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DNA Photoreactivating Enzyme from Placental Mammals. Origin and Characteristics†

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ABSTRACT: DNA photoreactivating enzyme in human leukocytes is concentrated in the phagocytotic monocytes and polymorphonuclear cells. Lymphocytes, erythrocytes, spleen, and serum contain little if any enzyme activity. Since bone marrow, which contains immature, nonphagocytotic monocytes and polymorphonuclear cells, also contains high levels of enzyme, it is unlikely that the enzyme in the mature cells resulted from bacteria engulfed by these cells. Photoreactivating enzyme is also found in murine, and in human cells in culture; confluent murine cells have higher specific activity than do rapidly growing cells. The leukocyte enzyme, which requires ultraviolet-irradiated DNA as substrate and visible light for catalysis, converts pyrimidine dimers in DNA to the corresponding monomers in the light-dependent reaction. The action spectrum for photoreactivation extends from 300 to 600 nm, with a peak at about 400 nm.

Ultraviolet light (220–300 nm) produces cyclobutylpyrimidine dimers[1] between adjacent pyrimidines on the same DNA strand. These dimers have been shown to be a major cause of death and mutation in prokaryotes and in the simple eukaryote *Paramecium* (Setlow and Setlow, 1973; Kimball, 1969; Sutherland *et al.*, 1967). The photoreactivating enzyme repairs dimer-containing DNA in a multistep reaction: the enzyme binds to the DNA, presumably at the dimer, forming a metastable complex (Rupert, 1962). On absorption of a photon in the range 300–600 nm, the enzyme catalyzes the photolysis of the cyclobutane ring, thus producing two monomer pyrimidines, and restoring biological integrity to the DNA (Setlow and Setlow, 1963).

Although the enzyme had been found in all groups of all phyla except the placental mammals (Cook and McGrath, 1967), a photoreactivating enzyme has recently been isolated from human leukocytes (Sutherland, 1974). This discovery is of particular interest as a potential analytical tool: the photoreactivating enzyme acts specifically and exclusively on pyrimidine dimers (Setlow and Setlow, 1963). Thus, if ultraviolet light (uv) induced biological damage can be reversed in a true photoenzymatic reaction, dimers were a major contributor to the production of the damage. In human cells a case of interest is the induction of skin cancer by ultraviolet light (Epstein, 1971).

We show here that the enzyme from leukocytes is of mammalian origin (rather than from bacteria engulfed by the cells), that the activity represents a genuine photoreactivating enzyme, and that the action spectrum for photoreac-

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[1] The name cyclobutadipyrimidine has also been suggested for the cyclobutylpyrimidine dimer (Madden *et al.*, 1973; Cohn *et al.*, 1974).

tivation by the enzyme extends from 300 to 600 nm, with a peak at around 400 nm. We have also found photoreactivating enzyme activity in cultured mouse and human fibroblasts.

Experimental Procedures

Preparation of Cells and Cell Extracts. (1) SPLEEN. Human spleen cells were obtained from Dr. G. A. Granger, Department of Molecular Biology and Biochemistry, University of California—Irvine. Spleens were teased into small pieces and suspended in 0.15 M NaCl and large clumps were removed. Cells were washed three times by suspension in 2 ml of 0.15 M NaCl, and centrifugation at 500*g* for 5 min. The cells were suspended in buffer E (0.01 M Tris (pH 7.0)–0.1 mM dithiothreitol–0.1 mM EDTA) and sonicated for 45 sec in a Kontes sonicator.

(2) HUMAN ERYTHROCYTE AND SERUM. Heparin-treated peripheral blood (10 cm^3) was centrifuged at 500*g* for 5 min. The supernatant serum and then the loosely packed leukocyte layer were removed by pasteur pipet. The erythrocyte pellet was suspended in 0.15 M NaCl, and centrifuged as before. The contaminating leukocytes were removed; the erythrocytes were suspended in 1.0 ml of buffer E and sonicated for 45 sec in the Kontes sonicator. The serum was centrifuged as before, to remove contaminating cells.

