SOME BIOCHEMICAL FACTORS IN X-RAY-INDUCED MUTATION IN BACTERIA

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Several years ago Witkin (1956, 1958) presented evidence which suggested that, in the case of ultraviolet light (UV) induced mutation in bacteria, “the time interval between the absorption of radiant energy and the production of stable genetic change can no longer be regarded as infinitesimal.” She pointed out that many postirradiation treatments alter the ultimate fate of a potential genetic change. Witkin suggested that protein synthesis is involved in mutation induction by UV since she found that amino acid supplementation increases mutation frequency response and that chloramphenicol, an antibiotic which inhibits protein synthesis, appreciably lowers the mutation frequency obtained with a given dose of UV. This type of postirradiation response was also found with ionizing radiations (KADA, BRUN and MARCOVICH 1960) as would be predicted by the existence of an appreciable “phenotypic” delay in X-ray-induced mutation (DEMEREC and LATARJET 1946). It is evident that the involvement of protein synthesis in mutagenesis must be restricted to certain mutation induction mechanisms since other mutations are not affected by identical postirradiation treatments (WITKIN and THEIL 1960).

Haas and Doudney (1957) presented evidence suggesting that the chemical basis for UV-induced mutation may involve nucleic acid precursors altered in vivo by UV. Subsequent studies by these workers indicate that ribonucleic acid (RNA) synthesis as well as protein synthesis is involved in the mutation induction process. It was found that various postirradiation treatments specifically affecting RNA or protein synthesis are effective in lowering markedly the mutation frequency response to ultraviolet light (Doudney and Haas 1958, 1959). Treatments of short duration reduced mutation frequency to a low level without significant modification of the time or rate of subsequent syntheses of RNA, deoxyribonucleic acid (DNA) and protein (Doudney and Haas 1960a,b). The evidence thus suggests that the mutation frequency decline process promoted by these specific agents is an active process, probably enzymatically mediated, and not dependent on delay in gross postirradiation macromolecular synthesis. The progressive loss of susceptibility of the potential mutations to these treatments is closely correlated with the progression of RNA synthesis in the culture. A rela-

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2 Postdoctoral Research Fellow supported by research training grant CRT-5047 from the National Institutes of Health.
tion exists between the amount of RNA synthesized at the time of chloramphenicol addition, the relative rate of deoxyribonucleic acid (DNA) synthesis in the presence of chloramphenicol and the induced mutation frequency observed after chloramphenicol challenge. To account for these facts the following hypothesis was proposed (Doudney and Haas 1960b): Recovery of the capacity to synthesize DNA after UV exposure requires the synthesis of RNA and protein; apparently the necessary RNA and protein must be formed prior to chloramphenicol addition in order for DNA to be replicated with consequent establishment of the mutation in the genome. When this mandatory RNA and protein synthesis has not occurred prior to chloramphenicol addition, the potentiality for mutation is lost, presumably through the active decline process. Thus the synthesis of RNA and protein has an active role in “fixing” the “potential mutation” prior to the synthesis of the new complement of DNA. The nature of the function of the RNA and protein in genetic replication and mutational change remains to be determined.

Recent studies strongly suggest that DNA synthesis is the terminal step in the mutation induction process. Loss of mutation photoreversibility is correlated with DNA synthesis in the culture (Haas and Doudney 1960; Doudney and Haas 1960b). It thus appears that the initial synthesis of DNA after UV exposure is the “copying” event leading to a photostable error in the genome. The phenotypic expression of UV-induced mutation to prototrophy is correlated with DNA synthesis in the culture (Haas and Doudney 1959). Mutation expression will not occur in the absence of DNA synthesis (Doudney and Haas 1960b). Furthermore, protein synthesis must follow DNA synthesis for phenotypic expression of the induced mutation (Haas and Doudney 1959). Chloramphenicol blocks mutation expression when added to the culture immediately following the initiation of postirradiation synthesis of DNA while allowing DNA synthesis to proceed. It thus appears probable that the functional mutated gene is formed with the initial replication of DNA following UV exposure but that protein synthesis is required for expression of the modified genetic character.

This paper describes research on the induction of mutation by X-rays utilizing techniques similar to those used in the UV-induced mutation studies. The effort is directed toward characterization of the processes involved in X-ray-induced mutation in relation to the postirradiation macromolecular synthetic activities of the irradiated bacteria. Some preliminary aspects of this study have been previously described (Kada, Doudney and Haas 1960).

**MATERIALS AND METHODS**

_Bacteriological techniques:_ An auxotrophic strain of _Escherichia coli_ (strain WP2) requiring tryptophan for growth was utilized in these studies. This strain (hereafter called the try strain) was kindly provided by Dr. Evelyn Witkin several years ago. In addition, several recently isolated auxotrophic strains requiring various amino acids were utilized. These strains were isolated after UV
irradiation of E. coli strain 15 thy, a thymine requiring strain.¹ These strains require (in addition to thymine) either proline, methionine, or both methionine and tyrosine. All strains are kept in stock culture on nutrient agar slants in the cold. Growth of the cultures for experimental purposes is in synthetic media containing 20 μg per ml of the required amino acid and, in case of the thymine-requiring strains, 20 μg per ml of thymine. Growth is for 16 hours at 37°C under vigorous aeration.

