PROTEIN SYNTHESIS AND THE INDUCTION OF MUTATIONS IN *ESCHERICHIA COLI* BY ALKYLATING AGENTS

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Induction of mutation in bacteria is a complex process. Cell division is almost always required after treatment with a mutagenic agent before the mutation is established and a variety of processes may occur during this time (Witkin, 1951). Witkin (1956), Doudney and Haas (1959), and Schwartz and Strauss (1958) have established the fact that protein synthesis must occur soon after ultraviolet irradiation to obtain the maximal number of reversions from auxotrophy to prototrophy. This synthesis is required for the fixation of the mutations induced by ultraviolet light.

It is possible that the complications described above are general characteristics of the mutation process but it is also possible that they represent peculiarities due to the mutagen used. The stabilization of the "mutagenic state" induced by ultraviolet irradiation requires protein synthesis but this may not be a characteristic of the mutation process in general. Mutations induced by caffeine occur in bacteria under conditions in which protein synthesis is not possible (Glass and Novick, 1959). Bromouracil is mutagenic for bacteriophage even when added along with chloramphenicol 10 min after infection, indicating that protein synthesis is not essential in initiating the series of events leading to mutation (Litman and Pardee, 1959).

Alkylating agents are efficient mutagens (Kølmark, 1956; Westergaard, 1957) and they have long been considered "radiomimetic." We have compared the mutagenic action of ultraviolet irradiation and the alkylating agents, ethyl sulfate and epichlorohydrin. Although there are similarities in the action of the two classes of mutagens, our results indicate that immediate protein synthesis is not essential for the establishment of mutations after treatment with either of the two alkylating agents tested.

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MATERIALS AND METHODS

A tryptophan-requiring mutant (*try*) of *Escherichia coli* strain B/r was used in these studies. The properties of this strain have been described previously (Schwartz and Strauss, 1958). We have used the change from tryptophan requirement to non-requirement as a measure of mutation. The number of revertants present in a particular sample was determined by plating a 0.1-ml portion of the bacterial suspension on plates of minimal medium (table 1) or on plates of minimal medium supplemented with broth as indicated below. The total number of viable cells was determined by plating on nutrient agar after dilution. All plates were aged 3 days at 37 C before use.

Cultures were grown in a minimal medium containing 0.3 g of sodium glutamate and 10 ml of nutrient broth per 100 ml of medium. Aerated cultures were grown in 100-ml amounts in Erlenmeyer flasks at 37 C for about 16 hr and started with a heavy inoculum taken from an 8-hr nutrient agar slant. The cells were collected and washed twice with 0.1 M $KH_2PO_4/NaPO_4$ buffer, pH 7.2, then suspended in the same buffer for treatment. After treatment with a chemical mutagen the cells were again harvested, washed, and resuspended in buffer. Cells treated with ultraviolet light were used without washing after treatment but were held in buffer after treatment for a time equivalent to that required to prepare the cells treated with a chemical mutagen. After treatment cells were incubated on a shaker at 37 C in the dark.

In many experiments a medium containing 0.3 per cent sodium glutamate was used. The glutamate contained several amino acids as impurities as demonstrated by paper chromatography. Although there were impurities in the glutamate used, the *try* strain never gave any visible growth on solid or liquid minimal medium containing 0.3 per cent of this preparation. Ethyl sulfate and epichlorohydrin (Wako Pure Chemi-

TABLE 1

Composition of media and sources of chemicals

Broth:	
Meat Extract (Kyokuto Seiyaku Co.)	10 g
Peptone ("Polypepton"-Daigo Brand, Wako Pure Chemicals)	10 g
NaCl (Matsunaga Chemical Co., Ltd)	2 g
Distilled water	1 L
(Adjust to pH 7.2)	
Minimal medium:	
Salts:	
$(NH_4)_2HPO_4$	2.5 g
KH_2PO_4	1.5 g
$MgSO_4 \cdot 7H_2O$	0.1 g
NaCl	5.0 g
Glucose (Chugai Seiyaku Kabushiki Kaisha)	3.0 g*
Sodium glutamate (Yashima Medicals, Ltd.)	3.0 g†
Distilled water	1 L
(Adjust to pH 7.2 with NaOH)	
Powdered agar	1.5%

All chemicals not otherwise indicated and DL-tryptophan from Wako Pure Chemical Industries, Ltd.

* Autoclaved separately.

