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DOSE MODIFICATION IN E. COLI BY A CONSTITUTIVE RADIOPROTECTIVE AGENT ISOLATED FROM M. RADIODURANS

by

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DISCLAIMER

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This dissertation is dedicated to my mother and in memory of my father.

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"What you see is what you get"
Geraldine Jones

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ABSTRACT

A constitutive low molecular weight radioprotective agent has been isolated from a colorless mutant of Micrococcus radiodurans. Organic separation with butanol and isoamyl alcohol demonstrates that it is hydrophilic in nature. Chromatographic resolution using Sephadex G-25 shows it to be comprised of two separate moities, both having a molecular weight between 1,000 and 5,000 daltons. The two appear to be either tautomeric forms or dimers of the same molecule. The high distribution coefficient indicates the probable presence of a heterocyclic ring subgroup. The agent is resistant to digestion by DNase, RNase, Pronase and Lipase.

Radioprotection of Escherichia coli B/r in the presence of extract is optimal when the sample is both aerobic and quiescent. Under these conditions the dose modification factor is 3.2. Preincubation in extract before irradiation in phosphate buffer results in no added protection. Incubation in extract after irradiation in phosphate buffer results in a dose modification of 1.3. Microscopic examination reveals that the protective agent causes the cells to aggregate and also causes nuclear condensation, loss of the periplasmic space and clustering of ribosomes in individual cells.

The effect of the radioprotective extract was also investigated in three mutant strains of E. coli B/r differing from one another at a given locus concerned with the repair of radiation-induced damage. The expression of two of the resistance genes, designated hcr and exr, was seen to be determined by the state of aerobiosis during irradiation; exr was expressed under aerobic conditions while hcr was expressed only during anaerobiosis. The gene which controls the formation of septae between

daughter cells after x-irradiation, designated fil, was expressed both in the presence and absence of oxygen. Extract increased the effect of both exr and hcr by approximately 12% while augmenting fil by 140%.

Alkaline sucrose density gradient centrifugation revealed that the radioprotective extract does not prevent the formation of single strand breaks in DNA, nor does it augment their repair. Extract apparently induces the formation of such lesions in fil^+ cells without exogenous ionizing radiation.

An hypothesis is advanced to explain radioprotection by extract based on differntial sites and/or types of damage incurred by the cell for aerobic and anaerobic conditions of irradiation.

INTRODUCTION

A. Historical Background

The quantitative response of a given organism to the lethal effects of ionizing radiation is subject to modifications over a wide range. For example, 30 kR inactivates E. coli B/r in the presence of the sulfhydryl binding agent N-ethylmaleimide (NEM) by 90% (Bridges, 1961) while irradiation in the presence of 2-mercaptoethylamine (MEA) requires 200 kR for a similar level of inactivation (Elias, 1961). Such perturbations in the absolute response of an organism to a given dose of radiation are termed dose modifications. They may be classified with respect to the time of administration of radiation and encompass a wide range of physiological and biochemical manipulations. The radioresponse of bacteria has been extensively investigated as a function of such treatments and will serve as an illustration of various dose modification procedures.

Modification of radioresponse by treatment before irradiation.

Hollander et al (1951) demonstrated that E. coli B was more radioresistant if grown in batch culture in nutrient broth supplemented with glucose than in broth alone. Stapleton and Eagel (1960) found a direct correlation between radioresistance and the final pH of the medium in which the cells were grown and subsequently irradiated. Variations in dose response as a function of culture age and stage has been demonstrated in both eucaryotes (Dewey, 1965) and bacteria (Stapleton, 1956; Morton and Haynes, 1966). The same effect was noted for log phase cells which had been deprived of a required nutrient 90 minutes prior to UV irradiation (Hanawalt, 1966). The transition from log to stationary phase does not

effect the lesion produced either qualitatively or quantitatively, but rather the cells' ability to bypass or repair it. Micrococcus radiodurans, when irradiated during conditions which define balanced growth, was more radioresistant at higher growth rates (Freedman and Bruce, 1972). That pretreatments other than those which alter the gross physiological state of the cell can modify the radioresponse was demonstrated by Axelrod and Adler in 1968. It was shown that recipient cells which had undergone hfr chromosome transfer were more resistant than cells which had undergone either simple F^+ transfer or in which no mating has occurred.

Treatments during irradiation.

Modifications of cellular radioresponse of the largest magnitude are the result of changes in environment during the irradiation. Drastic changes in the survival of the cell are brought about by reducing the concentration of O_2 below 10 uM/liter (Howard-Flanders and Alper, 1957). This effect is probably due to the amount of dissolved O_2 and not the concurrent cellular anaerobiosis. The obligate anaerobe, Clostridium botulinum, is fully viable at a concentration of 15 uM O_2/liter and yet the level of survival of the facultative anaerobe, E. coli B/r, at that concentration is the same as fully aerobic cultures. Quantitatively, dose modification in N_2 varies between 1.0 and 3.

The quality of the radiation can also cause large differences. Radiations of high linear energy transfer (the energy lost per of track of the primary ionizing particle) such as alpha particles and protons are usually more efficient for killing than x-rays or electrons and has prompted several workers to hypothesize that only double strand breaks incurred in DNA after such exposure are lethal to most cells

since these particles are vastly more effective in producing such lesions (Munson, et al, 1967). Exogenous chemicals added during irradiation can also have a marked effect. Radioprotectors have been known since 1942 (Dale), the most effective, 2-mercaptoethylamine, being discovered in 1949. Since then several thousand compounds have been screened but very few effective agents found (Bacq, 1965). Agents which have the opposite effect, termed radiosensitizers, have been demonstrated in bacteria (Bridges, 1960), mammals (Franks, et al. 1964) and tissue culture (Alexander, 1961). Whereas the chemical structure of the most effective radioprotectors follows the general scheme of SH- $(CH_2)_n$-R_2N where n = 2 or 3 and R = H^+ or NH_2 , radiosensitizers are more diverse. Included in this class of compounds are such non-related compounds as N-ethylmaleamide, iodoacetamide, p-hydroxymercuribenzoate (Bruce and Malchman, 1965) and quinones (Bruce, Mahoney, Thomas and Gersten, 1972). The molecular mechanism for both radiosensitizers and protectors is not firmly established. Even the presence of NaCl has been shown to sensitize bacteria (Elias, 1965).

Post irradiation treatments.

Treatments which effect viability after irradiation are just as varied and seemingly non-related. For example, Stapleton (1961) demonstrated that recovery was greater for E. coli B/r incubated at 6° than 18° but then decreased symetrically between 18 and 37°. Freedman and Bruce (1972) showed that survival of M. radiodurans whose generation time was lengthened by plating on limiting synthetic media was enhanced. Delaporte's (1951) observation that survival in yeast is higher when the cells are plated at higher concentrations (that is, when there were clumps of cells on the plate) prompted Fischer et al. (1965) to investigate the

existence of a similar division promoting extract from E. coli B/r. Such extract, when added to E. coli B after irradiation, overcome permanent division deficiencies which result from the ionizing radiation. Chemically such extracts may be phospholipid (Fischer, 1965) or nucleoprotein (Korgaonkar and Raut, 1967) in nature.

1. Dose Modification in Bacteria.

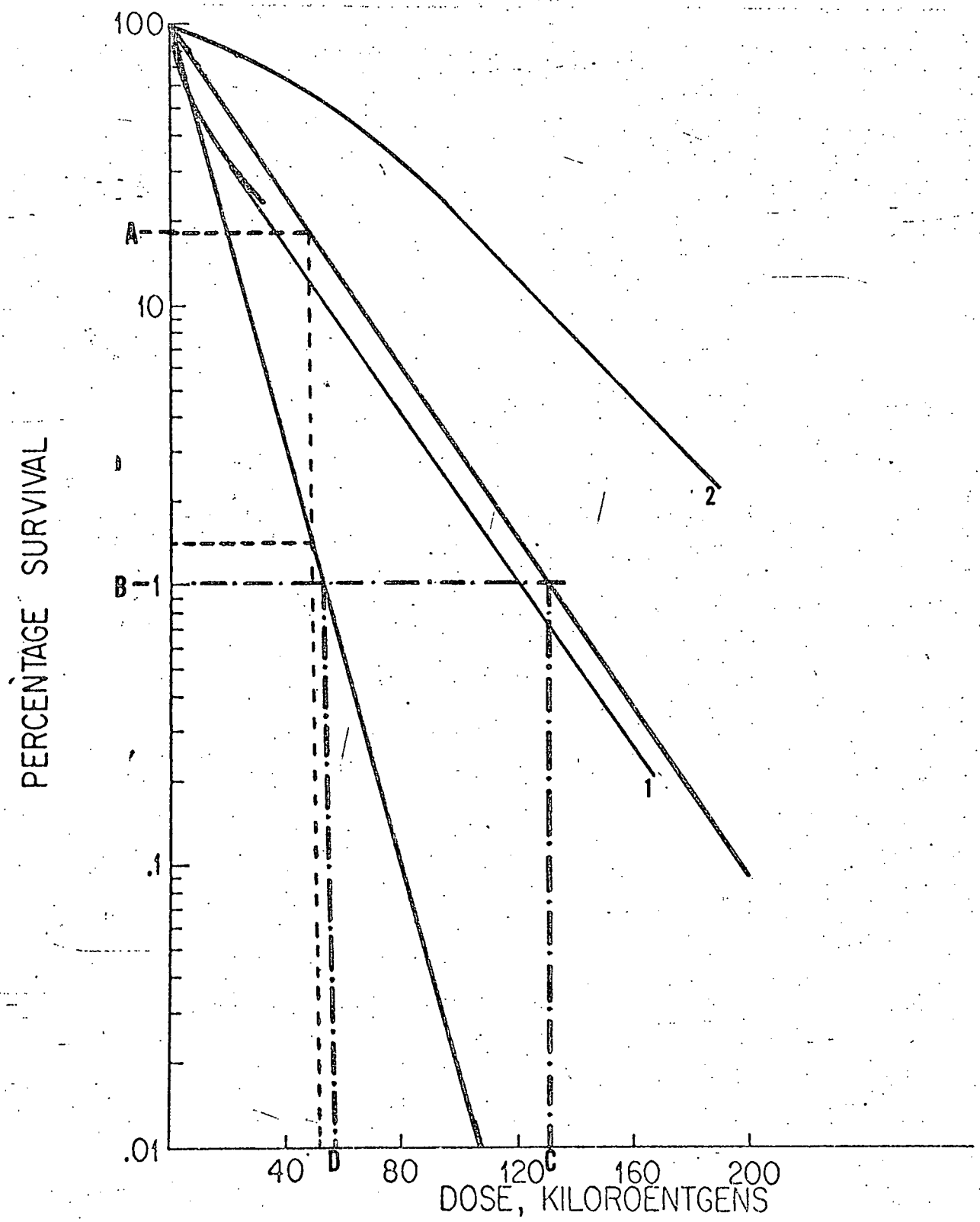
The analysis of a given dose modification relates the number of viable cells after each dose increment for treated and untreated samples. The dose-effect relationship that describes the number of viable cells as a function of dose for a given condition is called a survival curve. An example is shown in Fig. 1. The abscissa is a linear expression of cumulative dose delivered, the ordinate is logarithmic and describes the percentage survivors or viable cells for each dosage level delivered. Such curves can assume any of three distinct shapes; concave up (reduced survival at low doses, type 2 in Fig. 1) concave down (elevated survival at low doses, type 1 in Fig. 1) or straight (exponential).

Certain relationships which will be used throughout the description of this work will be defined as follows:

1) Dose Modification Factor (DMF). The ratio of the dose required to bring about a given level of survival in the presence of an agent which modifies cell viability to that dose which causes the same viability in the absence of the agent. This is represented as C/D in Fig. 1. If the DMF is greater than 1, the agent is referred to as a radioprotector, if the DMF is less than 1, the agent is termed a radiosensitizer.

2) Oxygen Enhancement Ratio (OER). The dose modification factor when the agent tested is nitrogen.

Figure 1. Sample survival curve denoting the parameters of protection used in the course of this work.


100
A
10
B
1
.1
.01
PERCENTAGE SURVIVAL
1
2
40
D
80
120
C
160
200
DOSE, KILOROENTGENS

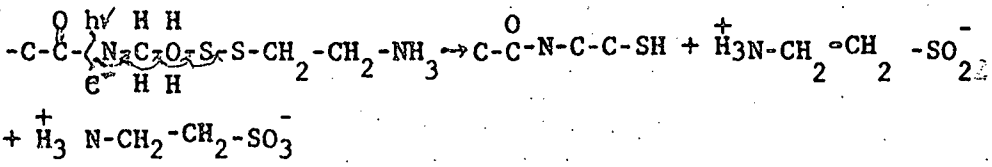
3) Pq (50). The ratio of survivors at a given dose (50 kR in this case) for cells irradiated in the presence and absence of a dose modifying agent. This is represented as A/B.

In order to understand the mechanisms involved in dose modification, it is necessary to investigate the primary chemical interactions caused by ionizing radiations. A site where a potentially lethal chemical event is induced is referred to as a target. Radiochemical modification is thought to occur in two ways, by direct and indirect action. Direct action (Alper, 1956) is the deposition of energy in a target molecule which results in either an excitation or ionization. Indirect action is the formation of a lesion in the target by the action of active species formed through the radiolysis of water (Bacq, 1965). Active species may include oxidation agents such as e- (hydrated electron) and H_2O_2, or free radicals ($H^{\cdot}$, $OH^{\cdot}$, $H_2\cdot$. $HO_2\cdot$).

A possible mechanism for dose modification which presupposes that the target of interest is a specific protein is the Mixed Disulfide Hypothesis of Eldharn and Pihl (1958). The model describes the formation of a disulfide linkage between the protein and a small sulfhydryl molecule. Radioprotection from indirect action is thought to occur by the following reaction: $HO_2^{\cdot}$, $OH^{\cdot}$, etc. + $H_3N\text{-}CH_2\text{-}CH_2\text{-}S\text{-}S\text{-}R \longrightarrow SH\text{-}R + H^{+}_3N\text{-}CH_2\text{-}CH_2\text{-}SO_2^{-}$ + $H^{+}_3N\text{-}CH_2\text{-}CH_2\text{-}SO_3^{-}$

1) Here, free radicals formed as the radiolysis products of water attack a protein which is bound by a disulfide linkage to a radioprotective molecule. Cleavage of the disulfide bond results in an intact reduced protein and an oxydized radioprotective compound.

Reactions concerning direct action are:



2) The energy added to the protein by the quantum of radiation is transferred by covalent bonds and eventually results in the cleavage of the disulfide linkage.

An increasing body of information indicates that the critical target within the cell is the DNA rather than a protein species. Mixed disulfides cannot explain the increased resistance afforded by chromosome transfer for example. A correlation between the number of phosphodiester backbone scissions and viability has been established for both E. coli B_{s-1} (McGrath and williams, 1966) and phage ØX 174 (Tessman, 1959).

The qualitative universality of radioprotection by hypoxia has led to the formulation of a mechanism based on the scavenging of oxygenated free radicals. However, Town and Smith (1971) have demonstrated that the damage induced in DNA under both aerobic and anaerobic conditions is quantitatively comparable. Recent evidence from the work of Davies (1967) has shown that a mutant of Chlamydomonas exhibits little or no increased survival under anaerobic conditions. Since the quantitative yield of free radicals is comparable over very wide ranges of viability for a given dose, it seems likely that survival is a function of either a cell's ability to repair such lesions or to chemically modify them and render them non-lethal.

Damage and repair of DNA.

Repair of damage DNA is a complex multistep process that involves the action of several specific enzymes before it is completed. Bacterial

repair systems were first demonstrated by Setlow and Duggan (1964) where the excision of ultra-violet induced photolesions (pyrimidine dimers) from DNA was noted. Repair of x-ray induced damage to DNA was shown by McGrath and Williams (1966).

A great deal of work at the molecular level in microbial radiobiology has been concerned with investigation of DNA repair systems. The basic reaction scheme includes:

(1). Recognition of damage--in UV irradiated cells this is represented by thymine-thymine dimers (the formation of a cyclobutane ring at the 5', 6' position of adjacent nucleotides), thymine-cytosine dimers, and cytosine-cytosine dimers.

(2). Dimer excision (UV only)--the scission of the phosphodiester bonds and removal of the photoproduct. The DNA at this stage is comparable to that which has undergone base damage or phosphodiester backbone scission by the action of ionizing radiation.

(3). Gap widening--the formation of single-stranded regions by the action of nucleases at a gap previously formed.

(4). Base insertion and polymerization--The proper bases are matched by hydrogen bonding requirements to nucleotides on the intact strand and incorporated, probably by the action of Kornberg polymerase.

(5). End rejoining--by the action of polynucleotide ligase.

Neither the specific radiochemical lesion nor the site of damage within the cell is as clearly defined for ionizing radiation as for UV. The production of specific UV photoproducts in DNA both in vitro (Beukers, 1959) and in vivo (Setlow, et al., 1965) has been demonstrated as the sole lesion within the cell that arises from biologically effective doses. Ionizing radiation on the other hand causes several distinct

kinds of damage to DNA including:

1. scission of the phosphodiester bonds
2. depurination
3. base degradation
4. base liberation
5. oxidation of the sugar moeity
6. crosslinkage with protein

Recent evidence (Burrel, et al. 1971; Cramp and Watkins, 1972) has implicated that a site of damage for ionizing radiation other than the bacterial chromosome is located at the membrane. Alper (1962) has suggested that there are two distinct types of damage resulting from ionizing radiation; Type O is associated with irradiations where oxygen is present, Type N where oxygen is absent. She further infers that Type O damage is localized at or near the membrane, while Type N is found at the bacterial chromosome. Dean et al. (1969) have suggested that there are two qualitatively different lesions produced in DNA in aerobic vs. anaerobic irradiation while Achey and Whitfield (1968) feel that the decreased amount of damage during anaerobic irradiations reflects a decrease in the number of free radicals formed. The existence of a DNA ligase for rejoining DNA damaged by scission of the phosphodiester bond under defined anaerobic conditions during irradiation and incubation post irradiation has also been demonstrated (Town and Smith, 1971).

While a macromolecular analysis has proven fruitful in studies with UV (see review by Hanawalt, 1965), the complex nature of the site and type of the lesion induced by ionizing radiation may lead to investigation of damage which is biochemically exciting yet biologically insignificant in

terms of viability. For example, the elegant technique of McGrath and Williams (1966) allows one to isolate and quantitate the production and repair of single strand breaks in the bacterial and eucaryotic chromosome induced by X or gamma rays. Much of the information gleaned from such studies indicates that these lesions are not lethal to most cells (Munson, et al., 1967). Notable exceptions are the single stranded DNA virus ØX-174 (Tessman, 1959) and the B_s series of E. coli (McGrath and Williams, 1967), where the production of single strand breaks is directly correlated to survival.

Other repair schemes have been elucidated. Rupp and Howard-Flanders (1968) have demonstrated the repair of UV lesions in excision deficient cells and have hypothesized the existence of a post replicative repair system. Briefly, proper base insertion is brought about by using newly synthesized DNA as a template for damage regions in a recombinational event. A third type of repair operative during or after x-irradiation which involves a single ligase reaction to close phosphodiester bond scissions has been found in M. radiodurans (Dean et al., 1969) and E.coli K_{12} (Towns and Smith, 1971).

It can be seen that the ability of the cell to overcome the lethal effects of ionizing radiation relies on the coordinated activity of many complex biochemical reactions at many levels. The radioresponse of E. coli has been shown to be genetically controlled by at least three genes (Witkin, 1967). Host cell reactivation (hcr) denotes the ability of the cell to repair the DNA of UV irradiated bacteriophage. It is therefore associated with the reapir processes described earlier. Exr denotes the presence of a "mutation prone" base insertion mechanism to fill the gaps

produced in DNA by the action of endogenous nucleases after the induction of damage. Fil is the ability of the cell to form crosswalls after irradiation and therefore form colonies instead of multinucleated filaments.

