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Detection of *in Vivo* Protein Photooxidation by Means of Tritium Loss from the Histidine Moieties* (32735)

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In vitro protein photooxidation with methylene blue was discovered to involve preferentially the histidine moieties (1). To detect photodamage to histidine and other amino acids, proteins were acid-hydrolyzed, and assays for the various amino acids were undertaken by chemical and microbial methods. These methods are laborious and are inconvenient when many samples are required as, for example, in cell survival-time studies. A rapid and sensitive presumptive test for the photooxidation of protein histidine is desirable. Such a test has been devised and is described in this report. It takes advantage of the tritium lost from tritiated histidine during photooxidation. Tritium detected in cell filtrates correlates with photodamage to the cellular protein histidine, if the suspensions are kept cold and are compared with suitable controls. Furthermore, this tritium loss is a more sensitive test for photodynamic action than is cell death.

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Materials and Methods. Escherichia coli was cultivated in MYE medium, was harvested during exponential growth (log cells), was washed in buffer, and was suspended in buffer, usually at a cell concentration of 5×10^8/ml. Photosensitizer (methylene blue at 5 μg/ml, acridine orange at 5 μg/ml, or benzo(*a*)pyrene at 1 μg/ml) was added, the suspension was placed in a small petri dish, and was allowed to equilibrate for 0.5 hour at 6°C before illumination. Media composition and other details have been given elsewhere (2). Illumination and all subsequent manipulations were at 6°C. Two controls were employed: cells held in darkness with photosensitizer (dark control), and cells illuminated in absence of photosensitizer (illumination control). The dyes were illuminated with two 17-inch, 15-watt "daylight" fluorescent tubes (G. E. F15T-D) in a desk type reflector 28 cm above the suspension. This gave an incident dose of 80 ergs per mm^2 per sec according to a YSI-Kettering model 65 radiometer. The hydrocarbon was administered in colloidal form and was illuminated with 355 mμ ultraviolet also at 80 ergs per mm^2 per sec (2).

Bacterial protein was labeled during growth by including in the MYE medium a tritiated

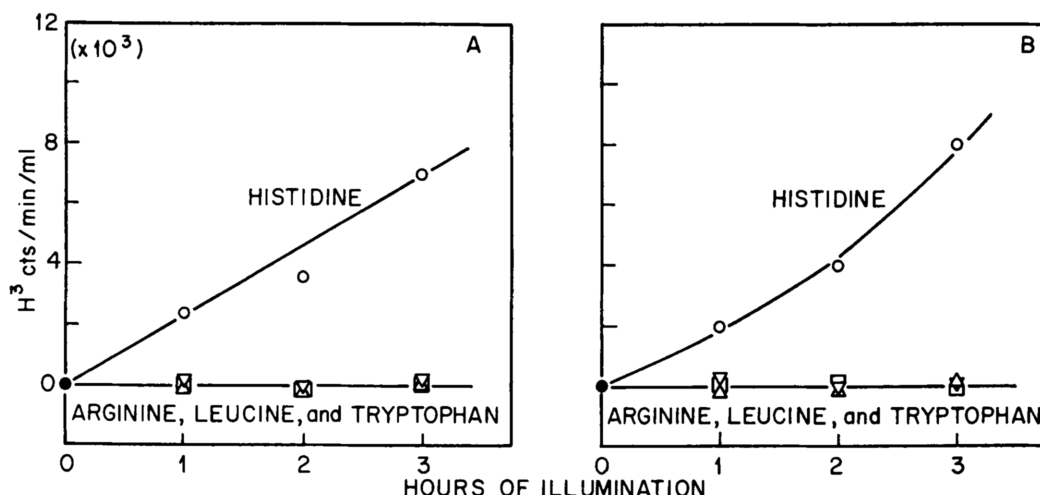

FIG. 1. Preferential loss of histidine-tritium from *E. coli* protein *in vivo* with benzo(*a*)pyrene as photosensitizer. Each assay on 1 ml of suspension containing 5×10^8 cells. In A the cells were centrifuged and radioassays were made on the supernatant. In B cells were first sonicated, the protein precipitated with cold 10% TCA, then was centrifuged, and radioassays were made on the sonicate supernatant. In both figures the background radioactivity and any radioactivity lost by the illumination controls has been subtracted. The dark controls, not plotted, lost no tritium.

