APURINIC ENDONUCLEASE ACTIVITY REMAINS CONSTANT DURING EARLY DROSOPHILA DEVELOPMENT

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An endonuclease activity that acts on alkali-labile lesions in x-irradiated PM2 DNA and recognizes apurinic lesions in heat/acid treated DNA has been partially purified from Drosophila melanogaster *embryos and its specific activity monitored throughout early development. The enzyme activity also showed a low level of activity on UV-irradiated DNA. The saturation kinetics observed with both x-irradiated and apurinic PM2 DNA substrates were similar. The endonuclease activity exhibited a broad pH optimum between pH 6 and 8.5 and was almost completely inhibited by 100 mM NaCl, 0.1 mM EDTA, 2 mM $CaCl_2$ and 10 mM NEM. The reaction was not completely dependent on the presence of Mg^{++}cation, but optimum activity was obtained at a concentration of 0.1 mM; concentrations greater than 1 mM Mg^{++} were inhibitory. The specific activity of the apurinic endonuclease, partially purified from several stages of embryonic and early larval development, remained the same. Unfertilized eggs exhibited a reduced level of this presumptive repair activity.*

INTRODUCTION

Apurinic/apyrimidinic (AP) endonucleases that incise at AP lesions in DNA are ubiquitous in nature and have been isolated from a wide variety of sources (see Lindahl, 1979, for review). The abundance of such enzymes must be related to the common occurrence of AP lesions which may be generated in DNA by various mechanisms: by spontaneous cleavage of the labile N-glycosylic bond (Lindahl and Nyberg, 1972), by heat/acid treatment (Lindahl and Andersson, 1972), by x-rays (Isildar *et al.*, 1981), after base alkylation (Zoltewicz *et al.*, 1970), by the action of DNA N-glycosylases on damaged purines (see Lindahl, 1979, for review) or pyrimidines (Haseltine *et al.*, 1980; Radanay and Friedberg, 1980; Demple and Linn, 1980; Katcher and Wallace, 1983) and by the action of uracil DNA N-glycosylase on misincorporated dUMP (Tye *et al.*, 1977). The potential importance of enzymes

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2. Abbreviations: AP endonucleases = Apurinic/apyrimidinic endonucleases.

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capable of acting at AP sites in DNA is borne out by the observations that unrepaired apurinic lesions are lethal (Verly, 1974; Kundrna *et al.*, 1979; Schaaper and Loeb, 1981) or mutagenic (Schaaper and Loeb, 1981) and that depurination decreases the fidelity of DNA synthesis *in vitro* (Shearman and Loeb, 1977; 1979).

The developmental profiles of several enzymes involved in DNA metabolism in Drosophila have been reported. A DNA polymerase and an exonuclease activity were shown to decline sharply during the course of Drosophila embryonic development (Margulies and Chargaff, 1973), and a stage dependent variation in total deoxyribonuclease activity with undefined substrate specificity was shown in larvae, pupae and adults (Boyd, 1967). An enzyme activity capable of repairing specific DNA lesions has been demonstrated at a defined stage of Drosophila development; in this study the presence of a Nuclease that recognizes uracil in DNA was found in crude extracts of third instar larvae (Deutch and Spiering, 1982).

We have characterized a partially purified AP endonuclease and monitored the specific activity of this enzyme activity in unfertilized eggs, four embryonic stages and early first instar larvae. A remarkable constancy in the level of this presumptive repair enzyme activity was observed throughout early Drosophila development. Preliminary accounts of some of these results have been presented (Wallace *et al.*, 1979; Margulies and Wallace, 1980).

MATERIALS AND METHODS

Strains

The inbred Oregon-R, wild-type and the f2, Y^S • In EN f v y • y^L y^+/C(1) RM y v bb/0 *Drosphila melanogaster* strains were obtained from Bowling Green State University. *Alteromonas espejiana* strain Bal 31-14 (thymine requiring auxotroph) and PM2 bacteriophage were obtained from H.B. Grey.

Collection of eggs

Flies were reared either on "instant" Drosophila medium (Carolina Biological Supply Co.) or on a cornmeal-brewer's yeast medium which was used for most of the developmental aspects of this work. Standard procedures were employed to insure that the deposited eggs were freshly fertilized. To obtain massive amounts of eggs for the purification and characterization of the enzyme, eggs were collected in a population cage over a period of 7-8 or 16-18 hours on a sugar-agar medium seeded with live yeast. To obtain eggs of various developmental stages, the collections were made in baskets over a 2-hour period and the oviposited eggs were either washed and frozen immediately (Stage 1) or allowed to age for different intervals. If insufficient quantities of eggs (less than 0.5 g) were obtained from a particular stage in a single 2 hour egg laying, two or more 2 hour periods were used and aged similarly; these were then pooled at the time of homogenization. Embryos of the following ages were obtained for enzyme isolation: 1-3 hour (0-2 hour collection with 1 hour allowance for washing

and freezing); 6-8 hour; 11-13 hour; 16-18 hour. Also young first instar larvae were harvested 27 hours after the 2 hour egg laying period ended. These developmental periods will be referred to as Stages 1-5 respectively. Eggs were collected for at least two, but often for three to four of these stages in a single day from one egg laying population of flies. Hatching curves performed with control eggs indicated the correct timing of embryonic or larval age. Under our conditions (25°C incubation), the hatching peak of larvae was after 24 hours of development from the end of the 2 hour egg laying period. Usually larvae hatched from 90% of the eggs in control plates. The age of development (in hours) was calculated from the midpoint of the 2 hour egg laying period.

