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Deoxyribonucleic Acid Repair Synthesis in Permeable Human Fibroblasts Exposed to Ultraviolet Radiation and *N*-Acetoxy-2-(acetyl amino)fluorene[†]

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ABSTRACT: We have studied deoxyribonucleic acid (DNA) repair synthesis in permeable, confluent normal and repair-deficient human fibroblasts using [³H]dCTP, BrdUTP, dATP, and dGTP as substrates. In this system repair synthesis occurred as judged by the following criteria: (1) labeled nucleotides were incorporated into parental DNA ($\rho = 1.73$ g/cm³ in alkaline CsCl); (2) incorporation into parental DNA was negligible in the absence of damage and increased 10-20-fold in normal cells following exposure to ultraviolet radiation (UV) or the direct-acting chemical carcinogen *N*-acetoxy-2-(acetyl amino)fluorene; and (3) damage-dependent DNA synthesis was absent in preparations made from excision repair-deficient human diploid fibroblasts (xeroderma pigmentosum cells, complementation group A). The reaction was

linear for 10 min and continued for at least 1 h. Repair synthesis was stimulated at least fivefold by the addition of 5 mM ATP. It was strongly Mg²⁺ dependent, inhibited by NaCl, and only partially dependent upon the addition of exogenous dNTPs. The pH optimum in Tris-HCl buffer was 7.6. Following damage (UV), labeled deoxycytidine and dCMP were incorporated into parental DNA but at reduced levels (38 and 88%, respectively) compared to dCTP. The addition of β -NAD⁺ (the naturally occurring isomer) or α -NAD⁺ (a competitive inhibitor) had little effect on repair synthesis in this system. By saturating the system with dNTPs and using published estimates of patch size, we calculated that in this system a normal cell can put in a minimum of 100-900 repair patches/min.

Although recent studies have greatly increased our understanding of DNA repair processes (Hanawalt et al., 1978, 1979; Nichols & Murphy, 1977; Setlow, 1978), the detailed molecular events of eucaryotic excision repair are still unclear. Investigations of the role of chromatin structure in repair (Mortelmans et al., 1976; Bodell, 1977; Cleaver, 1977; Smerdon et al., 1978; Smerdon & Lieberman, 1978a; Tlsty & Lieberman, 1978) have emphasized the importance of studying molecular mechanisms in systems which closely approximate conditions in the living cell. Studies of DNA replicative synthesis in permeable mammalian cells and nuclei (Seki et al., 1975; Krokan et al., 1975; Tseng & Goulian, 1975; Berger & Johnson, 1976) have prompted us, as well as others (Ciarrocchi & Linn, 1978; Smith & Hanawalt, 1978), to use this approach to study DNA repair. In this paper, we report our initial observations on DNA repair synthesis in permeable normal and xeroderma pigmentosum fibroblasts (XP) treated with ultraviolet radiation and the chemical carcinogen *N*-acetoxy-2-(acetyl amino)fluorene (NA-AAF)¹. We have

examined in detail the criteria for repair synthesis, cofactor requirements, and the rate of repair synthesis in this system.

Materials and Methods

Cell Culture and Preparation of Prelabeled Cells. Human diploid fibroblasts (IMR-90; Institute for Medical Research, Camden, NJ; Nichols et al., 1977) and xeroderma pigmentosum fibroblasts (XP12BE, complementation group A; American Type Culture Collection, Rockville, MD) were used between passages 15 and 24. Fibroblasts were grown to confluence as previously described (Amacher et al., 1977). To label parental DNA, we split cells (1:2 or 1:3) and exposed them to 2.5 nCi/mL [¹⁴C]thymidine (50-60 mCi/mmol, New England Nuclear or Amersham) during growth. The medium was changed, and the cells were allowed to come to confluence. Conditions were adjusted so that the specific activity of the DNA was in the range of 100-200 cpm/ μ g. Cells were used within 10 days after reaching confluence.

UV Irradiation and Chemical Damage. Confluent cells, prelabeled with [¹⁴C]thymidine, were treated with 10⁻⁴ M BrdUrd (Sigma) (final concentration) for ~3 h prior to damage. Incidental exposure to room lighting was minimized. To damage the cells with UV, we removed the medium and washed the cells once with ice-cold phosphate-buffered saline (PBS) (2.7 mM NaH₂PO₄, 13.1 mM Na₂HPO₄, 135 mM

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¹ Abbreviations used: NA-AAF, *N*-acetoxy-2-(acetyl amino)fluorene; Me₂SO, dimethyl sulfoxide; XP, xeroderma pigmentosum; dNTPs, the four deoxyribonucleoside 5'-triphosphates; PBS, phosphate-buffered saline.

