Split-dose Irradiation of *Escherichia coli* in the Absence and Presence of Mercaptoethylamine

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SUMMARY

A single dose of γ -radiation to *Escherichia coli* B gave a semi-logarithmic dose-response curve. A second irradiation 3, 6, or 18 hr after a priming dose of 8 krad showed that the cells had become more radio-sensitive during the incubation between irradiations.

When mercaptoethylamine was present during irradiation, protection against the lethal effects of the γ -radiation occurred. After a priming dose to the bacteria in the presence of the radio-protective chemical, and incubations for 3, 6, or 18 hr in its absence, a second irradiation in the absence of the chemical showed them to be much more radio-resistant than the normal unprotected *Escherichia coli* B. This indicates that the presence of the protector during irradiation alters the type of damage induced or the development and repair of that damage.

INTRODUCTION

In many types of cell significant differences can be demonstrated between the survival curves obtained following single and split-dose irradiations with γ rays. These differences may reflect repair of radiation damage during the interval between split-dose irradiations, synchronization of cells in a certain stage of the cell cycle at the time of the second irradiation, or changes in metabolism which affect the response of the cells to a second irradiation (Elkind & Sutton, 1959; Elkind, 1966).

Radioprotective chemicals are agents which by their presence during irradiation decrease the effects of sparsely ionizing radiations on living cells (Bacq, 1965). Post-irradiation repair of radiation damage induced in the presence of protector has received little attention, despite the possibility that the protective chemical may modify the initial radiation injury in such a way that the post-irradiation restoration may also be modified.

The response of *Escherichia coli* B to single and split-dose irradiations in the absence and presence of the chemical protector mercaptoethylamine (MEA) was therefore investigated, in order to determine the effects of the protector on the development of damage following irradiation.

METHODS

An inoculum of *Escherichia coli* B from a Difco nutrient agar slope was transferred to 500 ml. of Oxoid CM. 1 nutrient broth. After shaking vigorously, this was incubated at 37° for 24 hr to obtain a culture in stationary phase (Elias, 1961). The bacterial concentration was then about 2×10^8 bacteria/ml. A 45 ml. volume of the culture was centrifuged and the bacteria added to 45 ml. of an aerated Oxoid CM. 1 nutrient broth + 0.85 % saline mixture (with a broth: saline ratio of 1:100) at 0°.

For experiments not involving MEA this suspension was diluted 1/100 in the saline broth mixture and left for 1 hr at 0°. When the bacteria were to be protected, the chemical MEA was added to the suspension to give a final concentration of 0.04 M (Ginsberg, 1966) and left for 1 hr at 0° before irradiation. The dilution of these protected bacteria by 1/100 occurred after irradiation to minimize any effects of the protector in the later part of the experiment.

Aeration of the bacterial culture and suspensions was done by continuous shaking during the incubations before irradiation and between irradiations.

The suspensions were irradiated at 0° in a conical flask with a cotton-wool stopper, using a 9000 C ⁶⁰Co-γ source, giving a dose of 815 rad/min. as estimated by Fricke ferrous sulphate dosimetry. Serial samples were taken from the suspension after known irradiation doses, and the culture was aerated by shaking each time a sample was taken (at approximately 5 or 10 min. intervals).

The samples from the irradiated bacterial suspensions were diluted, plated out in triplicate on Difco nutrient agar plates and incubated for 24 hr at 37°. Those colonies which were visible to the naked eye were counted, and the criterion of survival of an irradiated bacterium was taken as the ability to form a visible colony.

Recovery of the cell suspensions was investigated following initial radiation doses of 8 krad in the absence of MEA and 12 krad (in the presence of MEA): these doses gave about 20 to 30 % survival. In one experiment a dose of 12 krad was also given to a culture lacking MEA. The non-protected samples were incubated in the saline broth mixture for 3, 12, or 18 hr between the two irradiations, at a temperature of 18° which gives a reasonable opportunity for restoration (Stapleton, 1955; Hollaender & Stapleton, 1953). Samples which had been irradiated in the presence of MEA were immediately centrifuged at 0 to 1° and resuspended in the saline + broth mixture; this was done either one or three times. The suspension was then incubated at 18° for 3, 12, or 18 hr before the second irradiation in the absence of MEA.