After collection or aging, eggs were washed extensively with water in a siliconized beaker, rinsed twice with Triton X-NaCl solution (0.01% Triton X − 0.7% NaCl), collected on a nylon mesh filter (65 μ), fitted on a Millipore apparatus, and washed again with Triton X-NaCl, water, and 10 ml of 0.05 M glycylglycine, pH 7.5 containing 10% glycerol. The eggs were air dried for a few minutes, weighed and quick frozen in dry ice-acetone. The wet weight of collected eggs representing any particular stage ranged from 0.3-1 g. However, within a single day's experiment, the relative quantity of eggs collected for the various developmental stages was similar. When 7-8 or 16-18 hour periods were used for the collection of eggs, the wet weight was usually 1-2 g. Frozen eggs were stored at −60°C for 4-7 days before enzyme isolation. For collection of young 1st instar larvae, the oviposited eggs were washed quickly as described above, then transferred from the nylon filter to a beaker containing a small amount of water and distributed immediately with a bristle brush to petri dishes (100 or 150 mm diameter) containing sugar-agar medium with dead Fleishman's yeast (4% sucrose, 2% agar, 1% yeast) at a density of about 1000-2000 eggs per petri dish respectively. After 27 hours from the end of the 2 hour egg laying period, 1st instar larvae were harvested with a brush and washed three times in a beaker at which time the floating egg skins were decanted. Larvae were then collected and washed on a nylon filter as described for eggs. Control samples taken from the filter showed that 85-88% of the larvae had hatched by this time. Freezing and storage was the same as for eggs. The wet weight of larval preparations ranged from 0.45-0.55 g.

For collection of unfertilized eggs, female virgins were mated with sterile XO males to facilitate oviposition (Schultz, 1956). This was accomplished by mating wild-type females with males of a compound X • Y genotype, namely Y^S • X • Y^L. The F_1 X • X • Y females and XO males were then mated and eggs were collected for 2 hour periods. Only 3% of the collected eggs in control petri dishes showed development. The unfertilized eggs were washed and frozen as already described for fertilized eggs.

DNA Substrates

^{3}H-DNA from PM2 phage was prepared as previously described (Wallace *et al.*, 1981). DNA concentrations for different preparations ranged from 4.5-25 μg per ml.

Specific activities ranged from 31,000-56,000 cpm/μg. For x-ray damaged DNA substrates, ^{3}H PM2 DNA (33-83μl) was irradiated in 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 50 mM KI in air at room temperature by a Phillips x-ray generator having a beryllium window tube operated at 60 kVp and 2.5 mA. The dose rate ranged from 15.5 to 23 krad/min. Apurinic ^{3}H PM2 DNA substrate was prepared by heating at acid pH as described by Lindahl and Andersson (1972), to induce from 1-2 apurinic sites per PM2 molecule as determined by exposure to alkaline conditions (glycine buffer, pH 13) for four hours followed by sedimentation analysis on alkaline sucrose gradients. UV irradiation was carried out with a General Electric germicidal lamp powered through a Sola constant voltage regulator. The intensity was 2.4 $Jm^{-2}sec^{-1}$, as determined by a Blak-Ray ultraviolet dosimeter.

Endonuclease Reactions

Aliquots (0.2 μg) of treated or untreated ^{3}H PM2 DNA were incubated with or without Drosophila enzyme in a 50 or 100 μl reaction mixture containing 50 mM Tris, pH 7.5; $MgCl_2$, usually 0.25 mM or as specified in the figure legends. In assays with x-irradiated DNA, additional Mg^{++} was added to the reaction mixture in order to overcome chelation by EDTA present in the substrate. In some reactions, but not in developmental studies of embryonic stages, calf thymus DNA was added as competing DNA. The amount added was dependent on the amount of enzyme protein in the reaction and was kept at a constant ratio of either 6:1 or 2.5:1 μg of DNA to enzyme protein. Enzyme dilutions were always made in a 20-25% glycerol buffer, 50 mM with respect to glycyl-glycine pH 7.5, and 1 mM with respect to dithiothreitol. The reaction was incubated at 37° for 10 minutes and terminated by the addition of 4 M KCl to a final concentration of 1 M. Usually, EDTA was added to chelate all the Mg^{++} before the reaction was quick frozen and stored at -60°C in preparation for sucrose gradient sedimentation analysis. A control for nonspecific endonuclease activity was run for each reaction using untreated PM2 DNA as the substrate while keeping the rest of the conditions constant.

The endonucleolytic activity was assayed by the proportion of Type I PM2 DNA converted to the nicked Type II DNA determined by sedimentation analysis as previously described (Wallace *et al.*, 1981). The data were normalized to the treated DNA without enzyme and corrected for any non-specific endonuclease activity.

Other Methods

DNA agarose was prepared by a modification of the method of Schaller *et al.* (1972), substituting 4% w/v agarose for 8%. Protein determinations were carried out with the Biorad Protein Assay (Biorad Laboratories).

Enzyme Isolation

The procedures to be described are essentially the same whether enzyme isolation was performed using relatively large quantities of eggs 1-18 hours of age or smaller quantities of embryos or larvae of more defined developmental age.

For enzyme purification, 5-7 grams of eggs, 1-18 hours of age when frozen, were suspended in four parts w/v of extraction buffer containing 50 mM glycylglycine pH 7.5, 0.35M sucrose, 5 mM $MgCl_2$, 0.5 mM EDTA and 1 mM dithiothreitol (Buffer A) and homogenized with 10-12 strokes in a glass Teflon grinder. The homogenate was spun at 300 g for 10 minutes and the supernatant spun again at 1,500 g for 15 minutes. The pellet, constituting a crude nuclear fraction, was resuspended in 50 mM glycylglycine, pH 7.5, 5 mM $MgCl_2$, 10% glycerol and 1 mM dithiothreitol (Buffer B) and sonicated with a microtip in a Branson Sonicator at 50% pulse, setting 3 for four 20-second periods. The sonicated nuclear fraction and the supernatant cytoplasmic fraction were spun separately at 27,000 g for 30 minutes in order to remove lysosomes, and the supernatants were subsequently filtered through glass wool. Protein determinations were done routinely on each fraction to check the efficiency of extraction. These were typically 3 and 17 mg/ml for the nuclear and cytoplasmic fractions respectively. Both fractions were then pooled (Fraction I), adjusted to 10% glycerol, 2 mM EDTA and 1 M KCl, and spun at 180,000 g for 75 minutes. To the supernatant (Fraction II), PEG 6000 was added to a final concentration of 10% w/v to precipitate nucleic acids (Alberts and Herrick, 1971). Fraction II was spun for 30 minutes at 12,000 g. The supernatant, Fraction III, which had a little more than one half of the protein concentration of Fraction II, was applied to a DNA agarose column (see below).

