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Repair of depurinated DNA *in vitro* by enzymes purified from human lymphoblasts

(methyl methanesulfonate/apurinic endonuclease/exonuclease/excision/bacteriophage T7)

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ABSTRACT **Alkali-labile lesions introduced into T7 DNA by treatment with methyl methanesulfonate were removed and the DNA was repaired by incubation with DNA polymerase α and nuclease from a human lymphoblastoid line followed by the addition of DNA ligase. The nuclease preparation contains both apurinic endonuclease and 5′-3′ exonuclease activities. Dinucleotides appear to be the first product of exonuclease action. Repair of methyl methanesulfonate-induced damage can occur by the insertion of only a few nucleotides per lesion as *in vivo*.**

Two different pathways for DNA excision repair have been reported in mammalian cells. Nucleotide excision repair (1), e.g., of UV-induced damage, results in the insertion of "patches" of about 100 nucleotides in human cells (2). In contrast, repair of methyl methanesulfonate (MeMes)-induced damage by the apurinic excision repair pathway results in patches of three to four nucleotides (2, 3). The reason for this difference in patch size is not known, but it seems reasonable to suppose that the different pathways use enzymes with different processive character (4). To study the factors involved in determining patch size, we are attempting to reconstruct repair systems *in vitro* that duplicate the *in vivo* characteristics of a particular repair pathway. In this paper we describe a system that carries out small patch apurinic repair using nuclease and polymerase activities derived from a human lymphoma cell line.

MATERIALS AND METHODS

Cell Culture. The human Burkitt's lymphoma line Daudi (5), obtained from a biopsy and provided by G. Klein, was adapted to spinner culture and grown in RPMI 1640 medium with 16% fetal calf serum (GIBCO) to a cell density of 1–1.5 × 10^6/ml. Cells were collected, washed, and stored at −70°.

Partial Purification of DNA Polymerase α and Nuclease Activity. Daudi cells (100 g) in 20 mM potassium phosphate, pH 7.4/10 mM 2-mercaptoethanol/20% glycerol (buffer A; all phosphate buffers were the potassium salt at pH 7.4; all contain mercaptoethanol and glycerol unless so noted) were disrupted in a nitrogen bomb. The cell supernatant was precipitated with ammonium sulfate and the precipitate (30–75% saturation) was dialyzed against buffer A and applied to a 2.5 × 26-cm DEAE-cellulose column (DE52) to remove nucleic acid. The column was equilibrated and eluted with 0.2 M phosphate. The protein peak fractions were dialyzed against buffer A, applied to a hydroxylapatite column [2.5 × 13.5 cm, Bio-Gel HTP (BioRad)], and eluted with a linear 0.02–0.5 M phosphate gradient. Pooled fractions containing polymerase activity were concentrated with polyethylene glycol 6000 and chromatographed on Sephadex G-100 (1.5 × 110 cm) with 0.1 M phosphate. The DNA polymerase peak fractions were combined, dialyzed (50 mM phosphate), applied to a small (6-ml bed volume) hydroxylapatite column, and eluted with 0.05–0.3 M phosphate. Pooled peak fractions were applied to 6 ml of DNA-agarose (6) equilibrated with 10 mM phosphate/20 mM KCl/10 mM 2-mercaptoethanol/10% glycerol and eluted with a linear 0.02–0.3 M KCl gradient in the same buffer. After elution at about 0.2 M KCl the enzyme was concentrated by dialysis against 60% glycerol/10 mM potassium phosphate, pH 7.4/100 mM KCl/10 mM 2-mercaptoethanol and stored at −20°. Identification of enzyme as an α polymerase (7) was on the basis of size (>100,000) and sensitivity to *N*-ethylmaleimide. Activity (8) at the time of these experiments was about 200 units/mg of protein.