Each experiment was repeated three times, except for the 12 hr incubation after irradiation in the presence of MEA (repeated twice) and the experiments involving the preincubation of MEA (once), and three repeated centrifugations before incubation (once).

RESULTS

Single-dose experiments

The effects of a single dose γ-irradiation on bacterial survival were examined. When MEA was not present (Fig. 1*a*) the dose-response took the form of a single exponential function, with the D_0 (i.e. the dose required to reduce the number of surviving bacteria by 63 %) being 6.5 ± 0.6 krad.

When the bacterial suspension was incubated with MEA at 0° for 1 hr, centrifuged, resuspended in a saline broth mixture without MEA and immediately irradiated at 0°

the form of the dose response curve (Fig. 1c) did not differ significantly from that of the bacteria which had not been pre-incubated with MEA (Fig. 1a). The D_0 was in this event 6 ± 1 krad. This indicates that after the single centrifugation and resuspension the concentration of MEA associated with the bacteria was not sufficient to alter their dose response significantly.

Fig. 1. Single-dose survival curves after the ^{60}Co - γ irradiation of *Escherichia coli* B in the absence and presence of mercaptoethylamine. (a) No mercaptoethylamine present during irradiation; (b) Irradiation in the presence of 0.04 M-mercaptoethylamine; (c) irradiation after one washing of the bacteria following 1 hr preincubation with 0.04 M-mercaptoethylamine. The curve of (a)—no mercaptoethylamine present during irradiation—is superimposed in this graph, thus ---.

In the presence of MEA an initial rapid drop in survival predominated until about 12 krad and at higher doses the decrease in survival with dose became slower, with D_0 equal to 13 ± 2 krad. Extrapolation of this slower exponential decrease to the ordinate gave an intercept at about 35 to 45 % (Fig. 1b). This suggests that two different populations coexist in the presence of MEA: first, a population comprising 55 to 65 % of the total with a D_0 not greatly different from that of unprotected bacteria, and secondly, a population comprising 35 to 45 % of the total with D_0 double that of unprotected bacteria. The dose-reduction factor for this second population is thus about 2 (i.e. D_0 in presence of MEA/ D_0 in absence of MEA).

Split-dose experiments: no MEA.

The effects of splitting the irradiation dose by 3, 12, or 18 hr were examined (Fig. 2). The curves for the second irradiation all have a 'shoulder', i.e. the slope of the curve increases with dose (the single dose curve is shown for comparison). The slope of the dose-response curve at low doses was not significantly different from that of the single dose curve, except after an 18 hr interval when the initial D_0 was about 9 krad. At second doses higher than about 20 krad the slope of the curve was much steeper, with the D_0 lying between 3.0 and 4.0 krad in each instance; this demonstrates a sensitization of the bacteria to the radiation at high doses, relative to their sensitivity as demonstrated by the single-dose experiments.

The effect of a second dose of radiation 12 hr after an initial dose of 12 krad (i.e. the same dose as given to protected bacteria) was similar to that after an initial dose of 8 krad.

Split-dose experiments: MEA present during first irradiation

The dose response curves for a second irradiation in the absence of MEA (following incubations at 18° for 3, 12, or 18 hr after the first irradiation in the presence of MEA) again demonstrated a shoulder after low doses (Fig. 3). At higher doses (greater than 20 to 25 krad) the curve became exponential, approximately parallel to the single-dose curve in the absence of MEA, and the D_0 values for the three curves at a high dose all lay between 5.5 and 7 krad. Thus no sensitization occurred in this case, above the radio-sensitivity of the bacteria to a single dose.

Fig. 2. The effect of a second ^{60}Co - γ irradiation on the survival of *Escherichia coli* B. No mercaptoethylamine was present during irradiation. The single-dose response curve of (a) —no mercaptoethylamine present during irradiation—is superimposed on the graphs for comparison thus ---. (a) 3 hr interval between irradiations, following a primary dose of 8 krad; (b) 12 hr interval between irradiations, following a primary dose of 8 krad; (c) 18 hr interval between irradiations, following a primary dose of 8 krad; (d) 12 hr interval between irradiations following a primary dose of 12 krad.

