ULTRAVIOLET LIGHT-INDUCED MUTATION AND DEOXYRIBONUCLEIC ACID SYNTHESIS IN *ESCHERICHIA COLI*

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Exposure of a bacterial population to a mutagenic dose of ultraviolet light generally does not establish phenotypically mutant cells immediately. Rather, a period of subcultivation of the irradiated cells is required before newly induced mutants can be detected (Demerec, 1946). It has been postulated (*see* Witkin, 1956, for a recent discussion) that the delay between irradiation and phenotypic expression can be accounted for by the time required for three processes: (1) the establishment of a stable, heritable alteration of the genotype, (2) the segregation, if necessary, of the mutant genome from the influence of dominant, non-mutant elements, and (3) the "expression" of the mutant genotype in an altered phenotype. This hypothetical fractionation of the over-all delay is supported in part by the finding, immediately after irradiation, of an interval of instability with respect to the potentiality for mutation. During this period it is possible to influence the ultimate yield of mutants by appropriate manipulations. This interval is, in some cases, demonstrably distinct from the period of growth still required for the completion of phenotypic expression (Lieb, 1959). During this labile phase the mutagenic effects of ultraviolet light can be reduced or photoreversed by exposure to white light. Cultivation of the cells in the dark leads to a fixation of the mutagenic effect with loss of susceptibility to photoreversal (Matney, Shankel, and Wyss, 1958; Doudney and Haas, 1959).

Witkin (1953, 1956) and Berrie (1953) have shown that the yield of mutants is also depressed by unusually high or low incubation temperatures. The period of temperature sensitivity is relatively short, lasting about one-third of the postirradiation lag phase.

Witkin (1956) has clarified the long-known dependence of the mutational process upon postirradiation nutritional supplementation (Demerec, 1946; Demerec and Cahn, 1953). She has shown that efficient mutation induction requires, or at least is associated with, a high rate of protein synthesis immediately following irradiation. This conclusion rests upon the findings that a full complement of amino acids is the essential nutritional element required and that exposure to chloramphenicol, known to be a specific inhibitor of protein synthesis, depresses the yield of mutants. Again, these factors exert their influence over only a short time period following irradiation. After this time the mutant yield is fixed and no longer susceptible to change by prolongation or cessation of treatment.

Haas and Doudney (1957) and Doudney and Haas (1958, 1959) extended Witkin's findings to a better understanding of the kinetics of the amino acid and chloramphenicol effects. In addition they have discovered that certain effects upon the mutation yield are brought about by pre- and postirradiation incubation in the presence of various purine and pyrimidine nucleosides and their structural analogues. They interpret their findings as suggesting a role for ribonucleic acid (RNA) synthesis in the process of ultraviolet-induced mutation.

Subsequently, Witkin (1958) has shown that the duration of the period of amino acid dependence and chloramphenicol sensitivity increases linearly with increasing doses of ultraviolet light. A similar extension of the sensitive period may be produced by incubation of the irradiated cells in caffeine. On the basis of the known ability of ultraviolet light to block deoxyribonucleic acid (DNA) synthesis in *Escherichia coli* strain B/r, the bacterium used, and the fact that caffeine is an inhibitor of nucleic acid synthesis, Witkin reasons that the termination of the period of mutational lability involves nucleic acid and probably DNA synthesis. Thus, synthesis of RNA, protein, and DNA have all three been implicated in the immediate postirradiation events in a fairly specific manner. These events serve, on the one

hand, to implement the mutagenic effect of ultraviolet light or, on the other hand, to dissipate its energy in a nonmutagenic fashion. The present paper seeks a more precise evaluation of the role of DNA synthesis in the mutagenic process. In this study, use is made of the thymine-requiring *E. coli* strain 15T⁻ in which DNA synthesis is uniquely regulated by the supply of thymine in the culture medium (Cohen and Barner, 1954 and later papers).