amino acid. The radiochemicals were purchased from Nuclear Chicago Corporation and they were stated to be generally, not uniformly, tritiated. When parallel cultures were labeled with different amino acids, the label in each culture was adjusted so that the incorporation into cell protein by the respective amino acid was at the same specific radioactivity. (The histidine-1-^{3}H was administered at a concentration of 1 μC/ml culture; about half of this radioactivity was incorporated into the cells and analyses of cell fractions showed that over 96% of the label was in protein.) Each culture was inoculated to give 5×10^7 cells/ml. After the culture density had risen to 3×10^8 cells/ml, a 200-fold excess of unlabeled amino acid was added, and the culture was allowed one further doubling. This addition was made to minimize label remaining in the intracellular amino acid pool. The cells were then harvested as described.

During the experiments 0.1-ml aliquots were diluted and were spread on YE agar plates to determine the viable cell titer (2). Death was defined as the inability of a cell to form a colony on this medium after overnight incubation at 37°C.

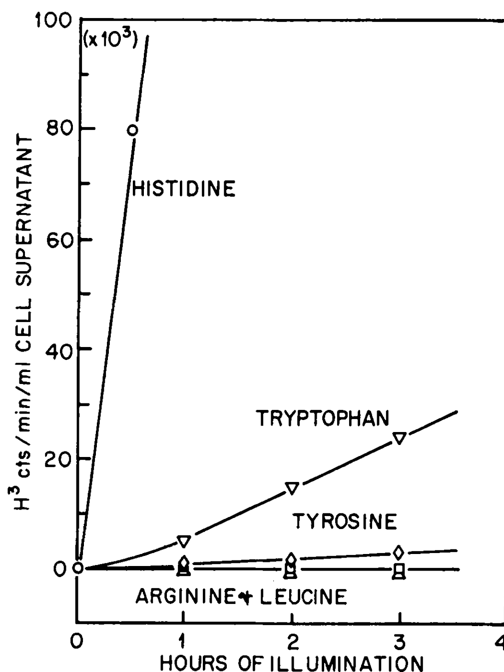

FIG. 2. Preferential loss of histidine-tritium from *E. coli* protein *in vivo* with methylene blue as photosensitizer. Each assay was on 1 ml of suspension containing 5×10^8 cells. Background radioactivity has been subtracted. Neither the illumination controls nor the dark controls lost tritium, and they have not been plotted.

