ACTION OF MEMBRANE ACTIVE ANTIBIOTICS ON THE SURVIVAL OF X-IRRADIATED *Escherichia coli* B/r

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ABSTRACT.

When E. coli strain B/r was X-irradiated in the presence of oxygen and incubated immediately after irradiation in a non-lethal concentration of penicillin, significant additional killing of the cells was observed. It was concluded that the observed enhanced killing was due to an interaction of penicillin and X-rays at the specific site of action of penicillin, since radiation energy is believed to be deposited randomly within the cells. The critical lesions probably occurred in the membrane moiety.

In this study, membrane active antibiotics were substituted in place of penicillin.

Polymixin B sulphate and Cerulenin showed similar effects as penicillin but not Tetracyline. It was therefore suggested that the latent radiation induced lesion can be detected by a range of membrane perturbing agents and the enhanced lethality observed does not depend entirely on the specific action of penicillin. On the extent of enhanced killing, penicillin showed a larger oxygen enhancement ratio from 2.8 to 4.9 compared to Polymixin 2.8 to 3.9 and Cerulenin from 2.8 to 3.8. The additional killing observed in penicillin was possibly through a combination of induction of filaments and direct membrane damage. However, in terms of the concentrations of these antibiotics required to cause the oxygen dependent membrane damage, Penicillin was 17M while Polymixin required 16M. Cerulenin on the other hand, required about 10^{3}M to achieve the same level of inhibition. Polymixin and Penicillin were about equally effective on exposing the membrane damage while Cerulenin was the least effective of the antibiotics used. The significance of this findings in relation to better understanding of the mechanisms of radiation damage is discussed.

INTRODUCTION.

Several attempt have been made since the discovery of X-rays to identify targets of radiation action in cells. Since then, much progress has been made towards a better understanding of the mechanisms which lead to radiation damage. A knowledge of these mechanisms is important in determining for-example, the possible hazards of radiation injury and protection to man and for the improvement of the uses of radiation

Damage by ionizing radiation, leading to cell death is believed to occur primarily through random deposition of energy in vital cellular macromolecules which are referred to as targets. The nature of such targets, therefore has to be unique and indispensable for cell proliferation, since the viability of cells is measured by their ability to divide and originate colonies.

There have been several schools of thought about such targets, which when damaged by radiation lead to the observed cell death. Because of its central role in cell replication, Deoxyribonucleic acid (DNA) was considered the most important type of molecule in the cell. Data abound in the literature on the role of DNA and mechanisms of action on it by radiation that lead to cell death. However, results of several other works[1,2,3,4] had come out with evidences in support of the role of membrane damage in radiation induced cell death.

A somewhat different approach to the problem of identifying radiation induced damage in cell membrane was adopted by Gillies, Obioha and Rathnajothi (1979)[5] in studies on E.coli B/r. The work was based on the assumption that if radiation does produce lethal lesions which are unique to the bacterial envelop (membranes and peptidoglycan layer), then post irradiation treatment of such cells with a chemical agent which is known to act specifically on a particular site within the cell envelop might result in an interaction of damage by radiation

and the agent at this site.
Although there are doubts about the specificity of action of many antibiotics, it has long been established that penicillin inhibits specifically the synthesis of the peptidoglycan layer[6,7] which is sand-witched between and attached to the outer[8] and inner membranes of the cell envelop of E.coli[9,10] Using this model, Gillies et.al[5] observed an enhanced killing of E.coli B/r cell by a non lethal concentration of penicillin provided the cells were irradiated in the presence of oxygen.
It was therefore concluded that under aerobic conditions, X-rays induced latent lesions which interacted with penicillin damage and the site of such action was likely to involve the peptidoglycan layer of bacterial cell envelop, since penicillin is known to inhibit the synthesis of this layer. However, it does not necessarily follow that the interaction was confined entirely to this layer since the peptidoglycan layer is known to be closely associated with the membranes of the cell envelop.