The composition of the synthetic minimal medium used is as follows: (NH₄)₂SO₄, 1.0 g; KH₂PO₄, 3.0 g; MgSO₄·7H₂O, 0.1 g; sodium citrate, 0.5 g; K₂HPO₄, 7.0 g; 1000 ml of water; glucose, 0.2 percent, sterilized separately.

The 16 hour culture is held at 6°C for one hour, for purposes of culture synchronization. The bacterial cells are then washed two times, by centrifugation and resuspension, with cold 0.9 percent saline and then resuspended in cold minimal medium without glucose (the volume being adjusted to about 1.5 × 10¹⁰ cells per ml).

Exposure of the bacteria to X-rays is accomplished as follows: 10 ml quantities of the cell suspension are put into 50 ml Erlenmeyer flasks. Each flask is placed in the middle of a specially designed plastic container and packed in ice. The plastic container is fitted directly over the apperature of the X-ray machine. The radiation factors for the General Electric “Maxitron 250” machine used are as follows: 250 kvp, 30 ma, 1 mm of aluminum added filtration. The dose rate in air at the locus of the cells is approximately 2860 r per minute.

The typical experiment is accomplished as follows: The bacterial cells are irradiated, usually with 10 kr of X-rays, resulting in a level of survival of approximately 20 percent. The ice-cold suspension of irradiated cells is then diluted 50 times with fresh, warm (37°C) minimal medium supplemented with a mixture of amino acids (DL-alanine, L-arginine, DL-aspartic acid, L-cysteine hydrochlorate, L-glutamic acid, glycine, L-hystidene hydrochrole, DL-isoleucine, DL-leucine, L-lysine hydrochrole, DL-methionine, DL-phenylalanine, L-proline, DL-serine, DL-threonine, L-tyrosine, L-tryptophan and DL-valine). Amino acids were present in the liquid medium at a concentration of 10 μg per ml except for the essential amino acids of the strain used (50 μg per ml). The antimetabolites, chloramphenicol (10 μg per ml) or 6-azauracil (50 μg per ml), are added to this medium in several experiments, as indicated. The cultures are then incubated, with satisfactory aeration, in Erlenmeyer flasks placed on a rotary shaker at 37°C. At intervals (usually ten minutes) a portion of the cell suspension is removed, chilled quickly in an ice bath and then washed two times with cold 0.9 percent saline solution. The cells are finally concentrated by resuspending in cold minimal medium (the volume being reduced by twenty to fiftyfold as compared to the irradiated incubation suspension). Samples of the cell suspensions (usually

¹ This strain is commonly referred to in the literature as 15—. The 15 thy designation is adopted in this paper, as being more appropriate to a commonly accepted standard nomenclature in bacterial genetics.
0.1 ml) are plated on two basic sorts of agar medium, for purposes of assaying mutations (HAAS and DOUDNEY 1959):

(a) Minimal agar medium without supplementation. This medium permits the formation of colonies from bacterial cells whose prototrophic character is already established at the time of plating.

(b) Minimal agar medium supplemented with 2.5 percent nutrient broth. This medium permits the metabolic processes involved in mutation induction and depending on amino acids to take place and also provides for the phenotypic expression of the mutated cells through provision of the required amino acid.

In case of the thymineless, amino acidless polyauxotrophs, these media are supplemented with the required growth factors, other than the requirement which is under study for reversion.

All plates are incubated for three days at 37°C and the colonies scored. The frequency of mutation is then calculated, taking into account the titre of viable cells determined on the supplemented agar medium after suitable dilution of the cell suspension, and the spontaneous mutations which exist both in the suspension before irradiation and after residual growth on the mutation assay medium used (either a. or b.). In general, the number of surviving bacteria plated for purposes of scoring induced mutations ranged between $4 \times 10^8$ and $7 \times 10^8$ per plate for the try strain and between $1 \times 10^8$ and $3 \times 10^8$ for strain 15 thy met tyr. Identical unirradiated controls were plated in order to determine the number of spontaneously-arising revertant bacteria per plate. This number ranged between 0–10 colonies per plate on minimal agar medium and 10–25 colonies per plate for the minimal agar medium supplemented with nutrient broth. This figure was subtracted from the total number of colonies observed with the irradiated cells (which ranged between 100 and 300 colonies per plate) before calculation of the induced-mutation frequency. Survival is determined by plating at an appropriately higher dilution on minimal agar medium containing 2.5 percent nutrient broth.

Chemical determinations: Culture samples, taken from the incubated suspension for analysis of RNA, DNA and protein are precipitated with 0.25 N perchloric acid in the cold. The nucleic acids are hydrolyzed by incubation in 0.5 N perchloric acid for 50 minutes at 70°C. Analysis for DNA is done by the diphenylamine method of BURTON (1956). For the evaluation of RNA, the ultraviolet absorption at 260 m$\mu$ and 290 m$\mu$ is determined (VISSER and CHARGAFF 1948), and the amount of DNA, as determined by BURTON analysis of the same sample, is then subtracted with correction for extinction coefficients. Protein is determined by the FOLIN method (LOWRY, ROSEBROUGH, FAAR and RANDALL 1951).

