The Development of Radiation-resistant Cultures of *Escherichia coli* 1 by a Process of 'Growth-irradiation Cycles'

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SUMMARY

The process whereby bacterial cultures are alternately grown and then irradiated (γ -radiation) through several 'cycles' has been studied as a means of developing radioresistant cultures of *Escherichia coli* 1. The nature of the environment at the time of irradiation influenced the extent of development of radioresistance. Radioresistance development was higher when the bacteria were irradiated in an organic environment than in an inorganic environment. It is not thought that radioresistant mutants would be produced in high numbers by this type of process during a dose-fractionation procedure of food irradiation.

INTRODUCTION

The development of radiation-resistant strains of micro-organisms may play an important role in determining the effectiveness of the radiation-processing of foods. The work of Gunter & Kohn (1956) suggested that when a cell population consists of a mixture of comparatively sensitive and resistant cells, radiation can be expected to select the more resistant cells. Several authors have shown that radiation-resistance may be induced in microbial populations where radiation survivors are repeatedly grown and exposed to further doses of radiation. Erdman, Thatcher & MacQueen (1961) reported that specific bacteria of public-health significance could be induced to develop radiation-resistance by the repeated γ -irradiation of radiation survivors in cultures when using doses which destroyed a high proportion of the population. Erdman *et al.* (1961) developed radioresistance in *Escherichia coli* and *Streptococcus faecalis* cultures, but not in *Clostridium botulinum* type E or *Salmonella gallinarum*. Gaden & Henley (1953) obtained an increase in resistance of *E. coli* strains B and B/R by repeated γ -irradiation of survivors (although the same workers obtained an increased radiation-sensitivity with *E. coli* strain 15). Increased radioresistance, which was a stable character, was developed in *Saccharomyces cerevisiae* by Maisin, Lambert & van Duyse (1955) by repeated exposure of survivors of X-irradiation. Luckiesh & Knowles (1948) with *E. coli*, showed an increase in resistance to ultraviolet (u.v.) radiation among u.v.-irradiation survivors. Both Erdman *et al.* (1961) and Maisin *et al.* (1955) found that the development of radioresistance in cultures occurred in a step-wise fashion; and Erdman *et al.* (1961) and Gaden & Henley (1953) showed that during the course of resistance-development a plateau was attained beyond which no further increase in resistance to a constant dose could be obtained.

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Erdman *et al.* (1961) showed that the radioresistance developed in cultures of *Streptococcus faecalis* and *Escherichia coli* was a 'true' radiation-resistance, and not a resistance to the possible toxic effects of reaction products from free radicals produced during the irradiation. Following the attainment of resistance-development plateaus, the *S. faecalis* and *E. coli* cultures were 1.6 times and 1.9 times more resistant than the respective parent cultures. Subsequently, Dr I. E. Erdman (1962; personal communication) showed that it was possible to extend the degree of radioresistance in such cultures by increasing the radiation doses at each plateau level. The development of the ' γ ' series of radioresistant *E. coli* strains by Erdman (cited by Idziak & Thatcher, 1964) was based on this procedure, the dose being increased at each ' γ ' level (or plateau) during multiple cycles of growth and irradiation to produce several distinct radioresistant cultures showing a progression in increased radioresistance. The details of the experimental procedure published by Erdman *et al.* (1961) were limited and were not in total agreement with those given in the personal communication from Dr I. E. Erdman. The purpose of the experiments described in the present paper was to establish a reproducible procedure for carrying out a series of growth-irradiation cycles on cultures of *E. coli*; and also to investigate the effect of growth medium and irradiation environment on radioresistance development. The work was not concerned with obtaining a maximal resistant development, but rather to compare radioresistance development in three well-defined series of experiments using the same radiation dose and the same limited number of cycles in each series. No attempt was made to reproduce the extreme radioresistance found by Erdman.