† When used.

cal Industries, Ltd.) were used as chemical mutagens (Westergaard, 1957); the ethyl sulfate was purified before use by vacuum distillation. Since these substances are nonmiscible with water, they were dissolved in ethanol and the ethanolic solution was added to the bacteria suspended in 0.1 M phosphate buffer, pH 7.2. Addition of the ethanolic ethyl sulfate solution to this suspension resulted in the appearance of a series of fine globules on the surface of the liquid but these globules dissolved after a short period of agitation. Usually 0.8 ml of ethyl sulfate solution (0.8 ml of ethyl sulfate plus 4.2 ml of ethanol) was added to 100 ml to give a 0.01 M solution. The added ethanol was not necessary to obtain the mutagenic effect. Mutants were obtained when bacteria suspended in water were treated with ethyl sulfate, but the pH often dropped low enough as a result of hydrolysis of the mutagen to cause appreciable cell death. The experimental treatment was therefore carried out in buffer by magnetically stirring a suspension of bacteria held at 37 C; alternately the bacteria were gently shaken in buffer on a reciprocating shaking machine.

The source of ultraviolet light was a "Toshiba" 15-watt sterilizing lamp with a maximal output at about 2600 A. Cultures in buffered suspension were irradiated in petri dishes at a distance of 60 cm from the ultraviolet source and the cultures were gently swirled by hand during the treatment.

DL-Tryptazan was a gift of Dr. H. R. Snyder, 5-methyl tryptophan was purchased from Mann Biochemicals, and the chloramphenicol was a product of the Sankyo Company.

RESULTS

Ethyl sulfate was mutagenic at a concentration of 0.01 M and the mutagenic effect was measurable without appreciable cell death (figure 1).

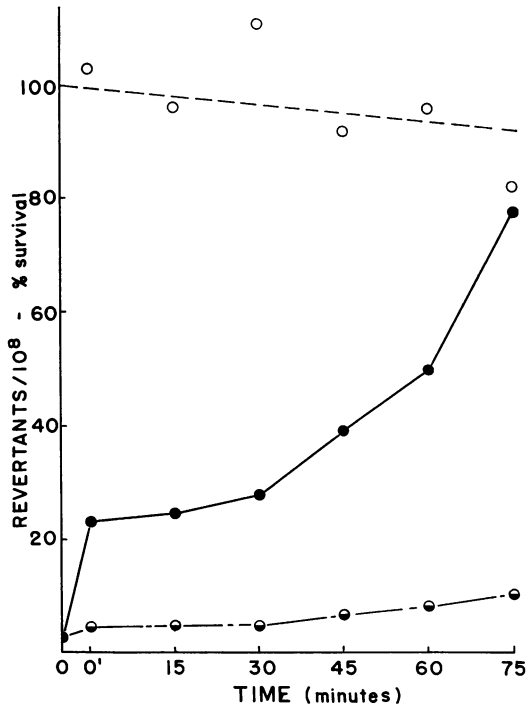

Figure 1. Mutagenic effect of diethyl sulfate as a function of the time of treatment. Twenty-five-ml samples of a suspension of 6×10^9 *try* cells were treated with 0.01 M ethyl sulfate as described in the text. All cells were washed with buffer before plating; 0′ refers to the set washed immediately after the addition of mutagen. *Open circles* indicate survival, *filled circles* indicate reversions per 10^8 cells obtained after plating on minimal plus glutamate plus 0.05 per cent broth, *half-filled circles* indicate reversion frequency after plating on minimal medium.

Larger amounts of epichlorohydrin were required to produce *try* reversions (figure 2). It was not possible to obtain really accurate curves of effect as a function of the time of treatment because of the ambiguity in the exact time of treatment introduced by the necessity of centrifuging and washing cells following treatment with chemical mutagens. Addition of mutagen followed by immediate centrifuging and washing gave an appreciable number of mutants (figure 1). It was possible to obtain a smooth curve if this treatment was omitted and plating was done directly (on the assumption that the mutagen would be diluted sufficiently by the large volume of medium on the plate to halt its action) but when this was done the recovery of revertants was higher than without washing.

It is generally accepted that comparisons of the mutagenic effect of different agents must measure mutation at the same locus since the response of different alleles to different mutagens may differ (Westergaard, 1957). Stadler and Yanofsky (1959) have shown that a *try* mutant of *E. coli* strain K-12 may yield either back mutations or suppressor mutations after ultraviolet irradiation. They also showed that suppressor mutations were more common among the ultraviolet-induced revertants. We also observed both small and large colonies on our plates (figure 3). Our large colonies yielded large colonies after subculture on minimal medium, the small colonies generally yielded small colonies. The growth rate of the small colonies in liquid minimal medium was definitely lower than the growth rate of material obtained from the large colonies. Both types of colonies were obtained after treatment with ultraviolet light, ethyl sulfate, or epichlorohydrin and the proportion of small to large colonies obtained was about the same after treatment with either ultraviolet light or ethyl sulfate. In one experiment, after 60 sec of ultraviolet irradiation about 36 to 40 per cent of the colonies were small; after ethyl sulfate treatment 47 to 50 per cent of the colonies were small. In other experiments the results were even closer. We therefore suppose that we are studying the same type(s) of mutation in both cases. Quantitative estimates were not made of the small to large colony ratio after treatment with epichlorohydrin.