The apurinic endonuclease plus 5′-3′ exonuclease activity was purified from the same Daudi cell extract used for polymerase preparation. Purification was followed by an assay in which MeMes-treated DNA, α polymerase, and a mixture of nucleoside triphosphates were incubated with nuclease. The combination of endo- plus exonuclease activity converts apurinic sites in MeMes-treated DNA into a polymerase substrate (see below). Eluates from the first hydroxylapatite column (above) were assayed for nuclease and the peak fractions were concentrated against polyethylene glycol 6000 and chromatographed on a 1.5 × 110-cm Sephadex G-100 column with 0.1 M phosphate. Separate calibration of the column indicated that nuclease activity was eluted at molecular weights of 50,000–60,000. Nuclease-containing fractions were pooled, dialyzed against 75 mM phosphate, applied to a hydroxylapatite column (5 ml packed bed volume), and eluted with a 0.075–0.35 M phosphate gradient. Peak activity fractions were pooled, concentrated by dialysis against 0.1 M phosphate/10 mM 2-mercaptoethanol/65% glycerol and stored at −20° for use as nuclease in these experiments. Two protein bands were observed on sodium dodecyl sulfate/polyacrylamide gel electrophoresis of 15 μg of this preparation. A detailed description of the enzyme purifications will be published elsewhere.

DNA ligase prepared from bacteriophage T4-infected *Escherichia coli* (9) was a gift of K. Agarwal. The enzyme was further purified by passage through a single-strand DNA-agarose column (6) before use to remove polymerase and nuclease activities.

Sucrose Gradients and Determination of Molecular Weight. Alkaline sucrose gradient centrifugation was as described (10). Centrifugation was at 4° except for a 15-min incubation at room temperature to hydrolyze apurinic sites (10).

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Abbreviation: MeMes, methyl methanesulfonate.

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Number average molecular weights (11) were calculated from the relationship $M_n = \Sigma c_i / \Sigma(c_i/M_i)$, in which c_i is the radioactivity in the ith fraction and M_i is calculated from the relationship $M = k(d/\omega^2 t)^{1/a}$ using the value of a determined by Studier (12) and k, a constant determined by calibration of a gradient with intact phage T7 DNA.

Preparation and Alkylation of Phage T7 DNA. Coliphage T7 was grown on *E. coli* strain BB as described (13). [^{3}H]DNA was prepared by addition of [^{3}H]thymidine (Schwarz Bioresearch Inc.) to a final concentration of 2 μCi/ml, 2 min after infection at a multiplicity of infection of 1. DNA was prepared by exhaustive phenol extraction of a phage suspension purified through a CsCl gradient and alkylated as described (14). T7 DNA (500–800 μg/ml) was incubated at 37° with an equal volume of 25 mM MeMes in 50 mM phosphate buffer, pH 7.4. After the desired reaction time, the mixture was diluted 1:10 with ice-cold 50 mM phosphate buffer, pH 7.4, and dialyzed overnight against the same buffer. The DNA solution was then concentrated with *sec*-butanol (15) to approximately 200–500 μg of DNA per ml.

Since the exact level of alkylation is difficult to control, we measured breaks and apurinic sites directly on a DNA alkylated for 10 min as described and then heated for 1 hr at 70°. Incubation in alkali at 37° gives the total number of apurinic sites plus breaks. Treatment with S1 nuclease followed by neutral sucrose sedimentation (4) or formamide denaturation followed by neutral sucrose sedimentation in buffer containing formaldehyde (14) with intact T7 [^{3}H]DNA standard gives the total number of DNA breaks since apurinic sites are resistant to both treatments. We found that this particular substrate had 16 alkali-labile (apurinic) sites and 0.3 break per T7 strand measured by either formamide denaturation or S1 nuclease treatment.

