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An Endonuclease from *Escherichia coli* That Acts Preferentially on UV-Irradiated DNA and Is Absent from the *uvrA* and *uvrB* Mutants*

(DNA repair/DNA-protein binding/membrane filtration)

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ABSTRACT **At least two endonucleolytic activities that preferentially incise ultraviolet (UV)-irradiated DNA exist in extracts of *E. coli*. These two activities can be separated by phosphocellulose chromatographic fractionation. The subject of this paper is one of these activities, which elutes from phosphocellulose with 0.25 M potassium phosphate, pH 7.5. This endonucleolytic activity specific for UV-irradiated DNA is absent from partially purified extracts of *uvrA* and *uvrB* mutants, which are defective in excision of pyrimidine dimers, but is present in normal amounts in the *uvrC* excision-defective mutant. The enzyme binds very tightly and specifically to UV-irradiated DNA. Binding can be prevented by prior treatment of the irradiated DNA with photoreactivating enzyme. This binding activity is absent in *uvrA* and *uvrB* mutants, but present in *uvrC* and *uvrD* mutants.**

Excision repair of UV-induced cyclobutane-type pyrimidine dimers in *Escherichia coli* is currently thought to be a four-stage process (1). Initially, an endonucleolytic incision is made in the vicinity of the dimer. This is followed by exonucleolytic removal of the dimer and several adjacent nucleotides. Nucleotides are reinserted into the resultant cavity by a polymerase, using the opposite, intact strand as a template, with the continuity of the repaired region restored by polynucleotide ligase.

Mutants of *E. coli* with markedly reduced levels of polymerase I (2) and polynucleotide ligase (3, 4) are substantially more UV-sensitive than the wild type. *UvrA*, *uvrB*, and *uvrC* mutants do not excise dimers (5) and presumably are defective in either the endonucleolytic or exonucleolytic steps of excision repair.

Endonucleolytic activities specific for UV-irradiated DNA have been isolated and purified from *Micrococcus luteus* (6, 7) and *E. coli* infected with phage T4 (8, 9). These activities are absent from some UV-sensitive mutants (8, 10). Takagi *et al.*, have described an activity in extracts of *E. coli* that specifically incises UV-irradiated DNA (11). However, since this activity was reported to be present in extracts of all studied UV-sensitive mutants, the role of this enzymatic activity in excision repair was not clear.

In this paper, we describe the partial purification and some of the properties of an endonucleolytic activity, specific for UV-irradiated DNA, that is absent from some UV-sensitive strains of *E. coli*.

Abbreviation: RFI DNA, replicative form I DNA.

* This is paper 6 of a series entitled "Enzymatic Repair of DNA." Paper 5 is Hamilton, L., Mahler, I. & Grossman, L., *Biochemistry*, in press. Paper 4 is ref. 10. This paper is contribution no. 942 from the Graduate Department of Biochemistry, Brandeis University.

METHODS

DNA. Replicative form I [^{3}H]DNA (RFI DNA) of ϕX174 was prepared by methods described by Schekman *et al.* (12). This method involves ethidium bromide–CsCl density gradient centrifugation followed by a neutral sucrose gradient. A substantial amount of material absorbing 260-nm light, presumably unlabeled RNA, is removed in the sucrose gradient. In many of the preparations used in these experiments, the final gradient was omitted. This omission had no detectable effect on the assays described below.

UV-Irradiation. The ϕX RFI [^{3}H]DNA was irradiated in a stoppered quartz cuvette at 280 nm with a monochrometer described previously (6). In general, irradiation was to an average dose of 9000 ergs/mm^2. The incident dose was adjusted to compensate for self-absorption.

Enzymes. M. luteus UV-endonuclease was purified as described (6). This enzyme binds to DEAE–cellulose, as opposed to another UV-specific endonuclease that has little or no affinity to DEAE–cellulose (13). Yeast photoreactivating enzyme (EC 4.1.99.3; deoxyribocyclobutadipyrimidine pyrimidine-lyase), purified through the DNA cellulose step (14), was generously provided by H. Werbin.