In this paper, a number of membrane active antibiotics have been substituted in place of penicillin to see which of them would produce similar effects as penicillin on the survival of aerobically irradiated cells of E.coli B/r.
The choice of these antibiotics depended largely on their known sites of action. These included, Polymixin B sulphate which alter the permeability of the outer membrane layer and may bring about release of cytoplasmic contents[11,12]. Cerulenin specifically inhibits fatty acid synthesis in E.coli[13.] While tetracycline, which although does not affect membrane layer directly, has been shown to inhibit the synthesis of envelop proteins in E.coli[14].

MATERIALS AND METHODS

Bacterial strain

Cells of E.coli B/r were obtained from the laboratory stock and grown to stationary phase by incubation in Oxoid nutrient broth at 37°c for 24hr.The cells were harvested by centrifugation, re-suspended in 0.67M phosphate buffer (pH 7.2) and the concentration adjusted to 5 x 10^7 cell/ml for irradiation. Viable counts were made on plates of BAB after overnight incubation at 37 C.

Irradiation

X-rays were obtained from a Marconi 250 Kev CP therapy machine at a dose rate of 2 Gy/min. The bacteria were irradiated under aerobic or anaerobic conditions as described by Alper and Gillies (1972)[15].

Sources of Antibiotics.

Polymixin B sulphate, Cerulenin and Tetracycline (crystalline) were all purchased from Sigma Laboratories, London and stored desiccated below 0°C.

Determination of non-lethal concentration of the antibiotics.

Stock solutions of the antibiotics were diluted in flasks containing cooled BAB (50°C) to obtain different concentrations. The flasks were thoroughly shaken and the contents allowed to settle. They were then poured onto sterile petridishes (about 20mls in each plate) and left to set. Un-irradiated cell suspensions were diluted appropriately and samples spread on pieces of cellophane lying on BAB/antibiotic using the method of Alper and Gillies[11]. To terminate treatment with the antibiotic, the pieces of cellophane were transferred from BAB/antibiotic to fresh BAB containing no antibiotic, at hourly intervals up to a maximum of 4h and incubation continued at 37°C overnight. Viable colonies were counted and survival calculated. Using this method, the concentration of the antibiotic which did not cause significant killing of the bacteria over 4h period was determined.

Treatment with the antibiotics after irradiation.

For post irradiation treatment with Polymixin and Tetracycline requiring incubation on cellophane, the method used was as described for un-irradiated cells above. For post-irradiation incubation with Cerulenin, the cells were centrifuged out of the buffer and re-suspended in 10mls of Nutrient Broth containing Cerulenin at the concentration which did not cause killing of un-irradiated cells during the 4h incubation at 37°C. The suspension was incubated with shaking in water bath for

4h at 37^{oC}.Samples of the culture were withdrawn at the beginning of the incubation period at hourly intervals, diluted appropriately in buffer, plated on BAB and incubated overnight after which viable colony counts were made.

RESULTS

The concentration of the antibiotic which did not cause significant killing of un-irradiated cells.

Survival of E.coli B/r in various concentrations of Polymixin, Cerulenin and Tetracycline are shown in figures 1, 2 and 3 respectively. These were Polymixin 16g/ml, Cerulenin 1mM, and Tetracycline 5g/ml. In all cases, lower concentrations of the antibiotics allowed cell division while higher concentrations killed the cells as shown in the diagrams.

Effects of post irradiation incubation with the antibiotics.

The effects of incubation of irradiated cells of E.coli B/r on BAB/Polymixin, BAB/Tetracycline and Nutrient Broth/Cerulenin are shown in figures 4,5 and 6 respectively. For E.coli B/r incubated in Polymixin and Cerulenin, the survival decreased during 4h incubation in cells which were aerobic during irradiation but not in those which were anaerobically irradiated. It is also seen that the decrease in survival is more extensive the higher the radiation dose levels. However, there was no significant differences in survival when cells which were similarly treated were incubated on BAB/Tetracycline as shown in figure 5.

Figures 7 and 8 illustrate the dose response relationships (survival curves) of the cells incubated in Polymixin and in Cerulenin respectively. These responses are represented in form of semi-logarithmic plots of the percentages of cells surviving against the dose of radiation in gray. The Enhancement ratios for Polymixin and Cerulenin were increased from 2.8 to 3.9 and 3.8 respectively.