Preparation of 5′-^{32}P-Labeled T5 DNA (16). Coliphage T5 DNA was a gift of S. Desi and S. B. Weiss. The 5′ termini were removed by incubating 500 μg of T5 DNA in 10 mM Tris·HCl, pH 7.85/50 mM NaCl with 10 units of bacterial alkaline phosphatase (P. L. Biochemical) for 30 min at 65°. After 30 min another 5 units of alkaline phosphatase were added to the reaction mixture and the incubation was continued for 30 min. After two extractions with buffer-saturated phenol, this DNA was labeled at the 5′ termini with polynucleotide kinase (T4-infected *E. coli*) and [γ-^{32}P]ATP. The reaction mixture (1 ml) contained 175 μg of phosphatase-treated T5 DNA, 12 mM 2-mercaptoethanol, 20 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 10 nmol of ^{32}P (2.25×10^8 cpm/pmol), and 250 units of polynucleotide kinase. After 30 min at 37° an additional 100 units of polynucleotide kinase was added and the incubation was continued for 30 min. The reaction was stopped by adding EDTA solution to a final concentration of 20 mM and cooling. Excess ATP was removed by overnight dialysis against 0.5 M NaCl/10 μM EDTA/10 mM Tris·HCl, pH 7.5 and finally against 0.1 M NaCl/10 μM EDTA/10 mM Tris·HCl, pH 7.5.

Preparation of Col E1 DNA. Double-stranded supercoiled Col E1 DNA was prepared from *E. coli* JC411 as described (17). Labeled Col E1 was prepared by the addition of chloramphenicol (150 μg/ml) to a culture and then, after 4 hr, the addition of [*methyl*-^{3}H] thymidine (2 μCi/ml; 60 Ci/mmol) and 250 μg of deoxyadenosine per ml. Incubation was continued for 12 hr and the DNA was extracted. Apurinic sites were produced in Col E1 DNA by reducing the pH to 5.1 with HCl and heating for 20 min at 70°. The pH was then brought back to pH 7.5. This procedure induces one to two apurinic sites per molecule (18).

Endonuclease Assay. Col E1 DNA containing apurinic sites was incubated with nuclease in a 100-μl reaction mixture containing 50 mM Tris·HCl (pH 7.85), 2 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 50 μg of bovine serum albumin per ml, and 0.26–1.2 μg of nuclease. The reaction was stopped by the addition of EDTA, and the products were analyzed by electrophoresis in 0.5% agarose gel containing 0.5 μg of ethidium bromide per ml as described (19).

Exonuclease Assay. T5 DNA labeled with ^{32}P at the 5′ termini was incubated in a 100-μl reaction mixture with 50 mM Tris·HCl (pH 7.85), 5 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 50 μg of bovine serum albumin per ml, and 0.03–0.6 μg of enzyme. At the end of the reaction period the DNA was precipitated by adding 100 μl of 17% perchloric acid containing 100 mM sodium pyrophosphate. The precipitate was collected on a Whatman G F/C glass fiber filter and washed with 25 ml of 5% trichloroacetic acid. The filter was then washed with 5 ml of ethanol and dried and radioactivity was determined. The reaction products were analyzed by homochromatography as described (20). At intervals, 5 μl of reaction mixture was applied to a DEAE-cellulose plate and developed with an RNA digest. The plate was covered with x-ray film, exposed overnight, developed, and then scanned with a Joyce–Loebl densitometer.

Nucleotide Incorporation into DNA. Control or treated salmon sperm, T5, or T7 DNA in a reaction mixture containing 50 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 50 μg of bovine serum albumin per ml, and 50 μM each of dATP, dGTP, dCTP, and dTTP (^{3}H, 91 cpm/pmol each) was incubated with DNA polymerase and/or nuclease at 37°. KCl (25 mM) and 2.5 mM potassium phosphate, pH 7.4, were added either separately or as part of the enzyme preparation. At the end of the reaction period the DNA was precipitated with 17% perchloric acid containing 100 mM sodium pyrophosphate. Bovine serum albumin (100 μg) was added as a coprecipitant. The precipitate was collected on a Whatman G F/C glass fiber filter and washed with 25 ml of 5% trichloroacetic acid followed by 5 ml of ethanol. The filters were dried and the radioactivity was determined by liquid scintillation counting.

RESULTS

Apurinic Endonuclease Activity. Col E1 DNA is not a substrate for either nuclease or polymerase preparations (Fig. 1). Even at very high nuclease concentrations (3 μg) there was no significant nicking of DNA (gels *1–4*). However, when DNA containing two to three heat-induced apurinic sites per molecule was used, a very small quantity of enzyme (0.26 μg) was enough to convert most of the apurinic DNA to nicked circles (upper bands, gels *6–8*) in 30 min, indicating that the endonuclease activity requires apurinic sites as a substrate.

