNA from a variety of sources, including bacterial and mammalian DNA. Enzyme activity against DNA from a homologous source, viz., a bovine endothelial cell line, is shown in Figure 2. The obvious evidence of degradation suggests that the calf thymus endonuclease does not play a role in bovine cells similar to that of

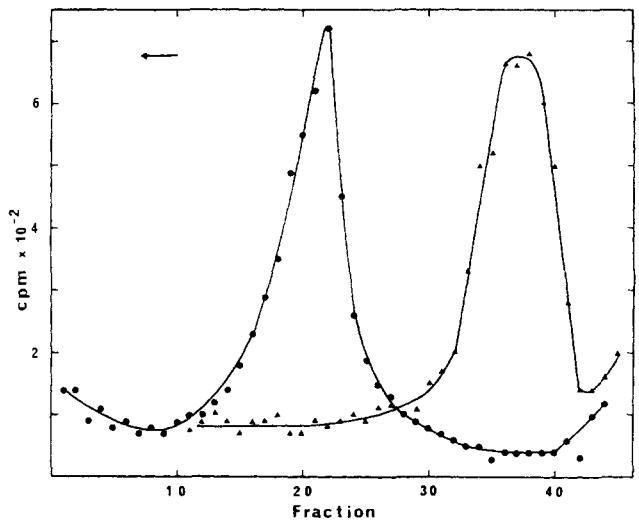

FIGURE 4: Resolution of the products of degradation of T3 DNA by alkaline sucrose density gradient centrifugation, at 50 000 rpm for 2 h using an SW56 head. Untreated DNA (●); DNA incubated with 10 μg/ml enzyme for 60 min (▲).

bacterial restriction enzymes. The number-average molecular weight of the limit degradation products was about 0.8×10^6. For characterization of the enzyme, bacteriophage T3 DNA has been used as substrate because of its isolation in essentially intact form. The sedimentation coefficient ($s_{20,w}^0$) of native T3 DNA was found to be 32.5 S, which is in good agreement with 32 S (±0.8) reported by Studier (1965) for intact T7 DNA. T3 and T7 are related bacteriophages having similar molecular weights and base compositions (Davis and Hyman, 1971).

In experiments with T3 DNA as substrate, single-strand but no double-strand breaks were detected after incubation for periods up to 1 h (Figure 3). Reaction is specific for double-stranded T3 DNA since no degradation of single-stranded T3 DNA was observed under the same conditions. Enzymic reaction with T4 DNA was qualitatively similar to that with T3 DNA inasmuch as single-strand but not double-strand breaks were observed. From Figure 3 it is evident that the limit of degradation of T3 DNA appears to be slightly less than 10% of the initial single-strand chain length, as judged by sedimentation in alkaline sucrose gradients. A tenfold higher enzyme concentration gave a similar rate of degradation (Figure 3) so that an enzyme concentration of 15 μg/ml is adequate for limit degradation within 1 h.

Size of Single-Stranded DNA Fragments Formed. Results of the sedimentation analysis of degradation products of T3 DNA on alkaline sucrose gradients are shown in Figure 4. DNA in the control sample had a number-average molecular weight of 1.3×10^7. Fragments resulting from limit digestion (60 min, see Figure 3) sedimented in a broad zone with a number-average molecular weight of about 0.8×10^6. Thus, an average of approximately 15 breaks per T3 DNA single strand have occurred.

A disadvantage of sedimentation analysis of enzyme-treated DNA is the incomplete resolution of polynucleotide fragments. Since Hayward and Smith (1972) have reported the separation by agarose gel electrophoresis of polynucleotides with molecular weights in the range 10^6 to 4×10^7 and with size differences as small as 5%, this method was adopted in addition to sedimentation analysis of polynucleotide fragments. Upon electrophoresis of an aliquot of the reaction products from the experiment shown in Figure 4, three peaks were observed

