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THE BIOLOGICAL IMPORTANCE OF ULTRAVIOLET LIGHT INDUCED  
DNA-PROTEIN CROSSLINKS IN *ESCHERICHIA COLI* 15 TAU

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## SUMMARY

The sensitivity of *Escherichia coli* 15 TAU to killing by ultraviolet light (2537 Å) when grown for various times under several nutritionally restricted conditions (+T-AU; -T+AU; -T-AU) has been measured. Concurrent measurements were made on the amount of DNA crosslinked to protein by these same doses of ultraviolet light. There appears to be a direct correlation between the amount of DNA crosslinked to protein by a given dose of ultraviolet light and the intrinsic sensitivity of the cells to killing by ultraviolet light under the several growth conditions studied. These results strongly suggest that the crosslinking of DNA and protein by physiological doses of ultraviolet light must play an important role in the loss of viability of ultraviolet light irradiated cells.

## INTRODUCTION

The discovery of thymine dimers by BEUKERS AND BERENDS<sup>1</sup> stimulated a renaissance in the study of the effects of ultraviolet light upon cells. The thymine dimer has proved to be the major single photoproduct produced in DNA by ultraviolet light. The biological importance of this lesion has been demonstrated<sup>2</sup>. Cells can repair this lesion both by photoreactivation mechanisms<sup>3</sup> and by dark recovery mechanisms<sup>4,5</sup>. The exponential appearance of papers concerning the biological importance of the thymine dimers has almost blinded us to the fact that about 30-50 % of the ultraviolet light induced lesions are not, in fact, thymine dimers<sup>6</sup>. Therefore, there must be other lesions produced in DNA which are of biological importance. One such lesion is the crosslinking of DNA and protein in *Escherichia coli* by physiological doses of ultraviolet light<sup>7,8</sup>. This lesion is not related to thymine dimerization and is not modified by photoreactivation<sup>9-11</sup>. The crosslinking of DNA and protein therefore cannot be an artifact of the secondary attachment of the photoreactivating enzyme subsequent to radiation. The crosslinking of DNA and protein is also observed in *E. coli* B<sub>8</sub> (Hill) (ref. 11) a strain which is apparently devoid of dark recovery enzymes<sup>6</sup>. These results seem to eliminate the possibility

that crosslinking is an artifact due to the subsequent attachment of thymine dimer excision enzymes following radiation. There may be other repair enzymes, however, of which we are as yet unaware. The best evidence that the response is the direct crosslinking of DNA and protein by ultraviolet light is that this response can be demonstrated *in vitro* with purified DNA and protein under conditions precluding the presence of repair enzymes<sup>9-11</sup>. We have recently isolated a mixed photoproduct of uracil and cysteine (identified as: 5-S-cysteine, 6-hydrouracil) and hope that it may serve as a model for the mechanism by which protein and DNA are crosslinked by ultraviolet light<sup>12</sup>.

The present paper deals with experiments designed to determine the biological importance of this crosslinking phenomenon. The intrinsic sensitivity of *E. coli* 15 TAU to killing by ultraviolet light can be varied by switching the cells from complete growth medium to various nutritionally restricted growth media. If the crosslinking of DNA and protein is important in the killing of cells then the magnitude of this response should fluctuate in the same manner as the change in the sensitivity of the cells to killing by ultraviolet light. Cells were therefore grown under varied nutritionally restricted growth conditions and were assayed for cell survival and for the amount of DNA crosslinked to protein by a given dose of ultraviolet light *versus* time of growth under the specified condition. The results of these experiments strongly suggest that the crosslinking of DNA and protein by physiological doses of ultraviolet light must play an important role in the loss of viability following the ultraviolet light irradiation of cells.

