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# Characterization of Deoxyribonucleic Acid Repair Synthesis in Permeable Human Fibroblasts<sup>†</sup>

Steven L. Dresler, John D. Roberts,<sup>‡</sup> and Michael W. Lieberman\*

**ABSTRACT:** We have extended our permeable cell system for measuring DNA excision repair [Roberts, J. D., & Lieberman, M. W. (1979) *Biochemistry* 18, 4499-4505] so that steps of the repair process, beginning with incision and extending at least through the "rearrangement" of repaired nucleosomes which follows repair synthesis, all take place in permeable cells. In the revised protocol, human fibroblasts are made permeable, damaged with UV or chemicals in suspension, and incubated with a reaction mix containing ATP and the four deoxyribonucleoside triphosphates, one of which is labeled with <sup>32</sup>P. By reducing the exogenous dNTP concentration to 3  $\mu$ M and including 15 mM KCl in the reaction mixture, we have greatly reduced background incorporation in undamaged cells without significantly reducing repair synthesis. This permits us to measure repair synthesis without separating it from replicative synthesis by isopycnic centrifugation. Repair synthesis in this system is very similar to that occurring in intact cells: in response to DNA damage, nucleotides are incorporated into DNA of parental density (when analyzed by the BrdUrd density shift technique), incorporation increases with increasing DNA damage, synthesis is dependent on the presence of all

four dNTPs, and the system accurately reflects the genetic UV repair deficiency of xeroderma pigmentosum (XP) cells. Furthermore, as has been observed in intact cells, repair-incorporated nucleotides in these permeable cells are initially overrepresented in staphylococcal nuclease sensitive regions of chromatin and are subsequently redistributed to give a nearly uniform distribution between nuclease-sensitive and -resistant regions. The UV dose curve of permeable cells differs somewhat from that of intact cells; however, the dose curve for permeable cells treated with *N*-methyl-*N*-nitrosourea is very similar to that of intact cells. Repair synthesis in UV-damaged, permeable normal and XP cells is stimulated by addition of *Micrococcus luteus* UV endonuclease, indicating that the damaged DNA is accessible to exogenous repair enzymes and suggesting that incision, or an obligatory preincision step, is rate limiting for excision repair in these permeable cells. Repair synthesis in this system is inhibited by aphidicolin, but not by high levels of dideoxy-TTP, suggesting involvement of DNA polymerase  $\alpha$  in excision repair. Novobiocin is also inhibitory at concentrations known to inhibit both DNA polymerase  $\alpha$  and the HeLa cell type II DNA topoisomerase.

**E**lucidation of the mechanisms of basic cellular processes such as DNA replication and transcription (e.g., Parker et al., 1978; DePamphilis & Wasserman, 1980) has been greatly facilitated by the use of subcellular systems capable of faithfully reproducing these functions. Several subcellular systems for studying DNA excision repair have recently been described (e.g., Ciarrocchi & Linn, 1978; Smith & Hanawalt, 1978; Roberts & Lieberman, 1979). As fully characterized, these systems use cells which are intact when exposed to DNA damaging agents and are subsequently disrupted. In such systems, the early stages of excision repair may not be accessible to biochemical manipulation. Several published experiments do indicate, however, that excision repair can occur in cells damaged after disruption (Smith & Hanawalt, 1978; Ciarrocchi et al., 1979). We have modified our previously described system (Roberts & Lieberman, 1979) by routinely damaging cells after they are made permeable and have established that steps of the excision repair process beginning with incision and extending at least through rearrangement of repaired nucleosomes take place in permeable cells. The characteristics of repair synthesis in permeable cells, including the distribution of repair-incorporated nucleotides in chro-

matin, are very similar to those of repair synthesis in intact cells. We have improved the sensitivity and rapidity of the assay so that this system now seems well adapted for studies of the mechanism of DNA excision repair.

## Materials and Methods

**Cell Culture.** Human diploid fibroblasts (AG1518; Institute for Medical Research) and xeroderma pigmentosum fibroblasts from complementation groups A (XP12BE; American Type Culture Collection) and G (XP2BI; Institute for Medical Research) were grown in Dulbecco's modified Eagle's medium, supplemented with 5% fetal calf serum and 5% newborn calf serum, at 37 °C in 10% CO<sub>2</sub>. Cells were diluted 1:2 or 1:3 and placed in either plastic tissue culture dishes or glass roller bottles, and the DNA was uniformly labeled during growth with 3 nCi/mL [<sup>14</sup>C]dThd<sup>1</sup> (50-60 mCi/mmol) giving a <sup>14</sup>C specific activity of 200-400 cpm/ $\mu$ g of DNA. The medium was replaced after 6-8 days, and cells were allowed to come to confluence and were used for experiments between 14 and 21 days after passage.

**Damaging of Permeable Cells with UV and Chemicals.** Confluent cells, prelabeled with [<sup>14</sup>C]dThd, were scraped from tissue culture plates or roller bottles, washed with ice-cold 2.7

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<sup>1</sup> Abbreviations: dThd, thymidine; UV, ultraviolet; MNU, *N*-methyl-*N*-nitrosourea; Cl<sub>3</sub>CCOOH, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; ddTTP, 2',3'-dideoxythymidine 5'-triphosphate; SN, staphylococcal nuclease; dNTPs, 2'-deoxyribonucleoside 5'-triphosphates; BrdUTP, 5-bromo-2'-deoxyuridine 5'-triphosphate; BrdUrd, 5-bromo-2'-deoxyuridine; XP, xeroderma pigmentosum; bp, base pairs; PCA, perchloric acid.

mM NaH<sub>2</sub>PO<sub>4</sub>, 13.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 135 mM NaCl, and 4.9 mM KCl, pelleted at 260g, and resuspended at a concentration of  $\sim 1 \times 10^7$  cells/mL in buffer A [10 mM Tris, pH 7.6 (at 37 °C), 4 mM MgCl<sub>2</sub>, 1 mM EDTA, 250 mM sucrose, 3 mM dithiothreitol] at 4 °C. The cell suspension was placed in a Dounce homogenizer and subjected to five strokes with a loose-fitting (A) pestle and kept at 4 °C for 30 min. To damage with UV, the suspension was spread in a layer 1 mm thick in either a plastic petri dish or the wells of a microtiter plate and, with gentle shaking, was exposed to radiation from a germicidal (G15T8) lamp at 4 °C until the desired UV dose was reached. To damage with MNU, the permeable cell suspension was brought to 40 mM Tris (pH 7.6, at 37 °C), 8 mM MgCl<sub>2</sub>, 15 mM KCl, 5 mM ATP, and the desired concentration of MNU (with a freshly prepared stock of 250 mM MNU in 10 mM potassium phosphate, pH 6.0) and was placed at 37 °C for 15 min. The cells were pelleted, washed once in cold buffer A, and resuspended in the original volume of buffer A at 4 °C.