2. Dose Modification by Constitutive Agents.

Organisms exhibit widely varying resistance to the lethal effects of ionizing radiation. The most sensitive strain isolated to date Escherichia coli B_{s12} (Alper, 1968) is inactivated 90% by 2.6 kR, while 600 - 700 kR are required to inactivate the highly resistant tetracoccus Micrococcus radiodurans (Anderson, 1956). Mammals are considerably more sensitive by a factor of 10^2 - 10^3, although nematode worms (inactivation dose = 48.96 kR) and other invertebrates are more resistant (Bacq, 1961).

The organism most resistant to the lethal effects of ionizing and ultraviolet radiation is the bacterium M. radiodurans. Inactivation of resistant mutants requires 1500 kR (Lewis, 1971). In comparison, E. coli B/r, a bacterium of average resistance, is inactivated to the same level by only 50 kR. M. radiodurans has a generation time of 90 minutes when grown under optimal conditions. It has been grown in synthetic media but requires supplementation of amino acids, nucleotides, and vitamins (Raj et al, 1956). Its growth requirements are not surprising since it is found naturally as an intracellular parasite. Microscopically each cell in the tetrad is 1-2 u in diameter and is gram positive. The cell membrane is characterized by a complex series of pegs and hexagonal pores (Thornley, 1965). Crosswall formation between cells is apparently incomplete. The deep red pigment is carotenoid in nature but has been shown not to confer radioresistance (Mathews and Krinsky, 1965).

The cell has an unusually high sulfhydryl content and prompted Bruce (1965) to implicate this as at least partially responsible for its radioresistance. Although the DNA of the cell undergoes both x-ray (Dean et al, 1966) and UV damage (Setlow and Duggan, 1964) comparable to other bacteria, its ability to overcome this is apparently far superior. Recent evidence (Bonura and Bruce, 1972) has demonstrated that M. radiodurans is very efficient in the repair of single strand breaks (phosphodiester bond scission) by ligase action immediately after or during x-irradiation.

One reason for the radioresistance of M. radiodurans is the existence of a constitutive radioprotective agent (Bruce, 1964). The active compound was pressure dialysable and hence had a molecular weight of less than 10,000 and was stable to (and in fact enhanced by) autoclaving. The presence of a contaminating radiosensitizer was also shown. A compound extracted in a similar manner resulted in a 10-fold increase in the number of transformants produced in M. radiodurans (Moseley, 1966). Purification of the agent by paper chromatography was accomplished by Serianni and Bruce, (1970) and radiochromatograms showed it to be non-sulfhydryl in nature. The dose modification exceeded that of MEA at optimal concentration. The extract was assayed in E. coli B/r.

Cellular radioprotective extracts of quite a different nature were isolated from E. coli B/r. It had been noted that the ability of the cell to form crosswalls during fission was severly impaired post x-irradiation and resulted in very large, multinucleate filaments. Fischer (1965) isolated a high molecular weight phospholipid and Korgaonkor and Raut (1967) a "nucleoprotein" both of which apparently act by similar mechanisms to overcome this filament formation in E. coli B after

exposure to ionizing radiation. Since the division promoting extract was most effective when irradiated cells were plated in its presence, it is more closely related to those post irradiation treatments mentioned earlier. The Micrococcus extract which must be present during the irradiation and therefore more closely resembles MEA.

However, little is known about the mechanism of dose modification by extracts of M. radiodurans. In view of the various types of damage caused by ionizing radiation, several possible modes of modification can be envisioned. The extract is in some way related to the very efficient repair system of M. radiodurans? It is somehow related to the complex cell wall and membrane of the cell? The mode of action is similar to known radioprotectors or does it in some way mimic non-specific physiological manipulations of the cell?

An analysis of the radioprotective effects of extract from M. radiodurans in the B series of Escherichia coli would provide some answers to these questions and perhaps clarify the complex nature of dose modification at the various levels. This cell line can be used to discern qualitatively the type of lethal damage incurred by exposure to ionizing radiation (Alper, 1968).

Attempts to correlate dose modification by synthetic radioprotectors with the level of accuracy or efficiency of repair of damage have been unsuccessful. Witkin (1967) demonstrated that enhanced viability is associated with both mutation proof and mutation prone mechanisms. Ginsberg and Webster (1971) showed that while the amount of damage sustained by DNA after x-irradiation could be modified by MEA, this bore no correlation to the viability of the cell. Lohman et al (1970) found that MEA actually induces breaks in DNA in unirradiated cells,

and that this could be overcome by washing the cells with NEM, a known radiosensitizer. It was hoped that a similar type of macromolecular analysis using an endogenous radioprotector would not be subject to artifactual complications and would therefore be directly related to macromolecular repair processes associated with viability.

Experimentation with an endogenous dose modifying agent offers a distinct advantage over similar work with exogenous chemicals; there is a direct correlation between the quantity of the agent and those processes which directly effect the cell's ability to overcome radiation damage. No such statement can be made for other agents or treatments with the possible exception of the nitrogen effect, which may deserve special consideration (Alper, 1962).

For this reason, the dose modification afforded by extracts of Micrococcus radiodurans was studied in depth.

B. Objectives

The work described was designed to fulfill the following objectives:

1. To separate and purify the active agent with characterization and identification of the molecule.
2. To determine the optimal conditions for radioprotection by the extract.
3. To determine the effect of the extract at the gene level in E. coli.
4. To determine the effect of the extract in the repair of damaged DNA at the macromolecular level.

Results from such an analysis would provide additional information concerning the mechanisms of action of bacterial systems which respond to damage caused by ionizing radiation.

MATERIALS AND METHODS

A. General

Organisms used.

Five strains of E. coli were used in the course of this work. All are complete autotrophs and were isolated on the basis of their radioresistance. The parent strain for all mutants was E. coli B originally isolated by Bronfenbrenner (1920). Mutants of increased radioresistance are E. coli B/r (CSH) isolated by Witkin in 1946 and E. coli B/r (ORNL) a substrain of B/r (CSH) which has slightly higher resistance (Adler and Engel, 1961). Strains of increased radiosensitivity were E. coli Bs-1, isolated by Hill (1958) and E. coli B III-10 isolated by Witkin (personal communication). The cells represent a five-fold (aerobic) to 15-fold (anaerobic) increase in resistance to the lethal effects of ionizing radiation and their order of radioresistance is B/r (ORNL)$\geq$B/r (CSH)$\geq$B$\geq$ B III-10$\geq$Bs-1.

The source of the extract was M. radiodurans, PH2, (Howell unpublished results) a colorless mutant of the wild type isolated by Anderson of the University of Oregon. The mutant lacks the pigment characteristic of the parent and is about 1.5 x more radioresistant under aerobic conditions (Goldstein, unpublished results).

Culture conditions.

All cells were grown at 31^{o} C with vigorous shaking to stationary phase in complete media to an O.D. at 650 mu of 1.9 - 2.2 when measured in a Beckman Model DB Spectrophotometer. Unlabelled E. coli were grown in nutrient broth and M. radiodurans PH2 in yeast-hydrolyzed casein (YHC). Labelled E. coli were grown in M-9 basal media (Anderson, 1946) supple-

mented with 250 mg deoxyadenosine/liter and 10 µl of H^3 (tritium) thymidine whose activity was 1 mCi/ml. The complete composition of all media is presented in Appendix I. Plates were made by adding agar to each of the above media to a final concentration of 2%.

B. Extract Preparation, Purification and Molecular Characterization

Extract preparation.

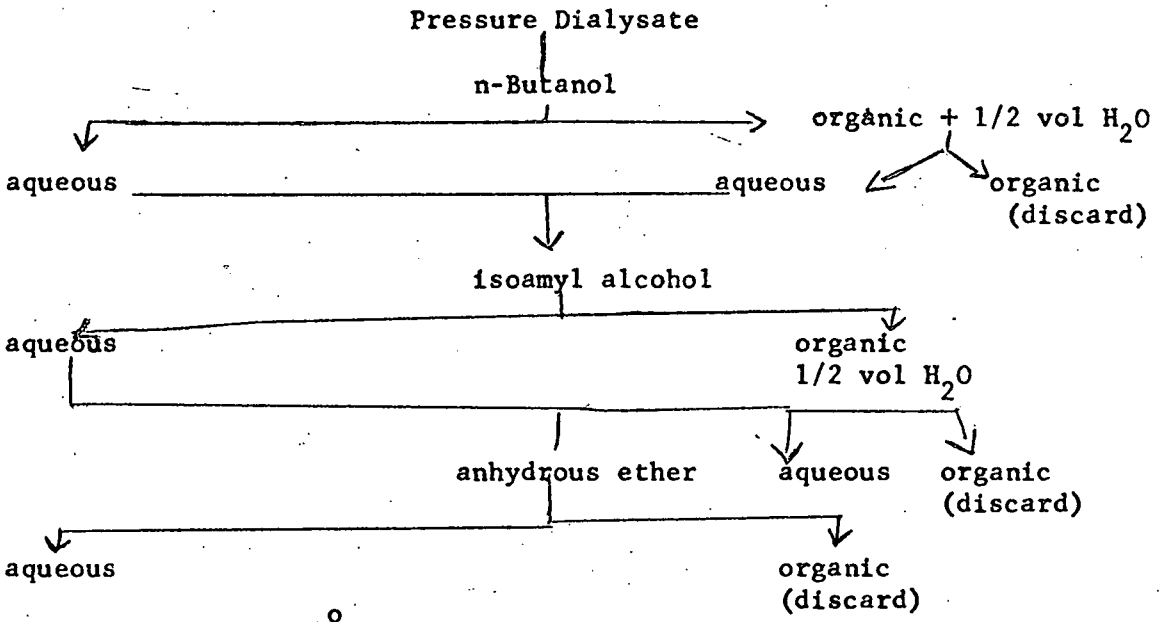
Ten 1 liter batch cultures of stationary M. radiodurans were harvested in a continuous flow centrifuge at 25,000 x G. The pellet was washed three times in distilled water and resuspended at a concentration of 1 gm wet weight cells per ml distilled water. The suspension was lysed by passing it three times through a French Pressure Cell (Amer. Inst. Co., Silver Springs, Md) operated at 10,000 -16,000 psi. Breakage was 90 -95% complete as determined by viable cell counts. The resulting pressate was centrifuged at 25,000 x G for 12.5 minutes to remove cell debris and unlysed cells. The supernatant was collected and the pellet resuspended in 10-20 ml distilled water per tube. This was centrifuged again and the supernatant pooled with the first; the pellet was discarded. The supernatant was pressure dialyzed overnight in ½ inch visking dialysis tubing at 8 psi using filtered air. The tubing retains molecules whose weight is greater than 10,000-15,000 daltons. The dialysate was collected and held at approximately 4°C to retard bacterial contamination.

Extract fractionation and concentration.

The extract, as prepared above, was fractionated with a series of organic solvents as outlined in Fig. 2. The sequence of N-butanol or isoamyl alcohol washes does not effect the final activity of the extract,

Figure 2. The schematic representation of the organic partition of the pressure dialysate of crude extract isolated from M. radiodurans PH 2. The aqueous layer was cleared of organic solvents by washing with anhydrous ether before concentration to 5.0 ml by flash evaporation at 60 C.

Figure 2



flash evaporation 60°

5 ml extracted dialysate

which is approximately 80% that of the non-fractionated. Considerable material (presumably lipid) partitions into the organic phases. Failure to remove this results in high viscosity when the material is concentrated. Viscous solutes can cause large aberrations in the elution profile obtained with Sephadex. The final yield of fractionated extract was 5 ml/80-100 gms cells (wet weight).

Sephadex chromatography: high resolution technique.

A 0.9 x 100 cm chromatography column was prepared using G-25 fine grade Sephadex (Pharmacia Co$_{\beta}$,Uppsala, Sweden) which had been swelled and sterilized in distilled water by autoclaving at 110°, 10 psi for 90 minutes. The gel was allowed to settle and small diameter beads were removed by suction from the supernatant. The hot slurry was poured into a presterilized column as quickly as possible to minimize the chance of bacterial contamination. The gel bed was packed and several volumes of sterile water were eluted through a .45 u in-line millipore filter to stabilize it. The column and collecting apparatus were maintained at 4°C.

The void volume (V_o) was determined using Dextran 2000 (Pharmacia) a dye with a molecular weight of 2×10^6 daltons. The total elution volume (V_t) was determined using adenosine monophosphate or MEA and monitoring the optical density at 2580 Å and 2400 Å respectively in a Beckman model DB spectrophotometer (Beckman Inst). Adenosine monophosphate has chemical properties which cause it to be retained by the gel to a greater extent than would be predicted if only molecular weight and shape were considered (Gelb, personal communication). G-25 Sephadex retains molecules of smaller than approximately 5,000 daltons.

The column was eluted with distilled water at an operating pressure of 12 ". An in line millipore filter (0.45 u) was inserted between the reservoir and column, and the resulting flow rate was 2 ml/hr. Resolution under these conditions was good, since both the high molecular weight dye and the adenosine monophosphate chromatogrammed as sharp bands. V_o and V_t (AMP) were determined as 45 and 90 ml respectively.

A 0.5 ml aliquot of extract was gently layered on the gel bed and allowed to diffuse into the gel. The dead volume (the space between the top of the gel and the top of the column) was filled with sterile water until all air bubbles had been removed. Collection was started immediately in 24 drop fractions into lyophilization tubes using a fraction collector (Buchler Inst.) with an integrated drop counter. After collection of the entire elution profile each sample was shelled in dry ice ETOH and lyophilized. The resulting powder was resuspended in 0.5 ml distilled water and bioassayed.

Sephadex chromatography: high yield technique.

A 2.5 x 100 cm G-25 fine grade sephadex column was prepared and calibrated as described previously. The operating pressure was adjusted to 30" and the resulting flow rate was 8-10 ml/hr. Values for V_o and V_t were determined as 170 and 550 ml respectively. Other parameters necessary for characterizing the behavior of the solute in the gel are "a" (the dry weight of gel) = 100 gms and Wr (the water regain) = 2.5. Two ml of crude extract were layered on the gel bed and the effluent collected in 320 drop fractions. These were reduced to 1-2 ml by flash evaporation and lyophilized. Each sample was resuspended to 1 ml with distilled water and bioassayed.

Bioassay.

The fractions from the sephadex chromatography were warmed to room temperature and 100 ul aliquots transferred to a vial using an Eppendorf pipette (Brinkman Inst.). To each aliquot was added 10 ul 0.67 M PO_4 buffer and 10 ul E. coli B/r at a concentration of 2×10^9 cells/ml. The mixture was drawn by capillary to the mark of a 100 ul disposable pipette (Corning, TC ½ % accuracy) and sealed. The micropipettes were placed at the circumference of a circular holder which was bolted to the x-ray unit. The samples were rotated at 51 rpm and incubated 15 minutes in the dark. They were irradiated for 17.6 minutes at 250 KVCP and 15 ma on a Westinghouse Coronado Therapy X-ray Unit. The beam was filtered through 40 ± 1 mm air, 0.1 mm soda lime glass and the inherent filtration of the machine at the point of maximum dose rate.

After irradiation, each micropipette was discharged and rinsed several times in a tube containing 9.9 ml PO_4 buffer. Each was serially diluted to a final cell concentration of $5 - 50 \times 10^2$ cells/ml and plated in duplicate on nutrient plates. The plates were counted after overnight incubation at 31° C.

The inherent variable of time spent in micropipettes before and after irradiation in buffer varied between 10-20 minutes each. Buffer controls treated in the same manner showed no significant alteration in radioresponse caused by this. There was no increase in cell counts if the plates were incubated for more than 18 hours.

Enzyme analysis of extract.

Concentrated Tris-HCl was added to extract to a final concentration of 10^{-2} molar. DNase (Worthington) RNase (Sigma) Pronase (Sigma) and Lipase (Worthington) were added to a final concentration of 10 ug/ml.

The solution containing DNase was further supplemented with 10^{-2} mM $MgSO_4$. Four samples were formulated to the following contents scheme for each enzyme

1. extract + enzyme
2. extract only
3. enzyme only
4. neither extract nor enzyme

Equal numbers of cells were added to each and the number of survivors for each treatment after 50 kR was compared to unirradiated controls and the result expressed as a % survival.

Electron microscopy.

Cells were incubated at a concentration of 1×10^9/ml in either extract or buffer for 15 minutes and then centrifuged at 25,000 x G for 1 minute. The cellular pellet was dehydrated by gently resuspending for 5 minuted each in 70%, 90%, 95% and 100% ethanol before fixation in 3% glutaraldehyde and 2% osmium. The fixed pellet was imbedded in Epon A and baked at 60° C for 24 hours. It was sectioned in a Sorval MT-2 ultramicrotome to a 500-750 Å thickness using a diamond knife. The sections were stained in uranylacetate and lead citrate and examined in a Mitachi HU-11-C electron microscope at 50 kV.

C. Optimization of Protection - Experiments Using Survival Curves

Dosimetry and irradiation conditions.

All irradiations other than that used in the bioassay of the sephadex chromatography were performed in the same chamber. A conical 1 ml glass centrifuge tube was placed in a lucite holder which was secured to the x-ray unit. The TSD was held constant for all irradiations

and was such that the glass tube could be removed for sampling without upsetting the geometry of the chamber. The chamber was situated so that the TSD measured to the center of the centrifuge tube was 665 mm and the beam was filtered through 34.5 mm air, 1 mm lucite, 1 mm glass, and the inherent filtration of the machine. Dosimetry was performed using the method of Weiss (1952). The dose rate for a 250 KVCP, 15 ma beam under these conditions was 10 $\pm$ 0.3 kR/minute. Operation of the machine is such that the first 6 seconds of irradiation is performed at 250 KVCP and 7.5 ma. The dose delivered during this period is 0.7 kR and in all experiments times of irradiation were corrected for accordingly. Since the dose rate was held constant, the total dose was a function of time. The dose delivered is the same between 20-200 ul of solution in the tube, and it is assumed to be equivalent in volumes less than 20 ul.

Survival curves.

The low yield of purified extract after Sephadex chromatography necessitated that subsequent experiments be performed using semi-micro techniques. Methods were developed that could generate 5-10 data points using samples with a total volume of 120 ul or less.

Washed cells at a concentration of 2×10^9 were diluted 1:10 in the solution to be irradiated. Two 5 ul aliquots were sampled for each data point and carried through separate serial dilutions. Each dilution was plated in duplicate and were generally such that between 50 and 500 colonies arose per plate. The variation between duplicate plates was approximately $\pm$ 2%, between duplicate samplings it was $\pm$ 5% and between experiments, $\pm$ 5%. The variation in dose to yield a given level of survival was $\pm$ 10%. Each experiment was repeated at least once so that each point represents the average of a minimum of 8 plates.

Survival curves for E. coli B/r were generated under the following conditions:

Type I Cells in extract or buffer vigorously bubbled before and during irradiation with oxygen (line air).

Type II Cells in extract or buffer bubbled with nitrogen before and during irradiation.

Type III Cells in extract or buffer left quiescent for 15 minutes before and during irradiation.

Type IV Cells in extract or buffer bubbled for 15 minutes with nitrogen and then overlaid with mineral oil to prevent diffusion of oxygen and left quiescent for 15 minutes before and during irradiation.

Effect of post-irradiation incubation in extract.

Complete survival curves were generated for post-irradiation incubation in extract. Cells were irradiated in buffer and 5 ul aliquots incubated in 10 ul of extract for 5-15 minutes prior to serial dilution and plating. The survival curve was compared to that for a buffer control.

D. Optimization of Protection - Experiments Using Protection Quotient

Effect of pre-incubation in extract.

For pre-incubation studies, E. coli B/r were incubated in extract for 15 minutes at a concentration of 2.2×10^8 cells/ml. The cells were centrifuged at 25,000 x G, the extract decanted, and the cells resuspended in an equal volume of PO_4 buffer. They were incubated for an additional 15 minutes and irradiated to 40 kR. The % survival was calculated and compared to buffer controls treated in the same way.

Radiotoxic effects of extract.

