*PHOTOREACTIVATING-ENZYME ACTIVITY IN METAZOA**

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Photoreactivation of ultraviolet (UV) radiation damage to organisms has been demonstrated in many plant and animal phyla.[1] In a few cases, this repair process has been shown to involve a light-dependent photoreactivating enzyme which monomerizes UV-induced pyrimidine dimers in DNA.[2, 3] However, the biological phenomenon of photoreactivation does not necessarily indicate the involvement of a photoreactivating enzyme, since "indirect photoreactivation," not mediated by such an enzyme, has also been described.[4] In most cases where biological photoreactivation has been observed, a photoreactivating enzyme has not yet been demonstrated and the mechansim of reactivation is not known.

In the sea urchin *Arbacia punctulata*, the eggs and zygotes are photoreactivable whereas the sperm are not.[5] In this same species,[6] we have found that photoreactivating-enzyme activity is demonstrable both in egg and in testis. Such a distribution of photoreactivating activity between sperm and testis is unexpected, since the shed sperm are exposed to sunlight in the shallow waters of the open sea, whereas the testis is always well shielded from both UV and visible radiations of the sun by a virtually opaque covering. The demonstration of a light-dependent enzyme in an internal tissue (i.e., one that is always in the dark) led us to inquire whether such a finding would be unique to this one species or this one tissue. A more general distribution of the enzyme would suggest that it might serve some function other than photoreactivation. Accordingly, we have conducted a survey on the distribution of photoreactivating-enzyme activity in various organs of several species representing the higher phyla of metazoa. In addition, we have fractionated frog liver, an internal organ of a vertebrate with a reasonably high level of photoreactivating activity, to determine the subcellular organelles with which the activity is associated.

Materials and Methods.—Tissues from the various metazoan species were homogenized in solutions of approximately isotonic potassium chloride (0.1 molar for amphibians, 0.15 molar for mammals, 0.5 molar for arthropods and echinoderms) containing 0.005 molar glutathione, 0.002 molar ethylenediaminetetraacetate (EDTA), 0.005 molar potassium phosphate buffer, pH 7.0, and 20% (v/v) glycerol. Substitution of sodium chloride for potassium chloride or of mercaptoethanol for glutathione did not affect the activity.

The tissues were placed in four volumes of appropriate medium and homogenized either with 8 to 10 passes in a Potter-Elvehjem homogenizer or (for muscle) with a Virtis homogenizer or (for skin) by grinding with sand. The enzyme was then more completely solubilized by two 30-sec treatments of the homogenate with a Bronwill "Biosonik" sonicator at a power setting of 60. Sonication for up to 5 min did not diminish the activity of the extracts. The crude homogenates were centrifuged at 2000 $\times$ *g* for 15 min, and the supernatant fractions were assayed for photoreactivating activity. All preparative procedures were performed at 0–4°C.

Activity was measured by the assay which Muhammed[7] developed from the experiments of Goodgal, Rupert, and Herriott,[8] i.e., by the ability of cell extracts to increase, in the light, the biological activity of UV-irradiated transforming DNA. A tissue extract was incubated at 37°C under "blacklight" illumination (320–420 mμ; 7000 ergs mm^{-2} min^{-1}) with transforming DNA from a streptomycin-resistant strain of *Hemophilus influenzae*. The DNA had been irradiated to 1% survival of the streptomycin marker. A 6000-fold excess of calf thymus DNA, which does

not interfere with the reaction between photoreactivating enzyme and irradiated DNA,[9] was added to the reaction mixture to protect the transforming DNA from nucleases usually present in the crude tissue extracts. Following incubation, the activity of the transforming DNA was assayed on streptomycin-sensitive organisms. Under the conditions employed, DNA that was maximally photoreactivated yielded an increase in the number of transformants by a factor of approximately 20 over identically treated controls kept in the dark. The unit of activity is defined as the factor increase in the number of transformants, minus 1, after 5 min of photoreactivation. In all cases identified as positive in Table 1 (below), the activity was reproducible, light-dependent, and abolished by heating the crude extract to 65°C for 10 min.