It has been reported that supplementation of minimal medium with small quantities of broth can lead to an increase in the number of revertants obtained (Demerec and Cahn, 1953) and we have obtained this effect with our *try* strain. The response to broth supplementation obtained

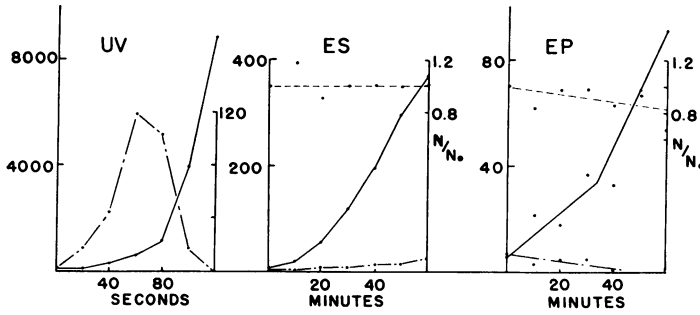

Figure 2. Try reversions as a function of the time of treatment with three different mutagens. *UV* (Ultraviolet) = 10 ml of a suspension of 10^9 *try* cells per ml were irradiated as described in the text. Survival equaled 0.6 after 40 sec, 0.12 after 80 and 0.012 after 100 sec. *ES* (ethyl sulfate) = 100 ml of a suspension of 10^9 *try* cells per ml were stirred in buffer made 0.01 M with respect to ethyl sulfate. One-ml portions were removed and plated directly without washing. *EP* (epichlorohydrin) = 100 ml of a suspension of 7×10^8 *try* cells per ml were stirred with buffer made 0.05 M with respect to epichlorohydrin (1 ml of a solution of 3 ml of ethanol and 2 ml of epichlorohydrin was added but the material did not go completely into solution). One-ml portions were removed and plated without washing.

Ordinates: left hand axis = reversions per 10^8 viable cells; right hand axis = reversions per 10^8 for ultraviolet treated material plated on minimal, = fraction survived for ethyl sulfate and epichlorohydrin.

Abscissa = time of treatment.

Solid line represents reversions per 10^8 after plating on minimal plus glutamate plus 0.05 per cent broth; alternate *long and short dashed line* represents mutants on minimal medium; *short dashed line* represents survival.