Growth-irradiation cycle. A growth-irradiation cycle is defined as the process initiated by the inoculation of the medium, the subsequent growth of the culture, the irradiation, followed by the immediate inoculation of fresh medium with the irradiated bacteria. It may be represented thus:

METHODS

Organisms. The test organism used for these studies was the strain of *Escherichia coli* 1 used by Erdman *et al.* (1961) and later by Dr I. E. Erdman (personal communication). This culture had been isolated from food, and was kindly supplied by Dr I. E. Erdman of the Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Canada, in 1962. Stock cultures of the organism were maintained on TGY (Tryptone-glucose-yeast extract) agar slopes, stored at 4°, and subcultured monthly.

Growth media and culture method. Cultures were grown in 60 ml. medium in 500 ml. Erlenmeyer flasks incubated at 37°, with aeration provided by shaking the flasks on reciprocating-shaker water baths (The Mickle Laboratory Engineering Co., Gomshall, Surrey) at 100 oscillations/min. Growth media used were: (a) nutrient broth (Difco

Bacto) + 0.3 % (w/v) yeast extract (YE; Difco Bacto, dehydrated); (b) mineral salts medium, M 63, + 0.4 % (w/v) D-glucose (AnalaR). The composition of M 63 mineral salts medium (all chemicals AnalaR grade) was: KH_2PO_4 , 0.1 M; $(\text{NH}_4)_2\text{SO}_4$, 0.02 M; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001 M; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.00001 M, in de-ionized water, adjusted to pH 7.0 with 6 N-KOH. The above media were solidified, when required, by the addition of 1.5 % (w/v) Ion Agar No. 2 (Oxoid).

Colony-counting medium, Tryptone-glucose-yeast extract agar (TGY). Bacto tryptone (Difco) 5.0 g.; dehydrated Bacto yeast extract (Difco) 3.0 g.; D-glucose 1.0 g.; de-ionized water to 1 l.; pH 6.8.

Irradiation, and growth-irradiation cycle procedures. Gamma-radiation was provided by a ^{60}Co source. A laboratory unit (the 'Hotspot' Mk IV, U.K.A.E.A. design no. 0497) of nominal activity 1084 Ci. was supplied by the U.K.A.E.A.

Samples (30 ml.) of cultures grown to the stationary phase were irradiated in a specially constructed glass vessel. The vessel, 5 cm. diam. and 7.5 cm. high, was equipped with gassing inlet and outlet ports in the removable lid. Irradiations were performed at room temperature under aerobic conditions, oxygen (British Oxygen Co. Ltd.) being bubbled into the suspension before and during the irradiation at the rate of 0.4 l./min.

A dose of 15 krad was selected for use in the cycling procedure, and 1 ml. samples were removed aseptically from the irradiation vessel before and after the radiation dose was given. These samples were transferred to 60 ml. fresh medium, and the cultures (which became the control (c) and test (t) cultures) were incubated in the standard manner. The test culture had now undergone one growth-irradiation cycle. After incubation for 12 hr the irradiation process was repeated on the test culture and a 1 ml. sample removed to fresh medium; the control culture was also sampled into fresh medium. The cycling process was repeated until 7 growth-irradiation cycles had been completed. Cultures were stored on plates of the appropriate solid medium, and test and control cultures at this stage were designated t 7 and c 7, respectively.

It was found essential to adopt rigorous aseptic techniques throughout each series of cycles to avoid contamination of master cultures. All procedures, apart from irradiations, were done in a previously fumigated laboratory.

Estimation of growth. Growth was measured as the increase in extinction at 600 m μ by using a Unicam S.P. 600 spectrophotometer and 1 cm. glass cells, reading against a sterile medium blank. For growth estimations during the cycling procedures, duplicate cultures were used to eliminate the chance of contaminating master cultures of the control and test by repeated sampling during the incubation.

Radiosensitivity estimates, by colony counts. Suspensions of organisms were irradiated at a population density corresponding to an E_{600} of 1.5. Colony counts were made by the pour-plate method, with TGY agar as the recovery medium. Plates were incubated at 37° and colonies counted after 24 hr, and checked after further incubation. Counts were made in triplicate.

RESULTS

Growth-irradiation cycle procedures were designed in three series so that growth medium and irradiation environment could be varied.