Typical results of the transformation assay with two preparations from *Arbacia* are shown in Figure 1*A*. After incubation of the transforming DNA with extract I in the dark there was a decrease in transforming activity despite the excess calf thymus DNA. With the same extract in the light, transforming activity was increased 20-fold over the sample incubated in the dark. Extract II showed more nuclease activity and less photoreactivating activity than extract I. In Figure 1*B* is shown the disappearance of thymine-containing cyclobutyl dimers from UV-irradiated *Escherichia coli* DNA (for assay method, see refs. 3 and 10) when such DNA was exposed to the same two *Arbacia* extracts under the same conditions of illumination. Since other conditions of the two assays were not identical (e.g., the DNA's were from different organisms and had been irradiated with different UV doses, etc.), a strict quantitative comparison cannot be made between Figures 1*A* and *B*. Nevertheless, it is evident that the *Arbacia* extract which was more effective in enhancing transformation was also more effective in splitting pyrimidine dimers.

Results.—Phylogenetic distribution of photoreactivating-enzyme activity: The tissues tested for activity are listed in Table 1. The specific activities of most of the tissues listed as positive were in the range of 1–10 units/mg protein. *Gecarcinus* testis (30 units/mg protein) was the most active preparation, whereas adult chick brain (less than 0.2 units/mg protein) was the least active of tissues giving consistently positive results.

TABLE 1

PHOTOREACTIVATING (PR) ACTIVITY IN METAZOAN TISSUES WHEN ASSAYED WITH ULTRAVIOLET-IRRADIATED TRANSFORMING DNA FROM *Hemophilus influenzae*

Animal	Tissues with PR activity	Tissues without PR activity
Echinodermata		
Sea urchin (*Arbacia punctulata*)	Testis; eggs Ovary + eggs	
Arthropoda		
Flower moth (*Anagasta kühniella*)	Abdomen of adult female	
Land crab (*Gecarcinus lateralis*)	Testis; ovary Epithelium Somatic muscle Heart muscle	Midgut gland
Vertebrata		
Teleost (*Haemulon sciurus*)	Cells of dorsal fin in tissue culture[12]	
Toad (*Bufo marinus*)	White blood cells	Blood serum Red blood cells
Frog (*Rana pipiens*)	Skeletal muscle Cardiac muscle Sciatic nerve Brain; liver	
Vertebrata (cont'd)		
Chicken	Primary fibroblasts Whole 4-day embryos Adult brain	Kidney Liver Skeletal muscle Egg white Egg yolk
Mouse		Skin
Rabbit		Skin
Rat		Liver Skeletal muscle Heart muscle Ovary Testis Brain Kidney
Calf		Fetus (3-cm)

Our results with *Anagasta* represent the first demonstration of photoreactivating-enzyme activity in insects. In a previous attempt, A. Muhammed and J. E. Trosko[11] obtained negative results with extracts prepared from *Drosophila* at all phases of the life cycle.

Of the six tissues tested from the land crab, all but the midgut gland (hepatopancreas) gave positive results; of eight amphibian tissues tested, six gave positive results. (The very slight activity in toad red cells could be accounted for by contamination with active white blood cells.) These data from the land crab and amphibia indicate that the enzyme activity is not localized in any particular tissue. The lack of tissue specificity is corroborated by the fact that activity is found in teleost cells which have been in tissue culture for at least 7 years,[12] since cells in culture generally tend to lose tissue-specific enzymes and to retain those which are "shared by all cells of an organism regardless of differentiation."[13]

Although whole chick embryos and chick fibroblasts, which were a primary cell line from chick embryo tissue (Grand Island Biological Co.), both showed high activity, adult chicken tissue showed little or none. Only slight activity was demonstrable in adult brain; reproducibly positive results could not be obtained in any of the other chicken tissues tested.

We were unable to demonstrate the enzyme in any mammalian tissue. Rat liver, skeletal muscle, cardiac muscle, ovary, testis, brain, and kidney were all negative in the standard assay. So were the skins of black rabbit, a black mouse, and a white mouse; the black animals were tested because melanocytes are known to re-

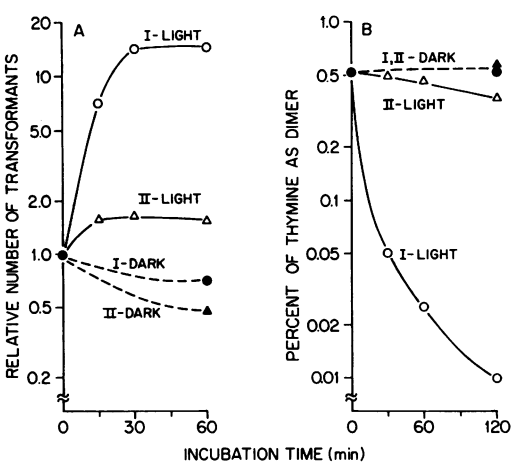


FIG. 1.—Photoreactivating activity of two extracts from *Arbacia*. Extract I had been freshly prepared from ovary, and contained 1 mg/ml protein; extract II was prepared from testis and contained 0.3 mg/ml protein.

