SOME BIOCHEMICAL ASPECTS OF THE POSTIRRADIATION MODIFICATION OF ULTRAVIOLET-INDUCED MUTATION FREQUENCY IN BACTERIA[1]

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WITKIN (1956, 1958) has cited considerable evidence which indicates that, in bacteria, ultraviolet light (UV) induced mutation is irreversibly established only after an appreciable delay. Her studies suggest that the induction process is completed during the period prior to the first postirradiation cell division and is dependent on the availability of amino acids. WITKIN suggested that protein synthesis is involved in mutation induction since she found that chloramphenicol (Chl), an antibiotic which blocks protein synthesis, appreciably lowers the mutation frequency obtained with a given dose of UV.

HAAS and DOUDNEY (1957) presented evidence suggesting that the chemical basis for UV-induced mutation involves nucleic acid precursors altered *in vivo* by UV. Later studies (DOUDNEY and HAAS 1958, 1959a) indicate that ribonucleic acid (RNA) synthesis, as well as protein synthesis, is involved in the mutation induction process. These results led to the suggestion that RNA and protein are involved in replication of deoxyribonucleic acid (DNA). As one possibility it was suggested that the RNA is modified through incorporation of the UV-altered purine or pyrimidine and that this modification in RNA specificity leads to a change in the genetic specificity of the DNA subsequently formed.

Recent studies (HAAS and DOUDNEY 1959, 1960) demonstrate that DNA synthesis is the final step in the mutation induction process. These studies further indicate that protein synthesis must follow DNA synthesis for phenotypic expression of the induced mutation. Chl blocks mutation expression when added to the culture immediately following postirradiation synthesis of DNA. It is clear that the *functional* mutated gene is formed with DNA replication.

Although the basis of UV-induced mutation has been investigated intensively during the last decade, a number of points remain to be elucidated before any hypothesis for mechanism can be established with certainty. In the first place, the nature of the initial receptor of the UV energy which leads to mutation must be established. While the data of HAAS and DOUDNEY (1957) is most readily interpreted as indicating the UV chromophore to be nucleic acid precursors present in the cell at the time of irradiation, the alternate hypothesis that the genetic material absorbs the UV which leads to mutation receives widespread

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support. Also in question is the biochemical basis for the reversibility (or prevention) of mutation following UV exposure (i.e., whether the lowering of mutation frequency by various postirradiation treatments is due to an active metabolic event or, conversely, to the physical or chemical "decay" of a "premutational state"). A third factor which must be clarified concerns the nature of the involvement of RNA and protein synthesis in mutation induction and in DNA replication.

The present report describes experiments designed to elucidate the nature of the processes leading to postirradiation decline in induced mutation frequency and the involvement of RNA and protein synthesis in DNA replication and in mutation induction. Efforts to characterize the nature of the mutational chromophore will be described in another report.

MATERIALS AND METHODS

The mutations studied result in reversion of the tryptophan requirement of *Escherichia coli* strain WP2 ($tr^- \rightarrow tr^+$). The mutation assay media used was minimal agar medium supplemented with 2.5 percent Difco nutrient broth (DOUDNEY and HAAS 1958). The low level of amino acids supplied by the nutrient broth allows a somewhat limited degree of mutation induction, and the tryptophan present permits the unreverted tr^- cells to make enough divisions to develop small, but visible, colonies at proper dilutions, and also enables mutation expression in the reverted tr^+ cells. The techniques followed have been previously described (HAAS and DOUDNEY 1957; DOUDNEY and HAAS 1958, 1959a). Briefly they are as follows. The culture is grown for 15.5 hours at 37°C with aeration in 50 ml portions of minimal medium supplemented with 0.2 mg per ml of DL-tryptophan. It is then held at 6°C for one hour, the cells centrifuged down in the cold, and resuspended in fresh medium at 37°C. This culture is grown for an additional period of 50 minutes, then chilled to halt further growth. The cells are centrifuged down again, washed once in cold 0.9 percent saline, and finally resuspended in cold minimal medium. Unirradiated suspensions are adjusted to contain about 6×10^8 colony-forming organisms per ml. UV irradiation is for ten seconds at a distance of 30 cm (964 ergs per mm^2). After UV exposure the sample is diluted one to four into appropriately supplemented growth medium and incubated for the indicated time interval at 37°C on a reciprocal shaker prior to plating at appropriate dilutions onto mutation assay agar medium. The plates are incubated for three days at 37°C and scored for total survivors (tr^-) and induced revertants (tr^+). The usual precautions to prevent photoreversal are observed subsequent to UV exposure.

Culture samples, taken at appropriate intervals during the postirradiation incubation period for analysis of RNA, DNA and protein, are precipitated and washed with 0.5 N perchloric acid. Nucleic acids are hydrolyzed by incubation in perchloric acid for 50 minutes at 70°C (OGUR and ROSEN 1950). Analysis for DNA is that of BURTON (1956). For RNA determination, the ultraviolet absorption at 260 mμ and 290 mμ is determined (VISSER and CHARGAFF 1948), and the

amount of DNA, as determined by the Burton analysis of the same sample, is then subtracted with correction for extinction coefficients. Protein is determined by the Folin method (LOWRY, ROSEBROUGH and RANDALL 1951).

EXPERIMENTAL RESULTS

Mutation frequency decline and macromolecular synthesis: The concept has been advanced that the level of mutation frequency induced by a given UV dose is related to the timing of DNA synthesis following irradiation. One investigator (WITKIN 1956, 1958) proposed that delay in DNA synthesis would increase mutation since more time would be available for protein synthesis, presumably involved in mutation induction. Others proposed that delay in DNA synthesis would decrease mutation since more time would be available for a hypothetical unstable "premutational" state to revert to a state not leading to mutation induction (KIMBALL, GAITHER and WILSON 1959). Both points of view imply that DNA synthesis is the terminal event in mutation induction.

Previous work has demonstrated that UV does cause a delay in DNA synthesis (KELNER 1953). Recently, several workers have shown that resumption of DNA synthesis following UV exposure requires prior synthesis of RNA and protein (HAROLD and ZIPORIN 1958; DOUDNEY 1959; DRACULIC and ERRERA 1959). Incubation with inhibitors blocking either RNA or protein synthesis, prior to initiation of DNA synthesis, leads to an additional lag in DNA synthesis during the time the necessary RNA and protein is formed. Many of the agents and conditions which block RNA or protein synthesis also promote a marked lowering in the expected induced mutation frequency. Whether the mutation frequency decline promoted by these agents can be ascribed to delay in DNA synthesis, or to an active decline process promoted by the inhibitors and independent of delay in DNA synthesis, remains to be resolved. If a quantitative correlation can be established between the effect of these agents on mutation frequency and their effect in delaying DNA synthesis, then the hypothesis suggested by KIMBALL and co-workers will receive considerable support. However, if decline in mutation frequency is independent of the effect on DNA synthesis, the hypothesis that mutation frequency decline is an active and enzymatically conditioned process (DOUDNEY and HAAS 1958) will appear more plausible.