For the developmental studies, 0.3-1 g of eggs of various developmental stages, or of larvae, were homogenized in the same way, except that aprotonin, a protease inhibitor, was added to the homogenization buffer at a concentration of 25 μl/ml. Moreover, after it was established that the AP endonuclease activity found in Fraction II of the various embryonic stages studied was the same in the presence or absence of the nuclear fraction, only the cytoplasmic fraction was used for further enzyme purification. Fraction I therefore became the supernatant obtained from the 27,000 g spin following homogenization. Protein concentrations were performed routinely on Fraction I; these ranged from 13-18 mg/ml for embryonic stages in different preparations, and from 11.5-13 mg/ml in larval extracts; these values were quite reproducible in different experiments. Purification through Fraction III was as described above; the only egg stage that was not purified beyond Fraction II was that involving unfertilized eggs, as discussed in the Results.

DNA Agarose Chomatography

Fraction III was diluted with Buffer C (50 mM glycylglycine, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol) to a KCl concentration of 0.15 M and immediately loaded onto a DNA agarose column previously equilibrated with Buffer C in 0.15 M KCl. The column bed volume was about twice the weight of eggs being processed (10 ml column for 5-7 g of eggs) and the flow rate ranged from one to two column volumes per hour. After washing the column with at least five column volumes of equilibration buffer, the enzyme was eluted in a stepwise fashion with Buffer C containing 0.3, 0.45, 0.6 and 1 M KCl at a flow rate of 1.2 column

volumes per hour. Four fractions of one half column volume each were collected at each salt concentration (Fraction IV) into tubes containing 80% glycerol in Buffer C such that each fraction contained a final concentration of 25% glycerol. Aliquots were removed for conductivity measurements and for enzyme assays and the remainder of each fraction was concentrated by dialysis against Buffer C made 20% or 30% w/v with respect to PEG 20,000. The concentrated fractions were adjusted to 50% glycerol and stored at −20°C. Protein concentrations in these fractions ranged from 67-292 μg/ml before concentrating and from 225-850 μg/ml after concentrating in PEG. The stored enzyme was stable in this state for many months without significant loss of activity.

Once the elution pattern on DNA agarose was determined (Figure 1), a somewhat modified procedure was used for the elution of AP endonuclease from the preparations of various developmental stages. Here, Fraction III was diluted in Buffer C with 20% glycerol to a 0.15 M KCl concentration, loaded onto a DNA agarose column at a flow rate of three column volumes per hour and eluted at the same flow rate with three and a half column volumes of Buffer C in 1 M KCl into one tube containing 80% glycerol buffer. Biorad protein determinations showed that 97% of the protein bound to DNA agarose was eluted from the column in this manner. The total amount of protein applied to a column from various embryonic stages was usually in the range of 20-30 mg, but as little as 9 mg of Fraction III could be chromatographed successfully using a bed volume of DNA agarose roughly twice the gram amount of eggs collected and homogenized. Consistently, 3-4% of the applied protein, irrespective of the stage studied, bound to DNA agarose and was subsequently eluted with the high salt buffer. In the case of larval Fraction III preparations, 13-15 mg of protein were applied to DNA agarose. The protein concentrations of the resulting DNA agarose eluates ranged from 60-250 μg/ml before concentration and from 140-540 μg/ml after concentration and dialysis in PEG.

RESULTS

Partial purification of apurinic endonuclease activity: Fraction II, obtained after ultracentrifugation in 1 M KCl, contained an endonuclease activity capable of incising x-irradiated (0.34 breaks/molecule), apurinic (0.7 breaks/AP site) and, to a lesser extent, UV-irradiated DNA (0.05 breaks/dimer) (data not shown). No AP endonuclease activity was detected in the crude extracts (Fraction I) in the presence of 5 mM Mg^{++} due to the high levels of non-specific endonuclease activity. Apurinic DNA was nicked by Fraction I when the Mg^{++} concentration was lowered to 0.25 mM (see Figure 2); the AP activity measured under these conditions was about 50-60% of the activity found in Fraction II.

In Figure 1 it can be seen that the bulk of the endonuclease active on apurinic, x-irradiated or UV-irradiated DNA eluted from DNA agarose between 0.18 and 0.4 M KCl (Fraction II). Two small peaks of activity were seen at about 0.6 M KCl with x-irradiated and UV-irradiated DNA. Given the broad elution profile, typical of DNA