An equal number of cells were incubated for 15 minutes in aliquots of extract that had been irradiated to 50-300 kR and unirradiated controls. The samples were serially diluted in PO_4 buffer, plated on nutrient plates and incubated overnight. The data are expressed as number of cells in irradiated extract divided by the number of cells in concurrent unirradiated buffer controls.

Effect of initial cell concentration on protection by extract.

E. coli B/r (5×10^9 cells) were diluted between 10^0 - 5×10^{-3} and incubated in either extract or buffer for 15 minutes. The cells were irradiated to 50 kR under Type III conditions, serially diluted, plated and incubated overnight on nutrient plates. The % survival was calculated using unirradiated buffer controls plated for each cell concentration.

Effect of extract concentration on protection.

Equal numbers of E. coli B/r were added to individual aliquots of extract which had been serially diluted with distilled water up to 1/256 of its original concentration. The % survival was determined for each dilution and the data expressed as % survival in extract/% survival in buffer to normalize variations in incubation times.

Effect of 2-mercaptoethylamine on extract.

Complete survival curves were generated for 0.044 M solutions of MEA (Evans Chemetics) in the presence or absence of extract under conditions where the radioprotective effect of each was optimal (Type III).

E. Alkaline Sucrose Density Gradient Centrifugation

Solutions of 5% (W/v) and 20% (W/v) optically pure sucrose (Swartz Mann) were prepared in .1 N NaOH. 4.8 ml linear gradients were formed in a gradient building apparatus by mixing increasing volumes of the 5% sucrose with decreasing volumes of the 20%. The gradients were built in ½ by 2 inch cellulose nitrate tubes which had been thoroughly washed in several voumes of EDTA, rinsed with distilled water and siliclad treated before air drying. A 100 ul lytic lamellae of 0.5 N NaOH + 0.05% sodium lauryl sulfate was gently layered atop each gradient just before 10 ul samples were layered on.

Cells used in gradient analysis were grown in continuous label M-9 media (see Appendix I) with 20 uCi/ml H^3 thymidine. The cells were washed several times in PO_4 buffer to remove label not associated with high molecular weight DNA. They were then resuspended to 1/5 their original volume and 10 ul of this suspension was added to 25 ul of extract or buffer. Samples were irradiated to 60 kR and were sampled either immediately after irradiation or after incubation in buffer or nutrient broth.

E. coli normally lyse completely and immediately when placed in 0.5 N NaOH with 0.05% SLS. The presence of extract prevented this however, and lysis could only be effected by incubating for 10 minutes in 10^{-2} Tris-EDTA buffer containing 5 mg/ml lysozyme before layering in the lytic lamellae. Cells in both buffer and extract were lysed in this manner.

The cells were allowed to lyse on the top of the gradient for 20 minutes before the tubes were centrifuged in order to optimize lysis and present the formation of a lamellar inversion. Centrifugation was

performed in the SW-39 head at 30,000 RPM for 100 minutes at 20°C in a Beckman Model L-2 ultracentrifgue. After centrifugation, the tubes were clamped in a continuous pressure apparatus and the bottom pierced with a hypodermic needle. The samples were collected in 10 drop fractions directly in scintillation vials. Fluor (Triton X-100, see Appendix I) was added to each and they were counted in a Packard Liquid Scintillation Counter (Packard Instruments Co., Inc.).

Analysis of each gradient in terms of molecular weight (weight average) and single strand breaks (McGrath and Williams) was performed in a CDC 6400 computer using the program of Bonura.

RESULTS

A. Isolation and Characterization of the Protective Extract

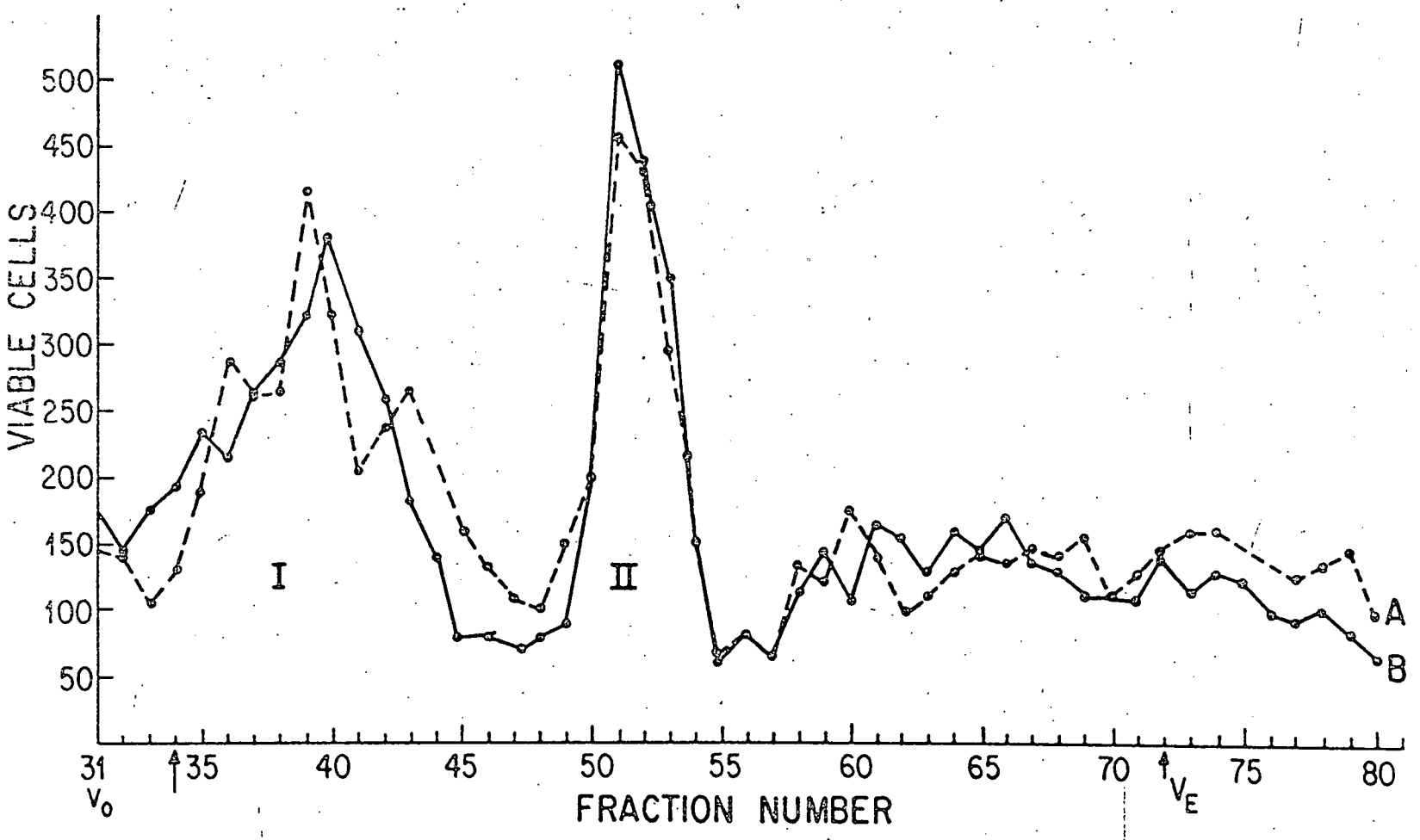
The bioassay for protection in fractions collected after sephadex chromatographic separation of the organically partitioned extract was designed so that all fractions received an equal number of cells initially and were irradiated to the same dose. The presence of a radioprotective dose-modifying agent would therefore be reflected as an increased number of survivors in certain fractions when compared to either fractions without the agent or to buffer controls, treated in the same way.

Crude extract isolated from Micrococcus radiodurans exerts a dose modification factor of 1.2 (Bruce, 1964) to 3.8 (Serianni and Bruce, 1968) in E. coli B/r. Irradiation of cells in PO_4 buffer under the conditions of the bioassay resulted in a survival of 3%. An estimation of the Pq (The ratio of % survival in the presence of a dose modifiying agent to the % survival in 0.067 M PO_4 buffer at a given dose) at that level of survival showed that a DMF of 1.2 would have a Pq = 2, and a DMF of 1.5 would have a Pq = 3. This calculation assumes a dose rate of approximately 2 kR/minute)obtained from % S in E. coli under conditions similar to this) and that the survival of E. coli as a function of total dose in extract or buffer, can best be described by an exponential function. Any fraction which had a Pq = 2 was considered to have a radioprotective agent.

High resolution technique.

The ability of G-25 sephadex to isolate the active protective component and the reproducibility of the bioassay is shown in Fig. 3. Each point represents the average viable cell count of duplicate plates

Figure 3. The bioassay of organically partitioned extract from M. radiodurans PH 2 eluted through a 0.9 X 100 cm sephadex G-25 column at a flow rate of 2 ml/hr and collected in 24 drop fractions. Each point is the average viable cell count of duplicate plates for individual fractions exposed for 17.6 minutes to x-irradiation. The solid line (—) is the first assay of the elution profile; the dotted line (--) is the repeat of the bioassay after the fractions were stored for 24 hrs. at 4 C.


VIABLE CELLS
500
450
400
350
300
250
200
150
100
50
I
II
A
B
31
V_0
35
40
45
50
55
60
65
70
V_E
75
80
FRACTION NUMBER

for each fraction. The void volume (V_o) and total elution volume (V_e) are indicated. The total elution volume was measured previously using MEA. Collection and bioassay were continued well past the total elution volume in order to insure that the Pq of the bioassay itself does not approach 2 and also that any compounds within the extract that might have unusual binding characteristics to the sephadex would be eluted.

The extract separates into two distinct radioprotective peaks. Both have a DMF of approximately 1.8 since this Pq is 2.6 - 3.0. Both A and B were analyzed by the procedure described in Materials and Methods. Plot B is a duplicate experiment using fractions from the column assayed in A that had been stored 48 hours at $4^{\circ}C$.

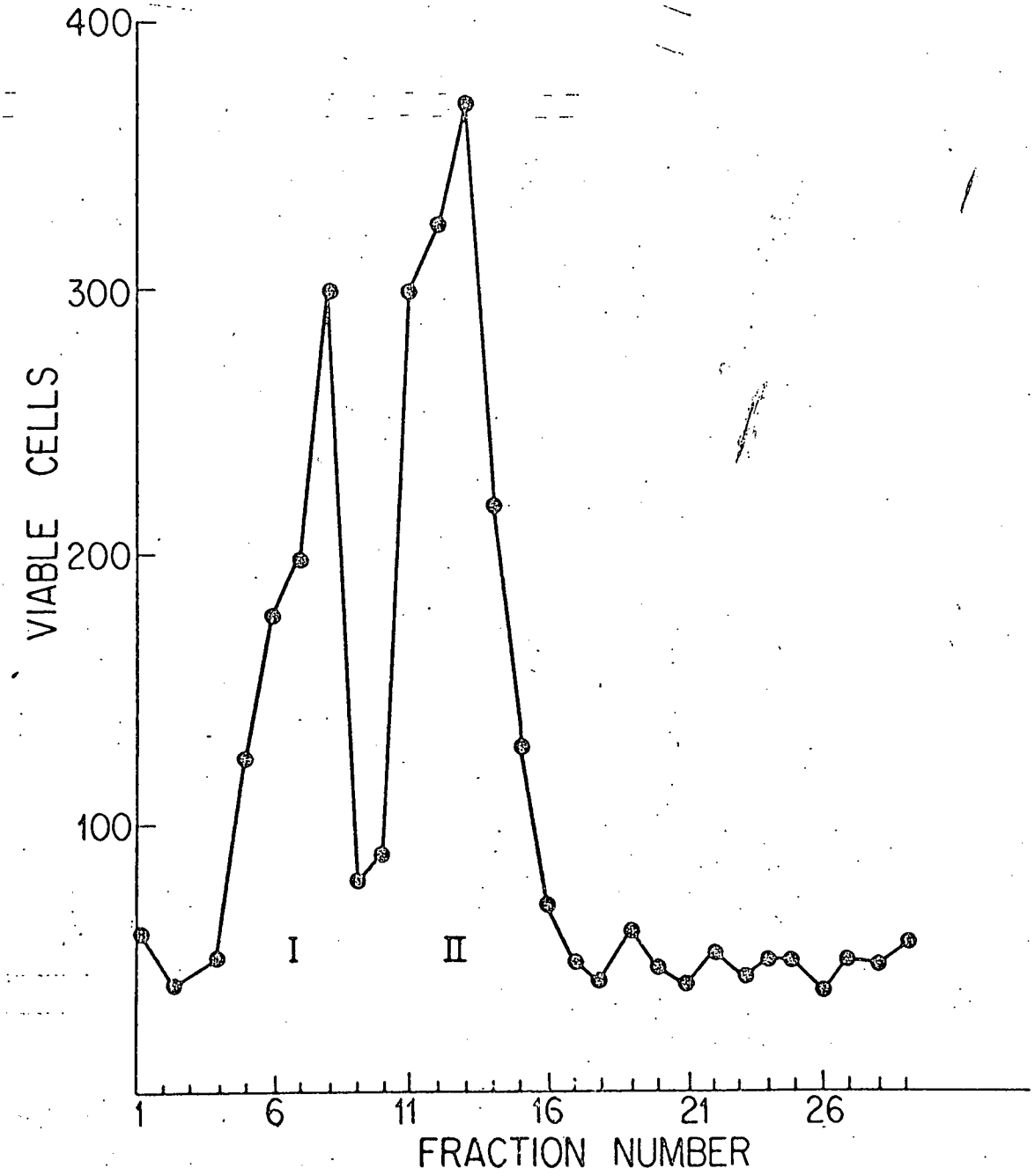
The variation in viable cells for duplicate plates of a given fraction exceeded 10% of the average for a given set only once in 48 separate events. Fully 85% of the samples had deviations that were less than 10% of the mean cell count per fraction.

High yield chromatography.

In experiments using the 2.5 x 100 cm column, a degree of resolution was sacrificed in order to obtain usable amounts of purified extract. The profile was collected in only 28 fractions instead of the 40 required by the technique described before and it would be expected that the peaks would elute closer to one another than with the high resolution technique. That this was in fact true is demonstrated by Fig. 4. The protective agent again elutes as 2 peaks, although the number of fractions separating them is reduced to 2. The DMF for the radioprotective peaks is 2.1.

The increase in net protection in the peak is probably due to the

Figure 4. The bioassay of the elution profile of organically partitioned extract from M. radiodurans eluted through a 2.5 X 100 cm sephadex G-25 column at 8 - 10 ml/hr and collected in 320 drop fractions. Each point represents the average viable cell count of duplicate plates for individual fractions exposed for 17.6 minutes to x-irradiation.


400
300
200
100
VIABLE CELLS
I
II
1
6
11
16
21
26
FRACTION NUMBER

increased concentration of the agent in a given sample. Whereas a 0.5 ml aliquot of crude extract was resuspended to 5 ml/fraction in the high resolution technique, 2 ml of crude extract is concentrated to 1 ml fractions in the high yield separation. Bruce (1964) had shown that the degree of protection afforded by crude Micrococcus radiodurans extract is highly concentration dependent. While storage of the extract at $4^{o}C$ resulted in reproducible profiles of radioprotection, freezing and/or thawing caused large variations in the protective ability of peak I. only under conditions where the fractions were quick-frozen and thawed at room temperature and then refrozen between experiments could the presence of both protective peaks be demonstrated reproducibly.

The homogeneity of peak II was determined by pooling the fractions comprising it, concentrating them to 0.5 ml and rechromatographing on the 2.5 x 100 cm column. The samples were concentrated to 0.5 ml fractions and were bioassayed as before. The resulting profile is shown in Fig. 5.

Resolution of peak II into two radioprotective peaks with the same elution characteristics as before can be interpreted in several ways:

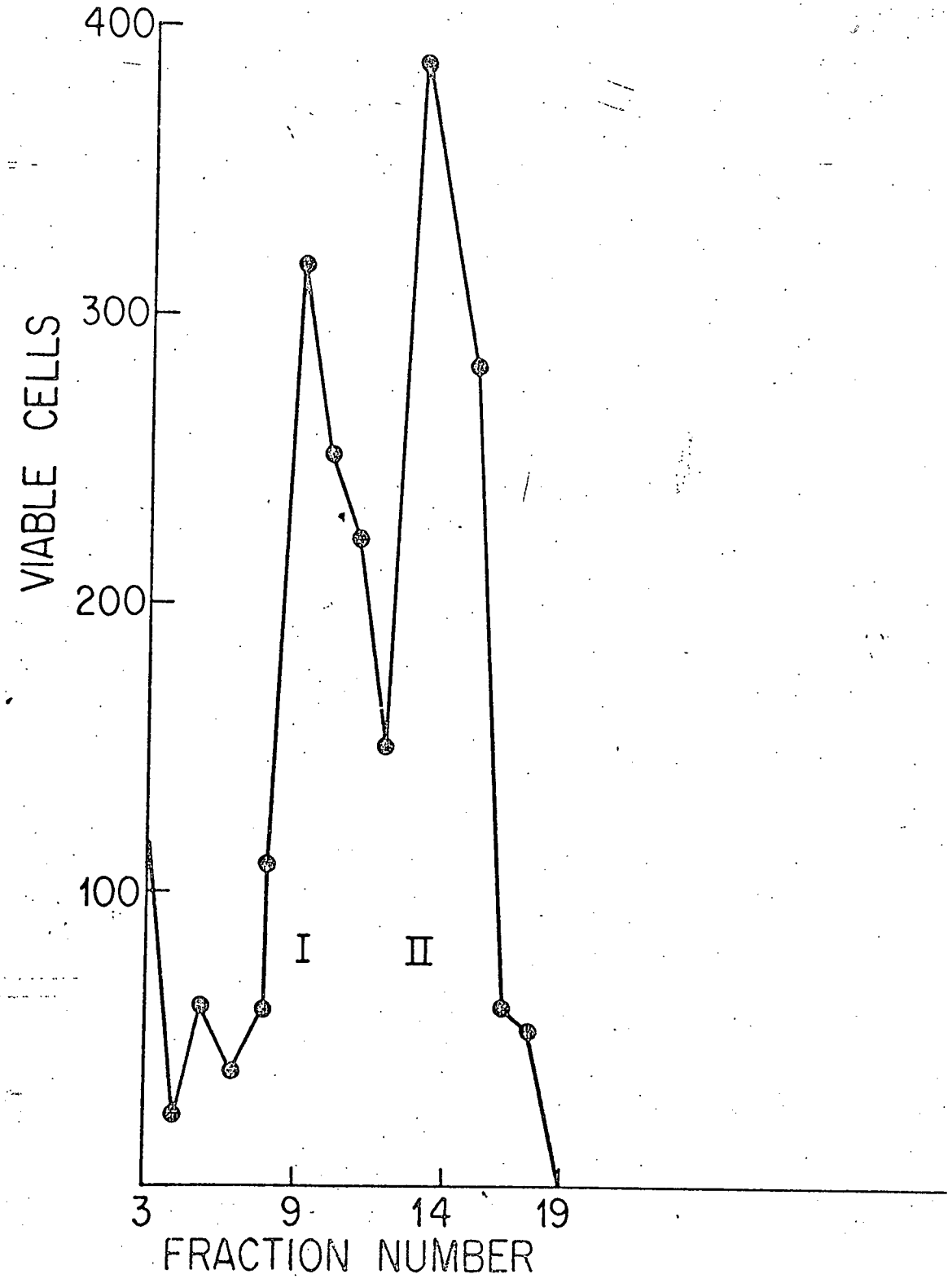
1. The original separation failed to resolve two distinct molecular species whose molecular weight and binding characteristics caused them to elute in the same fractions.

2. Molecules in peak II undergo some form of condensation to form an active radioprotector with a higher molecular weight.

3. The radioprotective agent is present in two forms, each of which has different chromatographic properties.

The first of these can be dismissed since the column was able to give good resolution of known marker materials used in calibration.

Figure 5. The bioassay of the elution profile of peak II after it was pooled, concentrated to 0.5 ml, chromatographed on a 2.5 X 100 cm sephadex G-25 column and collected in 320 drop fractions. Each point is the average viable cell count for duplicate platings of individual fractions exposed for 17.6 minuts to x-irradiation.