#### METHODS AND MATERIALS

##### *Preparation of cells*

*E. coli* 15 TAU (obtained from P. C. Hanawalt) was maintained on Difco nutrient agar slants at 4°. This mutant requires thymine, arginine and uracil, and was grown in a buffered salts-glucose medium supplemented with these compounds<sup>13</sup>. Inoculum cultures were prepared by introducing a loop of cells from a slant culture into liquid growth medium and incubating overnight at 37° or over the weekend at room temperature. Two or three ml of this culture were then added to 100 ml of fresh medium at 37° and incubation was continued in a 37° shaking water bath for 5-6 h until a cell concentration of  $1 \cdot 10^8$ - $3 \cdot 10^8$  was reached. The culture was then filtered through a membrane filter 90 mm in diameter (Schleicher and Schuell Co., Keene, N. H.; No. B6;  $0.4 \mu$ ). The cells were washed in and resuspended in the medium under investigation to give a concentration of cells of about  $5 \cdot 10^7$  per ml. The filter and all media were prewarmed to 37°. The total time required to change media was less than 60 sec. Incubation was continued at 37° and viable counts were made at intervals by diluting aliquots with 0.1 M phosphate buffer (pH 6.5) to a concentration of about  $10^8$  cells/ml and then plating 0.01-0.30 ml on the surface of Difco nutrient agar plates prewarmed to 37°. The cells were gently spread over the surface of the agar with a glass wand. Overnight incubation at 37° gave rise to countable colonies.

*Ultraviolet light irradiations*

Ten ml of cells were placed in a 100 mm diameter Petri dish and irradiated for various times with shaking under an unfiltered General Electric germicidal lamp (G8T5). The output at 2537 Å was 750 erg/mm<sup>2</sup> per min at a distance of 43 cm as measured by uranyl oxalate actinometry<sup>14</sup>.

*Radioactive thymine*

When required by the experiment, [2-<sup>14</sup>C]thymine (Calbiochem, Los Angeles) at 14.8 mC/mole was used undiluted to satisfy the requirement for thymine (2 µg/ml).

*Isolation and assay of radioactive DNA*

The procedures previously outlined<sup>7</sup> were used for the isolation and assay of radioactive DNA with the following modifications which proved necessary for this strain of *E. coli*. Unless otherwise indicated the cells were irradiated in their growth medium and then 7.5 ml was immediately pipetted into 8.0 ml of 4 % sodium lauryl sulfate (recrystallized from alcohol)-0.07 M sodium citrate, pH 7. This solution was mixed occasionally over 30 min at room temperature and then placed in the cold room (4°) overnight. Next morning, the samples were brought to room temperature, heated at 60° for 5 min and then stirred slowly on a magnetic stirrer for 30 min at room temperature. This modification was necessary because this strain proved more difficult to lyse than the strains used previously<sup>7</sup>. From this point on, the regular procedure was followed.

## RESULTS

*The ultraviolet light sensitivity of cells grown for various times (-T+AU)*

The cells were grown in radioactive medium( $\gamma$ ), [<sup>14</sup>C]thymine and while in log phase were quickly changed to medium containing no thymine (see METHODS). The culture was continued at 37° and at various times two aliquots were removed. One aliquot was used as the unirradiated control both for cell counts and for the extraction of DNA while the other one was irradiated with 480 erg/mm<sup>2</sup> of ultraviolet light at 2537 Å and then processed in the same manner.

As observed by others<sup>13,15-17</sup>, there is little death for the first 30-40 min of growth in the absence of thymine but thereafter, the cells begin to die at a very rapid rate (maximum half life of 7 min). After 100 min of growth in the absence of thymine (0.5 % survival), the rate of thymineless death falls off markedly (Fig. 1).