Results of experiments designed to differentiate between the alternatives posed above are presented in Figures 1, 2 and 3, and in Table 1. These experiments measure the effect of increasing periods of immediate postirradiation treatments which block RNA or protein synthesis on the mutation frequency and on the subsequent synthesis of DNA in the culture. The mutation frequency is assayed in this case when the relative amount of DNA has just doubled. However, it makes little difference when the sample is plated *after* the indicated interval of treatment since the mutation frequency observed upon plating remains at approximately the same level until the first cellular division (usually after 100 minutes incubation). Figure 1 demonstrates the effect of 10, 20 and 25 minutes incubation in the presence of the uracil analogue, 6-aza uracil. This analogue has

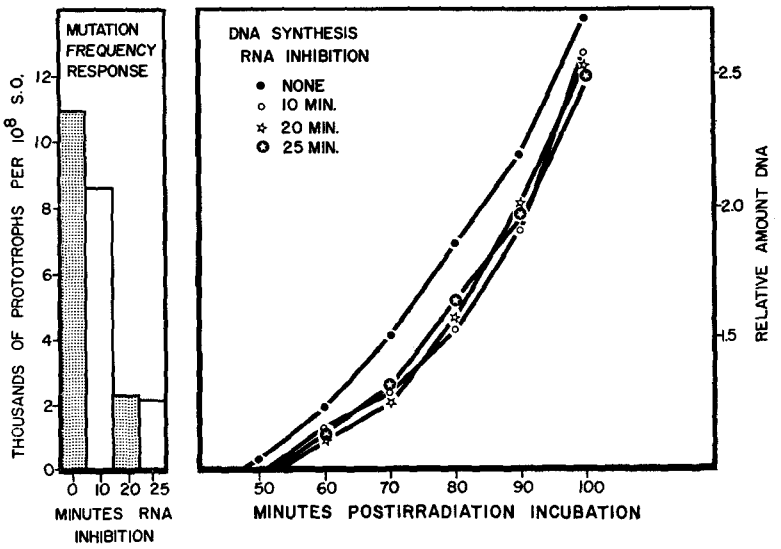

FIGURE 1.—Effect on yield of prototrophs, and on subsequent DNA synthesis in *Escherichia coli* strain WP2 (tryptophan requiring), of incubation with 6-aza uracil (5 mg per 100 ml medium) for varying time intervals following UV irradiation (*RNA inhibition*). The 6-aza uracil inhibition was reversed after the indicated period of incubation in liquid growth medium (minimal medium + casein hydrolysate, 2 mg per ml and 0.05 mg per ml DL-tryptophan) + 6 AU by the addition of uridine (5 mg per 100 ml). The increase in DNA relative to unincubated controls was followed during subsequent incubation. The times given are from the start of incubation following UV irradiation, and include both incubation periods (+ 6 AU and after reversal with uridine). Mutation frequency was assayed by plating after 80-minutes incubation, and prior to cell division (at 90–100 minutes), on nutrient broth supplemented mutation assay agar. Mutation frequency does not vary with plating from 20–90 minutes. No effect of 6 AU on survival was noted.

been shown to block RNA and protein synthesis and also to promote mutation frequency decline (DOUDNEY and HAAS 1959b). After the indicated intervals of incubation the effect of 6-aza uracil was reversed by the addition of uracil. Figure 2 demonstrates the effect of incubation of the culture for 10, 15, 20 and 25 minutes in the absence of nitrogen. This treatment also promotes rapid mutation frequency decline (DOUDNEY and HAAS 1958). After the indicated period of incubation, a mixture of amino acids is added to the medium. Figure 3 demonstrates the effect of depriving this tryptophan-requiring auxotrophic strain of tryptophan for 10, 20 and 25 minutes before adding the amino acid. Similar experiments carried out with chloramphenicol addition (Table 1), and with the addition of the amino acid analogue, β-thienylalanine (results not given), show comparable results. In the case of Chl addition, the cells were removed from the Chl by rapid centrifugation, washed and resuspended in fresh Chl-free medium.

These experiments demonstrate that a wide variety of conditions and agents which lead to lower mutation frequency through blockage of RNA or protein synthesis during the immediate postirradiation incubation period produce little

or no effect on subsequent DNA synthesis. There is a very slight delay in DNA synthesis in some experiments which seems to occur with "short-period" (five minute) holding of irradiated cultures under conditions where RNA or protein synthesis cannot take place. However, this delay is not correlated with the greater proportion of the mutation frequency decline observed. Only with treatment of at least 20 minutes duration is the maximum amount of decline in mutation frequency obtained. This decline is not associated with any further change in the pattern of DNA synthesis.

Similar results are observed for RNA and protein synthesis in the culture. Net RNA synthesis begins only after 25–30-minute postirradiation incubation, and net protein synthesis after 30-minute incubation. Treatments during the first 20 minutes which promote maximum mutation frequency decline do not appreciably modify the subsequent synthesis of RNA and protein by the culture.

Dinitrophenol (DNP), which interferes with oxidative phosphorylation, has been shown to prevent decline in mutation frequency (HAAS and DOUDNEY 1958; DOUDNEY and HAAS 1959a). This suggests that the decline process depends on energy yielding transformations. Table 1 demonstrates that, while the decline promoted by Chl is prevented by the addition of DNP, there is no effect of DNP on subsequent DNA synthesis. DNP interference with the decline in mutation

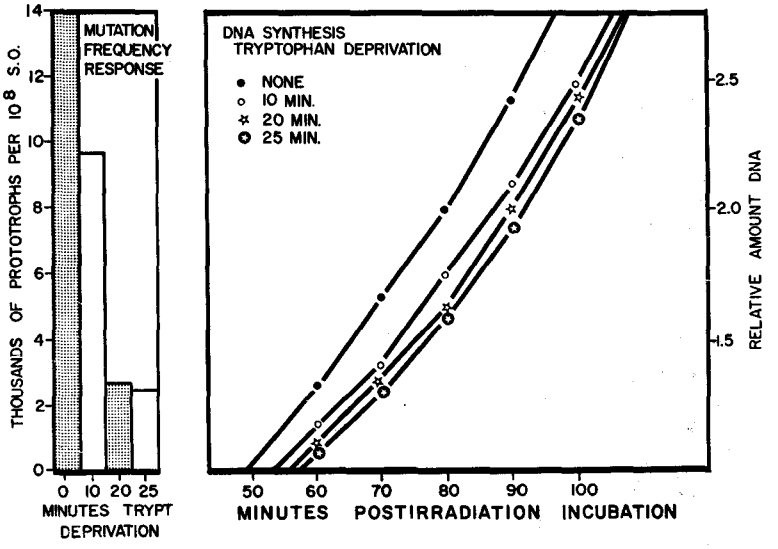

FIGURE 2.—Effect on yield of induced prototrophs, and on subsequent DNA synthesis in *Escherichia coli* strain WP2 (tryptophan requiring) of incubation without tryptophan for varying time intervals following UV irradiation. The incubation medium was minimal plus a mixture of 19 amino acids excluding tryptophan. DL-tryptophan was added (0.05 mg per ml) after the indicated intervals of incubation. The times given are from the start of incubation following UV irradiation, and include the incubation periods both without and with tryptophan. Mutation frequency was assayed by plating after 80-minutes incubation and prior to cell division (at 90–100 minutes) on nutrient broth supplemented mutation assay agar. Mutation frequency does not vary with plating from 20–90 minutes. No effect of tryptophan deprivation on survival was noted.