NaCl, and 4.9 mM KCl, pH 6.8). The cells were then irradiated (Smerdon et al., 1978); unless otherwise indicated, the total dose of UV was 10 J/m². NA-AAF was dissolved in 0.1 mL of anhydrous Me₂SO (Pierce Chemical; silylation grade) and added to each dish (20 mL). Control dishes received 0.1 mL of Me₂SO alone. The carcinogen was left on the cells for 20 min, at which time the medium was removed and the cells were washed with cold PBS.

Harvesting and Permeabilization of Cells. After irradiation or treatment with chemical carcinogen, the cells were harvested in the cold by scraping with a rubber policeman and rinsing with cold PBS. Cells were pelleted by centrifugation (258g, 10 min, 2 °C) and then gently vortexed in permeabilizing buffer (~0.18 mL/dish), containing 0.01 M Tris-HCl, 0.25 M sucrose, 1.0 mM EDTA (pH 7.6), 4.0 mM MgCl₂, and 15 mM dithiothreitol (Sigma), final pH 8.3 at 4 °C (Berger & Johnson, 1976; Seki & Oda, 1977a,b). The cells were suspended by using the loose-fitting pestle of a dounce homogenizer (Kontes). The suspension from two plates (0.4 mL; ~50 µg of DNA) was transferred to a 15-mL Corex tube and placed on ice for ~0.5 h. Preparations of IMR-90 and XP cells were examined by phase contrast and electron microscopy. Nuclei of the permeable cells were swollen and had a slightly "washed out" appearance. The cytoplasm was vacuolated and contained dilated, "washed out" rough endoplasmic reticulum. The mitochondria were often condensed and stained darkly, and in most cases plasma membranes were indistinct. No free nuclei or cell ghosts were seen. Cells which were incubated for 30 min in the reaction mixture at 37 °C were in general similar to freshly prepared cells maintained at 4 °C with the possible exception that some XP cells showed mild margination and clumping of chromatin.

DNA Repair Synthesis Incubation. DNA repair synthesis incubation was begun by adding 0.2 mL of DNA synthesis mix to each 0.4-mL aliquot of cells and placing the tubes in a 37 °C shaking water bath. Unless otherwise indicated, the final incubation mixture contained 38 mM Tris-HCl (pH 7.8 at 37 °C), 125 mM sucrose, 5.0 mM ATP, 8.7 mM MgCl₂, 133 µM each of the dNTPs (dATP, dGTP, and BrdUTP), 0.5 mM EDTA, 7.3 mM dithiothreitol, and 6.5 µM 5-[³H]dCTP (156 µCi/mL, 20–27 Ci/mmol; Amersham). The pH of the final reaction mixture was 7.6. In a few experiments, 5-[³H]deoxycytidine (28 Ci/mmol, Amersham) or 5-[³H]-deoxycytidine 5'-monophosphate (26 Ci/mmol, Amersham) was substituted for 5-[³H]dCTP. When NAD⁺ was to be added, a stock solution (50 mM, pH 6.4) of NAD⁺ was prepared no more than 48 h prior to its use and stored at -20 °C. The length of time between damage and the start of the DNA repair synthesis incubation was consistently between 1.75 and 2 h. Except for time course studies, incubation was at 37 °C for 30 min. The reaction was stopped by diluting the mixture to 10 mL with cold buffer A (0.01 M Tris-HCl and 0.32 M sucrose, final pH 8.2 at 4 °C) and placing the diluted reaction mixture on ice.

Isolation and Digestion of Nuclei. Cells in the diluted reaction mixture were pelleted by centrifugation at either 6000g or 12000g for 10 min at 2 °C, resuspended by vortexing in 10 mL of buffer B (buffer A + 0.5% Triton X-100), and repelleted as before. The wash in buffer B was repeated, followed by a wash in buffer A and then by a wash in 0.01 M Tris-HCl (pH 8.2 at 4 °C). The nuclear pellet was resuspended by vigorous vortexing in 1.6 mL of 0.01 M Tris-HCl and digested with proteinase K (EM Laboratories) (300 µg/mL, final concentration) and sodium dodecyl sulfate (Bio-Rad) (1%, final concentration) either for 10–13 h at 37

°C or for 2–7 h at 47 °C. The DNA was sheared by 10 passages through a 23 gauge needle.

Preparation of CsCl Density Gradients. Alkaline CsCl gradients were prepared by adding 4.6 g of CsCl (EM Laboratories) and 0.45 mL of 1.0 M K₂HPO₄, pH 12.5, to the DNA samples (2.0 mL). The volume was brought to 4.5 mL, and the solution was centrifuged at 12000g for 15 min at 12 °C. The clear phase was collected and adjusted to a density of 1.74 g/cm³; 4.2-mL aliquots were centrifuged to equilibrium at 50 000 rpm (at least 6 h) in a Sorvall TV865 vertical rotor. The gradients were fractionated, absorbance at 260 nm (Gilford 250 spectrometer) was measured, and the peak fractions were pooled. The volume was brought to 4.5 mL with alkaline CsCl ($\rho = 1.74$ g/cm³), and the samples were centrifuged as described above. This gradient was collected directly into scintillation vials, and each fraction (0.2 mL) was diluted with 0.5 mL of H₂O, mixed with 6.0 mL of Insta Gel (Packard), and counted in a Beckman LS-345 liquid scintillation spectrometer using standard double-label techniques (Smerdon et al., 1978; Kobayashi & Maudsly, 1974; Horrocks, 1974).