Assays for UV-Endonuclease. 1. "Nicking" assay. This assay, which is a slight modification of an assay described by Center and Richardson (15), measures the extent of endonucleolytic activity by following the conversion of RFI DNA to the "nicked" form II DNA by denaturing the DNA with high pH followed by neutralization. RFI DNA is conserved in this procedure, while the form II material is dissociated into at least two single-stranded pieces. The RFI DNA passes through nitrocellulose membrane filters, while the single-stranded DNA is retained. After incubation, a reaction mixture of 0.3 ml is flooded with 2 ml of 0.9 M NaCl–0.1 M K_2HPO_4–0.025 M EDTA, previously titrated to a pH of 12.1 with NaOH. The denatured DNA mixture is neutralized with 0.2 ml of 1 N HCl and filtered through a Scheleicher & Schuell B-6 filter (25 mm) at a flow rate of about 10 ml/min. The reaction tube is washed twice with 2 × SSC (0.3 M NaCl–0.03 M Na citrate). The filters are dried and counted in Liquiflor (New England Nuclear Corp.) in a Packard Tri Carb scintillation counter.

2. "Binding" assay. The *E. coli* and *M. luteus* UV-endonucleases bind preferentially to UV-irradiated DNA. This binding can be measured by a filtration technique similar to that of Riggs *et al.* (16). In our method, an aliquot of enzyme is mixed at 0° C with ϕX174 form I or form II [^{3}H]DNA. After a 10-min incubation at 0°, the 0.3-ml mixture is flooded with 5 ml of ice-cold 2 × SSC and immediately filtered at a rate of about 10 ml/min through a 25-mm HAWP Millipore filter. The reaction tube is washed once with 5 ml of ice-cold 2 × SSC and the filter dried and counted in Liquiflor. This assay, though somewhat less sensitive than the "nicking" assay, has the advantage of very low control values. In addition, there is little nonspecific binding to unirradiated DNA in partially purified extracts of *E. coli*, while there is substantial nonspecific endonucleolytic activity.

Standard Assay Conditions. ϕX174 RFI [^{3}H]DNA (0.05 μg, 10,000 cpm) was added to a buffer (10 mM Tris·HCl, pH 7.6–80 mM NaCl–1 mM EDTA–1 mM 2-mercaptoethanol) along with UV-endonuclease. In the "nicking" assay, incubation was for 30 min at 37°; in the "binding" assay, incubation was for at least 10 min at 0°. The final reaction volume was 0.3 ml.

Bacterial Strains. E. coli B, at late logarithmic phase, was purchased as a frozen paste from Grain Processing, Muscatine, Iowa. *E. coli* strains AB 1157 (UV normal), AB 2500 (*uvrA*), AB 2499 (*uvrB*), and AB 2498 (*uvrC*) were obtained from the Coli Genetic Stock Center of Yale University. These four strains are isogenic except for the UV-sensitivity loci and thymine requirements. The *uvrA* and *uvrB* strains could easily be distinguished by the fact that partial reactivation of UV-irradiated λp *uvrB* (obtained from Dr. M. Gottesman) was observed on the *uvrB* strain, while none was seen on the *uvrA* strain. *E. coli* strain N-145 (*uvrD*) was obtained from Dr. E. Siegle of Tufts University. Strain HRH was obtained from Dr. Colin Clark.

Cells were grown in 12 liters of TYS medium (10 g of Bactotryptone, 5 g of yeast extract, and 5 g of NaCl per liter) under forced aeration in a Brunswick Micro Ferm Laboratory Fermentor to a concentration of about 10^9 cells per ml and harvested with a Sharples continuous flow centrifuge.

