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Some Effects of Visible Light on *Escherichia coli*[1]

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Light above 400 nm had selective effects on *Escherichia coli* ML-308: several processes or enzymes were strongly inhibited, whereas others were relatively unaffected. There was a correlation between the inhibition of respiration and the inhibition of active uptake of glycine. However, phenylalanine uptake did not show such a correlation. The decrease in adenosine 5'-triphosphate level during the first few minutes of illumination resembled the inactivation kinetics of phenylalanine uptake. The results suggest that phenylalanine uptake may not depend greatly on oxidative energy and may depend on the adenosine 5'-triphosphate level. The results for glycine suggest either that its active uptake and respiration involve a common photosensitive component or alternately, that only the respiratory chain contains the photosensitive component, and that glycine uptake is coupled almost exclusively to respiration. The critical photochemical lesion does not involve D-lactate dehydrogenase, succinate dehydrogenase, or L-α-glycerophosphate dehydrogenase since their inactivation rate is markedly lower than that for respiration.

Visible light can be inhibitory or lethal to a wide variety of procaryotic or eucaryotic microorganisms. It can also have deleterious effects on several tissues of higher plants and animals (28). The primary physiological processes affected and the molecular mechanisms and photosensitizers involved have not, in general, been definitively established. It has been shown that cytochromes can be destroyed (9) or that respiration can be inhibited without affecting viability (23).

Recently it was observed that light above 400 nm had marked differential effects on active transport processes in *Escherichia coli* (4). For example, rates of inactivation of glycine transport differed from those for methyl β-D-thiogalactoside and phenylalanine. The photochemically induced lesions were considered to be useful in identifying molecules either directly involved in active transport or in associated energy coupling processes. These photochemical studies resulted from our earlier attempts to use an aromatic nitroazidé derivative of galactose as a photoaffinity label for proteins of the lactose permease. During these studies light intensities sufficient to cause photodecomposition of the label with half times of approximately 5 min were also found to inactivate the lactose permease in the absence of the label.

The present study had two aims: (i) delineation of the molecular mechanism of photodamage in *E. coli*, and (ii) utilization of the photoeffects to obtain information of molecular characteristics of active transport and energy-coupling processes.

MATERIALS AND METHODS

Culture conditions. *E. coli* ML 308 ($i^-y^+z^+a^+$) was grown at 37 C on medium 63 supplemented with 0.4% (wt/vol) glycerol as previously described (4). Cells were harvested by centrifugation, washed once, and then suspended in growth medium to a concentration of 0.8 to 0.9 mg/ml (dry weight). Cell suspensions were immediately used for illumination. Cell dry weight was measured turbidimetrically using a standard curve relating salt-free dry weight to absorbance at 600 nm.

Protein determination. Protein content was measured by the Lowry method (20) using bovine albumin (Miles Laboratories) as standard.

Illumination of cells. Cell suspensions (0.8 to 0.9 mg/ml [dry weight]) were illuminated using a 450-W mercury arc (Hanovia type 679A). A diagram of the apparatus used is shown in Fig. 1. The lamp was kept in a cylindrical pyrex vessel with a jacket through which a 1-mM solution of 1,4-di-(2-phenyloxazoyl)-*p*-bis-[2-(5-phenyloxazoyl)]benzene (POPOP) in 1:2 (vol/vol) toluene-*n*-propanol was circulated. This solution, which was passed through cooling coils at 24 to 26 C was used to filter out light below 400 nm. The

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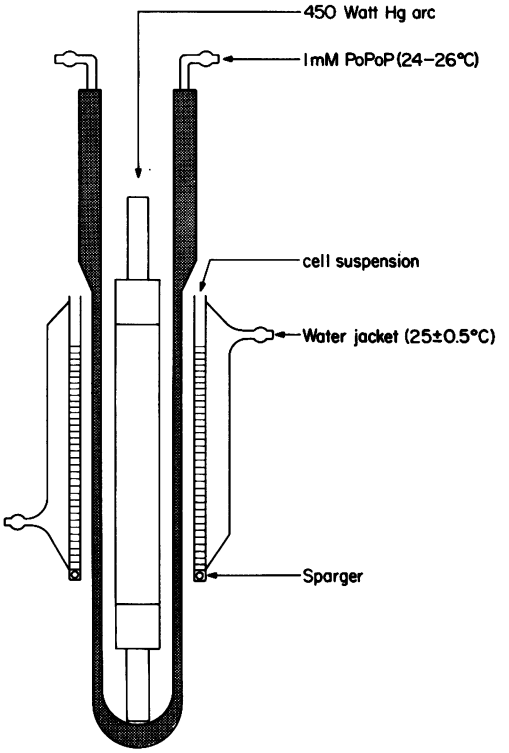