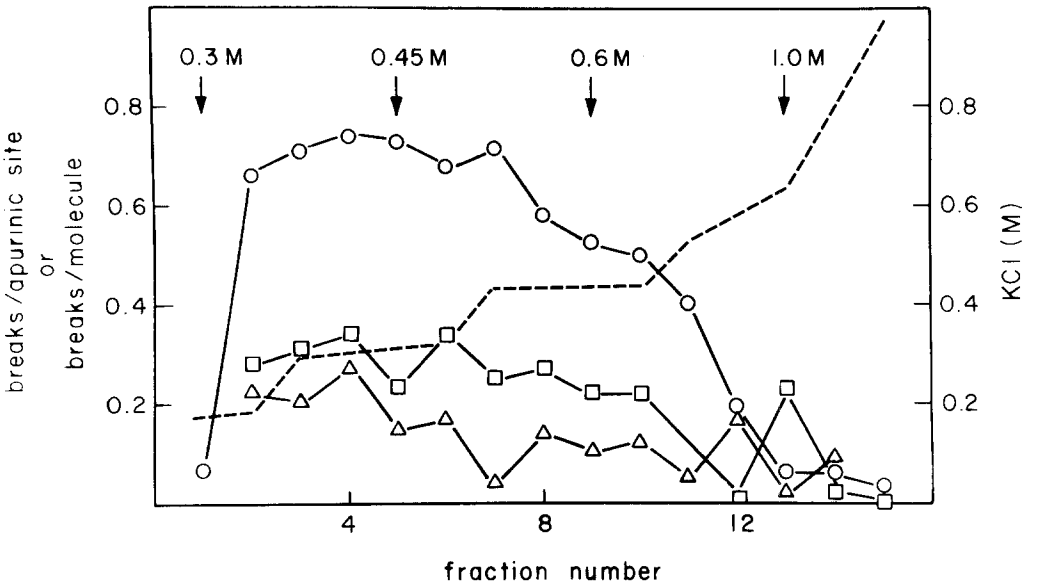

FIGURE 1. DNA Agarose Chromatography. Endonuclease fractions (5 μl) were added to reaction mixes containing 0.2 μg apurinic DNA, 1.6 AP sites/molecule (○), 1.25 mM $MgCl_2$ and 5.5 μg calf thymus competing DNA. Reactions with 0.2 μg x-irradiated DNA, 23 krads (□), or 0.2 μg UV-irradiated DNA, 1,300 Jm^{-2} (◇), contained 1.25 mM $MgCl_2$, 11 μg competing calf thymus DNA and 10 μl of each Fraction. In all reactions through Fraction 12, KCl was adjusted to a final concentration of 60 mM. All values were corrected for controls with untreated DNA with or without enzyme, and for treated DNA without enzyme. Untreated DNA typically had 0.1 breaks per molecule; there was no change in UV-irradiated DNA without enzyme, whereas apurinic DNA and x-irradiated DNA had 0.3 and 0.5 breaks/molecule respectively. The nonspecific activity against untreated DNA ranged from 0.1-0.14 breaks/molecule per 5 μl enzyme fraction and from 0.03-0.24 breaks/molecule per 10 μl enzyme. The untreated DNA used as a control for apurinic DNA activity was in 1 mM EDTA, 10 mM Tris instead of 10 mM sodium citrate, 100 mM NaCl. Enough Mg^{++} was added to overcome chelation by 0.2 mM EDTA in these reactions. The dasked line represents KCl concentration.

agarose affinity chromatography, it cannot be determined whether the endonuclease activity measured here represents one or more enzyme species. It should be noted that a similar pattern of endonuclease activity on apurinic DNA was observed when the PEG concentrated Fraction IV eluates were assayed with constant subsaturating amounts of protein. We have estimated the degree of purification of the AP endonuclease in Fraction IV to be a roughly forty fold, with a 52% recovery of the enzyme protein.

Requirements for activity and reaction optima: With AP DNA, the endonuclease activity (Fraction IV) exhibited a very broad pH optimum with maximum activity observed between pH 6 and 8.5. The pH optimum with x-irradiated DNA was similar.

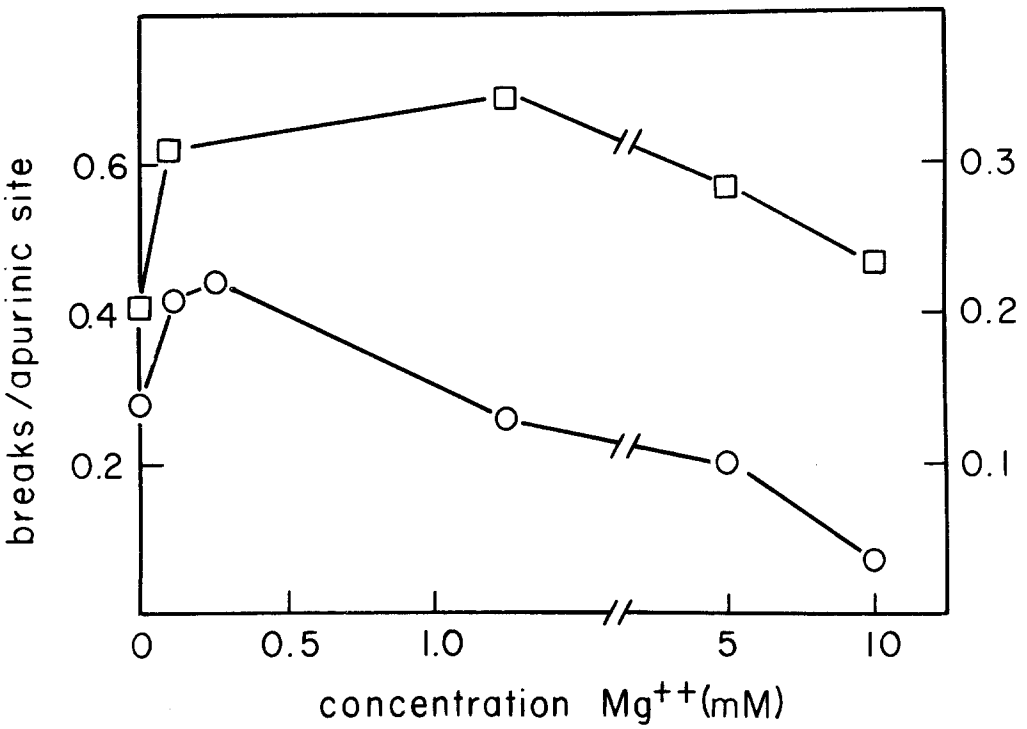

FIGURE 2. Effect of $MgCl_2$ on Endonuclease Activity. Samples 0.5 μg (□) left ordinate, or 0.25 μg (○) right ordinate, of the 0.34 M eluate, Fraction IV, were added to apurinic DNA (1 site/molecule) respectively in reaction mixes as described in the text and Figure 1.

One of the notable features of the Drosophila apurinic endonuclease activity is that it is not absolutely dependent on the presence of Mg^{++} cation (Figure 2); in fact 5 mM and higher concentrations of Mg^{++} were inhibitory. At low protein concentrations even 1.25 mM Mg^{++} was inhibitory. Optimum activity was obtained with 0.1 mM Mg^{++} which enhances the nicking of apurinic DNA by about 40%.

