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Nonphotoreactivating Repair of Ultraviolet Light-Damaged Transforming Deoxyribonucleic Acid by *Micrococcus lysodeikticus* Extracts[1]

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ABSTRACT

ELDER, ROBERT L. (Johns Hopkins University, Baltimore, Md.), AND ROLAND F. BEERS, JR. Nonphotoreactivating repair of ultraviolet light-damaged transforming deoxyribonucleic acid by *Micrococcus lysodeikticus* extracts. J. Bacteriol. **90**:681–686. 1965.—Extracts from *Micrococcus lysodeikticus* repair *Haemophilus influenzae* transforming deoxyribonucleic acid (DNA) damaged by ultraviolet light radiation. The repair is demonstrable over a wide dose range, with a constant dose reduction factor for a given concentration of DNA. The active component in the crude extract may be separated into a heat-stable dialyzable and a heat-labile nondialyzable component. The dialyzable fraction contains at least one component which appears to limit the maximal level of repair. Mg^{2+} ions are required for the repair process.

The marked resistance of *Micrococcus lysodeikticus* to ultraviolet (UV) light can be the result of a nonphotoreactivating repair mechanism (Elder and Beers, 1965). Photoreactivation, the reversal of short wavelength UV light damage by post-irradiation exposure to long wavelength light, was first noted by Kelner (1949, 1953). The enzymatic nature of the photoreactivation reversal of UV light damage on transforming deoxyribonucleic acid (tDNA) with an extract from baker's yeast was shown by Rupert (1962*a*, *b*). Rupert (1962*a*) proposed that the photoenzyme repaired an UV "lesion" on the tDNA which, if not reversed, would not permit expression of the genetic trait assayed in the recipient cell.

Dark-cell repair mechanisms have been demonstrated in *Escherichia coli* (Sauerbier, 1961). However, in vitro repair by *E. coli* extracts has not been demonstrated in the absence of photoreactivating light. In contrast, *M. lysodeikticus* extracts do repair UV light-damaged tDNA (Elder and Beers, 1964).

This paper presents some of the characteristics of this nonphotoreactivating repair system which uses the tDNA of *Haemophilus influenzae*. While these studies were in progress, Rörsch, Van der Kamp, and Adema (1964) demonstrated repair of replicated form (RF)-DNA by an extract of this microorganism.

MATERIALS AND METHODS

Sd *Haemophilus influenzae* resistant to 1,200 μg/ml of streptomycin (Merck & Co., Inc., Rahway, N.J.), and a streptomycin-sensitive strain of the same cell were kindly supplied by C. S. Rupert, Department of Biochemistry, Johns Hopkins University. Spray-dried cells of *M. lysodeikticus* were obtained from Miles Chemical Co., Elkhart, Ind.

H. influenzae tDNA was isolated from streptomycin-resistant cells (1,200 μg/ml) by the procedure recommended by Goodgal and Herriott (1961). The isolated tDNA has a ϵ260 mμ to ϵ230 mμ ratio of 2.35 and ϵ260 mμ to ϵ280 mμ ratio of 2.22 and was stored as a concentrated microgram per milliliter stock solution in 0.015 to 0.015 citrate-saline solution at 4 C.

UV irradiation of tDNA. tDNA suspended in 0.015 M NaCl at a concentration of 4 μg/ml was irradiated with a 15-w General Electric germicidal lamp described elsewhere (Elder and Beers, 1965) for the required exposure dose. The tDNA was irradiated while on a shaking water bath at a depth of 0.5 cm. The 260-mμ transmission was in excess of 95%.

Transformation assays. The direct method of assaying the tDNA activity as recommended by Goodgal and Herriott (1961) was used throughout

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the experimental program. tDNA carrying the streptomycin marker was introduced into Brain Heart Infusion broth (Difco) to which had been added hemin (10 μg/ml; Eastman Chemical Products, Inc., Kingsport, Tenn.), and 2 μg/ml of nicotinamide adenine dinucleotide (NAD) (Nutritional Biochemicals Corp., Cleveland, Ohio). Streptomycin-marked tDNA (0.1 ml) was added to 2.8 ml of Brain Heart Infusion broth and 0.1 ml of competent cells to give a final cell concentration of approximately 5×10^7 per milliliter. Streptomycin-sensitive competent receptor cells were prepared by the method recommended by Goodgal and Herriott (1961), and were stored at −60 C until required. (Competence is defined as a capacity of the cell to undergo transformation.) Competent cell preparations which were thawed immediately before being used showed a competence level ranging from 0.1 to 0.2% after a 6-week storage period. After addition of the competent cells, the mixture was incubated with aeration for 2 hr at 37 C. During this incubation, both DNA uptake and expression of the streptomycin marker occurred, thus allowing direct plating into Brain Heart Infusion agar which contained 250 μg/ml of streptomycin (Goodgal and Herriott, 1961). All dilutions of *H. influenzae* cells were made in 3.5% Eugonbroth (Difco).