Preparation of Enzyme. Frozen cells were suspended in 50 mM KA buffer (50 mM potassium phosphate, pH 7.5–5 mM 2-mercaptoethanol–1 mM EDTA) at room temperature to a concentration of 1 g of cells per 10 ml of buffer by use of a Waring blender. Two milligrams of solid lysozyme chloride per gram of cells was added and the suspension was incubated at 37° for 30 min. After the suspension was cooled to about 4°, it was sonicated for 5 min at full power with a Branson Sonifier. The temperature of the suspension remained below 30°. Preparative volumes of extract (about 1 liter) were centrifuged at 15,000 rpm in the no. 19 rotor of a Spinco model L-65 centrifuge for 3 hr. A small amount of 50 mM KA buffer was added to fill the centrifuge bottles. Analytical quantities of extract (about 60 ml) were centrifuged in a Sorvall SS-34 rotor for 1 hr at 15,000 rpm. These extracts were never diluted.

After the centrifugation, the clear supernatant fluids were saved and either used immediately or frozen. UV-specific endonucleolytic activity in crude extracts does not appear to be affected by freezing. The protein concentration of extracts prepared in this manner ranged from 9 to 14 mg/ml.

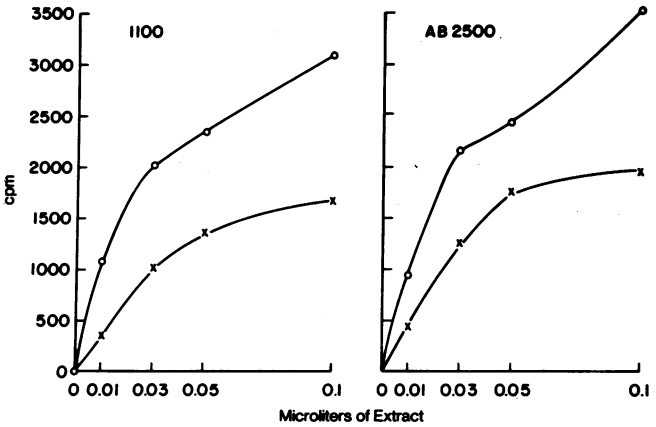

FIG. 1. Endonucleolytic activity specific for UV-irradiated DNA in crude extracts of *E. coli*. "Nicking" assays of various amounts of crude extracts of *E. coli* 1100 (UV-resistant) and AB2500 (UV-sensitive) were done as described in *Methods*. Each point is the average of two assays. A control incubation with no added extract has been subtracted from each point. The specific activity of the ϕX174 RFI DNA was 2 × 10^5 cpm/μg. (●) Irradiated DNA; (×) unirradiated DNA.

For preparative work, one liter of crude extract was brought to 0.15 M potassium phosphate by the addition of 0.11 volume of 1 M potassium phosphate, pH 7.5, and passed through a 6.5 × 12-cm column of DEAE–cellulose equilibrated with 0.15 M KA buffer (0.15 M potassium phosphate, pH 7.5–5 mM 2-mercaptoethanol–1 mM EDTA). The column was washed with 0.15 M KA buffer and all material with detectable A_{280} was collected, concentrated with 70% of saturation ammonium sulfate, dialyzed against 50 mM KA buffer in a Dow hollow fiber beaker dialyzer, and then applied to a 2.5 × 13-cm phosphocellulose column. After the column was washed with 50 mM KA buffer until no detectable A_{280} was observed in the flow-through material, the enzyme was eluted with a 500 ml (total volume) of 50 mM KA–0.5 M KA (0.5 M potassium phosphate, pH 7.5–5 mM 2-mercaptoethanol–1 mM EDTA) buffer gradient.

