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Factors Influencing the Photodynamic Action of Benzo[*a*]pyrene on *Escherichia coli*

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ABSTRACT

Death of *Escherichia coli* resulted when a buffer suspension was exposed simultaneously to colloidal benzo[*a*]pyrene (BP) and 355-mμ illumination. Neither hydrocarbon nor illumination alone caused death; oxygen had to be present. The survival curve had a shoulder, and then death proceeded exponentially with time. Death rate was independent of temperature between 6 and 32 C. The duration of the shoulder, however, decreased slightly with increase in temperature. The shoulder was not due to delay in BP entering the cell. Death was influenced by the composition of the medium in which the cells were grown prior to illumination. The amount of BP bound to the cells was determined after three ethyl alcohol-ether extractions. Appreciable binding occurred in the presence of 355-mμ illumination with air, and relatively little binding occurred under nitrogen; very little binding occurred in the dark with nitrogen or air. At the outset, rate of binding under illumination with air was not temperature-dependent, but with time it became strongly temperature-dependent. Binding under illumination with nitrogen was temperature-independent. Bound BP was associated primarily with cell protein. Cells in growth medium resisted death and BP binding. At 21 and 32 C, deoxyribonucleic acid damage occurred during exponential death. No damage was detected at 21 and 32 C in the dark with BP, under illumination in absence of BP, or under illumination with BP in a nitrogen atmosphere.

In vivo photodynamic action with polycyclic, aromatic hydrocarbons such as benzo[*a*]pyrene (BP), 3,4-benzyprene, has been studied in protozoa, for example, with *Paramecium caudatum* by Doniach (5). However, the in vivo site(s) of action of these compounds has not been elucidated in any microorganism, nor have critical studies been carried out with bacteria. We wished to learn the disposition of BP within the cell. Detection of the intracellular substrates for photodynamic action brought about by BP should point to this compound's binding site(s). The work summarized here describes some of the factors which affected photodynamic death of *Escherichia coli*. The configuration of the survival curves is discussed, and factors that influence BP binding and deoxyribonucleic acid (DNA) damage are described.

MATERIALS AND METHODS

Bacteria. A transplant of *E. coli* B (Oak Ridge National Laboratory) was obtained from Howard Adler. *E. coli* m-4 was acquired from Roy Curtiss III. Spontaneous mutants of this strain with deficiences for leucine and uracil were selected by a modification of Davis' (4) penicillin method and with the recycling technique described by Lubin (16) followed by replicate plating (15). A thymine requirement was introduced by the aminopterin method of Okada, Homma, and Sonohara (21) with subsequent selection for a low thymine requirer (9). This triple auotroph will be designated strain Ma.

Media and reagents. Buffer contained 0.6% $Na_2HPO_4 \cdot 7H_2O$ and 0.15% $NaH_2PO_4 \cdot H_2O$ (*p*H 7.1). In addition to buffer, minimal (M) medium contained 0.1% NH_4Cl, 0.05% KCl, 0.03% Na_2SO_4, 0.02% $MgCl_2 \cdot 6H_2O$, 0.3% glucose, 66 μg/ml of L-leucine, 34 μg/ml of uracil, and 10 μg/ml of thymidine. For plating, M medium was solidified with 1.6% agar. Buffer was employed as diluent. Minimal-yeast extract (MYE) medium was of the same composition as M medium, except that it contained 0.07% yeast extract (BBL), it lacked the L-leucine (sufficient leucine was present in the yeast extract), and it had the uracil content reduced to 9 μg/ml (the uracil requirement was only partly met with the yeast extract). This medium had two advantages over M medium: it permitted faster growth, and the cells from it had a greater susceptibility to photodynamic action. Yeast extract (YE) agar contained 0.2% K_2HPO_4, 0.2% yeast ex-

tract, 0.5% Trypticase (BBL), 0.5% glucose, and 50 μg/ml of thymidine; it was solidified with 1.6% agar. This medium was used for plating *E. coli* Ma. Diluent lacked thymidine and agar. Nutrient broth (NB) medium contained 0.8% Nutrient Broth (Difco) and 0.5% NaCl. This medium, solidified with agar, was used for plating *E. coli* B. Diluent was NB medium.

A stock solution of BP in *N,N*-dimethyl formamide was prepared at a concentration of 100 μg/ml. The hydrocarbon was purchased from Calbiochem, Los Angeles, Calif., and reagent-grade solvent was from Fisher Scientific Co., Pittsburgh, Pa. A colloidal suspension of BP was prepared in buffer at 21 C a few minutes prior to use by blowing from a pipette sufficient stock BP solution to give 1 μg/ml of BP. Tritiated BP (in benzene solution) was purchased from Nuclear-Chicago Corp., Des Plaines, Ill. The labeled compound was placed in a small test tube, the benzene was evaporated, the H^3-BP was dissolved in stock unlabeled BP solution, and colloidal BP was prepared in buffer as described. All manipulations with BP were carried out in darkness or subdued lighting. Purity of BP and suitability of a tritium label for uptake studies have been verified (20). Tritium-labeled (methyl-H^3) thymidine and L-leucine-C^{14} were purchased from Nuclear-Chicago Corp.

