Effect of Chemical Modifiers on Inactivation and Mutation-Induction by Gamma Radiation in *Escherichia coli*

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SUMMARY

The effect of chemical sensitizing and protective agents on the rate of mutation induction by gamma radiation in strain WP2 of *Escherichia coli* B/r was studied. This organism will not grow in the absence of tryptophan but it mutates spontaneously at a very low rate, and under the influence of radiation at a much higher rate, to stable forms which can grow in the absence of tryptophan. Both inactivation and mutation-induction were apparently related to radiation dose by an exponential function in the absence of modifying agents; in their presence this was not always the case. The sensitizing agents oxygen and *N*-ethylmaleimide, and the protective agents cysteine, glycerol, dimethyl sulphoxide and thiourea, affected both the inactivating and mutation-inducing actions of radiation though not always to the same extent.

INTRODUCTION

Although there are several reports of conditions before, during, and after irradiation which affect the yield of mutations with higher organisms (see, for example, Burdette, 1961), there are few in which the number of mutations induced in bacteria has been shown to be altered by chemical agents present during irradiation. Anderson (1957) showed that oxygen enhanced the rate at which mutations at two different loci in *Escherichia coli* were induced by X-rays. A dose-modifying factor (see statistical section) of 3 was found both for reversions to purine independence and for inactivation with one strain. With a different streptomycin-dependent strain the dose-modifying factor for reversions to independence was only 1.3, whereas for lethality it was 2.5. There is a report (Hollaender, Billen & Doudney, 1956) that cysteamine protected against mutation induction, but as no experimental details were given the possibility that oxygen-depletion was responsible cannot be ruled out.

Following the demonstration of sensitization by *N*-ethylmaleimide (Bridges, 1960, 1961, 1962*a*) and of protection by dimethyl sulphoxide (Bridges, 1962*b*) and thiourea (Bridges, 1963) when inactivation of colony-forming ability was the criterion of radiation damage, the influence of these compounds, together with the familiar modifiers oxygen, glycerol and cysteine, was investigated using an additional criterion of radiation effect, namely mutation.

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METHODS

Experimental

Reversions to tryptophan independence in strain WP2 of *Escherichia coli* B/r (kindly given by Dr C. O. Doudney) were studied. It is possible to use the same plating medium for total counts and for reversion counts with this organism. This is desirable because the survival of *E. coli* B/r and its response to chemical modifiers is known to be markedly influenced by the post-irradiation plating medium (Stapleton, Sbarra & Hollaender, 1955; Alper & Gillies, 1958; Freeman & Bridges, 1960). The medium used was that of Haas & Doudney (1957) which was essentially an inorganic salts solution with ammonium ions as nitrogen source and glucose as carbon source. To this was added a small amount of Oxoid no. 1 dehydrated nutrient broth (final concentration: 0.625 g./l.). A surface plating technique was used. At a suitable dilution the tryptophan-dependent organisms produced small but visible colonies after 2 days' incubation at 30°. When undiluted suspension was spread over the plate the tryptophan-dependent organisms quickly formed a background smear of growth and revertants appeared later as typical coliform colonies. Mutants were counted after 5 days' incubation at 30°. Stationary phase organisms were used for these experiments. They were washed off a 20 hr. culture on nutrient agar (Oxoid) with 10 ml. 0.067 M-phosphate buffer (pH 7), centrifuged and resuspended in 15 ml. buffer with or without the chemical modifier. Irradiations were begun 6–10 min. after resuspension and were given in five or six fractions, samples being removed for mutant and viable counts after each dose fraction. Suspensions with cysteine were left for 20 min. before irradiation. ⁶⁰Co γ-radiation was given at room temperature in a glass vessel at the rate of 4500 rad/min.; air or oxygen-free nitrogen was bubbled through the suspension for 6 min. before and throughout irradiation.

Statistical

The number of induced mutants per 10⁸ survivors (*G*) for a given dose is given by

$$G = \frac{M}{N} - \frac{M_0}{N_0} \times 10^8,$$

where *M* is the number of mutants/ml. in the irradiated suspension, *M*₀ the number of spontaneous mutants/ml. in the unirradiated suspension, *N* the number of survivors/ml. in the irradiated suspension and *N*₀ the number of viable organisms/ml. in the unirradiated suspension (Anderson, 1957). The expression corrects for pre-existing spontaneous mutants and also for those produced spontaneously during growth on the low concentration of organic nutrients on the plates (Kada, Brun & Marcovich, 1960). The number of surviving organisms (*N*) was found to be related to radiation dose (*x*) by an exponential equation $N = Ae^{-Bx}$ except in the presence of the protective agents. By fitting a line to the observed survival data using this equation it was possible to obtain a calculated mean value for the number of survivors (*N*) at each dose. This mean value is less subject to variation than the corresponding observed value at any particular dose and was therefore used in calculating the mutation rate *G* (except with the protective agents).