400
300
200
100
VIABLE CELLS
I
II
3
9
14
19
FRACTION NUMBER

Additionally if peak II were made up of separate molecular species with similar elution characteristics, rechromatogramming should again fail to resolve them. Clearly this is not the case.

In cases 2 and 3, the radioprotective extract could be thought to exist in the form $Ex \rightleftharpoons Ex^*$ where Ex^* is either a stearic conformation of extract or is the condensation product of extract with itself or some ligand.

Porath (1961) has shown that the behavior of a solute in a gel formation can be described by the distribution coefficient $K_d = \frac{V_e - V_o}{V_i}$ where V_e equals the elution volume of the solute, V_o equals the void volume and V_i equals the volume of the stationary phase. If K_d is greater than unity, thre is adsorption of the solute to the metrix; if K_d equals 0 (that is the solute elutes with the void volume), there is total exclusion.

The value for V_i is easily calculated for the 2.5 x 100 cm column since Vi = aWr, where a = weight of gel in grams and Wr is the water regain in ml/gram. Substituting known values Vi = 100 x 2.5 = 250. Using this value, Kd can be calculated for both peaks and is:

$$\text{Peak I} = \frac{140}{250} = .56$$

$$\text{Peak II} = \frac{410 - 170}{250} = .96$$

Since the distribution coefficient of the radioprotective peak II is so high, the molecule is either very low molecular weight with no ring subgroups (e.g. NaCl) or it adsorbs to the G-25 gel. The compound elutes in the void volume (Kd = 1) of columns prepared using G-10 fine grade sephadex which has a retention coefficient of 1,000 daltons. It seems unlikely therefore that the compound is very low molecular weight.

The lower distribution coefficient (0.56) might represent the equilibrium reached when the molecular weight of the compound is just retained by the exclusion volume of the gel (Kd = 1) and the adsorption of ring groups (Kd = V_t) to the gel (Gelb, personal communication).

The elution profile of peak II was compared to that of cyanocobalamine (Vitamin B_{12}, Sigma Chem. Co.) which is known to bind chemically to sepahdex G-25. It resulted in an elution profile similar to that obtained for the extract. The molecular weight might therefore be as high as 1350 daltons and result in a profile which would give a much lower molecular weight estimate if chemical binding to the gel were not considered.

All subsequent bioassays exhibited the characteristic bimodal pattern of protection. Since the presence of the first peak appeared to be a function of the molecular state of the second, it was the latter that was used in all subsequent experiments. For these, the second peak was pooled and reduced to 2 ml by flash evaporation and lyophilized. The resulting powder was resuspended to 800 ul in distilled water and frozen in a bath of dry ice and ethanol. Any precipitate that remained after resuspension was removed by millipore filtration. All the protective capacity was shown to reside in the soluble fraction.

Enzyme assay.

Characterization of the radioprotective agent was attempted using enzyme digestion. As shown in Table I, the activity of the extract is not significantly effected by DNase, RNase, Pronase or Lipase. It seems likely that the agent is not an oligonucleotide. It is retained by sephadex G-25 and therefore the molecular weight must be less than 5,000. If it has a molecular shape and binding characteristics simila

Table I

Enzyme Analysis of Extract

Enzyme	K	% S in Extract alone "A"	% S in Extract + Enz. "B"	$\frac{B}{A} K \times 100$
DNase	1.25	6.4	4.4	84.4%
RNase	1.21	5.6	5.0	109%
Pronase	1.46	5.9	4.2	105%
Lipase	1.02	4.5	4.6	102%

$$K = \frac{\%\ S \text{ in buffer}}{\%\ S \text{ in buffer + enzyme}}$$

to Vitamin B_{12}, it could be approximately 1300 daltons. This implies an oligonucleotide of only 3-4 bases and there is no evidence that such molecules are radioprotective. Similarly, if it were a polypeptide, it would be comprised of only 10 amino acids and radioprotection by such compounds (e.g. oxytocin, Bacq, 1965) is reported only in whole body mammalian studies. The organic purification procedure should serve to separate lipids from the crude extract and the result obtained with lipase seems to verify this.

Enzyme analysis demonstrates that the molecule of interest is of low molecular weight and therefore cannot be a specific protein or polynucleotide.

B. Dose Modification in E. coli B/r

Survival curves for E. coli B/r (CSH) under the conditions previously described are shown in Figs. 6 - 9. The dose modification for the various conditions are summarized in Table II. The dose modification factor was calculated at 1% S. In all cases, protection was reflected by a change in slope rather than by the formation of a shoulder. In this figure N = nitrogen, O = oxygen, A = agitated, Q = quiescent, B = Buffer and E = extract.

Nitrogen exerts its largest effect in samples which are agitated before and during irradiation. Quiescence of buffer samples reduces the net dose modification by simultaneously increasing resistance in aerobic and decreasing it in anaerobic conditions. The net effect is a reduction in nitrogen dose modification. The complete reduction of the nitrogen effect when comparing ENQ (Type IV)/EOQ (Type III) is due almost entirely to the increased resistance of cells in unbubbled extract in the presence of oxygen. Quiescence has no effect on cells irradiated in

Figure 6. Survival curves for E. coli B/r irradiated under conditions of aerobiosis and agitation. The circles (○) are cells irradiated in buffer; the triangles (△) are cells irradiated in extract. Each point is the average of at least 8 plates from at least 2 experiments.

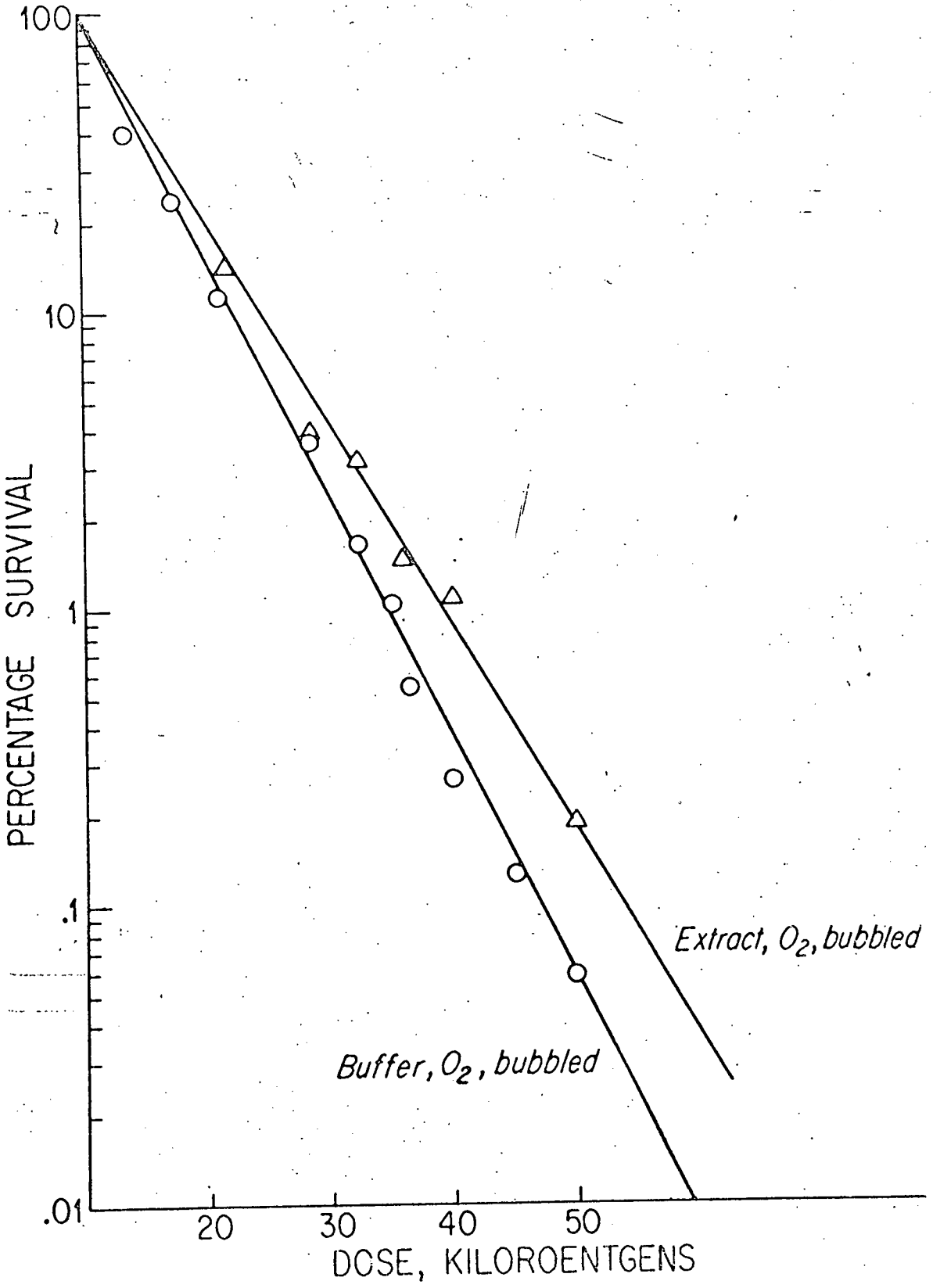

100
10
1
.1
.01
PERCENTAGE SURVIVAL
20
30
40
50
DOSE, KILOROENTGENS
Extract, O_2, bubbled
Buffer, O_2, bubbled

Figure 7. Survival curves for E. coli B/r irradiated under conditions of anaerobiosis and quiescence. The circles (O) are cells irradiated in buffer; the triangles (△) are cells irradiated in extract. Each point is the average of at least 8 plates from at least 2 experiments.

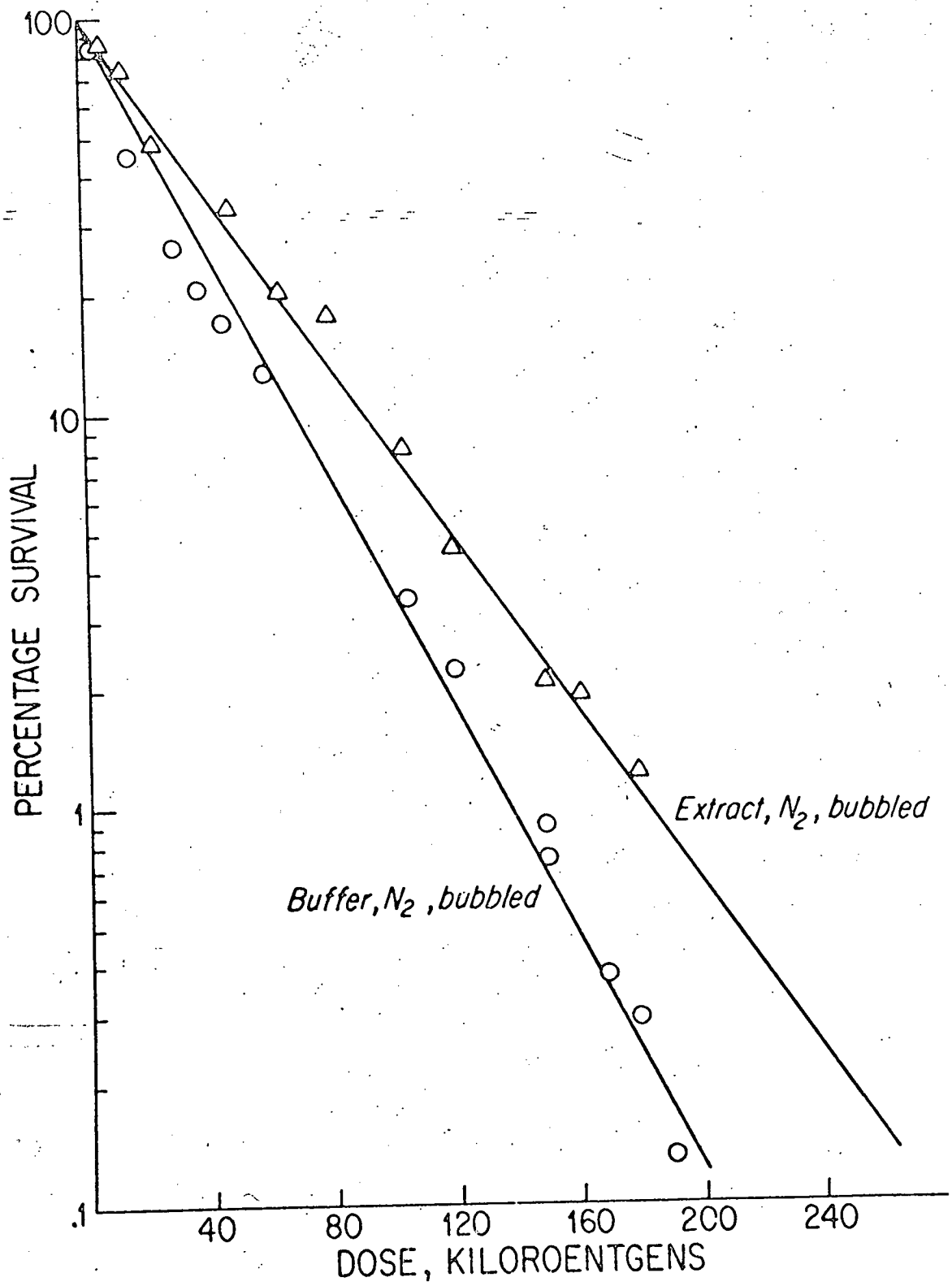

100
10
1
.1
PERCENTAGE SURVIVAL
40
80
120
160
200
240
DOSE, KILOROENTGENS
Extract, N_2, bubbled
Buffer, N_2, bubbled

Figure 8. Survival curves for E. coli B/r irradiated under conditions of anaerobiosis and quiescence. The circles (O) are cells irradiated in buffer; the triangels (△) are cells irradiated in extract. Each point is the average of at least 8 plates from at least 2 experiments.

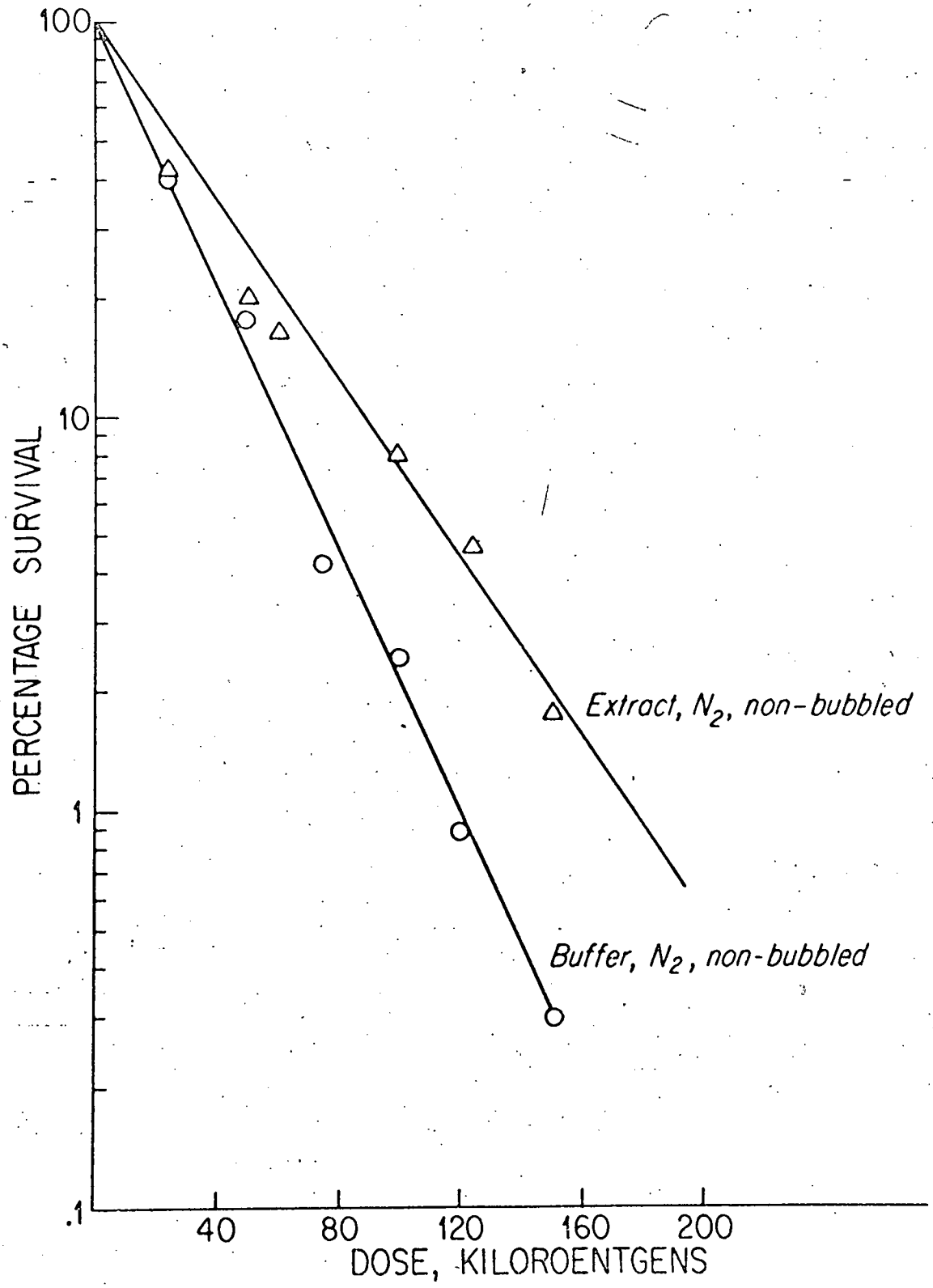

100
10
1
.1
PERCENTAGE SURVIVAL
40
80
120
160
200
DOSE, KILOROENTGENS
Extract, N_2, non-bubbled
Buffer, N_2, non-bubbled

Figure 9. Survival curves for E. coli B/r under conditions of aerobiosis and quiescence. The circles (O) are cells irradiated in buffer; the triangles (△) are cells irradiated in extract. Each point is the average of at least 8 plates from at least 2 experiments.