Scintillation mixtures for radioassays were the following: 175 g of naphthalene, 7 g of 2,5-diphenyloxazole, and 0.375 g of 1,4-bis-2-(4-methyl-5-phenoxazolyl)-benzene in 1 liter of 1,4-dioxane; 4 g of 2,5-diphenyloxazole and 0.1 of 1,4-bis-2-(4-methyl-5-phenoxazolyl)-benzene in 1 liter of toluene.

Cultivating and harvesting bacteria. Cells from a refrigerated stock slant were inoculated into 5 ml of MYE medium and were incubated overnight without forced aeration. In the morning, the culture was diluted 50-fold with MYE and incubated at 38 C with aeration. Growth was monitored nephelometrically with a Coleman model 9 Nephocolorimeter. The cells were harvested by centrifugation (4,000 × *g*, 5 min) after three or four mass doublings, at 5×10^8 to 1×10^9 cells per milliliter, always during exponential growth. They were washed three times with warm buffer in the centrifuge and were resuspended in the colloidal BP suspension.

Illumination. Two 15-w General Electric Blacklight fluorescent tubes (no. F15T8-BLB) were employed in a desk-type stand. Of their emission, 90% was between 330 and 380 mμ, with peak emission at 355 mμ. (BP absorbs strongly in this range.) New bulbs in our reflector delivered 60 ergs per mm^2 per sec at a distance of 280 mm and contributed little heat to the suspension. Incident illumination was monitored during the experiments with a dose-rate meter described by Jagger (11). Small petri dishes (50 mm in diameter, 15 mm deep) contained 5- to 10-ml suspensions. Flasks were used to contain larger volumes. To ensure homogeneity and ample oxygen for full photodynamic action, the bacterial suspensions in the flasks were bubbled with air during illumination. When anaerobic conditions were desired, bubbling was with oxygen-free nitrogen (nitrogen passed through 300 g of pyrogallic acid dissolved in 1 liter of 10 N KOH) and was begun 30 min before illumination. Dishes were illuminated from above with the Pyrex covers in place. Flasks were illuminated from below. Unless stated to the contrary, the experiments were carried out at room temperature (21 C). Temperatures above ambient were maintained in a constant-temperature chamber, and a temperature of 6 C was maintained by means of an ice bath.

Assays for cell death, for BP binding, and for DNA damage. Cell death will be defined as loss of colony-forming ability. Death was determined by agar plate count. A suitably diluted 0.1-ml sample was spread over the agar surface. The plates were incubated at 38 C for 18 to 24 hr with YE and NB agars and for 2 days with M agar.

Tritium label (supplied as H^3-BP) which remained in the cells after three extractions with 75% ethyl alcohol-diethyl ether (1:1, v/v) will be defined as "bound." (Negligible label could be removed from the cells by additional ethyl alcohol-ether extractions.) Bound BP was determined by placing a sample of suspension, usually 1 ml, containing 5×10^8 cells in a 1-ml glass centrifuge tube and centrifuging it at 5,000 × *g* for 3 min in a Misco micro-centrifuge (Microchemical Specialities Co., Berkeley, Calif.). The supernatant fluid was removed with a Pasteur pipette; the pellet was suspended in 0.6 ml of ethyl alcohol-ether (35 C) and was centrifuged for 2 min, and the supernatant fluid was likewise discarded. Two additional extractions were carried out. The pellet was then washed once with 0.6 ml of buffer, and it was finally suspended in 0.33 ml of buffer with the aid of a Pasteur pipette. Duplicate 0.1-ml samples were placed in 5 ml of dioxane scintillation mixture in glass vials. Counts were made to a statistical accuracy of 5% with a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). In all tables and figures herein, radioactivity has been corrected for background and dilution, and it is expressed usually as counts per minute in the cells from 1-ml test suspensions.

Alkali-labile bonds in single-stranded DNA were detected by the technique of McGrath and Williams (Nature, *in press*). A 0.1-ml amount of illuminated or control suspension (containing 2×10^7 to 5×10^7 *E. coli* Ma cells labeled with tritiated thymidine) was placed in a test tube at 38 C, and 0.03 ml of 30% sucrose [in 0.6 M tris(hydroxymethyl)aminomethane buffer, *p*H 8.1] and 0.02 ml of lysozyme (20 mg/ml) were added. After 5 min, the tube was transferred to an ice bath and 0.04 ml of 1% ethylenediaminetetraacetic acid was added. (Protoplasts formed immediately.) An 0.025-ml sample was lysed in a 0.1-ml (0.5 N) NaOH overlay on a sucrose gradient (5 to 20% sucrose, *p*H 12) at 5 C in a cellulose nitrate tube (0.5 × 2 inches). Centrifugation was at 5 C for 2 hr at 30,000 rev/min in a model L-1 Beckman ultracentrifuge. The tube was punctured with a no. 27 needle, and 2-drop samples (approximately 0.2 ml) were collected on a series of Whatman filter paper discs (2.3 cm × 3 mm). These were dried and then placed in a large beaker; they were washed 10 min once with cold 10% trichloroacetic acid, twice with cold 95% ethyl alcohol, and finally once with acetone. The dried discs were placed in glass vials containing 5-ml toluene scintilla-

tion mixtures and were assayed for tritium as described.