(*A*) Transformation assay as described in the text. *Hemophilus* DNA had been irradiated with 2442 ergs/mm² at wavelength 254 mμ.

(*B*) Loss of thymine-containing cyclobutyl dimers from *E. coli* DNA when incubated with the same extracts. *E. coli* DNA had been irradiated with 1000 ergs/mm² at wavelength 280 mμ.

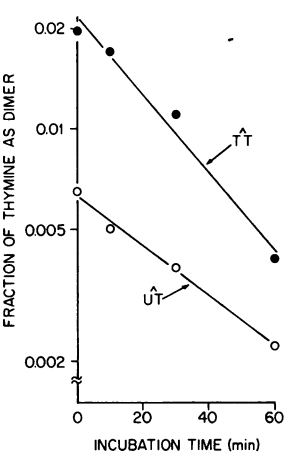


FIG. 2.—Loss of thymine-thymine (T^T) and cytosine-thymine (detected as uracil-thymine, U^T) dimers from UV-irradiated *E. coli* DNA exposed to frog-liver extract and light. Frog-liver extract contained approximately 3 mg/ml protein. *E. coli* DNA had been irradiated with 5000 ergs/mm² at wavelength 280 mμ. No dimers are lost on incubation in the dark.

spond to other photic stimuli. Since the extracts from rat liver, rat brain, and skin of black mouse did not inhibit yeast photoreactivating enzyme, it is unlikely that the negative results with these extracts alone were due to inhibitors. Extracts were frozen, thawed, and reassayed to determine whether the enzyme might be contained in lysosomes. Because of the sensitivity of the yeast enzyme to high concentrations of phosphate,[7] rat liver and brain were homogenized and assayed in media which contained tris (hydroxymethyl) aminomethane/hydrochloric acid buffer instead of phosphate buffer. Rat testis and brain were also assayed in media which contained 0.01 molar magnesium chloride and no EDTA. Since we found more activity in fetal than in adult chicken tissues, tissue from a 3-cm fetal calf was also tested. In none of these attempts were we able to demonstrate photoreactivating-enzyme activity in mammals.

All the tissues listed in Table 1 were tested with the transformation assay. To investigate the activity of a typical extract in greater detail, we have examined the ability of a frog-liver extract to monomerize thymine-containing cyclobutyl dimers in UV-irradiated *E. coli* DNA (Fig. 2). In this experiment, we chromatographically distinguished between thymine-thymine ($\widehat{\text{T-T}}$) dimers and uracil-thymine ($\widehat{\text{U-T}}$) dimers, the latter arising from deamination of cytosine-thymine dimers during the preparative hydrolysis of the DNA.[14] $\widehat{\text{T-T}}$ dimers are lost from DNA treated with frog extract and light at nearly twice the rate at which $\widehat{\text{U-T}}$ dimers are lost. Moreover, the activity of the frog-liver extract is nondialyzable. In these respects, the frog extract behaves very similarly to the more fully characterized photoreactivating enzyme from yeast.[15]

Subcellular distribution of photoreactivating activity in frog liver: To determine whether the photoreactivating activity of frog liver is associated with any particular organelle, we fractionated tissue homogenates and assayed the fractions for photoreactivating activity, cytochrome oxidase activity,[16] DNA,[17] and protein.[18] Data from a representative experiment are given in Table 2. The four fractions assayed were obtained as follows: (a) *Whole homogenate:* To 0.65 gm liver were added 3.2 ml of solution A (=0.17 molar sucrose, 0.002 molar calcium chloride, 0.002 molar magnesium chloride). The tissue was gently homogenized with six passes in a Potter-Elvehjem homogenizer, and 1.5 ml of the whole homogenate was removed and added to 1.5 ml of solution B (=0.001 molar EDTA, and 0.01 molar potassium phosphate buffer, pH 7). (b) S_1: The remaining 2.1 ml of the original homogenate were spun at 2000 × *g* for 15 min, and the supernatant was added to an equal volume of solution B. In S_1 we expect to find nearly all of the soluble en-

TABLE 2

DISTRIBUTION OF PHOTOREACTIVATING ACTIVITY, CYTOCHROME OXIDASE ACTIVITY, DNA, AND PROTEIN AMONG THREE FRACTIONS OF HOMOGENIZED FROG LIVER

Fractions prepared as described in the text. Figures in parentheses are per cent of whole-homogenate activity recovered in each fraction.

