(1976), *Biochemistry 15,* 407-414.
Hewish, D. R., Smith, K. K., and Spencer, J. H. (1976), *Virology 74,* 363-376.
Jay, E., Bambara, R., Padmanabhan, R., and Wu, R. (1974), *Nucleic Acids Res. 1,* 331-353.
Jovin, T. M., Englund, P. T., and Bertsch, L. L. (1969), *J. Biol. Chem. 244,* 2996-3008.
Kamen, R. (1972), *Biochim. Biophys. Acta 262,* 88-100.
Kaptein, J. S. (1977), Ph.D. Thesis, McGill University.
Kaptein, J., and Spencer, J. H. (1976), *Proc. Can. Fed. Biol. Soc. 19,* 120.
Ling, V. (1972a), *Proc. Natl. Acad. Sci. U.S.A. 69,* 742-746.
Ling, V. (1972b), *J. Mol. Biol. 64,* 87-102.
Oertel, W., and Schaller, H. (1972), *FEBS Lett. 27,* 316-320.
Oertel, W., and Schaller, H. (1973), *Eur. J. Biochem. 35,* 106-113.
Petersen, G. B., and Reeves, J. M. (1969), *Biochim. Biophys. Acta 179,* 510-512.
Richardson, C. C., Schildkraut, C. L., Aposhian, H. V., and Kornberg, A. (1964), *J. Biol. Chem. 239,* 222-232.
Sanger, F., and Coulson, A. R. (1975), *J. Mol. Biol. 94,* 441-448.
Sanger, F., Donelson, J. E., Coulson, A. R., Kossel, H., and Fischer, D. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1209-1213.
Sanger, F., Donelson, J. E., Coulson, A. R., Kossel, H., and Fischer, D. (1974), *J. Mol. Biol. 90,* 315-333.
Sanger, Fair, G. M., Barrell, B. G., Brown, N. L., Coulson, A. R., Fiddes, J. C., Hutchison, C. A., III, Slocombe, P. M., and Smith, M. (1977), *Nature (London) 264,* 687-695.
Schekman, R. W., Iwaya, M., Bromstrup, K., and Denhardt, D. T. (1971), *J. Mol. Biol. 57,* 177-199.
Shleser, R., Ishiwa, H., Mannes, B., and Tessman, E. S. (1968), *J. Mol. Biol. 34,* 121-129.
Spencer, J. H., and Boshkov, L. (1973), *Can. J. Biochem. 51,* 1206-1211.
Sulkowski, E., and Laskowski, M., Sr. (1971), *Biochim. Biophys. Acta 240,* 443-447.
Tate, W. P., and Petersen, G. B. (1974), *Virology 57,* 77-84.

Multiple Thymine Dimer Excising Nuclease Activities in Extracts of Human KB Cells†

Kem H. Cook and Errol C. Friedberg*

ABSTRACT: Crude extracts of human KB cells grown in suspension culture contain enzyme activity that catalyzes the preferential excision of thymine-containing pyrimidine dimers from UV-irradiated *E. coli* DNA specifically incised adjacent to dimer sites. Fractionation of KB cell crude extracts reveals the presence of three such activities with distinct affinities for both DEAE-cellulose and phosphocellulose. One of the activities (activity B) is distinguished by its $s_{20,w}$ (2.6) and isoelectric point (9.0) from the other two (activities A and C) which have similar $s_{20,w}$'s (3.0-3.2) and isoelectric points (6.0). All three differ in their extent of stimulation by divalent cation and inhibition by NaCl or a sulfhydryl group inhibitor. These results indicate that multiple 5′ → 3′ dimer excision nuclease activities exist in human cells; however, there is as yet no direct evidence that these enzymes are functional in nucleotide excision repair in vivo.

The molecular mechanism of nucleotide excision repair of DNA, specifically the excision of thymine dimer-containing nucleotides from UV-irradiated DNA, has been extensively studied in prokaryote systems (for recent reviews, see Grossman, 1974; Friedberg, 1975; Grossman et al., 1975). In all cases reported, endonucleases catalyze hydrolysis of phosphodiester bonds 5′ with respect to dimers in DNA (Kushner et al., 1971; Minton et al., 1975; Braun et al., 1976). Subsequent exonucleolytically catalyzed dimer excision, therefore, requires degradation of DNA in the 5′ → 3′ direction. In studies with cell-free systems of *E. coli*, the 5′ → 3′ exonuclease activities associated with DNA polymerases I and III catalyze thymine dimer excision (Kelly et al., 1969; Friedberg & Lehman, 1974; Pawl et al., 1975; Livingston & Richardson, 1975). In addition, both exonuclease V (Tanaka & Sekiguchi, 1975) and exonuclease VII of *E. coli* (Chase & Richardson, 1974), neither of which are associated with DNA polymerases, promote dimer excision from specifically preincised DNA in vitro. Enzyme activities comparable to some of these have been reported in extracts of *M. luteus* (Kaplan et al., 1969, 1971; Hamilton et al., 1973), and, in phage T4 infected *E. coli*, two phage-coded dimer excising 5′ → 3′ exonucleases have been identified (Ohshima & Sekiguchi, 1972; Friedberg et al., 1974; Shimizu & Sekiguchi, 1976).

Studies on the enzymology of thymine dimer excision have also been carried out using mammalian cell-free systems (for recent review, see Friedberg et al., 1977). A number of investigators have reported endonuclease activities that discriminate between unirradiated and heavily UV-irradiated DNA (Bacchetti et al., 1972; Van Lancker & Tomura, 1974; Tomura & Van Lancker, 1975; Brent, 1975; Bacchetti & Benne, 1975; Duker & Teebor, 1975); to our knowledge, however, none of these enzymes have been shown to catalyze phosphodiester

† From the Laboratory of Experimental Oncology, Department of Pathology, Stanford University, Stanford, California 94305. *Received September 15, 1977.* Studies supported by Research Grants CA-12428 from the U.S. Public Health Service and NP-174 from the American Cancer Society, and by Contract No. EY-76-S-03-0326 with the U.S. Energy Research and Development Association. E.C.F. is the recipient of U.S. Public Health Service Research Career Development Award No. 5K04-CA 71005-03. K.H.C. was supported by Training Grant CA-09151 from the U.S. Public Health Service.