EXPERIMENTAL RESULTS

Mutation expression and deoxyribonucleic acid synthesis: HAAS and DOUDNEY (1959) have shown a close correlation following UV exposure of the try strain
between recovery of DNA synthesis and mutation expression as measured by plating on unsupplemented minimal agar medium. They irradiated the cells with UV and then incubated in minimal medium supplemented with casein hydrolysate and tryptophan, the required amino acid. Under these conditions, the delay in DNA synthesis induced by irradiation is of sufficient duration to distinguish the initiation of synthesis of this macromolecule from the initiation of other macromolecular synthetic activities, such as RNA synthesis. By plating at periodic intervals on minimal agar medium, it was determined that a great majority of the ultraviolet-induced mutations become expressed (i.e., capable of forming colonies on minimal agar medium) only following DNA replication.

Experimental conditions which permit the realization of similar experiments in X-ray-induced mutation were devised. Synchronized cultures were irradiated with a dose of ten kr of X-rays and incubated at 37°C in minimal medium supplemented with a mixture of amino acids prior to plating (Figure 1). There is no measurable increase in the amount of DNA during the first ten-minutes incubation. During this period the synthesis of RNA and protein proceeds. Samples of the culture at proper dilution were plated either on minimal agar medium supplemented with 2.5 percent nutrient broth, or on unsupplemented

![Figure 1](image-url)

**Figure 1.**—Mutation induction and expression in *E. coli* strain WP2 following exposure to 10 kr X-rays. The culture was incubated, following exposure, in minimal medium supplemented with a mixture of amino acids including the required amino acid, tryptophan, and then plated at appropriate dilution at the indicated times, either on minimal agar medium (mutation expression) or on minimal agar medium supplemented with 2.5 percent Difco nutrient broth (mutation induction). The DNA curve represents the amount of DNA, relative to unincubated controls, which has been formed at the times of plating.
minimal agar medium. The results demonstrate that, in the first ten minutes, an appreciable portion of the total yield of mutations is expressed, as measured on minimal agar medium. These results are quite different from the results observed with UV, where only a very small proportion of the total yield of induced mutations are expressed prior to the initiation of DNA synthesis in the culture (Haas and Doudney 1959).

**Experiments with thymineless, amino acidless polyauxotrophs:** Synchronized cultures of strains 15 thy met and 15 thy pro were carefully washed with minimal medium to eliminate contaminative thymine and were then irradiated with 5 or 10 kr of X-rays. The irradiated cells were incubated at 37°C in a medium containing amino acids, including those essential for each strain, but no thymine. The cells were then harvested and plated on minimal medium supplemented with thymine, after washing to remove the amino acids. In the strains tested, a net increase in mutation frequency was observed, compared to that found prior to postirradiation thymineless incubation (Table 1). By measurements before and after incubation, it was determined that measurable net DNA synthesis did not occur during this period. It is to be noted that RNA and protein increase during this period while DNA synthesis is blocked.

Additional studies were carried out with a polyauxotroph requiring thymine, methionine, and tyrosine (Figure 2). The culture was exposed to 10 kr of X-rays, then incubated in medium containing a mixture of amino acids including methionine and tyrosine but no thymine. Samples of this culture were plated, after washing, on minimal agar medium supplemented with methionine and thymine and on minimal agar medium supplemented with methionine, thymine and 2.5 percent nutrient broth. Approximately half of the induced prototrophs (tyr+) are expressed with 20 minutes incubation. The control cells plated on minimal plus nutrient broth indicate the maximum mutation response observed. An additional 20 minutes incubation in the absence of thymine produced no more expressed prototrophs. This experiment makes it evident that, while approximately half of the prototrophs induced may be expressed in the presence of the required amino acid without the thymine required for DNA synthesis, the other half of the induced prototrophs require thymine, in addition to the amino

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<table>
<thead>
<tr>
<th>Strain</th>
<th>Minutes of incubation</th>
<th>Mutation frequency per 10^6 survivors</th>
<th>Relative amount</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>DNA</td>
</tr>
<tr>
<td>15 thy pro</td>
<td>0</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>5.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>3.0</td>
<td>0.9</td>
</tr>
<tr>
<td>15 thy met</td>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
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<tr>
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<td>30</td>
<td>30.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The preplating postirradiation incubation medium contained a mixture of amino acids including the amino acid required by the strain (methionine or proline) but no thymine. Plating was on minimal agar medium supplemented with thymine.

**Mutation expression in the absence of thymine in two biauxotrophic strains of Escherichia coli**
The effect of blockage of DNA synthesis through thymine deprivation on mutation induction and expression in *E. coli* strain 15 thy met tyr, a polyauxotroph requiring thymine, methionine and tyrosine, after exposure to 10 kr X-rays. The tyrosine requirement reversion was followed. The culture was incubated, following exposure, in minimal medium supplemented with a mixture of amino acids but with no thymine added. Samples at appropriate dilution were plated at the indicated times on minimal agar medium supplemented with thymine and methionine (mutation expression, black bars) or minimal agar medium supplemented with thymine, methionine and 2.5 percent Difco nutrient broth (mutation induction, light bars). In Experiment No. 2 the culture was starved for thymine in the above described incubation medium for 20 minutes prior to X-ray exposure.

Acid, for expression. In this strain, that proportion of the mutants expressed in the absence of thymine may correspond to that half of the mutants expressed before measurable DNA synthesis in the try strain.

The possibility exists that there is sufficient endogenous thymine retained in the irradiated bacterial cell under these conditions to allow synthesis of the DNA which might be required for mutation induction even in the absence of added thymine. In order to eliminate this possibility, the bacterial culture was starved in medium without thymine for 20 minutes prior to radiation exposure, and the above experiments repeated. As is demonstrated in Figure 2, the results are practically identical to the results obtained in the case of cells not starved for thymine prior to radiation exposure. Approximately one half of the cells are expressed in 20 minutes in absence of thymine. Further incubation in the absence of thymine does not lead to additional expression of the prototrophic phenotype.