MATERIALS AND METHODS

Bacterial strain. *E. coli* strain 15T⁻ was originally obtained from S. S. Cohen and has been maintained in this laboratory for a number of years. The mutation chosen for investigation was that of reversion from dependence to independence for a specific nutrient. By the use of ultraviolet irradiation coupled with selection by penicillin (Lederberg, 1950) a number of auxotrophs requiring amino acids were obtained. Repetition of this procedure yielded strains with multiple requirements. The strain finally selected for investigation required tryptophan, methionine, and arginine for growth in addition to the initial requirement for thymine. Originally it was planned to investigate back mutations from each of these requirements, but subsequent study revealed that the tryptophan and methionine mutations were technically unsuitable on the basis, respectively, of low ultraviolet inducibility and "leakiness." All work to be reported here involves only the mutation from arginine dependence to independence.

As reported by Ryan, Fried, and Mukai (1955) for a histidine requiring mutant of *E. coli* strain 15, our organism initially proved to be prone to lysis following ultraviolet irradiation. By repeated irradiation and subculture of the survivors we isolated a derivative apparently unchanged in other properties but which did not show ultraviolet-induced lysis. This was the strain finally used.

Media. Culture media were based on the B. D. Davis mineral salts solution described by Lederberg (1950). The following individually sterilized supplements were added to the concentrations per ml given: glucose, 4 mg; thymine, 0.025 mg; DL-methionine, DL-tryptophan, and L-arginine, 0.2 mg each. The above constituted a minimal liquid medium. The addition of separately sterilized agar to 1.5 per cent concentration gave a minimal plating medium. A supplemented liquid medium was prepared by the further addition of the 15 additional natural amino acids listed by Witkin (1956) to give a final concentration of 20 μg per ml each of the L-isomer. Supplemented agar was of the same qualitative composition but the concentration of each amino acid of the supplement was reduced to 1 μg per ml. Reduction of the arginine content of the two types of plating media to 1 μg per ml gave what we shall designate as minimal mutant plating medium and supplemented mutant plating medium. The compositions of these media are based on empirical determinations which will be described subsequently.

Preparation of cultures. All experiments were performed on cells in the logarithmic phase of growth. These were obtained by dilution of an overnight stationary-phase culture into fresh medium and incubation until the cells were at least two divisions out of the lag phase. All incubation was in 20 to 80 ml of medium in 300-ml Erlenmeyer flasks. The flasks were suspended on a wrist-action shaker in a 37 C water bath. Cultures were maintained at all times within a twofold range of optical density (0.4 to 0.8) by periodic dilution with fresh medium. When washed cells were required, washing was performed on the surface of a membrane (Millipore) filter. It was repeatedly demonstrated that washed cells could be flushed from the filter surface and resuspended completely quantitatively. All washing and diluting was done with the Davis mineral salts solution.

Nucleic acids. For nucleic acid determinations, cells were first extracted with 5 per cent cold trichloroacetic acid. The residue from this extraction was then treated with 0.5 N perchloric acid for 15 min at 70 C. Samples of this latter extract were assayed for DNA by the Burton (1956) modification of the diphenylamine reaction for deoxypentose and for RNA by the orcinol reaction (Volkin and Cohn, 1954).

Irradiation. Ultraviolet irradiation was performed on 10-ml portions of washed cells suspended to a standard optical density of 0.4 in a 15-cm petri dish. The suspension was shaken by hand during exposure. The ultraviolet source was a 15-watt Westinghouse Sterilamp with an intensity of 100 microwatts per cm^2 at the standard distance of 40 cm.

Induced mutations. The general procedure

followed was that of Demerec and Cahn (1953) in which the mutant yield is maximized by striking an empirical balance between the number of cells plated and the amount of nutritional supplement incorporated in the plating medium. In the experiments to be described, mutant counts of 50 to 100 per plate could be obtained from 1×10^6 survivors of a 10- to 20-sec dose of ultraviolet light. When plating this number of cells, it was determined that supplemented agar of the composition described would provide a full yield of mutants.