0006-2960/78/0417-0850$01.00/0 © 1978 American Chemical Society

bond hydrolysis either generally or specifically at pyrimidine dimers. Indeed, in one case in which this particular question was studied (Bacchetti et al., 1972), enzymatic photoreactivation of the DNA did not remove substrate sites for the endonuclease. In order to avoid selecting for enzyme activities that are not dimer specific and hence may not be involved in thymine dimer excision in living cells, we have previously monitored nuclease activity in cell-free extracts by measuring the selective loss of thymine dimers from acid-precipitable UV-irradiated DNA (Duncan et al., 1975; Mortelmans et al., 1976). This assay measures both DNA incision and the subsequent excision of dimer-containing nucleotides. We have also shown in preliminary experiments that the excision step can be conveniently assayed independently of DNA incision by using UV-irradiated DNA previously incised at dimer sites by incubation with the dimer specific endonuclease of phage T4 (Duncan et al., 1975; Cook et al., 1975). In the present study we have carried out partial purifications of 5′ → 3′ dimer excising nuclease activities found in extracts of the human cell line KB. During fractionation, this activity separates into three distinct forms that are differentiated by a variety of physicochemical criteria.

Materials and Methods

Antibiotic-antimycotic solution (100×), glutamine, autoclavable modified Eagle's medium for suspension (F-18), calf serum, and trypsin were purchased from Gibco, Santa Clara, Calif. Trizma base, Hepes,[1] α-chymotrypsinogen A, *p*-chloromercuriphenylsulfonic acid, and phenylmethanesulfonyl fluoride were purchased from Sigma Chemical Co. [^{3}H]-Methylthymine, [^{3}H]methylthymidine, and [^{3}H]uridine were purchased from New England Nuclear Co. DE-52 was obtained from Whatman Inc., Clifton, N.J., and Cellex-P from Bio-Rad. Ampholine carrier ampholytes were obtained from LKB Instruments. MN Sil-G plastic backed silica gel plates were obtained from Brinkman Ins., Inc., Westbury, N.Y.

Cell Growth. Human KB cells were obtained from the American Type Culture Collection. All manipulations of cells followed standard tissue culture sterile technique and were performed in a Baker Edgegard sterile hood. Cultures were grown in suspension in autoclavable modified Eagle's medium (for suspension) supplemented with 30 mM Hepes buffer, 24 mM sodium bicarbonate, 10% calf serum, and 2 mM glutamine. In addition, cultures contained 100 units of penicillin, 100 μg of streptomycin, and 0.25 μg of fungizone per 500 mL of medium. Suspension cultures were carried in 500-mL Bellco spinner flasks stirred at 100–200 rpm and maintained at a density of 2.0–3.0 × 10^5 cells/mL by daily dilution. For large scale growth of cells, cultures were grown in 6-L Bellco spinner flasks by progressively doubling the carrier culture to the required volume. Cultures were monitored for the presence of mycoplasma by the method used by Todaro et al. (1971).

^{3}H-Labeled E. coli DNA. [^{3}H]Methylthymine-containing *E. coli* DNA was purified by the method of Thomas et al. (1970) from exponentially growing *E. coli* 15 T$^-$ labeled for two generations with 25 μCi/mL of [^{3}H]methylthymine. One modification was introduced; proteinase K at 50 μg per mL was substituted for Pronase in the final proteolytic degradation step. The concentration of the DNA was determined spectrophotometrically.

Ultraviolet-Irradiated DNA. UV-irradiated DNA was prepared by irradiating ^{3}H-labeled *E. coli* DNA with 300 J/m^2 of 254-nm light from a GE G8T5 low-pressure mercury vapor lamp. This fluence, determined with an International Light Germicidal photometer, yielded about 1% of the thymine as thymine-containing pyrimidine dimers.

Bacteriophage T4 Enzyme Preparations. T4 UV endonuclease (fraction IV) was purified by the procedure of Friedberg & King (1971). PEG fractions of T4- and T4V$_1$-infected *E. coli* were also prepared by the method of Friedberg & King (1971).

Specifically Incised DNA. Specifically incised UV-irradiated DNA was prepared by incubating 500 μg of DNA with 400 μL of T4 UV endonuclease (30 units of fraction IV of Friedberg & King, 1971) for 4 h, or with 7.0 mL of a PEG fraction (2870 units of fraction II of Friedberg & King, 1971) for 8 h at pH 8.0 in 19 mM EDTA. Protein was digested by the addition of 50 μg/mL of proteinase K and the DNA was then extracted successively with phenol and chloroform–isoamyl alcohol (20:1) and exhaustively dialyzed against 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA. The extent of endonuclease catalyzed nicking was measured by incubating a 30-μL aliquot of the substrate with a saturating quantity (generally 450 μg as protein) of a PEG fraction of phage T4V$_1$-infected *E. coli* in the presence of 5 mM $MgCl_2$ and 1 mM *p*-chloromercuriphenylsulfonic acid. This extract does not contain active phage coded dimer-specific endonuclease activity, but does catalyze the selective excision of thymine dimer-containing oligonucleotides from preincised DNA (Friedberg et al., 1974). Following incubation, the dimer content of the acid-precipitable fraction was compared with that of a control incubation containing no extract. The extent of dimer excision is an indirect measure of the number of endonucleolytic incisions adjacent to pyrimidine dimers catalyzed by the T4 UV endonuclease. Generally, more than 90% of the thymine dimer sites were incised and such DNA was used as the substrate for measuring excising nuclease activity in extracts of KB cells.

Assay of Thymine Dimer Excising Nuclease Activity. Assays were performed in 100-μL incubations in 10 × 70 mm ignition tubes at 37 °C for 30 min. Unless otherwise specified, each assay contained a final concentration of 5 mM $MgCl_2$, 30 μM DNA (containing 1% of total thymine in pyrimidine dimers) and 20 mM Tris-HCl buffer (pH 7.5). Incubations were terminated by placing tubes on ice and immediately adding 10 μL of bovine serum albumin (5 mg/mL) and 140 μL of 10% trichloroacetic acid. Tubes were allowed to stand on ice for 15 min, the acid-insoluble pellet was collected centrifugally, and a 50-μL aliquot of the decanted supernatant was measured for radioactivity in 10 mL of scintillation cocktail (15.1 g of Omnifluor in 2 L of toluene and 1 L of Triton X-100). The inner side of the ignition tubes was dried to remove any remaining drops of trichloroacetic acid and the pellet was resuspended in 0.2 mL of 98% formic acid. The tubes were then sealed with an oxygen gas torch and the contents hydrolyzed by heating at 181 °C for 35 min. After hydrolysis, the tubes were cooled in a dry ice/acetone bath and opened. The hydrolysates were transferred to 13 × 100 mm culture tubes and dried in a fume hood under a gentle air stream. Each dried hydrolysate was suspended in 7.5 μL of water and spotted on silica gel thin-layer plates in three applications. The thin-layer plates were developed and fractionated, and radioactivity was measured as described by Cook & Friedberg (1976).