Exonuclease Activity. Nuclease solubilizes ^{32}P-labeled 5′ termini (Fig. 2). Heat denaturation of the substrate before incubation with enzyme greatly decreased the solubilization. The amount of digestion of the heat-denatured DNA could be due to partial renaturation during incubation. We scanned the autoradiographs of the homochromatography plates to estimate the proportion of different sized nucleotides among the reaction products. Dinucleotides accounted for 54–57% of the products, mononucleotides for 11–17%, and tri- and tetranucleotides for 26–30% at 5, 15, or 30 min of enzyme digestion.

Repair of Apurinic Sites in DNA. Nucleotides are incorporated into methylated, depurinated T7 DNA (approximately 20 apurinic sites per strand) incubated with nuclease and α polymerase (Fig. 3). Incorporation was linear with time up to 4 hr. No incorporation was observed with native DNA or when DNA polymerase or nuclease was used separately. Increasing

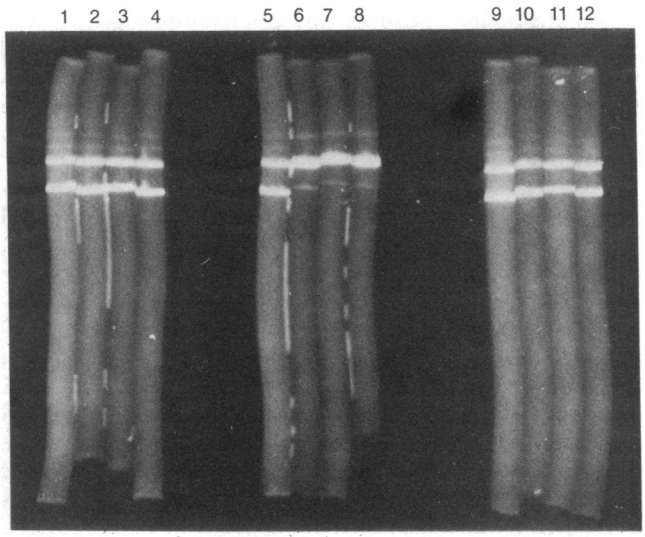

FIG. 1. Endonuclease assay with Col El DNA. Col El DNA (6 μg) was incubated with enzyme for 30 min at 37°. Approximately 1 μg of DNA was analyzed by 0.5% agarose/ethidium bromide gel electrophoresis as described (15). Electrophoresis was for 90 min at 5 mA/gel at room temperature. Gels were photographed under direct illumination with short-wave UV. Untreated DNA: (*1*) 0, (*2*) 0.24, (*3*) 0.62, and (*4*) 1.2 μg of nuclease. Apurinic DNA (two to three sites per molecule): (*5*) 0, (*6*) 0.25, (*7*) 0.62, and (*8*) 1.2 μg of nuclease. Untreated DNA: (*9*) 3.2 and (*10*) 6.4 μg of polymerase. Apurinic DNA: (*11*) 3.2 and (*12*) 6.4 μg of polymerase. The upper main band represents nicked DNA; the lower band represents intact Col El supercoils.

the number of apurinic sites increased template activity (Fig. 4). Salmon sperm DNA, sheared to give an average size of 2×10^7 daltons, was reacted with MeMes at various concentrations