DNA was extracted by the method of Marmur (17) after cell lysis with Duponol. After each spooling, the DNA on the glass rod was passed through three changes of ethyl alcohol-ether to remove unbound BP. In some experiments, the degree of deproteinization was monitored by means of radioassay of protein labeled (during cell growth) with L-leucine-C^{14}. Protein was also assayed by the Folin-Ciocalteau method described by Layne (14). DNA was assayed by the Burton (3) modification of the Dische reaction.

RESULTS

Appreciable death occurred only when the cells (in buffer) were exposed simultaneously to the three components: BP, 355-mμ illumination, and air (Fig. 1). The survival curve has a shoulder, but then death proceeds exponentially.

The duration of the shoulder is influenced by the composition of the medium in which the cells were grown prior to illumination. It is shorter in Fig. 1, where the cells had been grown in liquid YE medium (0.2% yeast extract), than in the other figures where the cells were from MYE medium (0.07% yeast extract). In M medium (no yeast extract), the shoulder is extended. An amino acid supplement to M medium increases the growth rate as much as added yeast extract, but it does not alter the shoulder. Thus, duration of the shoulder is not a function of the growth rate during cultivation. Cells from the shoulder show lengthening of lag upon subsequent cultivation (Fig. 2).

Death rate during the exponential death phase was proportional to the dose rate of 355 mμ. Moreover, if log survivors were plotted against total dose, there was consistency with the Bunsen-Roscoe reciprocity law at dose rates from 25 ergs per mm^2 per sec to 70 ergs per mm^2 per sec. Death rate was independent of initial cell concentration between 5×10^6 and 5×10^9 cells per milliliter. Above 10^9 cells per milliliter, death was proportionally less than at lower cell concentrations. This was not due to a limiting concentration of BP or oxygen, because raising the BP concentration to 20 μg/ml or forcibly aerating the suspensions did not alter survival. (Maximal death rate at all cell concentrations was attained at 1 μg/ml of BP.) The improved survival above 10^9 cells per milliliter may have been due to cellular synthesis

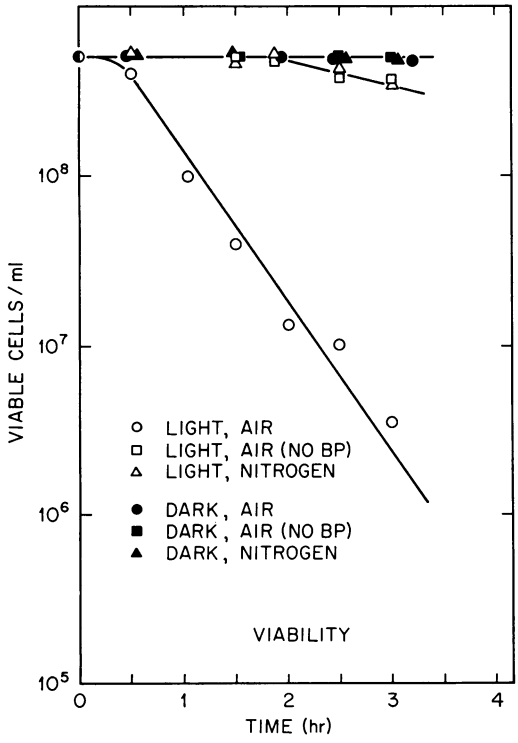

FIG. 1. *Viability of Escherichia coli Ma under various circumstances. Buffer suspensions containing 1 μg/ml (2 μc/ml) of benzo[a]pyrene and 5 $\times$ 10^8 cells per milliliter were bubbled at 32 C and received incident illumination at 50 ergs per mm^2 per sec.*

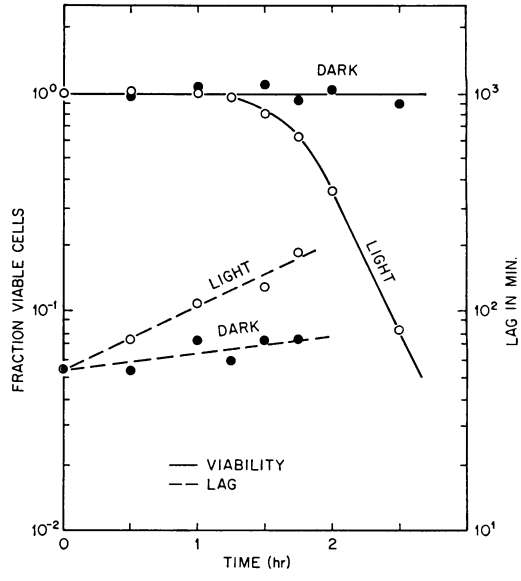

FIG. 2. *Evidence for cell damage in absence of cell death during 355-mμ illumination. Escherichia coli B, suspended to give 10^7 cells per milliliter in buffer containing 1 μg/ml of benzo[a]pyrene, was exposed to 70 ergs per mm^2 per sec. Periodically, 3-ml amounts were transferred to 3-ml amounts of double-strength NB medium in the dark; the growth was monitored nephelometrically and by means of plate counts. Lag times were estimated from the resulting growth curves.*