Another experiment was carried out along these lines with the same strain. However, in this case, after 40 minutes incubation thymine was added to the
preplating liquid growth medium and both mutation expression and DNA synthesis followed (Figure 3). In this experiment incubation for 20 minutes was sufficient for expression of half of the mutations. Incubation was continued for an additional 20 minutes in absence of thymine, without further expression. The mutation frequency then doubles in the next 20 minutes following the addition of thymine and initiation of DNA synthesis. Figure 3 further demonstrates that there is no measurable net DNA synthesis in 40 minutes in the absence of thymine, but that when the thymine is added, DNA synthesis is initiated in correlation with the increased mutation response. These experiments support the hypothesis that, while approximately one half of the mutations induced by X-rays at this locus can be expressed phenotypically in the absence of DNA synthesis, the other half of the induced mutations require DNA synthesis for expression. The experiments do not eliminate the possibility that thymine derived from degradation of cellular DNA might feed minor new DNA synthesis, thus supporting expression in the absence of added thymine. The role of cellular turnover of DNA in the case of spontaneous mutation in stationary phase cultures has been studied (Ryan 1959). It was finally concluded that the mutations resulted from errors in replication of genetic DNA which was in the process of

![Figure 3](image-url)

**Figure 3.**—Mutation expression and DNA synthesis in the absence and presence of thymine in *E. coli* strain 15 thy met try, a polyauxotroph requiring thymine, methionine and tyrosine, after exposure to 10 kr X-rays. The tyrosine requirement reversion was followed. The culture was incubated, following exposure, in minimal medium supplemented with a mixture of amino acids, including methionine and tyrosine, the required amino acids, but with no thymine added. At 50 minutes incubation, thymine was added to the culture. Samples at appropriate dilution were plated at the indicated times on minimal agar medium supplemented with methionine and thymine. The 60 minute control was plated on minimal agar medium supplemented with methionine, thymine and 2.5 percent Difco nutrient broth. The DNA values given represent the DNA measured at the times of plating as compared to unincubated controls.
turnover in the nondividing cell. It is very difficult to establish definitive proof which eliminates the last possibility that DNA turnover is involved in the X-ray-induced mutation mechanism which proceeds in the absence of thymine. In our case, the incubation period necessary for mutation expression is very short and normal metabolism is more or less blocked or modified because of the irradiation. Breakdown of nucleic acids and release of mononucleotides into the surrounding medium has been observed with incubation or holding of cells exposed to X-ray, although the nature of the process is not clear. For example, STUY (1960) irradiated certain strains of E. coli with high doses of X-rays and observed that about half of the cellular DNA, determined by the diphenylamine method, is lost with incubation. In our case, the dose used is relatively low and the measured amount of DNA remains practically constant during the postirradiation incubation period. If there exists DNA degradation products which are excreted into the medium to provide the thymine necessary to feed DNA synthesis at a low level, they should be eliminated by incubating the cells in a medium which permits degradation of the nucleic acid, followed by washing the cells to remove the surrounding medium containing the degradation products prior to addition of the required amino acid to the culture. Experiments of this type, where the cells are incubated for a period in the absence of the required amino acid to allow any possible degradation of DNA to take place and then washed to remove hypothetical degradation products of DNA, were carried out. This treatment does not prevent the expression of the expected frequency of mutation in the absence of thymine when the required amino acid is added. The results suggest that the synthesis of DNA is blocked in the thymineless strain during thymine-free incubation and that the increase in mutation frequency occurs in the absence of DNA replication.

Thymineless induction of mutation: COUGHLIN and ADELBerg (1956) have described an effect of incubation in the absence of thymine on reversion of the histidineless locus of E. coli strain 15 thy his. They found that the frequency of prototrophic cells to viable bacteria increased considerably after incubating the cells in thymine-free synthetic medium. Although the absolute number of prototrophs remained constant during thymine-free incubation, some evidence was reported to eliminate the possibility that differential killing of auxotrophic cells, as compared to prototrophic cells, though thymineless death might account for the observed increase in mutation frequency.

In our experiments with X-ray-induced mutation of the tyrosineless locus in E. coli strain 15 thy met tyr the period of incubation in the thymine-free medium is too brief to induce thymineless death. It is improbable therefore that the observed increase in mutation frequency is due to an effect of the thymineless condition comparable to that observed by COUGHLIN and ADELBerg (1956). However, an experiment was undertaken with this strain in order to observe the effect of the thymineless condition on reversion of the tyr locus. The organism was incubated in the thymine-free minimal medium containing the required amino acids, methionine and tyrosine, producing considerable "thymineless
inactivation” (Table 2). In spite of the lethal effect, the mutations observed decreased at the same rate as the parent population and the mutation frequency per viable cell remained constant indicating that no change in mutation frequency is produced by the thymineless incubation. It is thus obvious, at least in the case of this strain, that we can ignore any possible contribution of thymineless-induced mutations or of selection of spontaneous prototrophs, through thymineless death, in the studies of X-ray-induced mutation in the thymineless condition. Further it is evident that little or no thymineless induction of mutation is observed at this locus, as was reported by COUGHLIN and ADELBERG (1956) in the case of reversion of the histidine requirement studied by them.