(3) MOUSE L-CELLS; HUMAN FIBROBLASTS. Rapidly growing and density-inhibited cultures of three mouse 3T3 lines (Swiss #1, Swiss #2, and Balb/c) were the kind gift of Dr. Dennis Cunningham, Department of Medical Microbiology, University of California—Irvine. Each dish was washed three times with 5 ml of cold 0.15 M NaCl, and the cells were scraped into 3 ml of 0.15 M NaCl. The cells were centrifuged at 500*g* for 10 min, suspended in buffer E, and sonicated for 45 sec in the Kontes sonicator. Human neonatal fibroblasts were obtained from Dr. Jeffrey Clark, Department of Biological Chemistry, California College of Medicine, University of California—Irvine. Human embryonic muscle and skin fibroblasts were purchased from Flow Laboratories. Normal human fibroblast lines CRL 1222 and CRL 1126 were obtained from the American Type Culture Collection. The human cell lines were harvested, washed, and sonicated by the procedure described for mouse fibroblasts.

(4) BOVINE BONE MARROW. Fresh bovine bone marrow was obtained with the kind cooperation of Mr. David Dukes, Gold Pak Packing Co. The marrow was pushed from the bone with a Teflon rod, teased into small pieces, suspended in 0.15 M NaCl, and centrifuged at 500*g* for 10 min. The supernatant solution and floating debris were removed, and the cellular pellet was washed by resuspension in 0.15 M NaCl and centrifugation as before. The cells were suspended in buffer E and sonicated for 45 sec in the Kontes sonicator.

Human Leukocytes. Freshly drawn human peripheral blood (35 cm^3) was treated with 500 units of heparin; then 12 cm^3 of Plasmagel (Bellon Laboratories) was added and the suspension was allowed to settle at 37° for 45 min. The supernatant was centrifuged at 500*g* for 5 min; 10^8 cells from the resulting pellet were suspended in 1 ml of a 17.5% Ludox solution (see below) and placed in the bottom of a cellulose nitrate tube for a Beckman Model L SW50 rotor. A stock Ludox solution was prepared from 35 ml of Du Pont Ludox HS, 10 ml of 0.2 N HCl, 50 ml of 0.17 M HCl, and 50% polyvinylpyrolidone. The pH was adjusted to 7.5 and the volume brought to 100 ml. Solutions of 17, 14.5, 13, 11.5, 10, and 8.5% Ludox were prepared from this stock. One cubic centimeter of each of 17, 14.5, 13, 11.5, and 10% solutions was layered gently on top of the cell suspension to form a discontinuous gradient; then the 8.5% solution was added to fill the tube. The mixture was centrifuged at 10,000*g* for 30 min in the SW50 rotor of the Beckman Model L-50. The five bands of cells were removed with a pasteur pipet, resuspended in 1640 medium, and centrifuged at 500*g* for 5 min. A sample of each band was removed for microscopic examination (Runge, 1973); the rest was washed three times by suspension in 0.15 M NaCl and centrifugation at 500*g* for 10 min. The cells were suspended in buffer E, and sonicated for 45 sec in the Kontes sonicator.

Assay for Photoreactivation. This assay has been described in detail elsewhere (Sutherland and Chamberlin, 1973). Briefly, cell extracts were added to assay mixtures containing 0.2 ml of 0.02 M phosphate buffer, (pH 7.2), 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.01 M $MgCl_2$ plus 10–30 pmol of ^{32}P-labeled T7 DNA. One sample was exposed to broad spectrum photoreactivating light from a 150-W spot lamp (30 min at a rate of 1000 W/m^2); a duplicate was kept in the dark. All mixtures were digested to a mixture of mononucleosides, inorganic phosphate, and dimer-containing oligonucleotides (Setlow *et al.*, 1964) by the sequential addition of 25 μg of DNase I, then 10 μl of 1 M Tris buffer, (pH 8), and 50 μl of a mixture containing 1 μg of alkaline phosphatase and 100 μg of snake venom phosphodiesterase in 0.05 M Tris (pH 8) and 0.01 M $MgCl_2$. After 1 hr the digestion was stopped by the addition of 10 μl of 0.062 N HCl and 1 ml of a solution containing 4% acid-washed Norit in 0.1 M sodium phosphate–0.1 M sodium pyrophosphate (pH 6). The mixture was filtered through a Whatman GF/C filter; the radioactivity retained on Norit is a direct measure of the dimer content of the DNA. Photoreactivation of dimers was calculated by subtracting the dimer content of the sample exposed to photoreactivating light from that of the duplicate sample kept in the dark; enzyme activity is expressed in picomoles per milligram per hour. Protein was determined by the Lowry method using a bovine serum albumin standard (Lowry *et al.*, 1951).