Series A

Growth medium: nutrient broth + 0.3 % (w/v) yeast extract. Irradiation environment: bacteria irradiated in the growth medium.

Establishment of the radiation dose to be used during the process. Figure 1 shows the results of the radiosensitivity estimate made on 30 ml. of a culture in the stationary phase of growth. Survival (%) is plotted on a $\log_{10}$ scale against the dose. The D_{10} value (decimal reduction dose) for bacteria under these conditions was 4.8 krad,

Fig. 1

Fig. 2

Fig. 1. Dose-survival relationship for *Escherichia coli* 1 (the parent strain). Bacteria were grown to the final stationary phase, with shaking at 37°, in nutrient broth + 0.3 % (w/v) yeast extract, and irradiated in the growth medium. Survivors were estimated by the pour-plate method with TGY agar as recovery medium and incubated at 37° for 24 hr. Each point represents the mean value of counts in triplicate.

Fig. 2. Post-irradiation growth of the parent strain of *Escherichia coli* 1. Bacteria were grown to final stationary phase in nutrient broth + 0.3 % (w/v) yeast extract, then irradiated in the medium. Samples (1 ml.) were removed from unirradiated (control) and irradiated (15 krad) suspensions and transferred to 60 ml. nutrient broth + 0.3 % (w/v) yeast extract in 500 ml. Erlenmeyer flasks. Flasks were incubated, with shaking, at 37°. Samples were removed aseptically at intervals for E_{600} determination against a medium blank. ○, Control; ●, irradiated—15 krad.

and a dose of 15 krad inactivated slightly over 99.9 % of the original population; this dose was selected as the radiation dose to be used during subsequent growth-irradiation cycle procedures. Erdman *et al.* (1961) irradiated cultures at doses giving over 99 % mortality in parent cultures.

Figure 2 shows the effect of 15 krad on the bacteria under the conditions of series A, assessed by post-irradiation growth. This was done to establish at what time the bacteria again approached the stationary phase of growth so that they could be re-irradiated. After incubation for 12 hr bacteria surviving irradiation had passed through the exponential growth phase and approached the stationary phase. It was decided that cultures could be re-irradiated 12 hr after the initial irradiation and subculture. Throughout subsequent cycling procedures, irradiations were done after incubation for 12 hr.

Fig. 3

Fig. 4

Fig. 3. Dose-survival relationships for control and test cultures of *Escherichia coli* 1 following 7 complete growth-irradiation cycles (series A). Bacteria were grown to final stationary phase in nutrient broth + 0.3% (w/v) yeast extract and irradiated in the growth medium. Survivors were estimated by the pour-plate method with TGY agar as recovery medium, incubated at 37° for 24 hr. Each point represents the mean value of counts in triplicate. ○, Control culture; ●, test culture.

Fig. 4. *Escherichia coli* 1. Development of radioresistance during the growth-irradiation cycle process, as demonstrated by post-irradiation growth (series A). Samples (1 ml.) were removed from the cultures immediately following the 1st, 3rd and 7th irradiations (15 krad), and also from the unirradiated controls. Samples were transferred to 60 ml. nutrient broth + 0.3% (w/v) yeast extract and flasks were incubated, with shaking, at 37°. During the incubation, samples were removed for the determination of E_{600} against a sterile medium blank. ○, ●, 1st cycle; □, ■, 3rd cycle; △, ▲, 7th cycle. Open symbols, irradiated; closed symbols, unirradiated controls.

Growth-irradiation cycle procedure in series A. The established growth-irradiation cycle procedure was done for 7 complete cycles. The control (c 7) and test (t 7) cultures were subcultured on TGY agar and stored at 4°. The two cultures were visually pure, and did not differ from each other in colonial or cellular morphology. The dose survival

curves in Fig. 3 show a marked development in radioresistance in the t 7 culture compared with the c 7 culture. D_{10} values were, respectively, 7.3 krad and 5.3 krad, and according to the difference in these values the increase in radioresistance was 38 % after 7 cycles. The control D_{10} value was found to be increased from 4.8 to 5.3 krad following 7 cycles without irradiation. No real explanation can be suggested for this phenomenon, and the increase did not affect the result since % increase in radioresistance was derived from test and control D_{10} values after 7 complete cycles.