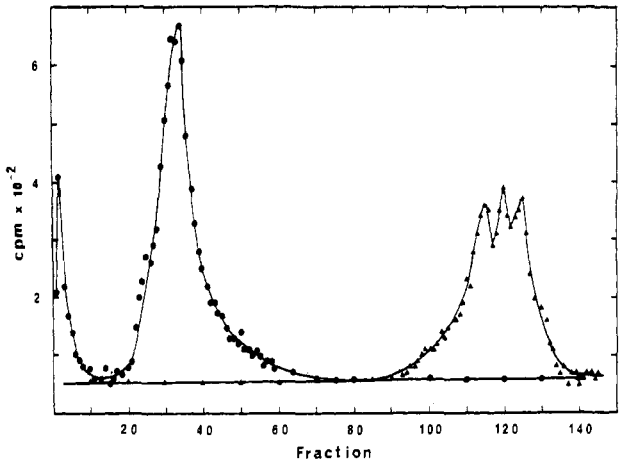

FIGURE 5: Separation of products of endonuclease degradation of T3 DNA by agarose gel electrophoresis. Procedure described in Methods. Untreated DNA (●); DNA incubated with 10 μg/ml enzyme for 60 min (▲).

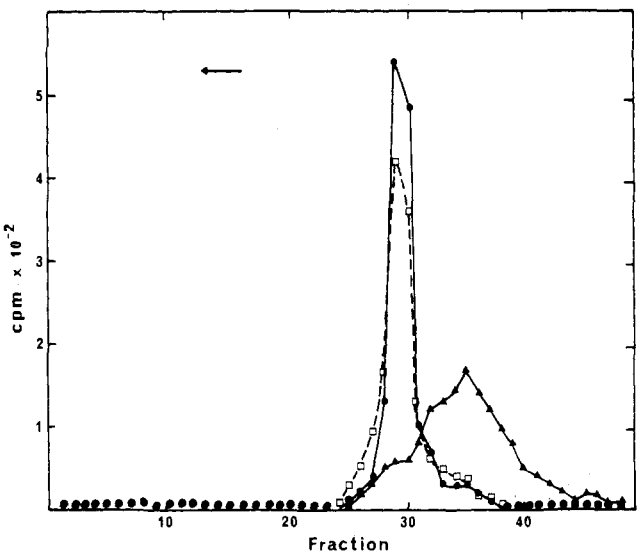

FIGURE 6: Conditions for formation of double-strand breaks in T3 DNA. Description of the methods of termination of enzyme reaction appears in Results section. Untreated DNA applied directly to gradient (●); untreated DNA extracted from incubation mixture with phenol prior to application (□); enzyme treated DNA extracted with phenol (▲).

(Figure 5). This implies the presence of at least three classes of fragments.

Position of Breaks on Opposite Strands. In the results so far presented, no evidence of double-strand breaks in T3 phage DNA has been observed. However, on termination of the enzymic reaction by gentle extraction with phenol, instead of by rapid cooling, degradation of T3 DNA was detected by sedimentation in neutral sucrose gradients (Figure 6). The molecular weight was reduced to about 8×10^6: approximately two double-strand scissions per molecule are indicated. Control DNA extracted with phenol showed no such evidence of breakdown. The observed dependence of result on the mode of reaction termination would be consistent with the formation of some single-strand breaks on opposite strands but in sufficiently close proximity for the intervening double-stranded region to come apart by DNA shearing during phenol extraction. Double-strand scissions of this kind were not seen with λ DNA under comparable conditions, although single-strand breaks were formed in this substrate.