*Mutation expression and macromolecular synthesis:* HAAS and DOUDNEY (1960) suggested that, with an amino acid deficient strain, the fraction of induced reversions capable of phenotypic expression at a given time following application of the inducting agent can be determined by plating identical samples of the culture on minimal agar medium and on minimal agar medium supplemented with a low level of the required amino acid. It was assumed that the amino acid is necessary for the appearance of the missing enzyme involved in the synthesis of the amino acid required by the strain and controlled by the mutated gene. It was suggested that this technique may serve as a means for differentiating those processes involved in gene replication and mutation from those involved in gene action (i.e., phenotypic expression).

These techniques are utilized in the following studies of expression of X-ray-induced reversion of the try strain. A typically biphasic expression curve is found, as is often observed when the X-ray exposed cells are plated onto minimal agar medium (Figure 4). It is further demonstrated that either chloramphenicol, an antibiotic which blocks protein synthesis, or 6-azauracil, a uracil analog which blocks RNA synthesis as well as protein synthesis, prevents mutation expression. Further studies have demonstrated that holding the irradiated cells at 2°C prevents mutation expression. The absence of an energy source (glucose) or the addition of dinitrophenol also inhibits this phenomenon. The necessity of the essential amino acid, tryptophan, has been demonstrated in these studies.

**TABLE 2**

<table>
<thead>
<tr>
<th>Minutes of incubation</th>
<th>Percent inactivated</th>
<th>Colony-forming bacteria plated</th>
<th>Average number of tyr* colonies found per plate</th>
<th>Frequency of tyr* per 10^8 tyr bacteria</th>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>2.4 × 10^8</td>
<td>14</td>
<td>5.6</td>
</tr>
<tr>
<td>30</td>
<td>14.5</td>
<td>2.1 × 10^8</td>
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</tr>
<tr>
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<tr>
<td>90</td>
<td>68.4</td>
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<td>4.6</td>
</tr>
<tr>
<td>120</td>
<td>86.2</td>
<td>3.3 × 10^7</td>
<td>2</td>
<td>4.6</td>
</tr>
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</table>

The culture was incubated in minimal medium containing a mixture of amino acids including methionine and tyrosine for the indicated times and then plated on minimal agar medium supplemented with thymine and methionine. As determined by plate washing and replating techniques, no residual divisions of the tyr cells occurred on this medium after plating.
It is therefore concluded that the phenotypic expression of the gene modified by X-ray requires RNA and protein synthesis, in order to permit growth on minimal agar medium.

**Mutation induction and macromolecular synthesis:** It is evident from the above results that, if we are to study mutation induction as opposed to expression of the modified phenotypic character, we must by some means provide for those metabolic processes involved in phenotypic expression and requiring the amino acid in which the strain is phenotypically deficient in synthesis. This may be accomplished by plating on minimal agar supplemented with a low level of the amino acid required. In practice, the supplementation of 2.5 percent nutrient broth to the minimal agar medium has proved satisfactory to meet this need.

If we add chloramphenicol at the start of incubation and then incubate for increasing periods of time, no appreciable effect is observed on the frequency of mutations, when the cells are plated on minimal medium supplemented with nutrient broth containing the tryptophan required for expression (Figure 5). On the other hand, it is evident that chloramphenicol interferes with the expression of the mutations as measured by plating on minimal agar medium. Thus, we can conclude that, while most of the potential mutations are not susceptible
to chloramphenicol in the sense of being unstable immediately following exposure, their expression is prevented by chloramphenicol. Here again we have a departure from the results with UV where most of the mutations are sensitive to chloramphenicol when this antimetabolite is added immediately (Doudney and Haas 1958, 1959).

Next, the effect of adding chloramphenicol after a slight period of incubation (ten minutes) was studied. These results make it clear that a period of incubation involving protein synthesis is necessary for the mutations to become sensitive to chloramphenicol. Thus, when chloramphenicol is added at ten minutes incubation, about half of the total number of mutations are sensitive to chloramphenicol and are lost through some sort of "mutation frequency decline" process. The other half appear not to be sensitive to chloramphenicol and are not affected by incubation in the presence of chloramphenicol. It is further demonstrated that in the first ten minutes incubation, that half of the mutations which is not affected by chloramphenicol becomes expressed, as measured by plating on minimal agar medium. Thus, we can conclude that the half of the mutations which is not sensitive to chloramphenicol is comparable to that half which is expressed in the first ten minutes in the absence of DNA synthesis, as described
above. Thus after ten minutes incubation following exposure, the population of potential mutations may be divided into two classes rather equal in number: those which are completely expressed and are not sensitive to chloramphenicol and those which are not expressed and are sensitive to chloramphenicol.

**Effect of chloramphenicol and 6-azauracil on survival:** When either chloramphenicol or 6-azauracil is added immediately after X-ray exposure, a marked lethal effect is observed (Table 3). However, when these antimetabolites are added after ten minutes incubation following radiation exposure this lethal effect is not apparent. Thus, it is evident that, in ten minutes, certain synthetic processes have taken place in repair of the X-ray damage, which leaves the bacterial cell as typically insensitive to any lethal effects of chloramphenicol or 6-azauracil as unirradiated cells. The point to be emphasized, as far as the mutation studies are concerned, is that the changes in the survival produced by these compounds are not correlated with the genetic effects observed. Thus, while the level of survival drops considerably when either chloramphenicol or 6-azauracil is added immediately following radiation exposure (Table 3), the mutation frequency remains constant (Figure 5). On the other hand, with addition of these compounds after ten minutes incubation, the level of survival remains constant while the mutation frequency changes markedly. It is evident therefore that the change in mutation frequency observed with these treatments begun after ten minutes is not merely a reflection of change in survival, nor is the stability of the potential mutations immediately following radiation exposure related to an effect of chloramphenicol or 6-azauracil on the survival of the cells.