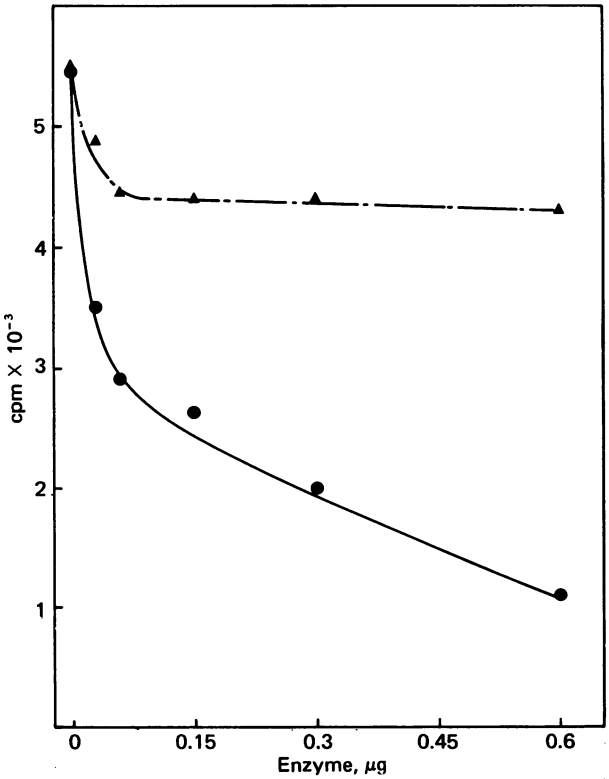

FIG. 2. Exonuclease assay using 5′-^{32}P-labeled T5 DNA. T5 DNA (0.4 μg), native (●) or denatured by heating for 10 min at 100°, (▲) was incubated with enzyme for 15 min as described in the text.

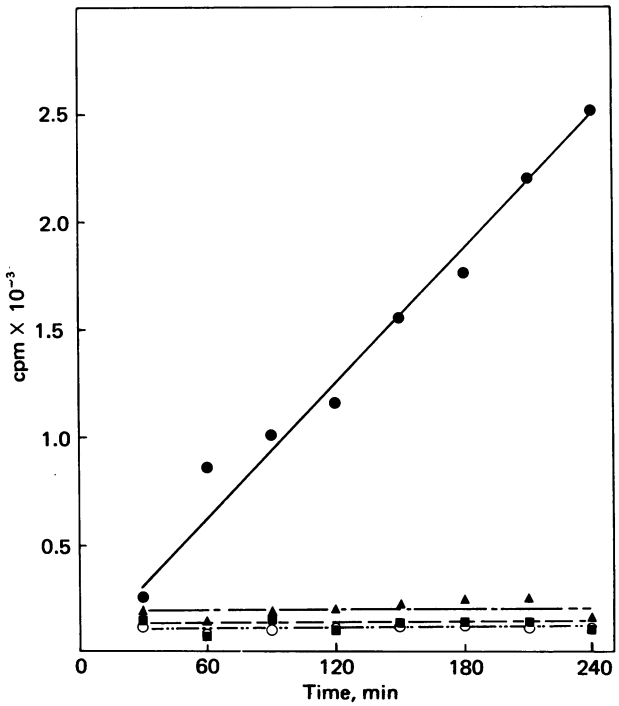

FIG. 3. Incorporation of nucleotides in apurinic T7 DNA. T7 DNA was alkylated with MeMes (12.5 mM for 20 min at 37°) and heated for 60 min at 70° as described in the text. Nondepurinated DNA (13 μg) or depurinated T7 DNA (16 μg) was incubated with either 16 μg of DNA polymerase and/or 3 μg of nuclease. At the indicated times, 20 μl of the total 200 μl of reaction mixture was withdrawn for determination of nucleotide incorporation as described in the text. ▲, Untreated DNA in complete reaction mixture. MeMes-treated DNA: ●, in complete reaction mixture; ■, without polymerase; ○, without nuclease.

and then heated. The rate of nucleotide incorporation increased as a function of the MMS concentration.

Our system also uses T5 DNA as a substrate (Fig. 5). This molecule has five unique strand interruptions (21) but no apurinic sites. We calculate that about seven nucleotides were incorporated per interruption per 30 min (see below). Neither the DNA polymerase nor the nuclease preparation used separately produced any incorporation of nucleotide into acid-precipitable material.

These data indicate that the nuclease and polymerase preparations together carry out a nick translation reaction with either apurinic or nicked DNA as a substrate. In order to eliminate the possibility that the polymerase and nuclease together produced some sort of strand displacement, we decided to see whether the structure produced was susceptible to ligase and whether the product of the repair reaction had the properties of an undamaged T7 DNA molecule with apurinic sites removed.