Dimer Determination in [^3H]Cytosine-Labeled DNA. Cell extracts or purified enzyme were added to 0.2 ml of 2 mM of phosphate buffer (pH 7.2), 0.1 mM EDTA, 0.1 mM dithiothreitol, and 1 mM $MgCl_2$ containing 200 pmol of [^3H]cytosine-labeled *E. coli* DNA. After exposure of the samples to photoreactivating light, they were chilled and the DNA was precipitated by the addition of ice-cold trichloroacetic acid to a final concentration of 10%. After 15 min, the samples were centrifuged for 15 min at 15,000 rpm in the SS34 rotor of the Sorvall RC2-B centrifuge. The supernatant was decanted, and the samples were hydrolyzed in 0.2 ml of formic acid at 175° for 30 min in a sealed tube. The formic acid was removed by evaporation, and the samples were spotted on thin-layer chromatograms and chromatographed in butanol–acetic acid–water (40:6:15) as previously described (Sutherland and Sutherland, 1969). The chromatograms were sliced, eluted with water, and counted in a dioxane-based scintillation fluid in a scintillation counter.

Action Spectra. A 1000-W high-pressure Hg arc served as a light source; the Hg lines were isolated using interference filters or a J4 monochromator (Model H-20). Expo-

TABLE I: Photoreactivating Enzyme Distribution in Human Leukocytes.

Cell Source	Protein (mg/ml)	Photoreact. Enzyme Act. (pmol/mg per hr)
Total leukocytes	3.22	7.95
Band A, lymphocytes	0.27	0.02
Band B, lymphocytes	0.111	3.70
Band C, lymphocytes	0.062	0.13
Band D, monocytes	0.066	21.3
Band E, polymorphonuclear cells	0.258	92.5

sures were adjusted to give equal photon fluxes by inserting neutral density filters before the interference filters and adjusting the current to the arc, by changing the monochromator slit width, or by adjusting the monochromator wavelength setting slightly. A 37-mm diameter quartz lens (75 mm focal length) formed a reduced image of the monochromator grating on the sample. Intensities were measured with a Hewlett-Packard thermopile.

Standard assay mixtures (Sutherland and Chamberlin, 1973) in Pyrex tubes were irradiated from above. The sample temperature was maintained at 37° by a stirred water bath.

Results

Distribution of the Enzyme in Human Blood. The level of photoreactivating enzyme can vary greatly in different cell types of an organism (Cook and McGrath, 1967). Since a photoreactivating enzyme has been found in human leukocytes (Sutherland, 1974), it is important to determine the distribution of enzyme in the different cells comprising the mixed leukocyte population tested by Sutherland. We thus separated the leukocytes by sedimentation through a preformed Ludox gradient into five cell fractions, A (top most) through E (bottom most). Fractions A, B, and C contained lymphocytes, fraction D monocytes, and E polymorphonuclear cells (PMN).[2] Fractions D and E contained relatively high levels of photoreactivating enzyme activity (see Table I), 21.3 and 92.5 pmol/mg per hr, respectively. In some experiments a low level of enzyme activity was also detected in one lymphocyte fraction. Since the volume of fractions in Table I was equal, the fractionated cells accounted for all the enzyme activity. We also tested human spleen, which contains large numbers of lymphocytes; very little photoreactivating enzyme activity could be detected. Thus the photoreactivating enzyme occurs mainly in the PMNs and monocytes, and perhaps at a low level in some lymphocyte populations.

We also examined other components of whole human blood: serum and erythrocytes (see Table II). Both contained enzyme activity at such low levels (0.23 and 0.12 pmol/mg per hr, respectively) that it is likely that a few contaminating monocytes or PMNs could be the source of the observed activity.

Photoreactivating Enzyme in Bone Marrow. Since the majority of the photoreactivating enzyme activity was found in the monocytes and PMNs, and since these cells

[2]Abbreviation used is: PMN, polymorphonuclear cell.

TABLE II: Photoreactivating Enzyme in Human Cells.