Calculation of the thymine dimer content of the DNA was done by subtracting an average background from the dimer region of the thin-layer chromatogram (determined by averaging the minimum value on either side of this peak) and dividing the corrected value of dimer radioactivity by the total

[1] Abbreviations used: PEG, poly(ethylene glycol); EDTA, ethylenediaminetetraacetate; UV, ultraviolet; BME, β-mercaptoethanol; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

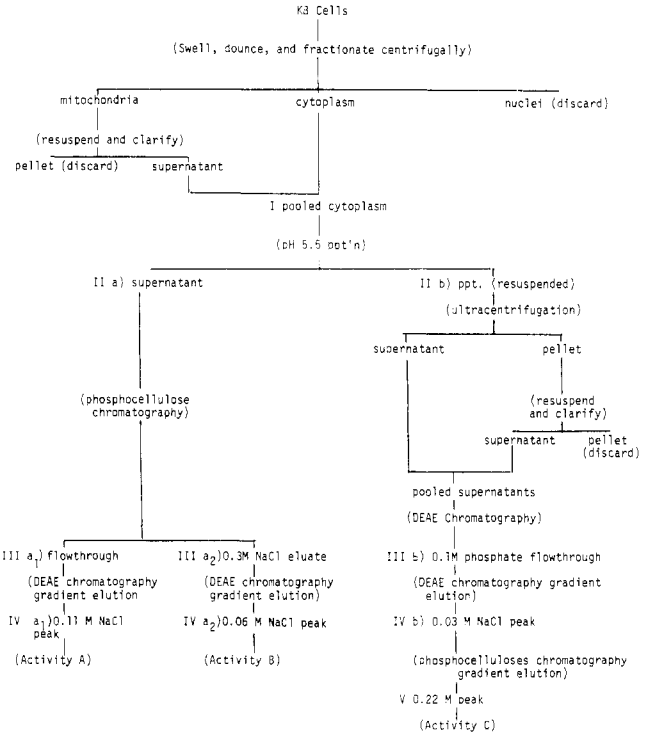

FIGURE 1: Flow scheme of dimer excising nuclease purification procedure. Complete details are described in the text.

radioactivity in thymine monomers. This gave a value for the ratio of radioactivity in dimers to radioactivity in thymine monomers. Thymine dimer excision was expressed as the percentage decrease in the ratio of thymine dimer to thymine monomer in test samples relative to control incubations with no added enzyme. A decrease in this *ratio* always indicates selective excision of thymine-containing dimers irrespective of the extent of total DNA degraded. Thus, random nonspecific degradation of DNA that includes loss of dimers does not alter the ratio of thymine dimer to thymine monomer in the acid-insoluble fraction. Similarly, extensive nonspecific degradation that does not include dimers will increase the ratio and mask any concomitant selective dimer excision occurring.

One unit of excision nuclease activity is defined as the amount of enzyme needed to release 1.0 pmol of thymine-containing pyrimidine dimers (TT and CT), under the usual assay conditions in 30 min. This was calculated by measuring total thymine released as dimers and correcting for the relative distribution of TT and CT in UV irradiated *E. coli* DNA (Rahn & Hosszu, 1969).

Ion-Exchange Matrices and Column Chromatography. Preswollen DEAE-cellulose (DE52) was prepared by defining. Cellex P (a phosphocellulose) was prepared for enzyme chromatography as suggested by the manufacturer. Each resin was equilibrated with a tenfold concentration of the appropriate buffer and columns were poured in the cold in 1.0 M sodium chloride in the starting buffer. Columns were washed with starting buffer until the conductivity and the pH of the effluent were that of the buffer. Fractions of the appropriate size were collected with a Gilson Mini-escargot fraction collector. Effluent salt concentration was determined by measuring the conductivity or by determining the refractive index. Effluent was monitored for absorption at 280 nm with a Gilson UV-RP monitor.

Enzyme Purification. A typical purification procedure is described below and a flow chart of the procedure is shown in Figure 1. After the growth of the cells all steps were carried out at 0 to 4 °C.

Eighty-four liters of KB cells grown in suspension to a density of 300 000 cells per mL was harvested at 5000 rpm using a Szent-Gyorgyi continuous flow rotor. A total of 91 g of cells was added to 810 mL of lysis buffer (5 mM potassium phosphate, pH 8.5, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM BME, 1 mM phenylmethanesulfonyl fluoride, and 1% isopropyl alcohol) and the cells were allowed to swell for 20 min. The cells were disrupted in a 50-mL Dounce homogenizer with 8 to 12 strokes of the tight-fitting pestle. Microscopic examination revealed that greater than 95% of the cells were disrupted by this procedure. The homogenate was clarified by pelleting the nuclei at 800*g* for 10 min and then pelleting the mitochondria at 13 000*g* for 20 min. The mitochondrial pellet was washed with 90 mL of lysis buffer and the supernatant was pooled with the cytoplasmic fraction (fraction I).

Fraction I (990 mL) was dialyzed against two changes of 9 L of acetate buffer (0.2 M sodium acetate, 1 mM EDTA, 1 mM BME, pH 5.5) for 12 h each. The precipitate that formed was collected by centrifugation at 13 000*g* for 20 min, resuspended in acetate buffer, and recentrifuged, and the supernatants were pooled (fraction IIa).

Fraction IIa (1190 mL) was dialyzed against two changes each of 8 L of buffer A (10 mM sodium phosphate, pH 7.2) for 12 h. This dialysate was loaded onto a 5.0 × 30 cm phosphocellulose column equilibrated in buffer A. Absorption of the effluent at 280 nm was monitored and 12.5-mL fractions were collected. The column was washed with 750 mL of buffer A and then eluted with 0.3 M sodium chloride in buffer A. The flow-through fractions containing thymine dimer excision activity were pooled (fraction $IIIa_1$). Those fractions containing activity eluted with salt were pooled as fraction $IIIa_2$.

Fraction $IIIa_1$ (980 mL) (phosphocellulose flow-through) was adjusted to pH 7.0 by the slow addition of 1.8 mL of 1.0 M monobasic sodium phosphate. The fraction was loaded onto a 2.5 × 60 cm DEAE-cellulose column equilibrated with buffer B (10 mM sodium phosphate, pH 7.0). The effluent was collected in 9.5-mL fractions and the absorption at 280 nm was monitored. The column was washed with 600 mL of buffer B and eluted with a linear sodium chloride gradient constituted by 500 mL of buffer B and 500 mL of 1.0 M sodium chloride in buffer B. The dimer excision activity eluted as a symmetric peak centered at 0.11 M sodium chloride. Active fractions were pooled as fraction IVa_1 and dialyzed against 10 mM Tris-HCl buffer (pH 7.5). This fraction contains activity A.