Analysis of CsCl Gradients. The ¹⁴C counts per minute per microgram of DNA was determined for each experiment from DNA purified in a CsCl density gradient. DNA was measured by optical density at 260 nm using a molar extinction coefficient of 20 mL/(mg cm) for double-stranded DNA and 26.7 mL/(mg cm) for single-stranded DNA. The amount of ³H incorporated during repair synthesis was calculated by subtracting the average number of ³H counts per minute in fractions not containing parental DNA (e.g., Figure 1B, fractions 2–6 and 15–19) from the ³H counts per minute present in the ¹⁴C peak fractions (parental DNA). The ³H/¹⁴C ratio in the four or five peak fractions was then converted to femtomoles of dCMP per microgram of DNA.

Results

DNA Repair Synthesis in Normal and Repair-Deficient Human Fibroblasts. Confluent cells prelabeled with [¹⁴C]-dThd were damaged, harvested, permeabilized, and incubated in a reaction mixture containing [³H]dCTP and BrdUTP (see Materials and Methods). The DNAs from UV-irradiated and unirradiated normal and XP (complementation group A) fibroblasts were banded in alkaline CsCl. DNA banding at parental density ($\rho = 1.73$ g/cm³) was recentered in alkaline CsCl, and the DNA rebanding at parental density was used to quantitate repair synthesis (Figure 1). In the absence of damage (Figure 1A) little dCMP is incorporated into parental DNA in normal cells, whereas UV irradiation (10 J/m²) results in about a 20-fold increase in incorporation into parental DNA (Figure 1B). No increase in repair synthesis is seen in preparations from the XP cells (parts C and D of Figure 1). Repair synthesis in control cells increases rapidly with dose up to ~5 J/m², and higher doses stimulate little additional incorporation (Figure 2A). Doses up to 40 J/m² do not stimulate incorporation in XP cells. Exposure of normal fibroblasts to increasing concentrations of NA-AAF increases the incorporation of ³H up to eightfold, while XP fibroblasts show very little repair synthesis after exposure to the carcinogen (Figure 2B). These dose-response relationships are similar to those observed for intact cells [e.g., Tlsty & Lieberman (1978), Stein et al. (1976), Pero et al. (1976), Ahmed & Setlow (1977), and Smerdon & Lieberman (1978b)]. The incorporation of dCMP begins immediately upon incubation at 37 °C and shows essentially a monotonic time dependence over at least 1 h (Figure 3). The reaction had the same time dependence and approximately the same initial slope whether

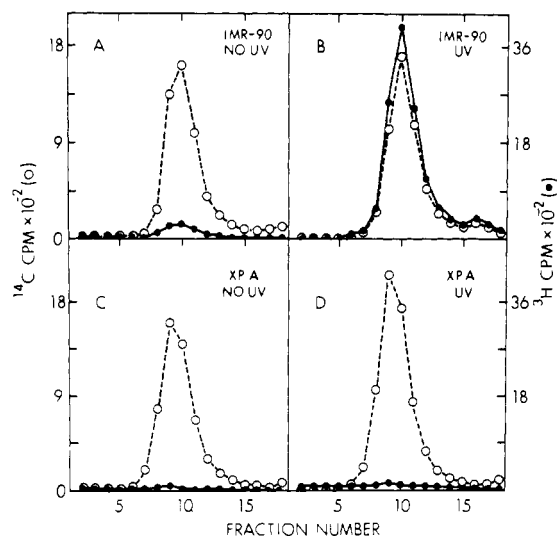


FIGURE 1: Repair synthesis in permeable IMR-90 and xeroderma pigmentosum (complementation group A) cells. After irradiation and incubation (see Materials and Methods), DNA was isolated in an alkaline CsCl gradient and parental density DNA was rebanded in a second alkaline CsCl gradient. Fractions were assayed for radioactivity as described. Fraction 1 contains material from the bottom (most dense) portion of the gradient. (○) ^{14}C ; (●) ^3H . (Panel A) IMR-90 cells, no UV; net repair synthesis (see Materials and Methods) 1.6 fmol of dCMP/ μg of DNA. (Panel B) IMR-90 cells, 10 J/m 2 of UV; net repair synthesis 35.1 fmol of dCMP/ μg of DNA. (Panel C) XP cells, no UV; net repair synthesis 0.5 fmol of dCMP/ μg of DNA. (Panel D) XP cells, 10 J/m 2 of UV; net repair synthesis 0.4 fmol of dCMP/ μg of DNA.