Cell Type	Protein (mg/ml)	Photoreact. Enzyme Act. (pmol/mg per hr)
Total leukocytes	3.22	6.78
Erythrocytes	2.72	0.12
Serum	0.51	0.23
Spleen	1.71	0.02

function in phagocytosis, it might be possible that the source of the observed enzyme activity was bacteria engulfed by these cells (E. Friedberg, personal communication). We thus tested cells from bovine bone marrow, a source of immature monocyte and PMNs which do not yet participate in phagocytosis. Table III shows the results of such an assay: cell extracts contained photoreactivating enzyme at a specific activity of 16.4 pmol/mg per hr. The enzyme from bone marrow was partially purified by the procedure developed for the human leukocyte enzyme (Sutherland, 1974). Thus, we conclude that the enzyme is present in nonphagocytosing mammalian cells and is not the result of bacterial engulfment by the monocytes and PMNs.

Enzyme Levels in Cultured Murine Cells. Cells in culture often cease production of enzymes present in the intact animal. Determination of the biological role of the enzyme in mammalian cells can best be carried out on cells in culture. We thus obtained cells from three murine 3T3 lines and determined the photoreactivating enzyme activity in each. Rapidly growing cells contained specific activities of 15.2, 56.0, and 32.8 pmol/mg per hr (see Table II). Thus, murine cells do contain photoreactivating enzyme at reasonably high levels. In bacterial cultures, stationary phase cells contain a higher level of photoreactivating enzyme than do cells in logarithmic growth. We thus tested confluent culture of the same cell lines for enzyme activity; in two lines (Swiss #1 and Balb/c) enzyme activity was considerably increased in stationary cultures. In Swiss #2, however, the activity in stationary phase was only slightly higher than in rapidly growing cells. Thus, determinations of enzyme activity in cultured cells should be performed on cells in comparable growth phase, and, for maximal activity, in cells which are no longer growing rapidly.

Photoreactivating Enzyme in Human Cells in Culture. The evaluation of the role of the photoreactivating enzyme in DNA repair is of special importance in human cells. We have tested four human fibroblasts and find that photoreactivating enzyme is present at a reasonably high specific activity (400-650 pmol/mg per hr). Since photoreactivating enzyme activity is present in the chick but not in the adult chicken, we examined the possibility that enzyme synthesis might cease in cells derived from older individuals. However, we found that cells from individuals aged 8, 33, and 73 years contain 112, 89, and 76% as much activity, respectively, as did those from neonatal and embryonic cells. Furthermore, over a time span of ten cell passages, we have observed no change in the enzyme level in these lines; thus, the enzyme seems to be produced at a stable rate in human cells in culture.

Dimer Monomerization by the Human Photoreactivating Enzyme. A true photoreactivating enzyme monomerizes cyclobutylpyrimidine dimers in DNA. The enzyme activity

TABLE III: Bovine and Murine Photoreactivating Enzyme.

Cell Source	Line	Growth State	Protein (mg/ml)	Photoreact. Enzyme Act. (pmol/mg per hr)
Bovine bone marrow			0.43	16.4
Mouse 3T3	Swiss #1	Nonconfluent	0.26	15.2
		Confluent	0.42	88.7
	Swiss #2	Nonconfluent	0.46	56.0
		Confluent	0.40	77.5
	Balb/c	Nonconfluent	1.36	32.8
		Confluent	0.49	91.0

from human cells has been shown to be a photoreactivating enzyme by the following criteria: (1) it requires uv-irradiated DNA as substrate; (2) light in the region 300–600 nm must be supplied to the enzyme–DNA complex; (3) the activity is trypsin-labile; (4) the enzyme causes the disappearance of [^{3}H]thymine-labeled pyrimidine dimers from DNA in a light-dependent reaction; and (5) in a photoreactivation assay using ^{32}P-labeled DNA, an indirect assay which measures total dimer content of the reaction mixture (Sutherland and Chamberlin, 1973), dimers disappear from DNA in the light but not in the dark.