Spontaneous mutations. Counts of ultraviolet-induced mutants must be corrected for any contribution due to spontaneous mutants already present in the experimental culture ("background" mutants) and for those mutants which arise spontaneously during growth on the plating medium. In the work described here no background mutants were ever present at any stage of the experiment in populations of the size plated (approximately 1×10^6 cells). However, the plating media supported an ultimate titer of approximately 5×10^8 cells per plate and from this population there emerged 0 to 3 colonies from spontaneously mutant cells. All induced mutant counts were corrected by subtracting the contribution from spontaneous mutants.

RESULTS AND DISCUSSION

State of DNA synthesis at the time of irradiation. In spite of the evidence accumulating to suggest that the mechanism of ultraviolet-induced mutation is indirect, that is, that the effect is mediated by an ultraviolet-produced poison, it is not possible completely to rule out direct mechanisms in which the DNA itself is the primary site of absorption of the ultraviolet energy. Under this latter hypothesis, the various experimental manipulations which influence mutant yields could be interpreted as influencing the probability of either the stabilization of the gene in mutant form or the nonmutagenic dissipation of a ultraviolet-produced metastable configuration. Witkin (1956) discussed a further modification of the direct-action hypothesis in which it was postulated that ultraviolet irradiation primarily produces a lethal lesion in the DNA. Under suitable conditions the lesion may be repaired but in a faulty manner such as to leave a viable but mutated structure.

It seemed reasonable that, if the ultraviolet light were producing changes directly in the bacterial chromosome, differences in mutability might be expected between cells actively engaged in the synthesis of DNA and those in which no DNA synthesis was occurring. If, for example, the bacterial chromosome should undergo a cyclical uncoiling and recoiling in the course of its replication, it might be expected that variation in susceptibility to alteration and post-ultraviolet stability would exist. Accordingly, an experiment was performed to measure changes in mutability during successive states of quiescence and activity of DNA synthesis.

Cells of the arginine-requiring strain of $15T^-$ (the methionine and tryptophan requirements will be ignored henceforth) were brought to the logarithmic growth phase in supplemented liquid medium as described. These cells were washed, resuspended to a titer of 6×10^8 per ml in the same medium but now lacking thymine, and incubated at 37 C with constant shaking. At zero time and at 10-min intervals, samples of the culture were removed, washed, and resuspended in the Davis salts solution for various determinations. Control viable counts were made by spreading 0.1-ml volumes of appropriate dilutions on the surface of supplemented agar. Spontaneous mutants were determined by spreading suitable dilutions on supplemented mutant agar plates. Ten-ml portions of the cell suspensions were irradiated with ultraviolet light and, after appropriate dilution, platings were made for total surviving cell count and for ultraviolet-induced mutants on the respective control and mutant agars. Thymine was added to the incubating culture at a time to be specified and the sampling was continued.

Figure 1 shows the results of a typical experiment which was repeated in its entirety five times and spot checked over suspected critical periods many more times. Details in the figure are kept to a minimum to allow the simultaneous comparison of all determinations. Quantitative data are given in the discussion which follows.

(1) Control count and time of thymine addition:—The time of thymine addition was set at 35 min on the basis of preliminary experiments which showed that thymineless death (Barner and Cohen, 1956) began at this time and that a measure of synchronous division could be obtained following thymine addition at this time.

As seen from the control count curve (*F*) of figure 1, no increase in count occurred during the 35-min period of thymine starvation. Following the addition of thymine an additional 40 min of incubation elapsed before any division occurred. At this time division began at a rapid rate with three doublings of the population occurring in approximately 60 min. This is to be compared with the normal division time of an untreated culture in the same medium which was determined to be 37 min. After the period of increased rate of division there was a gradual deceleration until the normal rate was reached. The viable count in this culture ranged between 1.5×10^8 and 1.2×10^9 cells per ml. Curve *F* represents the calculated progress of a theoretical, undiluted culture, as do all the curves of figure 1. It appears that a measure of synchrony in division was achieved in this experiment, although it was not as spectacular as that found by Barner and Cohen (1956) with the thymine starvation technique.