**Measurement of Repair Synthesis in Permeable Cells.** Small portions of the cell suspension (0.03–0.1 mL), either damaged or undamaged, were mixed at 4 °C with half the volume of a concentrated reaction mix to give the following concentrations: 40 mM Tris (pH 7.6, at 37 °C), 8 mM MgCl<sub>2</sub>, 15 mM KCl, 5 mM ATP, 3  $\mu$ M TTP, 3  $\mu$ M dCTP, 3  $\mu$ M dGTP, 3  $\mu$ M dATP, 20  $\mu$ Ci/mL [ $\alpha$ -<sup>32</sup>P]dCTP (410 Ci/mmol), 167 mM sucrose, 2 mM dithiothreitol, and 0.67 mM EDTA. (This final solution is subsequently referred to as "reaction mix".) Samples were placed at 37 °C for the time desired, and the reaction was stopped by adding a large volume (usually 5 mL) of ice-cold buffer B1 [10 mM Tris, pH 7.6 (at 37 °C), 320 mM sucrose, 0.5% Triton X-100] and centrifuging at 1600g for 20 min at 4 °C. In most experiments (and unless otherwise indicated), the resulting nuclear pellet was resuspended in 1 mL of 1% sodium dodecyl sulfate, precipitated by adding 1 mL of 10% Cl<sub>3</sub>CCOOH and 0.1 M tetrasodium pyrophosphate, and collected on a glass-fiber filter (Whatman GF/C). The filter was washed twice with 5% Cl<sub>3</sub>CCOOH and 50 mM tetrasodium pyrophosphate, twice with 0.1 N HCl, and twice with 70% ethanol and was dried. The radioactivity was determined by scintillation counting in 3a70 (Research Products International). In a few experiments, BrdUTP was substituted for TTP in the reaction mix, and the final nuclear pellet was washed twice with buffer B1, washed three times with 10 mM Tris (pH 7.6, at 37 °C), and digested in 1 mL of 10 mM Tris (pH 7.6) with 300  $\mu$ g/mL proteinase K at 37 °C for 16 h to release the DNA, which was analyzed by isopycnic centrifugation in alkaline CsCl as described (Smerdon et al., 1979).

**Determination of <sup>14</sup>C Specific Activity of DNA.** Cells were washed twice with buffer B1 and twice with 10 mM Tris, pH 7.6, and then analyzed by one of the following methods. Method 1: DNA was released by proteinase K digestion as described above and isolated by isopycnic centrifugation in neutral CsCl (Smerdon et al., 1978). DNA concentration of the peak was determined from UV absorbance, and radioactivity was determined by liquid scintillation counting in either Instagel (Packard) or 3a70. Method 2: The washed pellet was dissolved in 5 mM NaOH, an equal volume of 8.8% PCA was added, and the sample was heated to 70 °C for 20 min and centrifuged at 2300g for 15 min at 4 °C. DNA content of the supernatant was determined by the diphenylamine assay (Burton, 1968) using calf thymus DNA as a standard. Radioactivity of the supernatant was determined by liquid scintillation counting in 3a70. Appropriate corrections were made

for differences in counting efficiency between methods 1 and 2.

**Calculation of Repair Synthesis.** After correction for background and crossover, the <sup>32</sup>P/<sup>14</sup>C ratio for each sample was calculated. For examples analyzed by isopycnic centrifugation, the total <sup>32</sup>P and <sup>14</sup>C radioactivity in the central four to five fractions of the parental density peak ( $\rho \sim 1.73$  g/cm<sup>3</sup>) was used for this calculation. The known <sup>32</sup>P specific activity of the dCTP in the reaction mix and the <sup>14</sup>C specific activity of the bulk DNA (determined as described above) were used to convert the <sup>32</sup>P/<sup>14</sup>C ratios to dCMP incorporation per unit DNA. Repair synthesis was taken to be the difference between incorporation in corresponding damaged and undamaged samples.

**Isolation and Staphylococcal Nuclease Digestion of Nuclei.** For chromatin studies, nuclei were prepared by washing the permeable cells 3 times with buffer B2 [10 mM Tris, pH 8.0 (at 25 °C), 1 mM CaCl<sub>2</sub>, 250 mM sucrose, 0.5% Triton X-100], twice with buffer B2 without Triton X-100, and once with buffer B3 [10 mM Tris, pH 8.0 (at 25 °C), 0.1 mM CaCl<sub>2</sub>, 250 mM sucrose]. The final pellet was resuspended in buffer B3 to a concentration of  $2.2 \times 10^6$  nuclei/mL. Nuclei were digested at 37 °C with 4 units/mL staphylococcal nuclease (Worthington). Samples of the digestion mix were taken at various times, placed on ice, and brought to 9 mM EDTA to stop the digestion. For determination of the extent of digestion, samples were precipitated with 6.6% (v/v) perchloric acid for 20 min on ice and centrifuged at 15000g for 5 min at 4 °C, and the radioactivity of the supernatants was determined. The fraction of radioactivity released was determined at each time point by dividing the acid-soluble radioactivity by the total radioactivity present in the nuclei [determined as described by Smerdon et al. (1978)]. Some SN digestion samples were treated instead overnight at 37 °C with 300  $\mu$ g/mL proteinase K and ethanol precipitated, and the DNA fragments thus obtained were separated by electrophoresis on 2.8% agarose gels in neutral buffer (Smerdon et al., 1978). The gel lanes were divided into 2-mm sections which were melted in 2.5 mL of H<sub>2</sub>O and assayed for radioactivity by liquid scintillation counting after addition of 6.0 mL of 3a70. The relation between migration distance and fragment length was determined by simultaneous electrophoresis of the *Hae*III restriction fragments of  $\phi$ X174 RF. Sizes of the restriction fragments were assigned by using the nucleotide sequence of  $\phi$ X174 (Sanger et al., 1978).