RESULTS

Proof of an enzyme's role in cellular metabolism ultimately rests on the analysis of enzymatic activity in mutants of the cell. It is to be anticipated that an endonuclease specific for UV-irradiated DNA would be absent from some excision-defective strains of *E. coli*. However, it has not been possible to detect any difference in the specific activity of UV-endonuclease between crude extracts of wild-type and the excision-defective *uvrA* strain of *E. coli* (Fig. 1). Similar levels of UV-specific endonucleolytic activity have also been observed in crude extracts of *uvrB* and *uvrC* excision-defective mutants. These results are in accord with those of Takagi *et al.* (9). One of the several possible interpretations of these results is that more than one UV-specific endonucleolytic enzyme is present in extracts of *E. coli;* a major activity that is not involved in excision repair and a minor activity that is involved in such repair. Presumably the major activity is present in sufficient quantity to mask the presence or absence of the "true" UV-endonuclease. Indeed, two endonucleolytic activities that act upon UV-irradiated DNA in preference to unirradiated DNA have been shown to be present in extracts of *M. luteus* (17).

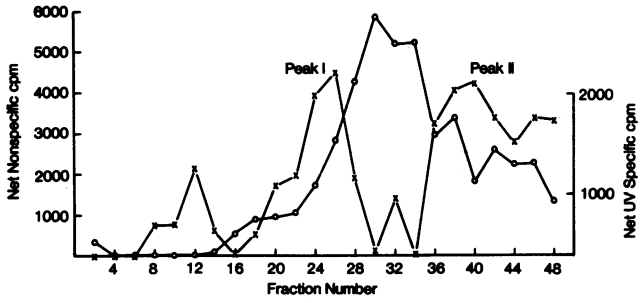

FIG. 2. Elution pattern of endonucleolytic activity from phosphocellulose. A crude extract previously passed through DEAE-cellulose was applied to a 2.5 × 13-cm column of phosphocellulose. After extensive washing, it was eluted with a gradient of 0.05 M KA to 0.5 M KA buffer. Endonuclease activity was measured by the "nicking" assay. (○) Nonspecific endonucleolytic activity; *left-hand scale.* (×) UV-specific endonucleolytic activity; *right-hand scale.*

To separate the UV-endonuclease responsible for the initial incision of the excision repair sequence from the presumed major masking activities, we applied partially purified extracts of *E. coli* B to a phosphocellulose column and eluted them with a gradient of increasing potassium phosphate (pH 7.5) concentration. The elution pattern of UV-specific endonuclease was determined by the RFI "nicking" assay. Two major peaks of UV-specific endonuclease activity were observed with an intervening peak of nonspecific activity (Fig. 2). The small peak of activity at fraction 12 in this experiment is not normally observed and has not been characterized. The peak eluting at about 0.25 M phosphate, referred to here as peak I, could be distinguished from the other activity (peak II) eluting from the phosphocellulose at about 0.35 M in two ways.

(*1*) First, when the fractions of the gradient of Fig. 2 were assayed by the filter-binding method, the pattern of Fig. 3 is observed. Specific binding to UV-irradiated DNA is found only in the region of the gradient occupied by the UV-specific endonucleolytic activity in peak I. The activity of peak-II material does not show any specific binding to irradiated DNA under these assay conditions. The *M. luteus* UV-endonuclease also binds to UV-irradiated DNA (Braun, unpublished results). The binding of the enzyme to UV-irradiated DNA can be shown to be due to the effects of cyclobutane pyrimidine dimers by the observation that photoreactivation of the irradiated DNA eliminates binding sites (Fig. 4). Photoreactivating enzyme from yeast is believed to act specifically on cyclobutane pyrimidine dimers in UV-irradiated DNA (18). Photoreactivation of binding sites for the UV-endonuclease is also an extremely sensitive method of the assay of the photoreactivating enzyme. In the experiment of Fig. 4, the photoreactivation of 10^{-15} moles of dimers can easily be detected.