Fraction	Photoreactivating activity (for units, see text)	Cytochrome oxidase activity (μM/min/ml)	DNA (μg/ml)	Protein (mg/ml)
Whole homogenate	48.5	1.58	215	12.7
S_1	10.0 (21%)	0.52 (33%)	7 (3%)	8.0 (63%)
S_2	None (0%)	0.56 (35%)	3 (1.4%)	2.5 (20%)
P	34.6 (71%)	0.61 (39%)	218 (101%)	5.2 (41%)
Total recoveries	44.6 (92%)	1.69 (107%)	228 (106%)	15.7 (124%)

zymes, most of the lysosomes and microsomes, and a substantial fraction of the mitochondria. (c) *S_2:* The pellet from S_1 was resuspended in solution A to a volume of 2.0 ml, and recentrifuged at 600 × *g* for 6 min; the supernatant from this second spin was added to an equal volume of solution B. In S_2 we expect to find mitochondria which had been sedimented in the previous centrifugation, and perhaps a few nuclei. (d) *P:* The pellet from S_2 was again brought to 2.0 ml and 2.0 ml of solution B were added. This sample should contain some mitochondria and nearly all nuclei of the original homogenate. The four samples, now all in solutions of comparable composition, were sonicated as usual and assayed, with the results given in Table 2.

Fraction P contained about one third of the total protein and nearly all of the DNA. About one third of the mitochondria, as detected by the cytochrome oxidase assay, were also in this final pellet. The specific activity of the photoreactivating enzyme was seven times greater in the pellet than in the combined supernatants. Hence most of the photoreactivating activity appears to be associated with nuclei. In six experiments of this type, in which homogenization was carried out in media of low ionic strength, between 71 and 91 per cent of the total activity was sedimented with the nuclei, while the remainder appeared in the first supernatant solution.

There appears to be little, if any, photoreactivating activity specifically associated with the mitochondria of this tissue. For example, sample S_2 in Table 2 contains one third of the total cytochrome oxidase but no detectable photoreactivating activity. In other experiments, we have centrifuged fractions equivalent to S_1 at 100,000 × *g* for 60 min. All cytochrome oxidase activity is sedimented by such centrifugation, whereas all photoreactivating activity remains in the supernatant. Hence the activity in S_1 appears to be soluble, and not associated with any cellular organelle.

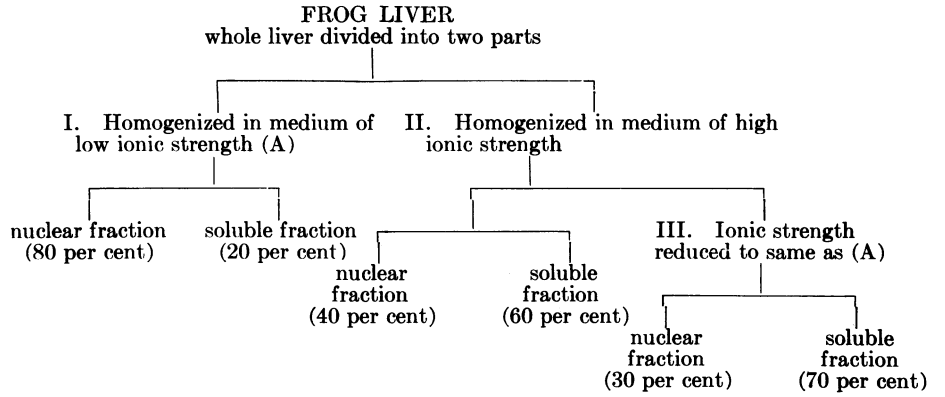
Although most of the photoreactivating activity was recovered in the nuclear fraction if the tissue were homogenized in the sucrose-calcium-magnesium medium, we had observed that if the tissue were homogenized in 0.1 molar potassium chloride buffered with 0.01 molar phosphate buffer, pH 7, then 60 to 90 per cent of the total photoreactivating activity was recovered in the first supernatant fraction (results of four experiments). The enzyme was clearly not bound to nuclei when the cells were disrupted in media of the higher ionic strength. Since such a solution more nearly approximated the cell interior than did the sucrose-calcium-magnesium solution, this result raised the possibility that the enzyme was in fact soluble, and became bound to nuclei only when the cells were disrupted in media of low ionic strength. To test this possibility, we have attempted to bind the soluble enzyme to nuclei by lowering the ionic strength of the suspending media.