In the type of experiment described above it is impossible to separate the

mutagenic from the lethal action of radiation; in some of the experiments reported here, up to 96% of the bacteria were killed. In consequence, when it is desired to correlate mutation-induction with dose, it is necessary to correct the observed number of mutants to allow for those killed. The assumption is frequently made that the newly induced mutants have the same radiation sensitivity as the parent strain. When this assumption is valid a plot of G against dose is a real measure of the rate of mutation-induction. When the assumption is invalid, a plot of G against dose represents an unknown function of the true mutation rate.

The relation of G with respect to dose (x) was not linear in these experiments. Three mathematical relations were applied. The first, $G = ax^n$ is implicit in the way that Kada, Brun & Marcovich (1960) plotted their data but has no theoretical basis. The second $G = ax + bx^2$ is what might be expected if there are separate 'one-hit' and 'two-hit' components. The third, $G = ae^{bx}$ represents an exponential relation with dose. Analyses were performed with all three models; the third expression although having no theoretical basis was found to fit the data most accurately and consistently and was used for testing significance. None of the relations, however, was very satisfactory.

Where the dose-response data for inactivation and mutation-induction could be fitted with exponential curves, the effectiveness of the modifier (expressed as an enhancement or protection ratio) was given by the ratio of the slope of the fitted exponential curve for the modified state to that for the anoxic control, the largest figure being taken as the numerator. Where exponential curves could not be fitted, enhancement or protection ratios were the ratios of the doses of radiation needed to produce a stated effect under modified and control (anoxic) conditions. When the modified dose-response curve can be made to fit exactly over the control curve merely by multiplying the doses by a constant factor, the effect of an agent is said to be dose-modifying.

RESULTS

Sensitizing agents

Measurements of lethality and mutation-induction were performed simultaneously on the same suspensions and with the same plating medium. It was suspected that *N*-ethylmaleimide (NEM) might affect the mutation rate by changing the post-irradiation development and expression of mutants which are known to be dependent upon metabolism (Kada *et al.* 1960; Kada, Doudney & Haas, 1961). NEM inhibits certain energy-giving reactions and, with the technique used, was bound to be carried over to some extent on to the plating medium. Experiments in which NEM was added immediately after anoxic irradiation, however, did not show any significant change in the induced-mutation rate. It may therefore be assumed that any change in mutation rate observed with NEM is a result of its being present during irradiation.

Three experiments in which the effects of oxygen and NEM were investigated are combined in Fig. 1, where the surviving fraction and G are plotted against dose of radiation. The fitted lines represent an exponential function of dose.

Neither NEM nor oxygen had any effect on the spontaneous mutation rate. NEM and oxygen enhanced both radiation-induced mutation and inactivation, and the enhancement ratios are shown in Table 1. For inactivation the enhancement

Table 1. *Enhancement ratios for the effects of oxygen and N-ethylmaleimide on Escherichia coli WP2*

N-Ethylmaleimide (NEM) was used at 0.0005 M, oxygen at the concentration of air-saturated solution.

	Enhancement ratio		
	For inactivation	For mutation-induction	
		Based on slopes	Based on doses for which $G = 300$
Oxygen	8.2	2.4	2.1
NEM	2.1	1.8	1.7
Oxygen + NEM	3.1	2.4	1.9

Fig. 1

Fig. 2

Fig. 1. Irradiation of *Escherichia coli* WP2: effect of oxygen (air-saturated solution) and 0.0005 M-*N*-ethylmaleimide (a) on inactivation, (b) on mutation-induction. The lines represent fitted exponential functions.

Fig. 2. Irradiation of *Escherichia coli* WP2 under anoxia: effect of 0.1 M-cysteine (a) on inactivation, (b) on mutation-induction. The lines have been fitted by eye.

ratios, based on slopes of survival curves, were greater for oxygen and for NEM + oxygen (which were identical) than for NEM alone. A similar pattern was shown for mutation induction when the enhancement ratios were based on the slopes of the fitted exponential functions except that the ratios were smaller, indicating that neither oxygen nor NEM enhanced mutation-induction to the same extent as inactivation. When, on the other hand, the enhancement ratios were based on the doses needed to produce a given effect (e.g. $G = 800$) an additional fact emerged, namely that the mutation enhancement ratio for oxygen + NEM was significantly less than that for oxygen alone. Examination of the data reveals that treatment with NEM + oxygen is not strictly dose-modifying. One interpretation of the data is that a constant small proportion of mutants is not expressed when NEM is present under aerobic conditions.