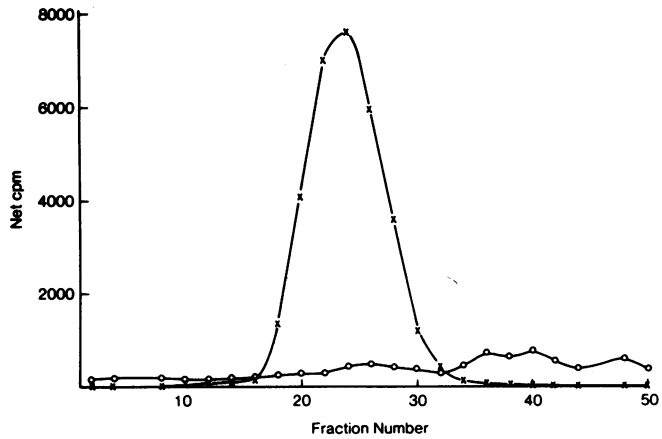

FIG. 3. Elution pattern of DNA-binding activity from phosphocellulose. The gradient depicted in Fig. 2 was assayed by the "binding" assay described in *Methods.* (○) Nonspecific binding activity; (×) UV-specific binding activity.

(*2*) A second distinction between activities associated with material of peak I and peak II is the observation that, like one of the *M. luteus* UV-endonuclease activities, the peak-I endonucleolytic activity is markedly stimulated by NaCl (19) (Fig. 5). Peak-II activity is only slightly stimulated by salt.

Several mutants of *E. coli* were grown to late logarithmic phase, harvested, lysed, and tested for the presence of activity coinciding with the properties of the material in peak I in two different ways. In one series of experiments, the crude extracts were applied directly to small phosphocellulose columns. Protein was eluted with a phosphate gradient, and UV-specific binding activity was assayed. Fig. 6 shows that little UV-specific binding activity was detected in phosphocellulose chromatograms of extracts of AB 2500 (*uvrA*) or AB 2499 (*uvrB*), while substantial activity was found in AB 2498 (*uvrC*). By this method, normal levels of UV-specific binding were also found in phosphocellulose chromatograms of N 145 (*uvrD*) (20) and HRH, a mutant with unusually high levels of host-cell reactivation (21).

A second method of isolating the peak-I activity was suggested by the elution pattern of the enzyme from Sephadex

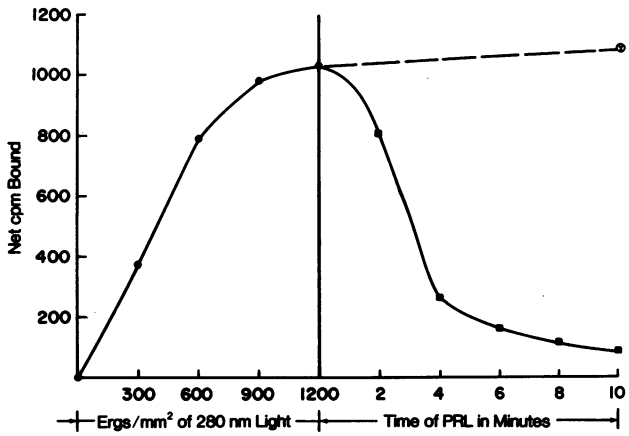

FIG. 4. Effect of photoreactivation on the binding of the *E. coli* endonuclease; 12.5 μg of φX RFI [³H]DNA were irradiated in 0.17 M NaCl–0.02 M potassium phosphate, pH 7.5 (0.5 ml final volume) in a 2-mm-thick quartz cuvette. At intervals, 20-μl aliquots were removed and assayed for ability to bind with *E. coli* UV-endonuclease in the standard binding assay. At 1200 ergs/mm² of 280-nm light, 5 μl of yeast photoreactivating enzyme was added to the remaining 0.3 ml in the cuvette and was irradiated at 37° at a distance of 11 cm from two Sylvania F15T8BLB bulbs. At the indicated times, 20-μl samples were assayed at 0° by the standard binding assay. Each point is the average of two assays. (●) Irradiated with 280-nm light; (■) photoreactivation enzyme added, irradiated with photoreactivating light (*PRL*); (⊗) photoreactivating enzyme added, incubated for 10 min at 37°, no photoreactivating light.