As can be seen in Table I, 100 mM NaCl inhibited the endonuclease activity by 84%. Low concentrations of EDTA (0.1 mM) were almost completely inhibitory as was 10 mM NEM, a sulfhydryl blocking reagent. $CaCl_2$ at a 2 mM concentration caused an 81% inhibition of the enzyme activity. The effect of EDTA and Ca^{++} can perhaps be explained on the basis of chelation of and competition with Mg^{++} respectively, if Mg^{++} were bound endogenously to the enzyme. On the other hand, EDTA might also be chelating another essential cofactor such as Zn^{++}. The effect of NEM points to the importance of sulfhydryl groups in the activity of the enzyme.

Substrate specificity: The endonuclease activity has been shown to attack three different substrates (Figure 1); of these, apurinic DNA was most efficiently nicked. The saturation kinetics with an apurinic substrate containing 1.4 lesions per molecule are shown in Figure 3. The partially purified endonuclease saturated this substrate at 0.5 μg of protein. The nonspecific activity was relatively low and increased less

TABLE 1
Inhibition of Drosphilia Endonuclease by NaCl, EDTA, N-ethylmaleimide and $CaCl^2$

For the EDTA and NEM reactions, samples (0.5μ) of the 0.32 M eluate, Fraction IV, were added to reaction mixes containing apurinic DNA (1 site/molecule), previously dialyzed against 10 mM Tris pH 7.5. Similarly 0.23 μg and 0.25 μg of the 0.32 M eluate, Fraction IV, were used for the $CaCl^2$ and NaCl reactions respectively. The Mg^{++} concentrations were 0.1 mM for NEM and 0.25 mM for the NaCl and $CaCl^2$ reactions.

Reaction Parameters	Breaks per Apurinic Site
Complete system	0.5
Complete plus 100 mM NaCl	0.08
Complete system	0.62
Complete plus 0.1 mM EDTA	0.04
Complete plus 10 mM NEM	0.05
Complete system	0.37
Complete plus 2 mM $CaCl^2$	0.07

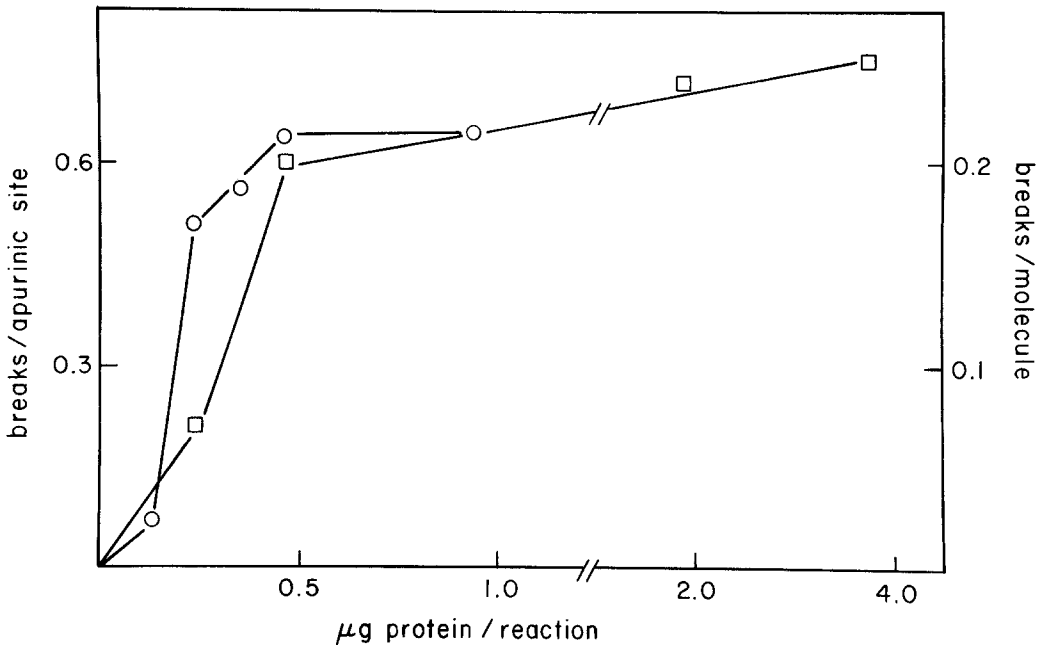

FIGURE 3. Comparative Saturation Kinetics Between X-irradiated and Apurinic DNA Substrates. Samples of the 0.3 M eluate, Fraction IV, were added to x-irradiated DNA, 22 krads (□) right ordinate, 0.65 mM $MgCl_2$; or apurinic DNA, 1.7 sites/molecule (○) left ordinate. 0.25 mM $MgCl_2$; in reaction mixes as described in the text and Figure 1. Nonspecific activity ranged from 0.03 to 0.1 breaks/molecule; the specific activity shown was corrected for nonspecific activity.

steeply at higher protein concentrations. Figure 3 also shows a comparison of the saturation kinetics between x-irradiated and apurinic PM2 DNA substrates. The reaction approached saturation with the x-irradiated substrate at the same protein concentration as with the apurinic substrate. These studies suggest that there is no unproductive binding of the enzyme to other lesions such as single-strand breaks in the x-irradiated DNA. Activity on uv-irradiated DNA can be accounted for by AP lesions produced by the high doses used to make the substrate.

To investigate the nature of the lesions that are recognized by the enzyme activity in x-ray-damaged DNA, irradiated DNA, with or without prior incubation with endonuclease, was analyzed by neutral and alkaline sucrose gradient sedimentation following exposure in the latter case to pH 13 for 14 hours. This alkaline treatment results in a quantitative conversion of all apurinic and apyrimidinic sites to breaks (Lindahl and Andersson, 1972). The number of breaks per molecule determined under alkaline conditions in x-irradiated DNA was the same in the presence or absence of enzyme (Table 2).