Fraction $IIIa_2$ (320 mL) (phosphocellulose bound) was dialyzed against 7 L of buffer B twice for 8 h each and was loaded onto a 2.5 × 30 cm DEAE-cellulose column equilibrated with buffer B. The column was washed with 300 mL of buffer B. Absorption of the effluent was monitored at 280 nm and 4.9-mL fractions were collected. Thymine dimer excising activity eluted as a symmetric peak at 0.06 M salt when the column was developed with a 500-mL linear sodium chloride gradient (0 to 0.5 M salt) in buffer B. Active fractions were pooled as fraction IVa_2. This fraction was dialyzed against 10 mM Tris-HCl buffer (pH 7.5) and contains activity B.

The pH 5.5 precipitate (fraction I) was resuspended in 133 mL of buffer C (0.2 M potassium phosphate, 1 mM EDTA, 1 mM BME, pH 8.5) and allowed to stand for 8 h with occasional mixing. Buffer C (67 mL) with 70% sucrose (w/v) was then added and the mixture was centrifuged with an overlayer of buffer C containing 11.7% sucrose (w/v). Centrifugation was for 90 min in a 60 Ti rotor at 40 000 rpm at 4 °C using a L3-50 Beckman ultracentrifuge. The fatty pellicle which floats

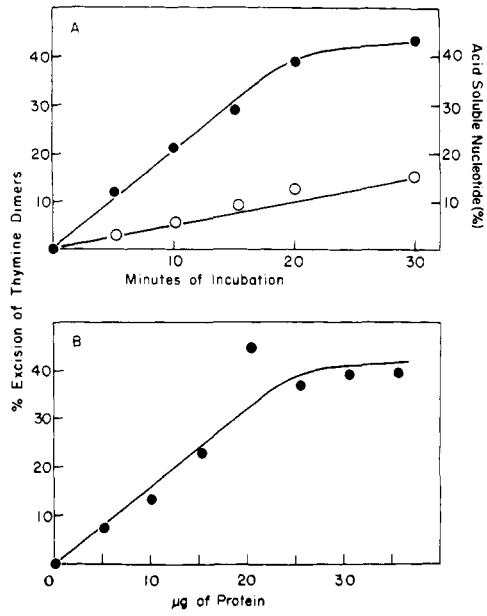

FIGURE 2: Loss of thymine dimers from the acid-insoluble fraction of UV-irradiated DNA. KB cells were harvested and washed twice with 0.15 M NaCl, 10 mM Tris-HCl buffer (pH 8.0). The pellet was resuspended in 0.15 M NaCl, 10 mM Tris-HCl buffer (pH 8.0) and 1 mM BME and sonicated with the small probe of a Biosonic II sonifier for three pulses of 10-s each. Incubations and the termination of reactions were performed as described in the text. In the experiment shown in A, 36 μg of extract as protein was added and incubation was for the times indicated. Solid circles represent percent of dimers excised; open circles represent percent of radioactivity released into the acid-soluble fractions. The incubation shown in B was for 25 min with the indicated amounts of protein. Solid circles represent percent of dimers excised.

on the overlayer was discarded and the supernatant saved. The pellet was resuspended in 75 mL of buffer C with 23.4% sucrose (w/v) and recentrifuged. The supernatants were pooled and designated fraction IIb. This fraction was diluted by the addition of 1 volume of 1 mM EDTA and 1 mM BME and loaded onto a 5 × 20 cm DEAE-cellulose column equilibrated in buffer D (0.1 M potassium phosphate, 1 mM EDTA, 1 mM BME, pH 8.5). The column was washed with 1200 mL of buffer D and fractions (22 mL) were collected. Enzyme activity in the flow-through was pooled as fraction IIIb and was dialyzed against 10 L of 10 mM sodium phosphate buffer, pH 6.8, with 1 mM EDTA and 1 mM BME for 8 h. This fraction was loaded onto a 2.0 × 30 cm DEAE-cellulose column equilibrated with buffer A. The column was washed with 300 mL of buffer A and eluted with a linear salt gradient (0–0.5 M NaCl) in 1 L of buffer A. Active fractions (fraction IVb) were pooled and dialyzed against 4 L of buffer B twice for 6 h each. Eighty milliliters was then loaded onto a 2.0 × 30 cm phosphocellulose column equilibrated with buffer B. The column was washed with 300 mL of buffer B and eluted with a 0–2.0 M linear sodium chloride gradient in 600 mL of buffer B. Fractions (4.5 mL) were collected and fractions containing enzyme activity pooled as fraction V. Fraction V was dialyzed against 10 mM Tris-HCl (pH 7.5) and contains activity C.

Isolectric Focusing. Isolectric focusing was performed in a J-tube apparatus with an inner diameter of 1.0 cm (Weller et al., 1968) in a 6-mL sucrose gradient held at 2 °C in a cooling water bath. Gradients were made by layering ten equal 0.5-mL steps over the cathode solution (61.5% sucrose w/v and 0.26 M NaOH) starting with 47% sucrose and 3.0% (pH 3.5–10) ampholyte and finishing with a 6.7% sucrose w/v and 1.0% (pH 3.5–10) ampholyte. The sample to be focused was used to make the intermediate layers. The gradients were overlayered with anode solution (0.188 M sulfuric acid) and current was applied with a Power Design Inc. (Palo Alto) Model 1556B power supply at 2 mA until a voltage of 550 V was acquired. Voltage was then held constant until the current fell below 0.3 mA (about 72 h). Samples were collected with a micropipet from the top of the gradient and assayed for enzyme activity.

TABLE I: Separation of KB Dimer Excising Activities.

	A	B	C
pH 5.5	Soluble	Soluble	Insoluble
[NaCl] to elute from phosphocellulose (M)	0.0	0.15	0.25
[NaCl] to elute from DEAE (M)	0.11	0.06	0.03

Ultracentrifugation. Sedimentation was performed by a modification of the method of Martin & Ames (1961). Linear, 4.3 mL (20% to 40%), glycerol gradients were prepared in a Brinkman 10-mL gradient maker and layered with 100-μL samples. Centrifugation was at 56 000 rpm in an SW56 rotor for 37 h at 4 °C. The standard tubes contained 350 μg each of α-chymotrypsinogen A and bovine serum albumin. After centrifugation the tubes were punctured and 10-drop fractions collected from the bottom. Absorption at 280 nm was determined in each fraction of the standard tubes and enzyme activity assayed in aliquots of appropriate fractions as described above.