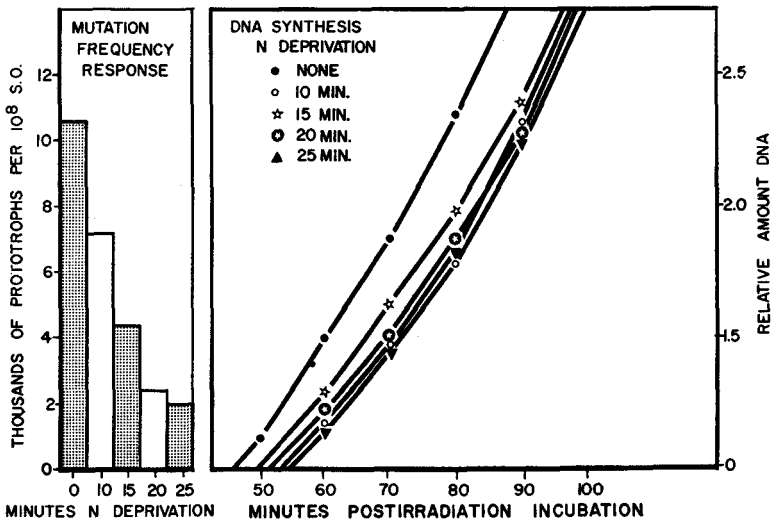

FIGURE 3.—Effect on yield of prototrophs, and on subsequent DNA synthesis, in *Escherichia coli* strain WP2 (tryptophan requiring) of incubation in nitrogen-free medium (minimal medium from which the ammonium sulfate was omitted) for varying time intervals following UV irradiation. Enzymatically digested casein hydrolysate (2 mg per ml) and DL-tryptophan (0.05 mg per ml) were added after the indicated interval of incubation. The increase in DNA relative to unincubated controls was then followed during subsequent incubation. The times given are from the start of incubation following UV irradiation, and include the incubation periods both without and with amino acids. Mutation frequency was assayed by plating after 80-minutes incubation and prior to cell division (at 90–100 minutes) on nutrient broth supplemented mutation assay agar. The mutation frequency does not vary with plating from 20–90 minutes. No effect of nitrogen deprivation on survival was observed.

frequency promoted by the other treatments gave similar results as to DNA synthesis. It therefore appears possible to selectively interfere with the decline process without modifying subsequent DNA synthesis.

From the experimental results reported above it is evident that the delay in DNA synthesis following UV exposure, as observed by HAROLD and ZIPORIN (1958), DOUDNEY (1959) and DRACULIC and ERRERA (1959) when RNA or protein synthesis is blocked, is not related to the mutation frequency decline observed. In fact, as will be discussed later, the delay in DNA synthesis, as observed by these investigators, appears only when *net* RNA or protein synthesis in the culture is interrupted (after 25–30-minute incubation); the RNA and protein requisite to DNA synthesis appears to be formed with the initial *doubling* of RNA in the culture. It is quite clear that delay in DNA synthesis cannot be held responsible for reducing the induced mutation frequency following UV irradiation.

Mutation fixation and mutation frequency decline: Since it has been shown that susceptibility of the "potential mutation" to treatments leading to lower mutation frequency is lost with increasing periods of postirradiation incubation (WITKIN 1956; DOUDNEY and HAAS 1958), it is apparent that the blockage to

RNA or protein formation leads to decline in mutation frequency *only* provided some significant metabolic event has not taken place. This event we have termed "mutation fixation". We define mutation fixation specifically, though arbitrarily, as that biosynthetic event which removes the potential mutation from susceptibility to all treatments which promote this *specific* decline process. We wish to make it quite clear that we do not regard mutation fixation as the final step in the induction of mutation; rather, the final step unquestionably appears to be the initial postirradiation synthesis of the genetic complement of DNA. The fact that certain drastic treatments (i.e., incubation in saline [LIEB 1960]) interfere with mutation induction after the event, defined as mutation fixation, has occurred does not invalidate this concept. Incubation in saline leads to extensive reorganization of cellular metabolism, including breakdown of RNA and requirements for RNA and protein synthesis before DNA synthesis can take place (DOUDNEY and HAAS, unpublished data). It is therefore not surprising that the "potential mutation" may be abolished by such treatments prior to DNA replication and establishment of the mutation in the genome.

A correlation between RNA synthesis and mutation fixation in UV-irradiated cultures has been described previously (DOUDNEY and HAAS 1959a). The postirradiation period required for the progression of mutation fixation in the culture can be followed by addition of Chl after varying postirradiation incubation intervals. Incubation in the presence of Chl is of sufficient duration to permit "complete" mutation frequency decline to take place where the mutation has not been "fixed"; those potential mutations which are fixed will not be affected. This Chl

TABLE 1

Effect of dinitrophenol on "mutation frequency decline" promoted by chloramphenicol and on subsequent DNA synthesis

Minutes with Chl	DNP present	tr^+ cells per 10^8 tr^- cells	Relative amount DNA after incubation Minutes of incubation				
			45	60	75	90	105
0	—	112	1.1	1.5	2.1	2.7	3.5
	+	108	1.1	1.4	1.9	2.6	3.5
10	—	75	1.2	1.5	2.1	2.7	3.6
	+	102	1.1	1.5	1.9	2.3	2.9
15	—	40	1.1	1.4	2.0	2.8	3.5
	+	115	1.2	1.5	1.9	2.5	3.2
20	—	20	1.1	1.5	2.1	2.6	3.6
	+	104	1.1	1.4	1.9	2.4	3.3
25	—	16	1.2	1.5	2.0	2.6	3.3
	+	112	1.2	1.4	2.1	2.5	3.2

After UV exposure, the bacteria were suspended in liquid growth medium (minimal + caseni hydrolysate, 2 mg per ml and 0.05 mg per ml DL-tryptophan) containing 20 μg per ml chloramphenicol and $\pm$ 5×10^{-3} M dinitrophenol. Following the indicated periods of incubation at 37°C, samples of the cultures were rapidly centrifuged out of this medium, resuspended in fresh growth medium without Chl or DNP and incubation continued. All times given are measured from the start of incubation after UV exposure.

Cell division occurred after 80-minutes incubation in all samples. After the indicated treatment and prior to cell division, the mutation frequency upon plating does not vary significantly. Mutation frequency values given were obtained by plating on nutrient broth supplemented agar medium after 75-minutes incubation. DNA increase is relative to unincubated control. UV delayed DNA synthesis for 45 minutes in this experiment. No effect of Chl or DNP on survival was observed.

"challenge" technique demonstrates, therefore, that fraction of potential mutations which are irreversibly "fixed" at any given interval of incubation following UV irradiation.