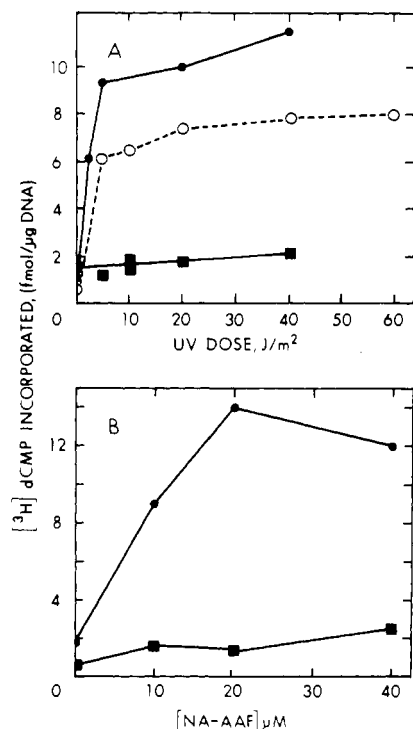


FIGURE 2: (A) UV-induced repair synthesis. Cells were irradiated at an incident dose rate of 1 W/m 2 , and repair synthesis was measured as described (see Materials and Methods and caption to Figure 1). (●) IMR-90 cells, standard incubation conditions (containing 5 mM exogenous ATP) + 80 mM NaCl; (○) IMR-90 cells (reaction mixture containing 2.5 mM ATP) + 80 mM NaCl; and (■) XP (group A) cells, standard incubation conditions + 80 mM NaCl. (B) Carcinogen-induced repair synthesis. Cells were exposed to NA-AAF for 20 min. (●) IMR-90 cells; and (■) XP cells.

or not the cells were preincubated at 37 °C for 10 min prior to the addition of the DNA synthesis mix. During the first

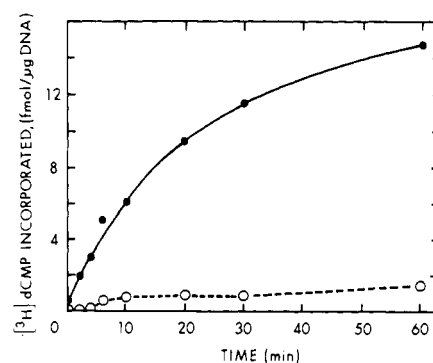


FIGURE 3: Time course of UV-induced repair synthesis in permeable IMR-90 cells. Cells were exposed to either 0 (○) or 10 (●) J/m 2 of UV and allowed to repair for the indicated times. Cells were prewarmed at 37 °C for 10 min prior to addition of the DNA synthesis mix.

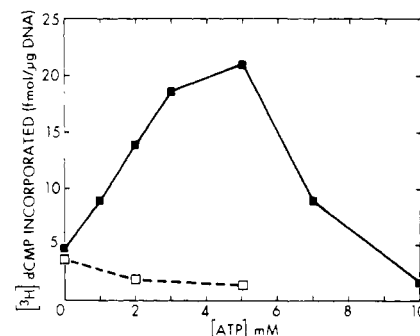


FIGURE 4: Effect of exogenous ATP on repair synthesis in permeable IMR-90 cells. The MgCl_2 concentration was 5.0 mM. (□) 0 J/m 2 of UV; and (■) 10 J/m 2 of UV.

Table I: Incorporation of ^3H in the Absence or Presence of Various Components in the Incubation Mixture^a

incubation conditions	% incorporation
complete system	100
-ATP	3-23
+7 mM ATP	52
+7 mM ATP, +7 mM MgCl_2	83
+10 mM ATP	10
+10 mM ATP, +10 mM MgCl_2	90
+15 μM [^3H]dCTP	96
+30 μM [^3H]dCTP	98
- [^3H]dCTP, + [^3H]dCMP (6.5 μM)	88
- [^3H]dCTP, + [^3H]dCyt (6.5 μM)	38
+ β -NAD $^+$ (5 mM)	93
+ β -NAD $^+$ (10 mM)	84
+ β -NAD $^+$ (5 mM) + nicotinamide (20 mM)	88
+ α -NAD $^+$ (5 mM)	112
+ α -NAD $^+$ (10 mM)	93

^a Confluent IMR-90 cells were exposed to 10 J/m 2 of UV, made permeable, and incubated in a DNA synthesis mixture at 37 °C for 30 min. The complete system contained 38 mM Tris-HCl, 125 mM sucrose, 5.0 mM ATP, 5 mM MgCl_2 , 133 μM each of dATP, dGTP, and BrdUTP, 0.5 mM EDTA, 2 mM dithiothreitol, and 6.5 μM 5-[^3H]dCTP. The pH of the final reaction mixture was 7.6. Repair synthesis was measured as described (see Materials and Methods).

10 min of the reaction, dCMP is incorporated into parental DNA at a rate of $\sim 2.4 \times 10^{-21}$ mol/(cell min) under conditions which are saturating for [^3H]dCTP and the three unlabeled dNTPs (see below). Because this calculation and others dealing with rates of incorporation (see below) assume that at saturation most or all of the pool is composed of [^3H]dCTP, these values are only approximate.

