Effect of Incubation with Bacterial Extract on the Survival of X-irradiated Bacteria

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SUMMARY

X-irradiated *Escherichia coli* B, a filament-forming strain, showed improved survival of X-rays after treatment with its own extract, either in liquid or on solid media. Extract-promoted recovery was not shown by a non-filament-forming strain *E. coli* B_{s-1}. We failed to observe liquid holding recovery of X-irradiated bacteria in the absence of extract. Most experiments were performed in liquid medium where the increased number of survivors depended on the incubation period and the extract concentration. In about 30 min. the increase reached a saturation value; the maximum number of survivors was obtained using a certain (optimal) concentration of extract which was then employed in all subsequent experiments. The activity of the extract was completely destroyed by heating at 52° for 40 min. No activity was detected in the DNA, RNA or ribosomal fractions of the extract. The soluble remainder, however, was fully active, suggesting that soluble protein was the active principle.

INTRODUCTION

The recovery of bacteria from ultraviolet light-induced damage is well established (Jagger, 1960; Castellani, Jagger & Setlow, 1964; Setlow, 1966; Kantor & Deering, 1967; Ganesan & Smith, 1968, 1969). Recovery from X-ray-induced damage has also been reported, after suboptimal growth conditions (Alper & Gillies, 1958, 1960), treatment with ribonuclease (Bhattacharjee, 1965), holding in buffer (Stapleton, Billen & Hollaender, 1953; Castellani, Magaouda & Trezza, 1968) and treatment with a bacterial extract (Adler, Fisher, Hardigree & Stapleton, 1966; Fisher, Adler, Shull & Cohen, 1969). Some authors found extract-promoted recovery only when the treatment was on an agar gel (Fisher *et al.* 1969), whereas others observed it only in liquid media (Korgaonkar & Raut, 1967). No success has previously been reported in observing extract-promoted recovery both on solid and in liquid media for the same strain of irradiated bacteria.

In this paper, we show that recovery from X-ray-induced damage in *Escherichia coli* B was promoted by bacterial extract on agar plates as well as in liquid media and that the soluble part of the extract, probably protein, was responsible.

METHODS

Preparation of the bacterial suspensions. The filament-forming bacterial strain *Escherichia coli* B and a non-filament-forming radiation-sensitive derivative *E. coli* B_{s-1} were routinely grown in the tris-glucose medium (Maaløe & Hanawalt, 1961). An over-

night culture was diluted 50 times with the same medium and grown with aeration at 37°. During the logarithmic phase the bacteria were collected by centrifugation, washed three times with ice-cold M15 phosphate buffer (pH 6.8) and resuspended in similar warmed buffer at between 10^8 and 10^9 organisms/ml. The X-ray sensitivity was unaffected by bacterial concentration within this range.

Preparation and estimation of cell components. Cell-free extract was obtained from protoplasts of *Escherichia coli* B formed by the penicillin method of Lederberg (1956) lysed in magnesium sulphate (0.01 M) and centrifuged at 5000 rev./min. in the cold. The supernatant, called the total cell-free extract (TCFE), was collected. The concentration of the bacterial extract was arbitrarily estimated by determining its refractive index with the Abbé refractometer, assuming that the specific refractive increment was 1.88×10^{-3} decilitre/g., equivalent to that of protein. From time to time, fresh extract was prepared and its concentration determined as above.

DNA of *Escherichia coli* B in the native form was extracted by the method of Marmur (1961) and assayed with a modified Dische reaction (Burton, 1956), using depolymerized calf-thymus DNA as the standard. The extinction was observed at 600 nm.

RNA was isolated by the phenol method from *Escherichia coli* B (Monier *et al.* 1962) and assayed at 660 nm. by the orcinol reaction (Mejbaum, 1939) with yeast RNA as standard.

Protein was estimated at 750 nm. by the method of Lowry, Rosebrough, Farr & Randall (1951) using lysozyme as standard. The DNA and RNA preparations contained below 3% protein.

TCFE after collection was centrifuged in a preparative ultracentrifuge at 92,739g for 180 min. following the technique of Anraku (1966). The supernatant containing RNA, DNA and soluble protein was collected. Sediment containing ribosomes was resuspended in phosphate buffer containing 0.01 M-MgSO₄. Both fractions were used for the treatment of irradiated bacteria.

The cell-fractions as well as TCFE were checked in each experiment for the presence of viable bacteria by plating 0.1 ml. If any visible colony was observed on such plates the experiments were rejected.

Irradiation of bacteria and determination of viable number. Bacteria were exposed to various doses of X-rays ranging from 1 to 24 krads in a brass holder in a small Petri dish in contact with ice. The X-ray dose from a Muller MG 150 tube run at 80 kV, 9 mA, as measured by the method of FeSO₄-dosimetry, was 6 krads/min. Viable bacteria were determined by plating 0.1 ml. aliquots of suitable dilutions on the surface of nutrient agar plates and counting colonies after 18 h. incubation at 37°. The initial viable titre of bacteria was determined from a non-irradiated sample.