Thus, the Drosophila endonuclease activity does not seem to recognize any lesions other than alkali-labile sites in x-irradiated DNA. Moreover, the enzyme-sensitive sites constituted 57% of the alkali-labile sites under conditions where the ratio of alkali-labile sites to single-stranded breaks was 1:3. This laboratory has obtained similar results with a partially purified AP endonuclease from *Saccharomyces cerevisiae* (Armel and Wallace, 1978).

Apurinic Endonuclease Patterns During Development

In order to make a valid comparison of the relative levels of AP endonuclease activity during the course of Drosophila development, partial purification through Fraction IV was carried out from eggs at each of the embryonic stages studied, as well as from early larvae. The particular ages of embryos were selected in order to compare endonuclease levels during developmental periods which differ greatly with respect to mitotic activity. For example, early in stage 1, eggs are undergoing the most rapid

TABLE 2
Recognition of Alkali-Labile Sites in X-irradiated DNA by Drosphilia Endonuclease

Samples (4 μg) of 0.32 M eluate, Fraction IV were added to reaction mixes containing x-irradiated DNA (15.5 Krad), 1.25 mM $MgCl_2$. Other conditions are as described in text. Reactions to be analyzed on alkaline sucrose gradients were stopped by the addition of 10 μl 200 mM EDTA.

			Breaks per Molecule	
	Neutral Gradients	**Alkali Gradients**	**Alkali-Labile Sites**	**Enzyme-Sensitive Sites**
X-irradiated PM2 DNA	0.32	0.74	0.42	—
X-irradiated PM2 DNA plus Enzyme	0.56	0.76	—	0.24

cleavage divisions recorded, 10 minutes per mitotic cycle; cells continue to actively divide until about twelve hours, at which time mitoses all but cease (Sonnenblick, 1950). The partial purification of the AP endonuclease was essential to allow resolution of the AP activity from contaminating non-specific nuclease activity which greatly increases in late embryos and larvae.

Preliminary experiments had shown that although the amount of AP endonuclease was easily measured using the cruder Fraction II from unfertilized eggs and from embryos up to thirteen hours of development, the reaction with Fraction II from older embryos and from larvae required the addition of calf thumus DNA to compete for nonspecific nucleases. Figure 4A illustrates the levels of Fraction IV AP endonuclease activity at various stages of development and compares the nonspecific nuclease activity at these stages. Figure 4B shows the levels of the nonspecific activity before DNA agarose chromatography. The nonspecific activity was reduced seven fold and eleven fold in embryonic Stages 3 and 4 respectively and fourteen fold in early larvae. Thus, purification by DNA agarose allowed for a clear comparison of the AP endonuclease levels per unit amount of protein (0.1 μg) during the course of embryonic development without the addition of competing calf thymus DNA. Further, since the amount of protein recovered in Fraction IV (3-4%) per mg protein present in Fraction I was constant in embryonic Stages 1 through 4, the AP endonuclease activity depicted in Figure 4A may be expressed per egg. The determination per 0.1 μg protein of Fraction IV represents the enzyme activity recovered from about 10 eggs (1 egg weighs 10 μg). These data show that although small fluctuations in enzyme levels were observed during development, the total AP endonuclease activity measured under the reaction conditions defined here remained essentially unchanged during the five periods of the life cycle of Drosophila which we have studied.

We were also interested in comparing the levels of this presumptive repair activity before and after fertilization. Since in unfertilized eggs and early embryos the amount of non-specific nuclease activity was negligible, the AP endonuclease activity could be determined from Fraction II. Figure 5 shows protein saturation curves before and after fertilization; the level of AP endonuclease activity in unfertilized eggs was about half of that found in Stage 1 embryos.

Figure 6A and B show the saturation kinetics using an apurinic DNA substrate with Fraction IV obtained from three embyronic stages (Stages 1, 2 and 4) and from early larvae (Stage 5) using enzyme preparations from two different experiments. In all cases, the levels of enzyme protein required for saturation (or near saturation) were similar.

In order to survey AP endonuclease activity in later stages of Drosophila development, extracts prepared from third instar larvae and pupae were processed through DNA agarose chromatography. However, specific AP endonuclease activity could not be detected in these stages because of the high level of nonspecific activity that still remained in Fraction IV. Further purification is required for the analysis of enzyme levels from these stages.

DISCUSSION

The properties exhibited by the Drosophila AP endonuclease activity reported here do not appear to be unique. The enzyme recognized AP lesions in x-irradiated DNA and apurinic sites in depurinated DNA. The activity, like most other eukaryotic AP