Gel Electrophoresis. Polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970).

Protein Determination. Protein was determined either by the method of Lowry et al. (1951) or by the method of Schaffner & Weissman (1973). Bovine serum albumin was used for standard curves.

Results

Assay of Dimer Excision Nuclease Activity. When a crude extract of KB cells previously frozen in buffer at −20 °C is incubated with UV-irradiated *E. coli* DNA, no selective loss of thymine dimers from the acid-insoluble DNA is observed (data not shown). This result confirms previous observations (Duncan et al., 1975; Mortelmans et al., 1976) that the endonuclease that recognizes pyrimidine dimers (or some factor required for endonuclease activity) is inactivated by freezing of either the cells or the extracts derived from frozen cells. However, preincubation of the DNA with T4 endonuclease V (Figure 2) produces a substrate from which a selective loss of thymine dimers occurs during subsequent incubation with extracts of frozen KB cells. As shown in the figure, the loss of dimers is linear with respect to both time of incubation and enzyme concentration within limits. Also shown in the figure is the relative amount of total degradation of the DNA to acid-soluble nucleotide. In a large number of experiments the total extent of degradation was usually less than 10% when 40–60% of the thymine dimers were excised. With crude extracts, the linearity of the assay falls off when about 40% of the thymine dimers are excised. With more purified fractions, the assay is linear up to 60–70% dimer excision. It is probable that dimer excision becomes limited by destruction of the substrate due to the combined effects of excision and nonspecific degradation of the DNA. Excision of thymine dimers from preincised DNA has an absolute requirement for divalent cation and is completely inhibited in the presence of 1–10 mM EDTA.

Evidence for Multiple Excision Nuclease Activities. In preliminary experiments a cytoplasmic fraction of KB cells

TABLE II: Purification of Dimer Excising Nuclease Activities.[a]

Fraction	Protein (mg/mL)	Units $\times 10^3$	Spec act. (U/mg)	Recovery[c] (%)	Purification[d] factor
Cell homogenate	7.7	102	14.6		
(I) Cytoplasm	4.4	109	27.4	107	
Activity A					
(IIa) Acid supernatant	2.2	43.6	22.7	98.9	
($IIIa_1$) Phosphocellulose flow-through	1.5	23.5	16.2	111.5	2.7
(IVa_1) DEAE 0.11 M peak	0.11	10.4	711	49.4	120.4
Activity B					
(IIa) Acid supernatant	2.2	43.6	22.7	98.9	
($IIIa_2$) Phosphocellulose 0.3 M step	1.2	25.1	66.1	111	11.2
(IVa_2) DEAE 0.06 M peak	0.24	9.8	305	43.26	51.7
Activity C					
(IIb) Resolubilized acid ppt	8.7	64.2	36.7	98.9	
(IIIb) 0.1 M phosphate DEAE flow-through	0.20	65.4	299	102	34.4
(IVb) DEAE 0.03 M peak	0.11	17.9	511	27.4	58.8
(V) Phosphocellulose[b] 0.22 M peak	0.11	7.5	866	11.6	99.6

[a] Activity A + activity B + activity C = total recovery = 27.0%. [b] Corrected for the fact that one-third of IVb was not fractionated on phosphocellulose. [c] Recovery is corrected for splitting of activities. [d] Purification is expressed as the purification over the calculated contribution to the total crude homogenate specific activity.

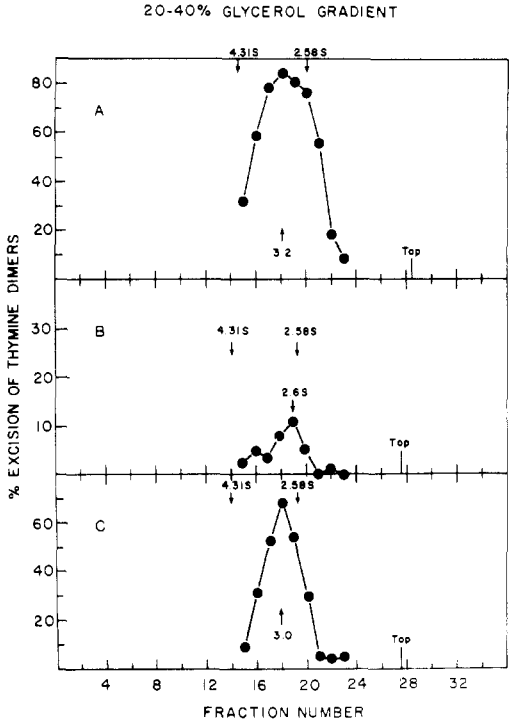

FIGURE 3: Sedimentation profiles of activities A, B, and C in glycerol gradients. One hundred and fifty microliters of fractions IVa_1 (activity A, 33 μg of protein), IVa_2 (activity B, 72 μg of protein), and V (activity C, 33 μg of protein) was sedimented for 37 h at 56 000 rpm in 20–40% glycerol gradients. Ten-drop fractions were collected and excision activity of 50-μL aliquots from appropriate fractions was measured. The positions of the bovine serum albumin and α-chymotrypsinogen A markers in the standard tube are shown for each activity. $s_{20,w}$ values were calculated from the standards assuming values of 4.32 S and 2.58 S for bovine serum albumin and α-chymotrypsinogen, respectively. Further experimental details are provided in the text.

prepared by homogenization in 10 volumes of buffer (5 mM sodium phosphate, pH 7.2, 1 mM EDTA, 2 mM $MgCl_2$, and 1 mM BME) was fractionated by phosphocellulose chromatography. Assay of the fractions for excision nuclease activity revealed a complex series of peaks (Friedberg et al., 1977). In addition, approximately 25% of the total activity failed to bind to the phosphocellulose. Comparable results were obtained using extracts prepared and maintained throughout the procedure in the presence of the protease inhibitor phenylmethanesulfonyl fluoride.

Further evidence for multiple dimer excising activities was provided by the results of systematic fractionation of the crude extract into three activities designated as activities A, B, and C (Figure 1). As indicated in Table I, activity C is differentiated from activities A and B by its response to acid precipitation at pH 5.5. The former activity is acid insoluble while the latter two are acid soluble. Activity C is further distinguished by different binding affinities for DEAE-cellulose and phosphocellulose (Table I). Activities A and B are distinguished by the observation that the former does not bind to phosphocellulose and also elutes from DEAE-cellulose at a higher ionic strength. During rechromatography on phosphocellulose each of three activities (separately tested) demonstrated the same relative binding affinity as described above. Activity B was found to elute from phosphocellulose at 0.15 M NaCl, but gradient elution provided little increase in purification because the major protein peak was eluted with it (data not shown). The purification of each activity together with unit recoveries is shown in Table II. Slab gel electrophoresis under denaturing conditions demonstrated that none of the three activities is homogeneous.