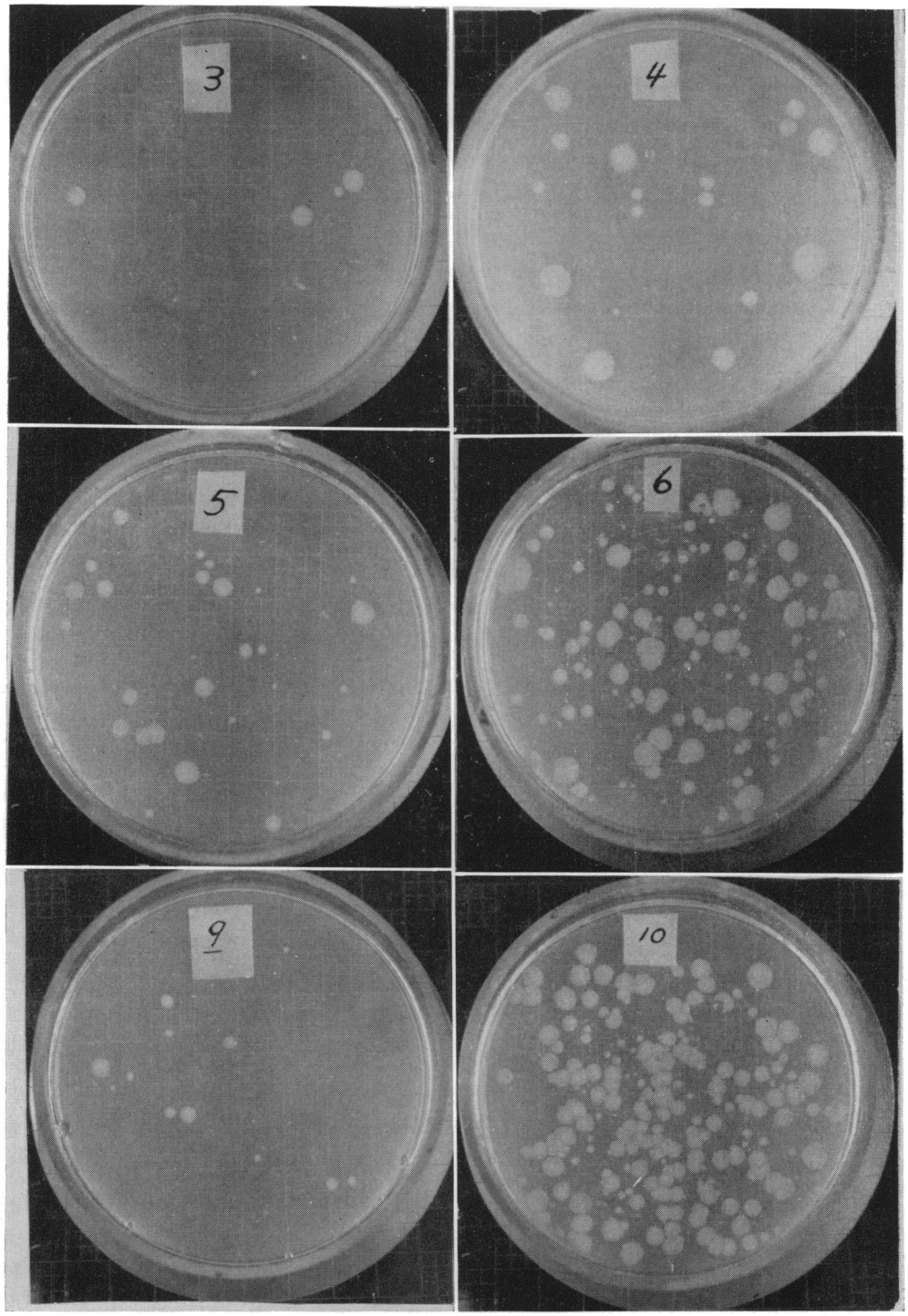

Figure 3. Small and large colonies among *try* revertants. Plates *3* and *4* show spontaneous revertants, plates *5* and *6* show the revertants obtained after treatment with ethyl sulfate for 15 min (this material was used for the data shown in figure 1) and plates *9* and *10* show the revertants obtained after treatment with ultraviolet for 20 sec (95 per cent survival). Odd numbered plates contain minimal medium, even numbered plates contain minimal medium supplemented with glutamate and 0.05 per cent broth.

TABLE 2

Effect of broth supplementation on the reversion of mutant try

Expt	Mutagen	Survival (%)	Broth Concentration (%)			
			0	0.01	0.025	0.05
1	Ultraviolet	97	11.2	29.0	45.7	84.8 mut/10^8
		(3.4×10^8)*	1.0	2.6	4.1	7.6 ratio
	Ethyl sulfate	97	16.0	15.1	22.3	56.4 mut/10^8
		(3.4×10^8)	1.0	0.9	1.4	3.5 ratio
2	Ultraviolet	59	12.8	36.0	88.8	161.0 mut/10^8
		(1.6×10^8)	1.0	2.8	6.9	12.6 ratio
	Ethyl sulfate	59	7.2	10.0	20.9	71.8 mut/10^8
		(1.6×10^8)	1.0	1.4	2.9	10.0 ratio

* Number of cells plated.

Recorded mutants per 10^8 cells, not corrected for the spontaneous frequency.

Ratio line (/) indicates the frequency of mutants at a particular broth concentration divided by the frequency obtained with no broth supplementation.

TABLE 3

Effect of posttreatment incubation on the frequency of try revertants

Incubation	Revertants/10^8 after			
	Ultraviolet treatment and plating on		Ethyl sulfate treatment and plating on	
	M + G*	M + G + B	M + G	M + G + B
None	4	46	21	58
M + G + T + B (10%)				
40 min	103	203	73	128
80 min	530	741	164	158

Recorded revertants per 10^8 viable cells corrected for the spontaneous frequency (2.7 on M + G, 4.2 on M + G + B).

Try cells were irradiated 20 sec with ultraviolet light or were treated for 15 min with ethyl sulfate. A portion of the resulting suspension of 2.6×10^9 organisms per ml (total) was plated directly (no incubation). Portions (0.5 ml) were added to 9.5 ml of medium and shaken in the dark at 37 C (40 and 80 min incubation). The cells were harvested and washed after incubation and 5.0 ml of buffer was added to suspend the cells before plating.