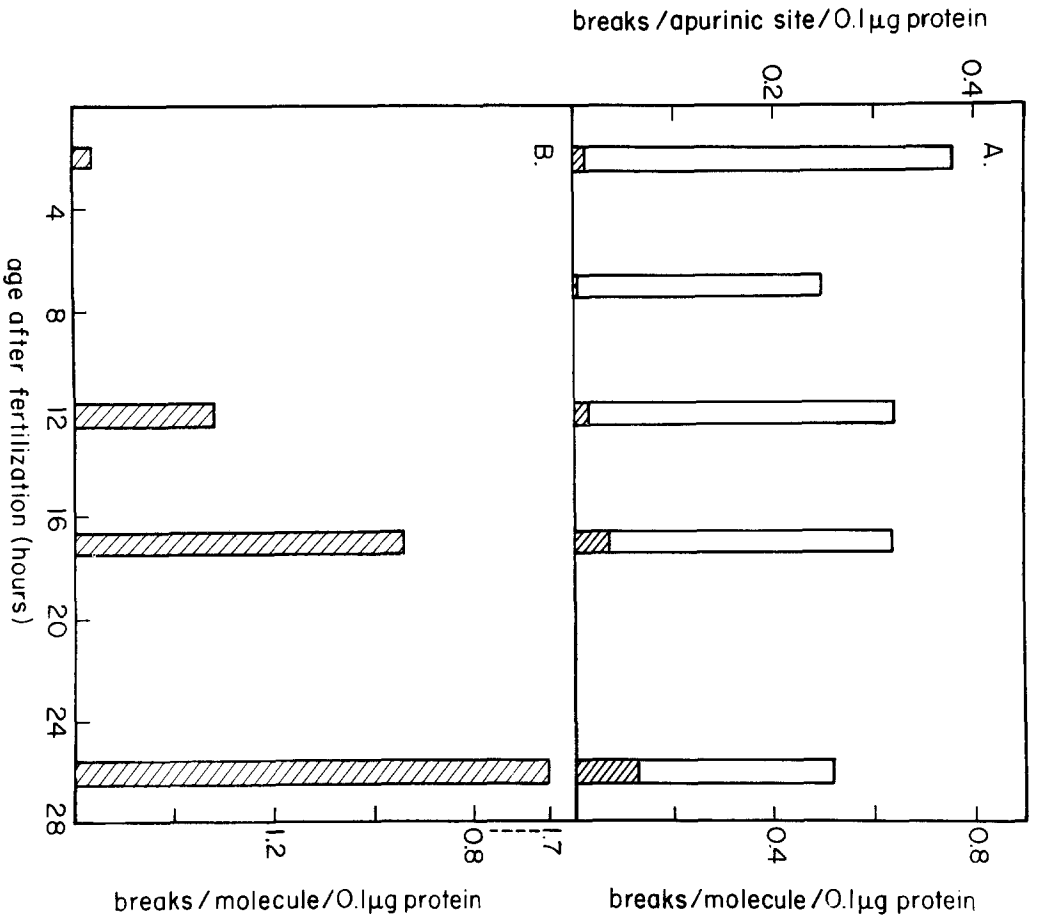

FIGURE 4. Patterns of Partially Purified AP Endonuclease and Nonspecific Endonuclease Activity During Development.

A. Dialyzed and concentrated Fraction IV was assayed using protein concentrations ranging from 0.05-0.4 μg per 50 λ reaction in the presence of 0.25 mM Mg^{++}. Data from three different experiments were pooled and the activities averaged in the linear range of the reaction. Apurinic DNA ranged from 1.4-2.1 sites/molecule in different experiments. Nonspecific activity was computed in the same way, except that untreated PM2 DNA was used as a substrate. Clear bars represent specific activity in breaks/AP site, hatched bars the nonspecific activity in breaks/molecule.

B. Fraction II (2 μg protein) from each stage was assayed under the same conditions as in A using untreated PM2 DNA (0.2 μg) except that the larval Fraction II was assayed in the absence of Mg^{++} and in the presence of 34 μg of calf thymus DNA.

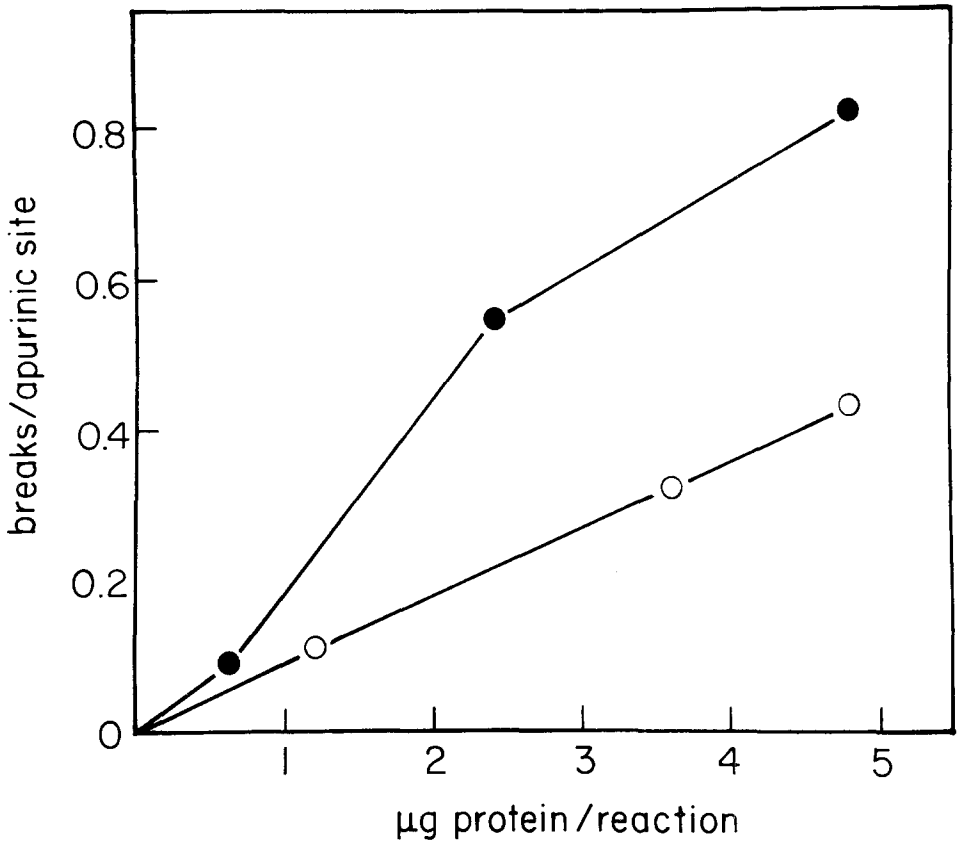

FIGURE 5. AP Endonuclease Activity From Unfertilized Eggs and Stage 1 Embryos as a Function of Protein Concentration.

Fraction II from unfertilized eggs (○) and from early embryos (●) was assayed in a 100 λ reaction under the same conditions as in Figure 4A, except competing calf thymus DNA was added in a ratio of 2.2:1 of DNA to protein assayed. AP DNA had 1.8 sites/molecule.

endonucleases previously isolated, was stimulated by Mg^{++}, inhibited by EDTA, and had a fairly broad pH optimum above neutral. It is unusual, however, for an AP endonuclease to be as inhibited by relatively low concentrations of Mg^{++} as was this one. One would suspect Drosophila to have more than one AP endonuclease, but it is possible that some are not strong DNA-binding proteins, as were the ones selected for here. Spiering and Deutsch (1981) have demonstrated two Drosophila AP endonuclease activities chromatographically separable on phosphocellulose. We have recently resolved the AP endonuclease activity described here into three peaks by phosphocellulose chromatography (results not shown). Thus the AP activity measured in these developmental studies represents the AP repair potential of at least three enzymes or isozymes.