DISCUSSION.

Damage by ionizing radiation, leading to cell death is believed to occur primarily through random deposition of energy in vital cellular macromolecules which are referred to as targets. The nature of such targets, therefore has to be unique and indispensable for cell proliferation since cells viability is measured by their ability to divide and originate colonies[16].

In the search for lethal radiation targets in cells, Gillies et.al[5] showed that X-irradiation of E.coli B/r in the presence of oxygen followed by incubation in a concentration of penicillin which on its own did not kill the cells, caused additional killing of the cells. Penicillin has no effect on the lethality of these cells when they were irradiated under anoxic conditions or with Ultra Violet (260nm).radiation. It was suggested that an X-ray induced lesion, sensitive to the presence of oxygen at the time of irradiation and probably located in the cell envelop caused the observed cell lethality in this strain.

Several other studies have demonstrated that the radiation induced lesion exposed by penicillin is associated with energy deposition within the membrane components For-example, it was shown that penicillin caused the same effect in E.coli B/r, irradiated under anoxic conditions in the presence of some highly lipophilic electron affinic radiation sensitizers[17] and that the antibiotic effects occurred in close proximity to the cell membrane since the efficiency of the radiosensitizers in combination with penicillin depends on the levels of their lipohilicity.

Support for this model (Gillies et.al[1)] has come from the work of Obioha et al[18] in which *m* and *K* constants of the Alper and Howard-Flanders oxygen equation was used to characterize the mechanisms of the lethal lesions in the membrane as well as the DNA. The results of this analysis demonstrate that any interaction of oxygen with sites of energy deposition in the DNA must play a very much smaller role in radio sensitization than does interaction with sites of energy deposition on the membrane.

Further support for this suggestion that penicillin affects the membrane layers in E.coli has come from the work by Hewamanna[19] and Gillies[20] in which the effects of penicillin were studied by measuring the synthesis of membrane macromolecules using labeled

precausors. It was observed that incubation with penicillin depressed the incorporation ^{14}C-acetate in aerobically irradiated E.Coli K_{12} cells and these corresponded with the enhanced killing observed when the cells which were similarly irradiated were incubated with penicillin. Analysis of the phospholipid components in this study showed that the depressed incorporation of ^{14}C-acetate occurred more in the phatdyiethamolamine (which is found mainly in the outer layers) than in other components of phospholipids.

These conclusions are further reinforced by the findings in the present study.
Polymixin and cerulenin enhanced the killing of cells which were aerobic during irradiation but not those irradiated under anoxic conditions. The oxygen enhancement ratios were effectively increased from 2.8 to 3.9 and 2.8 to 3.8 respectively. No enhanced killing was observed for cells which were irradiated under oxic or anoxic conditions and subsequently incubated with tetracycline.
It follows from the proposed model Gillies et al (1979)[5] that these lesions were occurring at the specific sites of action of these antibiotics which undoubtedly implicates membrane layers of the bacterial cell envelop.
Model membrane studies have shown that Polymixin inhibits lipid synthesis in E.coli and strong interactions between Polymixin and photidylethanolamine, phosphatidyl glycerol and cardiolipin had been demonstrated. It has also been shown that Cerulenin specifically blocked lipid synthesis in E.coli. Thus Polymixin, Cerulenin and Penicillin each inhibits lipid synthesis in E.coli which may be involved with the enhanced killing observed in aerobically irradiated cells.

Tetracycline on the hand, did not show any significant effect on cells which were either aerobic or anaerobic during irradiation. Although, tetracycline does not affect the synthesis of lipids, there are indications that it preferentially inhibits synthesis of envelop proteins in E.coli. The absence of enhanced killing by tetracycline indicates that inhibition of protein synthesis may not be associated with this radiation induced damage. Support for this suggestion has come from the work of Hewamanna (personal communication)[19] in which it was shown that the amount of protein synthesis occurring in aerobically or anaerobically irradiated cells of E.coli K_{12} was independent of the presence of penicillin.