Repair assay. Unless noted otherwise in the text, 1 volume of extract from *M. lysodeikticus* was mixed with 1 volume of 0.025 M $MgSO_4$, 1 volume of irradiated tDNA, and 1 volume of 0.01 M tris-(hydroxymethyl)aminomethane (Tris) buffer (*p*H 6.8) and incubated for 30 min at 37 C in a shaking water bath. At the end of a 30-min repair time, 2.8 ml of Brain Heart Infusion broth and 0.1 ml of competent cells were added for the 2-hr tDNA uptake and expression as noted above. For all controls which were not treated with the crude extract, an additional volume of Tris buffer (*p*H 6.8) was added.

All experiments were performed in subdued light. Attempts to increase the magnitude of repair by exposure to photoreactivating light were performed as recommended by Rupert (1960).

Net per cent repair is defined as the difference in the number of transformations of the extract-treated and nontreated UV-transforming DNA divided by the number of transformations obtained from an identical quantity of nonirradiated transforming DNA.

Preparation of extract. Cells (100 g) were suspended in 1 liter of 0.01 M phosphate (*p*H 7.2), warmed to 37 C, and held at this temperature for 1 hr. A 3.3 ml amount of 3.0 M NaCl was added (final concentration, 10 mM). The cells were then lysed by the addition of 10 ml of 0.01 M phosphate (*p*H 7.2) containing 200 mg of lysozyme (Worthington Biochemical Corp., Freehold, N.J.). The suspension was stirred continuously. When the mixture became very thick and resembled a curd, the mixture was rapidly cooled to 5 C or lower to stop the lysis. The lysed cells were centrifuged in 250-ml centrifuge tubes at 8,000 rev/min for 30 min at −10 C. The yellow-brown supernatant fluid was decanted and 5 M KH_2PO_4 was added to reduce the *p*H to 6.8 (usually about 7.5 ml). The liquid extract was frozen and stored at −10 C. No loss of activity occurred after these procedures.

This extraction procedure is similar to that described by Beers (1957) for the preparation of polyribonucleotide phosphorylase. Lysis of the cell wall is stopped before the protoplasts are lysed. Grinding with alumina, sonic rupture, or allowing the lysis to proceed longer than 30 min did not yield an active extract. The more drastic rupture of cells gave an extract which decreased the number of transformations from both irradiated and nonirradiated tDNA.

Fractionation of the crude extract. The extract prepared by the above procedure was dialyzed for 48 hr in 0.01 M Tris buffer (*p*H 6.8) (8× volume).

The dialyzed extract was fractionated on columns of diethylaminoethyl (DEAE) cellulose

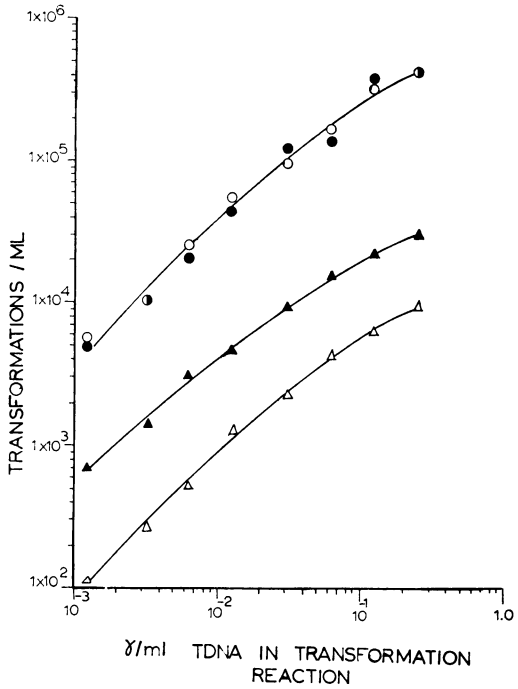

FIG. 1. *Maximal repair at varying tDNA concentrations. Symbols:* ○ = *nonirradiated tDNA with no extract;* ● = *nonirradiated tDNA plus extract;* ▲ = *irradiated tDNA plus extract;* △ = *irradiated tDNA with no extract. An amount (10 μg/ml) of tDNA was irradiated to 3,500 ergs/mm² and diluted to give the following micrograms in a 0.1-ml portion: 1.0, 0.5, 0.25, 0.125, 0.05, 0.0125, and 0.005. After irradiation, 0.1 ml of tDNA plus 0.1 ml of 0.025 M $MgSO_4$, 0.1 ml of 0.01 M Tris (pH 6.8), and 0.1 ml of crude extract were added. After a 30-min repair time, 2.8 ml of Brain Heart Infusion broth plus 0.1 ml of 2 × 10⁹ competent cells per milliliter were added for the transformation assay.*