It is interesting that the Drosophila AP endonuclease activity is recovered primarily from the cytoplasm. Thebodeau and Verly (1980), utilizing rat liver, showed that 90% of the apurinic endonuclease activity was associated with the nucleus. One possible explanation for this difference is that in rat liver the AP precursor enzyme,

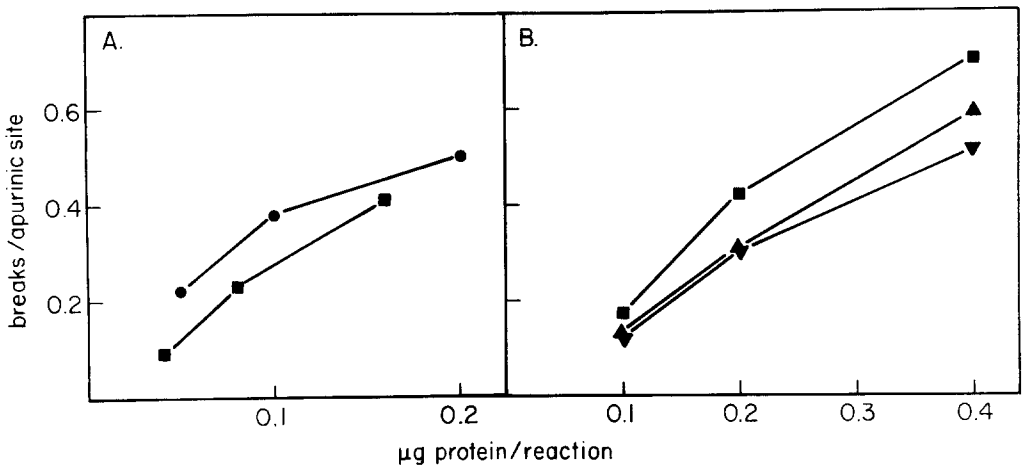

FIGURE 6. AP Endonuclease Activity During Embryonic and Larval Development as a Function of Protein Concentration.

In two different experiments, Fraction IV was assayed in a 50 λ reaction as in Figure 4A, using apurinic DNA with 1.4 and 1.8 sites/molecule in A and B respectively. In all three experiments specific activity is shown after correcting for nonspecific activity. Symbols: ● Stage 1; ■ Stage 2; ▲ Stage 4; ▼ Stage 5.

presumably present in the cytoplasm, is inactive on AP DNA while in the case of Drosophila either the cytoplasmic enzyme is the active enzymic species or it is an active nuclear precursor.

The potential assignment of a particular gene for the Drosophila AP endonuclease has been faciliated by the isolation of a number of mutagen-sensitive, presumptive repair-defective Drosophila mutants (for review see Smith *et al.*, 1980). Recently, Boyd's laboratory (Osgood and Boyd, 1982) has shown a reduced level of AP endonuclease activity in crude extracts derived from several tissues of *mei-9* and *mus(2)201* repair defective mutants. It remains to be shown whether the activity measured by Boyd in the crude form is the same as the more purified enzyme activity reported here.

The specific activity of the partially purified AP endonuclease, which was monitored from one hour after fertilization through several stages of embryonic development as well as to first instar larvae, showed very little variation. The only substantial difference was detected in unfertilized eggs which had about one half of the activity found shortly after fertilization. It is possible that this latter difference may be due to the genotypically different females used for the oviposition of unfertilized eggs, since the wild-type strain was outcrossed in order to obtain these XX•Y females which are heterozygous for a number of X-linked markers associated with the compound X•Y chromosome. However, it is more likely that the decreased level of AP endonuclease activity before fertilization is a developmental phenomenon, since the genotype of the XX•Y females is not associated with any known DNA repair defect.

O'Brien and MacIntyre (1978) have raised questions as to the validity of comparing enzyme levels at various developmental stages on the basis of specific activity since the total soluble protein level changes during the course of the life cycle of Drosophila. This is particularly true of stages such as puparium, later pupa and the adult stage when the amount of soluble protein fluctuates. However, we have investigated AP endonuclease levels in development over a much shorter period of time when the total protein concentrations showed very little variability. Moreover, the purification by DNA agarose chromatography resulted in very similar proportions of protein recovery vs. protein bound to the column for each stage analyzed, indicating that differential loss or recovery of protein is probably not a factor in our results.

The question also arises as to whether our findings can be correlated with the relative radiosensitivity of Drosophila embryos during the course of development. Such studies carried out up to nine hours of embryogenesis showed that 1-2½ and 4½-5½ hour-old embryos are most sensitive to x-irradiation (Bownes and Summell, 1977; Wurgler and Ulrich, 1976). Since the specific activity of the AP endonuclease activity measured here remained fairly constant during this time period, an additional observation merits attention. Our comparative study is based on expressing enzyme activity per unit protein. However, the DNA concentration per egg increases sharply during development, rising from 2×10^{-4} μg/egg in unfertilized eggs to 140×10^{-4} μg/egg in late embryos (Margulies and Chargaff, 1973). If the AP endonuclease activity were expressed as a function of DNA concentration, the ratio of repair enzyme to substrate would indeed be much higher in unfertilized eggs and early embryos than in later stages. It is also possible that the sum of AP endonuclease activities measured here is masking fluctuations of a particular AP endonuclease(s) that specifically functions in repair during development and might thus correlate with radiosensitivity.

In summary, the high constant level of AP endonuclease activity as measured per μg protein, which is present before fertilization, during embryogenesis and early larval development, is consistent with the idea that such presumptive repair enzymes are needed at high levels in the germ cells and during embryogenesis to protect the integrity of the genetic material.

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