(2) Optical density:—As shown by curve *D* of figure 1, changes in optical density give little clue to the progress of the viable count during the experiment. The rate of increase in cell material (presumably largely protein) was essentially constant at all times.

(3) DNA synthesis:—As found by Barner and Cohen (1956), a slight increase in DNA was seen immediately following the deprivation of thymine, but the cells then ceased to synthesize measurable increments. Curve *E* in figure 1 shows the increase in DNA content per unit volume of culture. It is seen that, upon the addition of thymine to the starving culture, DNA synthesis was promptly initiated at approximately twice the normal rate. The rate then gradually returned to normal as cell division began. Curve *B*, showing the calculated DNA content per cell, indicates that during the period of rapid synthesis the DNA content per cell approximately quadruples. This corresponds to the fourfold increase in cell size, indicated by the increment in optical density, occurring during the period when no cells were dividing. By the time of completion of the "burst" of division all cells are essentially back to normal with respect to size and DNA content.

(4) Ultraviolet survival:—Curve *C* of figure 1 depicts the changes found in the sensitivity of the culture to the lethal action of ultraviolet light. At each sampling time a standardized sample of the culture was irradiated for 10 sec. Initially this fixed dose of ultraviolet light permitted 50 per cent survival. As starvation for thymine proceeded, a rapid increase in sensitivity was observed. Just before thymine addition an ultraviolet dose of 10 sec reduced the survival to approximately 7 per cent. Separate determinations of survival curves for normal cells and for those starved for thymine for 35 min show a difference in slope of approximately 2. Upon the addition of thymine there is a rapid increase in resistance to a level somewhat above that of the starting culture. The undulation in the rising portion of this curve was consistently demonstrable. The changes in sensitivity to ultraviolet light described here are closely

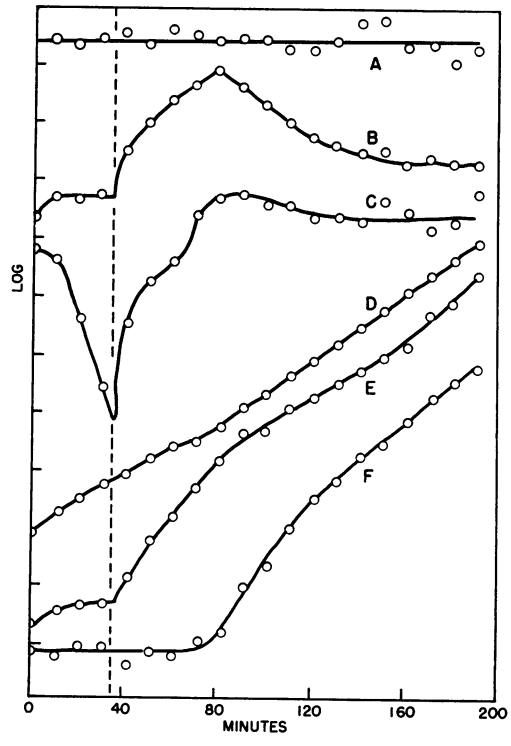

Figure 1. Log_2 relative values of: (*A*) ultraviolet-induced mutants per survivor, (*B*) DNA per cell, (*C*) ultraviolet survival, (*D*) optical density, (*E*) DNA per unit volume of culture, and (*F*) control viable count at intervals during thymine starvation and resupplementation. The vertical line at 35 min indicates the time of thymine addition. Each ordinate unit represents one doubling.

paralleled by those recently reported by Billen (1959) for X-irradiated *E. coli* strain 15T⁻. As will be demonstrated in a later section, it is of interest to note that irradiation followed by thymine starvation does not result in increased killing.

(5) Induced mutations:—Curve *A* of figure 1 shows that the frequency of arginine-independent mutants among the survivors of irradiation was constant throughout the experiment. A 10-sec dose of ultraviolet light yielded approximately 50 mutants per million survivors at every interval examined. At the end of the period of thymine starvation, when DNA synthesis had been stopped for 35 min, the cells had doubled in size and thus contained one-half the normal DNA content per unit of protoplasm. Upon the addition of thymine, DNA was synthesized more rapidly than the rest of the cell constituents, and just before the initiation of cell division each cell (plating unit) actually represented the equivalent of four normal cells. In spite of these changes in DNA/protoplasm and DNA/cell ratios, of the different degrees of activity in synthesis of DNA, and of the differences in susceptibility to ultraviolet killing, the cells remained uniform in sensitivity to the induction of mutation.