However, a definitive demonstration of true photoreactivation is the disappearance of pyrimidine dimers and the concurrent stoichiometric appearance of monomer pyrimidines. This determination is quite difficult in [^{3}H]thymine-labeled DNA, as newly monomerized pyrimidines cochromatograph with the vast excess of monomer pyrimidines which were never in dimers. However, an elegant procedure developed by Setlow *et al.* (1964) circumvents this problem. Figure 1 shows a schematic representation of this procedure: uv-irradiated [^{3}H]cytosine-labeled DNA is heated at 60° for 60 min. [5-6-Saturated cytosines deaminate upon heating to give uracil; thus (CC) and (CT) dimers deaminate to (UU) and (UT) dimers, respectively, while less than 1% of the C monomers are deaminated.] The DNA is photoreactivated, acid precipitated, hydrolyzed to constituent monomers and dimers by acid hydrolysis, and chromatographed.

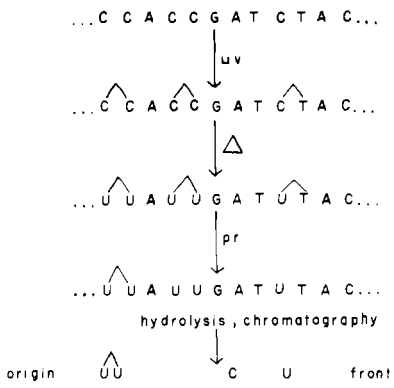

FIGURE 1: Scheme for direct detection of conversion of pyrimidine dimers to monomers by the photoreactivating enzyme. [^{3}H]Cytosine-labeled DNA (line 1) is irradiated with ultraviolet light to produce cytosine–cytosine (CC) and cytosine–thymine (CT) dimers (line 2). Upon heating, the cytosines in dimers are deaminated to uracils, although undimerized cytosines are largely unaffected (line 3). When the DNA is treated with photoreactivating enzyme and light in the wavelength range 300–600 nm, some of the dimers are monomerized (line 4). After hydrolysis and chromatography in butanol–acetic acid–water (40:6:15), dimers, cytosine (representing cytosines never in dimers), and uracil (representing the product of dimer monomerization) are easily separated (Setlow *et al.*, 1964).

Since uracil and cytosine have different chromatographic mobilities, monomer Us (the product of photoenzymatic monomerization of (UU) or (UT) dimers) may be easily detected. Figure 2 shows that in such an assay the human leukocyte enzyme indeed converts pyrimidine dimers to the corresponding monomers. Thus, the human enzyme meets all the criteria of being a true photoreactivating enzyme. Furthermore, over half the dimers in the DNA were photoreactivated by the enzyme.

Action Spectrum. Determination of the action spectrum of the human enzyme gives an indication of the wavelength range expected to be most effective in photoreactivation *in vivo*, and may also give information on the absorbing chromophore. We have determined the action spectrum of the

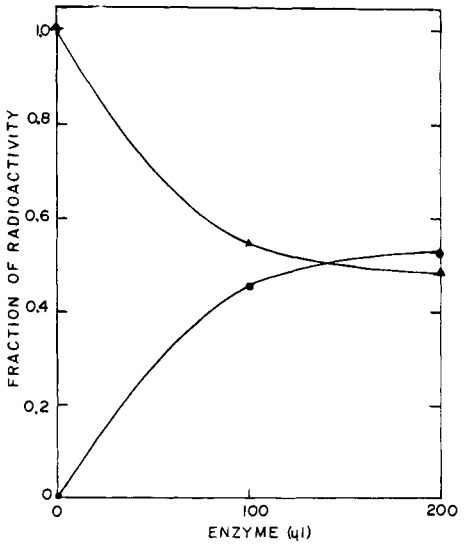

FIGURE 2: Concomitant disappearance of pyrimidine dimers (▲) and appearance of the corresponding monomers (●) produced by the human leukocyte photoreactivating enzyme. Fraction II enzyme (Sutherland, 1974) is added to uv-irradiated, [^{3}H]cytosine-labeled *E. coli* DNA, which had been heated to 60° for 60 min and exposed to photoreactivating light for 30 min. The DNA was precipitated with trichloroacetic acid, hydrolyzed with formic acid, and chromatographed in butanol–acetic acid–water (40:6:15). Chromatograms were sliced and counted in a liquid scintillation counter. The pyrimidine dimers uracil–uracil and uracil–thymine, derived from the deamination of cytosine–cytosine and cytosine–thymine dimers, respectively, are converted to monomer uracil and thymine. The detection of uracil indicates true dimer monomerization by the enzyme (see Figure 1).