**Chloramphenicol and 6-azauracil challenge:** A correlation between RNA synthesis and "mutation fixation" in UV-irradiated cultures has been previously described (DOUDNEY and HAAS 1959). Since it is apparent that susceptibility of the potential mutation to those treatments leading to lower mutation frequency is lost with increasing periods of postirradiation incubation (WITKIN 1956; DOUDNEY and HAAS 1958), it is evident that blockage to RNA or protein forma-

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### Table 3

**Effect of chloramphenicol and 6-azauracil on survival of X-irradiated Escherichia coli strain WP2**

<table>
<thead>
<tr>
<th>Minutes of incubation</th>
<th>Chloramphenicol Added at &quot;0&quot;'s time</th>
<th>Chloramphenicol Added after 10 minutes</th>
<th>6-Azauracil Added at &quot;0&quot;'s time</th>
<th>6-Azauracil Added after 10 minutes</th>
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<tr>
<td>0</td>
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<td>40</td>
<td>$1.6 \times 10^7$</td>
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<td>50</td>
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</tr>
<tr>
<td>60</td>
<td>$1.3 \times 10^7$</td>
<td>$8.6 \times 10^7$</td>
<td>$6.5 \times 10^7$</td>
<td>$1.4 \times 10^8$</td>
</tr>
</tbody>
</table>

The irradiated cells, divided into four cultures, are incubated in minimal medium supplemented with a mixture of amino acids including tryptophan. Chloramphenicol or 6-azauracil was added to a selected culture either at "0"'s time or after ten minutes incubation. Appropriately diluted samples were plated from each culture at the indicated times on minimal agar medium supplemented with 2.5 percent Difco nutrient broth.
tion leads to a decline in mutation frequency only provided some significant metabolic event has not taken place. This event we have termed "mutation fixation." Mutation fixation is defined specifically, though arbitrarily, as that biosynthetic event which removes the potential mutation from susceptibility to those treatments which promote the specific decline process involved. It should be made clear that mutation fixation is not regarded as the final step in the mutation induction process. Rather, the final step in mutation induction by UV unquestionably appears to be the initial postirradiation synthesis of the genetic complement of DNA.

The time required for mutation fixation can be quantitatively measured by short term challenge with chloramphenicol or other agents or conditions which interfere specifically with protein synthesis or RNA synthesis. This challenge procedure demonstrates that fraction of the potential induced mutations "fixed" at any given interval following UV exposure, in the sense that they are not susceptible to the specific mutation frequency decline process. Following the challenge period, which is carried out in liquid medium, plating is on minimal agar medium supplemented with 2.5 percent nutrient broth. This contains

![Graph](image)

**Figure 6.**—Effect of chloramphenicol challenge on mutation induction and expression in *E. coli* strain WP2 following exposure to 10 kr X-rays. Several identical cultures were incubated, following exposure, in minimal medium supplemented with a mixture of amino acids including the required amino acid, tryptophan. At the indicated times chloramphenicol was added to a selected culture and incubation continued in the presence of chloramphenicol for 50 minutes, a period of time sufficient to eliminate all chloramphenicol sensitive mutations. Following this period of incubation appropriately diluted samples of the cultures were plated on minimal agar medium (mutation expression) or minimal agar medium supplemented with 2.5 percent Difco nutrient broth (mutation induction).
sufficient tryptophan for the protein synthesis necessary for mutation expression, and thus eliminates mutation expression from consideration in the studies.

In Figure 6, a similar study of X-ray-induced mutation with the try strain using essentially the same techniques is demonstrated. The technique consists of adding chloramphenicol after different periods of incubation in minimal medium supplemented with amino acids, including tryptophan, and then continuing the incubation for 50 minutes prior to plating, a period of time sufficient to interfere with all chloramphenicol sensitive mutations. As this figure shows, and in agreement with the above described results, in the first ten minutes half of the mutations become sensitive to chloramphenicol. This sensitivity remains constant for some twenty minutes. Sensitivity to chloramphenicol is rapidly lost with further incubation. Similar results have been obtained using 6-azauracil (Table 4). Thus, it appears that RNA and protein synthesis are involved in the induction of this proportion of the X-ray-induced mutations, as is the case with UV-induced mutations.

The lower curve in Figure 6 demonstrates the results of treating samples of the culture in an identical manner with chloramphenicol, but plating on minimal agar medium instead of minimal medium supplemented with nutrient broth. This curve thus demonstrates only those prototrophs which, at the time of plating, are both expressed and insensitive to the chloramphenicol challenge. It can be seen that during that period in which one half of the mutations become sensitive to chloramphenicol, the other half are rapidly becoming expressed. In addition it is evident that expression of the half of the induced mutations sensitive to chloramphenicol follows the loss of sensitivity of these mutations to chloramphenicol.