Dependence upon ATP. Repair synthesis is strongly stimulated by the presence of ATP in the reaction mixture

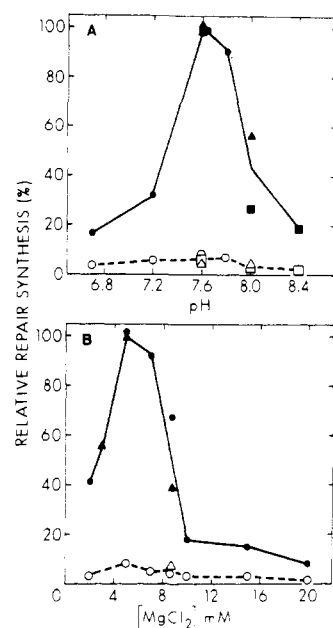


FIGURE 5: (A) Effect of pH on repair synthesis in permeable IMR-90 fibroblasts. (Open symbols) 0 J/m² of UV; and (closed symbols) 10 J/m². Different symbols represent different experiments which were normalized to the value at pH 7.6. The actual values at pH 7.6 for the three experiments were (●) 13, (■) 16, and (▲) 24 fmol of dCMP/μg of DNA. (B) Effect of MgCl₂ on repair synthesis in permeable IMR-90 cells. Different symbols represent different experiments which were normalized to the value at 5.0 mM Mg²⁺. (Open symbols) 0 J/m² of UV; and (closed symbols) 10 J/m² of UV. The amounts of incorporation at 5.0 mM Mg²⁺ for the different experiments were (●) 23 and (▲) 37 fmol of dCMP/μg of DNA.

(Figure 4; see also Figure 2A). The addition of 5 mM ATP (optimal conditions) stimulates incorporation at least fivefold. In different experiments, the amount of repair synthesis in the absence of ATP varied from 3 to 23% of the level of repair synthesis with 5 mM ATP (Table I). The reduction in repair synthesis at ATP levels higher than 5 mM was unexpected, though a similar inhibition of DNA replicative synthesis was observed in permeable mouse ascites cells (Seki & Oda, 1977a). A possible explanation is that free Mg²⁺ is required either for permeability or for repair (and replicative) synthesis, and, at high ATP concentrations, most or all of the Mg²⁺ may be bound to ATP. To test this hypothesis, we measured repair synthesis at three different ATP concentrations (5, 7, and 10 mM) and at Mg²⁺ concentrations equal to the ATP concentrations (i.e., with 5, 7, and 10 mM Mg²⁺, respectively). The Mg²⁺ and ATP were mixed prior to their addition to the cells. At the ATP concentrations greater than 5 mM, the addition of MgCl₂ at levels equal to the ATP concentration restored repair synthesis to nearly the level observed at 5 mM ATP and 5 mM MgCl₂ (Table I). This result suggests that decreased repair synthesis at high ATP concentrations is due to a depletion of free Mg²⁺ caused by ATP binding to the cation.

Effect of pH and Cations. Maximum repair synthesis in Tris-HCl buffer occurs at pH 7.6 (Figure 5A). Normal fibroblasts incorporated similar levels of dCMP in phosphate buffer as in Tris-HCl, though the optimum pH in phosphate was ~7.0 (data not shown). Therefore, the sharp decline in activity at low pH values in Tris-HCl may be due to the weak buffering capacity of Tris at pH values close to 7.0. The optimum Mg²⁺ concentration is between 3 and 7 mM at an ATP concentration of 5 mM (Figure 5B). NaCl at concentrations between 10 and 80 mM strongly inhibits repair synthesis, reducing incorporation to ~90% at 10 mM, 50%

at 40 mM, and 20% at 80 mM.

Deoxyribonucleotides. Repair synthesis in permeable cells is only partially dependent upon exogenous deoxyribonucleoside triphosphates; the omission of the three unlabeled dNTPs reduced the incorporation of label from [³H]dCTP (kept at 6.5 μM) to 64% of values obtained under standard conditions (133 μM of each unlabeled dNTP, 6.5 μM [³H]dCTP). Repair synthesis saturated at a 15 μM concentration of each of the unlabeled dNTPs and 6.5 μM [³H]dCTP. The value for 0 "dNTP added" is only approximate because no BrdUTP was added to shift replicative DNA to a higher density; however, the level of incorporation into parental DNA at 0 mM dNTPs in the absence of UV was very low, indicating that most of the synthesis after 10 J/m² UV was repair synthesis. When high concentrations (133 μM) of the three unlabeled dNTPs were used, increasing the concentration of [³H]dCTP from 6.5 to 30 μM did not result in increased incorporation (Table I), suggesting that the dCTP pool was saturated. [³H]dCMP or [³H]dCyt (each at 6.5 μM) supported repair synthesis, though at reduced levels (Table I); thus, the permeable cell system can probably phosphorylate these precursors.