Treatment of X-irradiated bacteria. Samples were usually divided into four parts after irradiation, each containing 1 ml. of suspension. To each of the tubes, TCFE at four different final concentrations (997.5, 1662.5, 2660 and 3325 µg./ml.) was added and the final volume was made up to 4 cm.³ by adding phosphate buffer. The suspensions were then shaken in a 37° water-bath shaker for 2 h. For comparison, irradiated cells were also held under conditions identical save for the absence of the bacterial extract. The number of viable bacteria was determined at 30 min. intervals. Similar experiments were also done with native, denatured and renatured DNA, native RNA, and native DNA and native RNA mixed in the ratio in which they existed in bacteria, and with ribosomal and soluble (protein) fractions.

For experiments with TCFE on plates, 0.1 ml. of X-irradiated suspension was spread after suitable dilution on agar plates in presence of TCFE. The concentration of TCFE was calculated as $\mu\text{g./ml.}$ of the medium in the agar plate. In order to prevent any decrease in survival due to radiation-induced peroxides, catalase⁵ was incorporated in all post-irradiation media at between 2 and 4 $\mu\text{g./ml.}$

Fig. 1. Change in viable count during incubation of bacteria in buffer. (a) Non-irradiated bacteria in presence of extract (⊗), catalase (●), extract + catalase (□). (b) Irradiated bacteria in presence of extract + catalase at dose of radiation: 1 krad (○), 1.5 krad (●), 3 krad (Δ), 6 krad (▲), 12 krad (▼).

RESULTS

Fig. 1 shows the effect of holding X-irradiated and non-irradiated bacteria in the presence of total cell-free extract (TCFE) in buffer at 37°. This is a typical experiment in which extract was employed at a concentration (defined above) of 1662.5 $\mu\text{g./ml.}$ Experiments at other concentrations gave broadly similar results and were used to construct the curves in Fig. 2. For bacteria irradiated with X-rays to the level of about 60% survival, an increase up to 100% viability was observed after 30 min. incubation. At the 50% survival level, a maximum of 90% recovery was attained. Below the 40% survival level, however, there was much less recovery, and below 0.1% survival none at all. The maximum increase in survival level was reached after about 30 min. incubation for all

X-ray doses, and for all the concentrations of TCFE used. Non-irradiated control bacteria did not show any change in viable count during incubation with the extract.

Fig. 2 shows the influence of the extract concentration on recovery. For all the doses of radiation there was an optimum concentration for recovery and this was used in the subsequent experiments. The time allowed for incubation was 1 h., which was sufficient for maximum recovery.

Fig. 2. Variation in the survival of irradiated bacteria after 1 krad (○), 1.5 krad (●), 3 krad (△), 6 krad (▲), 12 krad (▼), during a 1 h. incubation with different concentrations of bacterial extract.

Table 1. *Liquid holding of X-irradiated Escherichia coli B cells in phosphate buffer*

Initial X-ray survival (%)	% surviving after incubation period (min.)							
	In presence of catalase				In absence of catalase			
	30	60	90	120	30	60	90	120
57	57	57.5	56.8	57	52	51	51.8	51.5
50	49.6	49.8	51	49	45	44	44.8	45.2
21	21	21	20.5	20.8	9.5	10	10.5	9.5
7	7	7.2	7.5	6.8	2.7	2.5	3	2.5
0.25	0.3	0.25	0.24	0.25	0.03	0.02	0.025	0.025

In order to test for the involvement of liquid-holding recovery, X-irradiated bacteria were held for 2 h. at 37° in phosphate buffer with or without catalase (2 µg./ml.). Table 1 shows that X-irradiated bacteria without catalase gave the usual peroxide death, which reached a maximum in about ½ h.; with catalase viability was constant for the whole period, in contrast to the observations of Castellani *et al.* (1968), which showed 'liquid-holding recovery'.

Fig. 3. Effect of heating the extract at 52° on its ability to promote recovery after X-ray doses: 1 krad (○), 1.5 krad (●), 3 krad (△), 6 krad (▲) and 12 krad (▼). Irradiated bacteria were incubated for 1 h. with the optimum concentration of TCFE.

Table 2. Effect of extract of *Escherichia coli* B on X-irradiated *E. coli* B_{s-1}

Initial X-ray survival (%)	% surviving after different periods of incubation (min.)			
	30	60	90	120
31	32	29	32	31.5
20	20	18	21	20.5
6	6	7	5.8	5.8
0.35	0.4	0.35	0.30	0.32

The observed extract-promoted increase in X-ray survival shown in Fig. 1 was obtained with *Escherichia coli* B only. The same experiment repeated with *E. coli* B_{s-1} (Table 2) showed no increase in viability on incubation of bacteria in buffer containing catalase (2 µg./ml.) and/or the extract made from *E. coli* B. The experiment was performed with similar results for various extract concentrations, although results for only the optimum concentration, 1662.5 µg./ml., are shown in Table 2.

Fig. 3 shows the effect of heating the extract on its ability to promote recovery.