LIEB (1960) has questioned the validity of the mutation fixation concept, since she regards the amount of DNA formed during the Chl challenge period as significant in determining the level of induced mutation frequency observed. However, the level of mutation fixation can be related to postirradiation RNA formation under conditions where both RNA and DNA synthesis, as well as protein synthesis, are blocked during "challenge". The uracil analogue, 6-aza uracil, blocks net RNA, DNA and protein synthesis immediately after addition to the culture. Following UV irradiation, short-term incubation with this analogue produces rapid mutation frequency decline which is correlated with that produced by Chl (DOUDNEY and HAAS 1959b). As demonstrated (Figure 1), incubation with this analogue for a time period sufficient for maximum mutation frequency decline does not modify significantly the timing of subsequent DNA synthesis. Table 2 demonstrates that mutation fixation, measured by challenge with 6-aza uracil, is correlated with RNA synthesis as is mutation fixation when determined by Chl challenge. It is evident, therefore, that RNA synthesized after Chl addition has no role in mutation induction. These results would be expected only if *both* RNA and protein synthesis are involved in mutation fixation. Blockage of protein synthesis with Chl would prevent mutation fixation but would allow RNA synthesis to proceed. It is also evident that DNA synthesis during the challenge period is not involved in determining the level of mutation frequency since DNA synthesis does not occur in the presence of 6-aza uracil. Although DNA synthesis does occur in the presence of Chl, the level of mutation induction observed at a given time of Chl addition is identical with that obtained at the same time with 6-aza uracil challenge where no DNA synthesis occurs. Furthermore, the synthesis of DNA in the presence of Chl may be prevented with no modification in mutation frequency by addition of 6-aza uracil after 20-minute

TABLE 2

Relation of macromolecular synthesis to "mutation fixation" as measured by chloramphenicol and 6-aza uracil "challenge"

Minutes incubation prior to challenge	tr^+ cells per 10^6 tr^- cells		Relative amount macromolecule at challenge		
	6 AU challenge	Chl challenge	RNA	Protein	DNA
0	15.3	19.2	1.0	1.0	1.0
15	16.8	14.2	1.0	1.0	1.0
30	35.0	35.3	1.3	1.2	1.0
45	60.0	65.3	1.6	1.6	1.1
60	98.0	106.2	2.1	1.8	1.5
75	90.0	95.9	2.7	2.1	1.9

Following irradiation with UV, the bacteria were suspended in liquid growth medium and incubated at 37°C. At the indicated times either 6-aza uracil (5 mg per 100 ml) or chloramphenicol (20 μg per ml) was added to separate samples and incubation continued for 45 minutes before plating on nutrient broth supplemented agar medium. The RNA, protein and DNA content at the time of antimetabolite addition is given relative to unincubated controls. Cell division occurred after 85-minutes incubation. No effect of 6 AU or Chl on survival was observed in this experiment.

incubation in Chl. These results make it appear improbable that DNA synthesis in the presence of Chl is the basic factor influencing the level of mutation fixation, as contended by LIEB (1960).

An observation which should be emphasized in this regard is that, once the cells have been incubated in the presence of Chl for the minimum time necessary for the decline in mutation frequency to take place, additional incubation in the presence of this inhibitor does not modify the mutation frequency response (Table 3). The decline process is complete to the minimum level attainable within 30-minute incubation in Chl. It is thus evident that the observed number of "potential mutations" sensitive to Chl challenge decreases with the postirradiation incubation, not because of a decrease in rate of the mutation frequency decline process or other factors, but because the process of mutation fixation has rendered a portion of the potential mutations insensitive. The same arguments hold for comparable results observed with 6-aza uracil challenge.

It is evident that if RNA and protein are involved in DNA replication and in mutation fixation (DOUDNEY and HAAS 1958, 1959a), then addition of agents blocking RNA or protein synthesis at a given time of postirradiation incubation would block mutation fixation. Furthermore, DNA synthesis should be blocked where the RNA or protein necessary for DNA replication has not yet been formed. This hypothesis can be tested by use of chloramphenicol since this antibiotic blocks protein synthesis specifically and not DNA synthesis in cells possessing the complete DNA synthesizing mechanism (DOUDNEY 1960). Table 4 demonstrates that, at any given time of Chl addition following irradiation, the capacity of the culture to synthesize DNA without further protein synthesis is related to the amount of postirradiation RNA formed before the antibiotic is added. The culture attains the maximum rate of DNA synthesis in the presence of Chl when RNA has doubled prior to Chl addition. Furthermore, it is evident, from the same data, that a relation exists between mutation fixation and the amount of postirradiation RNA formed. This suggests that mutation

TABLE 3

Mutation frequency decline produced by chloramphenicol added after various postirradiation incubation periods

Minutes incubation prior to Chl addition	tr^+ cells per 10^8 tr^- cells after incubation in Chl Minutes in Chl							
	0	10	15	20	30	40	50	60
0	112.9	55.0	35.0	30.6	19.6	16.4	17.8	16.5
15	106.8	56.8	34.0	29.6	18.2	16.4	17.8	18.7
30	119.9	62.3	52.1	37.8	28.5	29.8	31.7	30.6
40	115.9	81.3	66.6	41.6	36.5	40.7	39.2	38.6
50	111.3	100.6	84.0	87.6	88.0	80.1	76.3	82.1
60	117.6	121.7	107.9	107.6	112.4	118.7	109.8	111.3

After UV irradiation, the bacteria were suspended in liquid growth medium and incubated at 37°C. Chloramphenicol (20 μg per ml) was added to separate samples at the indicated times of incubation, and the samples incubated in the presence of Chl for the times indicated before plating onto nutrient broth supplemented mutation assay agar. No effect of Chl on survival was observed.

TABLE 4

Relation between "mutation fixation", amount of RNA synthesized at chloramphenicol addition and subsequent rate of DNA synthesis in presence of chloramphenicol

Minutes incubation prior to Chl addition	Mutation fixation		RNA		DNA synthesis in Chl				
					Relative amount DNA Minutes incubation				
	tr^+ cells per 10^6 tr^- cells	Percent of max. frequency	Relative amount	Percent increase	60	70	80	90	Percent of max. rate in Chl
0	28.9	16.7	1.00	0	1.06	1.02	1.08	1.00	0
20	23.7	13.6	1.03	3	1.01	1.03	1.04	1.01	0
25	28.8	16.6	1.06	6	1.10	1.12	1.16	1.20	11
30	38.4	22.2	1.17	17	1.12	1.19	1.24	1.33	20
35	45.3	26.1	1.24	24	1.16	1.23	1.32	1.40	28
40	57.0	32.9	1.36	36	1.18	1.24	1.43	1.53	40
45	71.3	41.1	1.48	48	1.18	1.32	1.52	1.71	53
50	106.6	61.3	1.63	63	1.18	1.38	1.58	1.77	62
55	135.0	79.0	1.81	81	1.20	1.45	1.74	2.04	81
60	162.0	93.6	2.02	102	1.23	1.55	1.87	2.06	97
65	169.8	97.6	2.37	137	1.23	1.61	1.89	2.10	100

After UV irradiation the bacteria were diluted into liquid growth medium and incubated. At the indicated times, chloramphenicol was added to separate samples and incubation continued. RNA was determined at the time of Chl addition. Mutation fixation was determined by incubation with Chl for an additional 45 minutes (a period sufficient for complete Chl-promoted "mutation frequency decline") and then plating onto nutrient broth supplemented mutation assay agar. Relative increase in DNA was followed during incubation in Chl. The times given are measured from start of incubation following UV exposure. The *rates* of DNA synthesis were calculated for the period between 60 and 90 minutes, and the percent of the maximum rate in Chl then calculated. DNA synthesis in Chl is linear. The maximum rate of DNA synthesis in Chl was observed with 65-minutes incubation prior to Chl addition. No effect of Chl on survival was observed.

fixation requires the formation of RNA and protein made necessary for DNA replication by the UV irradiation. The data suggest that, if the necessary RNA and protein is formed prior to Chl addition, mutation frequency decline does not take place. The process of mutation fixation therefore appears to be related to the capacity of the cells to synthesize DNA rather than DNA synthesis *per se*.