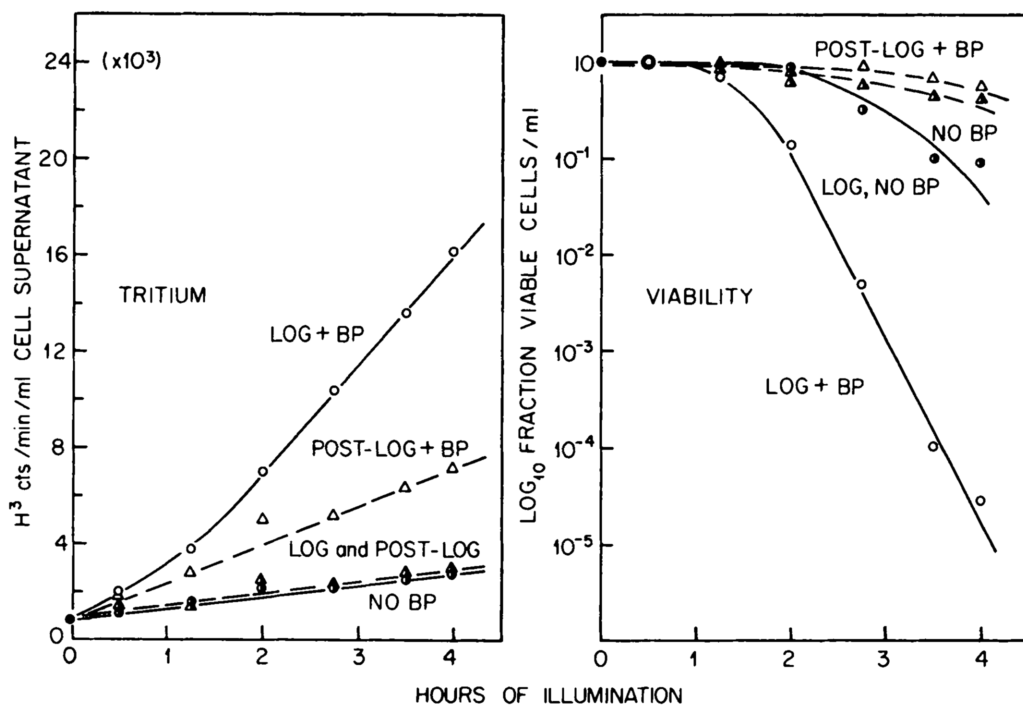

FIG. 3. Comparison of the photosensitivity of log and post-log *E. coli* Ma with benzo(a) pyrene as photosensitizer. The radioactivity and the cell mass in both suspensions were the same, but since log cells were twice the mass of post-log cells the concentration of the former was 4×10^8/ml whereas that of the latter was 8×10^8/ml. The dark controls, not plotted, underwent neither loss of tritium nor loss of viability. BP = benzo(a)pyrene.

After centrifugation, loss of tritium from the cells may be detected in either of two ways: by measuring the increase in radioactivity in the supernatant, or by measuring the decrease in radioactivity in the pellet. The former is most suitable for detecting low levels of tritium loss, and was the method generally employed. One-ml samples were spun in a microcentrifuge (2). The supernatant was pipetted from the pellet and 0.1 ml was added to 5 ml of dioxane liquid scintillation fluid (2) and was counted in the Tricarb Liquid Scintillation Counter (Packard Instrument Co.). Loss of tritium, if it occurs, ceases immediately after a sample is placed in the dark; therefore several samples were collected and were centrifuged at the same time. The results were plotted as counts per min per ml supernatant versus hours of illumination of the test suspension (Figs. 1, 3, and 4). Upon prolonged illumination the slopes decrease and the curves eventually plateau.

Measurements of the pellet were carried out after it was washed with buffer and was brought back to volume in buffer. The results were plotted as counts per min per ml of cells versus hours of illumination of the test suspension. The curve begins by decreasing in proportion to time (illumination dose), but it does not decrease to zero radioactivity. With our sample of histidine-1-^{3}H it became asymptotic to a retention value of 37%. Prolonged illumination with methylene blue did not further diminish the tritium retained by the cells, and at this level no histidine could be demonstrated in cell protein by chemical or microbiological assay. Thus, 63% of the incorporated tritium is *labile* and 37% is *stable*. If calculations are based on this *labile* tritium, a good correlation occurs between the tritium loss and the actual histidine loss (Table I). The 37% retained represents tritium which has been reincorporated (unlikely at 6°C), or which resides on some part of the histidine molecule not photooxidized as, for example,