* M = minimal medium, G = glutamate (0.3 per cent), B = broth (0.05 per cent in plating medium), and T = DL-tryptophan (40 μg per ml).

in our experiments depended on the mutagen used (table 2). *Try* cells treated with ultraviolet light gave an increased number of revertants at broth concentrations which were lower than those effective with ethyl sulfate-treated cells. This difference between the two mutagens will be noticed in all the data given in this report. Even at higher broth supplementation the increase in the number of revertants was greater for ultraviolet than for ethyl sulfate-treated cells and the difference was greater at higher ultraviolet doses. The survival of ethyl sulfate-treated cells in these experiments is reported as equal to that of the ultraviolet-treated cells (table 2). The agreement is undoubtedly spurious. The ethyl sulfate-treated cells were centrifuged and washed whereas the cells treated with ultraviolet light were plated directly as were the controls. Loss of cells during the centrifuging and washing process would contribute to the apparent lethality of the ethyl sulfate. When the controls were centrifuged and washed it was seen that the ethyl sulfate effects (at the doses we used) were produced without appreciable killing (figure 1).

Incubation of cells in a complex medium after treatment with either ultraviolet light or with ethyl sulfate resulted in a large increase in the number of revertant cells obtained (table 3). The response of the ultraviolet-treated cells to broth was greater than the response of the cells after

TABLE 4

Effect of incubation with tryptophan analogues on the reversion of mutant try

Incubation	Revertants/10^8 after					
	Ultraviolet treatment and plating on			Ethyl sulfate treatment and plating on		
	M	M + G	M + G + B	M	M + G	M + G + B
None	1.8	2.1	71.0	6.5	12.6	34.2
M + G	2.8	5.9	50.5	8.1	27.7	39.5
M + G + T	28.1	37.7	44.5	35.5	35.3	46.0
M + G + MT	1.7	5.6	24.9	18.0	70.7	112.0
M + G + TZ	1.0	2.9	13.8	6.6	54.4	160.0
M + G + 2.5%B	97.0	123.0	212.0	110.0	121.0	160.0
	65% survival			59% survival		

Recorded reversions per 10^8 viable cells corrected for the spontaneous frequency (on M = 1.0, M + G = 1.1, M + G + B = 1.5.) M = minimal medium, G = glutamate (0.3 per cent), B = 0.1 per cent broth (for plating), T = DL-tryptophan, MT = 5-methyl tryptophan, and TZ = tryptazan, all at a concentration of 40 μg per ml. Incubation was for 1 hr with shaking at 37 C. After incubation the cells were washed with buffer and between 2 and 5 × 10^8 cells were added to each plate.

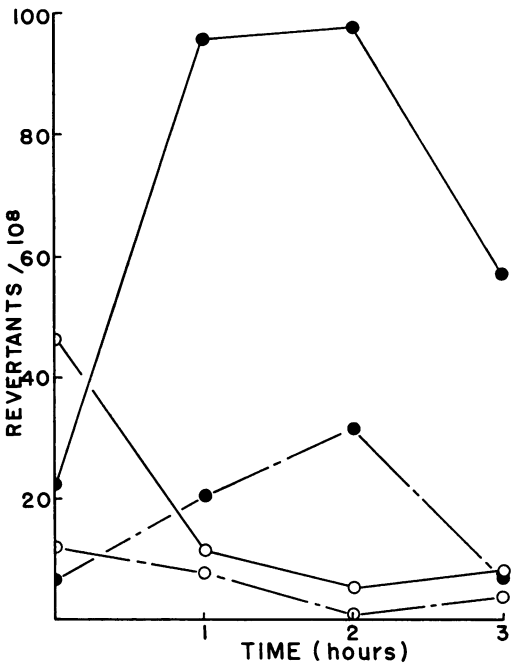

Figure 4. Effect of incubation in tryptazan-containing medium on the *try* reversion frequency. *Try* cells were treated with 0.01 M ethyl sulfate for 10 min (*solid circles*) or with ultraviolet for 25 sec (*open circles*) and 0.5 ml of the suspension (after washing and harvesting of the ethyl sulfate-treated material) was added to 9.5 ml of a liquid minimal medium containing 0.3 per cent glutamate and 40 μg/ml of DL-tryptazan. The cells were incubated with shaking at 37 C for the time indicated and were then harvested and plated. About 1.5 × 10^8 cells were plated except at 0 time when about 5 × 10^8 cells were plated. The viable cell count in the growth tubes is given as a function of time in figure 5*A*. *Solid lines* indicate material plated on minimal medium supplemented with glutamate and 0.05 per cent broth. *Long and short dashed lines* indicate the results with material plated on minimal medium.

ethyl sulfate treatment. The addition of glutamate to the plating medium increased the number of revertants obtained after treatment with the chemical mutagens but it had almost no effect on the number of revertants recovered after ultraviolet treatment.