Two alternative possibilities appear plausible for the involvement of RNA and protein in DNA replication based on the varying distribution of capacity for DNA synthesis in the presence of Chl among cells in the culture with incubation. On the one hand, the progressive acquisition of capacity of the culture to form DNA may be a rapid "all or none" phenomenon in each cell and based on the distribution of the requisite RNA and protein formation in time. According to this hypothesis, formation of RNA and protein necessary for synthesis of the total DNA complement of the cell must occur prior to Chl addition if DNA synthesis is to occur in that cell at all. For example, this might be the case if the RNA is involved in formation of an enzyme system required in DNA replication. To explain the kinetics of DNA synthesis according to this hypothesis, it would be necessary to assume that the time required for formation of the enzyme system in a given cell occupies only a small fraction of the time required for initial doubling of the RNA in the culture and that once the necessary enzyme is formed, that cell is capable of DNA synthesis at maximum rate. On the other hand, progressive gain in competence to synthesize DNA *in each cell* could be correlated with doubling of RNA in the culture, and it could be postulated that

the progressive formation of a rate-limiting enzyme system critical for DNA synthesis in the cell is involved.

If we adopt the point of view that the RNA and protein involved in DNA replication functions, not enzymatically, but directly in DNA synthesis (HAAS and DOUDNEY 1957; DOUDNEY and HAAS 1958, 1959a), then considerations similar to the above would apply. Each cell might rapidly synthesize the total RNA and protein involved, such synthesis requiring only a small part of the time needed for RNA doubling in the culture. Alternately, the RNA and protein required for DNA replication may be synthesized over the entire period required for doubling of RNA in the culture. The latter possibility has an intriguing implication. Partial formation of the RNA involved in DNA replication prior to Chl addition might lead *only* to replication of that part of the genome which corresponds genetically to the partially synthesized RNA. In such a case mutation frequency decline would take place only for that part of the genome which is not replicated, due to incomplete RNA formation. This suggests the possibility (which should be amenable to test) of "mapping" the genes along the length of the RNA as well as the DNA by this technique. This mapping would be based on *differential* mutation fixation of different prototrophic characters in a multiple-requiring auxotrophic strain. WITKIN (personal communication) has found evidence from preliminary experiments which indicates that mapping of genes along the replicating bacterial genome can be accomplished by the Chl challenge technique.

Reduction in mutation frequency by use of purine and pyrimidine analogues: Several purine and pyrimidine analogues have been shown to promote a decline in mutation frequency, but only after a considerable period of incubation (DOUDNEY and HAAS 1959b). Among these analogues are 5-hydroxyuridine (5-HU) and 6-mercaptopurine. The decline promoted by these analogues is correlated with doubling of RNA in their presence. It appears likely that they exert their effect through incorporation into RNA. The analogues do not block RNA synthesis, but convert synthesis from an "exponential" rate to a "linear" rate. This appears to be typical of those analogues incorporated into RNA or protein and interfering with macromolecular function. These analogues which do not block RNA synthesis do not promote the immediate mutation frequency decline *process*, as do analogues which are not incorporated, but are capable of blocking synthesis, i.e., 6-aza uracil (DOUDNEY and HAAS 1959b). We have suggested that the lower mutation frequency promoted by 5-HU is due to synthesis of "nonfunctional" or "rejected" RNA produced by analogue incorporation (DOUDNEY and HAAS 1959a). The mutagenic substrate (i.e., the UV-modified purine or pyrimidine) would be used up in the synthesis of this RNA according to this hypothesis.

Incubation in the presence of a mixture of amino acids immediately following UV exposure markedly increases the mutation yield (WITKIN 1956; HAAS and DOUDNEY 1957; DOUDNEY and HAAS 1958). The time interval required for amino acids to exert a measurable effect was shown by incubating the irradiated bacteria on a high level of amino acids for increasing intervals and then plating

on minimal medium containing a concentration of amino acids which is limiting to mutation frequency (DOUDNEY and HAAS 1959a). Such experiments established that amino acids sufficient to increase to maximum the induced mutation frequency are taken into the cell within 30-minute postirradiation incubation and prior to any measurable protein synthesis. Incubation in the presence of high levels of amino acids does not change the course of subsequent net protein synthesis, nor modify mutation fixation, although such treatment does prevent mutation frequency decline. Therefore, the amino acids taken into the cell must actually "do something" to increase mutation frequency response. We have termed this event "mutation stabilization" for convenience in reference. As one possibility it was suggested that the amino acids interact with nucleic acid precursors so as to "stabilize" temporarily the precursors (including the UV-modified precursors) for subsequent nucleic acid synthesis (DOUDNEY and HAAS 1959a). The possible involvement of nucleotide-amino acid complexes in both nucleic acid and protein synthesis has been previously suggested. However, it seems probable that "mutation stabilization" involves more than formation of amino acid-nucleotide complexes since the blockage of RNA or protein synthesis does not prevent formation of such complexes but does lead to mutation frequency decline. Since no net RNA or protein synthesis can be detected during this period, it may be that RNA and protein synthesis involved in mutation stabilization is quantitatively minor.

The timing of the loss of ability of 5-HU to reduce the level of mutation frequency has considerable bearing on our insight into the nature of the "premutational" state following UV irradiation. If loss of susceptibility to 5-HU promoted decline (which is found correlated with initial doubling of RNA) is correlated with the mutation stabilization process which occurs considerably earlier, then the data would suggest that, in order to reduce mutation frequency, the 5-HU must interact with amino acids in the same way as the hypothetical mutagenic nucleic acid precursors in mutation stabilization.

Figure 4 shows that the effect of 5-HU in lowering mutation frequency is lost in close correlation with the time course of mutation stabilization; 5-HU added after 30-minute incubation has no effect on the mutation yield. These data are in line with the hypothesis that the "potential mutation" exists initially as a modified nucleic acid precursor, and that both the modified precursor and 5-HU must undergo similar interactions with amino acids in order to exert their respective effects. On this basis, incorporation of the modified precursor leads to mutation, while incorporation of 5-HU leads to production of biologically ineffective RNA.

Other experiments were carried out with the objective of determining whether or not incubation with 5-HU for a time period sufficient to attain the lowest mutation frequency possible with this analogue would interefere with subsequent DNA synthesis. Such interference would be expected if the mechanism of 5-HU action in promoting the lower mutation frequency were, for example, formation of a defective enzyme incapable of functioning in DNA synthesis. The results of such experiments (Table 5) show that this hypothesis must be rejected. While

5-HU reduces mutation frequency in correlation with RNA synthesis, no significant delay in DNA synthesis is observed when the analogue is counteracted by addition of uridine at initiation of DNA synthesis. The RNA and protein formed in the presence of 5-HU must therefore be adequate for DNA synthesis, but inadequate for mutation induction. All enzymes required for DNA synthesis