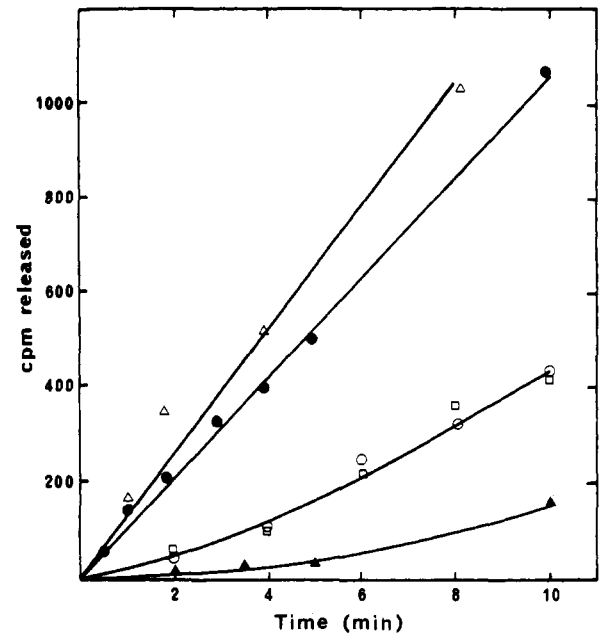

FIGURE 7: Action of spleen and venom exonucleases on DNA nicked by calf thymus endonuclease. Initial incubations contained 300 μl of endonuclease (10 μg), 50 μl of DNA (4 μg; 80 000 cpm), 70 μl of 0.1 M $MgCl_2$, 200 μl of 0.1 M Tris buffer, pH 8. Incubation was for 30 min at 37 °C; reaction was terminated by heating at 100 °C for 2 min. A 500-μl sample was taken and digested with either spleen or venom exonuclease under the conditions described by Laval and Paoletti (1972). Untreated DNA exposed to venom exonuclease (▲); endonuclease nicked DNA exposed to venom exonuclease (●); untreated DNA exposed to spleen exonuclease (□); endonuclease nicked DNA exposed to spleen exonuclease (○); endonuclease nicked DNA treated with alkaline phosphatase prior to exposure to spleen exonuclease (△), the relevant control being described essentially by the curve for untreated DNA exposed to spleen exonuclease.

Nature of the Ends Generated by Endonucleolytic Attack. Spleen and venom phosphodiesterases were used to determine which of the free ends produced by calf thymus endonuclease attack on T3 DNA contain the phosphate and hydroxyl groups, respectively. Spleen phosphodiesterase attacks free 5′-OH termini, liberating 3′-P mononucleotides, whereas the venom diesterase initiates hydrolysis at 3′-OH ends releasing 5′-P mononucleotides (Laval and Paoletti, 1972). Figure 7 shows that T3 DNA nicked by the endonuclease and then denatured acts as a good substrate for venom phosphodiesterase. Indeed the 15-fold increase in initial velocity is consistent with the number of fragments produced (Figure 3). Incubation of an aliquot of untreated DNA and the same limit T3 DNA degradation product with spleen phosphodiesterase resulted in a delayed release of similar, small amounts of acid-soluble material, possibly as a result of minor contamination of the diesterase with endonuclease (Bernardi and Griffe, 1964). Prior treatment of the limit T3 DNA degradation product with alkaline phosphatase before exposure to spleen phosphodiesterase did give rise to additional sites of attack by the spleen enzyme, a result consistent with the concept that 5′-phosphate ends are created by the nicking enzyme. We therefore conclude that the calf thymus endonuclease gives rise to 3′-OH and 5′-P ends.

Discussion

The enzyme from calf thymus studied in the present investigation has been shown to be a nicking endonuclease which makes a limited number of single-strand breaks in double-stranded DNA from a number of sources. Occasional dou-

ble-strand scissions in a defined substrate such as T3 DNA can be observed, but under conditions that would be conducive to shear-induced unwinding of a short, double-stranded region between two single-strand breaks, on opposite strands. An alternative, but less convincing postulate is that detection of such breaks under conditions which promote shearing might be explained by the removal of enzyme which maintained the duplex structure of DNA.

The inhibition of enzyme activity by EDTA suggests dependence of enzyme function on a divalent cation. The retention of activity on omission of Mg^{2+} from the reaction mixture implies that the enzyme contains bound cation in sufficient quantity. Inhibition of endonuclease activity by added Ca^{2+} is a result at variance with that for an endonuclease from nuclei of rat liver cells (Burgoyne et al., 1970). Furthermore, the present enzyme is not DNase II (Young and Sinsheimer, 1965) because of the nature of the ends formed and because of the limited number of breaks made (Figure 3). The broad pH range for activity of this endonuclease contrasts with that of the nicking enzyme induced in *E. coli* by bacteriophage T4 (Ando et al., 1969), which has a narrow range around pH 6. The enzyme is thermolabile at and above 45 °C but has been stable on storage at −20 °C for up to 5 months.