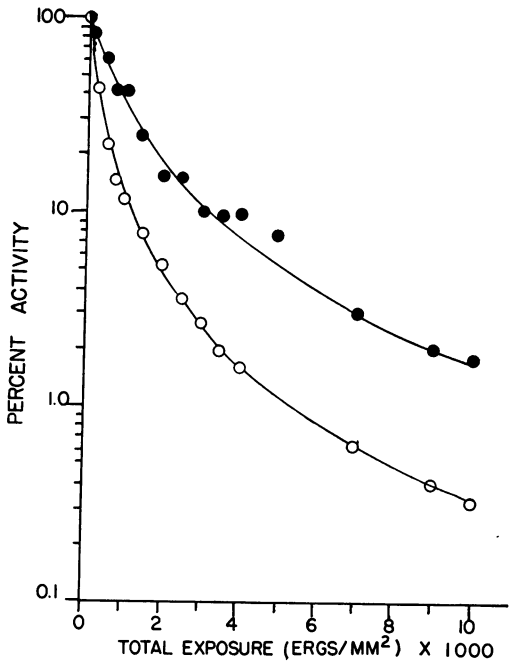

FIG. 2. *Maximal repair at various exposure doses. Symbols:* ● = *tDNA plus extract;* ○ = *tDNA and no extract. An amount (0.1 ml) of 2 μg/ml of tDNA was added to 0.1 ml of 0.01 M Tris buffer (pH 6.8) plus 0.1 ml of 0.025 M $MgSO_4$ plus 0.1 ml of crude extract. After a 30-min repair time at 37 C, 2.8 ml of Brain Heart Infusion broth plus 0.1 ml of 2.0 × 10^9 competent cells per milliliter were added for the transformation assay.*

previously washed with distilled water and 0.01 M Tris buffer (*p*H 6.8). After the addition of 25 ml of the extract, the column was eluted with 25-ml portions of 0.01 M Tris buffer (*p*H 6.8) containing 0.1, 0.2, 0.3, and 0.4 N KCl.

RESULTS

Treatment of UV-irradiated tDNA with the crude extract of *M. lysodeikticus* in the presence of Mg^{2+} ions resulted in an increase in the number of transformants over that of the control (Fig. 1). Treatment of nonirradiated tDNA with the extract did not increase the number of transformations. This net increase in the number of transformations is indicative of a repair of the UV light damage on the tDNA. The system parallels the photoenzymatic repair of irradiated tDNA reported by Rupert (1962*a*, *b*), but does not require photoreactivating light. Duplicate experiments performed in the presence of photoreactivating light did not increase the amount of repair. The repair of UV light-damaged tDNA may be assayed over the entire tDNA titration curve.

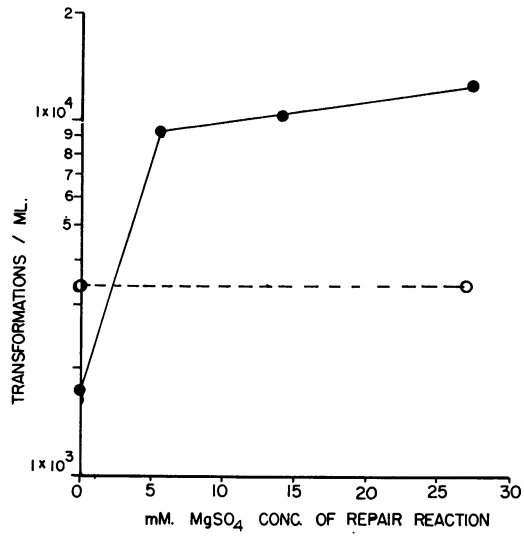

FIG. 3. *Effect of magnesium on maximal repair of irradiated tDNA. Symbols:* ○ = *irradiated tDNA with no extract;* ● = *irradiated tDNA plus extract. Each test contained 0.1 ml of 1.14 μg/ml of tDNA, 0.1 ml of crude extract, 0.1 ml of 0.01 M Tris buffer (pH 6.8), and 0.1 ml of $MgSO_4$. (Additional volume of Tris buffer replaced the extract in the untreated.) After the repair reaction, 2.8 ml of Brain Heart Infusion broth plus 0.1 ml of 5 × 10^8 competent cells per milliliter were added for the transformation assay.*