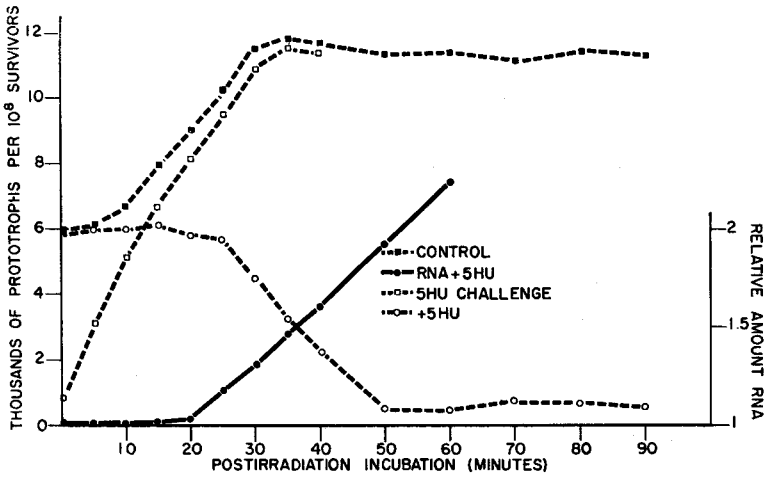

FIGURE 4.—Loss of effectiveness of 5-hydroxyuridine in decreasing mutation yield with incubation following UV irradiation. The control curve demonstrates the mutation frequency observed with plating on nutrient broth supplemented mutation assay agar after the indicated time of incubation in minimal medium supplemented with casein hydrolysate (2 mg per ml) and DL-tryptophan (0.05 mg per ml). The + 5HU curve demonstrates the effect on mutation frequency of incubation in this same medium supplemented with 5-hydroxyuridine (5 mg per 100 ml). The RNA + 5HU curve demonstrate relative RNA increase in the presence of 5-hydroxyuridine during the same period. The 5HU challenge curve demonstrates the effect upon mutation frequency of addition of 5-hydroxyuridine at the indicated time (rather than zero time), and incubating to the 90-minute postirradiation incubation time prior to plating. No effect of 5-hydroxyuridine on survival was noted.

TABLE 5

Effect of incubation with 5-hydroxyuridine on mutation yield and on DNA synthesis

Incubation time (minutes)	*tr*+ cells per 10^8 *tr*− cells			DNA (relative amount)		
	Control	5-HU	5-HU, uridine added at 60 min	Control	5-HU	5-HU, uridine added at 60 min
0	62.0	66.0	65.8	1.0	1.0	1.0
20	65.6	57.5	59.3	1.0	1.0	1.0
40	68.9	31.5	33.6	1.0	1.1	1.0
60	64.3	18.2	16.8	1.3	1.2	1.2
80	70.1	15.7	15.2	1.9	1.3	1.6
100	53.8	18.6	15.1	2.6	1.4	2.3

After UV irradiation the bacteria were suspended in growth medium ± 5-hydroxyuridine (5 mg per 100 ml). The mutation frequency and increase in DNA relative to unincubated controls was determined for the indicated times of incubation. To an identical set of 5-HU containing samples, uridine (5 mg per 100 ml) was added at 60 minutes incubation to counter the analogue. No effect of 5-HU on survival was observed.

either must have been present following UV exposure or were formed in the presence of 5-HU. The hypothesis that the RNA required is intimately involved in transfer of genetic information fits the 5-HU data since introduction of 5-HU into this RNA could lead to defective RNA. It would be presumed that, in turn, defective DNA unable to perform its obligatory genetic role would be synthesized as a consequence of the defective RNA; the cell would then revert to the "old" DNA for its genetic information. If this is true, the expected mutation yield would not be observed even though RNA and DNA synthesis took place as usual. Only through further experimentation can this possibility be established or rejected.

DNA synthesis in the mutation induction process: We have previously reported experiments which strongly suggest that DNA synthesis is the terminal event in mutation induction (HAAS and DOUDNEY 1959, 1960). These experiments also indicate that DNA synthesis must be followed by a period of protein synthesis if the induced mutations (reversion of the tryptophan requirement in this case) are to be expressed. It seems quite probable that, in these experiments with strain WP2, we are dealing with the initial action of the reverted gene and that the increase in mutation frequency observed on minimal agar after increasing periods of incubation in complete medium is due to the appearance of the missing enzyme involved in tryptophan synthesis which is controlled by the mutated gene. The most reasonable explanation for the results of the experiments is that the final step in the process of UV-induced mutation requires synthesis of DNA but that the mutation cannot express itself phenotypically until the gene has had time to establish the production of the required enzyme.

We have recently carried out experiments with thymine- and amino acid-requiring double auxotrophs of *E. coli* in which the mutation followed was reversion of the amino acid requirement. It was found that absence of thymine prevents mutation expression in these strains but does not lead to mutation frequency decline as determined by subsequent plating onto amino acid- and thymine-containing medium. These results appear to be a direct demonstration of the necessity for DNA synthesis in mutation induction. It is quite clear from the experiments that delay in DNA synthesis due to lack of thymine does not modify mutation frequency in the absence of the conditions which promote the active decline process. These studies of mutation induction in thymine-requiring strains of *E. coli* will be fully described in a separate communication.

The reality of the relation of mutation induction to DNA synthesis may be assessed by testing for the photoreversibility of the mutation after DNA synthesis. One would not expect that the "potential mutation" could be photoreversed after final incorporation of the "genetic change" into the DNA. A number of workers using various mutational systems and organisms have reported that photoreversibility of UV-induced mutation is lost with postirradiation incubation (KELNER 1949; NEWCOMBE and WHITEHEAD 1951; NOVICK and SZILLARD 1949; MATNEY, SHANKEL and WYSS 1958). A similar effect has been reported from studies of the tryptophan-requirement reversion in *E. coli* strain WP2 (DOUDNEY and HAAS 1959a). Figure 5 demonstrates that, for the major

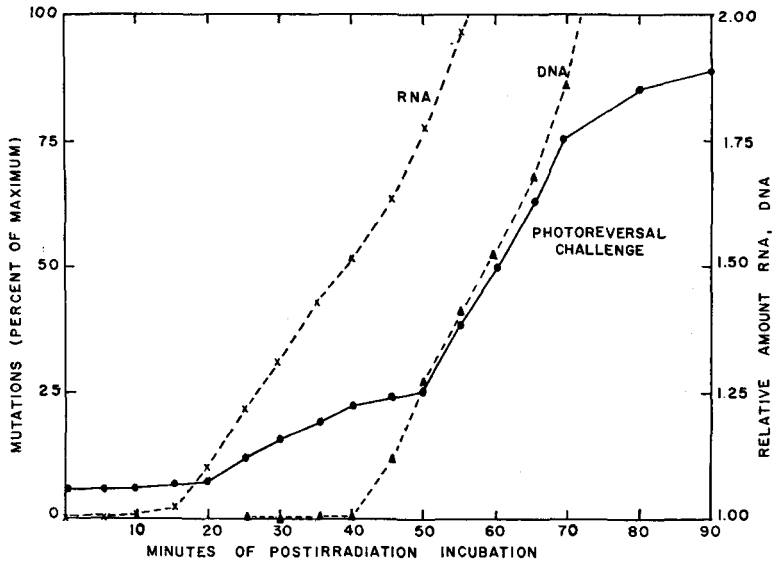

FIGURE 5.—Relation to RNA and DNA synthesis of loss of susceptibility of "potential mutations" to photoreversal with postirradiation incubation. At the indicated times of incubation in liquid growth medium, samples of the culture were chilled and held in ice bath at 2°C either in photoreversing light (two General Electric 500 watt projection bulbs at a distance of six inches) or in the dark for one hour (a period of white light exposure giving maximum photoreversal) prior to plating on nutrient broth supplemented mutation assay agar. At the same times RNA and DNA relative to unincubated controls were determined with identical samples. Maximum mutation frequency observed for control cells (no photoreversal) was approximately 90 prototrophs per 10^6 surviving organisms. Survival following this dose of UV was approximately 50 percent. The white light treatment resulted in photoreactivation to the 70 percent level of survival when applied without incubation. This effect was not apparent after 20-minutes incubation.

portion of the reversions induced, loss of photoreversibility is correlated with the progression of DNA synthesis. Loss of photoreversibility occurs at a rapid rate with initiation of DNA synthesis; at the time that DNA has doubled approximately 85 percent of the potential mutations are no longer photoreversible. The initial loss of photoreversibility, occurring at 20–40-minute incubation and prior to initiation of DNA synthesis, may represent some physical or chemical transformation of the potential mutation to a nonphotoreversible state through a process not connected with DNA synthesis. In this regard, the initial loss of photoreversibility appears to be correlated with initiation of RNA synthesis in the culture, although this relation may be fortuitous.