## Results

**DNA Repair Synthesis following UV Irradiation of Permeable Normal Human Fibroblasts.** Confluent normal human fibroblasts (AG1518), prelabeled with [<sup>14</sup>C]dThd, were harvested, made permeable, damaged, and incubated with reaction mix containing BrdUTP in place of TTP. Samples of DNA isolated from UV-irradiated and unirradiated cells were analyzed by isopycnic centrifugation in alkaline CsCl (Figure 1). In undamaged cells (Figure 1A), there was little incorporation of [<sup>32</sup>P]dCMP into DNA of parental density (i.e., cosedimenting with the <sup>14</sup>C bulk label). Following UV irradiation (Figure 1B), a large amount of [<sup>32</sup>P]dCMP incorporation into parental density DNA was observed, indicating that DNA excision repair was taking place in these cells.

The reaction mix used in these experiments was modified from that which we have used previously (Roberts & Lieberman, 1979) by reduction of the dNTP concentrations and inclusion of KCl. These changes greatly reduce the incorporation (mostly due to replication) in undamaged cells (see Figures 5 and 6 below), permitting the calculation of repair

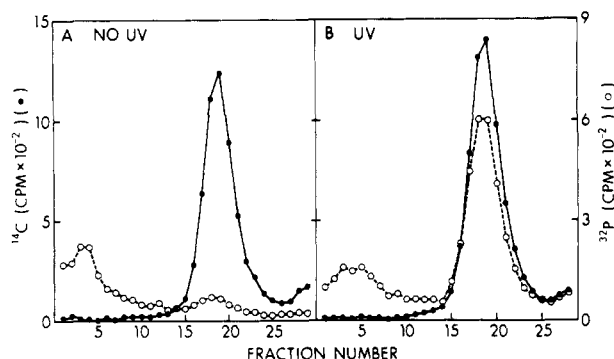


FIGURE 1: Incorporation of dCMP induced by UV irradiation of permeable cells. Permeable normal human fibroblasts were damaged and incubated with reaction mix containing BrdUTP in place of TTP (see Materials and Methods); DNA was isolated and analyzed by isopycnic centrifugation in alkaline CsCl. Fractions are numbered beginning with the most dense. (●)  $^{14}\text{C}$ ; (○)  $^{32}\text{P}$ . (A) No UV; dCMP incorporation in parental peak, 1.4 fmol/ $\mu\text{g}$  of DNA. (B) 170  $\text{J}/\text{m}^2$  UV; dCMP incorporation in parental peak, 20.2 fmol/ $\mu\text{g}$  of DNA.

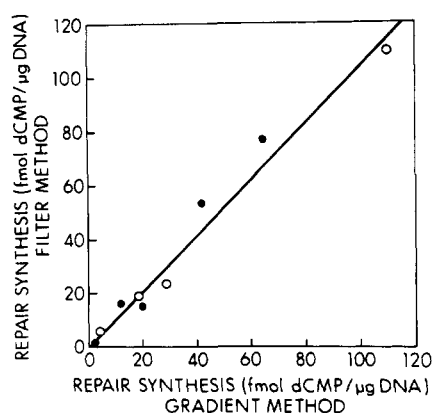


FIGURE 2: Comparison of repair synthesis determined by measurement of dCMP incorporation into isolated parental density DNA with that determined by measurement of total  $\text{Cl}_3\text{CCOOH}$ -precipitable [ $^{32}\text{P}$ ]dCMP incorporation. Permeable normal human fibroblasts, irradiated with either 20 (●) or 200  $\text{J}/\text{m}^2$  (○) of UV, were incubated for various times with reaction mix containing BrdUTP in place of TTP. Samples were divided, and repair synthesis was determined both by separation of repair and replicative incorporation on alkaline CsCl gradients ("gradient method") and by measurement of total incorporation into  $\text{Cl}_3\text{CCOOH}$ -precipitable material collected on glass-fiber filters ("filter method"; see Materials and Methods for details).

synthesis from measurements of total [ $^{32}\text{P}$ ]dCMP incorporation in damaged and undamaged cells. In one experiment (Figure 2), we divided the samples following the synthesis incubation and measured repair synthesis by both separation of replication and repair incorporation on alkaline CsCl gradients ("gradient method") and by determination of  $^{32}\text{P}$  incorporation into  $\text{Cl}_3\text{CCOOH}$  precipitable material collected on glass-fiber filters ("filter method"). The values obtained by the two techniques were very similar, and correlation analysis (Daniel, 1978) of the data yielded a correlation coefficient of 0.99. Although the filter method gave values for repair synthesis which were, on the average, about 5% higher than those obtained by the gradient method, we believed that, under these reaction conditions, the filter technique measures repair synthesis with sufficient accuracy to be useful for most purposes.

Using this simple  $\text{Cl}_3\text{CCOOH}$  precipitation-filtration technique, we have investigated the effect on repair synthesis of varying UV dose. In permeable cells irradiated with UV in suspension, dCMP incorporation increased with dose up to about 50  $\text{J}/\text{m}^2$  (Figure 3); doses above this induced little

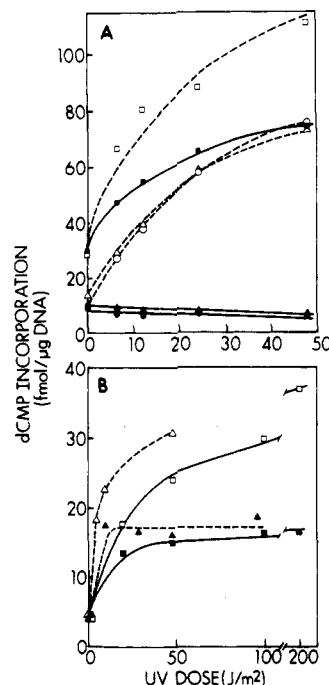


FIGURE 3: (A) UV-induced repair synthesis in permeable normal (AG1518) and xeroderma pigmentosum (complementation groups A and G) fibroblasts. Permeable normal cells (■, □), XP group A cells (▲, △), and XP group G cells (●, ○) were damaged with the indicated dose of UV and incubated for 15 min with either complete reaction mix (see Materials and Methods) (■, ▲, ●) or complete reaction mix plus 130 units/mL (final concentration) *M. luteus* UV endonuclease (□, △, ○), and acid precipitable dCMP incorporation was determined as described (Materials and Methods). (B) UV-induced repair synthesis in permeable AG1518 cells damaged in various ways. Permeable cells damaged in suspension at 4 °C and incubated for 15 min with complete reaction mix (as under Materials and Methods) (■) or with reaction mix containing 130 units/mL (final concentration) *M. luteus* UV endonuclease (□). Intact cells irradiated as a tissue culture monolayer at 4 °C and either made permeable and incubated with reaction mix for 15 min (▲) or preincubated in cell culture medium at 37 °C for 10 min and then made permeable and incubated with reaction mix for 15 min (△). Each point is the average of two determinations.