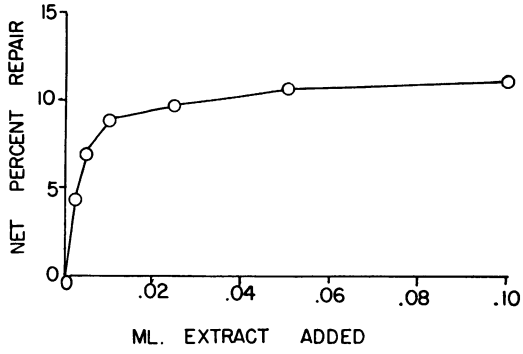

FIG. 4. *Effect of the quantity of extract on maximal repair. Cells were incubated in 0.1 ml (0.571 μg/ml) of tDNA (irradiated at 3,500 ergs/mm²) plus 0.1 ml of 0.025 M $MgSO_4$ plus the noted volume of crude extract. All samples were adjusted to a total volume of 0.4 ml by adding 0.01 M Tris buffer (pH 6.8). After 30 min, 2.8 ml of Brain Heart Infusion broth plus 0.1 ml of a suspension containing 10^9 competent cells per milliliter were added for the transformation assay.*

However, the degree of repair was lower for the high-concentration samples (for which a higher concentration of irradiated DNA was in contact with the fixed amount of cell extract prior to

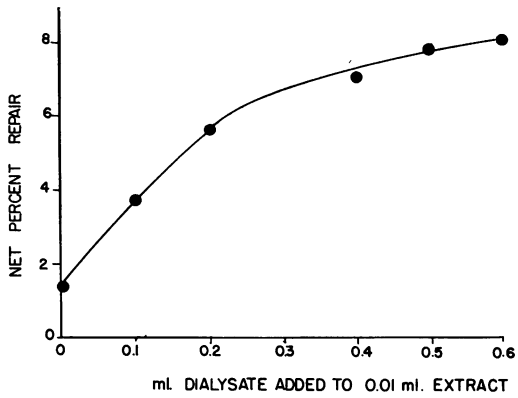

FIG. 5. *Effect of the quantity of dialysate on maximal repair. Each repair reaction contained 0.1 ml of a 2% crude extract suspension plus 0.1 ml of 0.025 M $MgSO_4$ plus 0.1 ml (2 μg/ml) of irradiated tDNA plus the noted volume of dialysate. Each tube was adjusted to 1.0 ml by adding the appropriate amount of 0.01 M Tris (pH 6.8). An amount (2.8 ml) of Brain Heart Infusion broth plus 0.1 ml of 10^9 competent cells were added for the transformation assay. (A more concentrated Brain Heart Infusion broth was added to correct for dilution.) Control tubes were similarly adjusted.*

assay). The repair of the UV light damage, assayed for doses ranging from 250 to 9,000 ergs/mm², gave a constant dose reduction factor of 0.36 (Fig. 2).

The apparent *p*H optimum for the repair process under the experimental conditions employed was 6.8. Mg^{2+} increased the extent of repair by almost one order of magnitude (Fig. 3). The concentration of the divalent cation required for maximal effect was high for a typical cofactor requirement in an enzyme-catalyzed reaction (0.03 M). The mechanism of action by Mg^{2+} is complicated by the apparent protective effect it has on the number of transformants obtained with irradiated or nonirradiated tDNA mixed with the crude bacterial extract. In the absence of Mg^{2+}, but in the presence of the extract, the number of transformants was decreased to less than 50% of the control. Ca^{2+}, Ba^{2+}, and Fe^{2+} did not stimulate the repair process. No similar specificity existed for the anions SO_4^{2-}, acetate, and NO_3^-.

The extent of repair of tDNA increased with increasing amounts of the extract to a maximal value (Fig. 4). However, the kinetic significance of the extent of maximal repair observed after 30 min of incubation of the irradiated tDNA with the extract is complicated by the fact that longer incubation times with suboptimal amounts of the extract did not give the same extent of repair. This can be corrected by adding dialysate of the crude extract to the suboptimal quantities of extract (Fig. 5).