Fraction IVa_1 containing activity A (Figure 2) is insensitive to freezing at −20 °C and is stable for at least 2 weeks at 0 °C in 10 mM Tris-HCl buffer, pH 7.5. Fraction IVa_2 containing activity B is insensitive to freezing at −20 °C but loses about 20% of its activity in 1 week when stored at 0 °C. The same is true of fraction V containing activity C.

Characterization of the Thymine Dimer Excising Nuclease Activities. The three partially purified activities were characterized in order to detect further possible physical or functional differences. Each was sedimented in glycerol gradients and their respective $s_{20,w}$ values estimated relative to that of bovine serum albumin and α-chymotrypsinogen A. The results (Figure 3) indicate values of 3.2, 2.6, and 3.0 for activities A, B, and C, respectively. The $s_{20,w}$ value of activity B is sufficiently distinct in repeated experiments to suggest that it is a protein different from the other two. Assuming that all three activities are spherical proteins we estimate approximate

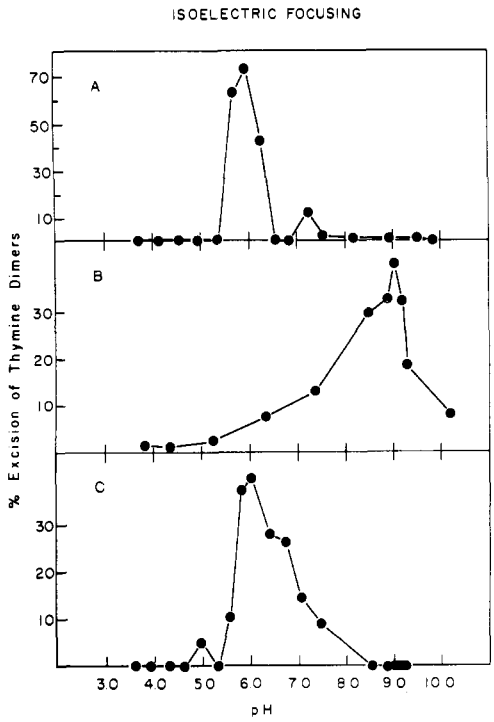

FIGURE 4: Isolectric focusing activity profiles of KB cell thymine excising nucleases. In each case 1.0 mL of the relevant activity (110 μg of activity A, 240 μg of activity B, and 110 μg of activity C) was used to construct the pH gradient as described in the text. Following focusing, individual 300-μL fractions in the case of activities A and B and 200-μL fractions in the case of activity C were collected. The pH (shown as solid circles) of each fraction was measured at 4 °C. Fifty-microliter aliquots were incubated with final concentrations of 50 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$ and 2.9 nmol of DNA (as nucleotide) in a volume of 150 μL. Incubations were at 37 °C for 30 min.

TABLE III: Effect of Various Divalent Cations as Cofactor.

Cation	Concn (mM)	% of maximum activity[a] Activity A	Activity B	Activity C
Mg^{2+}	1	89	100.0	86
	3	100	92	
	5	69	88	100
	7	49	75	
	10			91
	15		31	78
Mn^{2+}	1	88	99	46
	3	59	86	46
	5	38	73	29
	7	38	60	13
Ca^{2+}	1	18	2.2	0.0
	3	5.8	0.0	0.0
	5	9.5	0.7	26.4
	7	0.0	0.0	0.0

[a] Assays were carried out under the conditions described in the text, except that the indicated amount of divalent cation was present as the chloride salt rather than the standard 5 mM $MgCl_2$. Assays of activity A contained 5.5 μg of fraction IVa_1 (50 μL); of activity B, 14.0 μg of fraction IVa_2 (60 μL); and of activity C, 6.6 μg of fraction V (60 μL).

molecular weights of 38 000, 27 000, and 34 000, respectively. The isolectric focusing points of each activity are shown in Figure 4. Activity B (pI = 9.0) is clearly distinct from activities A and C, both of which have pI values of 6.0.

The differences in sedimentation velocity and isolectric point clearly suggest that activity B is a molecular entity distinct from activities A and C. In an effort to determine possible distinguishing features between the latter two activities, the effects of divalent cations, sodium chloride, and SH group inhibitors on dimer excising activity were measured. The effect of three different divalent cations is shown in Table III. None of the activities effectively utilize Ca^{2+} as a cofactor and in each case Mg^{2+} is at least as effective or more effective than Mn^{2+} at equivalent concentrations. Activity A has a Mg^{2+} optimum at 3 mM and approximately 50% optimal activity at 7 mM Mg^{2+}, while activity C has a very broad optimum between 1 and 10 mM Mg^{2+}. The activity of activity A is approximately equal in the presence of either Mg^{2+} or Mn^{2+} at 7 mM. However, activity C is significantly more active in the presence of Mg^{2+} than Mn^{2+} at concentrations of either cation greater than 5 mM. Both activities A and B are progressively inhibited by salt and retain about one-half of their original activity at 75 mM sodium chloride. Activity C loses 25% of its optimal activity between 10 and 20 mM salt but shows no further inhibition even at 75 mM sodium chloride.

All three activities are sensitive to total inhibition by *p*-chloromercuriphenylsulfonic acid at 5 mM; however, at lower concentrations of this reagent, the activities show different degrees of inhibition. At both 10 μM and 50 μM activity A shows only about 25% inhibition while activity B is 80 and 90% inhibited and activity C 55% and 75% inhibited at these two concentrations of the SH group inhibitor.

The substrate specificity of the three dimer excising nuclease activities was examined by incubating each with single- and double-stranded unirradiated and UV-irradiated DNA. The results are shown in Table IV. Activities A and C degrade unirradiated or UV-irradiated single-stranded DNA to acid-soluble products, but not double-stranded DNA. Activity B fails to degrade either single- or double-stranded DNA to acid-soluble nucleotide under the incubation conditions employed. Further studies on the substrate specificity of each activity and on their mechanism of action on specifically incised UV-irradiated DNA will be reported elsewhere.