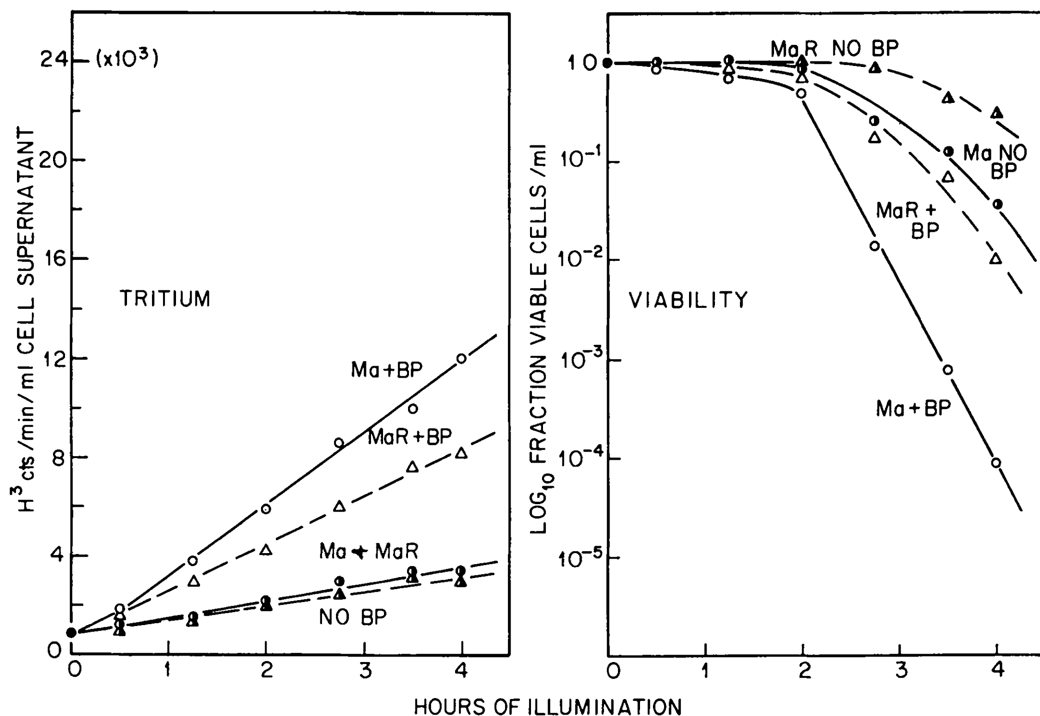

Fig. 4. Comparison of the photosensitivity of log *E. coli* Ma (wild-type) and log *E. coli* MaR (photoresistant mutant). The radioactivity and the cell mass in both suspensions was the same; the cell concentration of both was 5 × 10^8/ml. The dark controls, not plotted. underwent neither loss of tritium nor loss of viability. BP = benzo(*a*)pyrene.

that which may be afixed to the protein backbone through the peptide bond. The same result was obtained by another method in which centrifugation is not required. A 0.1 ml sample of suspension was added to 0.2 ml of 2% formalin. Then 0.1 ml of this was placed directly on Whatman filter paper discs (2.3 cm × 3 mm), the disc were air-dried, were washed with two changes of cold 10% TCA, with two changes of 75% ethanol, and finally with acetone to aid final drying. The discs were placed in 5 ml of toluene liquid scintillation fluid (2) and counted. In this method the acid-insoluble material remains on the discs, whereas supernatant tritium is lost. Apparently, the supernatant tritium is in a volatile form, possibly in water, so is lost during the first drying. The same retention versus time curve was obtained, and 37% of the tritium remained in the cells. Washing the discs was not necessary, but washed discs gave higher counts, probably because the solids (such as the phosphate buffer) which otherwise would absorb radioactivity were washed out.

TABLE I. Correlation Between Loss of Histidine-Tritium and Loss of Biologically Active Histidine from *E. coli* Protein *in Vivo*.

Hours of illumination	Fraction of the *labile* tritium remaining in the cells[a]	Fraction of the protein histidine remaining in the cells[b]
0	1.0	1.0
0.5	0.83	0.90
1	0.67	0.65
2	0.32	0.35
4	0.[c]	0.

[a] Determined by the paper disc method.

[b] Determined by microbiological assay with *E. coli* strain $15_{H^-T^-L^-}$.

[c] 63% of the *total* tritium has been lost and 37% remains in the cells. See "Materials and methods."

It is important in the three methods just described that all manipulations be carried out in the cold and away from bright illumination.

At room temperature dark reactions may occur and obscure the results. Drying the paper discs, however, required an elevated temperature. The formalin treatment prevented subsequent dark reactions during drying.

Photoresistant mutants were obtained by cycling the cells between 6°C buffer illuminated with photosensitizer and 37°C MYE medium in darkness. Single colony isolates were made to YE stock slants.

Results and Discussion. Cells with their protein labeled with histidine-^{3}H lose tritium into the milieu, whereas cells labeled with arginine-^{3}H, leucine-^{3}H, or tryptophan-^{3}H lose no measurable tritium under the same circumstances (Fig. 1A). If the cells are first sonicated and their protein precipitated with TCA, then the supernatant assayed, the result is the same (Fig. 1B). The result in 1A, therefore, cannot be attributed solely to photodynamic action on an intracellular histidine pool, but must involve protein histidine.

With methylene blue instead of benzo(*a*)-pyrene as photosensitizer, the histidine-tritium loss is much more rapid (compare the units in Fig. 2 with those in Fig. 1A). Here, loss of some tryptophan–tritium and a trace of tyrosine–tritium are also detectable. Although histidine was preferentially attacked in the *in vitro* study (1), some photooxidation of tryptophan and a very slight photooxidation of tyrosine also occurred. Thus the result (Fig. 2) is not in conflict with it. Benzo(*a*)pyrene at higher illumination doses may show similar activity upon tryptophan and tyrosine. But higher dose rates are not practicable with our illumination source, and we wished to avoid long-term exposure for fear the cells might then undergo significant alterations in physiology during the experiment. Increasing the benzo(*a*)pyrene concentration to above 1 μg/ml does not increase the rate of photodamage.