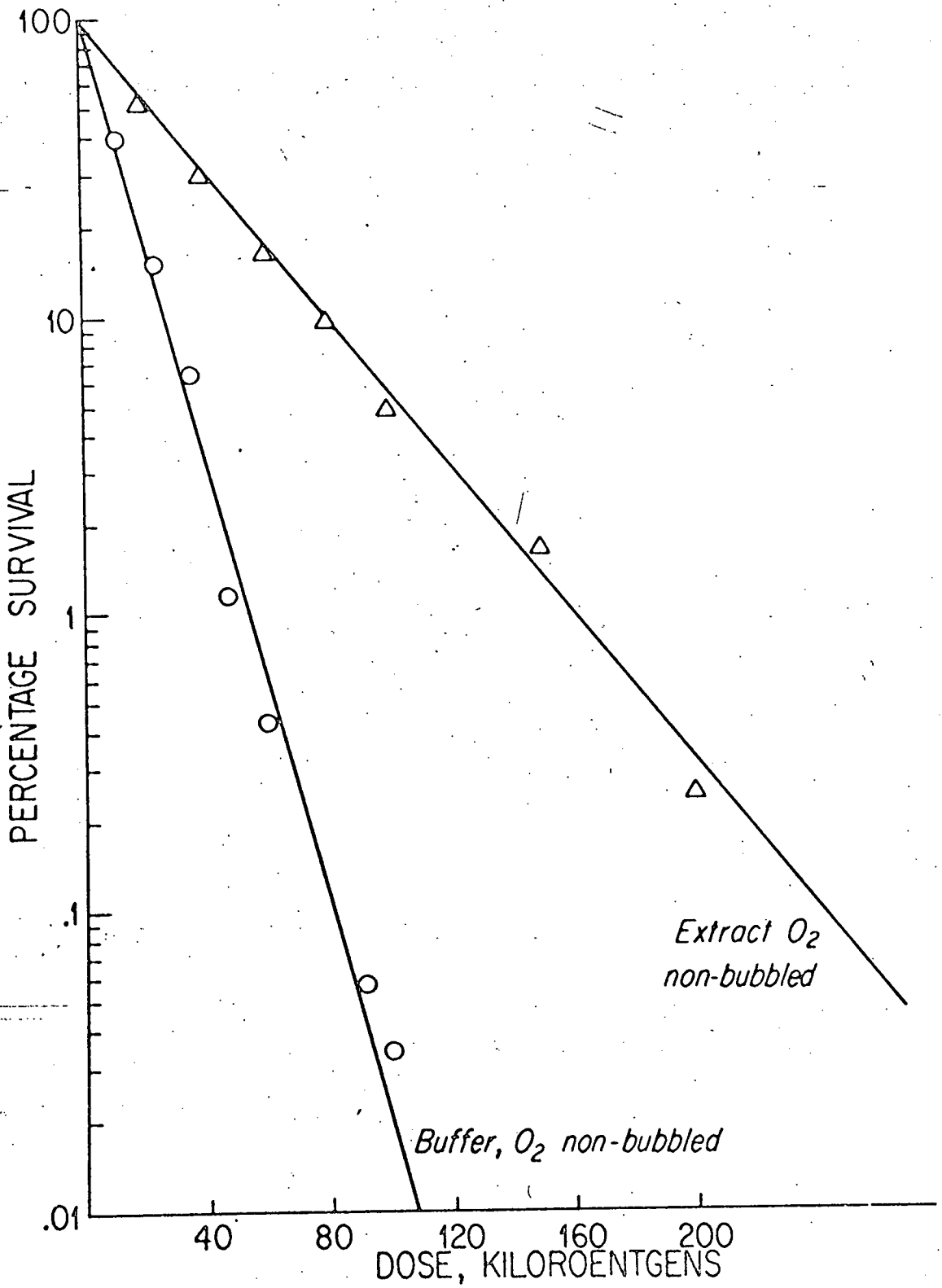

100
10
1
.1
.01
PERCENTAGE SURVIVAL
40
80
120
160
200
DOSE, KILOROENTGENS
Extract O_2
non-bubbled
Buffer, O_2 non-bubbled

Figure 10. Survival curves for E. coli B/r irradiated in buffer and then incubated for 5 or 15 minutes in buffer (O) or extract (△) before plating. Each point is the average of at least 8 plates from at least 2 experiments.

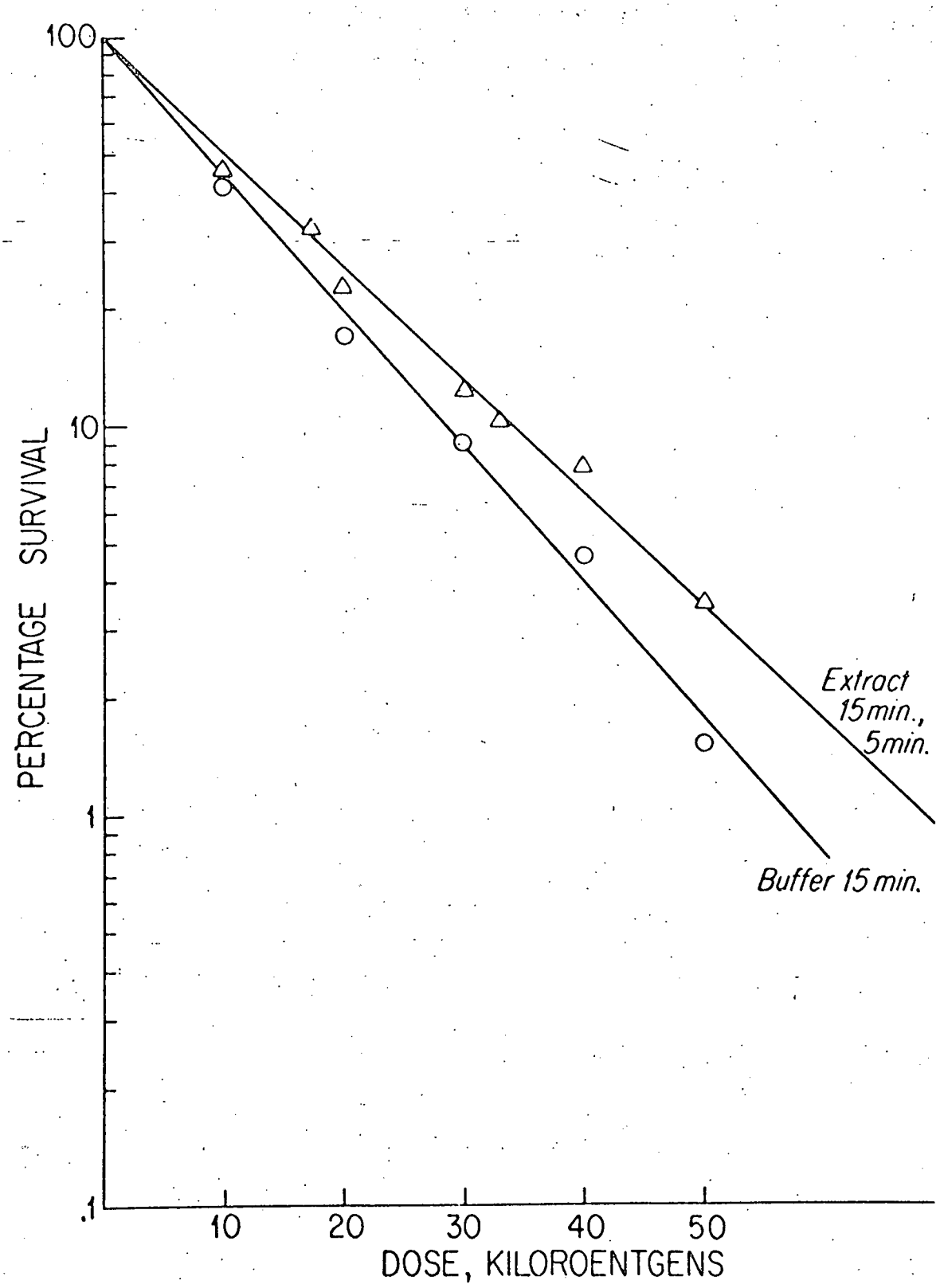

100
10
1
.1
PERCENTAGE SURVIVAL
10
20
30
40
50
DOSE, KILOROENTGENS
Extract
15 min.,
5 min.
Buffer 15 min.

Table II

Dose Modification in E. coli B/r For Nitrogen, Quiescence, and Extract

Dose Modifying Agent	Calculation Used	D.M.F.
Nitrogen	B N A / B O A	2.8
	E N A / E O A	3.1
	B N Q / B O Q	2.2
	E N Q / E O Q	1.1
Quiescence	B N Q / B N A	1.1
	E N Q / E N A	0.9
	B O Q / B O A	0.9
	E O Q / E O A	2.8
Extract	E N A / B N A	1.3
	E N Q / B N Q	1.4
	E O A / B O A	1.2
	E O Q / B O Q	3.3

E = Extract
B = Buffer
O = Oxygen
N = Nitrogen
A = Agitated (bubbled)
Q = Quiescent

buffer. Only in the presence of extract under Type III (EOQ) conditions does it increase survival. Radioprotection by extract seems to have two distinct levels of activity. The first is low level (DMF = 1.2 - 1.5) and is independent of both aerobiosis and agitation. The more pronounced effect is under conditions of aerobiosis and quiescence. A shift from aerobiosis to anaerobiosis to give a dose modification analogous to nitrogen in the absence of exogenous oxygen cannot be ruled out, and will be discussed in length later. The conditions for maximal radioprotection described here are similar to those for 2-mercaptoethylamine (Elias, 1960).

Parameters of radioprotection in E. coli B/r.

Preincubation of cells in extract prior to irradiation in buffer afforded no additional radioprotection. This is shown in the table below:

Condition	Dose, kR	% S	PQ	DMF
Incubation in ext.	540	2.28 $\pm$ 1.5	1.16 $\pm$ 0.25	1.08
Incubation in buff.	540	2.11 $\pm$ 1.3	-	1.00

Post incubation of cells in extract.

Irradiation of cells in buffer followed by incubation in extract for either 5 or 15 minutes post irradiation resulted in increased radioresistance. The survival curve for such a treatment is shown in Fig. 10. Included for reference is the figure is the survival curve of cells irradiated in buffer with no post incubation. Incubation in buffer alone increases resistance by a DMF of 1.2, while incubation in extract results in an additional DMF of 1.2. Known radioprotectors do not enhance survival unless they are present during irradiation. The increased

survival when compared to non-incubated buffer controls may be analogous to the restoration processes reviewed by Latarjet (1954).

Effect of extract concentration.

Cells irradiated to a given dose in different concentrations of extract have widely differing survival as shown in Fig. 11. The dependence of protection on extract concentration has been demonstrated by Bruce (1964) in extract whose source was wild type Micrococcus radiodurans. Here high concentrations protected most efficiently and low extract concentration actually sensitized the cells to a slight degree. In this study survival in extract was never less than buffer controls. Again it can be seen that maximal protection is afforded under aerobic, quiescent conditions.

Effect of initial cell concentration.

The effect of initial cell concentration (N_o) on survival after a given dose is shown in Fig. 12. The Pq for N_o between 5×10^5 and 5×10^7 remains constant at 2.4 and corresponds to a dose modification factor of 1.3 under these conditions. This falls within the 1.2 - 1.5 range noted for survival under Types I, II, IV conditions. The DMF at a concentration of 2×10^9 is approximately 2.0, well below the optimal DMF of 3.26 noted in the survival curves. This and the extract concentration curve show that radioprotection by extract is not solely a function of the specific activity of extract on a per-cell basis and may reflect the presence of an artifact introduced when the original washed cells were concentrated 10-fold before dilution in extract or buffer.

Protection of E. coli B/r in the presence of extract therefore

Eigure 11. Effect of extract concentration on dose modification in E. coli B/r irradiated to 50 kR. The ordinate is the relative extract concentration; the abscissa is the ratio of survival in extract to that in buffer irradiated under the same conditions. (□) Nitrogen and agitation ; (○) air with agitation; (△) air and quiescence. Each point represents the average of 8 plates from 2 experiments.

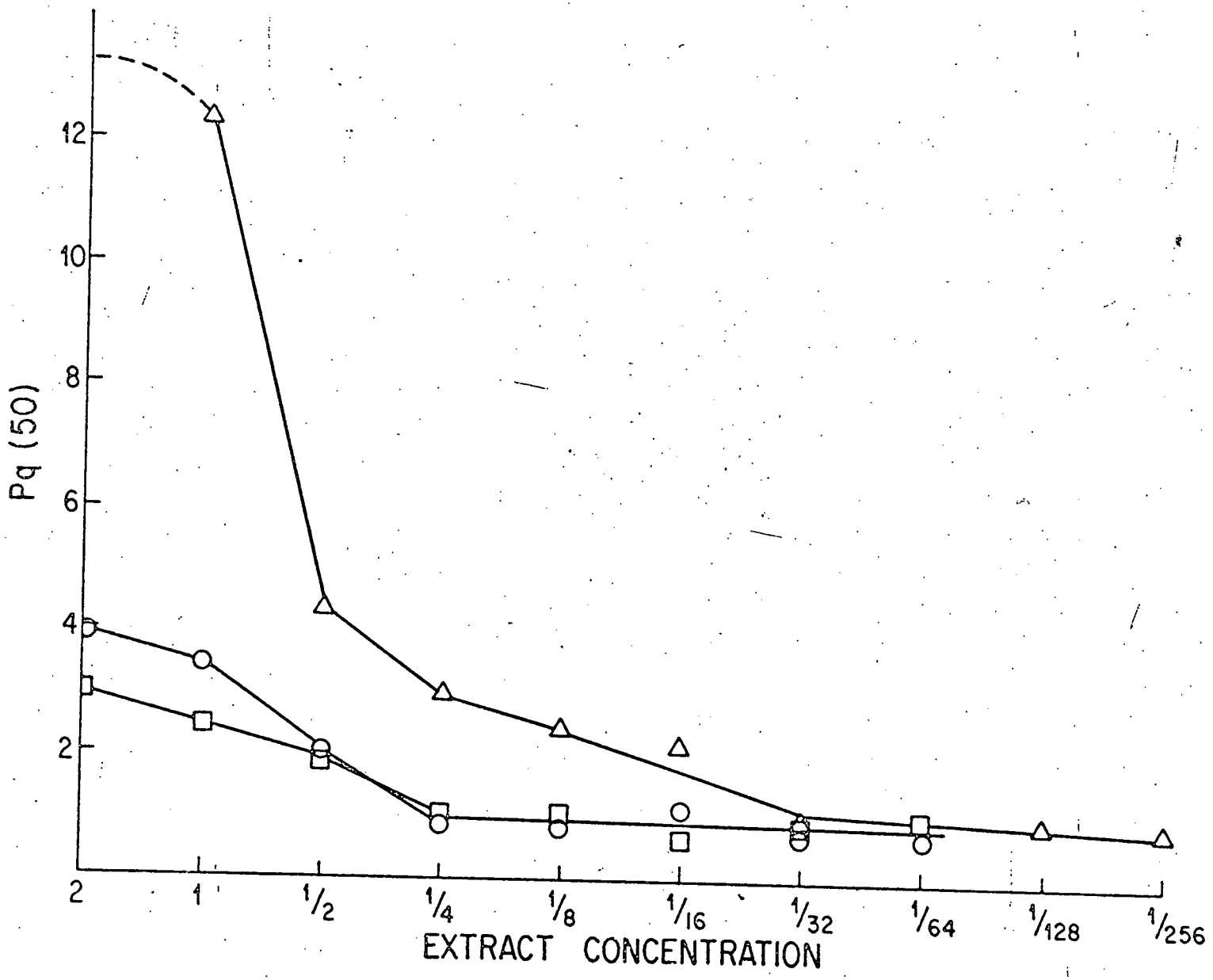
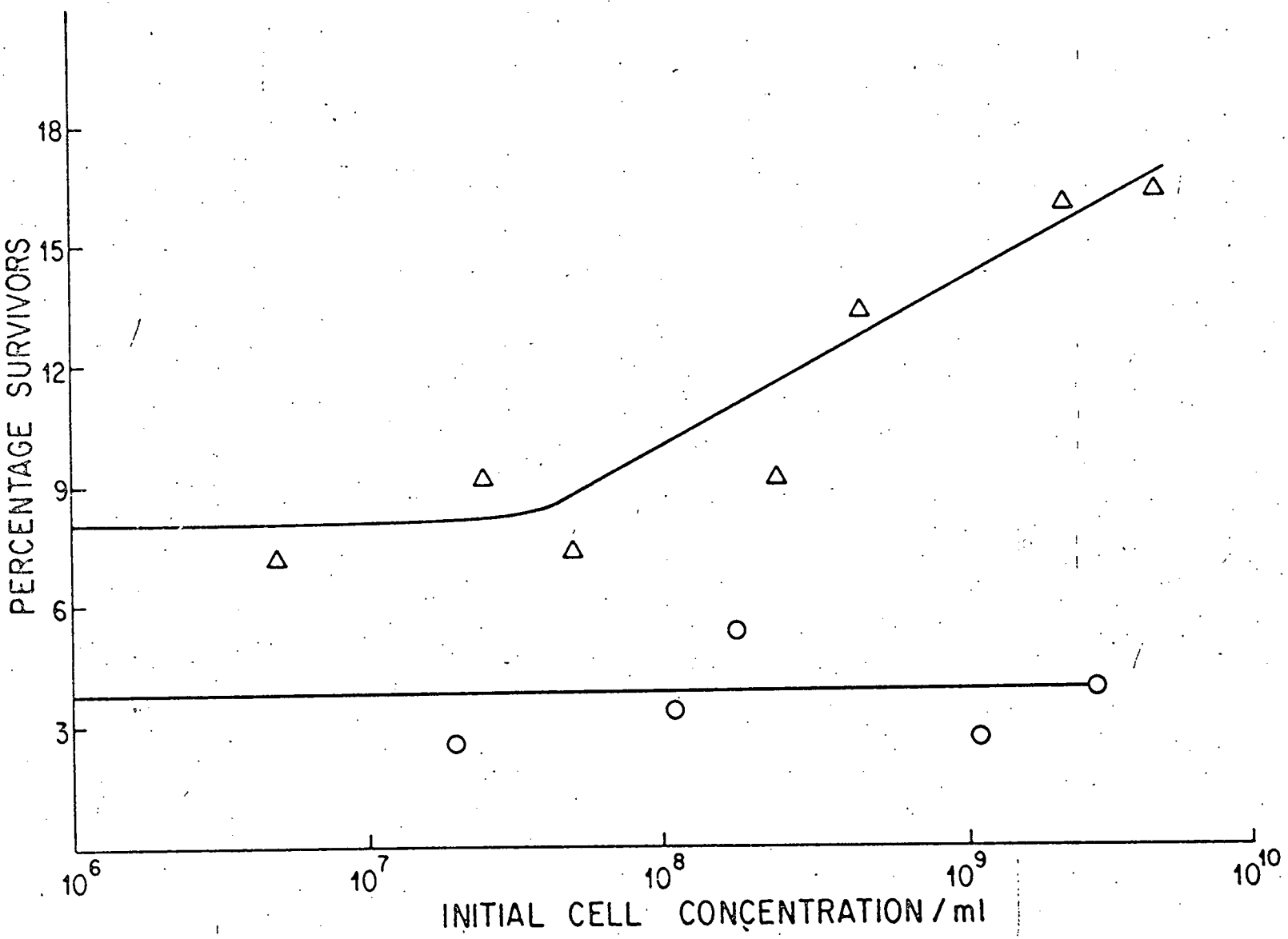

Pq (50)
12
10
8
6
4
2
2
1
1/2
1/4
1/8
1/16
1/32
1/64
1/128
1/256
EXTRACT CONCENTRATION

Figure 12. The effect of initial cell concentration on survival after exposure to 50 kR of x-rays. The triangles (△) represent the percentage survival in extract under conditions of aerobiosis and quiescence; the circles (O) are buffer controls treated in the same way. Each point is the average of at least 8 plates from at least 2 experiments.


PERCENTAGE SURVIVORS
18
15
12
9
6
3
10^6
10^7
10^8
10^9
10^{10}
INITIAL CELL CONCENTRATION / ml

depends on the following conditions:

1. The extract must be presend during the irradiation
2. The extract must be as concentrated as possible
3. There must be at least 5×10^8 cells present initially
4. Irradiation must be carried out in the absence of exogenous O_2 and/or agitation.

Other experiements with E. coli B/r.

Toxicity of extract.

The unusual cell concentration curve might be interpreted to mean that a minimum number of cells are required to overcome (or at least invalidate) lethality from toxic radiolysis of the extract. That this is not the case is shown in Fig. 13. It can be readily seen that no stable toxic products are produced in extract even at doses well beyond those of biological interest for studies with E. coli.

Effect of 2-mercaptoethylamine.

Survival curves were generated for cells in 2-mercaptoethylamine both alone and in the presence of extract (Fig. 14). Included for reference are survival curves for cells in buffer alone and extract alone. It can be seen that the presence of extract has no effect on dose modification by MEA, but the presence of MEA decreases radioprotection by extract. It seems likely that both compete for the same active site within the cell, and that 2-mercaptoethylamine is preferentially bound. It is not clear whether both act by an analogous mechanism, although the conditions for optimal protection are similar. The dose modification factor for radioprotection by MEA is consistant with that reported by Serianni and Bruce (1968).

Figure 13. Toxicity of irradiated extract. The ordinate is the dosages to which the extract was exposed; the abscissa is the ratio of viable cells of an aliquot of cells diluted into extract and buffer. Each point is the average of the ratio of 8 plates from 2 experiments.