Comparison of efficacy of the three antibiotics with penicillin

The four antibiotics were used at concentrations that did not kill un-irradiated cells but were large enough to inhibit division of the cells for at least several hours. Of the three antibiotics that enhanced radiation sensitivity of E.coli B/r, penicillin required 17M to inhibit cell division for about 4hours.On the other hand, 16M of Polymixin was required to enhance sensitivity of the cells.
It follows therefore that polymixin and penicillin were approximately equally effective when the cells were exposed to the antibiotics. However, Cerulenin was the least effective, requiring about 10^{3}M to achieve the same level of enhanced killing of the cells. Despite the different kinds of other properties of these agents and the wide range of concentrations required, their effects on aerobically irradiated cells seem to be similar.
It is evident from these observations that the properties of these antibiotics have helped to widen the scope of understanding of the structure and functions of biological membranes and it is hoped that the present study might serve as a foundation for further investigation on the effects of these antibiotics on higher cells especially mammalian cells. It might be interesting to note that at present survival curves for Chinese hamster lung V79 cells have already been obtained with polymixin B but however, no effect of the antibiotic on aerobically X-irradiated cells was observed for the methods used (Khokhar, personal communication)

Although these antibiotics have been useful in detecting radiation induced lesions in cells irradiated under aerobic conditions, other agents may be helpful in bringing to light radiation damage in anoxic cells. However, they do show that

there are forms of latent lesions induced in the cell membranes after irradiation which might be explored as means of bringing about further improvement in the treatment of malignancy by sensitizing agents used in conjunction with radiotherapy.

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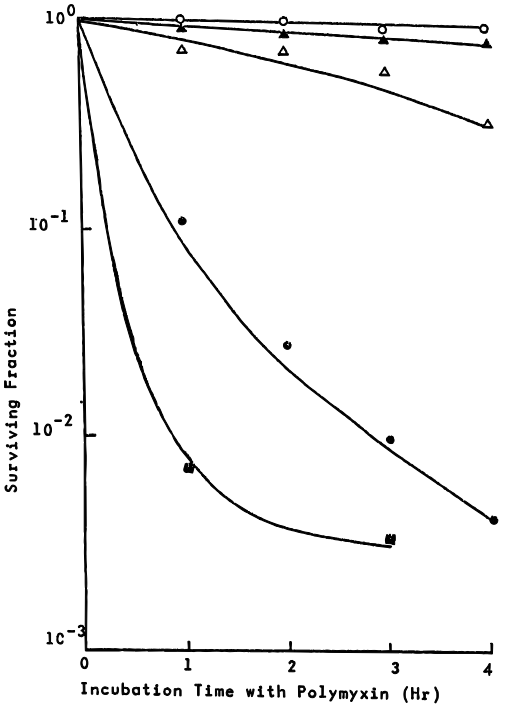


Figure I Survival of E.coli B/r on BAB containing the following concentrations of polymyxin
- O - 10μ M
- ▲ - 16 "
- Δ - 20 "
- ● - 30 "
- ■ - 40 "

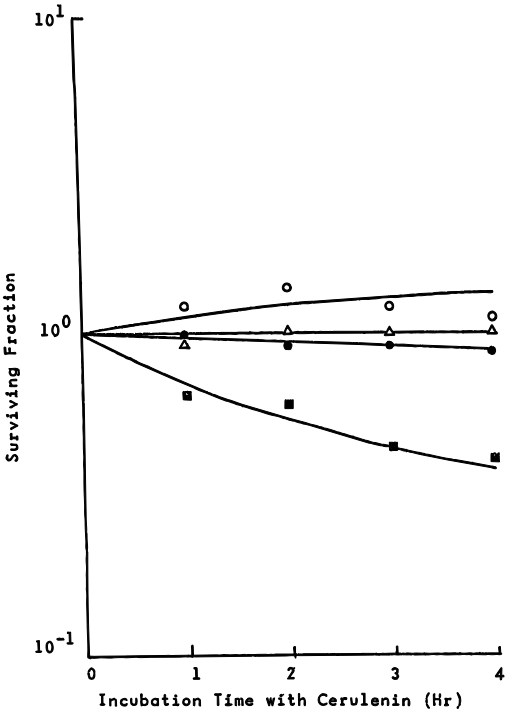


Figure 2 Survival of E.coli B/r in suspension in nutrient broth containing the following concentrations of cerulenin:
- O - 0.4 mM
- Δ - 0.8 "
- ● - 1.0 "
- ■ - 1.6 "