additional incorporation. We have compared this UV dose curve with the dose curves for cells damaged according to two protocols previously described in the literature (Figure 3B). Cells irradiated as a cell culture monolayer at 4 °C, then harvested, made permeable, and incubated with reaction mix [similar to Roberts & Lieberman (1979)] showed a sharp increase in dCMP incorporation up to 10  $\text{J}/\text{m}^2$  and no additional increase as the dose was increased further. The plateau level of incorporation in these cells was very similar to that for permeable cells irradiated in suspension. Cells irradiated as a cell culture monolayer, incubated for 10 min at 37 °C prior to being harvested, made permeable, and incubated with reaction mix [similar to Ciarrocchi & Linn (1978)] showed higher levels of dCMP incorporation which increased up to 48  $\text{J}/\text{m}^2$ , the highest dose used.

To investigate the molecular basis for the differences between these dose curves, we also examined the UV dose curve for normal cells made permeable, irradiated in suspension, and incubated with reaction mix containing *Micrococcus luteus* UV endonuclease (a gift of Dr. L. Grossman), which incises DNA at the sites of pyrimidine dimers (Figure 3). At every dose, these cells showed more damage-dependent dCMP incorporation than cells which were treated identically except that UV endonuclease was not present in the reaction mix. The dose curve for cells incubated with synthesis mix containing UV endonuclease did not plateau at 50  $\text{J}/\text{m}^2$  but

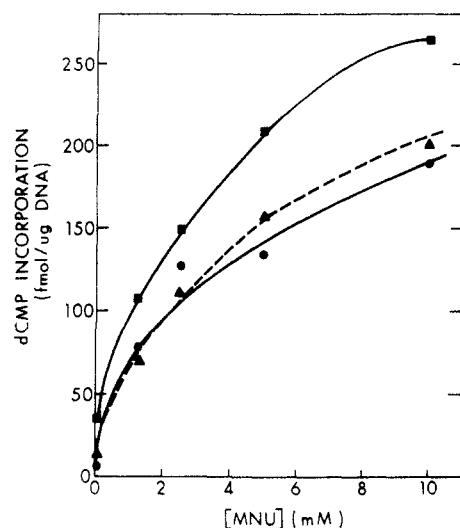


FIGURE 4: MNU-induced repair synthesis in permeable normal and xeroderma pigmentosum fibroblasts. Permeable normal cells (■), XP group A cells (▲), and XP group G cells (●) were damaged with MNU at the indicated concentration (Materials and Methods) and incubated for 15 min with reaction mix, and  $\text{Cl}_3\text{CCOOH}$ -precipitable dCMP incorporation was determined. Each point is the average of two determinations.

continued to increase to  $200 \text{ J/m}^2$ , the highest dose tested.

**Repair Synthesis following UV Damage to Permeable Xeroderma Pigmentosum Cells.** UV irradiation of permeable xeroderma pigmentosum cells (complementation groups A and G) produced no increase in dCMP incorporation (Figure 3A) even at a dose of  $48 \text{ J/m}^2$ . Addition of *M. luteus* UV endonuclease to the reaction mix alleviated the XP repair defect and allowed the permeable XP cells to attain essentially normal levels of repair synthesis in response to UV (Figure 3A).

**Repair Synthesis following Chemical Damage to Permeable Cells.** Permeable normal human fibroblasts, damaged with MNU for 15 min at  $37^\circ\text{C}$ , washed with buffer A, and then incubated with reaction mix, showed high levels of dCMP incorporation (Figure 4) which increased with dose of MNU up to  $10 \text{ mM}$ , the highest concentration used. The dose curve is similar to that for repair synthesis following MNU damage of intact normal human fibroblasts (P. Policastro and M. W. Lieberman, unpublished data). As has been found in intact cells [see Friedberg et al. (1979)], the response of permeable XP cells to MNU is essentially the same as that of permeable normal cells (Figure 4).

**Optimal Conditions for Repair Synthesis in Permeable Cells.** Optimal concentrations for several components of the reaction mix are similar to those previously described (Roberts & Lieberman, 1979):  $8 \text{ mM MgCl}_2$ ,  $5 \text{ mM ATP}$ , pH 7.6 (at  $37^\circ\text{C}$ ), in Tris-HCl buffer (data not shown). We have investigated in detail the dependence of UV-induced dCMP incorporation on the concentration of several salts (Figure 5). Addition to the reaction mix of KCl up to  $15 \text{ mM}$  produced a 50% decrease in incorporation in unirradiated cells, but no decrease in incorporation in irradiated cells. Increasing the concentration of KCl above  $15 \text{ mM}$  sharply reduced incorporation in damaged cells as well.

The dependence of UV-induced dCMP incorporation on the concentration of  $\text{NH}_4^+$  [added as  $(\text{NH}_4)_2\text{SO}_4$ ] was identical with the dependence on  $\text{K}^+$  (Figure 5). Added NaCl stimulated incorporation in both irradiated and unirradiated cells at low concentrations ( $5$ – $10 \text{ mM}$ ), and then inhibited as the concentration was increased further (Figure 5). The inhibitory effects of NaCl have been reported by previous authors (Ciarrocchi & Linn, 1978; Roberts & Lieberman, 1979), but none