The constancy of mutability in the face of major differences in the amount and degree of synthetic activity of DNA at the time of irradiation suggests that DNA does not become involved in the mutational process immediately upon irradiation.

Time of DNA involvement in the mutational process. Since it is conceptually imperative that the mutagenic process ultimately result in a stable, reproducible alteration of the genetic apparatus of the cell and presumably in the DNA of that system, the search was continued for a stage in the mutagenic process where an interruption of DNA synthesis would alter the results. Attention was focused on the early postirradiation interval during which the mutagenic process is susceptible to alteration by a variety of experimental techniques. It was reasoned that the termination of the period of lability or indecision with regard to mutation might be the result of the establishment of a stable change in DNA. If DNA synthesis were then required for this termination, it should be possible to prolong the labile period in irradiated cells of *E. coli* strain 15T⁻ by withholding thymine.

If cells are grown in a medium containing a mixture of amino acids, irradiated, and plated on a minimal medium lacking amino acid supplement, the yield of mutants is low. If, however, the irradiated cells are subcultured for a time in the presence of amino acids, the mutations become "fixed" as defined by the acquisition of indifference to the challenge of plating on a minimal medium (Witkin, 1956). By progressively increasing the duration of the postirradiation exposure to the amino acid supplement before minimal plating, a series of increasing values of mutant yield is obtained. From these values a curve of "mutation fixation" can be plotted (Doudney and Haas, 1958).

Our question, then, is whether mutation fixation, as defined, requires DNA synthesis. The following experiment was performed.

Cells were harvested from the logarithmic phase of growth in liquid medium supplemented with amino acids. A standard suspension of washed cells was irradiated to 20 per cent survival and then divided into two equal portions. One portion was incubated at 37 C in the same supplemented medium. The second portion was handled in identical fashion except that thymine was initially omitted from the culture medium, to be added after 35 min incubation. At zero time and at intervals thereafter samples of each culture were removed, the cells washed, diluted, and plated. Platings were made on minimal medium devoid of amino acid supplement except for the three amino acids specifically required by the organism. Total counts were determined on this medium containing 200 μg per ml of arginine; mutant counts were determined on the same medium containing 1 μg per ml of arginine. Figure 2 illustrates the results of a typical experiment. It may first be noted that during the incubation time of 90 min there was no measurable change in the total cell counts in either the thymine-containing subculture or in the culture from which thymine was withheld for the first 35 min (curves *A* and *B*, respectively). Referring now to curve *C*, showing the yield of arginine-independent mutants per 1×10^6 survivors, it is seen that immediate postirradiation plating on minimal agar results in a relatively low yield of mutants, in this instance approximately 10 mutants per million viable cells plated.

However, when the minimal agar challenge is preceded by increasing periods of incubation in a medium containing amino acid, increasing numbers of mutants become fixed until a maximal yield of approximately 50 mutants per million survivors is obtained. The resultant data are plotted to give a typical curve of mutation fixation. In the absence of thymine, as shown by curve *D* of figure 2, mutation fixation occurs only to a slight extent. Upon the addition of thymine, however, mutation fixation is promptly initiated and continued until the maximal yield is reached. Direct measurements of DNA synthesis were considered to be of doubtful value in these experiments because approximately 80 per cent of the cells are dead. The thymine effect is considered adequate to establish that mutation fixation in this case is actually associated with DNA synthesis.

DNA synthesis and "mutant frequency decline." In a reverse experiment, cells are grown in a medium containing a mixture of amino acids, washed, irradiated, and incubated now in a minimal medium lacking the general amino acid supplement. Samples are plated at intervals on agar supplemented with amino acids. Upon deferring the plating for increasingly longer intervals during the course of incubation, one observes a progressive decline in the number of mutants recovered. Doudney and Haas (1958) have introduced the expression, "mutation frequency decline," to designate this phenomenon.