Bacteria which had MEA present during the first irradiation were therefore less sensitive to a second dose than the bacteria which were not incubated with MEA during the first irradiation. To investigate the possibility that some MEA may remain in the bacteria after one centrifugation and resuspension, thereby causing protection during the

second irradiation, the bacteria in one experiment were centrifuged and resuspended three times at the beginning of a 12 hr interval at 18°: the results of a second irradiation of these bacteria (Fig. 3*d*), showed once again a shoulder, and a final D_0 of 6 krad.

Cell division during split-dose experiments

The suspending medium during the interval between irradiations was always the saline-broth mixture. No bacterial division occurred in any suspension which had MEA present during the first irradiation, nor in the suspension without MEA which was left for 3 hr between irradiations, as estimated by plating-out techniques.

Fig. 3. The effect of a second ^{60}Co - γ irradiation in the absence of MEA on the survival of *Escherichia coli* B after a priming dose of 12 krad in the presence of 0.04 M-mercaptoethylamine. The single-dose response curve of Fig. 1 (*a*)—no mercaptoethylamine present during irradiation—is superimposed on the graphs for comparison, thus ---. (*a*) 3 hr interval between irradiations; bacteria washed *once* after first irradiation; (*b*) 12 hr interval between irradiations; bacteria washed *once* after first irradiation; (*c*) 18 hr interval between irradiations; bacteria washed *once* after first irradiation; (*d*) 12 hr interval between irradiations; bacteria washed *three times* after first irradiation.

The bacteria initially irradiated in the absence of MEA had increased in number by factors of 10 and 32 at the end of 12 and 18 hr incubations respectively, representing concentrations 4 and 12 times as great as that of the bacterial suspension before irradiation. Thus the increase cannot be caused merely by repair of damage during incubation (Stapleton, 1955) but must involve cell division.

Toxicity of MEA

Because MEA is a toxic chemical, a test was made to determine whether it was damaging the bacteria, thereby affecting their measured survival. They were incubated with MEA at 0 to 1° for 1 hr, and were then diluted and plated out.

The concentration of 0.04 M-MEA (used in these experiments) did not measurably affect survival; no significant decrease in survival occurred at concentrations up to 0.25 M.

DISCUSSION

The dose-response curve for the single-dose irradiation in the presence of MEA demonstrated that there were probably at least two different populations of bacteria, one showing little protection and one showing considerably greater protection. This is an unusual finding, as other workers appear to find a fairly uniform protection of all the cells (Elias, 1961; Ginsberg, 1966; Comroy & Adler, 1962), and it may be caused by differences in the organisms' abilities to take up MEA according to their cell cycle stage at the beginning of incubation with MEA, before division stops (Ginsberg, 1966). The use of high concentrations, relative to the amount of protective chemical, may contribute to this effect. There were about 2×10^8 bacteria per ml. in this study, whereas other workers have used between 4×10^4 and 10^8 bacteria per ml. (Elias, 1961; Hernadi, Valyi-Nagy, Nagy & Seney, 1962; Kohn & Gunter, 1959; Ginsberg, 1966), and therefore they may have had excess MEA present, thereby allowing all the bacteria to take up sufficient MEA to induce radio-protection.

Dose-reduction factors depend on many variables (Elias, 1961; Ginsberg, 1966, Comroy & Adler, 1962): the dose-reduction factor of 2 found for protected bacteria in this study cannot therefore be compared directly with those found by other workers but it may be related to the magnitude of the oxygen effect in these bacteria under these experimental conditions (Comroy & Adler, 1962).

The possibility of variations in oxygen concentrations during the experimental procedures must be considered, as Alper & Moore (1967), using slightly different experimental conditions, found an oxygen enhancement ratio of 2.84 for *Escherichia coli* B. Any consumption of oxygen during the autoxidation of cysteamine should be maximum during the incubation prior to irradiation, when aeration occurs. Decrease of oxygen concentration due to the γ -irradiation should be small, as the dose-rate is low enough to allow diffusion of oxygen into the bacteria (Dewey & Boag, 1959). The oxygen consumption of the bacteria, although finite at 0° during the 10 min. irradiation periods, should not be sufficient to induce significant anoxia. The possibility that the oxygen concentrations may alter slightly during irradiation cannot therefore be excluded, but significant decrease in the dose-response curves would require a comparatively high degree of anoxia, with an oxygen partial pressure of the order of 1% or less (Howard-Flanders & Alper, 1957; Dewey & Boag, 1959).