Incubation of T7 DNA with 12.5 mM MeMes for 10 min followed by heating for 1 hr at 70° in pH 7.4 buffer gave a substrate with about 10 alkali-labile sites per strand (Fig. 6). Since only about 0.3 S1 susceptible site is present per strand in similarly treated substrates, most of the alkali-labile sites are apurinic. Incubation with nuclease followed by ligase and ATP did not result in joining. Incubation with nuclease and polymerase (plus nucleoside triphosphates) was similarly ineffective. Incubation of alkylated DNA with nuclease and polymerase for 30 min followed by the addition of T4 ligase and ATP and incubation for an additional 30 min resulted in the loss of 9 of 10 alkali-labile sites per strand. Since the original substrate

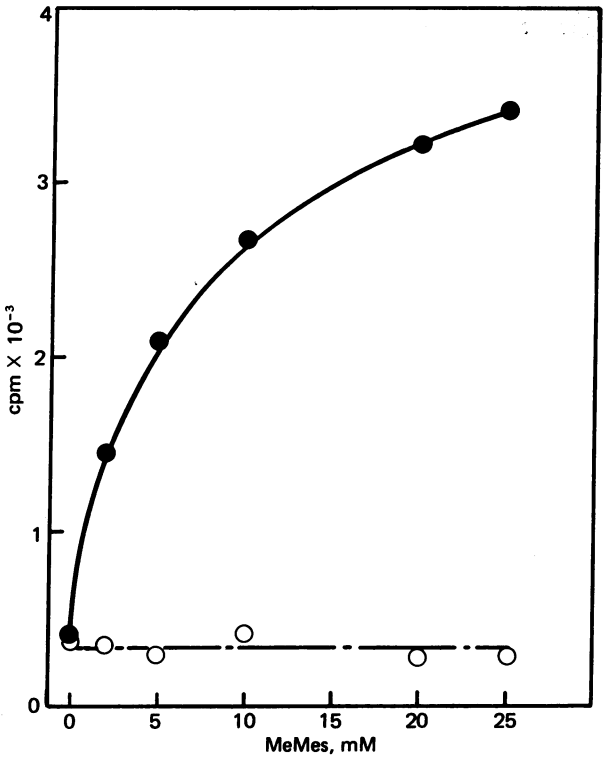

FIG. 4. Nucleotide incorporation into salmon sperm DNA treated with various MeMes concentrations and then depurinated for 1 hr at 70°. DNA was alkylated and depurinated as described in the text. Treated salmon sperm DNA (5 μg) was incubated in a 50-μl reaction volume with 5 μg of DNA polymerase and 1.25 μg of nuclease. Samples were incubated at 37° for 30 min and nucleotide incorporation was determined on the complete 50-μl reaction mixture. ○, No enzyme; ●, nuclease and polymerase.