The response of these cells to a constant dose of ultraviolet light delivered at various times after switching to a thymineless medium indicates that the ultraviolet light sensitivity of these cells remains essentially normal for about the first 10 min but thereafter, the sensitivity of the cells to ultraviolet light killing increases markedly. By 50 min, the intrinsic sensitivity of the cells to killing by ultraviolet light (defined as: 1/surviving fraction) has increased 17-fold and this is at a time when only about 50 % of the cells have undergone thymineless death. Beyond 50 min, there is again an abrupt alteration in the intrinsic sensitivity of the cells to killing by ultraviolet

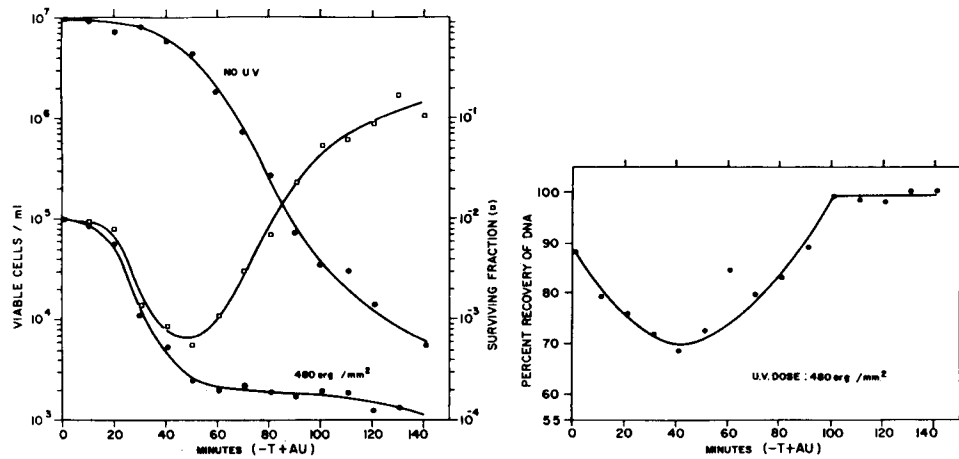


Fig. 1. The susceptibility of *E. coli* 15 TAU to killing and to the crosslinking of their DNA and protein by ultraviolet light (UV) at various times of growth under conditions of  $(-T+AU)$ . A logarithmic culture of *E. coli* 15 TAU was suddenly switched to a medium devoid of thymine by filtration and resuspension. At various times thereafter, two aliquots were withdrawn from the culture. One of these aliquots was irradiated with  $480 \text{ erg/mm}^2$  ( $2537 \text{ \AA}$ ) and then both aliquots were assayed for viable cells and for the amount of DNA that could be extracted free of protein. Reproduced with permission from: K. C. SMITH, *Photochem. Photobiol.*, 3 (1964) 415.

light. By 140 min, the surviving cells (0.06 %) are about 15 times more resistant to killing by ultraviolet light as compared to the log phase cells (time zero).

Fluctuations in the sensitivity of these cells to killing by ultraviolet light after growth for various times under conditions of  $(-T+AU)$  can also be seen in the

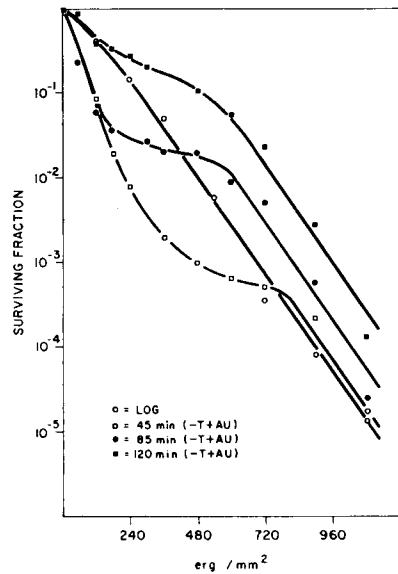


Fig. 2. Ultraviolet light survival curves for cultures of *E. coli* 15 TAU grown for various times under conditions of  $(-T+AU)$ . The "S" shaped survival curves are characteristic for mixtures of two populations of cells with different UV sensitivities (see Fig. 5).