FIG. 1. *Schematic diagram of apparatus used to illuminate cells.*

thickness of the POPOP solution was 1 cm. The principle of operation of this filter is based on the fact that POPOP absorbs strongly in the region of the 360-nm line of mercury ($\epsilon \approx 4.3 \times 10^4$) and remits this light as fluorescence between 400 to 500 nm with a peak at about 420 nm. Light of wavelengths below 366 nm are absorbed by the glass, the toluene, and also the POPOP. The absence of appreciable intensity below 400 nm was demonstrated using a monochromator and photomultiplier. Taking the intensity at 435 nm as 100%, there was a peak at 404 nm 3% of intensity, whereas that for all mercury lines down to 239 nm was less than 10^{-5}%.

The cells were kept in an annular shaped pyrex vessel which fitted closely around the POPOP jacket (Fig. 1). The outer surface was jacketed and maintained at 25 ± 0.5 C. Temperature of the suspension during a run did not vary from the mean by more than 0.5 C. The suspension was stirred by passing air through a stainless steel sparger. The pyrex vessel was fitted to a height of 10 cm and at this height approximately 80 to 90% of the light coming through the POPOP filter impinged on the cell suspension. The path length of light through the annular vessel was 0.5 cm and its inner surface was 1.2 cm from the outer surface of the lamp. Incident light intensity was 200 mW/cm^2 (YSI Radiometer).

Volumes of 3 to 4 ml were removed at selected time intervals during the course of illumination and chloramphenicol was added to give a final concentration in each volume of 80 μg/ml. Transport assays were carried out immediately in growth medium (medium 63 plus glycerol). Initial rates of uptake were measured 30 s after the addition of radioactive substrate (4).

Respirometry. Oxygen consumption of cell suspensions (0.16 to 0.18 mg/ml [dry weight]) was measured in growth medium at 25 C using a Clark type oxygen electrode (Yellow Springs Instrument) and expressed as a percent of that in nonilluminated cells.

ATP measurement. ATP (adenosine 5'-triphosphate) extracts were prepared by the method of Forrest and Walker (10). A volume of cell suspension (2.7 mg [dry weight]) containing about 1.4 μg of ATP was centrifuged at 8,000 × *g* for 10 min and the resulting pellet was suspended in 0.5 ml of H_2SO_4 (0.3 M). The acidified suspension, allowed to stand for 40 to 60 min at room temperature, was then neutralized with 0.6 M NaOH. The suspension was centrifuged and the clear supernatant was retained as the ATP extract.

ATP content was determined by the method of Prydz and Frøholm (25) using the luciferase-luciferin system. The reaction mixture (1.0 ml) contained 0.8 ml of reconstituted extract of firefly tails (Worthington) in 0.05 M $KH_2A_sO_4$ and 0.02 M $MgSO_4$ (pH 7.4), and 0.2 ml of ATP extract. Full scale deflection of a Beckman DB spectrophotometer was set with 1.0 μg of ATP. Standard ATP solutions (0.1 to 1.0 μg) were assayed with daily samples.

Enzyme assays. Cell samples illuminated from 0 to 60 min were sedimented at 5,000 × *g* for 10 min, suspended in a 0.5 volume of 0.05 M PO_4 buffer (pH 7.6), and sonicated for 4 min at 0 C with a Branson Sonifier cell disruptor. Enzyme activities in illuminated samples were expressed as a percent of that in nonilluminated controls.

ATPase (EC 3.6.1.3) activity (26) was measured as the amount of ^{32}Pi released during the incubation with γ-^{32}P-ATP of sonically treated cells prepared in growth medium. The reaction mixture (1.2 ml) contained 1.5 mM $MgCl_2$, 3.0 mM ATP disodium salt (Sigma), and 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.5). The final specific activity of γ-^{32}P-ATP (New England Nuclear) was approximately 3.6 Ci/μmol. The reaction was initiated by adding 0.2 ml of sonicated cell preparation (125 μg of protein). After 15 min of incubation at 25 C, the reaction was terminated by the addition of 0.5 ml of 5% (vol/vol) $HClO_4$. ATP in the perchloric acid supernatant fluid was removed by absorption to Norit A charcoal as described by Crane and Lipmann (8). The radioactivity of the released ^{32}Pi, corrected for acid hydrolysis of ATP, was measured in a scintillation counter using Aquasol (New England Nuclear) as scintillation fluid.