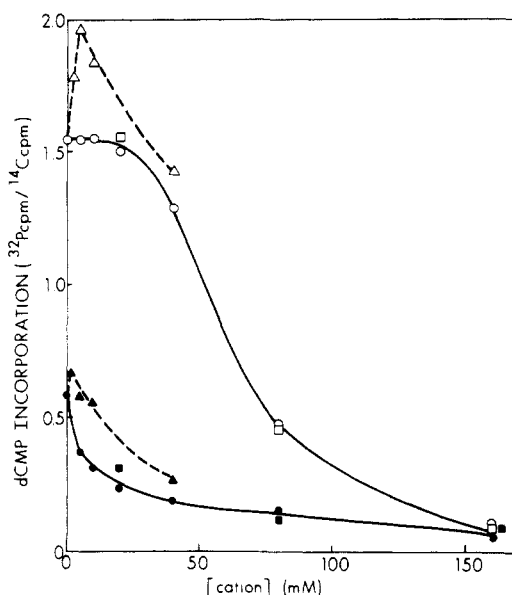


FIGURE 5: Effects of KCl, NaCl, and  $(\text{NH}_4)_2\text{SO}_4$  on repair synthesis. Permeable AG1518 cells, either undamaged (●, ■, ▲) or irradiated with  $168 \text{ J/m}^2$  UV (○, □, △), were incubated for 30 min with reaction mixes modified from that described under Materials and Methods as follows: (●, ○) KCl adjusted to the final concentration indicated; (■, □) no KCl and  $(\text{NH}_4)_2\text{SO}_4$  added to give the indicated final concentration of  $\text{NH}_4^+$ ; (▲, △) no KCl and NaCl added to the indicated final concentration. Each point is the average of two determinations.

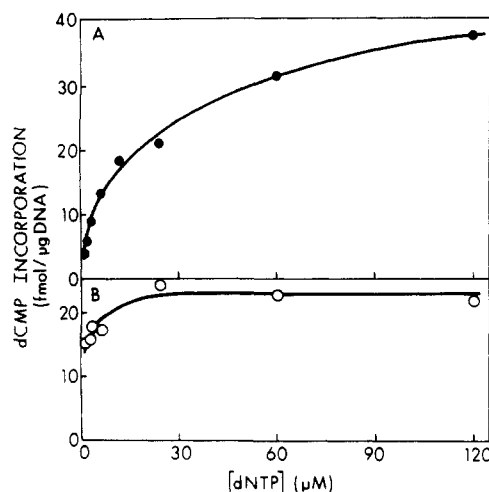


FIGURE 6: (A) Effect of dNTP concentration on incorporation in undamaged permeable cells. Permeable normal cells were incubated for 15 min at  $37^\circ\text{C}$  with reaction mix containing the indicated final concentrations of dATP, dGTP, dCTP, and TTP.  $\text{Cl}_3\text{CCOOH}$ -precipitable dCMP incorporation was determined. Each point is the average of two determinations. (B) Effect of dNTP concentration of UV-induced repair synthesis. Permeable normal cells, unirradiated or irradiated with  $100 \text{ J/m}^2$  UV, were incubated for 15 min with reaction mix containing the indicated concentration of dATP, dGTP, dCTP, and BrdUTP. DNA was analyzed by isopycnic centrifugation in alkaline CsCl. Values on the ordinate are net repair synthesis, calculated as described under Materials and Methods.

have previously examined low enough NaCl concentrations to observe the stimulatory effect we described here.

Increasing the concentration of deoxyribonucleoside triphosphates in the reaction mix increased dCMP incorporation into undamaged permeable cells over the entire range examined,  $1$ – $120 \mu\text{M}$  (Figure 6A). In contrast, UV-induced repair synthesis increased with dNTP concentration only up to about  $20 \mu\text{M}$  and then remained constant up to  $120 \mu\text{M}$ , the highest level examined (Figure 6B). A double-reciprocal plot (not

Table I: Effect on Repair Synthesis of Various Modifications of the Reaction Mixture<sup>a</sup>

modification	repair synthesis (%)
none (control)	100
-TTP	16
-unlabeled dCTP <sup>b</sup>	56
-dGTP	18
-dATP	95
-TTP, dGTP, dATP, unlabeled dCTP <sup>b</sup>	7
-ATP	0
-ATP, +GTP	0
+GTP	107
+1 mM spermidine	53
+53 $\mu$ M novobiocin	95
+530 $\mu$ M novobiocin	49
+1060 $\mu$ M novobiocin	0
+2.6 mM nalidixic acid	130
+150 $\mu$ M dideoxy-TTP	89
+25 $\mu$ M aphidicolin	10

<sup>a</sup> Permeable AG1518 cells, unirradiated and irradiated with 20 or 40 J/m<sup>2</sup> UV, were incubated for 15 min with reaction mixes modified as indicated from that described under Materials and Methods. Repair synthesis was determined as under Materials and Methods. Each value is the average of two determinations.

<sup>b</sup> Unlabeled dCTP was omitted, but 20  $\mu$ Ci/mL [ $\alpha$ -<sup>32</sup>P]dCTP was present, giving a final dCTP concentration of 57 nM.

shown) of the data for incorporation into undamaged permeable cells was linear and yielded an apparent  $K_m$  for dNTPs of 8  $\mu$ M. The data for UV-induced repair synthesis in permeable cells (which covered an insufficient range of dCMP incorporation to justify analysis by double-reciprocal plot) indicate that the apparent  $K_m$  for dNTPs for that process is less than 1  $\mu$ M (i.e., incorporation at 1  $\mu$ M is greater than half of the maximal incorporation). Repair synthesis in our permeable cell system is strongly dependent, however, on added dNTPs. Omission of all four dNTPs (except for the tracer amount of [<sup>32</sup>P]dCTP) reduced repair synthesis to 7% of the control level (Table I). Omission of either TTP or dGTP alone also greatly reduced repair synthesis. Omitting only unlabeled dCTP from the mix reduced repair synthesis by about half; apparently the 57 nM dCTP present due to the presence of [ $\alpha$ -<sup>32</sup>P]dCTP was sufficient to support a significant level of repair synthesis in the presence of the other three dNTPs at 3  $\mu$ M. Omission of dATP had little effect, but a fairly high level of dATP might still have been present due to either metabolism of the ATP present or contamination of the added ATP with dATP.

**Time Course of UV-Induced Repair Synthesis.** Incorporation following UV irradiation was linear for 20–30 min and continued for 60 min (Figure 7). The duration of incorporation was not altered by varying the UV dose. The time course was not prolonged by adding fresh 5 mM ATP, fresh 3  $\mu$ M dNTPs (including 20  $\mu$ Ci/mL [ $\alpha$ -<sup>32</sup>P]dCTP), fresh complete reaction mix, or *M. luteus* UV endonuclease at 30 or 60 min or by additional UV irradiation at 30 or 60 min.