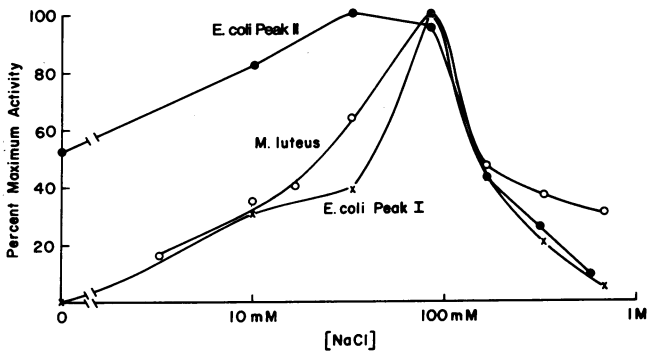

FIG. 5. Salt dependence of *E. coli* and *M. luteus* endonucleases. Assayed in 10 mM Tris·HCl, pH 7.6–1 mM EDTA–1 mM 2-mercaptoethanol and the indicated concentration of NaCl. (×) 20 μl of *E. coli* enzyme in 0.5 M potassium phosphate, pH 7.5, was assayed, yielding a final added 0.0033 M phosphate concentration. (○) The *M. luteus* enzyme was sufficiently concentrated so that essentially no salt was added to the assay with the enzyme. Each point is the average of two assays, and an unirradiated DNA control value was subtracted from each point.

G-75. In 0.1 M potassium phosphate, pH 7.5, the activity eluted in several peaks. The peak of activity most retarded by Sephadex G-75 eluted at a rate indicating a molecular weight of less than 14,000. Similar studies with peak-II activity imply that this enzyme has a molecular weight of about 20,000. The peak-I endonuclease would thus be expected to pass through a molecular sieve with a cutoff in the range of 14,000 molecular weight, while the activity of peak II would be retained. Spectrapor 2 brand dialysis tubing (Spectrum Medical Industries) appeared to be appropriate for this purpose, since it is capable of passing proteins of molecular weight of less than 14,000 (22). Pilot experiments with peak-I activity indicated that about 15% passed through the Spectrapor 2 tubing, while no detectable peak-II activity was observed to pass. Consequently, crude extracts of several *E. coli* mutants were forced through a single layer of this tubing under pressure, and the material passing through was assayed for UV-specific endonucleolytic activity. Fig. 7 shows that, once again, AB 2500 (*uvrA*) and AB 2499 (*uvrB*) lack appreciable UV-specific endonucleolytic activity, while AB 2498 (*uvrC*) and AB 1157 (UV-resistant) appear to have significant and equal amounts of enzymatic activity.

On the basis of these two experiments, and the substantial similarity of the activity associated with peak I with that of the *M. luteus* UV-endonuclease, we have concluded that this activity represents the enzyme responsible for the initial step of excision repair in *E. coli*.

DISCUSSION

Our finding that a UV-endonuclease is absent from extracts of *uvrA* and *uvrB* excision-defective mutants while the enzyme is present in *uvrC*, *uvrD*, and wild-type extracts is in accord with two results *in vivo*. According to Otsuji and Murayama (23), single-strand breaks are found in wild-type and *uvrC* cells after treatment with 7-methoxymitosene and decarbamoyl mitomycin C, while no evidence of incision was observed in *uvrA* and *uvrB* cells. Another indication of the *in vivo* function of the *uvrA*, *B*, and *C* gene products comes from the recent work of Seeburg and Johansen, who showed that superinfecting irradiated lambda DNA was not incised by *uvrA* and *uvrB*