arising from "cross feeding." [Harrison and Lawrence (10) demonstrated that *Aerobacter aerogenes* at high concentration in buffer may elute sufficient cell material to serve as nutriment.] Indeed, cells suspended in M medium in lieu of buffer did not die, but they grew at a diminished rate and resisted BP binding. M medium was not protective per se, because at 6 C the cells died in it when illuminated at 355 mμ.

Cell lysis did not accompany cell death; there was little change in turbidity, and tritium label in DNA (incorporated during growth as H^3-thymidine) was not released into the buffer during or after exposure.

Binding of BP to the cells occurred in the presence of illumination, but it was relatively slight under nitrogen (Fig. 3). Very little binding occurred in the dark, and it increased only slightly after the first few minutes. The dark-bound radioactivity is 0.008% of the radioactivity available in the buffer suspension; this is equalivalent to 1.6×10^{-11} μg per cell or 3.8×10^4 molecules per cell. The light-bound radioactivity (in air) increased with time (Fig. 3), approximately doubling every 50 min.

The survival curve (Fig. 4) and the binding curve (Fig. 5) are little affected by the duration of the initial dark period. Moreover, when a suspension receiving low-intensity illumination was transferred to the dark, death ceased abruptly (Fig. 4), as did BP binding (Fig. 5). When illumination was renewed after an interposed dark period, death began without delay (curves Ia and Ib, Fig. 4). The loss of the shoulder in curve Ib was due to the previous illumination, not solely to cell aging (compare curves Ib and II). Above 80 ergs per mm² per sec, dying, on occasion, continued briefly after the suspension was transferred to the dark, but BP binding ceased immediately. Illuminating colloidal BP in the usual buffer, but without cells, at 200 ergs per mm² per sec for 6 hr at 35 C caused the characteristic yellow-green fluorescence of the colloid to diminish in intensity. Cells added to this BP in the dark were killed. However, "dark-death" has never been observed in the usual experiments at dose rates of 80 ergs per mm² per sec and below during the experimental period we have employed, up to 4 hr of illumination.

Little effect of temperature would be expected

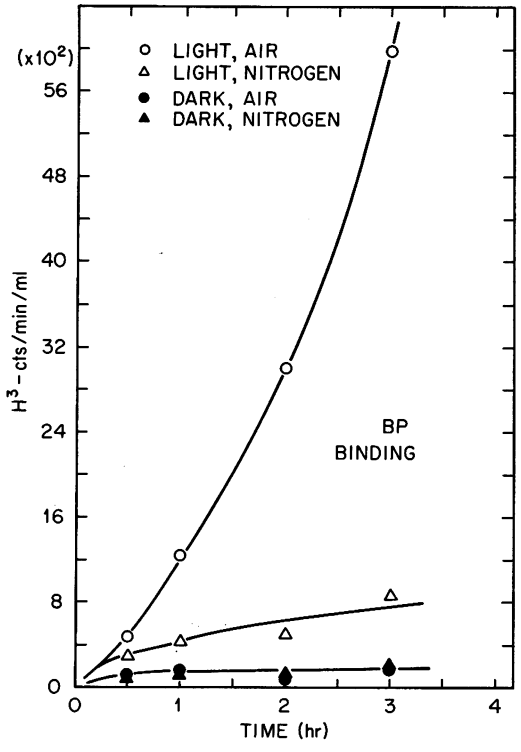

FIG. 3. *Benzo[a]pyrene (BP) binding by Escherichia coli Ma under various circumstances. Buffer suspensions containing 1 μg/ml (2 μc/ml) of BP and 5 × 10⁸ cells per milliliter were bubbled at 32 C and received incident illumination at 50 ergs per mm² per sec.*

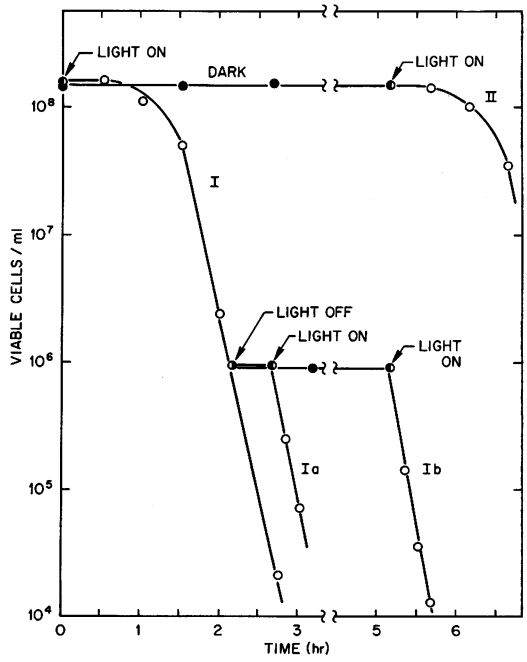

FIG. 4. *Viability as affected by an interposed dark period. Escherichia coli Ma in buffer containing 1 μg/ml of benzo[a]pyrene received illumination at 60 ergs per mm² per sec.*