β-Galactosidase (EC 3.2.1.23) activity was measured at 25 C by following the rate of hydrolysis of *o*-nitrophenyl-β-D-galactoside at 420 nm (16).

The method of Ne'eman et al. (22) was used to assay *p*-nitrophenylphosphatase activity in cell sonicates.

The assay of glucose-6-PO_4 dehydrogenase (EC 1.1.1.49) activity was similar to that described by

Scott and Cohen (27). The reaction mixture (3.0 ml) contained 2 mM D-glucose-6-PO_4 monosodium salt (Sigma), 15 mM $MgCl_2$, 50 mM Tris buffer (pH 8.0) and 0.2 ml of sonicated cell suspension, and was equilibrated for 10 to 15 min at 25 C. The reaction was initiated by the addition of 0.2 ml of 6 mM triphosphopyridine nucleotide monosodium salt (Sigma) in Tris buffer (pH 8.0). The initial 60-s reduction of triphosphophoryridine nucleotide was measured as the increase in absorbance at 340 nm.

Malate (EC 1.1.1.37) (7), succinate (7), D-lactate (EC 1.1.2.4) (3), and L-α-glycerophosphate (EC 1.1.99.5) (19) dehydrogenase activities were measured using the phenazine methosulfate coupled reduction of dichlorophenol indophenol as described by Arrigoni and Singer (1). The final concentration of phenazine methosulfate and dichlorophenol indophenol in the reaction mixtures was 0.001 M and 6×10^{-5} M, respectively. Dehydrogenases were first activated by incubating sonically treated cells with appropriate substrates at 25 C for 10 to 15 min in the presence of 2.3 mM KCN and 55 mM PO_4 buffer (pH 7.6); for malate dehydrogenase activation, 0.6 mM DPN (Worthington) was also present. The final substrate concentration of L-malate, disodium succinate, and lithium D-lactate was 20 mM; 30 mM disodium DL-α-glycerophosphate was used for the glycerophosphate dehydrogenase assay. Substrates were obtained from Sigma. The reactions, initiated by the addition of phenozine methosulfate and dichlorophenol indophenol, were measured by following the increase in percent transmittance at 600 nm. The initial 60-s activity of the dehydrogenases in unilluminated controls usually resulted in a 15 to 30% increase in transmittance.

Envelope fraction. After illumination as described above, cells were sedimented at 5,000 × *g* for 10 min, resuspended in 0.05 M potassium phosphate buffer at pH 7.0, and sonicated in an ice-water cooled tube for 10 min. Unbroken cells and debris were removed by centrifuging at 5,000 × *g* for 5 min. The resulting supernatant was centrifuged at 50,000 × *g* for 60 min and in some experiments at 100,000 × *g*, and the pelleted envelope fraction was washed twice in phosphate buffer.

Acrylamide gel electrophoresis. Electrophoresis and staining followed the methods of Weber and Osborn (30) except that the washed cells or envelope fraction were dissolved in 2% (wt/vol) sodium dodecyl sulfate, 0.02 M dithiothreitol, 8.0 M urea, and 0.01 M phosphate buffer at pH 7.0, and placed in boiling water for 2 min before layering on the gels.

Amino acid analysis. Washed cells or envelope fraction were hydrolyzed for 24 h in 6 N HCl at 110 C in evacuated sealed tubes. Analysis was carried out with a Beckman model 121 analyzer using the single column procedure (Beckman methodology brochure, A-TB-059A).

RESULTS

Light of wavelengths greater than 400 nm inhibits respiration, ATPase activity, and decreases the ATP level (Fig. 2). The inactivation rates differ markedly. After 10 min, respiration,

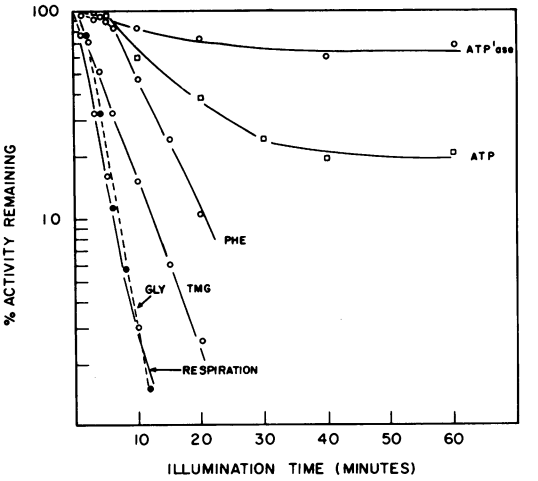

FIG. 2. *Inhibition by light of several processes in E. coli. GLY, PHE, and TMG refer to uptake of glycine, phenylalanine and methyl thio-β-D-galactoside.*

which shows one of the more rapid rates, decreases to 3% of the control value, whereas 83% of the ATPase activity and 65% of the ATP level remain. The inactivation rate for respiration correlates closely with that for glycine uptake (Fig. 2). During the first 15 min of illumination, the inactivation behavior of phenylalanine uptake and the decrease in ATP level (Fig. 2) show similarities in that small initial changes are followed by a rapid decline.