The complete experiment was as follows (see Table 3 for flow sheet). We divided a liver into two parts. The first (I) was homogenized in eight volumes of solution A (=0.17 molar sucrose, 0.0015 molar calcium chloride, and 0.0015 molar magnesium chloride) plus one volume of solution B (= 0.05 molar potassium chloride, and 0.01 molar phosphate buffer, pH 7). Thus, the cells in sample I were disrupted in medium of low ionic strength. The homogenate was centrifuged at 2000 × *g* for 15 min. The second part of the liver was homogenized in one volume of solution B, i.e., in a medium of relatively high ionic strength. This second homogenate was

TABLE 3

FLOW SHEET FOR EXPERIMENT SHOWING THAT PHOTOREACTIVATING-ENZYME ACTIVITY IN THE SOLUBLE FRACTION DOES NOT BECOME REASSOCIATED WITH THE NUCLEAR FRACTION IN MEDIA OF LOW IONIC STRENGTH

Percentages show how the total photoreactivating activity of a given sample was distributed between the pellet (nuclear fraction) and supernatant (soluble fraction) following centrifugation at 2000 × *g* for 15 min. Details of the experiment are given in the text.



subdivided into samples II and III. Sample II was immediately centrifuged. To III were added eight volumes of solution A, thus reducing the ionic strength of this suspension to the same value as sample I. Then sample III was stirred for 5 min prior to centrifugation. The supernatants and pellets of each sample were assayed for photoreactivating activity as before. In sample I (low ionic strength homogenization), 20 per cent of the activity and 3 per cent of the DNA were recovered in the supernatant, i.e., most of the activity was sedimented with the nuclei. In sample II (high ionic strength homogenization), 60 per cent of the activity and 3 per cent of the DNA were found in the supernatant, i.e., most of the activity was soluble. In sample III, where binding of the activity to nuclei should have occurred if it was going to occur at all, 70 per cent of the photoreactivating activity but no detectable DNA was recovered in the supernatant, i.e., the activity remained soluble. Therefore, it does not appear that the enzyme is in a soluble phase in the cells from which it becomes bound to nuclei only in media of low ionic strength. We conclude that most of the enzyme is normally concentrated in cell nuclei *in vivo*. Whether the approximately 20 per cent of the total activity which we invariably find in the soluble fraction is an artifact of isolation or is present in the soluble phase of intact cells we do not know.

The localization of photoreactivating activity primarily in nuclei appears to be true of *E. coli* as well as frog liver. In a mutant[19] of *E. coli* in which an eccentric cell division yields a small "minicell" containing no nuclear bodies and no DNA, the minicells contain no demonstrable photoreactivating activity.[20]

However, there is biological evidence from other tissues that photoreactivation, and very possibly the photoreactivating enzyme, functions in organelles other than the nucleus. Enucleated sea urchin eggs can photoreactivate UV-irradiated sperm with which they may be fertilized.[15] Photoreactivation of chloroplast development in *Euglena*[21] and of extrachromosomal mutations in yeast[22, 23] has also been described. In each of these cases DNA is found in the organelle where photoreactivation is found.

In summary, photoreactivating-enzyme activity is widely distributed among the metazoa (excepting mammals); the activity is not limited to any particular tissue, and is located in many tissues where a photoreactivating enzyme almost certainly cannot perform the only function of which we now know it to be capable, i.e., the light-dependent monomerization of UV-induced pyrimidine dimers. The widespread occurrence of the one or more enzymes responsible for this activity suggests that they might have an additional function, as yet unknown, one, perhaps, which may be an essential component of the nucleic acid metabolism of these animals.

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