The active component(s) of the crude extract could be precipitated by 40% saturated $(NH_4)_2SO_4$ or 40% (v/v) acetone. Fractionation on the DEAE columns resulted in the loss of activity which could be restored by adding the dialysate of the crude extract to the 0.1 N KCl eluate (Table 1).

AgCl at a concentration of 0.5 mM inhibited the repair process, but did not interfere with the transformation assay. Digestion with trypsin inactivated the crude extract; chymotrypsin did

TABLE 1. *Effect of the quantity of dialysate on DEAE fraction repair of irradiated tDNA**

Fraction	Transformations/ml	
	Nonirradiated tDNA	Irradiated tDNA (3,500 ergs/mm²)
Control, buffer only	2.4×10^5	5.8×10^3
Dialysate only (0.4 ml)	2.5×10^5	6.0×10^3
0.1 N KCl DEAE eluant + buffer	2.5×10^5	5.8×10^3
0.1 N KCl DEAE eluant + 0.05 ml of dialysate	—	7.5×10^3
0.1 N KCl DEAE eluant + 0.1 ml of dialysate	—	7.7×10^5
0.1 N KCl DEAE eluant + 0.2 ml of dialysate	—	9.8×10^3
0.1 N KCl DEAE eluant + 0.3 ml of dialysate	—	1.5×10^4
0.1 N KCl DEAE eluant + 0.4 ml of dialysate	2.3×10^5	1.1×10^4
Native extract only	2.5×10^5	1.2×10^4

* Each repair reaction contained the volumes of dialysate noted plus 0.1 ml of 0.1 N KCl DEAE eluant plus 0.1 ml of 0.025 M $MgSO_4$ plus 0.1 ml (2 μg/ml) of tDNA plus an appropriate volume of 0.01 M Tris buffer (*p*H 6.8) to give a final repair reaction volume of 0.7 ml. After a 30-min repair time, 2.8 ml of Brain Heart Infusion broth plus 0.1 ml of 2×10^9 competent cells per milliliter were added for the transformation assay.

not. A temperature of 100 C destroyed the activity of the crude extract and of the dialyzed extract. The dialysate was stable at 100 C for 10 min.

DISCUSSION

Repair of tDNA damaged by UV light radiation by an extract of *M. lysodeikticus* cells provides evidence for the hypothesis that these cells contain a nonphotoreactivating enzymatic repair system (Elder and Beers, 1965).

The enzymatic character of the bacterial extract is obscured by the probable presence of DNA-destroying components in the crude or partially purified extracts. One indication of this is the destruction of transforming ability of irradiated tDNA treated with the crude extract in the absence of Mg^{2+} ions (Fig. 3). Thus, it would appear that the inability to demonstrate a classical time extent of repair relationship may be the result of a competing tDNA-destruction process which is partially blocked by Mg^{2+}. Additional evidence for a competing system is the failure to obtain active extracts when the cells are extensively broken down by lysozyme or mechanical means. Presumably these conditions lead to the release of nucleases.

The enzymatic character of the extract is also suggested by its heat sensitivity and inhibition by trypsin and AgCl. At least one dialyzable heat-stable cofactor is required. The apparent stoichiometric requirement of this cofactor suggests that it may be an essential substrate for the repair process.

The repair of the UV light-damaged replicate form of 0174 DNA by extracts of *M. lysodeikticus* described by Rörsch et al. (1964) shows certain similarities to the system described in this paper. It is possible that other DNA-containing systems damaged by UV light can be repaired by extracts from these cells. It has been shown that UV irradiation produces intrastrand dimers between adjacent thymine residues (Setlow and Setlow, 1962). Wulff and Rupert (1962) demonstrated that the splitting of thymine dimers was associated with the photoreactivation repair of UV-irradiated transforming DNA. These results, as well as the subsequent work of Boyce and Howard-Flanders (1964) and Setlow and Carrier (1964), supported the concept that the error- or damage-correcting mechanism for reversing UV light damages is based upon thymine dimer excision. Boyce and Howard-Flanders (1964) showed that the difference in the UV light sensitivity of the mutant strain *E. coli* K-12 AB 1886 from the *E. coli* K-12 1157 UV-resistant parent strain was the inability to excise thymine dimers. It is not possible to correlate the data obtained in this study on the dark in vitro repair of UV light-damaged transforming DNA by an extract from *M. lysodeikticus*, and that of the enzymatic excision of UV-induced thymine dimers. Further investigation is required to determine whether the dark in vitro repair of UV-irradiated tDNA by extracts of *M. lysodeikticus* works in a similar manner.

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