Fig. 4. Survival of X-irradiated bacteria in presence of extract in buffer and on plates. 1, Initial viable bacteria; 2, X-ray survival in absence of extract; 3, X-ray survival after treatment with extract on plates; 4, survival after 1 h. holding in buffer in presence of extract (plating without extract); 5, plating of sample in 4 with extract; 6, sample in 4, washed and plated without extract; 7, sample in 4, washed and plated with extract.

Table 3. *Effect of different cell constituents on post-irradiation recovery in buffer*

Bacterial titre/ml.	X-ray dose (krad)	Treatment	Concentration ($\mu\text{g./ml.}$)	Survival on immediate plating (%)	Survival after 1 h. incubation (%)
1.05×10^9	1.0	Native DNA	99	55	56
	1.5	Native DNA	99	49	49
1.65×10^8	3	Native DNA	99	20	19.3
1.93×10^8	1.0	Denatured DNA	99	54.8	55.8
	1.5	Denatured DNA	99	46	46
1.19×10^8	3	Denatured DNA	99	21	20
2.27×10^8	1	Renatured DNA	99	56.2	56.8
	1.5	Renatured DNA	99	49.8	49
1.65×10^8	3	Renatured DNA	99	20	20
5.6×10^8	1	Native RNA	100	58	59
	1.5	Native RNA	100	47	46.5
4.65×10^8	3	Native RNA	100	19	19
4.4×10^8	1	Native (DNA & RNA)	40, 80	58.2	57
7.0×10^8	1.5	Native (DNA & RNA)	40, 80	48	47.5
	3	Native (DNA & RNA)	40, 80	20.5	21
1.67×10^9	1	Ribosomal protein	80	62.2	62.8
	1	Soluble protein	80	62.2	100
	1.5	Ribosomal protein	80	50.3	48.5
	1.5	Soluble protein	80	50.3	89.8
	3	Ribosomal protein	80	19	21.5
	3	Soluble protein	80	19	49

Here for each X-ray survival level the maximum percentage of survivors that could be attained with untreated extract was considered to be the initial point. With the increase in heating time there was a systematic decrease in the maximum number of survivors that could be attained after incubation, until after 40 min. or more there was no recovery at all.

Bacteria plated immediately after X-irradiation on nutrient agar plates in presence of TCFE showed an increase in survivors compared to those obtained without the extract (Fig. 4). The maximum effect was quantitatively similar to that shown in buffer for the same level of X-ray survivors. Bacteria first allowed to recover with TCFE in buffer and then washed did not show any subsequent effect of TCFE in the plating medium, suggesting that recovery in either liquid or on agar medium was probably due to the same cause.

Table 3 shows the results of holding the X-irradiated bacteria in buffer in the presence of different concentrations of various fractions of the TCFE. For the purpose of these experiments, the TCFE was considered to contain RNA, DNA, ribosomal protein and a soluble fraction (including soluble protein). It may be seen that no recovery occurred during incubation for 1 h. in presence of DNA, native, denatured or in renatured form, RNA in native form, ribosomal protein, or DNA and RNA in the ratio in which they were present in bacteria (RNA:DNA, 2:1, dry wt; Setlow & Pollard, 1962). Although Table 3 shows the result for only one concentration of the constituents, experiments with similar results were conducted with concentrations both below and above that given in the table. The soluble protein fraction, however, gave a clear and significant increase in number of survivors during incubation. The increase obtained with the optimal amount of soluble fraction was almost the same as that obtained with the total cell-free extract at its optimal concentration.

DISCUSSION

We believe that the increase in survival of X-irradiated *Escherichia coli* B observed with TCFE is the manifestation of a genuine recovery rather than division of surviving bacteria because of the following observations: (a) the presence of bacterial extract, catalase or both did not affect the survival of non-irradiated bacteria during incubation in phosphate buffer; (b) heated extract was inactive; (c) the increase in number of survivors of an X-irradiated bacterial population due to the presence of extract was the same in buffer or on an agar surface.

Adler *et al.* (1966) suggested that the extract-promoted restoration of X-ray-induced damage involved the division mechanism of bacteria. This is supported by our observation that *Escherichia coli* B, a filament-forming strain, showed the extract-promoted recovery, whereas *E. coli* B_{s-1}, a non-filament former, did not.

The results show that the active agent responsible for the increase in survival number was in the soluble fraction, possibly protein, although Adler and his associates thought that the activity might be associated with a large particle (Fisher *et al.* 1969). The removal of activity by heating at 52° is consistent with, but does not prove, the involvement of protein. Treatment with RNA or DNA did not influence the survival number in any way, in agreement with Fisher *et al.* (1969), although highly polymerized DNA did promote recovery of X-irradiated *Salmonella typhimurium* (Kanazir, Cecuk, Krajcinovic & Hudnik, 1959).

An interesting aspect of the experiments was that there was an optimum concentration of TCFE above which recovery was reduced. Such an optimum concentration has also been reported by other experimenters (Korgaonkar & Raut, 1967). Perhaps the enzymic balance in the extract was favourable only at the optimal concentration; below it the concentration of the active agent was too low, whereas above it some harmful enzymic activity was prominent. Another possibility is that some component of the extract at high concentrations inhibits the uptake of the active agent.

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