Effect of Pyridine Nucleotides. Because pyridine nucleotides are known to be involved in procaryotic repair and a role for ADP-ribosylation in eucaryotic repair has been postulated (see Discussion), we examined the effects of NAD⁺ and nicotinamide on DNA repair synthesis in our system. The addition of the naturally occurring NAD⁺ isomer β-NAD⁺ (0.1–10 mM) had little effect on the incorporation of dCTP in this system (Table I). The effects of two inhibitors of β-NAD⁺ were also examined. Neither the administration of α-NAD⁺ (up to 10 mM) nor the coaddition of nicotinamide (20 mM) and β-NAD⁺ affected repair (Table I). The addition of nicotinamide alone had a slight inhibitory effect on repair synthesis in our system (data not shown).

Discussion

The incorporation of [³H]dCTP in permeable human fibroblasts has been identified as repair synthesis on the basis of three criteria: (1) the labeled nucleotide is incorporated into parental density DNA; (2) incorporation is strongly dependent upon prior exposure of the cells to UV or to a chemical carcinogen known to damage DNA; and (3) incorporation depends upon the repair competence of the cells (i.e., exposure of XP cells to UV or NA-AAF does not stimulate incorporation of labeled nucleotides into parental density DNA). This latter result also rules out the possibility that the radiolabel which bands with parental density DNA results from an inhibition of chain elongation of replicating strands. Such an inhibition of replicative synthesis might produce short patches which could appear in parental DNA. In parallel experiments (data not shown) we have demonstrated that in the absence of damage most of the ³H label is incorporated into "heavy DNA" (ρ ~ 1.8 g/cm³). We have also found that changing the reaction conditions (pH, ATP, Mg²⁺, NaCl) does not appreciably increase the amount of ³H incorporated into parental density DNA from undamaged cells. These results provide evidence that the increase in incorporation under various conditions is not due to the slowing or early termination of replicative chain elongation and the appearance of these chains in light DNA. We have also demonstrated that in our system XP cells will carry out repair synthesis following treatment with the X-ray mimetic agent *N*-methyl-*N*-nitrosourea (J. D. Roberts and M. W. Lieberman, unpublished experiments). Because XP cells repair damage induced by X-ray mimetic agents (Cleaver, 1973; Regan &

Table II: Characteristics of Permeable Cell/Subcellular Repair Systems

	type of system		
	permeable cell ^a	lysed cell ^b	nuclei ^c
criteria for repair synthesis:			
(a) incorporation into parental DNA	yes	not examined	yes
(b) dependent on damage (UV)	yes	yes	yes
(c) absence in repair-deficient cells (XP-A)	yes	yes	yes
time course of repair synthesis	at least 60 min (linear for 10 min)	at least 60 min (linear for 15 min)	5 min
ATP dependence	stimulated at least fivefold by 5 mM ATP	stimulated 40% by 1-6 mM ATP	stimulated 100% by 5 mM ATP
% of max act. in absence of unlabeled dNTPs	64	56	60
inhibition by NaCl	50% inhibition at 40 mM	50% inhibition at 25 mM	not examined (100 mM NaCl present in assay)
effect of NAD ⁺	none	not examined	not examined
size of repair patch	not examined	not examined	not examined
estimate of no. of repair patches inserted per cell per min	100-900 (minimum)	not examined	~35 nucleotides
stimulation of XP by T4 UV endonuclease V	not examined	yes	yes
repair synthesis induced by chemicals	yes	yes	not examined
Mg ²⁺ optimum	~5 mM	7-14 mM	not examined
pH optimum	~7.6 (Tris-HCl)	6.9-7.3 (PO ₄ -citrate or Hepes-KOH)	not examined

^a Our results. ^b Results from Ciarrocchi & Linn (1978). ^c Results from Smith & Hanawalt (1978).

Setlow, 1974), these results indicate that the failure of XP cells to repair UV or NA-AAF lesions in our system is not the result of some peculiar sensitivity of XP cells to our experimental conditions.

Table II summarizes our results and those of Ciarrocchi & Linn (1978) and Smith & Hanawalt (1978). Although three different types of preparations were used, including a permeable cell system (this paper), a lysed cell system (Ciarrocchi & Linn, 1978), and isolated nuclei (Smith & Hanawalt, 1978), in general the results from the three investigations are similar. However, some major quantitative differences are apparent. The amount of stimulation by ATP addition varies among the three systems, with the permeable cell system showing the greatest stimulation. Also, the time course of repair synthesis is markedly shorter in isolated nuclei than in cell preparations; the nuclei showed very little repair replication after 5 min of incubation. However, repair synthesis in nuclei was stimulated by the addition of T4 endonuclease V, which specifically nicks DNA containing pyrimidine dimers. These results suggest that nucleotide-permeable cells and whole-cell lysates may retain the factors responsible for incision of DNA at or near pyrimidine dimers, whereas isolated nuclei have lost these factors. At present we have not investigated the ability of permeable cells to repair UV damage incurred *after* the cells have been removed from the dish and suspended in permeabilizing buffer at 4 °C. While such experiments might help to determine if permeable cells have the ability to incise DNA at damaged sites, they are technically difficult because of possible "shielding" effects when a slurry of cells is irradiated. However, results from this type of experiment might be interesting in view of a report (Smith & Hanawalt, 1978) that in nuclei little repair synthesis occurs unless the cells have been incubated at 37 °C in complete medium following irradiation and prior to harvest.