full survival curves (Fig. 2). All except the curve for log phase cells are distinctly "S" shaped. After 45 min of growth in the absence of thymine the majority of the cells are markedly more sensitive to ultraviolet light than are the log phase cells but there is also a population that is resistant to ultraviolet light. As the cells undergo thymineless death, the relative proportion of the remaining viable cells become composed largely of ultraviolet light resistant cells. The identification of the cells that are sensitive to thymineless death with those that are sensitive to ultraviolet light has been reported previously<sup>16</sup>. When the survival curves change so drastically in shape with time, it is apparent that experiments using a single dose of radiation (Figs. 1, 3, 4) can give different results depending upon the dose of ultraviolet light used. Thus, cells grown for 85 min in the absence of thymine would be found more sensitive to ultraviolet light than log phase cells at doses below 430 erg/mm<sup>2</sup>, and more resistant at doses above this value (Fig. 2).

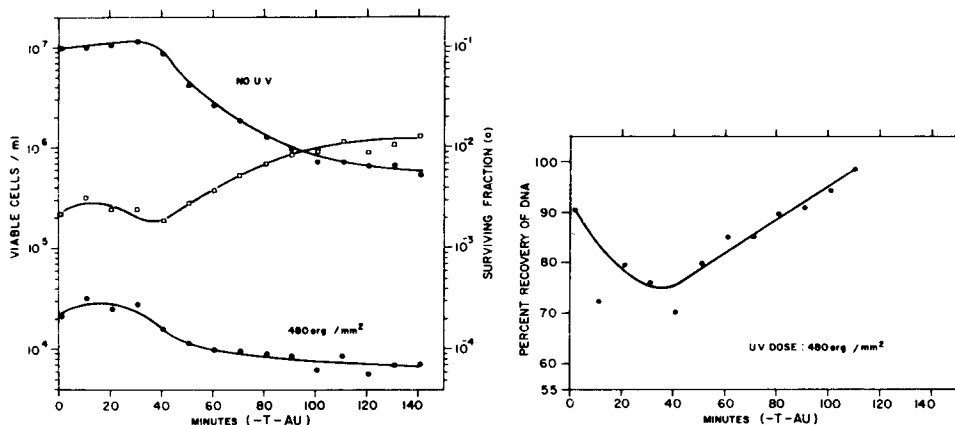


Fig. 3. The susceptibility of *E. coli* 15 TAU to killing and to the crosslinking of their DNA and protein by ultraviolet light (UV) at various times of growth under conditions of (-T-AU). A logarithmic culture of *E. coli* 15 TAU was suddenly switched to a medium devoid of thymine, arginine and uracil by filtration and resuspension. At various times thereafter, two aliquots were withdrawn from the culture. One of these aliquots was irradiated with 480 erg/mm<sup>2</sup> (2537Å) and then both aliquots were assayed for viable cells and for the amount of DNA that could be extracted free of protein.

If we now look at the curve for the ultraviolet light crosslinking of DNA and protein (right half of Fig. 1), we see that the thymineless state also produces a marked increase in the total amount of the DNA that becomes crosslinked to protein by a given dose of ultraviolet light, and therefore a greater loss in the recovery of free DNA. There is a marked increase in crosslinking for the first 40 min of incubation under conditions of (-T+AU) and thereafter the DNA of these cells becomes much less sensitive and finally more resistant to crosslinking than the DNA of log phase cells. The time course for the change in the amount of DNA crosslinked to protein is very similar to that for the changes in the sensitivity of these cells to killing by this same dose of ultraviolet light (left half of Fig. 1). The viability experiments, of course, only measure those cells in the culture that are still viable but the chemistry part looks at all the cells in the culture regardless of viability.