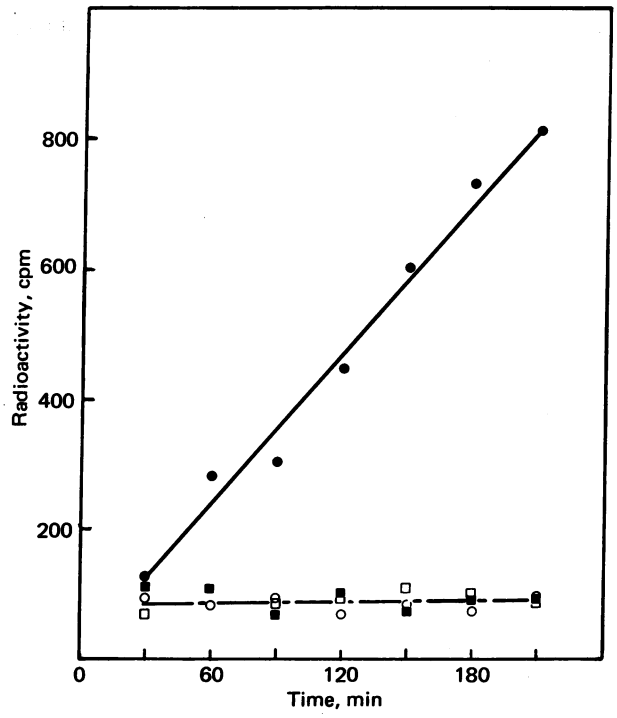

FIG. 5. Nucleotide incorporation into T5 DNA. The reaction conditions are similar to those described in the text. T5 DNA (31 μg) was incubated with 16 μg of DNA polymerase and/or 3 μg of nuclease in a 200-μl reaction volume at 37°. Samples (20 μl) were withdrawn at intervals as indicated for the determination of nucleotide incorporation described in the text. ●, Complete system; ■, without polymerase; □, without nuclease; ○, no enzyme.

contained only about a 0.3 break per strand, this result demonstrates the removal of apurinic sites and restoration of the integrity of the T7 molecule.

DISCUSSION

The repair of alkylated T7 DNA *in vitro* was reported by Verly *et al.* (22) using a mixture of bacterial enzymes. Masker (23) used *Micrococcus luteus* endonuclease and a crude extract of *E. coli* to repair UV-treated T7 DNA. These systems use a polymerase with 5′-3′ exonuclease activity; coordinated nick translation is built into the system. The problem is different when the polymerase has no nuclease activity since repair involves the coordinated activity of separate enzymes. We observed repair of apurinic sites at a ratio of protein in polymerase to nuclease of about 4:1. Repair was not observed at higher nuclease concentration, indicating that the system works in a coordinated fashion only when nuclease actually is limiting.

The linear incorporation of nucleotides into T7 as a function of time permits an estimate of the number of nucleotides incorporated per lesion at any specific reaction time. A T7 substrate with 40 apurinic sites incorporated 1.2 nucleotides per lesion in 30 min (Fig. 3). T5 DNA with five natural interruptions per molecule incorporated 7.1 nucleotides per lesion per 30 min (Fig. 5). Salmon sperm DNA incorporated 1.3 nucleotides per lesion per 30 min under conditions of enzyme excess (Fig. 4). The *in vitro* repair rate is at least roughly comparable to that observed *in vivo* since a similar Burkitt's lymphoma line, Raji, was reported to incorporate 1.7 nucleotides per lesion per hr after treatment at an MeMes dose of 0.25 mM (24). The data also indicate that the repair patches can be small, as expected for apurinic repair, but the approximations involved, particularly in the calculation of the number of breaks from sucrose gradient data, make it impossible to specify patch size precisely. Patch size in this *in vitro* system should depend on the time of ligase addition, and calculations on the basis of a 30-min reaction time (Fig. 6) are, of course, arbitrary. However, alkylation repair in lymphoma cells is rapid and a sizable fraction of the repair observed is complete by 30 min *in vivo* (25).

The separate activity of the enzyme preparations is consistent with their activity in the complete system. We determined endonuclease activity by measuring the radioactivity in broken and intact Col E1 circles eluted from gels. Our enzyme preparation breaks 1.4×10^{13} apurinic sites per μg of protein per 30 min. Exonuclease activity was 3.6×10^{11} 5′-^{32}P sites removed per μg of protein per 30 min (Fig. 2). DNA polymerase activity with activated salmon sperm DNA as a template was 6×10^{13} nucleotides added per μg of protein per 30 min. The alkylated T7 DNA (Fig. 6) had 20 apurinic sites per molecule, of which 18 were repaired in 30 min, or about 2.5×10^{12} apurinic sites. Both polymerase and endonuclease activities were sufficient to account for this repair activity. However the 1.9 μg of nuclease preparation by itself would only have removed 6.9×10^{11} free 5′ ends and therefore may be more active in the presence of polymerase, as demonstrated for the exonuclease fragment of *E. coli* polymerase 1 (26). We suppose that it is the exonuclease activity that is the limiting factor in our system.

Notwithstanding the observation that the initial exonucleolytic product is probably a dinucleotide and that small patches are involved, we think that the system can be properly characterized as carrying out nick translation (27). The product of nuclease and polymerase action is susceptible to DNA ligase, indicating that there are repaired sites without gaps between adjacent nucleotides after a period of reaction.