Interpretation of results obtained with mammalian DNA as substrate is rendered difficult by (i) the array of sizes of double-stranded molecules that arise from shearing during isolation, and (ii) the presence of a variable number of hidden single-strand nicks. Granted these reservations, the enzyme appears to introduce a limited number of single-strand breaks in mammalian DNAs, the fragments being of the same order of size as those produced using better defined substrates (Figure 2). In the case of T3 DNA these fragments averaged about 0.8×10^6 daltons in size, which is equivalent to about 2700 bases. Although there is heterogeneity with respect to chain length (Figure 5), these results imply that the enzyme has restricted sites of attack. We have shown that it gives rise to 3′-OH and 5′-P ends, but clearly more information is required concerning the actual nucleotide sequence at the attack sites: such work is currently in progress. If the site of attack is unique, this enzyme may be useful for DNA sequence analysis: either to provide specific initiation points for *E. coli* DNA polymerase I or, followed by S1 nuclease, as a means of introducing double-strand breaks at specific points in defined DNA molecules (Beard et al., 1973).

Any number of possible biological roles could be suggested for this endonuclease, for example, in DNA replication. While the fragments derived from attack by this enzyme are considerably larger than the size reported for Okazaki fragments in mammalian DNA replication (about 100 bases, Huberman and Horwitz, 1973), nicking may be required either ahead of or behind the growing fork to allow unwinding of the double helix (Blair et al., 1971). The enzyme could conceivably be involved in recombination since this process requires strand breakage and subsequent rejoining. Since this enzyme occurs in thymus, a remote possibility might be a role as is required in Baltimore's (1974) hypothesis for immunoglobulin gene variation.

References

Ando, T., Takagi, J., Kosawa, T., and Ikeda, Y. (1969), *J. Biochem. (Tokyo) 65,* 1.

Andrews, P. (1964), *Biochem. J. 91,* 222.

Baltimore, D. (1974), *Nature (London) 248,* 409.

Beard, P., Morrow, J. F., and Berg, P. (1973), *J. Virol. 12,* 1303.

Bernardi, G., and Griffe, M. (1964), *Biochemistry 3,* 1419.

Blair, D. G., Clewell, D. B., Sheratt, D. G., and Helinski, D. R. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 210.

Brookes, P., and Lawley, P. D. (1961), *Biochem. J. 80,* 496.

Burgoyne, L. A., Waquar, M. A., and Atkinson, M. R. (1970), *Biochem. Biophys. Res. Commun. 39,* 918.

Byars, N., and Kidson, C. (1970), *Nature (London) 226,* 648.

Counsilman, C., and Lavin, M. F. (1975), *Proc. Aust. Biochem. Soc. 8,* 88.

Davis, R. W., and Hyman, R. W. (1971), *J. Mol. Biol. 62,* 287.

Fredericq, E., and Oth, A. (1958), *Biochim. Biophys. Acta 29,* 281.

Friedberg, E. C., and Goldthwait, D. A. (1969), *Proc. Natl. Acad. Sci. U.S.A. 62,* 934.

Hayward, G. S., and Smith, M. G. (1972), *J. Mol. Biol. 63,* 383.

Huberman, J. A., and Horwitz, H. (1973), *Cold Spring Harbor Symp. Quant. Biol. 38,* 233.

Kidson, C. (1966), *J. Mol. Biol. 17,* 1.

Laval, J., and Paoletti, C. (1972), *Biochemistry 11,* 3604.

Lavin, M., Kikuchi, T., and Kidson, C. (1972), *Proc. Aust. Biochem. Soc. 5,* 33.

Ljungquist, S., and Lindahl, T. (1974), *J. Biol. Chem. 249,* 1530.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236,* 1372.

Melgar, F., and Goldthwait, D. A. (1968), *J. Biol. Chem. 243,* 4401.

Strauss, B. S., Coyle, M., and Robbins, M. (1968), *Cold Spring Harbor Symp. Quant. Biol. 33,* 277.

Studier, F. R. (1965), *J. Mol. Biol. 11,* 373.

Thomas, C. A., and Abelson, J. (1966), *Proced. Nucleic Acid Res.,* 553.

Young, E. T., and Sinsheimer, R. L. (1965), *J. Biol. Chem. 240,* 1274.