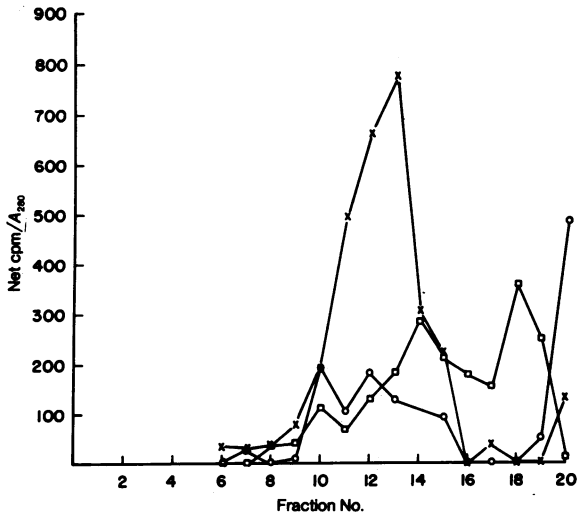

FIG. 6. Elution pattern of UV-specific binding from phosphocellulose of various UV-sensitive mutants. Crude extracts of *E. coli*, prepared as described in *Methods*, were applied directly to a 1 × 12-cm column of phosphocellulose equilibrated with 50 mM KA buffer. About 250 mg of protein was applied. The elution was with a 50-ml 50 mM KA to 0.5 M KA gradient. Twenty-five 2-ml fractions were collected and assayed by the binding assay. (○) AB2500 (*uvrA*); (□) AB2499 (*uvrB*); (×) AB2498 (*uvrC*).

hosts, while incision took place in wild-type and *uvrC* hosts (24). In their work, the apparent incision rate was lower in the *uvrC* host than in the wild type. This conflicts with our observation of equal enzymatic activity in both.

Recent data (Braun and Grossman, unpublished) indicate that *E. coli* polynucleotide ligase is capable of closing the incisions produced by the *E. coli* UV-endonuclease. Thus, it is possible that the reduced rate of incision observed by Seeburg and Johansen in the *uvrC* mutant is a reflection of an enhanced

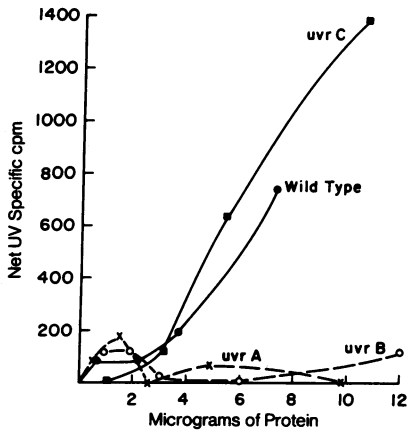

FIG. 7. UV-specific endonucleolytic activity of crude extracts passed through Spectrapor 2 tubing. Spectrapor tubing was opened by cutting it along one edge, soaked in water overnight, and installed in an Amicon pressure dialysis cell. Crude extract was placed in the cell and 40 lbs/inch^2 of nitrogen pressure was applied. Two-milliliter fractions were taken and assayed by the "nicking" assay. Protein concentration was estimated from the A_{233} and A_{224}. Each point is the average of two assays, and an unirradiated DNA control was subtracted from each point. (×) AB2500 (*uvrA*); (○) AB2499 (*uvrB*); (■) AB2498 (*uvrC*); (●) AB1157 (UV-resistant).

probability of the sealing of incisions produced by the UV-endonuclease. It is unlikely that mutations in the *uvrC* gene produce an inhibitor of the UV-endonuclease since these mutations are recessive to the wild type (25).

It is difficult to resolve the results presented in this paper with the observation that T4 UV-endonuclease (26) and the *M. luteus* UV-endonuclease (27) reactivate the biological activity of irradiated ϕX174 and ϕA RFI DNA to an equal extent in the *uvrA, B,* and *C* hosts; a result that appears to indicate the absence of this enzyme in all three mutants. We have also observed this result and are at a loss to explain its significance.

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