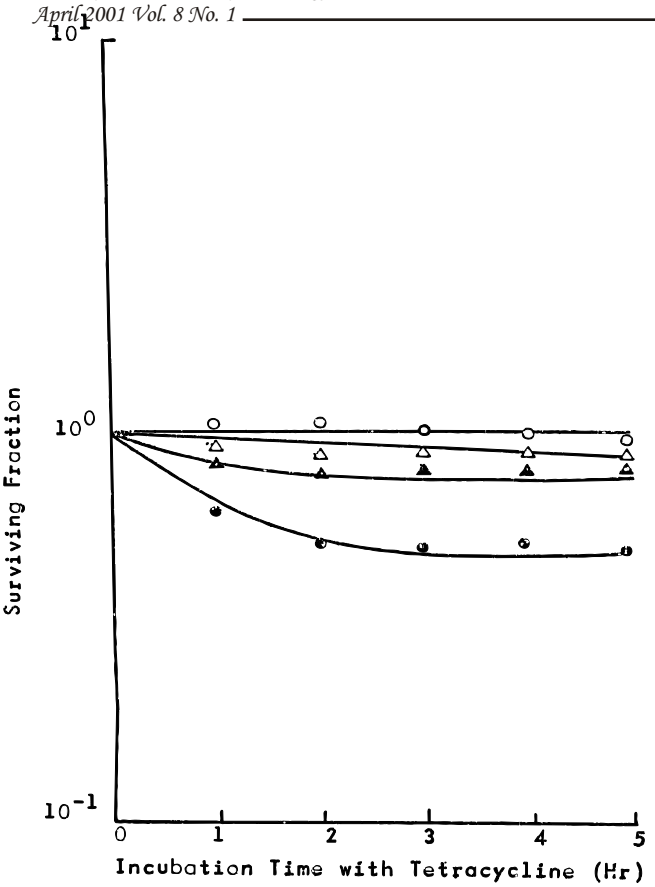


Figure 3 Survival of E.coli B/r on BAB containing the following concentrations of tetracycline
○ - 1 µg/ml
△ - 5 "
▲ - 10 "
● - 20 "