**Distribution of UV-Induced Repair Synthesis in Chromatin in Permeable Cells.** Normal human fibroblasts, which had been prelabeled with [<sup>14</sup>C]dThd, harvested, made permeable, and damaged with UV, were incubated with complete reaction mix for 15 min and chased for various times in reaction mix lacking [ $\alpha$ -<sup>32</sup>P]dCTP. Incorporation of dCMP into irradiated cells during the pulse period was 50.5 fmol/ $\mu$ g of DNA, and there was neither further incorporation nor loss of label during the 60-min chase period. (Incorporation into unirradiated cells during the 15-min pulse was 9.0 fmol/ $\mu$ g of DNA.) Nuclei prepared from cells following the pulse and chase periods were digested with staphylococcal nuclease to investigate the dis-

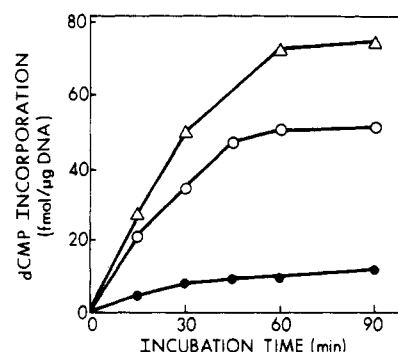


FIGURE 7: Time course of repair synthesis. Permeable AG1518 cells, irradiated with 0 (●), 40 (○), or 400 J/m<sup>2</sup> UV, were incubated with reaction mix for the times indicated. Each point is the average of two determinations.

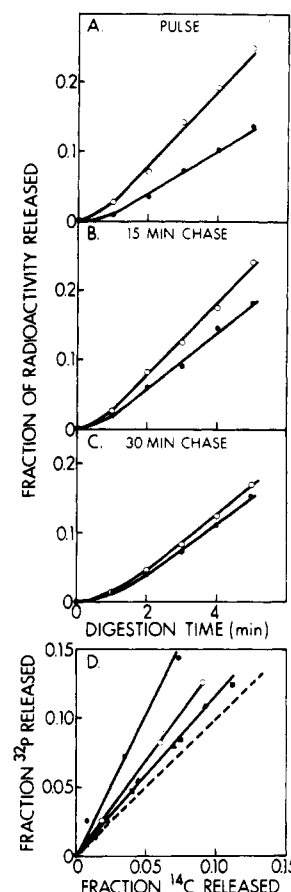


FIGURE 8: Staphylococcal nuclease digestion of nuclei from permeable cells labeled during repair synthesis and chased for various times. Permeable AG1518 cells were damaged with 400 J/m<sup>2</sup> UV, incubated for 15 min at 37 °C with complete reaction mix (pulse period), washed twice at 4 °C with reaction mix lacking <sup>32</sup>P label, and incubated for various times at 37 °C in reaction mix without <sup>32</sup>P (chase period). Nuclei prepared from cells immediately after the pulse (panel A) and after 15 (panel B) and 30 min (panel C) of chase were digested with staphylococcal nuclease, and the release of repair-incorporated nucleotides (<sup>32</sup>P labeled) (○) and bulk DNA (<sup>14</sup>C labeled) (●) was measured. In panel D, SN digestion data for nuclei prepared after the pulse (●), 15-min chase (○), and 30-min chase (■) are replotted, along with digestion data for nuclei prepared after a 60-min chase (▲). The dashed line in panel D represents equal SN sensitivity of repair and bulk labels.

tribution of repair synthesis in chromatin. Immediately following the pulse period (Figure 8A), repair-incorporated nucleotides (<sup>32</sup>P labeled) were released much more rapidly by SN than were the nucleotides of bulk DNA (<sup>14</sup>C labeled), indicating a large overrepresentation of repair synthesis in nuclease-sensitive regions of chromatin. This overrepresen-

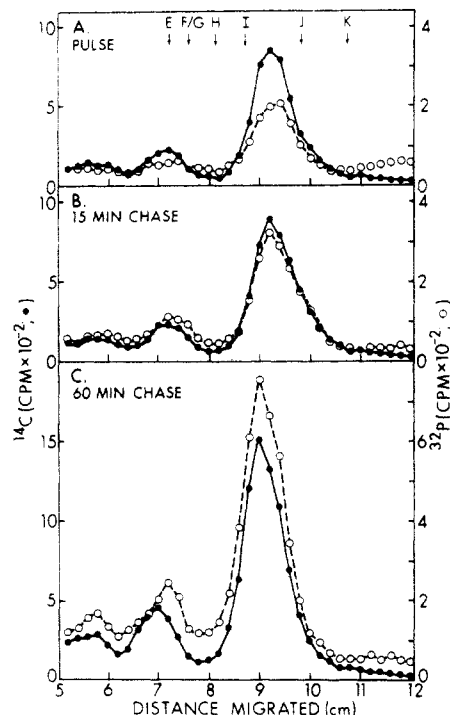


FIGURE 9: Agarose gel electrophoresis of SN-resistant DNA fragments. Nuclei isolated from permeable cells pulse labeled for 15 min during repair synthesis (panel A) or pulsed and then chased for 15 min (panel B) or 60 min (panel C) (see legend to Figure 7 for details of pulse-chase) were digested with SN until the following fractions of the bulk DNA ( $^{14}\text{C}$ ) were rendered acid soluble: (A) 0.10; (B) 0.14; (C) 0.13. The nuclease-resistant DNA fragments were prepared and analyzed by electrophoresis in 2.8% agarose. The gel lanes were sliced, and radioactivity was determined. (●)  $^{14}\text{C}$  bulk label; (○)  $^{32}\text{P}$  repair label. Arrows indicate the migration distances of *Hae*III restriction fragments of  $\phi$ X174 RF of the following sizes: E, 310 bp; F/G, 281/271 bp; H, 234 bp; I, 194 bp; J, 118 bp; K, 72 bp.

tation decreased during the chase period (Figure 8B), and by 30 min (Figure 8C), repair-incorporated nucleotides were released by SN only slightly faster than was the bulk of DNA. The relative SN sensitivities of repair-incorporated nucleotides and bulk DNA can be more easily compared in Figure 8D. The initial overrepresentation of repair-incorporated nucleotides in nuclease-sensitive regions was greater than 2-fold. It decreased rapidly during the first 15 min of chase and continued to decrease for 30 min. After 30 min, no further reduction in the SN sensitivity of the repair-incorporated nucleotides could be detected, and at 60 min, there remained a slight overrepresentation of these in the nuclease-sensitive portion of chromatin (Figure 8D).