We were interested in estimating the number of repair patches inserted by a cell during our *in vitro* incubation. During the first 10 min of incubation, dCMP is incorporated at a rate of $\sim 2.4 \times 10^{-21}$ mol/(cell min). Previous studies have indicated that after UV damage of mammalian cells, the excision repair process synthesizes repair patches between 35 and 100 nucleotides long (Smith & Hanawalt, 1978; Edenberg & Hanawalt, 1972; Regan & Setlow, 1974); using these values, we calculate that in our system between 100 and 200

repair patches are synthesized per IMR-90 cell per min during the first 10 min. With data from other experiments in which higher levels of incorporation were observed, this value would range from 300 to 900 repair patches/(cell min). This rate is probably a minimum estimate because it relies on the assumption that all of the dCMP incorporated comes from the labeled pool (see next paragraph). In addition, rates of repair synthesis might be faster in intact cells than in permeable cells or nuclei.

Like DNA replicative synthesis in permeable and subcellular eucaryotic systems (Krokan et al., 1975; Tseng & Goulian, 1975; Berger & Johnson, 1976; Seki & Oda, 1977a,b; Seale, 1977; Schlaeger, 1978), DNA repair synthesis in permeable human cells is strongly stimulated by the addition of ATP (Figure 4). Repair synthesis in procaryotes also appears to be ATP dependent [Moses & Moody, 1975; Thielmann, 1976; Yoakum & Cole, 1977; Dorson et al., 1978; Thielmann & Gersbach, 1978; however, see Moses & Richardson (1970)], although it is unclear whether the ATP is needed for the same or different functions in the two systems. In our permeable cell repair system, ATP may be utilized for (1) phosphorylation of precursors of dNTPs or NTPs, (2) incision of DNA at the site of damage, (3) ligation, or (4) some unidentified energy-requiring step(s). The first possibility seems unlikely because ATP is required even when dNTPs are in great excess. In addition, increasing the concentration of [³H]dCTP in the presence of excess dATP, dGTP, and BrdUTP does not increase incorporation as would be expected if the mode of action of ATP were to maintain the [³H]dCTP pool (Table I). Furthermore, [³H]dCTP is utilized more efficiently than [³H]dCMP or [³H]dCyt (Table I). However, our data do not rule out the possibility that repair synthesis involves an obligatory dNTP degradation step, followed by ATP-dependent rephosphorylation similar to that observed in bacteriophage T4 replication (Mathews, 1976; Reddy & Mathews, 1978). Alternatively, ATP may not be involved directly in the repair process but may function in maintaining the integrity of the entire system.

The addition of NaCl resulted in substantial inhibition of repair synthesis in our system. Whether NaCl disrupts the native structure of chromatin, inhibits an enzymatic process(es), or changes the permeability of cells to nucleoside triphosphates is unknown. However, the NaCl effect has been

observed in disrupted cells (Ciarrocchi & Linn, 1978), suggesting that this inhibition is probably not due to changes in permeability.

We were unable to demonstrate a role for pyridine nucleotide metabolism in excision repair of UV damage. Some investigators (Chambon et al., 1966; Burzio & Koide, 1970, 1971; Schacter & Burke, 1978) have suggested that the NAD⁺ may have a regulatory role in DNA replicative synthesis, while others (Miller, 1975; Smulson et al., 1977; Benjamin & Gill, 1978; Jacobson & Jacobson, 1978) have postulated a role for NAD⁺ in DNA repair. Recent studies (Claycomb, 1976; Kidwell & Burdette, 1974; Rechsteiner et al., 1976) have indicated that the formation of poly(ADP-ribose) may be the mechanism by which NAD⁺ exerts its regulatory effects. We find that under our conditions neither the naturally occurring active form (β -NAD⁺) nor a competitive inhibitor (α -NAD⁺) affects repair synthesis induced by UV; however, these studies do not rule out the possibility that NAD⁺ might have an effect on repair synthesis under conditions optimal for poly(ADP-ribose) formation (Berger et al., 1978). Furthermore, our studies do not exclude the possibility that the degradation of previously synthesized poly-(ADP-ribose) plays a role in repair or that pyridine nucleotide metabolism is involved in other types of repair processes.