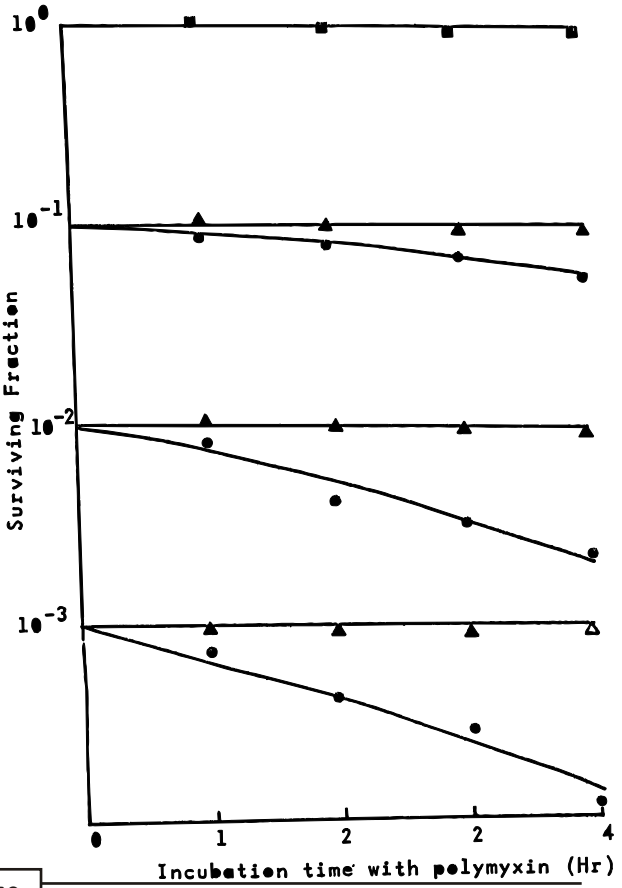


Figure 4 Survival of E.coli B/r, X-irradiated under oxic or anoxic conditions followed by incubation on BAB/polymyxin for 4 hours.
■ - unirradiated cells + polymyxin
▲ - N_2 + polymyxin
● - O_2 + polymyxin
Reading from the highest to the lowest level of survival the radiation doses delivered under aerobic conditions were 330, 560 and 840 Greys respectively. The corresponding doses under anoxia were 950, 1600 and 2400 Greys.

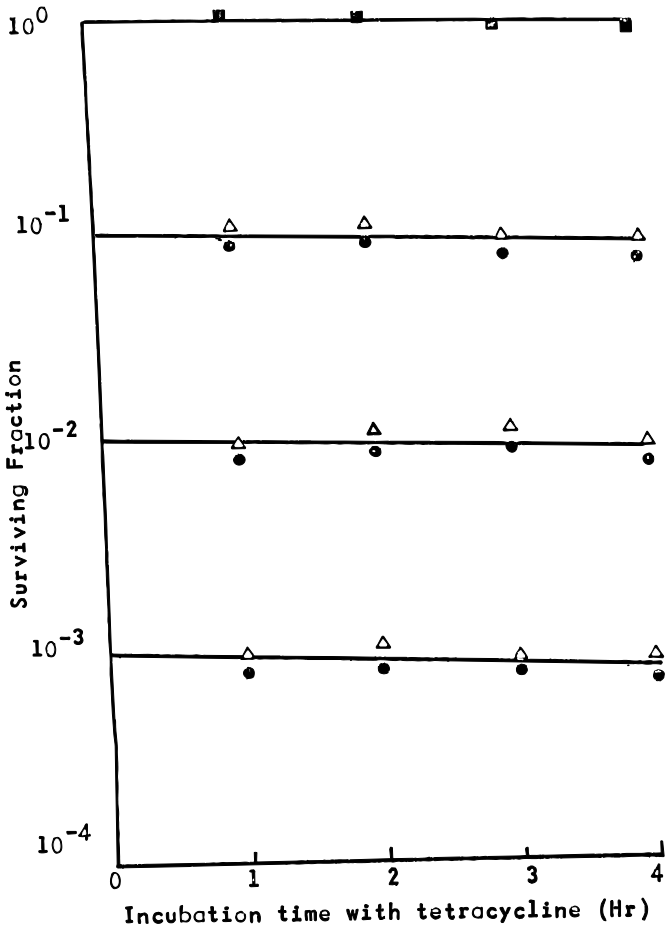


Figure 5 Survival of E.coli B/r to X-irradiation under oxic or anoxic conditions followed by incubation on BAD/tetracycline for 4 hours.
■ - unirradiated cells + penicillin
△ - N_2 + tetracycline
● - O_2 + tetracycline
Reading from the highest to the lowest level of survival the radiation doses delivered under aerobic conditions were 330, 560 and 840 Grays respectively. The corresponding doses under anoxia were 950, 1600 and 2400 Grays.