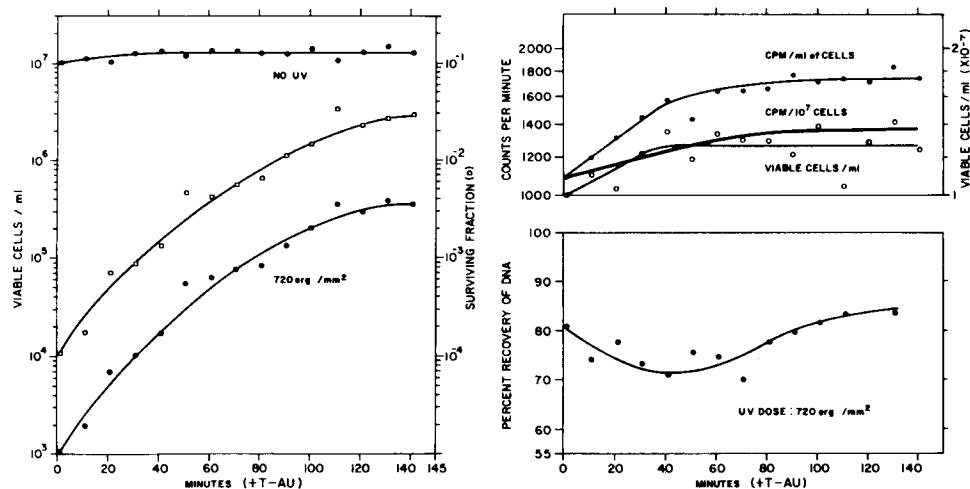


Fig. 4. The susceptibility of *E. coli* 15 TAU to killing and to the crosslinking of their DNA and protein by ultraviolet light (UV) at various times of growth under conditions of (+T-AU). A logarithmic culture of *E. coli* 15 TAU was suddenly switched to a medium devoid of arginine and uracil by filtration and resuspension. At various times thereafter, two aliquots were withdrawn from the culture. One of these aliquots was irradiated with 720 erg/mm<sup>2</sup> (2537 Å) and then both aliquots were assayed for viable cells and for the amount of DNA that could be extracted free of protein. The DNA content per ml of culture as well as the DNA content per cell was also determined.

We can conclude that the crosslinking of DNA and protein by ultraviolet light is not a random, non-specific process but changes under the same conditions that lead to the production of cells that are more sensitive to ultraviolet light killing. The near equivalence in the timing of changes in viability and crosslinking are suggestive that the crosslinking may play a significant role in the loss of viability of irradiated cells.

#### *The ultraviolet light sensitivity of cells grown for various times (-T-AU)*

The cells were grown in radioactive medium ([<sup>14</sup>C]thymine) and while in log phase were quickly changed to medium containing no thymine, arginine or uracil (see METHODS). The culture was continued at 37° and at various times two aliquots were removed and processed as described above for the experiment in which the cells were grown for various times (-T+AU).

A very slight increase in the number of cells was observed for the first 30 min but after about 40 min, the cells began to die of thymineless death but with a constantly changing (decreasing) rate of death and finally plateaued at about 5% survival at 140 min of growth (-T-AU) (Fig. 3).

The cells actually showed a slight increase in resistance to ultraviolet light killing during the first 20 min of growth at (-T-AU). By about 40 min, the sensitivity returned to normal or perhaps was very slightly increased in sensitivity. Beyond 40 min, the sensitivity of the cells to ultraviolet light killing steadily decreased such that by about 140 min the cells were 10-fold more resistant than the

log phase cells (time zero). The decrease in sensitivity at 10 min and increase at 40 min has also been observed in three other experiments not reported here.

If we now look at the curve for the ultraviolet light crosslinking of DNA and protein (right half of Fig. 3), we again see that the thymineless state has altered the response of the DNA of these cells to crosslinking. Under conditions of (-T-AU), the increase in sensitivity to crosslinking is not as great as under conditions of (-T+AU) nor is the sensitivity to killing by ultraviolet light. The cells also begin to recover sooner but at a slower rate than under conditions of (-T+AU), but they reach the sensitivity of log phase cells (time zero) at about the same time. Again we find a similarity in the timing and direction of change in surviving fraction and in crosslinking after a given dose of ultraviolet light delivered to cells grown for various times under a nutritionally restricted condition (-T-AU).