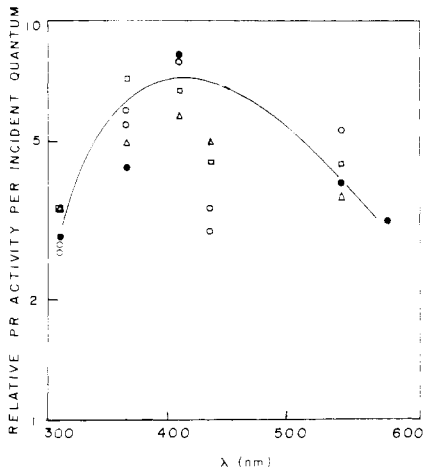

FIGURE 3: Action spectrum for photoreactivation by the enzyme from human leukocytes. The relative effect per incident quantum for photoreactivation by fraction II enzyme (Sutherland, 1964) was determined using light from a 1000-W Hg lamp passed through a J-4 monochromator. The incident intensity was determined by a Hewlett-Packard thermopile; the intensities were adjusted so that the time of irradiation at each wavelength was constant. Photoreactivation was assayed as ususal. Light intensities (in W/m^2) for the various mercury lines were 2.6 at 313 nm, 2.5 at 334 nm, 2.2 at 365 nm, 2.0 at 408 nm, 1.9 at 434 nm, 1.5 at 546 nm, and 1.4 at 577 nm.

enzyme from human leukocytes; the enzyme shows activity in the wavelength range 300–600 nm, with a peak at approximately 400 nm. This spectrum is red-shifted from those of the yeast and *Escherichia coli* enzymes, with maxima at about 366 nm (Jagger *et al.*, 1970; Sutherland *et al.*, 1973), and extends further into the visible region.

Discussion

Sutherland (1974) purified a photoreactivating enzyme from mixed populations of human leukocytes. Since we found that phagocytotic leukocytes contained the highest enzyme levels, we thought it imperative to seek photoreactivating enzyme in unequivocally mammalian sources, and to further characterize the mammalian enzyme. Photoreactivating enzyme is not present at the same specific activity in all leukocytes. The monocytes and polymorphonuclear cells contained the highest enzyme activity, 21.3 and 92.5 pmol/mg per hr, respectively, while the lymphocytes possessed very little (3.70 pmol/mg per hr or less). Both serum and erythrocytes had less than 0.2 pmol/mg per hr. Since the monocytes and PMNs are active in phagocytosis, it is possible that the enzyme present in the leukocyte was actually from bacteria engulfed by the cells. However, assays of fresh bone marrow showed that immature nonphagocytosing leukocytes also contained high levels of enzyme. Thus, we conclude that the enzyme is present in the cells of placental mammals and is not merely a contaminant.

Tests of the biological role of the enzyme would be most conveniently carried out on cells in culture. However, since not all genes expressed in the intact animal are expressed in cells growing in culture, it was important to determine whether cultured fibroblasts from placental mammals contained photoreactivating enzyme activity. We found that three lines of fibroblasts from the mouse contained photoreactivating enzyme at reasonably high specific activities (15–56 pmol/mg per hr). Further, since Setlow (1966) showed that the level of photoreactivating enzyme increases as bacteria go from logarithmic to stationary phase, we examined enzyme levels in both rapidly growing, nonconfluent, and in confluent cells. In two lines enzyme activity was increased as much as sixfold in the confluent cells; in the third confluent culture the enzyme activity was slightly higher in the confluent cells. Thus, for accurate comparison of enzyme levels, it is necessary to determine the growth phase of the cells and to examine cell cultures at different times to determine maximum activity.

Since the photoreactivating enzyme had previously been thought to be missing from placental mammals (for a review, see Cook, 1971), we felt it imperative to examine the activity from human cells to determine if it is a true photoreactivating enzyme. Previous work has shown that the human enzyme uses pyrimidine dimers in DNA as substrate, and, in the presence of photoreactivating light, causes their disappearance. A photoreactivating enzyme should meet these criteria and one more: it should convert pyrimidine dimers to monomer pyrimidines in the light-dependent reaction. Figure 3 shows that the enzyme does convert pyrimidine dimers to the constituent monomers and is thus by these criteria a photoreactivating enzyme.