One of the intriguing aspects of our findings is the apparent similarity of the pH and cofactor requirements for excision repair synthesis and replicative synthesis in mammalian cells (Tseng & Goulian, 1975; Berger & Johnson, 1976; Seki & Oda, 1977a). In the past, repair synthesis and replicative synthesis in eucaryotes have been investigated largely independently, although it is widely believed that in bacteria the two processes are closely related. Exploration of the possible relationships between repair synthesis and replicative synthesis in human cells and analysis of the cellular factors involved in these processes will be of interest.

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Location of the Stilbenedisulfonate Binding Site of the Human Erythrocyte Anion-Exchange System by Resonance Energy Transfer[†]

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ABSTRACT: The stilbenedisulfonate inhibitory site of the human erythrocyte anion-exchange system has been characterized by using several fluorescent stilbenedisulfonates. The covalent inhibitor 4-benzamido-4'-isothiocyanostilbene-2,2'-disulfonate (BIDS) reacts specifically with the band 3 protein of the plasma membrane when added to intact erythrocytes, and the reversible inhibitors 4,4'-dibenzamidostilbene-2,2'-disulfonate (DBDS) and 4-benzamido-4'-aminostilbene-2,2'-disulfonate (BADs) show a fluorescence enhancement upon binding to the inhibitory site on erythrocyte ghosts. The fluorescence properties of all three bound probes indicate a rigid, hydrophobic site with nearby tryptophan residues. The Triton X-100 solubilized and purified band 3 protein has similar affinities for DBDS, BADs, and 4,4'-dinitrostilbene-2,2'-disulfonate (DNDS) to those observed on intact erythrocytes and

erythrocyte ghosts, showing that the anion binding site is not perturbed by the solubilization procedure. The distance between the stilbenedisulfonate binding site and a group of cysteine residues on the 40 000-dalton amino-terminal cytoplasmic domain of band 3 was measured by the fluorescence resonance energy transfer technique. Four different fluorescent sulfhydryl reagents were used as either energy transfer donors or energy transfer acceptors in combination with the stilbenedisulfonates (BIDS, DBDS, BADs, and DNDS). Efficiencies of transfer were measured by sensitized emission, donor quenching, and donor lifetime changes. Although these sites are approachable from opposite sides of the membrane by impermeant reagents, they are separated by only 34-42 Å, indicating that the anion binding site is located in a protein cleft which extends some distance into the membrane.

The anion transport system of the human erythrocyte plasma membrane catalyzes bicarbonate for chloride exchange to facilitate CO₂ release in the lungs. A 95 000-dalton polypeptide, labeled band 3 for its relative mobility upon sodium dodecyl sulfate (NaDodSO₄)¹ gel electrophoresis, is responsible for this exchange [for reviews see Cabantchik et al. (1978) and Steck (1978)]. This polypeptide is present in the membrane as a dimer (Steck, 1972; Kiehm & Ji, 1977; Nigg & Cherry, 1979) and makes up approximately 25% of the total membrane protein (Fairbanks et al., 1971). Considerable research on the primary structure has shown that band 3 asymmetrically spans the bilayer with a 40 000-dalton amino-terminal domain in the cytoplasm and a 55 000-dalton carboxy-terminal domain which crosses the bilayer at least once and perhaps several times (Steck et al., 1976; Drickamer, 1976; Rao, 1979).

Although it is clear that this protein catalyzes one for one anion-exchange orders of magnitude faster than unidirectional

transport, the mechanism of this exchange is unknown. Some information about the structure and mechanism of this system has been obtained by using a class of stilbenedisulfonates which are not readily transported but act as high-affinity competitive inhibitors of anion exchange when added to the outside of intact erythrocytes (Maddy, 1964; Cabantchik & Rothstein, 1972, 1974). Two of these compounds, 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) and 4,4'-diisothiocyanodihydrostilbene-2,2'-disulfonate (H₂-DIDS), react specifically and irreversibly with approximately one site per band 3 monomer to completely inhibit anion exchange in intact erythrocytes (Lepke et al., 1976; Ship et al., 1977; Jennings & Passow, 1979). The reactive site can be approached from the outside of the cell with impermeant reagents (Cabantchik & Rothstein, 1974) and has been located on a 19 000-dalton tryptic fragment near the center of the polypeptide chain (i.e., in the amino-terminal third of the 55 000-dalton carboxy-terminal domain; Grinstein et al., 1978).

In this study we utilize the fluorescent properties of stilbenedisulfonates which interact reversibly [DBDS and BADs; see Table I (a)] or irreversibly (BIDS) with the anion-exchange system to characterize the inhibitory site. The integrity of this site is maintained on red-cell ghosts and on Triton X-100 solubilized and purified band 3. The distance from this site

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¹ Abbreviations used: DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonate; H₂-DIDS, 4,4'-diisothiocyanodihydrostilbene-2,2'-disulfonate; NaDodSO₄, sodium dodecyl sulfate; all other abbreviations are explained in Table I.