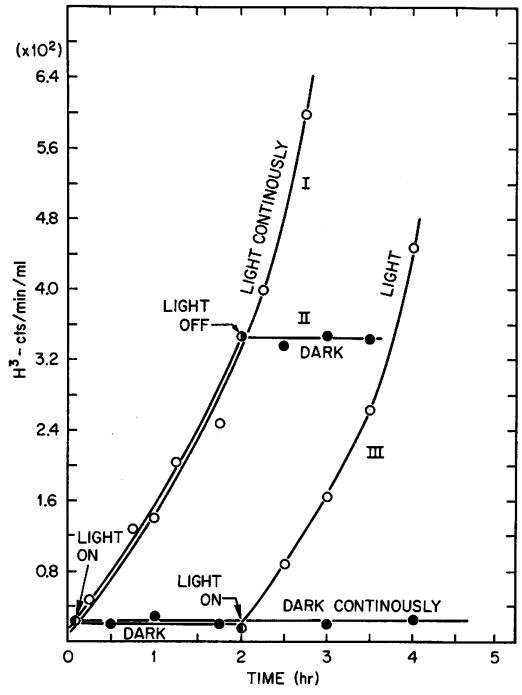

FIG. 5. *Benzo[a]pyrene (BP) binding as affected by duration of darkness prior to illumination. Escherichia coli Ma in buffer containing 1 μg/ml (4 μc/ml) of BP received illumination at 80 ergs per mm² per sec.*

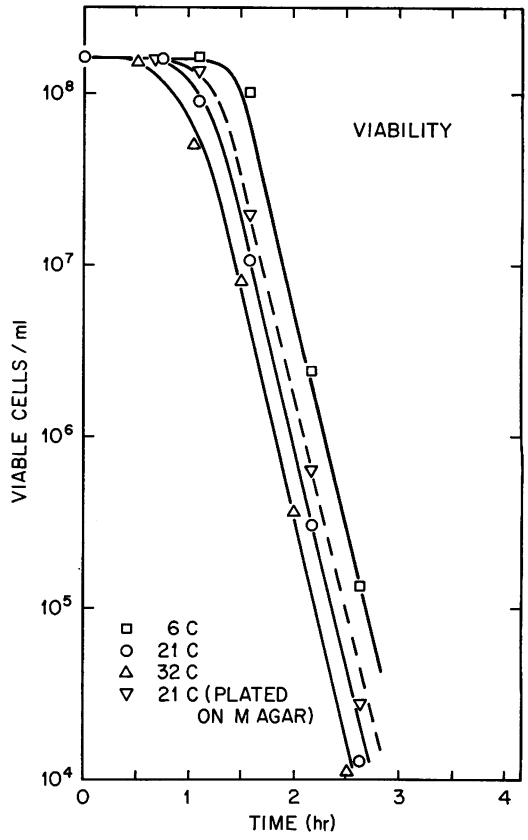

FIG. 6. *Effect of temperature on viability of Escherichia coli Ma. Buffer suspensions exposed to the air and containing 1 μg/ml (4 μc/ml) of benzo[a]pyrene were held at the temperatures indicated and received incident illumination at 70 ergs per mm² per sec.*

in a pure photochemical process. Indeed, during exponential death there was no difference in the death rate between 6 and 32 C; however, the shoulder decreased slightly with increase in temperature (Fig. 6). During the first 45 min, the rate of BP binding (in presence of air) was influenced relatively little by temperature, but, with time, rate of binding became strongly temperature-dependent (Fig. 7). Binding of BP under nitrogen, however, was not influenced by temperature between 6 and 32 C over a 3-hr period, and it followed closely the curve of "air-binding" at 6 C. Although survival during exponential death at any given moment was twice as great on M agar as on the usual plating medium, the death rate was the same (Fig. 6).

At 21 and 32 C, DNA damage occurred during exponential death. Extracted DNA could no longer be "spooled" [Marmur's method (17)]. Concomitantly, alkali-labile bonds in DNA appeared (Fig. 8). No damage was detected at 32 C in the dark with BP, under illumination in absence of BP, or under illumination with BP in a nitrogen atmosphere. Moreover, after illumination in air at 6 C for 2.5 hr, a condition which caused the cells to die (Fig. 6), the DNA could have nevertheless been spooled, and no alkali-labile bonds were detectable. However, if the cells after 6 C illumination were transferred to darkness at 32 C, alkali-labile bonds then appeared (Fig. 9).