The progressive development of radioresistance with increasing number of cycles was also apparent in the post-irradiation growth curves plotted during various cycles. Figure 4 shows that while the curves for the test culture are gradually displaced in time towards the y axis with increasing number of cycles, the curves for control cultures are not displaced. This provides a clear indication of the development of resistance, for it is to be expected that the more resistant cultures will be less affected by the same dose of radiation, and hence grow more quickly than the initial irradiated culture. The method also indicates that the development of radioresistance is progressive (though irregular) and suggests a process of selection. The time required for the test culture to reach a $\log_{10} E_{600}$ value of 1.5 was 10 hr after the first irradiation, but only 5.5 hr after the seventh irradiation.

Series B

Growth medium: nutrient broth + 0.3 % (w/v) yeast extract. Irradiation environment: M/15 phosphate buffer (pH 7.0).

This series of experiments was done as a control for series A, to determine the effect on radioresistance-development of irradiating bacteria in the presence of the organic medium and metabolic products. The experimental procedure was as in series A, except that before irradiation, the bacteria were harvested from the medium by centrifugation (6000 g for 10 min.), washed and suspended in the buffer to the original volume. Control cultures were similarly treated. Cultures were again taken through 7 complete cycles, and were then stored on TGY agar at 4°. Both c 7 and t 7 cultures appeared pure when examined by microscope, and were morphologically the same. D_{10} values calculated from the dose-survival curves for these cultures (Fig. 5) were 5.7 krad and 6.8 krad for the c 7 and t 7 cultures, respectively. The % increase in radioresistance according to these values was 19 %. The post-irradiation growth curves obtained during cycling in this series (Fig. 6) are comparable to those obtained in series A (Fig. 4); however, according to colony-count estimations the development of resistance in series B was in fact only half that in series A for the same number of cycles. This appreciably lower development of resistance in series B suggests that in series A there was an effect of the medium which resulted in an enhancement of resistance development when the bacteria were irradiated in the presence of the medium or metabolic products.

Series C

This series of growth-irradiation cycles was made with a medium lacking yeast extract (YE), to determine whether or not this supplement had any effect on resistance development. Haas & Doudney (1957) showed that cultivation in the presence of YE could increase the mutational response of *Escherichia coli* to u.v. radiation. The medium also lacked nutrient broth.

Growth medium: mineral salts medium M 63+0.4% (w/v) glucose. Irradiation environment: bacteria irradiated in the growth medium.

Growth-irradiation cycles were done in the standard way, and after seven completed cycles c 7 and t 7 cultures were subcultured on medium M 63-glucose agar and stored at 4°.

Fig. 5

Fig. 6

Fig. 5. *Escherichia coli* 1. Dose-survival relationships for control and test cultures following 7 complete growth-irradiation cycles, (series B). Bacteria were grown to final stationary phase in nutrient broth+0.3% (w/v) yeast extract, harvested from the medium by centrifugation and resuspended to the same cell density in M/15 phosphate buffer (pH 7.0) before irradiation. Other details as in Fig. 3. ○, Control culture; ●, test culture.

Fig. 6. *Escherichia coli* 1. Development of radioresistance during the growth-irradiation cycle process, as demonstrated by post-irradiation growth (series B). Samples (1 ml.) were removed from the bacterial suspensions in buffer immediately following irradiation (15 krads) in the 1st, 5th and 7th cycles. Samples were also taken from the corresponding unirradiated controls. The samples were transferred to 60 ml. nutrient broth+0.3% (w/v) yeast extract, which was incubated, with shaking, at 37°. Samples were removed at intervals during the incubation for determination of E_{800} against a sterile medium blank. ○, ●, 1st cycle; □, ■, 5th cycle; △, ▲, 7th cycle. Open symbols, irradiated; closed symbols, unirradiated controls.