Fig. 3. Irradiation of *Escherichia coli* WP2 under anoxia: effect of *m*-glycerol, 0.2 *m*-thiourea and 2 *m*-dimethyl sulphoxide (DMS) (a, c) on inactivation, (b, d) on mutation induction. The lines have been fitted by eye.

Protective agents

All the protective agents were tested under conditions of strict anoxia, thus eliminating oxygen-depletion as a cause of apparent protection. None of the chemicals tested affected the spontaneous mutation rate. The data are plotted in

Figs. 2 and 3 and the protection ratios are given in Table 2. In Fig. 3 (*c, d*) the results of two experiments with dimethyl sulphoxide and thiourea are combined. Two points emerge from these data. With the possible exception of cysteine, the treatments were not dose-modifying; protection was shown only at doses greater than 10–15 krads. With glycerol, for example, this ‘threshold’ effect was shown whether the compound was added 6 or 26 min. before irradiation, indicating that the threshold is not due to a requirement for pre-incubation with the compound. The second point is that glycerol, cysteine and thiourea protected against mutation-induction and inactivation to the same extent, whereas dimethyl sulphoxide was appreciably less efficient at protecting against mutation-induction than against inactivation.

Table 2. *Protection ratios for the effects of various compounds on Escherichia coli WP 2*

All irradiations were under anoxic conditions.

	Protection ratio	
	For inactivation. Based on doses for 10 % survival	For mutation-induction. Based on doses for which $G = 400$
Cysteine (0.1 M)	1.7	1.7
Glycerol (1 M)	1.9	1.8
Thiourea (0.2 M)	1.9	2.0
Dimethyl sulphoxide (2 M)	1.9	1.5

DISCUSSION

Sensitizing agents. In previous publications (Bridges, 1961, 1962*a*) it was shown that *N*-ethylmaleimide (NEM) enhances the lethal action of radiation only when present during irradiation; in this and other respects it resembles oxygen. It was suggested that NEM and oxygen are both able to combine with reactive sites which appear during irradiation on important biological molecules. In the absence of NEM and oxygen these sites are assumed to be restorable so that the molecule does not lose its biological integrity. The sites which react with oxygen are not known but it was suggested that those which react with NEM might be –SH groups or –S free radicals. If this hypothesis be correct, proteins must be intimately involved in sensitization, and hence in mutation, since they are the only large sulphur-containing molecules of importance to the cell.

The present results are somewhat difficult to reconcile with this hypothesis if mutations arise as a result of changes in deoxyribonucleic acid (DNA). Hill (1962) studied ultraviolet-induced reversions to prototrophy in *Escherichia coli* B/r (WP2) and concluded that the change from tryptophan dependence to independence was frequently unstable and might be due to the detachment of an episome from a chromosomal suppressor locus. Such a possibility illustrates one of the problems involved in the study of mutagenesis, namely the difficulty of knowing whether the mutations observed are chromosomal mutations or episomal changes. As systems in which mutants are easily induced tend to be chosen for investigation, it is possible

that they may be atypical. Biochemical reversions to prototrophy are certainly atypical in that they are beneficial under almost all conditions of growth. It is not known what episomes are composed of, apart from DNA, so that it is difficult to interpret results in terms of chemical changes.

There are several lines of evidence which indicate that chemical sensitization depends on the nature of the target molecule and is not a uniform and general phenomenon, for example: the effects of oxygen and NEM vary in extent from one species to another (Bridges, 1961; Cromroy & Adler, 1962); the magnitude of the oxygen effect can be markedly influenced by treatments after irradiation (Alper & Gillies, 1958); the enhancing effects of NEM and oxygen are different in magnitude when inactivation or mutation-induction are taken as the end results; and NEM enhances only 'type 3' (protein?) damage in *Serratia marcescens* (Dewey, 1963). These facts support the above hypothesis about the mechanism of sensitization since only in those molecules containing -S-S- bonds or masked -SH groups would radiation damage be enhanced by NEM. The importance to the cell of such molecules might be expected to differ in different organisms.

Protective agents. Four types of compound are known to protect cells from radiation damage by means other than oxygen depletion; aliphatic alcohols, dimethyl sulphoxide, thiourea and the cysteine-cysteamine group. Their mechanism of action is not known, but under anoxic conditions they all appear to protect largely against the same component of damage (Bridges, 1963). In contrast to sensitization the available evidence seems to indicate that chemical protection is a fairly uniform and general phenomenon (Cromroy & Adler, 1962; Bridges, 1963) and largely independent of the nature of the targets (Dewey, 1962). The present results with thiourea, glycerol and cysteine would seem to confirm this idea. With dimethyl sulphoxide, however, significantly less protection was afforded against mutation-induction than against inactivation. It is possible that dimethyl sulphoxide has difficulty in penetrating sufficiently near to the locus which determines tryptophan-dependence.

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