Figure 10 demonstrates that tritium label (H^3-BP) was lost in proportion to C^{14} label (C^{14}-leucine) during deproteinization of DNA. A small amount of BP bound to DNA could not be detected in this experiment. Table 1 summarizes an experiment where, after partial purification, the DNA was heated at 100 C for 30 min with 5% trichloroacetic acid. This treatment, which separated the DNA presumably from all the protein, reduced the ratio of illuminated-DNA counts per min to dark-DNA counts per min to almost unity (1.4), whereas the ratio of illuminated-protein counts per min to dark-protein counts per min remained high (9.5). The illuminated DNA had more associated protein (last line in Table 1).

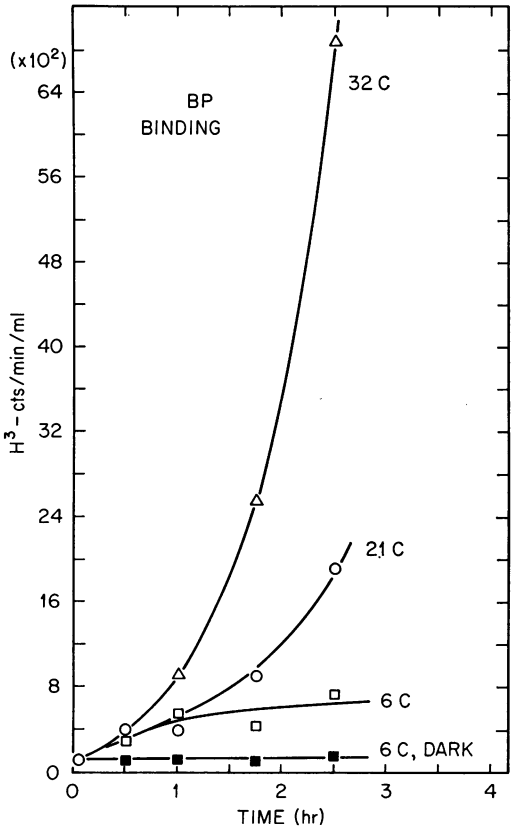

FIG. 7. *Effect of temperature on benzo[a]pyrene (BP) binding by Escherichia coli Ma. Buffer suspensions, exposed to the air and containing 1 μg/ml (4 μc/ml) of BP, were held at the temperatures indicated and received incident illumination at 70 ergs per mm² per sec.*

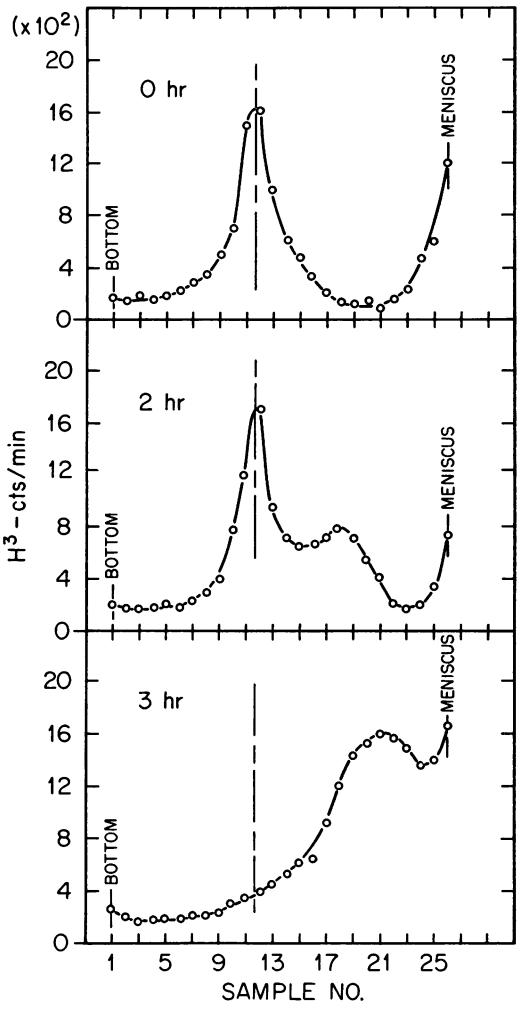

FIG. 8. *Radioactivity of tritium-labeled material in Escherichia coli Ma lysed and centrifuged on alkaline sucrose gradients after 32 C illumination for various periods of time. Cells labeled with tritiated thymidine during growth were washed and were suspended in buffer to give 4 × 10⁸ cells per milliliter in 1 μg/ml of benzo[a]-pyrene. Illumination was under air at 60 ergs per mm² per sec.*

When the radioactivity was recalculated in terms of unit mass protein, the ratio between illuminated- and dark-protein counts per min became 5. This is consistent with the same ratio obtained for whole cells (Fig. 7, curves for 6 C).