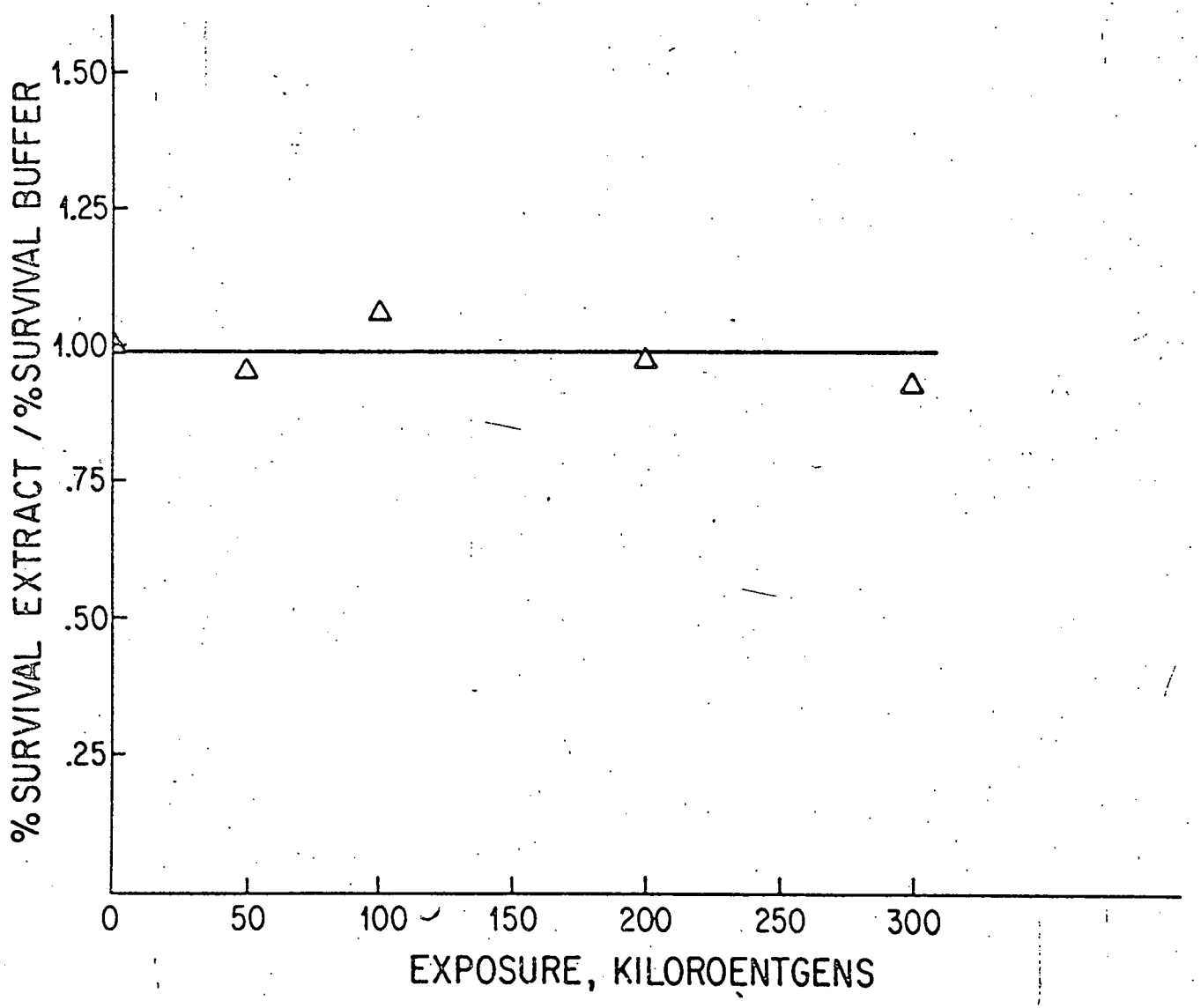
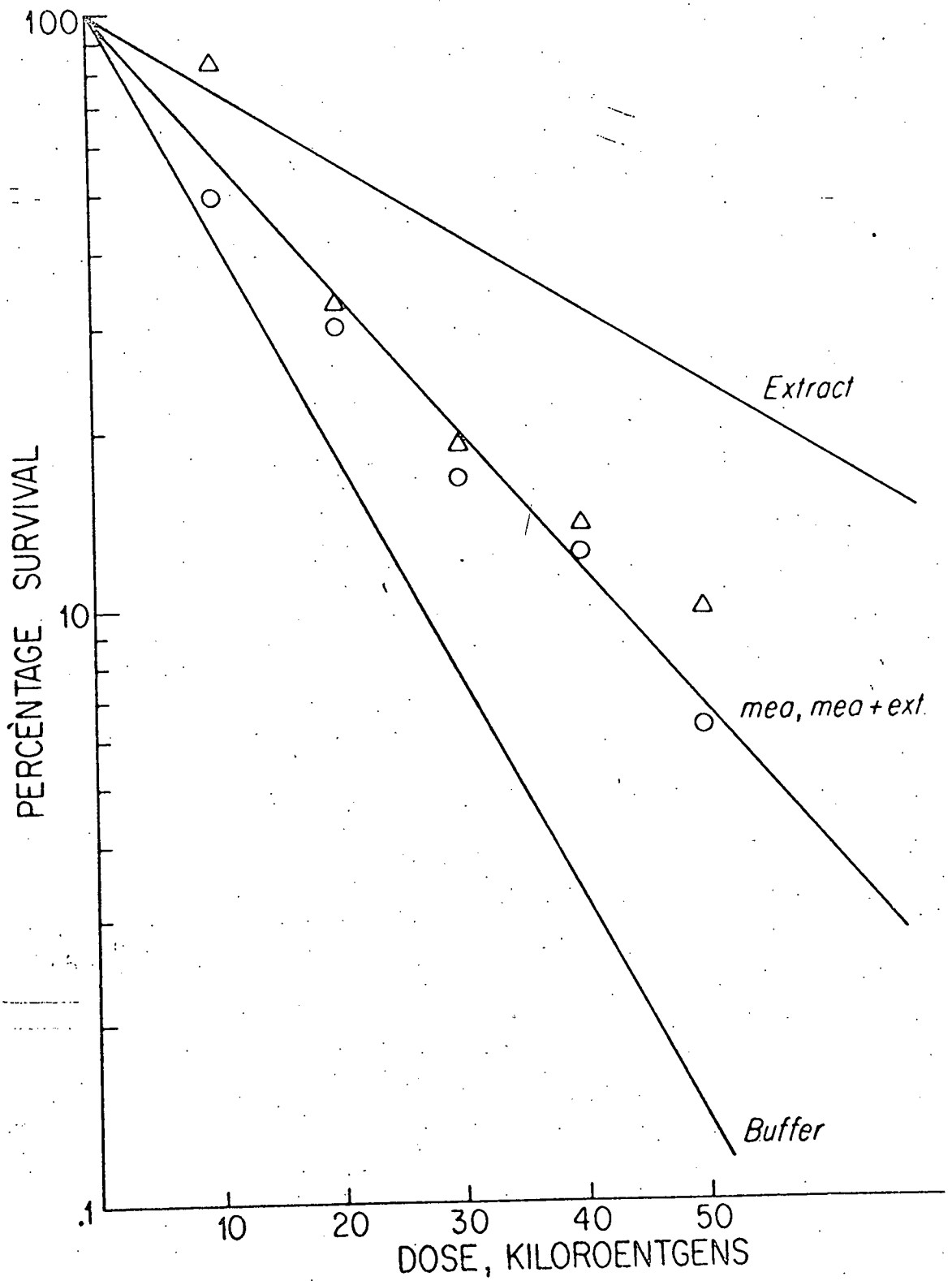

% SURVIVAL EXTRACT / % SURVIVAL BUFFER
1.50
1.25
1.00
.75
.50
.25
0
50
100
150
200
250
300
EXPOSURE, KILOROENTGENS

Figure 14. Effect of extract on dose modification by 2-mercaptoethylamine. E. coli B/r were irradiated in 0.044 M MEA in both the presence (O) and absence (△) of extract. Each point is the average of at least 8 plates from at least 2 experiments. All samples were irradiated under conditions of aerobiosis and quiescence. Survival curves for extract alone and buffer controls irradiated under the same conditions (Fig. 6) are included for reference.


100
10
.1
PERCENTAGE SURVIVAL
10
20
30
40
50
DOSE, KILOROENTGENS
Extract
meo, meo + ext.
Buffer

Microscopy.

Microscopic examination of E. coli B/r in the presence of extract showed that the cells tended to clump tegether. The aggregates are fragile, since dilution and mixing of a clumped fraction as performed routinely for the unirradiated control plates gave approximately the same viable count as buffer controls. Analysis of bubbled samples showed that there were fewer clumps, and fewer cells per clump.

Aggregation alone is not responsible for protection since cells clumped in extract made anaerobic and left quiescent for 15 minutes show only slightly increased resistance when compared to buffer controls. The optimal effect requires both O_2 and aggregation for expression in the absence of other data, no clear picture of the cause of the effect emerges. Interpretations must therefore remain speculative.

Electron micrographs of cells incubated in buffer are shown on plate 1 for magnifications of 12,000 X, 36,000 X and 129,000X. Plate 2 shows cells incubated in extract and viewed at the respective magnifications. The extract causes distinct cytological changes. The DNA (white, electron sparse areas) appears condensed and relatively isolated from the cytoplasm. The periplasmic space is reduced in the presence of extract, and the ribosomes (electron dense particles) appear clustered. No evidence of cytoplasmic bridging or pili could be found.

The histochemical events might be the result of cytoplasmic migration to the periphery of the cell. It is unlikely that it is the result of the fixation and staining procedures.

C. Effect of Extract at the Gene Level

Witkin (1967) ascribes the radioresistance of the E. coli B series to three genes: 1) Hcr denotes the presence of a repair system

Plate I. Electron micrographs of cells incubated in buffer.

1. 12,000 X
2. 36,000 X
3. 129,000 X

P = periplasmic space
D = DNA
R = ribosomes

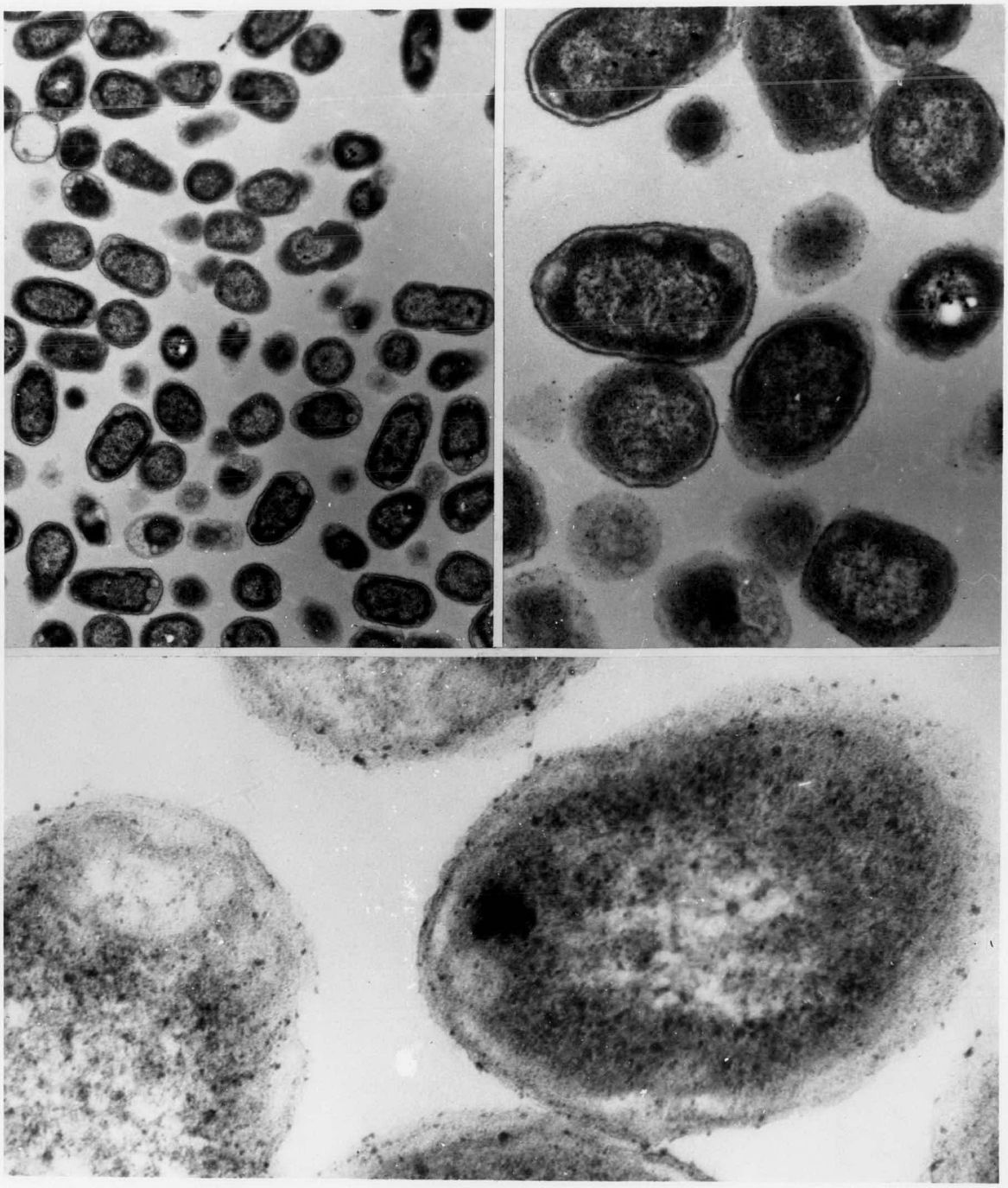
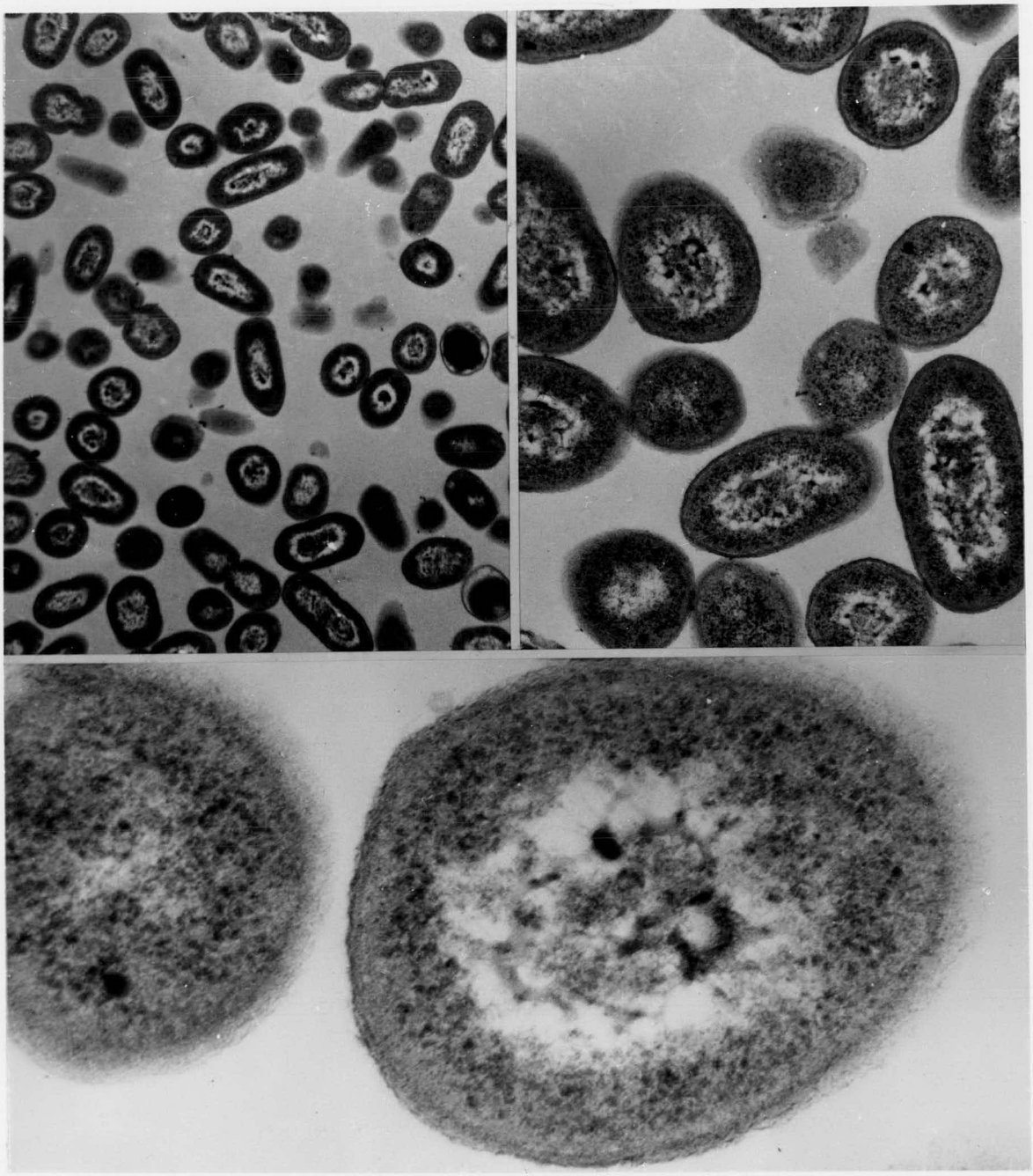


Plate II. Electron micrographs of cells incubated in extract.

1. 12,000 X
2. 36,000 X
3. 129,000 X

P = periplasmic space
D = DNA
R = ribosomes



and governs reactions concerning dimer recognition (UV), dimer excision (UV), nuclease, polymerase, and ligase activity. The last three processes are thought to occur for damage caused by UV, ionizing radiation and mutagens (Alkylating agents). 2) Exr is thought to act by filling gaps in the DNA formed by the action of nucleases in a relatively non specific (mutation prone) manner. 3) Fil^- denotes the inability of the cell to form large filaments after irradiation caused by a malfunction of septal formation (Adler and Hardigree, 1965).

The strains investigated, when arranged in order of increasing radioresistance, differ from one another at the exr, hcr and fil loci respectively as shown in Table III. Strain B/r (ORNL) is thought to be isogenic to B/r (CSH, Adler and Engel, 1961) and was included as a verification of extract effects in the latter.

The data are summarized in Table III. The DMF's were calculated at 1% S according to the following formulae:

$$\text{DMF } N_2 = \frac{LD_{99}\ \text{BNA}}{LD_{99}\ \text{BOA}}$$

$$\text{DMF EOA} = \frac{LD_{99}\ \text{EOA}}{LD_{99}\ \text{BOA}}$$

$$\text{DMF ENA} = \frac{LD_{99}\ \text{ENA}}{LD_{99}\ \text{BNA}}$$

$$\text{DMF EOQ} = \frac{LD_{99}\ \text{EOQ}}{LD_{99}\ \text{BOA}}$$

where E = extract, B = buffer, N = nitrogen, O = oxygen, A = agitated and Q = quiescent.

Complete survival curbes were generated for all strains for all conditions listed. In all cases, as previously, radioprotection was reflected by a change in the slope of the curve, and not through the

TABLE III

Effect of Extract on LD_{99} and DMF of Several Strains

E. coli Under Various Irradiation Conditions

Strain	Bs-1	BIII-10	B	B/r CSH	B/r ORNL
Genotype	exr^+hcr^- fil^+	exr^+hcr^- fil^+	exr^+hcr^+ fil^+	exr^+ hcr^+ fil^-	exr^+ hcr^+ fil^-
(a) B O A $LD_{(99)}$, kR	10.8	18.8	19.6	49	57.5
DMF	1.0	1.0	1.0	1.0	1.0
(b) B N A $LD_{(99)}$, kR	15	16.8	32.8	138	135
DMF	1.4	0.9	1.7	2.8	2.7
(c) E O A $LD_{(99)}$, kR	12	24.6	24.8	58	57.5
DMF	1.1	1.3	1.3	1.2	1.0
(d) E N A $LD_{(99)}$, kR	16.5	18.8	40.8	184	170
DMF	1.1	1.1	1.2	1.3	1.3
(e) E O Q $LD_{(99)}$, kR	13.2	21.8	26.4	160	186
DMF	1.2	1.2	1.3	3.3	3.2

a) Cells irradiated in buffer with continuous air bubbling
b) Cells irradiated in buffer with continuous nitrogen bubbling
c) Cells irradiated in extract with continuous air bubbling
d) Cells irradiated in extract with continuous nitrogen bubbling
e) Cells irradiated in extract without any bubbling

generation of a shoulder.