DISCUSSION

Several recent investigations suggest that UV induces mutation through at least two mechanisms (Witkin and Theil 1960; Kada, Brun and Marcovich 1960). In one type of mutation induction process, postirradiation treatments of

### TABLE 4

Effect of 6-azauracil challenge on mutation induction and expression in Escherichia coli strain WP2 following ten kr X-rays

<table>
<thead>
<tr>
<th>Time of addition of 6-azauracil (minutes)</th>
<th>Prototrophs per 10^7 survivors</th>
<th>Plated on minimal medium plus 2.5 percent nutrient broth</th>
<th>Plated on minimal agar medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>28.0</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>17.4</td>
<td>11.1</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>28.9</td>
<td>22.1</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>33.0</td>
<td>23.7</td>
<td></td>
</tr>
</tbody>
</table>

The X-irradiated cells in several identical cultures were incubated in minimal medium supplemented with a mixture of amino acids including tryptophan. At the indicated times 6-azauracil was added to a selected culture and incubation continued for 20 additional minutes (a period of time sufficient to eliminate all 6-azauracil sensitive mutations). Appropriately diluted samples were then plated on minimal agar medium supplemented with 2.5 percent Difco nutrient broth or on minimal agar medium.
various sorts (and specifically the interruption of RNA or protein synthesis) interfere with mutation induction. With this type of mutation, we may conclude that the initial chemical damage produced by the ultraviolet light is unstable and is subject to modification by postirradiation metabolic processes. It is clear from recent investigations of the mechanism of induction and expression of this type of mutation that while processes upon which mutation induction depends occur prior to DNA synthesis, the final step in mutation induction involves the synthesis of DNA (Haas and Doudney 1959). The expression of the phenotypic change which is the product of the induced mutation follows the initial post-irradiation synthesis of DNA and is dependent on protein synthesis. It has been suggested that the dependence of mutation expression on protein synthesis following DNA synthesis is related to the appearance of the missing enzyme involved in synthesis of the required amino acid and controlled by the mutated gene in the new DNA (Haas and Doudney 1959). The other type of UV induced mutation is not influenced significantly by postirradiation metabolic activities, but Witkin and Theil (1960) have inferred an involvement of protein synthesis in phenotypic expression of this type of mutation.

A similar division into at least two classes of mutations based on the nature of the induction mechanism appears to hold for X-ray-induced mutation. While a portion of X-ray-induced mutations are influenced by postirradiation conditions, another portion appear to be refractory to these treatments (Haas and Doudney 1958; Kada, Brun and Marcovich 1960). In the case of the two mutational sites studied here (reversion of the tyr locus in strain WP2 and reversion of the tyr locus in strain 15 thy met tyr) the effect of X-ray in inducing mutations seems to be divided rather equally between two mechanisms comparable to those described above. One type of X-ray-induced mutation mechanism seems to be unstable with various postirradiation treatments and dependent on DNA synthesis for induction while the other type appears to be refractory to postirradiation treatments and not to require DNA synthesis for induction.

These data support the hypothesis that the functional reversion is produced by X-ray in the gene without the necessity of DNA replication in one half of the X-ray-induced mutations. In experiments with the tyr reversion in strain WP2, incubation for ten minutes with tryptophan was sufficient to permit complete expression of half of the mutations, as measured by plating on minimal agar medium. During this incubation, RNA and protein synthesis proceed, but there is no measurable DNA synthesis. In the case of strain 15 thy met tyr, the tyr+ reversion took place in the absence of DNA synthesis in thymine-free medium in a comparable time period. It was shown that thymine starvation itself does not cause the increase in mutation frequency observed in this strain and further that endogenous thymine in the cell at the time of radiation does not seem to play any role in the observed increase in mutation frequency. The results make it clear that RNA synthesis and protein synthesis are involved in phenotypic expression of this type of mutation following X-ray exposure.

Since these studies seem to eliminate the possibility of participation of post-
irradiation DNA synthesis in mutation induction for this half of the induced mutations, it may be that the mechanism of action of the ionizing radiation in this case is by direct action on genes in situ. Thus, the classical notion of action of radiation in inducing mutations, based on direct hits of the radiation on genetic material, may be still valid for that portion of the mutations induced by ionizing radiation which do not require postirradiation metabolism for induction. It is interesting to note that the target size calculated for induction of reversion in strain WP2 is on the order of 10–30 atoms (Kada, Brun and Marcovich 1960). This was also found to be true for mutation to bacteriophage resistance of E. coli strain B/r (Demerec and Lajaret 1946). Therefore, it is not difficult to imagine that X-ray might produce mutation through direct physical action at the level of the functional structure of the gene, although the detailed physicochemical aspects of the process are not clear.

The similarity of mechanism of induction of half of the mutations induced by X-ray to those induced by UV is obvious. The increase in mutation frequency as measured by plating on minimal agar medium is dependent on the onset of new DNA synthesis in the case of this half of the X-ray-induced mutations, as was the case with the UV-induced mutations. Mutation expression is complete in both cases before the first postirradiation cell division. Furthermore, in view of the comparable effects of chloramphenicol and 6-azauracil, it is clear that RNA and protein synthesis are involved in the mutation induction process with both types of radiation.