Survival curves plotted in Fig. 7 show that D_{10} values for c 7 and t 7 cultures were 6.5 krads and 8.8 krads, respectively, representing an increase in radioresistance of 35%. Figure 8 illustrates the progressive development of radioresistance during the cycling procedure as seen by post-irradiation growth measurement. The survival curves for c 7 and t 7 cultures in Fig. 7 exhibit marked shoulders over the first part of the curves. The concentration of glucose in the growth medium was relatively high

(0.4 % w/v), so the shoulders in both curves may represent the established glucose-effect (Hollaender, Stapleton & Martin, 1951).

DISCUSSION

The radiation doses used throughout the cycling procedures were comparatively high (initially inactivating over 99 % of the population in each series), and it seems likely that the process of radioresistance development was one of selection rather

Fig. 7

Fig. 8

Fig. 7. *Escherichia coli* 1. Dose-survival relationships for control and test cultures following 7 complete growth-irradiation cycles (series C). Bacteria were grown to final stationary phase in mineral salts medium M63 + 0.4 % (w/v) glucose, and irradiated in the growth medium. Other details as in Fig. 3. ○, Control culture; ●, test culture.

Fig. 8. *Escherichia coli* 1. Development of radioresistance during the growth-irradiation cycle process, as shown by post-irradiation growth (series C). Samples (1 ml.) were removed from cultures in mineral salts medium M63 + 0.4 % (w/v) glucose immediately following the 15 krad dose in the 1st, 3rd and 7th cycles. The samples were inoculated into 60 ml. fresh medium, which was incubated with shaking at 37°. Samples from corresponding unirradiated controls were similarly treated. E_{600} values were determined against sterile medium blanks at intervals during the incubation. ○, ●, 1st cycle; □, ■, 3rd cycle; △, ▲, 7th cycle. Open symbols, irradiated; closed symbols, unirradiated controls.

than one of progressive radio-adaptation. It is proposed that variations in experimental technique (particularly radiation doses, growth media, irradiation environment, growth phase of cultures) may partly explain the variable findings of other workers; and further that this explanation may allay scepticism about the process of repeated irradiation of survivors as a method of obtaining increased and stable radioresistance. The progressive development in radioresistance (particularly in series A and C) appears to be step-wise, though irregular. The post-irradiation growth curves obtained during various growth-irradiation cycles suggest that there might be a

threshold number of cycles after which resistance development becomes more rapid. This is indicated by the fact that growth curves in the 3rd cycle were not far removed from those of the 1st cycle, while the positions of the curves during the 7th cycle in these cases showed a very marked resistance development between the 3rd and 7th cycles. Post-irradiation growth curves provided further evidence for the development of radioresistance as, in each case, a dose of 15 krad in the 1st cycle caused a phase of post-irradiation cell lysis, whereas in the 7th cycle the bacteria were not affected to the extent that a lytic phase was observed. The effect of 15 krad in the 7th cycle was merely to introduce an apparent post-irradiation lag phase before exponential growth proceeded. The post-irradiation growth curves also suggest that a plateau in resistance development was being approached for the constant dose of 15 krad after 7 cycles. Post-irradiation growth curves after 7 cycles approach the control curves (representing the growth of unirradiated cells), and it is unlikely that under such experimental conditions (immediately following a dose of 15 krad) post-irradiation growth curves would show a displacement past the unirradiated control growth curves.

The varying reports in the literature on the radiosensitivity of a particular species of micro-organism can often be accounted for by variation in factors such as the strain of organism, growth phase, and conditions before, during, and after irradiation. However, the results presented in the present paper show that there was a definite increase in radioresistance in each test culture, and that the radioresistance developed was genuine as in each case the radioresistance of the test culture was compared with that of the corresponding control culture (taken through the same cycling procedure, without irradiation). Thus % increase in radioresistance in each series, obtained after a limited number of cycles, was shown for the experimental conditions of each series. Variations in conditions among experimental series were kept to a minimum by establishing, and adhering to, an experimental procedure which was repeated in each series. In this way any differences between each series of experiments were purposely designed, and thus differences in results from one series to the next were assumed to be due to imposed experimental differences.