The strains can be divided in sub-sets based on their response to nitrogen (Alper, 1962). Analysis of the survival data will be in terms of those cells having a low oxygen enhancement ratio (OER), which by definition show little enhancement of survival when nitrogen is substituted for oxygen. Those with a high OER, E. coli Bs-1, B III-10, and B below to the former and the two B/r substrains to the latter.

Radioresponse in strains with a low OER.

All three strains exhibit qualitatively similar responses to protection (Types I and II) by extract. The dose modification for extract under conditions of agitation with nitrogen or oxygen or under quiescent anaerobiotic (Type IV) conditions is comparable and of a low order of magnitude, ranging from 1.1 to 1.35. There is no difference if the cells are aerobic or anaerobic and the extract effect is not manifested in quiescent oxygenated (Type III) conditions as it is in the B/r strains. The magnitude of the response is similar to conditions noted in earlier E. coli B/r (CSH) where high dose modification could not be demonstrated when continuous gaseous bubbling was used. Strain B III -10 is unusual since it exhibits no oxygen enhancement ratio. This is similar to results obtained in Chlamydomonas by Davies (1967).

Radioresistance in strains with a high OER.

The existence of two levels of the radioprotective effect of extract is E. coli B/r (CSH) was described earlier and the response is quite similar in the ORNL strain. The ORNL strain is more radioresistant under aerobic conditions when compared to CSH as was noted by Adler and Engel (1961), and does not exhibit any increased radioresistance in

the presence of extract when the sample is bubbled with O_2. Both strains demonstrate high order DMF's with extract under Type III irradiations only.

C. Effect at The Gene Level

The dose modification brought about by the inclusion of an active structural gene can be determined by comparing radioresistance between substrains under a given condition. The effect of exr can be measured by comparing the LD_{99} of a strain without the gene (Bs-1) to the LD_{99} of that strain with the gene (B III-10). Similarly, strains B III-10 and B are isogenic except at the hcr locus, and B and B/r differ only at the fil locus. Such a comparison for several irradiation conditions is summarized in Table IV.

The contribution of both exr and hcr depend on the state of aerobiosis since exr exerts its influence only when O_2 is present, while hcr acts when O_2 is absent. The fil gene is active under both, but is more pronounced during anaerobiosis.

The protection conferred by the extract on the genes under conditions where they are expressed is given by the following formulae and values derived in Table III.

$$\text{exr} = \frac{\text{DMF EOA}}{\text{DMF BOA}} = \frac{2.05}{1.74} = 1.2$$

$$\text{hcr} = \frac{\text{DMF ENA}}{\text{DMF BNA}} = \frac{2.2}{1.95} = 1.1$$

$$\text{fil} = \frac{\text{DMF EOQ}}{\text{DMF BOQ}} = \frac{6.0}{2.5} = 2.4$$

It is obvious that almost all of the enhanced viability is due to the action of the extract on the fil gene. The magnitude of the response for either hcr or exr is similar to the added resistance conferred in Bs-1 where both genes are absent.

TABLE IV

Dose Modification at the Gene Level

Gene	B O A	B N A	E O A	E N A	E O Q
exr (1)	1.74	1.1	2.05	1.1	1.6
hcr (2)	1.04	1.95	1.00	2.2	1.2
fil (3)	2.5	4.2	2.34	4.5	6.0

B = Buffer

E = Extract

N = Nitrogen

O = Oxygen

A = Agitated

Q = Quiescent

(1) Dose modification for exr is calculated by comparing the LD_{99} of *E. coli* Bs-1 to that of *E. coli* B III-10 under the same conditions.

(2) Dose modification for hcr is calculated by comparing the LD_{99} of *E. coli* B III-10 to that of *E. coli* B under the same conditions.

(3) Dose modification for fil is calculated by comparing the LD_{99} of *E. coli* B to that of *E. coli* B/r (CSH) under the same conditions.

It is supposed therefore that the protection afforded at the hcr and exr genes is not significantly different from that conferred to the rest of the genome or other possible targets within the cell. The product of this "non specified" protection and the dose modification at the fil^- gene (or processes it controls) is 1.2 (average) x 2.4 = 2.9 and corresponds closely to the net protection observed in fil^- strains.

The data do not exclude the possibility that the radioprotection afforded by the extract is caused by exr^+, hcr^+ or both working in concert with fil^-. The mutant strains of B/r needed to test this, namely exr^-, hcr^+, fil^- and/or exr^+, hcr^-, fil^- were not available.

D. Macromolecular Effects at the DNA Level

Alkaline sucrose density gradient centrifugation was used to quantitate degradation and restitution of DNA post irradiation. The technique permits the isolation of high molecular weight DNA which has not been degraded by hydrodynamic shear. Maintaining the gradient at pH 13 causes separation of the DNA into single strands and changes in the molecular weight reflect either the formation or repair of single strand breaks. The computer analysis of sedimentation profiles is summarized in Table V. All incubations post irradiation were carried out in the irradiation chamber.

Ginsberg and Webster (1970) were unable to demonstrate any correlation between radioprotection by MEA and the sedimentation profiles of DNA on alkaline sucrose gradients. Their observation would seem to hold for protection by extract. Case A is the only instance where radioprotection when assayed by survival is demonstrated. Yet here there is considerably more single strand breaks and/or degradation. Incubation in growth medium (C) partially overcomes this, but here degradation is

Table V

Alkaline Sucrose Density Gradient Sedimentation Analysis

Strain		% S	Irrad.	Incub.	Molecular Weight x 10^7 Unirrad.	0' post	40' post
A	B/r	.04	B/Q	B	15	4.6	5.2
	B/r	17	E/Q	B	16	3.4	3.8
B	B/r	.02	B/A	B	10	7.0	6.1
	B/r	.1	E/A	B	8.7	3.4	2.5
C	B/r	.04	B/Q	NUT	-	7.5	7.2
	B/r	17	E/Q	NUT	-	4.4	4.2
D	B	.01	B/Q	B	10	4.7	5.2
	B	.01	E/Q	B	3.6	2.8	5.1

B = Buffer

E = Extract

A = Agitated

Q = Quiescent

NUT= Nutrient Broth

still comparable to buffer controls. No repair was noted either in the presence or absence of growth medium as measured by a restitution to high molecular weight species. It is possible that the 40 minute post irradiation incubation was not sufficient to permit complete reconstitution. Survival after this dose ranges between less than 0.1% and 27% under the conditions of irradiation used.

Sedimentation analysis of E. coli B (Case D) shows that extract alone causes breaks and/or degradation, and that more single strand breaks are found in the presence of extract than in buffer controls post irradiation. The degradation of DNA in the unirradiated cells most probably is caused by the release of endogenous endonuclease and may be similar to DNA degradation after photosensitization of lysosomes in eucaryotes (Allison (1965).

Clearly there is no correlation between decreased damage (Achey and Whitfield, 1965) less degradation post irradiation (Dean, et al. 1969) or repair (McGrath and Williams, 1967) and enhanced viability in the presence of extract. Either there is a qualitative rather than quantitative change in the modification of damage, or the extract works to modify damage to a part of the cell other than its DNA.

The lytic procedure described earlier might render the DNA susceptible to hydrodynamic shear. This however is not the case since unirradiated buffer cells lysed without preincubation in lysozyme (on the lytic lamella) resulted in calculated molecular weights quite close to those obtained with lysozyme. Both are in agreement with the value obtained by McGrath and Williams.

In sedimentation profiles of cells irradiated in extract, the end of the peak was only 4 samples from the end of the gradient. While such

a distribution would have a greater effect on the number average molecular weight than the weight average molecular weight used here (Lett, 1970), variations caused by free diffusion of oligonucleotides cannot be ruled out. The data should therefore be recognized as being more qualitative than quantitative.

DISCUSSION

A. Endogenous Radioprotectors.

The existence of a constitutive low molecular wieght radioprotective agent in M. radiodurans has been previously demonstrated by Bruce (1964). The Pq at optimal concentrations was 2 after a 40 kR exposure, while autoclaving the extract increased its activity five-fold. All irradiations were performed under conditions of vigorous aeration. Serriani and Bruce (1968), using organic purification and two dimensional chromatography increased the specific activity of the extract such that the dose modification factor was 3.2. It was shown to be hydrophobic when partitioned between butanol and water, after having been extracted from late log phase cultures. No hydrophobic component could be found in extract isolated from stationary cells.

The extract used in the course of this work differs from that of Bruce in serveral respects. It was isolated from a mutant of the wild type M. radiodurans which lacked the deep red pigment of the parent. Matthews and Krinsky (1965) had been unable to demonstrate any radioprotective effect associated with this pigment. The aerobic radioresistance of the mutant is greater than the parent, but less than that of a mutant of the same strain isolated by Lewis (1971). It is quite possible that the mutation to PH-2 involved qualitative or quantitative modification of the mechanisms of the cell which respond to the lethal effects of ionizing radiation. The extract used here, when isolated from stationary phase cells is hydrophilic when partitioned between water and both iso-amyl alcohol and butanol. The work described by Serriani and Bruce does not rule out the possibility that the solubility of extract from M. radiodurans is somehow modified when its source is

stationary cultures. The work described by Bruce (1964) reported the likely existence of a radiosensitizing agent contaminating the extract. The experiments described in this work with "purified" extract could not demonstrate the presence of such a compound.

The nature of protection in this extract is different than that described by other workers using E. coli B/r as a source. Fischer et al. (1969) have demonstrated the existence of a system which overcomes the formation of filaments in fil^+ cells after irradiation. This division promoting fraction is effective when irradiated cells are plated in its presence. It is relatively high molecular weight heat labile, and associated with membrane. In comparison, extract components from M. radiodurans PH-2 are low molecular weight (below 5,000 daltons) and are heat stabile to at least 60°C. The nature of Adler's extract seems more related to those dose modifications by post irradiation treatments noted earlier. Furthermore, Adler tentatively identified the active component as phospholipase and lipase sensitive while the agent described here is unaffected by lipase.

A similar study by Korgaonkar and Raut (1967) established that a division promoting agent resides in the "nucleoprotein" component of cell free extracts of E. coli B/r. As in the case of Adler's extract, this effects fil^+ cells, and is active when added to cells during plating and subsequent incubation post irradiation.

Both extracts of E. coli B/r may act through a mechanism similar to the neighbor restoration phenomenon described by Delaporte in yeast (1951). In all these cases the viability of the cell post irradiation seems little affected. It is only the secondary effect of septal formation and ability to form visible colonies which modifies the magnitude of

of survival.

Radioprotection by exogenous synthetic sulfhydryl agents with the chemical formula SH-R-NH_2, where R is a carbon chain of no more than three, is analogous to that afforded by extract isolated from M. radiodurans PH-2. Both must be present during the irradiation for maximum effectiveness, are most effective without exogenous aeration (as was demonstrated for 2-mercaptoethylamine by Elias, 1961), and have high dose modification factors. Cromroy and Adler (1962) have shown that radioprotection by MEA is quantitatively higher in strain B/r ORNL than in either B or Bs-1. The same effect is noted for extract and will be described in greater detail later.

Radioprotection by extracts of M. radiodurans is therefore qualitatively different than that afforded by other cellular derivatives. It is similar to that of cell-free extracts of the parent strain and may be related to radioprotection by sulfhydryl compounds.

Chemical nature of the radioprotective agent.

Wile Sephadex Gel Column Chromatography is a useful technique for separation of various molecular species, it has limited application in describing the physico-chemical parameters of a given molecule. The distribution coefficient is a function of not only molecular weight (Andrews, 1964), but also the shape and chemical binding characteristics of the molecule (Porath, 1961).

The molecular weight range of the compound can be approximated. Since it is retained by sephadex G-25 and not G-10 it almost certainly is between 1,000 and 5,000 daltons. It is therefore not chemically similar to known sulfhydryl radioprotectors which are all low molecular weight. A comparison of the elution profiles of extract and Vitamin

B_{12}, shows that both have similar distribution coefficients. It is supposed, therefore, that the active molecule in extract possesses a hetercyclic ring and binds to the gel. This subgroup might be analogous to the corrin ring of Vitamin B_{12} which is the moiety principally responsible for the elution characteristics of the molecule (Gelb, personal communication). The molecular weight of the radioprotector might be as high as 1350 daltons. The inability to eliminate protective capacity by predigextion with DNase, RNase, Pronase, or Lipase indicates that either the active species is not nucleic acid, protein or lipid n nature, or that it is, but for some reason it is resistant to those enzymes. Other than the fact that activity is affected by slow freezing and/or thawing, little information conerning its chemical composition has been obtained.

B. Mechanism of Action of Extract

Analysis of damage to cells and their subsequent recovery from the lethal effects of ionizing radiation is complicated by the presence of numerous kinds of lesions produced at different sites within the cell. There is no clear correlation between any given lesion and viability. For example, ionizing radiation produces scission of the phosphodiester backbone in a strand of DNA, yet, except for the B_s series of E. coli and the single stranded viruses (Tessman, 1959) such lesions are non-lethal. This led to speculation that single strand breaks at or near a given point on both strands of DNA are lethal (Munson and Bridges, 1967) yet M. radiodurans almost certainly can repair this type of lesion. There are reports that repair of membrane damage (Cramp, 1972), RNA synthesis (Kitayama and Matsuyama, 1971) or de novo protein synthesis

(Billen and Hewitt, 1967) are the critical events needed for recovery.

The dose-response relationship of the B series of E. coli to ionizing radiation has been the subject of theoretical interpretations by Alper (1958). The theory is based on three major observations:

1) The Oxygen Enhancement Ratio, when plotted as a function of the LD_{90} (the dose necessary to kill 90% of the population) in various resistance mutants of the E. coli B substrain, can best be described by a straight line (Alper, 1968).

2) Those strains which exhibit low oxygen enhancement ratios are more effected by treatments before and after exposure to ionizing radiation than those administered during exposure.

3) Those strains which exhibit a high oxygen enhancement ratio are more effected by treatments administered during irradiation than those given either before or after exposure.

From this, the presence of oxygen or nitrogen during the irradiation is thought to define two resolvable types* of damage to the cell, "one of which occurs at the physico-chemical level to immediately modify a disturbance, and one which in some way modifies the biochemical results of this disturbance" (Alper, 1958). These are defined as follows:

(1) Type N damage - The principle type of damage incurred under anoxic conditions of irradiation. It is thought to occur at the nucleic acid level and is the predominant type of non-repairable damage in cells with a low OER. E. coli B. B III-10 and Bs-1 can be considered to be representative of cells exhibiting this kind of effect.

* the term type is not defined by Alper and will be used synonymously with site, lesion, damage, etc.

(2) Type O damage - The principal type of damage formed under aerobic irradiation conditions. Its site of action is not clear, although location at the cytoplasmic membrane has been implicated. Cells which exhibit a high OER have an efficient system for overcoming lethality caused by Type N damage, and killing under aerobic conditions is caused by an inability to cope with Type O lesions. E. coli B/r is a cell which exhibits survival patterns consistant with these criteria.

Table VI summarizes the differences for both types of lesion.

Table VI

	Type O	Type N
Strains of Interest	E. coli B/r	E. coli Bs-1, B III-10,B
Site of Damage	(membrane)	Nucleic Acid
Oxygen Enhancement Ratio	High	Low
Post, Pre irrad. trtmnts.	Ineffective	Effective
Presence During Irrad.	Yes	No

Alper (1968) has recently shown that each type of damage can interact with one another. For example, damage to the membrane may cause the release of lytic enzymes (DNase) found there which could then act to augment repair of damage in the DNA. This will be discussed at lenth later.

A possible site of Type O damage has been elucidated by Cramp et al. (1972). Isolation of DNA-membrane fragments using the technique of Okazaki (1965(and analysis of DNA synthesis in vitro as a function of the total dose delivered resulted in an oxygen enhancement ratio of 8. Whole cells did not demonstrate this order of OER and the result was interpreted to mean that the membrane was sensitive to aerobic irradiation and was the site of the Type O lesion. Burrell et al.

(1970) have shown that DNA is associated with membrane fragments when sedimented in neutral alkaline sucrose gradients and feel that repair of double strand breaks may rely on "multiple attachment sites". The implication of membrane required mediation of repair reactions in both these studies is consistent within the framework of Alper's theory. Similarly, Dean et al (1969) demonstrated that M. radiodurans is capable of rapairing single strand breaks during or immediately after anoxic irradiation, but not in the presence of oxygen. This was interpreted to mean that two different types of lesions were involved, although the alkaline sucrose gradient technique used could not qualitatively distinguish between them. This too, is interpretable in terms of Type N and Type O damage. In eucaryotes, Allison (1965) has shown that photosensitization of the lysosomal membrane followed by exposure to visible light initiates the enzymatic breakdown of DNA by endogenous nucleases.

Ionizing radiation produces both types of damage in cells. A definied aerobiotic condition favors the formation of one type of lesion over the other. Lethality under anaerobiosis is therefore a measure of the cells ability to overcome Type N damage since this is the type that predominates. Similarly, survival during aerobic irradiation is a measure of the recovery from Type O lesions. Quantitatively the shift from Type O to Type N survival occurs when the concentration of dissolved Oxygen is less than 10 mM/liter (Howard-Flanders and Alper, 1967).

Analysis of the type of damage modified by extract is complicated by the fact that protection is afforded under both Type N and Type O conditions. These will be treated separately in this discussion.

1. Modification of Type O damage

That the extract can act by a modification to Type O damage is

demonstrated in the table below which is reproduced from Table I. It summarizes the dose modification by extract under conditions of oxygen present initially but not continuously bubbled (quiescent) during exposure.

Strain	B_{s-1}	B III-10	B	B/r CSH	B/r ORNL
DMF	1.2	1.2	1.3	3.3	3.2

Extract must be present during the irradiation (DMF for extract added after exposure is 1.3 in B/r), oxygen must be present initially, the sample must not be exogenously bubbled with air to give optimal protection. Only the two resistant mutants respond to such modification and these are separable from the other strains by consideration of their high oxygen enhancement ratios. These are precisely the conditions defined by Alper as being representative of Type O modification.

Two mechanisms may be advanced to account for the radioprotection by extract under these conditions:

(1) The extract induces anaerobiosis either chemically by oxygen scavenging or through the respiration of the cell suspension.

(2) The extract chemically reacts with the Type O lesions and renders it non lethal, or modifies the cell's capacity to overcome or repair them.

Modification by the first mechanism implies that Type O damage is not produced in the presence of extract, whereas the second allows for the production of such damage and its subsequent repair.

A mechanism based on oxygen scavenging can be dismissed by consideration of Fig. 12. Here all concentrations of extract are constant yet the viability of the cells is dramatically different. If the extract scavenged oxygen or its associated aqueous free radicals, the relative

survival for all cell concentrations should be the same. Clearly this is not the case.

There is no direct evidence to rule out the possibility of the induced cellular anaerobiosis by the extract, although some definition is required of the term. In bacterial physiology a cell which is referred to as an obligate anaerobe is fully viable in medium with a dissolved concentration of oxygen of 15 nm/ml. Radiobiological anaerobiosis indicates the complete exclusion of dissolved oxygen in the irradiation medium. Howard-Flanders and Alper (1957) demonstrated that most of the survival response was due to the amount of dissolved oxygen and not the anaerobic state of the culture. There was no change in radioresponse until there was less than 10 nu oxygen/ at which point the cells' resistance increased dramatically to the total anaerobic response. It seems unlikely that cellular respiration alone could deplete the medium of dissolved oxygen in the 15 minute pre-irradiation incubation used in this work.