DISCUSSION

Although photodynamic killing of *Serratia marcescens* with erythrosine as photosensitizer appears to be a "single-hit" process (12), survival curves obtained with a variety of other bacteria show a shoulder prior to exponential kill. These bacteria include a mutant of *Rhodopseudomonas spheroides* with bacteriochlorophyl as endogenous photosensitizer (6), a colorless mutant of *Corynebacterium pointsettiae* photosensitized with toluidine blue (13), and colorless *Sarcina lutea* with endogenous and also toluidine blue photosensitizer (18, 19). The yeast, *Saccharomyces cerevisiae*, haploid as well as diploid and photosensitized with acridine orange, showed "multi-hit" survival curves (D. M. Freifelder, Ph.D. Thesis, University of Chicago, Chicago, Ill., 1959).

Paramecium caudatum, photosensitized with BP, showed a delay before harmful effects could be detected. This delay was inversely proportional to the duration of prior holding with BP in the dark (5), and it was therefore concluded (8) that death delay was probably a consequence of the time required for penetration of the protozoan cell by the hydrocarbon. However, other factors

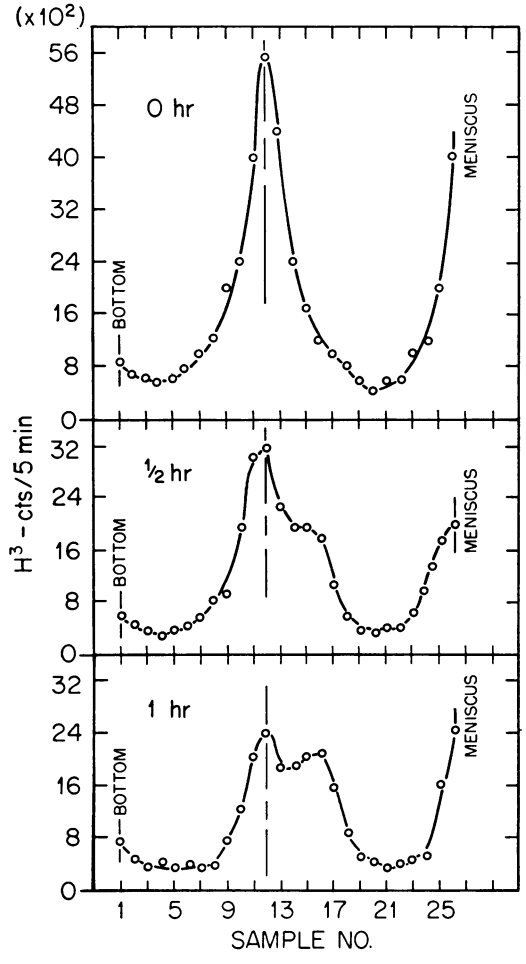

FIG. 9. *Radioactivity of tritium-labeled material in Escherichia coli Ma lysed and centrifuged on alkaline sucrose gradients after 6 C illumination for 2.5 hr with subsequent dark-holding at 32 C. The tritiated cells were prepared and were illuminated as in Fig. 8, but at 6 C. One gradient was prepared immediately after illumination (0-hr curve), and 0.6 ml of the illuminated suspension was added to 0.3 ml of triple-strength MYE medium at 32 C and held in the dark. Gradients were prepared from the dark suspension after 30 min and 1 hr (bottom two curves).*

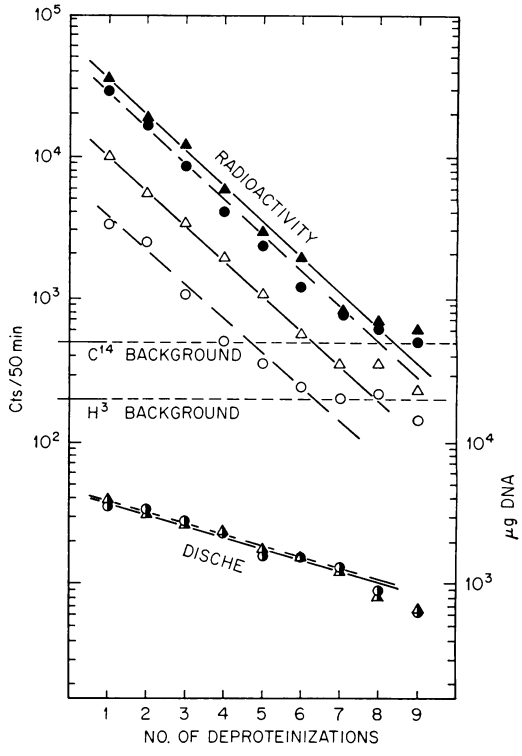

FIG. 10. *Loss of radioactivity from the DNA fraction during deproteinization. A quantity of 10^{12} log Escherichia coli Ma cells labeled with C^{14}-leucine during growth were washed and were suspended in 200 ml of duffer containing 1 μg/ml (3.0 μc/ml) of H^3-benzo[a]-pyrene (BP) and were illuminated at 80 ergs per mm² per sec for 2.5 hr under nitrogen at 32 C. An identical suspension was held in the dark. The cells were harvested, were extracted three times with ethyl alcohol-ether, and then were lysed, and the DNA was extracted. (RNase treatment was carried out between the third and fourth deproteinizations. Isopropanol precipitation was carried out between the fourth and fifth deproteinizations.) Solid symbols = C^{14} (protein); open symbols = H^3 (BP); solid curves = illuminated; broken curves = dark.*