Using electrophoresis in 2.8% agarose, we analyzed the nuclease-resistant DNA fragments produced during these SN digestions (Figure 9). Although there was neither any dCMP incorporation nor any loss of label during the chase period [i.e., there was no change in the ratio of  $^{32}\text{P}/^{14}\text{C}$  (repair label/bulk label) in total DNA], the  $^{32}\text{P}/^{14}\text{C}$  ratio in SN-resistant DNA fragments of the size found in nucleosome core particles (145–165 bp) increased during the chase. In samples digested to render approximately the same fraction of the bulk label acid soluble, which are directly comparable for this purpose (Smerdon & Lieberman, 1978a), the  $^{32}\text{P}/^{14}\text{C}$  ratio at 145 bp rose from 0.26 immediately following the pulse (Figure 9A) to 0.50 after 60 min of chase (Figure 9C). From these data we conclude that those repair-incorporated nucleotides which disappeared from the nuclease-sensitive portion of chromatin during the chase (see above) subsequently appeared in nucleosome core DNA, producing a more uniform distribution

of repair-incorporated nucleotides in chromatin.

**Effect of Some Low Molecular Weight Compounds on UV-Induced Repair Synthesis in Permeable Cells.** Repair synthesis in this system absolutely required ATP and would not utilize GTP in its place (Table I). Furthermore, addition of GTP to complete reaction mix (containing ATP) did not significantly increase repair synthesis. As has been previously reported (Seki et al., 1979), addition of 1 mM spermidine reduced repair synthesis by about half (Table I). Novobiocin, an inhibitor of prokaryotic and eukaryotic topoisomerases and of eukaryotic polymerase  $\alpha$  (see Discussion), inhibited repair synthesis, but nalidixic acid, another topoisomerase inhibitor, did not (Table I). Aphidicolin, a very specific inhibitor of DNA polymerase  $\alpha$  (Huberman, 1981), inhibited repair synthesis almost completely, but ddTTP, which inhibits polymerase  $\beta$  and  $\gamma$  more strongly than it inhibits  $\alpha$  (Edenberg et al., 1978; Waqar et al., 1978), inhibited repair synthesis only slightly even at 150  $\mu\text{M}$  (i.e., at a concentration which gave a ddTTP/TTP ratio of 50; Table I).

## Discussion

We have examined the DNA repair synthesis occurring when permeable normal human fibroblasts are irradiated with UV or damaged with MNU as a cell suspension and incubated with a synthesis mix containing Tris, KCl,  $\text{Mg}^{2+}$ , ATP, and the four dNTPs. Repair synthesis in these permeable cells is very similar to that occurring in intact cells: newly incorporated nucleotides are found in DNA of parental density (Cleaver, 1969; Smith & Hanawalt, 1976); nucleotide incorporation is dependent on damage and increases with increasing damage; nucleotide incorporation is dependent on the presence in the synthesis mix of at least three, and probably of all four, dNTPs, suggesting that all four dNTPs are incorporated during repair synthesis (Cleaver, 1973; Lieberman & Poirier, 1973). Excision repair in this system also accurately reflects the genetic composition of the cells used. Permeable xeroderma pigmentosum cells (complementation groups A and G), which are genetically deficient in repair of UV damage (reviewed in Friedberg et al., 1979), show no increase in incorporation following UV irradiation, but this defect is alleviated by addition to the reaction mix of *M. luteus* UV endonuclease, which incises DNA at the sites of pyrimidine dimers (Riazuddin & Grossman, 1977b; Haseltine et al., 1980). Such complementation has been observed by others in intact cells (Tanaka et al., 1975, 1977) and in subcellular repair systems (Ciarrocchi & Linn, 1978; Smith & Hanawalt, 1978). As has been found in intact cell studies [see Friedberg et al. (1979)], permeable XP cells repair MNU damage as efficiently as do permeable normal cells.

We have also found that the distribution of UV-induced repair synthesis in the chromatin of permeable cells is very similar to that seen in intact cells (Cleaver, 1977; Smerdon et al., 1978, 1979; Smerdon & Lieberman, 1978a; Williams & Friedberg, 1979; Bodell & Cleaver, 1981): Nucleotides newly incorporated by repair synthesis are overrepresented (by about 2-fold) in the nuclease sensitive fraction of chromatin, but with time, these nucleotides are "rearranged" so that they eventually are distributed nearly uniformly between nuclease-sensitive and -resistant regions. The redistribution of repair-incorporated nucleotides from nuclease-sensitive to nuclease-resistant regions of chromatin seems to occur slightly faster in these permeable cells than in intact normal human fibroblasts of the same type (Smerdon et al., 1979), but in both permeable and intact cells, a plateau is reached at which there is a persistent slight overrepresentation of repair-incorporated nucleotides in nuclease-sensitive regions. These chromatin



findings provide strong evidence that this system faithfully reproduces excision repair as it occurs in intact cells and raise the possibility that the system might be useful for studying the role of chromatin structure in DNA repair.

One difference between repair synthesis in our present permeable cell system and in intact cells is the UV dose curve. In our laboratory, UV-induced repair synthesis in intact human fibroblasts (IMR-90 and AG1518) irradiated as a cell culture monolayer typically increases up to a dose between 5 and 10 J/m<sup>2</sup> and then reaches a plateau (Smerdon & Lieberman, 1978b; S. L. Dresler and M. W. Lieberman, unpublished data). Cells irradiated as a monolayer and then made permeable and incubated with synthesis mix have been found to have a similar UV dose curve (Roberts & Lieberman, 1979; Figure 3B of this paper). In the present study, we have found that in cells damaged according to our current protocol, i.e., UV irradiation as a suspension of permeable cells, repair synthesis rises more slowly with increasing UV dose but eventually reaches almost the same plateau level as repair synthesis in cells irradiated as a monolayer at 4 °C before being made permeable. We believe it to be most likely that this difference is due to simple shielding of the permeable cells in suspension, but we cannot exclude an alteration in chromatin "damageability" induced when the cells are made permeable. The normal distribution of repair synthesis in chromatin (as described above) argues against the latter, however.