These authors envision two courses as being open to the irradiated and potentially mutant cell. On the one hand the primary mutagenic change produced by the ultraviolet light may be stabilized in the presence of amino acids until some terminal reaction occurs to fix the mutation in a form no longer dependent upon the presence of amino acids. Alternatively, in the absence of amino acids, the mutagenic potentiality may be diverted or dissipated in a nonmutagenic fashion leading to a progressive decline in the mutant frequency. The latter process is viewed, particularly on the basis of its temperature dependence and its sensitivity to dinitrophenol (Doudney and Haas, 1959), as an active metabolic process as opposed to a passive diffusion of poisons or a decay of a metastable chemical configuration. More specifically, they view mutation frequency decline as resulting from the active removal of an ultraviolet-altered precursor

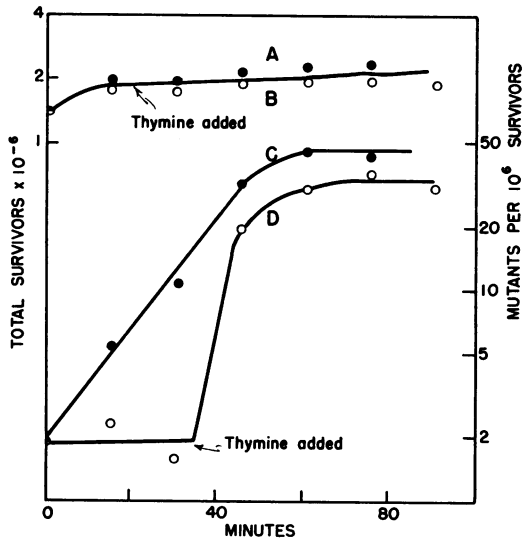

Figure 2. Influence of thymine on fixation of arg^+ mutants against amino-acidless challenge during postirradiation cultivation in amino acid medium. (*A*) Total surviving cell count, thymine continuously present; (*B*) as *A* except thymine omitted for first 35 min; (*C*) mutant frequency with thymine present continuously; and (*D*) mutant frequency when thymine added after 35 min.

of RNA which otherwise, in the presence of amino acids, would have acted as a mutagen. There is as yet, however, little evidence to exclude the involvement of DNA in the mutation frequency decline. The possibility remains that the course of mutation frequency decline might be dependent upon the amount of DNA synthetic activity during the labile postirradiation period.

Several experiments were performed to determine the rate and extent of mutation frequency decline in the presence and absence of thymine. Logarithmic growth phase cells were obtained in liquid medium supplemented with amino acids. The cells were washed, irradiated to approximately 20 per cent survival, and the suspension was then divided into two equal portions. One portion was subcultured at 37 C with continuous shaking in minimal liquid medium. The other portion was handled in the same fashion except that the culture medium lacked thymine. At zero time and at regular intervals thereafter samples of each culture were removed, washed, and diluted for plating. Total counts were determined by plating on agar supplemented