If the extract was inducing anaerobiosis, the qualitative response under optimal conditions should be similar to that afforded by nitrogen for all strains. A comparison of the figures for these respective conditions in Table I shows that this is not the case. Extract is 16-37% more effective than nitrogen in the two B/r strains, is 50% less effective in B and Bs-1 while in B III-10 it gives minimal protection, where nitrogen gives none and may even sensitize the cells.

For optimal protection of Type O damage, several conditions must be satisfied during the irradiation. The concentration of extract on a per cell basis must be as high as possible (Fig. 11), and the concentration of cells present initially must be greater than 2×10^8/ml.

If the concentration of dissolved oxygen is held constant at approximately 1350 n moles/ml, radioprotection by extract is reduced to 1.0-1.3. Calculations on the per cell concentration of oxygen required to demonstrate radioprotection by extract differes by several orders of magnitude when compared to the data of Howard-Flanders and Alper (1967). It is not clear what role, if any, is played by the clumping of cells in extract noted earlier. All the strains tested do aggregate in the presence of extract, but it is not known whether this is associated with a recovery process in the B/r series. No aggregation was noted for cells incubated in 2-mercaptoethylamine.

With respect to these parameters, radioprotection by extract is similar to that afforded by MEA, which is also optimally effective at high sub-lethal concentrations and under the aerobic conditions previously described. It also follows the qualitative strain dependence noted for the extract in the E. coli B series. The radioprotective effects of extract and MEA are not additive (Fig. 14) and it is possible that both compete for the same active site in the cell.

2. Modification of Type N damage

The presence of extract during anaerobic irradiations increases the survival of all cells tested, although its quantitative level of protection is higher in E. coli B and E. coli B/r. That it also augments survival after irradiation in buffer and incubation in extract is shown by Fig. 5. Dose modification of this type is operationally defined as acting on Type N lesions.

A cell which shows little capacity to repair or modify Type N damage (that is, one having a low oxygen enhancement ratio) should have

a negligible response to modification of that damage. This is demonstrated in strain B III-10 which undergoes little or no augmentation of survival by extract under anaerobic conditions. Strain B s-1 has been described as having at best a highly abortive DNA repair system by Freifelder (1966) and McGrath and Williams (1967). The oxygen enhancement ratio of 1.4 is probably due to a very sensitive Type O response which depresses the LD_{99} under aerobic conditions to 10.8 kR. This strain is also typified by a low extract enhancement.

Strains in which the presence of a Type N repair system has been noted by the repair of U.V. irradiated phage are designated hcr^+. Modification of damage in such strains in the presence of extract is 2.5 - 3.0 times higher than those which cannot modify damage to DNA. Therefore extract augments the action of functional repair systems for the modification of Type N damage.

3. Interaction of Type O and Type N damage

Alper does not exclude the possibility of interaction between the two types of lesions. That this can occur is shown by the work of Allison (1965) in eucaryotes and Cram et al. (1972) in bacteria. In both cases, induction of Type O damage led to a modification of the theoretical Type N target as measured by DNA degradation and polymerization respectively. Such interation is implicated by the viability studies performed here with extract.

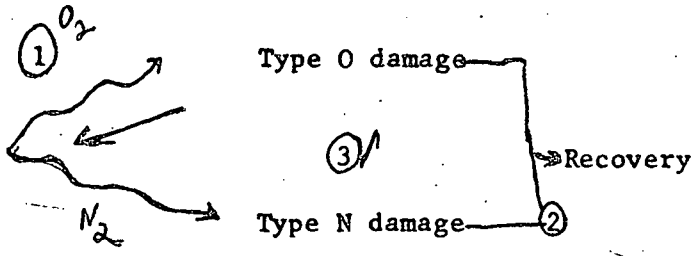
The B series substrains investigated can be arranged with respect to their quantitative resistance to a given type of damage. Using the symbol + to represent such resistance and * to represent additional increments of radioprotection above the buffer level they are as follows:

Strain	Presence of system		DMF by extract	
	Type O	Type N	Type O	Type N
Bs-1	-	+	1.2	1.1
B III-10	+	-	1.2	1.3
B	+	+	1.3	1.3
B/r (CSH)	++	++	3.3	1.2
B/r (ORNL)	++	++	3.2	1.0

The action of extract can best be described as modifying Type O damage only when both Type N and Type O repair systems are found in the cell, as they are in the B/r substrains.

The interaction of Type N and Type O damage may reside in the distribution of enzymes at or near the membrane which subsequently take part in the repair of damaged DNA. DNA polymerase which is concerned with the polymerization of single stranded regions of DNA which have been degraded by nucleases is almost certainly concerned with repair of damaged DNA. It has an absolute requirement for free 3'-OH sites. Any agent which induces their formation in DNA which has been otherwise damaged should increase the efficiency of the enzyme. Such nucleotidases reside at the periplasmic space between the cell wall and membrane (Neu and Chous, 1967). This is one of the regions modified cytologically by the extract (see electron micrographs). It should also be noted that extract causes a condensation of DNA although the implications of such a modification as relating to reapir are not clear.

Diagramatically, a simplistic model for the deposition of lesions and their subsequent modification might be:



Site 1 represents the modification of Type O damage in the B/r strains under conditions which are aerobic, but there is no exogenous bubbling with air. Site 2 is the augmentation of the DNA repair systems in hcr^+ strains. Site 3 represents a possible site of action to give a qualitatively similar Type N lesion where damage is incurred only at a Type O site.

C. Effect at the Gene Level

It has been stated before that strain B III-10 undergoes only Type N damage. This response is similar to one noted for Chlamydomonas by Davies (1967). It seems reasonable to conclude therefore that the addition of a resistance (or repair) gene to its genome would enhance its survival under anaerobic conditions, while having little effect aerobically. This is precisely the case when one investigates the hcr gene by comparing survival in E. coli B and B III-10. The gene has no effect in the presence of air (Table IV), but has a dose modification of 1.95 for anoxic irradiations. This gene is assayed for by monitoring the repair of Type N damage to UV irradiated phage in an undamaged host. Its level of action is therefore entirely at the DNA level. Alper (1967) has demonstrated this preferential action for hcr^+ in several of the B substrains.

The action of exr is thought to involve "mutation prone" gap filling in DNA which has been degraded by nucleases (Witkin, 1967).

It is thought to act after DNA semi-conservative replication and recombinational repair (Rupp and Howard-Flanders, 1968). However, the error prone mechanism advanced by Witkin has been challenged by Hill (personal communication) who suggests that Witkins' data may reflect a plating artifact. The data presented here indicate that exr acts preferentially to modify Type O damage (DMF = 1.74) and as such would be only indirectly concerned with DNA repair per se. The UV sensitivities, and therefore the modification of Type N damage of B III-10 and the Bs-1 strain are comparable (Witkin, personal communication) and in this way verify the anoxic survival data and interpretations.

Phenotypically, the presence of a fil^+ gene is characterized by the irreversible inability of an x-irradiated cell to undergo cross-wall formation and hence colony formation. This alone is not sufficient to define the radioresistance however, since both radiosensitive (Hill, 1958) and radioresistant (Stravic et al., 1968) mutants of fil^+ have been isolated. The mutation to fil^- alters the radioresponse in such a way as to render the cell qualitatively and quantitatively similar to other species of bacteria and has prompted Adler (1966) to suggest that B/r should be considered the parent.

The fil gene enhances viability after both aerobic and anaerobic irradiations. Its Type O and Type N dose modifications are 2.5 and 4.2 respectively. The interation of membrane associated reactions in modifications of Type N damage and its probable site of Type O lesions has been discussed earlier. In view of these considerations, it does not seem unlikely that modification of membrane processes would effect both types of damage.

A comparison of dose modification at the gene level in this work with values obtained by other workers is as follows:

	Oxygen		Nitrogen	
	Cromroy & Adler (1962)	Goldstein	Bridges & Munson (1967)	Goldstein
exr	[illegible]	[illegible]	12	1.1
hcr	2.7	2.0	1.36	1.95
fil	1.6	2.5	6.0	4.2

The combined effect of exr and hcr under aerobiosis was calculated by comparing the double mutant Bs-1 (Mattern, et al., 1966) with the parent. The differences noted can be attributed in part to at least two variables. Adler irradiated in 0.85 M NaCl, and Elias (1965) has shown that under such conditions, cells are more sensitive than PO_4 buffer controls. Also while the survival of both Bs-1 strains was comparable, the resistance of the B strains differed by about 25%. Although theoretically isogenic, there remains the possibility that E. coli B exhibits a differential strain response similar to that found in B/r (Adler, 1966).

Munson and Bridges calculated their effects in a series of E. coli B/r mutations designated the Wp2 line. Since it has been shown in this and other work that there can be an interaction between genes controlling different types of damage, such variations are not wholly unexpected. While the contribution of the hcr gene is diminished in Munsons' work, the qualitative nature of the gene effect is similar to that obtained here.

The effect of extract at the gene level is reflected by an increase

in their respective dose modifications. Augmentation of both exr and hcr are comparable, while that for fil is very much higher. The increases expressed as percentages were calculated using the following formulae:

$$\text{exr} = \frac{\text{DMF in bubbled, aerobic extract - DMF in bubbled aerobic buffer}}{\text{DMF in bubbled aerobic buffer}} \times 100$$

$$= \frac{2.05 - 1.74}{1.74} \times 100$$

$$= 14.4\%$$

$$\text{hcr} = \frac{\text{DMF in anoxic bubbled extract - DMF in anoxic bubbled buffer}}{\text{DMF in anoxic bubbled buffer}} \times 100$$

$$= \frac{2.2 - 1.95}{1.95} \times 100$$

$$= 12.8\%$$

Changes in the dose modification by fil$^-$ calculated in the same way for bubbled aerobic and anaerobic conditions are -6.8% and +7.1% respectively. However, under quiescent aerobic conditions fil is augmented by 140%, hcr by 20% and exr is decreased by 8%. Apparently the contribution to Type O recovery is assumed by fil in the absence of exogenous oxygen, and by exr in its presence. It is obvious that the greatest contribution by extract at the gene level is at the processes controlled by the fil gene.

The data do no differentiate between the contributions of a single gene and the net effect of two or more genes working together. They do demonstrate that interpretation of survival in terms of two distinct sites of damage is supported by genetic data. They also rule out interpretations which are based on preventing or repairing lethal damage

to DNA as well as those predicting protection of a functional repair system.

D. Effect of Extract on DNA - Sedimentation Analysis

The data in Table III indicate little correlation between either DNA degradation or repair under aerobic conditions. This is to be expected if the major modification effecting lethality occurs at some other site as predicted by Alper's hypothesis. The viability data and macromolecular analysis of DNA are consistant with other studies performed with known radioprotective agents.

Ginsberg and Webster (1969) could not correlate DNA breakdown in E. coli B/r or Bs-1 in the presence of MEA with respect to quantitative increases in viability. Lohman et al. (1971) have demonstrated that the presence of MEA in quantities necessary to enhance survival induced single strand breaks in the DNA of E. coli. The same effect is noted for extract in strain B. Using endogenous systems found in M. radiodurans , Fox and Hopkins (1970) could find no relationship between DNA breakdown and recovery.

Certain mechanisms concerning modifications of DNA by extract during aerobic irradiation are not supported by sedimentation analysis. The number of single strand breaks formed in the presence of extract is actually higher than buffer controls. Therefore radical scavenging already eliminated by viability studies, can be discounted at the molecular level. Similarly, extract does not augment existing repair systems to reconstruct damaged DNA to any great extent. The same effect was noted while considering the effect at the hcr gene. Nor does extract protect the DNA repair system from damage, since little or no reconstruction of

high molecular weight DNA could be noted in cells with a very efficient system (B/r) or one with a lower efficiency (B).

Alkaline sucrose density gradient centrifugation of DNA isolated from cells which have been incubated in extract does present an interesting implication with respect to the deposition of damage during aerobic irradiation and its subsequent repair. A comparison of unirradiated extract controls of E. coli B and E. coli B/r shows that extract alone is sufficient to induce single strand breaks in the former only. It seems likely, therefore, that the fil$^-$ gene in some way controls the stabilization of high molecular weight DNA and that single strand break analysis assays damage resulting from both direct exposure to ionizing radiation and induced enzymatic degradation.

The possible extract mediated release of nucleotidases has been discussed earlier. That the control of such enzymes is necessary for recovery has been demonstrated by Howard-Flanders and Theriot (1966) in E.coli K-12. Uncontrolled nuclease activity, termed reckless degradation, was shown to follow exposure of the cell to UV light. Dean et al (1972) have found that the endogenous nuclease activity in M. radiodurans is sufficient to degrade 80% of the cells' DNA within one hour, and its control is affected by post irradiation conditions. A corollary to this is that such activity must be tightly regulated by the cell.

The maintenance of high molecular weight DNA may also be a function of controlled DNA ligase activity. Town et al. (1971) have demonstrated the existence of such an enzyme which is responsible for the repair of single strand breaks of E. coli K12 irradiated anaerobically. It is possible that such an enzyme is inhibited by extract in E. coli B, but not B/r.

As has been described earlier, the sedimentation analysis performed here is not quantitative in nature. McGrath and Williams (1967) give a value of one single strand break per 3.3 kR exposure. The number of such breaks resulting from the 60 kR exposures used here should therefore be 15 - 20. Computer analysis of the profiles showed the number to be only 25 - 30% of the predicted value under all conditions tested. The reason for this remains obscure.

The site of action of extract is therefore other than that concerned with the deposition and repair of single strand breaks in DNA. Several possible mechanisms for radioprotection by extract present themselves:

1. If type O damage is concerned with DNA but not in the formation of single strand breaks, extract may prevent the deposition of this type of damage (e.g. depurination, base degradation, damage to the sugar moiety) or augment its repair. The lack of a precise analytical system at present prevents this type of investigation.

2. If Type O damage is to some site other than DNA within the cell, extract may work to modify the specific type of lesion formed or repaired.

3. Extract may act as a cofactor to augment natural cellular repair processes and increase their qualitative accuracy.

The quantitative role of this radioprotector in situ in M. radiodurans is still speculative at this time. The extremely high aerobic radioresistance of the cell and its relatively low oxygen enhancement ratio would imply that restorative processes involving mechanisms similar to those determined for radioprotection in E. coli are surely present. The isolation of the compound from unirradiated cells would also imply that such processes are necessary for the maintenance of

cellular integrity. Dose modification might therefore only be a secondary result of those processes that determine the viability of a living organim.

SUMMARY AND CONCLUSIONS

A constitutive low molecular weight radioprotective agent isolated from Micrococcus radiodurans PH-2 was shown to exert a considerable dose modification in E. coli. Crude extract was purified by organic separation and sephadex gel chromatography. Conditions for radioprotection were optimized with respect to the extract concentration, initial cell concentration, and state of aerobiosis. The mechanism of action was analyzed with respect to both survival and macromolecular modifications.

The major findings in this report concerning radioprotection in Escherichia coli by the agent isolated from M. radiodurans are the following:

1. The extract isolated from stationary cells is a hydrophilic molecule with a molecular weight between 1,000 and 5,000 daltons. It is resistant to digestion by DNase, RNase, pronase and lipase and is thought to contain a heterocyclic ring subgroup. Its physical characteristics are similar to a protective extract isolated from wild type M. radiodurans.

2. The radioprotective compound causes distinct cytological changes in E. coli B/r. Electron micrographs reveal nuclear condensation, the loss of the periplasmic space, and the clustering of ribosomes at the membrane. Aggregation of cells is also noted, although the contribution this makes to radioprotection is not clear.

3. Radioprotection by extract in Escherichia coli B/r is approximately 1.5 times greater than that observed for optimal concentration of 2-mercaptoethylamine. Optimal conditions for expression include maximum concentration of extract, the presence of oxygen, and non-

agitation of the sample during irradiation. Extract must be present during the irradiation, although post-irradiation incubation in extract results in a dose modification of 1.3. More than 5×10^8 cells must be present initially for optimal expression. Survival under these conditions can best be described by a straight exponential function with respect to accumulated exposure.

4. The radioprotective effects of extract and 2-mercaptoethylamine are not additive and indicate that both may compete for the same site within the cell. Optimal conditions for both are similar, as is the quantitative response in different substrains of E. coli B. Unlike MEA it is non toxic.

5. Radioprotection of a high magnitude is demonstrated in strains which do not form filaments (fil^-) after exposure to ionizing radiation. Low magnitude protection is noted in cells with low to medium oxygen enhancement ratios.

6. Extract neither affects the induction of single strand breaks, nor does it prevent post irradiation degradation or restitution of DNA in protected cells. Extract induces phosphodiester bond scission in at least one fil^+ strain. There is no correlation between macromolecular parameters or reduced or repaired damage and viability.

7. The expression of 2 of the 3 genes thought to be responsible for radioresistance in the B substrain of E. coli was shown to depend on the state of aerobiosis during the irradiation. Exr was expressed only under aerobic conditions while hcr required anaerobiosis. The third gene, fil^-, was expressed both with and without the presence of molecular oxygen. Extract was shown to amplify both exr and hcr by 12 - 14%, while augmenting the action of fil by 140%.

8, Analysis of radioprotection by extract is consistent with Alpers' hypothesis for the deposition and repair of damage induced by ionizing radiation as representing 2 resolvable types which depend on the conditions of irradiation. Mechanisms predicated on oxygen radical scavenging and augmentation of DNA repair processes are not supported. The possible interaction of the two sites for lesion deposition is also discussed.

These findings point to needed investigations to discern the nature of lethal damage to bacteria caused by sites other than the chromosome alone.

APPENDIX I

Composition of media and fluor used in the course of this study

Media

Nutrient Broth

8.0 grams dehydrated Nutrient Broth (Difco) per liter distilled water

M-9+ per liter

$Na_2HPO_4 \cdot 7H_2O$	13.0 grams
KH_2PO_4	3.0 grams
NH_4Cl	1.0 grams
Casamino Acids	2.5 grams

Adenosine monophosphate 250 mg

Autoclave, then add sterile per liter

1.0 ml 1M $MgSO_4$

2.0 ml 25% NaCl

0.3 ml 0.01 M $FeCl_3$ in 0.1 N HCl

10 ml 2% gelatin

5.0 ml 20% glucose

DNA labelling medium

M-9+ supplemented with 10 ul tritiated (H^3 1.00 mCi/ml) thymidine (New England Nuclear)

Triton X-100 Flour per liter

333 ml Triton X-100 (Packard Inst. Co.)

667 ml reagent grade toluene (Fisher Sci.)

8.25 grams PPO (Packard Inst. Co.)

0.25 grams M_2POPOP (Packard Inst. Co.)

APPENDIX II

Equations used in the analysis of the alkaline sucrose gradients

measurements made for each gradient were the total distance to the meniscus (H), the total number of fractions (N), and the H^3 activity of each fraction (r). The distance D from the meniscus for each fraction is represented as

$$D = \frac{1 \text{ X } N - (\text{fraction number} + 1)}{N} \quad (1)$$

The sedimentation coefficient was derived from a modification of the Burgi-Hershey (1963) equation as

$$S_{20,w} = \frac{B\ Di}{V^2 t + K} \quad (2)$$

for single stranda at $20^{\circ}C$ in water where D = the mean distance sedimented in cm, $B = 5.693 \times 10^{10}$, K = correction factor for deceleration = 1.21×10^{8}, V = angular velocity and t = time in hours. B was based on measurements for denatured T_4 phage.

The mean molecular weight was calculated from the relationship developed by Studier (1965).

$$S_{20,w} = 0.0528\ M_i^{0.4} \quad (3)$$

Values for the weight average molecular weight were determined by the equation of Lett (1970).

$$M_w = \frac{\Sigma wi\ Mi}{\Sigma wi} \quad (4)$$

where Mi is the mean molecular weight of the ith fraction and wi is the weight of DNA in the ith fraction which is proportional to the radioactivity in that fraction so that

$$Mw = \frac{\Sigma ri\ Mi}{\Sigma ri} \quad (5)$$

Calculations were made on a CDC 6400 computer using a program developed by T. Bonura.

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