To determine whether the mechanism by which broth incubation increased the number of revertants was the same in both cases we decided to use the tryptophan analogues. The analogue tryptazan can be incorporated into protein by *E. coli* to give an abnormal protein (Brawerman and Ycas, 1957); the analogue 5-methyl tryptophan acts as a competitive inhibitor but is not incorporated. It has previously been shown that incubation of ultraviolet-treated cells with tryptazan leads to an irreversible loss of mutations indicating the essential role of normal protein synthesis in the induction of mutation by ultraviolet (Schwartz and Strauss, 1958).

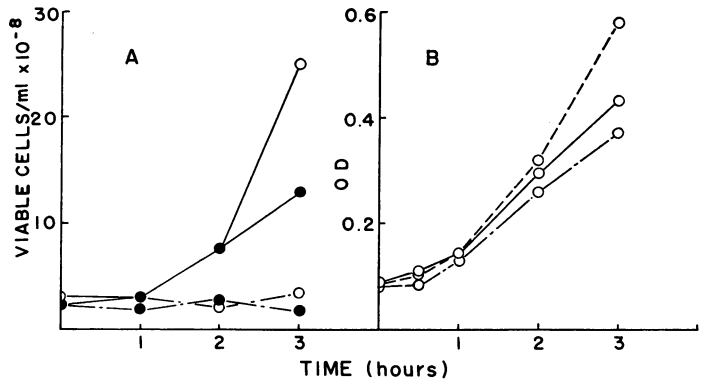

Figure 5. Growth rate of *try* cells after treatment with standard doses of ultraviolet and ethyl sulfate. *A*. Viable cells per ml as a function of time of incubation after treatment with 0.01 M ethyl sulfate for 10 min (*open circles*) or with ultraviolet for 25 sec (*closed circles*). *Solid lines* represent the results after incubation in 10 ml of minimal medium with glutamate and 40 μg/ml of DL-tryptophan. Alternate long and short dashed lines represent incubation in 10 ml of minimal medium with glutamate and 40 μg/ml of DL-tryptazan. *B*. Optical density (*OD*) at 700 mμ as a function of the time of incubation. The *solid line* represents untreated material, the *short dashed line* represents treatment with ultraviolet for 25 sec and the *alternate long and short dashed line* represents treatment with 0.01 M ethyl sulfate for 10 min. Incubation was at 37 C with shaking in minimal medium supplemented with glutamate and 40 μg/ml of DL-tryptophan. A and B represent different experiments performed at different times.

Both tryptazan and 5-methyl tryptophan decreased the yield of revertants obtained after treatment with ultraviolet light when plating was done on a solid minimal medium or on medium supplemented with broth (table 4). An entirely different result was obtained when the behavior of cultures treated with ethyl sulfate was studied. Incubation of ethyl sulfate-treated *try* cultures with the analogues increased the number of revertants obtained when plating was done on supplemented minimal medium. This increase in the number of revertants obtained after incubation of ethyl sulfate-treated cultures in tryptazan-containing medium was real and repeatable (figure 4). The number of revertants obtained after incubation of ultraviolet-treated cells with tryptazan decreased from the start of the incubation period, whereas the number of revertants obtained from ethyl sulfate-treated cultures increased for 2 hr after the start of incubation. There was no significant change in cell number when *try* cells were incubated with analogue after either treatment (figure 5). In our previous experiments we showed that tryptazan was not differentially toxic for revertants as compared to *try* cells (Schwartz and Strauss, 1958).

The difference between ultraviolet-treated cells and cells treated with an alkylating agent was also demonstrated by the response of ultra-

TABLE 5

Effect of incubation with tryptophan analogues on the reversion of mutant try after treatment with epichlorohydrin

Incubation Medium	Revertants/10^8 following Treatment with					
	Ultraviolet and plating on			Epichlorohydrin and plating on		
	M	M + G	M + G + B	M	M + G	M + G + B
None	3.8	4.7	75.0	0	4.6	65.0
M + G + T	70.0	72.0	88.0	1.6	10.1	149.0
M + G + MT	4.4	5.5	33.3	0	0	178.0
M + G + TZ	1.7	3.7	9.3	0	4.4	156.0
	65% survival			48% survival		

Recorded revertants per 10^8 viable cells corrected for the spontaneous frequency (0.6 on M, 0.9 on M + G, 1.0 on M + G + B). Abbreviations as in the legend for table 4, except that the broth supplementation on plates here equals 0.05 per cent. Incubation for 1½ hr as in the previous experiments in a total volume of 10 ml. Ultraviolet irradiation for 25 sec. Epichlorohydrin (0.6 ml) was added to 50 ml of suspension and the mixture was stirred at 37 C for 25 min. The suspension was then harvested, rinsed and made to 10 ml.