This similarity in the results with the two types of radiation does not necessarily mean an identity of the primary sites of mutation induction. Kada and Marcovich (personal communication) have carried out some experiments which favor the hypothesis of difference in primary sites for UV and X-ray-induced mutation. They have irradiated the cells under various physiological conditions which would vary the content of nucleic acids. These observations, in addition to other radiobiological observations, have led them to the supposition that RNA components of the cell contain the site for UV-induced mutation and DNA for X-ray-induced mutation. In the case of X-ray induction of reversion of the try locus of strain WP2, it was necessary to incubate ten minutes for the potential mutation to become sensitive to chloramphenicol or 6-azauracil in the case of that half of the mutations which are comparable to UV-induced mutation. This makes it clear that the gaining of sensitivity to chloramphenicol or 6-azauracil of the potential X-ray-induced mutation depends on RNA and protein synthesis subsequent to X-ray exposure. The possibility exists that the modification induced by X-ray on some primary site (e.g., on one or both strands of DNA) is transferred, in terms of genetic specificity, to some other material which is sensitive, metabolically, to the addition of 6-azauracil or chloramphenicol with the consequent blockage of RNA or protein synthesis. One possibility is that some specific ribonucleoprotein formed under control of the X-ray-modified DNA carries genetically modified information and that this ribonucleoprotein could then be involved in postirradiation DNA replication in formation of the stable genetic
character. This hypothesis is comparable to the hypothesis proposed for UV-induced mutation by Doudney and Haas (1959), where some specific RNA modified by incorporation of a UV-modified nucleotide precursor would pass its mutational specificity on to newly formed DNA. These hypotheses are in agreement with a scheme for UV and X-ray-induced mutation, where a convergence of the two mutagenic mechanisms, coming from different primary sites, is supposed (Kada, Brun and Marcovich 1960).

In any case, it is clear that we do not know the primary site or nature of the damage leading either to X-ray or UV-induced mutation. Establishment of the nature of the primary damage in this type of UV or X-ray-induced mutation which is dependent upon DNA replication is required before we can arrive at an understanding of the mechanisms involved in mutation induction. While the studies of Haas and Doudney (1957) suggest that the potential UV-induced mutation is initially in the form of a mutagenic nucleic acid precursor formed by the action of UV on purine or pyrimidine containing monomers, no conclusive evidence exists to support this hypothesis. The possibility remains that the initial UV damage is to the cellular DNA or to other sites (Doudney and Haas 1960a). Similar reservations concerning the nature of the primary site of X-ray-induced mutation must be retained.

This investigation makes it clear that one of the mechanisms of X-ray-induced mutation involves DNA replication in the irradiated cells as well as RNA and protein synthesis. The involvement of RNA and protein synthesis in DNA replication following UV exposure has been demonstrated (Harold and Ziporin 1958; Doudney 1959; Drakulic and Errera 1959; Doudney and Haas 1960a,b). The situation in the case of DNA replication following X-ray exposure is undoubtedly more complex though there is some evidence that similar mechanisms may be involved (Doudney 1956). Upon present evidence, it cannot be concluded that DNA is capable of transferring its genetic information to RNA either after X-ray or UV-irradiation, though this possibility must be considered in the evolution of hypotheses for DNA replication and genetic change. Further basic studies are needed to clarify the meaning of the RNA and protein involvement in DNA synthesis in irradiated cells before it will be possible to understand the mechanism of mutation induction.

Recent experiments have shown that cells harvested during that postirradiation period that the potential X-ray-induced mutation is sensitive to chloramphenicol (i.e., between 10 and 20 minutes incubation after X-ray exposure) are much more sensitive, in terms of lethality, to a second period of irradiation with X-ray than at any other phase of postirradiation development of the culture. We would suppose that the X-ray irradiated cells are at a critical phase in the course of repair of their DNA replication mechanism during this period.

**SUMMARY**

Recent studies have established that various postirradiation treatments specifically affecting RNA or protein synthesis are effective in lowering markedly the
mutation frequency response to ultraviolet light. Several lines of evidence indicate that DNA synthesis is the terminal event in UV-induced mutation, but that phenotypic expression of the genetic change involves protein synthesis subsequent to DNA synthesis. About half of the X-ray-induced reversions of the tryptophan requiring auxotroph, *E. coli* strain WP2, are lost, when the culture is incubated for 50 minutes with chloramphenicol or 6-azauracil, agents which block RNA or protein synthesis. However, unlike UV reversion, it appears that a short period of incubation following X-ray exposure before addition of chloramphenicol or 6-azauracil is necessary for development of the chloramphenicol or 6-azauracil sensitivity of the mutations. These mutations require RNA synthesis, protein synthesis and DNA synthesis for induction in a manner comparable to UV-induced reversion. The other half of the X-ray-induced mutations are not lost when incubated with chloramphenicol or 6-azauracil for 50 minutes prior to plating on tryptophan containing medium. These mutations are completely expressed when plated on minimal medium without tryptophan after a short period of incubation in tryptophan containing medium and before measurable DNA synthesis in the culture occurs. However, expression does not occur in the presence of 6-azauracil or chloramphenicol. In the case of a thymineless, tyrosineless polyauxotroph expression of the tyrosine reversion occurs in the absence of thymine for one half of the mutations while the other half of the mutations requires thymine for expression. The data suggest that with that half of the mutations which can take place in the absence of thymine, the functional reversion is produced by the X-ray in the gene, without the necessity of DNA replication, but that RNA and protein synthesis is required for gene action in phenotypic expression of the reverted character.

**LITERATURE CITED**