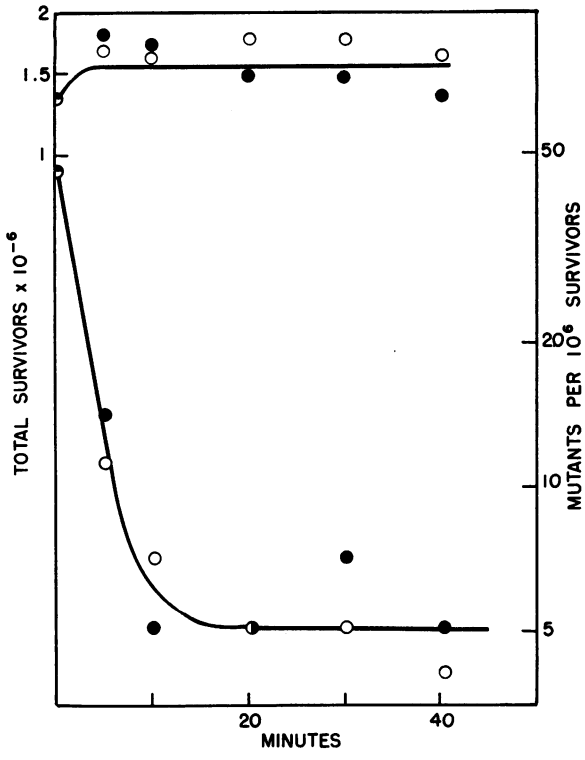

Figure 3. Mutant frequency decline during incubation in medium without amino acid, thymine present or absent. *Upper curve:* total viable cell count (*open circles*, thymine absent; *closed circles*, thymine present). *Lower curve:* mutant frequency (*open circles*, thymine absent; *closed circles*, thymine present).

with amino acids and mutant counts were determined on supplemented mutant agar. Results of a typical experiment are shown in figure 3. During 40 min incubation the total counts in the thymine-containing and thymineless cultures were constant and equal. The curves of mutant frequency decline illustrate a precipitous loss of potential mutants from approximately 45 per million survivors to less than one-tenth that value in 10 min. The presence or absence of thymine had no influence on the rate or extent of the mutation frequency decline. It may be objected at this point that the irradiation alone might have blocked DNA synthesis, leaving the cells indifferent to the presence or absence of thymine until that block could be surmounted. The demonstration, however, of the thymine effect in the mutation fixation experiments indicates that in the present experiment, in which the same ultraviolet dose was used, there is indeed a difference in the two cultures with respect to DNA synthesis.

The results described in this paper are interpreted as supporting the general hypothesis that ultraviolet-induced mutation is predominantly brought about through an indirect mechanism. Tentatively, it is postulated that some cellular constituents, possibly the RNA precursors proposed by Doudney and Haas (1959), are chemically altered by the absorption of ultraviolet energy. These mutagenic poisons then require the stabilizing presence of a complete amino acid mixture until the synthesis of new DNA has served to copy, incorporate, or otherwise fix a new and mutant configuration of the genome. In the absence of stabilizing amino acids, the mutagen is rapidly (figure 3) dissipated or diverted in a nonmutagenic fashion by a mechanism otherwise unknown but not involving DNA synthesis. These conclusions differ from those of Doudney and Haas (1959). Those authors investigated the mutational reversion of strain WP2 of *E. coli* from tryptophan dependence to independence. Curves of mutation

fixation were obtained by following the acquisition by the irradiated cells of resistance to the antimutagenic action of exposure to chloramphenicol. Simultaneous chemical determinations showed that the fixation curve obtained in this way could be superimposed on the curve of RNA increase in the culture. The initiation of measurable DNA synthesis lagged far behind in spite of the relatively low ultraviolet dose employed (75 per cent survival). From these data, Doudney and Haas concluded that mutation fixation as defined by resistance to chloramphenicol requires not DNA synthesis but RNA synthesis. This difference in results is surprising, since it had been tentatively assumed that the effects of chloramphenicol and of amino acid deprivation were the same, namely the blockage of protein synthesis. If the difference can be confirmed, preferably in a single strain, it will serve to divide the mutational process into further stages.

SUMMARY

An arginine-requiring mutant of the thymine-requiring *Escherichia coli* strain 15T⁻ was examined for ultraviolet-light-induced back mutation to arginine independence. During starvation for thymine when no deoxyribonucleic acid (DNA) was being synthesized and upon thymine supplementation when DNA synthesis was proceeding rapidly, the cells remained unaltered in their normal sensitivity to ultraviolet-induced mutation.

The fixation of mutation in a form no longer dependent upon the presence of an amino acid supplement was shown to be dependent upon the presence of thymine and thus of DNA synthesis.

The decline in mutant frequency brought about by incubation of irradiated cells in the absence of amino acids was shown to occur independently of the presence or absence of thymine.

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