*The ultraviolet light sensitivity of cells grown for various times (+T-AU)*

A culture of *E. coli* 15 TAU was grown overnight on radioactive medium ( $^{14}\text{C}$ thymine) and an aliquot of this was used to inoculate the radioactive medium to be used in the experiment. While this culture was in log phase, it was quickly changed to medium containing  $^{14}\text{C}$ thymine, but devoid of arginine or uracil (see METHODS). The culture was continued at  $37^\circ$  and at various times, two aliquots were removed and processed as described above for the experiments in which the cells were grown for various times (-T+AU) and (-T-AU). Two exceptions should be noted. The dose of ultraviolet light was increased to  $720 \text{ erg/mm}^2$ , and the increase in radioactivity per cell with time was followed. The latter was obtained by counting the radioactivity in the controls prior to the final step (addition of KCl) in the isolation of the free DNA and correlating this with the cell counts.

The results shown in Fig. 4 indicate that about 27 % of the cells were able to divide in the absence of arginine and uracil, but further division was inhibited and the cell number remained constant from about 30 min to 140 min (the end of the experiment). After incubation for 120 min under conditions of (+T-AU), there was an increase of 58 % in the amount of  $^{14}\text{C}$ thymine incorporated into cells per ml of culture and a 24 % increase in radioactivity per cell.

According to the interpretation of MAALØE AND HANAWALT<sup>13</sup>, there should be two populations of cells in this culture. One population should be composed of cells that have just divided but cannot begin another round of DNA synthesis without subsequent RNA and/or protein synthesis, which are blocked by the absence of arginine and uracil. The second population should be composed of cells that have finished doubling their DNA content but are prevented from dividing because of the absence of arginine and uracil.

From our data, it can be deduced that of the 27 % of the cells that divided after the culture was switched to conditions of (+T-AU), half of these went on to double their DNA but did not divide and the other half continued stabilized with one unit of DNA per cell. The argument for this conclusion is as follows. If the 27 % of the cells that divided were prevented from further replicating their DNA but the remainder of the cells finished replicating their DNA until they contained two components of DNA per cell, then at the end of 120 min 42.5 % of the cells should contain one component of DNA and 57.5 % should contain two components of DNA (Case I).

If, however, half of the cells that divided were able to initiate and complete a second round of DNA replication but did not subsequently divide, then there would be 21.2 % of the cells with one component of DNA and 78.8 % of the cells with two components (Case II). Our data are consistent with this latter conclusion (Table I).

TABLE I

CHANGE IN THE DNA CONTENT PER CELL AFTER GROWTH FOR 120 MIN (+T-AU)

	<i>log phase*</i>	120 min (+T-AU)*	Cells with 1 × DNA**	Cells with 2 × DNA**	Case I***	Case II†
Counts/min per ml cells	1096	1730	—	—	1521	1727
Counts/min per 10 <sup>7</sup> cells	1096	1362	761	1520	1198	1360

\* Data taken from the experiment in Fig. 4. See the text for further information.

\*\* Calculated from the data for log phase cells and the assumption that the average DNA content of log phase cells is 1.44, where a newly divided cell would have a DNA content of 1.0 and a cell just prior to division a content of 2.0 (ref. 13).

\*\*\* Calculated on the basis that the 27 % of the cells that divide after being switched to (+T-AU) cannot initiate another round of DNA synthesis and therefore would contain one complement of DNA. The population would therefore be composed of 42.5 % of cells containing 1 × DNA and 57.5 % of cells containing 2 × DNA. Compare these results with the experimental results in column 2.