At the conclusion of the incubation cells were washed and resuspended in buffer. About 2×10^8 cells were plated after ultraviolet, about 2×10^7 cells after epichlorohydrin.

TABLE 6

Effect of epichlorohydrin on the appearance of mutant try reversions

Treatment	Revertants Plated on		Viable Cells Plated $\times 10^{-8}$	Revertants/10^8 Plated on	
	M	M + G + B		M	M + G + B
None	16 12	26 24	2.2	6.4	11.4
Epichlorohydrin, added, cells harvested at once	0 1	56 39	1.7	0.3	28.0
Epichlorohydrin added, incubation 15 min before harvesting	0 0	63 52	1.5	0	38.3

Epichlorohydrin (0.6 ml of a 1:1 solution in ethanol) was added to 50 ml of bacterial suspension.

M = minimal medium, G = 0.3 per cent sodium glutamate, and B = 0.05 per cent broth.

TABLE 7

Effect of chloramphenicol on the reversion of mutant try

Mutagen	Incubation Medium	Viable Cells Plated $\times 10^{-8}$	Mutants/10^8 Plated on	
			M	M + G
Ultraviolet, 80% survival	None	3.5	5.0	7.3
	M + G + T	3.8	31.2	38.5
	M + G + T + Chl	3.0	0	5.4
Ethyl sulfate, 73% survival	None	3.2	6.2	18.1
	M + G + T	3.5	95.1	151
	M + G + T + Chl	2.8	23.0	63.7

Recorded values corrected for the spontaneous rate on M = 1.3, M + G = 1.3. M = minimal medium, G = 0.3 per cent glutamate, T = DL-tryptophan (40 μg per ml), Chl = chloramphenicol (20 μg per ml), and B = 0.05 per cent broth. Incubation was for 1 hr with shaking at 37 C in 10 ml of the medium indicated.

violet- and epichlorohydrin-treated cells to incubation with tryptophan analogues (table 5). Incubation with the analogues had no effect on cells treated with epichlorohydrin when plated on broth-supplemented medium. In this particular experiment we plated about 5 to 10 times more of the ultraviolet-treated cells than of the epichlorohydrin-treated. This might have made a difference in the quantitative results since, as Demerec and Cahn have shown (1953), the number of residual divisions on plates is determined, in part, by the number of cells plated. There was no obvious reason, however, why the difference in cell number should have affected the qualitative picture obtained.

Epichlorohydrin treatment resulted in a loss of revertants when plating was done on minimal medium immediately after treatment with the compound (table 6). Even the spontaneous revertants disappeared following epichlorohydrin treatment. There was an increase in the number of revertants obtained after treatment when plating was done on medium supplemented with broth and glutamate. We have no explanation for this behavior.

Chloramphenicol affected cells differently depending on whether they were treated with ultraviolet light or with ethyl sulfate (table 7). After ultraviolet irradiation, chloramphenicol completely inhibited the response of cells to incubation when they were plated on either minimal medium or on glutamate-supplemented medium. Incubation with chloramphenicol resulted in a decrease in the number of revertants obtained compared to cultures incubated with no chloramphenicol. However, in contrast to cells treated with ultraviolet light, cells treated with ethyl sulfate and then incubated with chloramphenicol showed an increase in the number of revertants recovered as compared to controls plated directly with no incubation.

The difference between the effects of treatment with ultraviolet light or with ethyl sulfate could not be accounted for by a difference in the onset of growth following treatment with the doses of mutagen used (figure 5). Cell division occurred at about the same time after treatment with either ultraviolet light or with ethyl sulfate and the turbidity of ethyl sulfate-treated cultures began to increase in minimal medium supplemented with glutamate and tryptophan at about the same time as ultraviolet-treated cultures and in approximately the same manner.

DISCUSSION

It has been supposed that protein synthesis is necessary for the fixation of the mutations in-

duced by ultraviolet light (Witkin, 1956; Doudney and Haas, 1959). In the absence of protein synthesis within a short time following irradiation or when abnormal protein is synthesized in the presence of tryptazan, the mutagen formed as a result of ultraviolet irradiation appears to be consumed without the production of mutation (Schwartz and Strauss, 1958). The same mechanism cannot account for the action of the alkylating agents. Formation of an abnormal protein in the presence of tryptazan undoubtedly does affect the *expression* of mutation induced by alkylating agents when plating is done on a minimal medium, but the potentiality of producing the mutation is not affected as can be seen when plating is done on a supplemented medium. Incubation with chloramphenicol under conditions that completely inhibit the increase in ultraviolet-induced mutations still permits a significant increase in the number of revertants recovered after treatment with ethyl sulfate. Since chloramphenicol is an inhibitor of protein synthesis it is necessary to conclude that protein synthesis is not required for the fixation of potential mutations induced by the alkylating agents. The protein synthesis required after ultraviolet irradiation for the production of the maximal number of reversions is, therefore, a characteristic of the mutagen used rather than of the general process of mutation.