Since the enzyme requires light for catalysis, the determination of the action spectrum for photoreactivation is a fundamental characterization of the enzyme. We find that the human leukocyte enzyme uses light in the wavelength range 313–577 nm, with a peak at 405 nm. This spectrum is red-shifted with regard to the yeast and *E. coli* enzymes, but is well within the range seen for other photoreactivating enzymes (see Jagger, 1967).

The demonstration that the enzyme converts pyrimidine dimers to constituent monomers in a light-dependent reaction confirms its designation as a true photoreactivating enzyme; its occurrence in bone marrow and in mouse fibroblasts indicates that it is of true mammalian origin. Our demonstration that the enzyme does occur in cells in culture and determination of the wavelength range most efficient for photoreactivation should provide the foundation for examination of the biological role of the enzyme in DNA repair in the cell.

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Slow Binding of D-Galactal, a "Reversible" Inhibitor of Bacterial β-Galactosidase†

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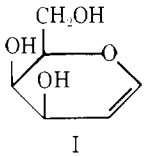
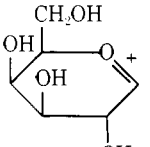
ABSTRACT: The inhibition of bacterial β-galactosidase by D-galactal was found to be reversible and time dependent. Rate constants observed for binding (2.7×10^2 sec^{-1} M^{-1}) and release (4.6×10^{-3} sec^{-1}) of galactal were found to be consistent with an apparent K_i of 1.4×10^{-5} M for D-galactal as a competitive inhibitor in imidazole-HCl buffer (0.1 M, pH 7.0) at 25°. Efforts to trap and analyze the galactal–enzyme complex were unsuccessful. The rate of formation of this complex was, however, found to be markedly reduced in the presence of deuterium oxide; substitution of deuterium oxide for solvent water also raised the apparent K_i for galactal by a factor of 2.1. The conventional competitive inhibitor isopropyl β-D-thiogalactoside, in contrast, showed no detectable lag in binding or release, and its K_i was not appreciably affected by the substitution of D_2O for solvent water. The rate of release of galactal can be largely accounted for by the rate of its enzymatic conversion to 2-deoxygalactose.

The effectiveness of D-galactal (I) as an inhibitor of β-galactosidase from *Escherichia coli* has been attributed to its resemblance to a half-chair intermediate (II) which may be formed during substrate transformation (Lee, 1969). A



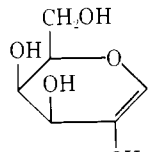
I



II

difficulty with this interpretation is that the position of unsaturation in galactal differs from that in the oxonium form of the carbonium ion, so that ring substituents are arranged differently with respect to coplanar atoms of the ring (Levvy and Snaith, 1972).

An alternative possibility would appear to be that substrate transformation may proceed by an elimination–addition mechanism, involving an unstable intermediate (III)



III

which resembles D-galactal in structure. To the extent that the enzyme stabilizes such an intermediate during catalysis, an analog of this intermediate might be expected to be tightly bound.

A third possibility is that a nucleophilic group on the enzyme may participate in a double displacement reaction, forming a covalent galactosyl–enzyme intermediate (Wallenfels and Malhotra, 1961; Viratelle *et al.*, 1969; Stokes and Wilson, 1972; Sinnott and Viratelle, 1973; Sinnott and Souchard, 1973). Galactal might form a covalent adduct resembling this intermediate.

The present studies were undertaken in order to provide further information about the mechanism of inhibition of β-galactosidase by D-galactal, and to attempt to distinguish between these alternatives if possible.

Materials and Methods

β-Galactosidase from *Escherichia coli* was purchased from Worthington as a crystalline suspension in 2.5 M ammonium sulfate. The enzyme was used after dialysis against a solution containing 0.1 M imidazole-HCl buffer (pH 7.0), 0.145 M NaCl, and 1 mM $MgSO_4$. D-Galactal, obtained from Raylo Chemicals Ltd., was recrystallized three times from ethyl acetate, mp 91–93° (lit. mp 104°, Overend *et al.* 1950). Isopropyl β-D-thiogalactoside (IPTG),[1] *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, 2-deoxy-D-galactose, and β-galactose dehydrogenase (from *Pseudomonas fluorescens*) were obtained from Sigma Chemical

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[1] Abbreviations used are: IPTG, isopropyl β-D-thiogalactoside; ONPG, *o*-nitrophenyl β-D-galactoside.