† Calculated on the basis that of the 27 % of the cells that do divide after being switched to (+T-AU) half of them can replicate their DNA and half cannot. The resulting population would therefore contain 21.2 % of cells containing 1 × DNA and 78.8 % of cells containing 2 × DNA. Compare these results with the experimental results in column 2.

This implies that the two daughter cells are not identical at the time of division. One is able to replicate its DNA in the absence of arginine and uracil while the other is not.

During the time of incubation under the condition of (+T-AU), there is a continuous increase in the resistance of the cells to killing by ultraviolet light to a maximum of 360-fold more resistant than the log phase cells (time zero). The radiation resistance of this population of cells has been explained on the basis of the presence of duplicate copies of the genetic information<sup>17-20</sup>, on the possibility that the DNA exists in a different and more radiation resistant state<sup>17,21</sup>, and on the basis that they are better able to cope with the initial lethal damage<sup>22,23</sup> (see DISCUSSION).

In Fig. 5, the survival curves are presented for log phase cells and for cells grown 120 min (+T-AU) as well as artificial mixtures of these two cultures. It can be seen that the experimental points are in reasonably good agreement with the curves that have been calculated for the two different mixtures of cells. The main characteristic of the curves generated from the mixed populations of cells is that they are "S" shaped. In this experiment, the survival curve for the cells grown for 120 min under conditions of (+T-AU) is drawn as a smooth curve without an "S" shape. From arguments cited above, this population of cells should contain 21 % of sensitive cells and 79 % of resistant cells. This would mean that the main curve should extrapolate to 0.8 and the survival curve should have a different slope above this value (it should be "S" shaped). It is apparent that sufficient points have not

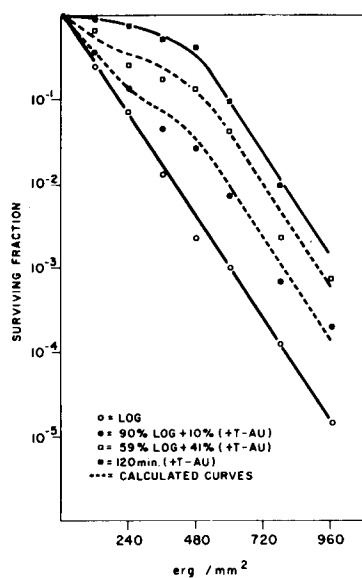


Fig. 5. Ultraviolet light survival curves for single and mixed cultures of *E. coli* 15 TAU. The curves for log phase cells and for cells grown 120 min under conditions of (+T-AU) are drawn to fit the experimental points. The other two curves were calculated from these by summing the per cent contribution of each curve for the indicated mixture of cells. The points represent the actual experimental results for such mixtures of the two cell populations.

been determined in the present experiment to prove whether or not 21 % of the cells are of a different sensitivity than the remainder but they are probably sufficient to indicate that 42.5 % of the cells are not more sensitive than the rest (otherwise an easily detectable "S" shaped curve would be generated) and it therefore offers further proof against Case I above.

The data for the crosslinking of DNA and protein (Fig. 4) show a very slight increase in sensitivity following the removal of arginine and uracil from the culture which returns to the normal value for log phase cells by about 90 min and thereafter becomes somewhat more resistant than for log phase cells. It is not expected that the continued replication of the DNA that occurs under conditions of (+T-AU) should alter the sensitivity of the DNA to crosslinking with protein by ultraviolet light. It is possible, however, that at the end of DNA replication the enzymes might leave the vicinity of the DNA and thus render the DNA somewhat more resistant to crosslinking. However, it is expected that in the absence of uracil initiated chains of messenger RNA would remain attached to the DNA and therefore a portion of the DNA would still be covered with protein (RNA polymerase). The fact that the alteration in the sensitivity of the DNA to crosslinking does not markedly differ from that for log phase cells even though the resistance to killing by ultraviolet light has increased markedly would seem to argue against the hypothesis that the increased resistance observed here is due to an alteration in the state of the DNA but would support the hypothesis that the resistance is due to the presence of duplicate copies of the genetic material and/or the presence of repair enzymes (see DISCUSSION).