The % increase in radioresistance following 7 growth-irradiation cycles was comparable in series C to that in series A. This indicates that the development of radioresistance in these experiments did not necessarily require the presence of yeast extract in the growth medium or irradiation environment. It would seem that the presence of organic metabolites and metabolic products, not necessarily from yeast extract, in the irradiation environment of series A and C may have influenced a greater development in radioresistance than in series B, where the irradiation environment was inorganic. This is supported by the fact that radioresistance developed in series A and C was approximately twice that developed in series B. The effect of enrichment of pre-irradiation culture media, particularly with glucose, leading to increased radioresistance has been widely reported (Hollaender *et al.* 1951; Stapleton & Engel, 1960). The results presented in the present paper, however, suggest that the nature of the environment at the time of irradiation becomes a contributing factor in radioresistance development by the process described. It is conceivable that the two organic environments (in series A and C), by virtue of their relative complexity, might be more mutagenic under irradiation than the inorganic buffer environment (series B).

The development of radioresistance by the growth-irradiation cycle process is

dependent on the growth of radiation survivors such that a large population density is reached before the next irradiation. The population attained might be expected to consist of bacteria representing a range in radiosensitivity. A progressive increase in radioresistance, obtained with increasing number of cycles, would require that this population should contain a high proportion of radioresistant bacteria which might be further selected by irradiation.

With the increased application of dose-fractionation processing in the irradiation of commercial goods, there arises the possibility that the repeated irradiation of the survivors of the initial dose might lead to the development of radiation-resistant micro-organisms through a mechanism analogous to that in the growth-irradiation cycle procedure. However, the results of the investigations reported in this paper suggest that it is unlikely that such a problem of resistance development will be encountered for two reasons: (a) development of resistance is dependent on several cycles: fractionation processes would rarely attain the number of irradiations warranted; (b) radioresistance development is dependent upon a substantial period of growth and multiplication of surviving bacteria between each irradiation. It is unlikely that a dose fractionation process would permit such growth of surviving bacteria, at least not to a high concentration, since inter-irradiation growth would require suitable conditions of temperature, pH value, nutrient supply and time. If dose-fractionation radiation-processing is applied to food products where it is conceivable that radioresistant development might be encouraged in radiation-survivors, particularly in unpackaged products, the danger could easily be averted by holding the products at low temperatures between successive radiation doses.

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REFERENCES

- ERDMAN, I. E., THATCHER, F. S. & MACQUEEN, K. F. (1961). Irradiation resistant mutants. *Can. J. Microbiol.* **7**, 207.
- GADEN, E. L. JUN. & HENLEY, E. J. (1953). Induced resistance to gamma irradiation in *E. coli*. *J. Bact.* **65**, 727.
- GUNTER, S. E. & KOHN, H. I. (1956). The effect of X-rays on the survival of bacteria and yeast. *J. Bact.* **71**, 571.
- HAAS, F. L. & DOUDNEY, C. O. (1957). A relation of nucleic acid synthesis to radiation-induced mutation frequency in bacteria. *Proc. natn. Acad. Sci. U.S.A.* **43**, 871.
- HOLLAENDER, A., STAPLETON, G. E. & MARTIN, F. L. (1951). X-ray sensitivity of *E. coli* as modified by oxygen tension. *Nature, Lond.* **167**, 103.
- IDZIAK, E. S. & THATCHER, F. S. (1964). Some physiological aspects of mutants of *Escherichia coli* resistant to gamma irradiation. *Can. J. Microbiol.* **10**, 683.
- LUCKIESH, M. & KNOWLES, T. (1948). Resistivity of *Escherichia coli* to ultra-violet energy ($\lambda 2537$) as affected by irradiation of preceding cultures. *J. Bact.* **55**, 369.
- MAISIN, J., LAMBERT, G. & VAN DUYSSE, E. (1955). Acquired radiation resistance by repeated exposures to radiations in *Saccharomyces cerevisiae*. *Acta Un. int. Cancr.* **10**, 137.
- STAPLETON, G. E. & ENGEL, M. S. (1960). Cultural conditions as determinants of sensitivity of *E. coli* to damaging agents. *J. Bact.* **80**, 544.