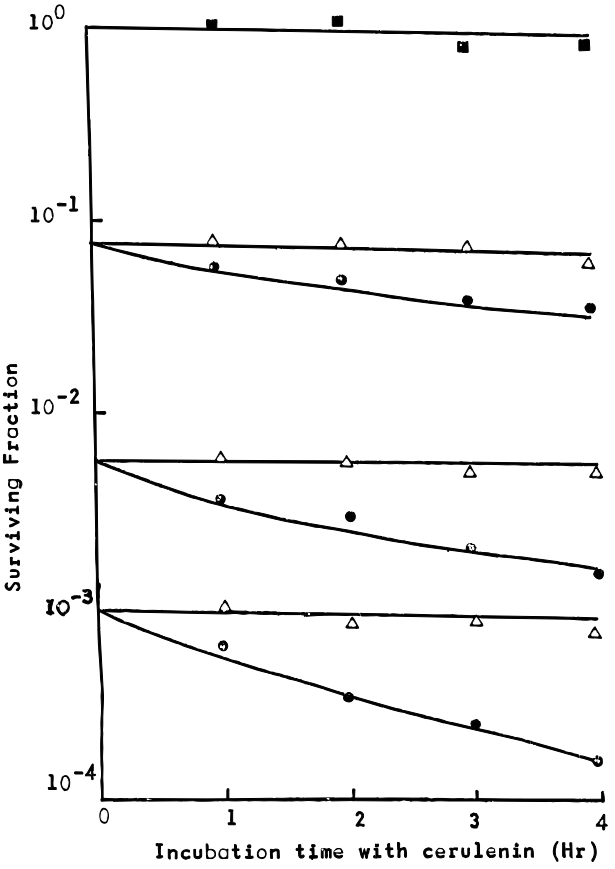
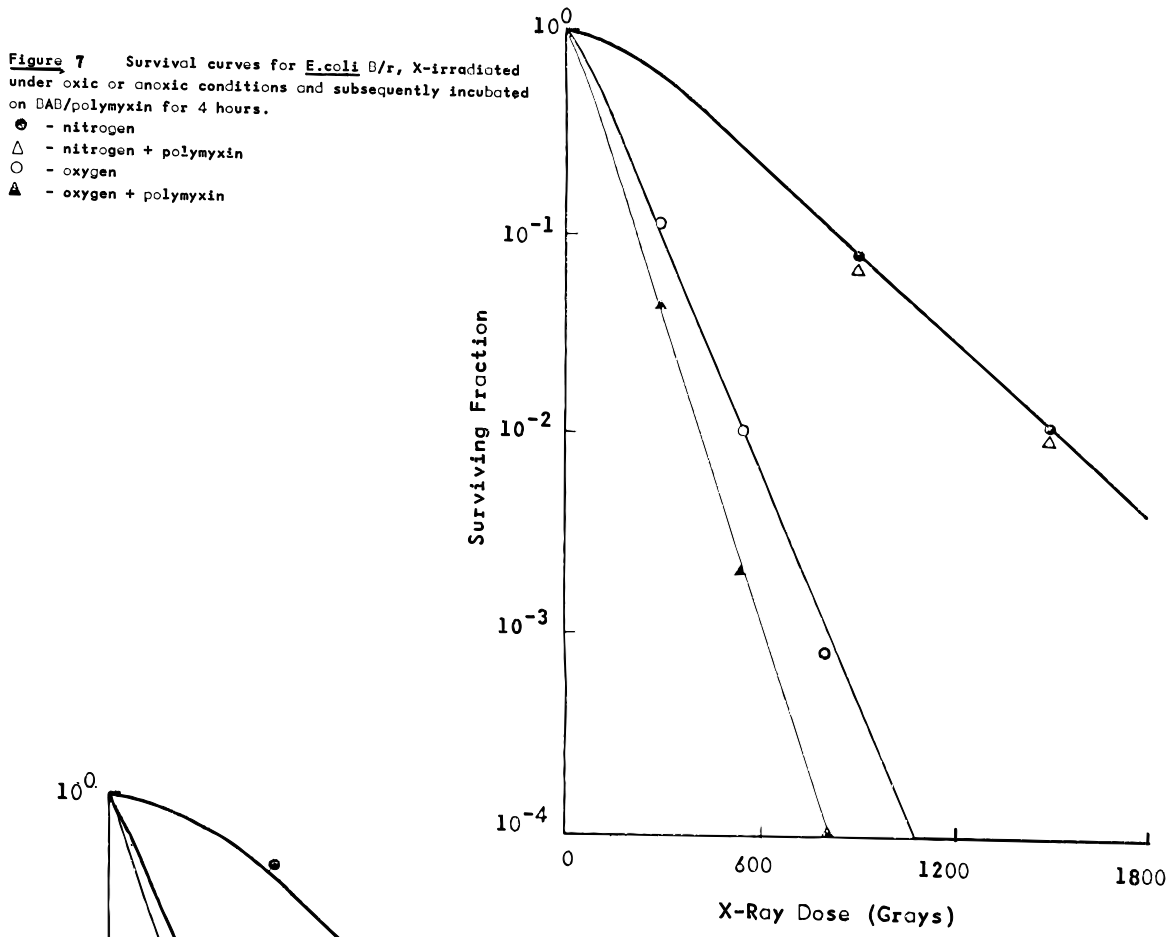