The three enzymes, D-lactate, L-α-glycerophosphate, and succinate dehydrogenase, were inactivated with similar kinetics (Fig. 3). These rates were slower than that for respiration and more rapid than that for phenylalanine uptake. The D-lactate and L-α-glycerophosphate dehydrogenases have been established to be flavoproteins (11, 18, 31), whereas the succinate dehydrogenase of *Eschrichia coli* is usually also considered to be a flavoprotein, by analogy with the corresponding enzyme isolated from other sources.

Although light had marked effects on a number of cellular functions, some enzymes were relatively unaffected. After 10 min of illumination, β-galactosidase, malate dehydrogenase, and glucose 6-phosphate dehydrogenase retained more than 80% of their activities, whereas 70% of the *p*-nitrophenylphosphatase activity remained (Fig. 3). Previous work showed that viability is also not affected appreciably during the first 10 min (4).

There was no correlation between an enzyme being membrane bound and its photosensitivity. The three flavoenzymes investigated (12, 31) and (G. W. Dietz, Abstr. Fed. Proc. **30**:1062,

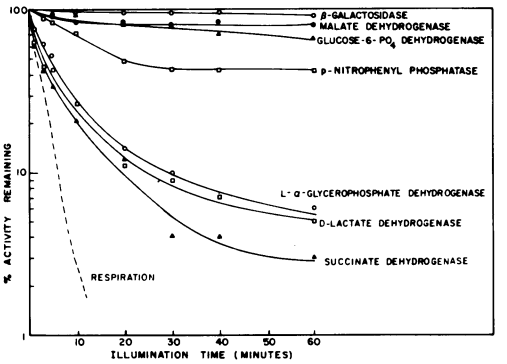

FIG. 3. *Light effects on several enzymes of E. coli. Respiration data taken from Fig. 2.*

1971), the ATPase (26), and malate dehydrogenase (6) are all membrane bound, but the latter two were only minimally affected by light. A correlation between location in the cytoplasm and photosensitivity cannot be established with the present data since only two cytoplasmic enzymes were investigated: β-galactosidase and glucose-6-phosphate dehydrogenase.

In an attempt to identify proteins altered by light, gel electrophoresis experiments and amino acid analyses were carried out on intact cells and cell-envelope fractions. The electrophoretic patterns of the 50,000 × *g* envelope fractions from control and illuminated cells showed no perceptible changes (Fig. 4). The molecular weight of the protein peaks 1, 2, 3, 4, and 5 were 98, 57, 46, 32, and 27 × 10^3, respectively, as estimated from the gel scan of standard proteins (scan a). Patterns of 50,000 × *g* envelope fractions illuminated for up to 80 min did not differ from those of unilluminated controls. The electrophoretic analyses did not reveal any differences between the patterns from illuminated and control 100,000 × *g* envelope fractions, nor in the corresponding patterns from intact cells.

The amino acid composition of the 50,000 × *g* envelope fraction from control and illuminated cells was very similar. Only methionine and cystine/2 appeared to increase on illumination (methionine from 0.3 to 0.7 and cystine/2 from 0.1 to 0.5 mol%). However, the significance of these changes is not clear because these amino acids were present in small amounts and are susceptible to the hydrolytic procedure. Envelope fractions prepared at 100,000 × *g* from control and cells illuminated for up to 80 min showed no marked changes in any of the amino acids. No changes in the proportions of amino acids of whole cells were observed after 0, 10, 20, and 40 min of illumination. Thus, if photodamage to cell or envelope proteins occurs, a large change in molecular weight does not take place, nor is there extensive alterations in the proportions of the analyzed amino acids.