Since the fluctuation in the sensitivity to crosslinking has about the same time constant as the increase in cell number, we postulate that the increase in crosslinking is a consequence of cell division. An increase in the ultraviolet light sensitivity of cells has been observed following division<sup>24</sup>. The subsequent decrease in crosslinking is due at least in part to half of the dividing cells initiating and completing a new round of DNA synthesis and part to the completion of DNA polymerase activity in the non-dividing cells.

*Ultraviolet light dose response curves for the extractability of DNA from cultures grown under various nutritionally restricted conditions*

Cultures of *E. coli* 15 TAU were grown in the presence of [<sup>14</sup>C]thymine to log phase and then filtered, washed, and resuspended in the appropriate medium and maintained in this medium (at 37°) for the times indicated (see METHODS). The stationary cultures were grown overnight. The cultures were collected by filtration and were resuspended in 0.1 M phosphate buffer (pH 6.5). 10-ml samples were irradiated with the doses indicated, the cells were recovered by centrifugation (4°) and then were subjected to the procedure used for the isolation of DNA. The data presented are the average results of at least two experiments (Fig. 6).

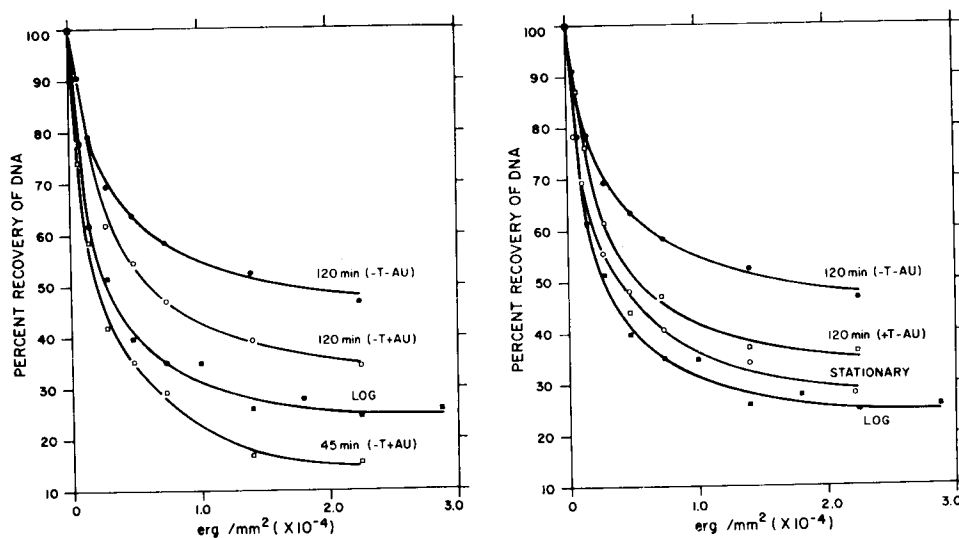


Fig. 6. The extractability of DNA from cultures of *E. coli* 15 TAU following irradiation with increasing doses of ultraviolet light. Log phase cultures were switched to a particular nutritionally restricted medium and maintained at 37° for the times indicated. Stationary cultures were grown overnight. The cultures were then harvested by filtration and resuspended in phosphate buffer. Aliquots were irradiated with different doses of ultraviolet light (2537 Å) and the amount of free DNA that could be extracted from these aliquots is plotted *versus* the dose of ultraviolet light. Left figure reproduced with permission from: K. C. SMITH, *Photochem. Photobiol.*, 3 (1964) 415.

In confirmation of the results in Fig. 1, both the absolute sensitivity of the DNA (initial slope) and the amount of DNA that is sensitive to crosslinking with protein are increased at 45 min of growth under conditions of (-T+AU), when