must be involved in death delay in other systems; for example, delayed entry of photosensitizer would not account for the shoulder on survival curves with an endogenous photosensitizer such as bacteriochlorophyl. Moreover, in the present work with *E. coli*, holding the organism with BP in the dark 5 hr prior to illumination did not alter the survival curve (Fig. 4), and the increase in binding of BP began at or near time zero (Fig. 3, 5, 7). Furthermore, a harmful result other than death, lengthening of lag (Fig. 2), also occurred on the shoulder of the survival curve apparently from time zero. The shoulder is not due to cell clumping, which we have never observed with our strains. The shoulder simply indicates that the cells were able to sustain a certain amount of damage before they succumbed.

In our experiments, it is unlikely that the cells in buffer underwent active repair. The effect of temperature on the duration of the shoulder was but slight, and a rise in temperature lessened rather than extended survival (Fig. 7). Indeed, even the presence of glucose extended the shoulder but

TABLE 1. *Distribution of radioactivity in DNA and protein*[a]

DNA fraction after	Total radioactivity[b] (H_3 counts per min)		Illuminated[c]/dark
	Dark	Illuminated	
Two deproteinizations	2.9×10^3	4.7×10^4	16
RNase treatment, a third deproteinization, and isopropanol precipitation	9.0×10^2	1.6×10^4	18
Hydrolysis of the isopropanol-precipitated DNA with hot 5% trichloroacetic acid			
Supernatant fluid	4.8×10^2 (2.8 mg of DNA)	6.5×10^2 (2.8 mg of DNA)	1.4
Pellet (dissolved in 1 N NaOH)	1.9×10^2 (0.14 mg of protein)	1.8×10^3 (0.26 mg of protein)	9.5

[a] Log cells of *Escherichia coli* Ma (8×10^{11}) were suspended in 200 ml of buffer containing 1 μg/ml (10 μc/ml) of BP and were illuminated in air at 80 ergs per mm^2 per sec for 2.5 hr at 6 C. An identical suspension was held in the dark. The cells were harvested, were extracted three times with ethyl alcohol-ether, and then were lysed, and the DNA was extracted. Samples were dried on filter paper discs, and these were assayed in toluene scintillation mixture.

[b] The sum of the counts in the last two lines does not equal that in the preceding line. This probably arises from two causes: absorption of protein radioactivity by the NaOH on the filter discs; adsorption of DNA radioactivity by the papers fibers, since trichloroacetic acid-digested DNA will be embedded more deeply in the paper discs than the long-strand native DNA.

[c] Ratio between counts per minute for illuminated DNA and DNA kept in the dark.

slightly. Furthermore, holding cells in the dark after illumination (Fig. 4) did not restore the shoulder. Growth is required to restore it. [Patrick, Haynes, and Uretz (22) observed that *S. cerevisiae* held in buffer at 30 C in the dark 4 days prior to plating did not recover from acridine orange photoinactivation.] Viability in growth medium, unlike buffer, was strongly influenced by temperature. This must be related to synthesis, because glucose alone does not alter viability. The distortions of survival curves at high cell density may arise from synthetic processes which were permitted through the accumulation of cell effluent serving as nutriment. Photochemical action on the effluent may also occur. [Burchard and Dworkin (2) obtained with *Myxococcus xanthus* at high cell density some survival curves of complex form.] Survival to germicidal ultraviolet is influenced by composition of plating agars (see, for example, reference 1). In our system, we observed apparent rescue of some cells by plating on M medium.

Three levels of binding have been discerned in our experiments. The first is low-level binding. It occurred in the dark and it showed little increase after the first few minutes in nitrogen or air. The cells did not die. The second type is high-level binding. It was brought about by illumination, and it increased exponentially with time. It occurred only in air, and it was strongly temperature dependent. Since death rate was temperature independent (Fig. 6), we conclude that high-level binding is ancillary to death. It may represent binding by a photoproduct of BP. The third type is intermediate-level binding. It was brought about by illumination also, but, after the first few minutes, it increased only slowly with time. It occurred in air at 6 C (Fig. 7). When it occurred under nitrogen (Fig. 3), it was influenced little by temperature.

This research does not indicate what manner of binding is responsible for cell death. However, it suggests that death is a pure photochemical process, since death was temperature-independent. Although most of the tightly bound BP is associated with the protein fraction, it is possible that a small (undetectable) amount is bound to DNA. [Ts'o and Lu (23) described in vitro BP covalent binding to DNA under anaerobic illumination.] The formation of alkali-labile bonds in DNA results from a reaction (possibly enzymatic) after initial photodynamic damage, because formation of these unstable bonds was temperature-dependent and occurred in the dark (Fig. 8 and 9).

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