Figure 6 .Survival of E.coli B/r to X-irradiation under oxic or anoxic conditions followed by incubation on BAD/cerulenin for 4 hours.
■ - unirradiated cells
△ - N_2 + cerulenin
● - O_2 + cerulenin
Reading from the highest to the lowest level of survival the radiation doses delivered under aerobic conditions were 370, 600 and 840 Grays respectively. The corresponding doses under anoxia were 1050, 1700 and 2400 Grays.

Figure 7 Survival curves for E.coli B/r, X-irradiated under oxic or anoxic conditions and subsequently incubated on DAB/polymyxin for 4 hours.
- ⊕ - nitrogen
- △ - nitrogen + polymyxin
- ○ - oxygen
- ▲ - oxygen + polymyxin



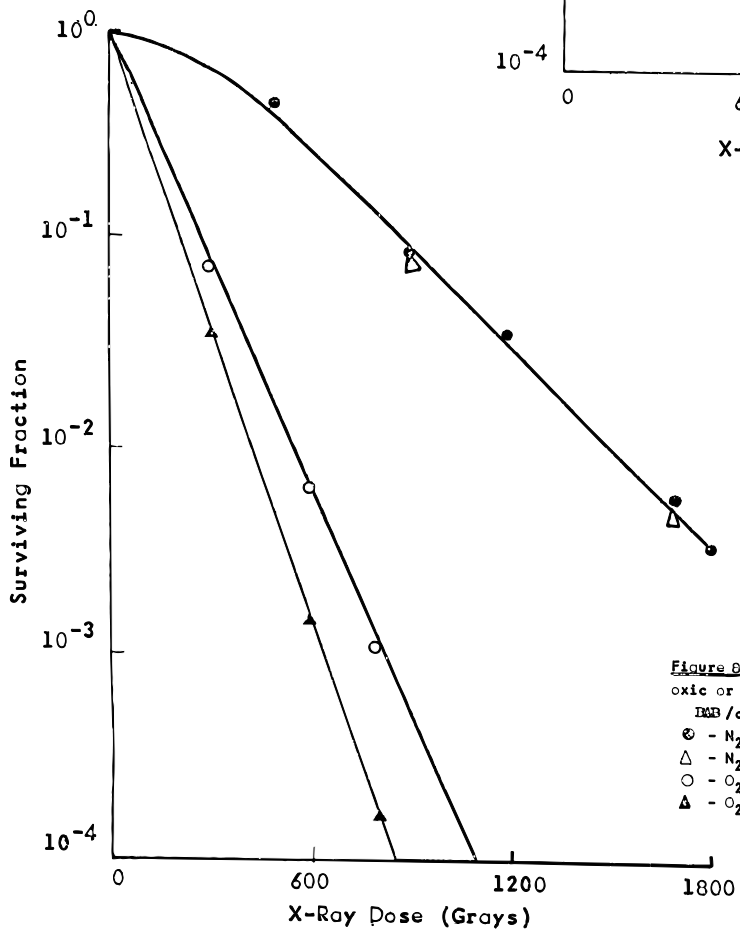


Figure 8 Survival of E.coli B/r, X-irradiated under oxic or anoxic conditions and subsequently incubated DAB/cerulenin for 4 hours.
- ⊕ - N_2
- △ - N_2 + cerulenin
- ○ - O_2
- ▲ - O_2 + cerulenin

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