The processes responsible for loss of photoreversibility have been studied by use of Chl and DNP. While Chl specifically blocks protein synthesis, DNP blocks most of the energy-requiring reactions of the cell (including RNA, DNA and protein synthesis). DNP blocks completely the loss of photoreversibility (Table 6); therefore, energy-requiring reactions must be involved in the process. However, Chl does not block loss of photoreversibility but does block

TABLE 6

Effect of chloramphenicol and dinitrophenol on loss of mutation photoreversibility

Incubation prior to photoreversal (minutes)	Control			DNP added at 45 minutes			Chl added at 60 minutes		
	tr^+ cells per 10^6 tr^- cells		DNA (rel. amount)	tr^+ cells per 10^6 tr^- cells		DNA (rel. amount)	tr^+ cells per 10^6 tr^- cells		DNA (rel. amount)
	Dark	Light		Dark	Light		Dark	Light	
0	119.2	4.9	1.0	. . .	. .	. .	. . .	. . .	. .
60	109.6	20.0	1.2	87.6	9.0	1.1	106.8	16.8	1.2
75	102.0	50.6	1.5	91.3	10.6	1.1	111.3	46.4	1.5
90	112.5	89.1	2.2	88.2	11.8	1.1	91.7	77.6	2.0
105	97.8	87.3	2.9	89.3	9.9	1.1	99.2	83.5	2.3

After UV irradiation the bacteria were incubated in liquid growth medium. To one set of three identical series of cultures, dinitrophenol (5×10^{-3} M) was added at 45-minutes incubation; to one other set chloramphenicol (20 μg per ml) was added at 60-minutes incubation. At the indicated times of incubation samples were taken, centrifuged, washed with cold minimal medium, recentrifuged and resuspended in minimal medium and held in an ice bath in the dark or exposed to intense white light for one hour (a period of time sufficient for maximum photoreversal). The increase in DNA relative to unincubated controls was determined at the times the samples were taken.

protein synthesis; it thus appears that protein synthesis is not involved in the process.

The question of involvement of protein synthesis in that earlier loss of photoreversibility which occurs prior to DNA synthesis cannot readily be tested since treatment with Chl during this period leads to some degree of "mutation frequency decline." However, DNP blocks this earlier loss of photoreversibility indicating that energy is required for the transformations involved.

These experiments suggest that the greater proportion of the potential mutations induced by UV exist in a state susceptible to photoreversal until DNA replication. Therefore, whatever the nature of the involvement of the potential mutation in the processes of mutation fixation (which occur prior to DNA synthesis), these transformations do not compromise the photoreversibility of the "potential mutation." This would seem reasonable if the potential mutation exists originally as a mutagenic nucleic acid precursor which may be "photoreversed" to a nonmutagenic condition. If this precursor is incorporated into RNA during mutation fixation as we have suggested, then it must be assumed that the mutagenic nucleotide remains photosensitive in the RNA. If, conversely, one assumes that the damage leading to mutation is induced directly in the DNA, either in one or both strands, then this DNA damage must be photoreversible until DNA replication. This question cannot be resolved until the nature of the chromophore for UV-induced mutation is established. The fact is clear, however, that the initial synthesis of DNA after UV exposure is the "copying" event leading to a photostable "error" in the genome.

DISCUSSION

Recent studies indicate that ultraviolet may induce mutation through at least two mechanisms (WITKIN and THIEL 1960; KADA, BRUN and MARXOVICH 1960). In one type of mutation induction, the initial damage is unstable and subject to modification by postirradiation treatments. It is this type of mutation induction

with which we are concerned in this paper. The second type of UV-induced mutation is stable and is not influenced significantly by postirradiation conditions. Similar considerations appear to hold for X-ray induced mutations. While a portion of the latter are influenced by postirradiation conditions, another portion is refractory to these treatments (HAAS and DOUDNEY 1958; KADA, BRUN and MARXOVICH 1960). Complete *expression* of this latter type of X-ray-induced mutation can be observed within a short period following irradiation and in the absence of DNA synthesis (KADA, DOUDNEY and HAAS 1960). RNA and protein synthesis is required for *expression* but apparently not for *induction*.

Since we do not know the chemical nature of the unstable condition which leads to UV-induced mutation at this time, we prefer to refer to it simply as the "potential mutation." While our studies suggest that the potential mutation is initially in the form of a mutagenic nucleic acid precursor formed by the action of UV on a purine or pyrimidine-containing monomer (HAAS and DOUDNEY 1957), 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.

It is quite evident that the potential mutation can be influenced by metabolic conditions. Conditions leading to mutation require RNA and protein synthesis which is quantitatively minor and apparently not related to the gross synthesis of these macromolecules in the culture. The term "mutation stabilization" has been used to indicate those interactions increasing or supporting mutation and occurring prior to any measurable net macromolecular synthesis. The effectiveness of mutation stabilization seems to determine the frequency of mutation under ordinary conditions. In this special sense, the original view of WITKIN (1956) that protein synthesis determines the level of mutation induction is valid. While the nature of mutation stabilization is not known, it is quite attractive to interpret it as a "templating" or preparatory process to gross macromolecular synthesis in the cell. This possibility is supported by the evidence that 5-hydroxyuridine must participate in a process correlated with mutation stabilization in order to lower the induced mutation frequency (presumably through subsequent incorporation into RNA).

If mutation stabilization does not occur, it is evident that an antagonistic or competitive process occurs which removes the potential mutation from pathways leading to mutation induction. This process we have termed "mutation frequency decline." The process appears to be enzymatic since a Q_{10} of approximately two is obtained based on the rate of mutation frequency decline in the culture at various temperatures of incubation (WITKIN 1956; DOUDNEY and HAAS 1958). Energy is required since DNP blocks the process, but it is clear that protein and RNA synthesis is not required in the process. DNA synthesis is evidently not required since the process occurs in the absence of thymine as deduced from studies of UV-induced reversion of the amino acid requirement in thymine- and amino acid-requiring double auxotrophs of *E. coli* (DOUDNEY and HAAS, unpublished results).