Discussion

Previous studies from other laboratories have identified single exonuclease activities in extracts of mammalian tissues which excise dimers. DNase IV has been purified and characterized by Lindahl and his colleagues from rabbit bone marrow and from rabbit lung (Lindahl et al., 1969; Lindahl, 1971a,b, 1972). This enzyme attacks DNA at 5′ ends containing either phosphate or hydroxyl end groups and degrades the double-stranded synthetic copolymer [d(A-T)] much more rapidly than single-stranded polymers or native or denatured DNA from natural sources. DNase IV is not detectably inhibited by the presence of pyrimidine dimers in native DNA exposed to high doses of UV irradiation. The ability of this exonuclease to effectively hydrolyze UV-irradiated substrates suggested that it might be capable of dimer excision from specifically incised DNA. This was borne out experimentally by incubating DNase IV with UV-irradiated DNA preincubated with a UV endonuclease activity from *M. luteus* (Lindahl, 1972).

Doniger & Grossman (1976) have described the properties of an exonuclease termed correxonuclease, isolated from human placenta. Like the UV exonuclease of *M. luteus* (Kaplan et al., 1971) and exonuclease VII of *E. coli* (Chase & Richardson, 1974), this enzyme hydrolyzes single-stranded unirradiated or UV-irradiated DNA. It initiates hydrolysis at

TABLE IV: Substrate Specificity of Thymine Dimer Excising Nuclease Activities.[a]

Activity	Nucleotide rendered acid soluble (pmol)				Thymine dimer excised (%)
	Denatured DNA	Native DNA	Denatured UV-irradiated DNA	UV-irradiated preincised double-stranded DNA	UV irradiated preincised double-stranded DNA
A	78.0	2.8	69.0	67.0 (0.26%)	4.3
B	0.77	2.05	3.39	36.0 (0.10%)	20.0
C	43.0	1.28	47.0	56.0 (0.29%)	10.5

[a] Incubations were performed for 15 min at 37 °C and contained 2.0 nmol (as nucleotide) of DNA, 10 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, and the following amounts of enzyme: activity A (fraction IVa_1), 3.3 μg of protein in 30 μL; activity B (fraction IVa_2), 7.2 μg of protein in 30 μL; activity C (fraction V), 3.3 μg of protein in 30 μL. UV DNA was prepared by irradiation at a total fluence of 1600 J/m^2. Denaturation was carried out by heating to 100 °C at pH 8.0 in 10 mM Tris-HCl buffer for 5 min and quenching rapidly in ice. The preincised UV-irradiated DNA was prepared as described in the text. Acid-soluble nucleotide and thymine dimer excision were measured as described in the text.

both the 3′ and 5′ termini of such DNA with equal facility, yielding 5′-phosphorylated oligonucleotides averaging four nucleotides in length. Unlike DNase IV, this enzyme is apparently inactive against intact native DNA but can initiate hydrolysis at single-strand nicks creating gaps 30–40 nucleotides long. Similarly, the enzyme can excise pyrimidine dimers with adjacent 5′ incisions, releasing 30–40 nucleotides/dimer excised.

In the present study we have selected for dimer excising nucleases with an assay that directly measured this function. Though somewhat tedious in its execution, this assay offers the theoretical advantage of including dimer excising enzymes that may fail to degrade UV-irradiated single-stranded DNA and excluding those which fail to selectively excise dimers from duplex DNA, irrespective of other catalytic activities. We have chosen cells growing in tissue culture as a source of material in order to avoid the use of heterogeneous cell populations which might conceivably contain distinct enzymes or isozymes. The other perceived disadvantages of tissues (particularly human tissues) as a source of enzymes is that genetic heterogeneity and differences in metabolic activity are not readily controllable.

We have operationally defined three activities in extracts of KB cells that can excise thymine-containing pyrimidine dimers from preincised UV-irradiated DNA. Based on our results to date we suggest that activity B represents an enzyme distinct from activities A and C. Physically, activity B can be distinguished by its distinct isolectric point and its smaller $s_{20,w}$ value. Enzymatically, activity B does not significantly degrade single-stranded DNA to acid-soluble products and behaves in a unique manner with respect to sulfhydryl sensitivity and chromatographic affinity for DEAE and phosphocellulose.

The physicochemical distinction between activities A and C is less clear cut. The physical parameters of sedimentation velocity and isolectric point do not distinguish these activities; however, their binding affinities for DEAE-cellulose and phosphocellulose are different. Activity A does not bind to phosphocellulose at pH 7.2, whereas activity C binds fairly strongly. Conversely, activity A binds to DEAE more avidly than does activity C. It should also be noted that activity A is soluble at pH 5.5 while activity C is not. Further differences we have observed are that the inhibition of activity A by 75 mM sodium chloride is twice as great as that of activity C, while activity A is less sensitive to 10^{-5} M *p*-chloromercuriphenylsulfonic acid. Finally the relative stimulations of the two activities by Mg^{2+} and Mn^{2+} are different. These distinctions are made in partially purified fractions and may change upon complete purification. Enzymatically these two activities have not yet been distinguished. They both degrade UV-irradiated or unirradiated single-stranded but not duplex DNA, and of course both activities excise thymine dimers in the 5′ → 3′ direction.

It is possible that activities A and C are heterogeneous forms of the same gene product, perhaps due to posttranslational modification of one form. Alternatively the modification may be artefactual due to nonspecific binding of some subcellular components, or due to proteolysis. Mammalian enzymologists are plagued by the presence of heterogeneous forms of what is apparently the same enzyme. A particularly well-documented case is that of DNA polymerase α (Holmes & Johnston, 1975). Recently Linsley et al. (1977) have described multiple forms of apurinic endonuclease from human placenta.

Each of the three activities described is under further study. The apparent limited ability of activity B to degrade single-stranded DNA to acid-soluble products suggests that it may be similar to DNase IV of rodent tissues (Lindahl, 1972). Alternatively the enzyme may be an endonuclease rather than an exonuclease activity. If so, it represents a novel enzymatic mechanism for dimer excision, since all previously described thymine dimer excising activities are exonucleases, presumably requiring a free terminus (created by endonucleolytic incision) to initiate excision. However, a single-strand specific endonuclease could readily subserve this function by recognizing the single-strandedness of the dimer containing segment of DNA rather than the presence of the free terminus. DNA incision may, however, be required to provide the necessary degree of single strandedness. Mammalian endonucleases capable of acting on single strand DNA have been identified (Pedrini et al., 1976; Otto & Knippers, 1976; Wang & Furth, 1977) but none of these activities has been examined for their ability to excise pyrimidine dimers from an appropriate substrate and all seem to have significantly higher molecular weights than that estimated for activity B. The properties of both activities A and C bear a close resemblance to those described for correxonuclease of human placenta (Doniger & Grossman, 1976); however, further detailed studies are required to establish their similarities or differences.