This conclusion is supported by the findings that bromouracil- and caffeine-induced mutations can occur in the absence of protein synthesis (Litman and Pardee, 1959; Glass and Novick, 1959), and by the difference in the photoreactivation of ultraviolet-induced damage as compared to damage induced by alkylating agents. Ultraviolet-induced effects can be photoreactivated (Jagger, 1958) and there is a correlation between the time during which these effects are subject to photoreactivation and the time in which the protein synthesis required for mutation fixation is effective. The effects of the alkylating agent, tertiary butyl hydroperoxide, could not be photoreactivated in Drosophila (Altenburg, 1958) and in some preliminary experiments we failed to obtain photoreactivation following treatment with ethyl sulfate although photoreactivation was obtained after ultraviolet treatment.

Notwithstanding the lack of a requirement for immediate protein synthesis following treatment with the alkylating agents, it does seem that some metabolic process leads to an increase in the number of mutants. An increase in the number of revertants recovered occurs after ethyl sulfate treatment when plating is done on glutamate-supplemented medium after incubation with tryptazan, and the increase even occurs on minimal medium after incubation with chloramphenicol. It would be presumptuous to attempt to identify this process or processes on the basis of our data. We suppose that after irradiation an unstable mutagen is formed which requires protein synthesis for its stabilization and then requires other metabolic processes to produce mutation (Doudney and Haas, 1959). We suppose that the product of reaction of the alkylating agents with their target substance in the cell is more stable and does not require protein synthesis to effect the mutation process. The homologue of ethyl sulfate, methyl sulfate, will alkylate the naturally occurring purine, hypoxanthine, at physiological pH and temperature *in vitro* to produce the stable mutagen caffeine (Koch, 1956) which produces mutation *in vivo* in the absence of protein synthesis (Glass and Novick, 1959).

In these experiments we used the number of revertants recovered per 10^8 cells as a measure of the effect of the various treatments on mutation. We attempted to plate equal numbers of cells (both total and viable) after ultraviolet treatment and treatment with the alkylating agents in the hope that any population effects on the plates would be the same. We have shown (figure 5) that when cells were incubated in liquid medium the ultraviolet-treated and alkylating agent-treated populations behaved in the same fashion as far as total population size was concerned. There was no significant change in cell number when *try* cells were incubated for up to 3 hr in medium containing tryptazan or chloramphenicol and there was no reason to suppose that revertant or wild-type cells would be able to multiply in the presence of these inhibitors. In previous experiments (Schwartz and Strauss, 1958) it was shown that there was no differential toxic effect of tryptazan on either *try* or revertant cells after ultraviolet treatment. Notwithstanding the incubation in liquid medium, therefore, there is no reason to suppose that selective mechanisms account for these results. Certainly there is no reason to suppose that these mechanisms operate in one way after treatment with ultra-

violet light but in another way after treatment with the alkylating agents.

On the basis of the evidence we have it is not possible to account for the *increase* in the number of revertants caused by incubation of ethyl sulfate-treated cells in a medium containing the analogue tryptazan when compared to incubation with tryptophan. Whatever the mechanism, it must be related to the ethyl sulfate treatment, for the analogue is not mutagenic by itself and does not have this effect when applied to ultraviolet-treated cells.

One of the serious difficulties in the interpretation of these experiments is our uncertainty about the mutations produced. Although the proportion of large to small colonies is similar after treatment with both mutagens, in the absence of genetic analysis it is not possible to know whether the "same" mutations are being induced. However, even if different mutations are induced, this fact would provide more evidence for the distinction in the mode of action of the two mutagens. Certainly the mutations induced by the chemical mutagens do not appear to require immediate protein synthesis for their fixation into the genome.

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SUMMARY

A comparison has been made of the effects of incubation in various media on the appearance of revertants of a tryptophan-requiring strain of *Escherichia coli* after ultraviolet irradiation and after treatment with the alkylating agents, ethyl sulfate or epichlorohydrin. Incubation of ultraviolet-treated cells with the amino acid analogue tryptazan or with chloramphenicol leads to a decrease in the number of revertants obtained. Incubation of cells treated with alkylating agents, with tryptazan, or with chloramphenicol does not lower the number of revertant cells obtained but tends to result in an increase. It is concluded that the protein synthesis required for the fixation of potential mutations after ultraviolet irradiation is a characteristic of the mutagen used, rather than of the mutation process in general.

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