We have also found that cells damaged according to a protocol similar to that of Ciarrocchi & Linn (1978), i.e., irradiated with UV as a cell culture monolayer and preincubated at 37 °C for 10 min before being made permeable (allowing early steps in excision repair to occur in the intact cell), have a UV dose curve very similar to that described by those authors and show levels of repair synthesis considerably higher than those found with either of the other two damage protocols we used (see above). Because repair synthesis took place in permeable cells under the same conditions for all three damage protocols, these data suggest that intact cells may have higher levels than permeable cells of one or more of the excision repair factors (e.g., those mediating damage recognition, incision, or excision) which act prior to nucleotide incorporation. The stimulation produced by *M. luteus* UV endonuclease (Figure 3) indicates that incision of the damaged DNA (or some preincision step which is bypassed by the *M. luteus* UV endonuclease) is the rate-limiting activity in these permeable cells and suggests that damage-specific incising activity may be reduced in permeable cells as compared to intact cells. Extreme lability of UV damage-specific incising activities in human cell extracts has been noted previously (Mortelmans et al., 1976; Waldstein et al., 1979) and was suggested by Smith & Hanawalt (1978) to be the reason that isolated nuclei show lower levels of UV excision repair than intact cells.

We have found that the effect of varying dNTP concentration on nucleotide incorporation by undamaged cells is very different from its influence on DNA repair synthesis. Incorporation in undamaged cells has an apparent  $K_m$  for dNTPs of about 8  $\mu$ M; the corresponding value for repair synthesis is less than 1  $\mu$ M. These data are consistent with the suggestion that hydroxyurea preferentially inhibits DNA replication in intact cells because replication is more sensitive than repair to the reduction in cellular deoxynucleotide levels induced by the drug (Cleaver, 1969). We have taken advantage of this differential effect in our permeable cell system; by reducing the dNTP concentration to 3  $\mu$ M we have greatly reduced incorporation by undamaged cells with little effect

on repair synthesis. This modification, together with addition of 15 mM KCl to the reaction mix, has yielded levels of nucleotide incorporation in unirradiated permeable cells which are a small fraction of the levels typically produced by repair synthesis. Thus we can now quickly and reliably measure repair synthesis by measuring acid-precipitable incorporation in unirradiated cells and in irradiated cells and taking the difference, without recourse to the laborious process of separating replicative incorporation from repair incorporation on alkaline CsCl density gradients.

We have used this system to investigate the effects of several compounds on DNA repair synthesis. Aphidicolin, a very specific inhibitor of DNA polymerase  $\alpha$  (Ohashi et al., 1978; Pedrali-Noy & Spadari, 1979), almost totally inhibited UV-induced repair synthesis in our system. We have observed a similar inhibition in intact cells (S. L. Dresler and M. W. Lieberman, unpublished data). Dideoxy-TTP, at a concentration giving a ddTTP/TTP ratio much higher than that required to inhibit DNA polymerase  $\beta$  (Edenberg et al., 1978; Waqar et al., 1978), produced only slight inhibition of repair synthesis in permeable cells. These data suggest, as do the data of a number of other investigators (Hanaoka et al., 1979; Berger et al., 1979; Ciarrocchi et al., 1979; Snyder & Regan, 1981), that DNA polymerase  $\alpha$  is involved in repair synthesis following UV damage.

Collins & Johnson (1979) have found that novobiocin, an inhibitor of bacterial DNA gyrase (Gellert et al., 1976; Sugino et al., 1978), of some eukaryotic DNA topoisomerases (Hsieh & Brutlag, 1980; Miller et al., 1981), and of eukaryotic DNA polymerase  $\alpha$  (Edenberg, 1980), reduces the number of single-strand DNA breaks produced in intact HeLa cells following UV irradiation. This finding presumably reflects a reduction of damage-specific incision activity. Mattern & Scudiero (1981) report that, in intact CHO cells, UV-induced DNA repair synthesis is inhibited both by novobiocin and by nalidixic acid, an inhibitor of bacterial DNA gyrase (Gellert et al., 1977; Sugino et al., 1977) and of the HeLa type II DNA topoisomerase (Miller et al., 1981). We also find that DNA repair synthesis in UV-irradiated permeable human fibroblasts is inhibited by novobiocin but is not inhibited by a high concentration of nalidixic acid. Previous authors have suggested that novobiocin inhibition of DNA excision repair is due to inhibition of a topoisomerase (Collins & Johnson, 1979; Mattern & Scudiero, 1981). Because DNA polymerase  $\alpha$  has been implicated in UV-induced repair synthesis (see above), and because DNA polymerase  $\alpha$  is inhibited by novobiocin (Edenberg, 1980) at concentrations similar to those which inhibit repair synthesis in our system, we believe that inhibition of DNA polymerase activity also must be considered as a possible mechanism of novobiocin inhibition of DNA repair synthesis. The recent finding that aphidicolin, another inhibitor of polymerase  $\alpha$ , increases the number of DNA single-strand breaks produced following UV irradiation (Snyder & Regan, 1981) suggests, however, that a mechanism other than polymerase inhibition is required to explain the reduction in single-strand breaks observed by Collins & Johnson (1979); novobiocin may act at more than one point in the repair pathway.

This permeable cell system offers several important advantages for DNA repair studies. First, the events preceding repair synthesis take place after the cell is made permeable and thus are potentially accessible to biochemical manipulation. We have found that damaged DNA in the chromatin of these permeable cells is accessible to exogenous macromolecules at least as large as *M. luteus* UV endonuclease



(11 000–15 000 daltons; Riazuddin & Grossman, 1977a), indicating the feasibility of this approach. Second, irradiation of the cells in suspension allows the study of excision repair of UV damage using cells grown in roller bottles, reducing the time and materials required for tissue culture. Third, by manipulating the components of the repair synthesis mix (e.g., dNTPs and KCl; see above), we have reduced the replicative background so that repair synthesis may be rapidly and reliably determined from measurements of acid-precipitable radioactivity in irradiated and unirradiated cells, allowing up to 150 assays to be performed conveniently in 1 day by one person. Fourth, use of a  $^{32}\text{P}$ -labeled nucleoside triphosphate has increased the sensitivity of the system so that 75 assays may be conducted by using the cells from a single standard (1170  $\text{cm}^2$ ) roller bottle. We believe that this system will make practical the molecular dissection of DNA excision repair.

#### Added in Proof

Using an assay which is based on the sensitivity of unligated repair patches to exonuclease III, we have recently demonstrated that ligation of UV-induced repair patches occurs in our permeable cell system (D. J. Hunting, S. L. Dresler, B. J. Gowans, and M. W. Lieberman, unpublished data).

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