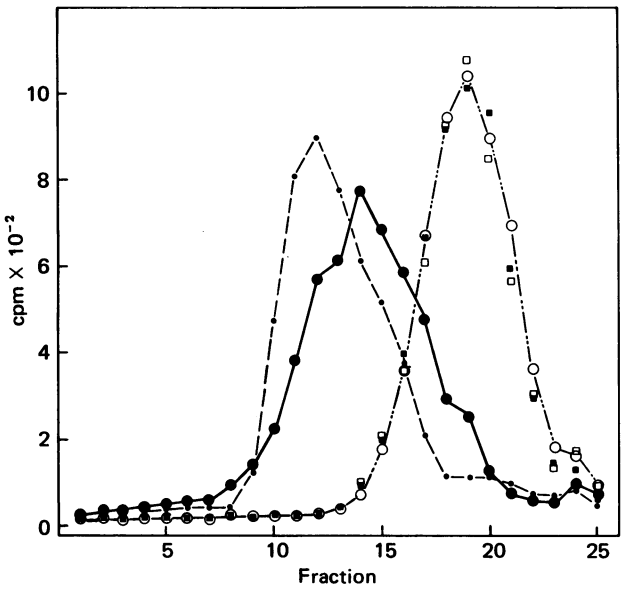

FIG. 6. Repair of alkali-labile sites in T7 DNA. T7 DNA (5.7 μg, 5600 ^{3}H cpm/μg), alkylated and depurinated to give 10 apurinic sites per strand, was incubated with 8 μg of DNA polymerase and/or 1.9 μg of nuclease as described (*Nucleotide Incorporation into DNA*) but with unlabeled nucleoside triphosphates. Incubation was for 30 min at 37°. Ligase (10 units) and ATP (final concentration 100 μM) were added where indicated. The reaction mixture was then incubated an additional 30 min at 30°, after which 200 μl of 0.2 M NaOH/0.1 M NaCl/20 mM EDTA/1% sucrose was added to stop the reaction. The mixtures were incubated for 15 min at room temperature and then 100 μl was layered on top of a preformed 5–20% linear sucrose gradient containing 0.3 M NaOH/0.5 M NaCl/10 mM EDTA. The gradients were centrifuged at 44,800 rpm for 2.5 hr at 5°. Samples were collected from the bottom of the tube on filter paper disks (Whatman 3MM), dried, washed with 5% trichloroacetic acid, 95% ethanol, and acetone, and dried; radioactivity was then determined. •---•, Untreated T7 DNA; ●—●, MeMes-treated DNA plus nuclease plus polymerase plus ligase (complete system); ○, complete system minus polymerase; □, complete system minus polymerase and minus ligase; ■, complete system minus ligase. The curve for the complete system (polymerase plus ligase) but without nuclease is not shown, but these results superimpose on the results for the complete system minus polymerase or minus ligase.

The nuclease preparation used in our studies has a least two different activities: an endonuclease activity specific for apurinic sites and a 5′-3′ exonuclease activity specific for double-stranded DNA. Our most purified sample shows two visible protein bands at 40,000 and 50,000 daltons on sodium dodecyl sulfate/polyacrylamide gel electrophoresis, and these sizes are consistent with estimates obtained by calibration of the Sephadex G-100 column used for purification. Although there have been reports of enzymes with both apurinic endonuclease and 3′-5′ exonuclease activity (28–30), we think that the assay system we used forced copurification of the two activities in our system. The assay with polymerase and apurinic DNA permitted fractionation of a system that would carry out *in vitro* repair but that was not necessarily optimal for isolation of separated enzymes.

The rate of hydrolysis of glycosidic linkages is greatly enhanced after treatment of DNA with alkylating agents. Apurinic sites also result from the spontaneous loss of purines (31). The apurinic repair pathway can maintain and restore the integrity of the DNA. While it is far from certain that the enzymes we obtained from lymphoma cells are physiologically involved in repair in normal human tissue, the system described does mimic properties required of the apurinic repair pathway *in vivo*.

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