DISCUSSION

The results clearly show that the effects of high intensity light can be selective; under conditions where several processes such as gly-

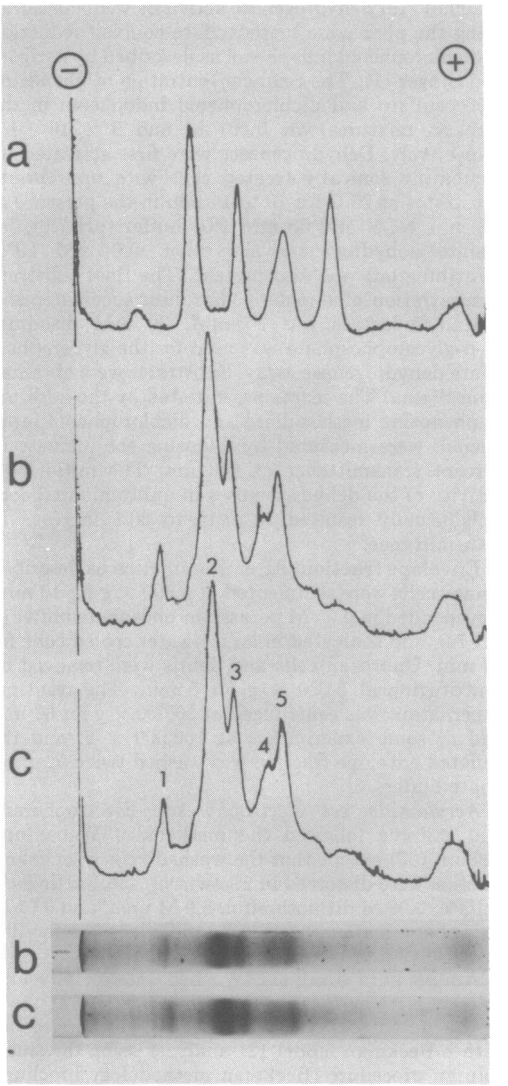

FIG. 4. *Polyacrylamide gel (7%) electrophoresis in sodium dodecyl sulfate and 8 M urea of envelope fractions prepared at 50,000 × g. Scan (a) is from calibrating standards (bovine serum albumin, ovalbumin, trypsin, hemoglobin); (b) envelope fraction from unilluminated cells; (c) envelope fraction from cells illuminated for 20 min.*

cine uptake and respiration are strongly inhibited, several membrane or cytoplasmic enzyme activities are relatively unaffected. Taken together with the absence of a marked decrease in viability during the first 10 min of illumination, the results suggest that the photochemical damage occurring during this interval is localized. The possibility that the damage is localized suggests that it could well be useful in probing cellular processes.

Systems inactivated at similar rates probably involve a common photochemical lesion. Thus, the similar rates shown by the three flavoenzymes investigated suggest that the damage involves the flavin moiety. Sensitivity of flavoenzymes to light of wavelengths greater than 400 nm has been found also by Codd (5).

Two explanations can account for the correlation between inhibition of respiration and uptake of glycine. Neither can be definitively established at present, but both are of intrinsic interest. One is that respiration and uptake of glycine involve a common photosensitive component. The other is that the photosensitive component is involved only in respiration, and uptake of glycine is closely coupled to respiration. In the event of close coupling between respiration and uptake of several substrates, the critical photosensitive component is not D-lactate dehydrogenase. This enzyme has been proposed to be a key factor in the coupling of oxidative energy to transport in *E. coli* vesicles (14). It cannot be the critical lesion because it is inactivated at a significantly slower rate than respiration (Fig. 2 and 3). For the same reason, succinate or L-α-glycerophosphate dehydrogenase, which have also been implicated in the coupling of oxidative energy to transport (3, 12, 31), cannot be the site of the critical lesion.

Energization of active transport in *E. coli* has been suggested to depend either on oxidative energy or ATP (2, 17). The present results, however, suggest that uptake of one substrate at least, phenylalanine, does not depend greatly on oxidative energy, and may exhibit a preference for energy derived from ATP. There is no correlation between rates of respiration and of phenylalanine uptake. Five minutes of illumination decreases phenylalanine uptake to only the 90% level, whereas respiration is decreased to the 15% level. On the other hand, light effects during the first 15 min on phenylalanine uptake and ATP level are similar (Fig. 2). It is of interest to note also that if the observed photoeffect on uptake of glycine reflects damage to respiration only, it would follow that energization of this uptake system is preferentially linked to respiration.

The identity of the critical lesions and photosensitizers would aid greatly in the interpretation of the effects observed. However, this identification is difficult at present. One reason is the occurrence of inactivations with differing rates, resulting probably from the simultaneous occurrence of a number of different reactions which proceed at different rates. Such behavior could result from involvement of several photosensitizers as well as different reactants. Possible candidates here, in addition to flavins, are quinones (13, 15, 21, 29) and cytochromes (9, 24). Identification of lesions and photosensitizers will probably require studies using monochromatic radiation. Preliminary experiments along these lines have been highly encouraging, greater selectivity being observed.

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