Experiments were standardized to give equivalent amounts of radiation damage (as measured by cell death) during the first irradiation, and after that the conditions were the same for both previously protected and unprotected organisms. Thus differences in cellular metabolism and radiation responses following a primary irradiation should therefore reflect differences in the type and development of damage induced by the initial irradiation rather than environmental factors after irradiation.

In the split-dose experiments in the absence of MEA the low D_0 obtained after high second doses suggests that the incubation between irradiations renders bacteria less capable of withstanding high radiation doses, while having little effect on their response to low doses. This may be in part due to the bacteria entering a growth phase following irradiation: the bacteria had not actually divided during the 3 hr incubation, but at 12 hr had divided on average 3.2 times. If this explanation is correct, it must be postulated that at 3 hr after 8 krad the bacteria are moving through the DNA synthetic and the G_2 period before division and are therefore rendered more sensitive to the radiation, although Stapleton (1955) found that the radio-sensitivity of *Escherichia coli* B/R only increased when the bacteria entered log. growth phase, and actually decreased at the end of the lag phase. Although it may be possible to investigate the importance of growth phase by allowing these irradiated bacteria to divide until they reach stationary phase (this requiring on average 9 to 10 divisions) and then determining the radiation response, there would have been considerable selection for bacteria showing little radiation damage and a consequent depression in the percentage of heavily damaged bacteria during the divisions; the final culture in stationary phase could not therefore be truly compared with the culture in stationary phase during the first irradiation. Also, considerable intracellular repair of damage may occur, thereby altering the radiation response.

The change in sensitivity after irradiation could also be caused by the development of radiation-induced changes in bacterial metabolism (not connected with division) which could change the organism's response to further radiation lesions.

The response of bacteria to a second irradiation, after irradiation in the presence of MEA, was significantly different from when MEA was not present during the first irradiation, and indicates that the chemical may not have been removed from the bacteria by the washing procedure. However, pre-incubation of the bacteria with MEA, followed by centrifugation and resuspension, did not induce radioprotection, and this suggests that the chemical is readily removed. A similar conclusion may be derived from the fact that repeating the normal washing procedure three times after the first irradiation did not significantly alter the dose-response curve for the second irradiation. Ginsberg (1966) has also shown, using ^{35}S -MEA, that the process of adsorption or absorption is readily reversible and that the chemical is rapidly removed from *Escherichia coli* by washing, when MEA-induced inhibition of DNA synthesis and division ceases immediately and radio-protection also ceases. It seems, therefore, that the additional protection observed in this study is unlikely to be caused by the presence of MEA.

Similarly, the lack of division after irradiation of the bacteria in the presence of MEA does not seem to be caused by a residue of the chemical remaining in the bacteria and it seems therefore that the type of radiation damage occurring in the presence of MEA differs from that occurring in its absence. This difference expresses itself to some extent in the inhibition of division, which may partially explain the protective action of the chemical, in accordance with the view that bacteria have a better chance of recovering from radiation damage if there is a delay in division following irradiation (Sinclair, 1966).

The development of the shoulder on the split-dose curves, with extrapolation numbers in the range 5 to 10, suggests that bacteria after being irradiated in the presence of MEA are altered in such a way that they can withstand a larger amount of radiation

damage before being killed. This is unlikely to be a result of cessation of division, as the bacterial culture for the single experiment in the absence of MEA was also in stationary phase, this latter observation is also confirmed by the experiment involving pre-incubation with MEA, for if the culture had been in the growth phase the chemical should have brought it to stationary phase, altering the dose response (Ginsberg, 1966). The development of the shoulder would thus appear to be the result of metabolic changes in the bacteria following the first irradiation, rather than the cessation of division.

When the second radiation dose was sufficient to decrease survival to a point on the exponential portion of the dose-response curve, the slope of the curves were not significantly different from the single-dose response curve. Thus, once the bacteria have accumulated their maximum amount of sublethal damage, the response as measured by survival is similar to that of previously unirradiated bacteria.

It would appear, therefore, that MEA protects the bacteria against the lethal effects of γ -irradiation, and this protection also alters the type of damage occurring in such a way that division is greatly inhibited, and the bacteria can withstand comparatively large amounts of radiation damage before the lethal amount of damage is reached.

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