Alternative possibilities (based on the hypothesis adopted as to the nature of the mutational chromophore) can be proposed for the mechanism leading to

mutation frequency decline. If one assumes that the potential mutation resides initially in the DNA existing at the time of UV irradiation, then the decline process may be ascribed to "repair" of the UV damage. The hypothetical repair mechanism could not involve RNA, DNA or protein synthesis, but would require energy. If the alternate view is taken that the potential mutation resides in a UV-modified nucleic acid precursor, then any process removing this precursor from the synthetic pathway of the nucleic acids involved in genetic replication would prevent mutation. In the case of the auxotrophic reversion the possibility that mutation frequency decline is due to a selective lethal effect caused by blockage of RNA or protein synthesis, on cells bearing potential mutations, cannot be eliminated although such selectivity seems extremely unlikely. In the case of the "color mutants" distinguished on EMB agar this possibility appears to be ruled out since such lethality was not observed with mutation frequency decline where the potential mutation-containing cells constituted a relatively large portion of the total survivors (DOUDNEY and HAAS 1958).

Whatever the nature of the mutation frequency decline process, it is evident that this process can be carried to completion without influencing materially the timing or the rate of subsequent macromolecular syntheses *definitely involved in mutation induction.* Treatments sufficient to reverse (or prevent) all susceptible mutations produce little or no effect on subsequent RNA, DNA or protein synthesis. For this reason, the processes involved either in mutation frequency decline or in mutation stabilization would appear to take place prior to the RNA and protein synthesis made necessary by UV irradiation to further DNA replication (HAROLD and ZIPORIN 1959; DOUDNEY 1959; DRACULIC and ERRERA 1959). It should be emphasized that these findings effectively rule out any hypothesis for mutation frequency decline based on treatments which modify only the timing of DNA synthesis so as to take advantage of decay of a "premutational" state at a constant rate (KIMBALL, GAITHER and WILSON 1959), or disappearance of "mutagens" at a rate *not influenced* by the treatment (LIEB 1960).

Postirradiation incubation in complete medium clearly leads to fixation of the potential mutation in the sense that blockage of RNA or protein synthesis will no longer promote mutation frequency decline after some synthetic event has occurred. Mutation fixation (measured by challenge procedures involving *specific* blockage of RNA or protein synthesis) appears to be correlated with initial doubling of RNA in the culture. There appears to be a relation of the amount of postirradiation RNA synthesized at the time of chloramphenicol addition to the rate of DNA synthesis in the presence of Chl and to the level of induced mutation obtained after Chl challenge. Only where the RNA and protein necessary for genetic replication is formed prior to Chl addition can DNA be produced. If this RNA and protein is not formed at the time Chl is added, the potential mutation is lost through the mutation frequency decline process. It seems quite likely that the RNA and protein involved in mutation fixation is identical with that involved in postirradiation recovery of the DNA synthesizing mechanism. It is also evident that acquisition of the *capacity* to synthesize DNA,

rather than DNA synthesis *per se*, is necessary for mutation fixation (since DNA synthesis during the Chl challenge period is unnecessary).

DNA synthesis appears to be firmly established as the terminal event in UV-induced mutation. Loss of susceptibility of the potential mutation to photoreversal appears to be intimately correlated with doubling of the DNA. Furthermore, mutation expression follows DNA synthesis and will not occur in its absence. Protein synthesis following DNA synthesis is necessary for mutation expression (HAAS and DOUDNEY 1959).

A number of hypotheses have been advanced in regard to the mechanism of mutation induction by UV. One possibility is that the UV damage occurs to the genetic DNA, and that gene action (the synthesis of RNA on the DNA template) in some manner "fixes" this damage. With subsequent DNA replication the functional mutated gene would be formed. It is difficult to explain a number of observations on this basis. The mutation frequency decline process cannot be reasonably accounted for; the 5-hydroxyuridine effect in decreasing mutation frequency remains unexplained, since it is difficult to see why RNA incorporating 5-HU would be less effective in fixing mutation according to this hypothesis than "normal" RNA. In our opinion the hypothesis for UV-induced mutation which most adequately reconciles the reported experimental data is that which we have previously advanced (DOUDNEY and HAAS 1958, 1959a). According to this hypothesis, DNA synthesis involves transfer of information from the parental DNA to the daughter DNA through an RNA-protein intermediate. On this basis, incorporation of a UV-modified RNA precursor into the RNA would lead to a "copying error" involving substitution of a different nucleotide pair in the subsequently formed daughter DNA.

Little experimental support at the molecular level exists for this indirect mechanism of DNA replication at present. A possibility which might be considered is that the DNA replicates under the usual circumstances according to the scheme proposed by WATSON and CRICK (1953), but following UV irradiation this mechanism is inactivated. In this case, the cell might revert to an alternate mechanism for DNA formation which involves an intermediate transfer of information to RNA. On this basis, we could account for most of the mutation induction data, as well as the necessity for RNA and protein synthesis in DNA replication following UV irradiation.

It is quite clear that none of the above hypotheses or speculations have been established and that a considerable amount of further experimentation is necessary before mutation induction will be understood. However, the investigations reported here indicate some of the directions which future experimentation must take. Moreover, in view of the mesmeric effect of the WATSON and CRICK hypothesis for DNA replication on research in this area, with its support at the enzymatic level by the definitive studies of KORNBERG and co-workers, it should be pointed out that no conclusive evidence has been reported to support this direct complementary scheme for DNA replication, either in *living systems* or for formation of *functioning* genetic DNA. Accordingly, other less direct mech-

anisms for DNA replication must be thoroughly investigated and considered. This is especially true in understanding UV-induced genetic change which clearly involves not only DNA synthesis, but also synthesis of the other primary information-bearing macromolecules of the cell, RNA and protein.

SUMMARY

Postirradiation treatments blocking RNA or protein synthesis lower markedly the ultraviolet-induced mutation frequency (reversion of tryptophan requirement) in *Escherichia coli* strain WP2. Treatments of less than 20 minutes reduce the mutation frequency to a minimal level without significant modification of the time or rate of subsequent synthesis of RNA, DNA and protein. Therefore, the mutation frequency decline process apparently is an active process, probably enzymatically mediated, and not dependent on delay in postirradiation macromolecular synthesis. The progressive loss of susceptibility of the potential mutation to these treatments is closely correlated with the progression of ribonucleic acid synthesis in the culture. A relation exists between the amount of RNA synthesized at the time of chloramphenicol addition, the relative rate of DNA synthesis in the presence of chloramphenicol, and the induced mutation frequency. Apparently, only when the RNA and protein necessary for genetic replication is synthesized prior to chloramphenicol addition will DNA be formed. When the RNA and protein involved has not been formed, the potentiality for mutation is lost through the active decline process.

Decline in induced mutation frequency is promoted by 5-hydroxyuridine after a considerable lag. This decline is correlated with the doubling of RNA in the culture suggesting that the analogue causes the decline through incorporation into the RNA. However, loss of *susceptibility* to the 5-hydroxyuridine-promoted reduction in mutation frequency occurs prior to measurable RNA synthesis and is correlated with the *mutation stabilization* process. Incubation with 5-hydroxyuridine of sufficient duration to prevent all sensitive mutations does not delay DNA synthesis.

Several lines of evidence indicate that DNA synthesis is the terminal event in UV-induced mutation. However, delay of DNA synthesis does not modify UV-induced mutation frequency in the absence of the active decline process unless drastic treatments are employed which grossly alter cellular metabolism. Loss of mutation photoreversibility is correlated with DNA synthesis in the culture. Dinitrophenol (but not chloramphenicol) blocks the loss of mutation photoreversibility.

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