At present it is not possible to assign biological functions to these activities. An endonuclease activity that recognizes pyrimidine dimer sites in UV-irradiated DNA or chromatin has been identified in crude extracts of human cells (Ducan et al., 1975; Mortelmans et al., 1976), but it is not known whether this enzyme creates a substrate for dimer excision that sufficiently resembles the model substrate we have used employing T4 endonuclease V. We have previously reported that the specific activity of total 5′ → 3′ dimer excising nuclease activity is normal in extracts of xeroderma pigmentosum fibroblasts (Cook et al., 1975). However, further studies are required to determine whether or not all three activities identified in KB

cell extracts are present in extracts of xeroderma pigmentosum cells.

Acknowledgment

The authors thank Mr. Paul Fisher, Dr. Duane Eichler, and Dr. Teresa Wang for helpful discussions and the sharing of large scale KB cell harvests. We are indebted to Ms. Margareta Brorsson for skilled technical assistance.

References

Bacchetti, S., & Benne, R. (1975) *Biochim. Biophys. Acta 390,* 285.

Bacchetti, S., van der Plas, A., & Veldhuisen, G. (1972) *Biochem. Biophys. Res. Commun. 48,* 662.

Braun, A., Radman, M., & Grossman, L. (1976) *Biochemistry 15,* 4116.

Brent, T. (1975) *Biochim. Biophys. Acta 407,* 191.

Chase, J. W., & Richardson, C. C. (1974) *J. Biol. Chem. 249,* 4553.

Cook, K. H., & Friedberg, E. C. (1976) *Anal. Biochem. 73,* 411.

Cook, K. H., Friedberg, E. C., Slor, H., & Cleaver, J. E. (1975) *Nature (London) 256,* 235.

Doniger, J., & Grossman, L. (1976) *J. Biol. Chem. 251,* 4579.

Duker, N. J., & Teebor, G. W. (1975) *Nature (London) 255,* 82.

Duncan, J., Slor, H., Cook, K., & Friedberg, E. C. (1975) in *Molecular Mechanisms for Repair of DNA* (Hanawalt, P. C., & Setlow, R. B., Eds.) p 643, Plenum Press, New York, N.Y.

Friedberg, E. C. (1975) *Photochem. Photobiol. 21,* 277.

Friedberg, E. C., & King, J. J. (1971) *J. Bacteriol. 106,* 500.

Friedberg, E. C., & Lehman, I. R. (1974) *Biochem. Biophys. Res. Commun. 58,* 132.

Friedberg, E. C., Minton, K., Pawl, G., & Verzola, P. (1974) *J. Virol. 13,* 953.

Friedberg, E. C., Cook, K. H., Duncan, J., & Mortelmans, K. (1977) *Photochem. Photobiol. Rev. 2,* 263.

Grossman, L. (1974) *Adv. Radiat. Res. 4,* 77.

Grossman, L., Braun, A., Feldberg, R., & Mahler, I. (1975) *Annu. Rev. Biochem. 44,* 19.

Hamilton, L., Mahler, I., & Grossman, L. (1974) *Biochemistry 13,* 1886.

Holmes, A. M., & Johnston, I. R. (1975) *FEBS Lett. 60,* 223.

Kaplan, J. C., Kushner, S. R., & Grossman, L. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63,* 144.

Kaplan, J. C., Kushner, S. R., & Grossman, L. (1971) *Biochemistry 10,* 3315.

Kelly, R. B., Atkinson, M. R., Huberman, J. A., & Kornberg, A. (1969) *Nature (London) 224,* 495.

Kushner, S. R., Kaplan, J. C., Ono, H., & Grossman, L. (1971) *Biochemistry 10,* 3315.

Laemmli, U. K. (1970) *Nature (London) 227,* 680.

Lindahl, T. (1971a) *Eur. J. Biochem. 18,* 407.

Lindahl, T. (1971b) *Eur. J. Biochem. 18,* 415.

Lindahl, T. (1972) in *Molecular and Cellular Repair Processes* (Beers, R. F., Herriott, R. M., & Tilghman, R. C. Eds.) p 3, Johns Hopkins University Press, Baltimore, Md.

Lindahl, T., Gally, J. A., & Edelman, G. M. (1969) *Proc. Natl. Acad. Sci. U.S.A. 62,* 597.

Linsley, W. S., Penhoet, E. E., & Linn, S. (1977) *J. Biol. Chem. 252,* 1235.

Livingston, D. M., & Richardson, C. C. (1975) *J. Biol. Chem. 250,* 470.

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

Martin, R. G., & Ames, B. N. (1960) *J. Biol. Chem. 236,* 1372.

Minton, K., Durphy, M., Taylor, R., & Friedberg, E. C. (1975) *J. Biol. Chem. 250,* 282.

Mortelmans, K., Friedberg, E. C., Slor, H., Cleaver, J. E., & Thomas, G. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 2757.

Ohshima, S., & Sekiguchi, M. (1972) *Biochem. Biophys. Res. Commun. 47,* 1126.

Otto, B., & Knippers, R. (1976) *Eur. J. Biochem. 71,* 617.

Pawl, G., Slor, H., & Friedberg, E. C. (1975) *J. Bacteriol. 122,* 34.

Pedrini, A. M., Ranzani, G., Pedrali Noy, G. C. F., Spadari, S., & Falaschi, A. (1976) *Eur. J. Biochem. 70,* 275.

Rahn, R. O., & Hosszu, J. L. (1969) *Biochim. Biophys. Acta 190,* 26.

Schaffner, W., & Weissman, C. (1973) *Anal. Biochem. 56,* 502.

Shimizu, K., & Sekiguchi, M. (1976) *J. Biol. Chem. 251,* 2613.

Tanaka, J. I., & Sekiguchi, M. (1975) *Biochim. Biophys. Acta 383,* 178.

Thomas, C. A., Berns, K. I., & Kelly, T. J., Jr. (1970) *Proced. Nucleic Acid Res.,* 535.

Todaro, G. J., Aaronson, S. A., & Rands, E. (1971) *Exp. Cell. Res. 65,* 256.

Tomura, T., & Van Lancker, J. L. (1975) *Biochim. Biophys. Acta 402,* 343.

Van Lancker, J. L., & Tomura, J. (1974) *Biochim. Biophys. Acta 353,* 99.

Wang, E. C., & Furth, J. J. (1977) *J. Biol. Chem. 252,* 116.

Weller, D. L., Heaney, A., & Sjogren, R. E. (1968) *Biochim. Biophys. Acta 168,* 576.