Tritium loss is compared with viability, and log cells are compared with post-log cells (Fig. 3). Post-log cells were harvested from MYE medium after overnight incubation. Note that tritium loss and viability loss correlate. Note also that loss of tritium is detectable earlier than loss of viability. Further, the slight killing that occurs under 355 mμ illumination in absence of photosensitizer correlates with a slight loss of histidine–tritium.

Germicidal (254 mμ) ultraviolet causes no loss of histidine–tritium from cell protein even at illumination doses several times greater than required to bring about 99% kill.

Tritium loss is compared with viability loss by log cells of wild-type and by log cells of a photoresistant mutant (Fig. 4). Again, tritium loss correlates with viability loss, and loss of tritium is detectable earlier than loss of viability. Observe that in this and the previous figure there is no "break" in the tritium curve to correspond to the "break" in the viability curve which occured between 1 and 2 hours.

With methylene blue or acridine orange as photosensitizer the results are the same, so figures have not been included: Tritium loss correlates with viability loss and post-log cells lose tritium and viability at a lower rate than log cells. The illumination controls, however, lose neither tritium nor viability. This reflects the nature of the illumination, namely, "daylight" versus "blacklight" (355 mμ UV). The latter is known to be slightly harmful in itself (3).

Although there is a correlation between degree of histidine–tritium loss and photokilling, this does not establish a causative role by the former. Other kinds of damage must be taken into consideration. For example, with methylene blue the guanine moieties of DNA are preferentially photooxidized *in vitro* (4) and *in vivo* (5). With acridine orange (6, 7) and benzo(*a*)pyrene (2) damage to DNA also may occur.

Summary. Measuring the loss of histidine–tritium from cellular protein provides a sensitive assay for *in vivo* protein photooxidation. The relative rates of tritium loss from the various protein amino acids *in vivo* correlate with the relative rates of photodamage known to occur to them *in vitro*: histidine>tryptophan>tyrosine>arginine and leucine. Moreover, the degree of loss of histidine–tritium during *in vivo* photodynamic activity correlates with the degree of photokilling.

The experiments with cell sonicates were suggested to us by Dr. W. E. Barnett.

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Influence of Cystine Supplements on the Incidence of Aortic Rupture in Rats Fed β-Aminopropionitrile* (32736)

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An appreciable difference in the incidence of aortic rupture has been encountered when *Lathyrus odoratus* seeds are fed to immature rats (1–4). It has been suggested that the concentration of protein in semisynthetic diets probably exerts some protective effect against the development of aortic rupture in *L. odoratus*-fed rats (2). In support of this hypothesis, supplements of casein, gelatin, and lactalbumin minimize both suppression of growth and severity of osteolathyrism (5, 6). Besides the concentration of protein in the diet, it appeared that the type of protein probably also exerts some influence on the production of tissue changes in lathyritic rats. Protein quality became a consideration when it was observed that at equivalent concentrations of BAPN, semisynthetic diets of crude casein were more protective against aortic rupture than commercial diets (7, 8). The type of protein also appeared influential in another study because only cottonseed meal and BAPN were able to produce fecal impaction and colonic atony (9). The beneficial effect of protein against BAPN intoxication is supported by other studies where supplements of *l*-cysteine or *l*-glutamine were found to be protective against growth suppression and osteolathyrism (10). Supplements of *dl*-methionine in a diet with insufficient casein, however, appeared to enhance the incidence of aortic rupture when rats were fed BAPN (7). The protective influence of amino acid supplements has been questioned in still other studies because they did not appear to minimize BAPN intoxication (6).

Additional studies on the relationship of amino acids or protein to BAPN intoxication are needed if this controversy is to be resolved. Such studies may also offer some explanation for the wide variation in the incidence of aortic rupture which has occurred in rats in experimental lathyrism.

The point of issue in this study was to establish whether the incidence of aortic rupture which is caused by the ingestion of BAPN could be reduced by supplementation of amino acid. At present, ample justification exists for controversy regarding the influence of protein or amino acid supplements on the development of osteolathyrism or angiolathyrism in immature rats. Comparable experimental conditions have not always been employed in experimental lathyrism when evaluating tissue alteration in rats. There have been variations in the duration of the assay period, concentration of lathyrogen, strain, age, and sex of rats, as well as the concentration of protein. Finally, the observations that suggested a beneficial effect from protein or amino acid supplementation have not satisfied statistical criteria because insufficient numbers of rats were employed.

Before attempting to study the relationship between aortic rupture and dietary manipulation in BAPN-fed rats, several arbitrary limitations seemed essential. We decided to

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