Title
EFFECTS OF pH AND ANOXIA ON THE CELL MORPHOLOGY AND RADIATION SENSITIVITY OF Escherichia coli

Author
Sargent, Thornton William

Publication Date
1959-02-27

UCRL 8668

UNIVERSITY OF
CALIFORNIA

Ernest O. Lawrence

Radiation Laboratory

EFFECTS OF pH AND ANOXIA ON THE CELL MORPHOLOGY AND RADIATION SENSITIVITY OF *Escherichia coli*

BERKELEY, CALIFORNIA

DISCLAIMER

UCRL-8668
Biology and Medicine

UNIVERSITY OF CALIFORNIA
Lawrence Radiation Laboratory
Berkeley, California

Contract No. W-7405-eng-48

EFFECTS OF pH AND ANOXIA ON THE CELL MORPHOLOGY AND RADIATION SENSITIVITY OF *Escherichia coli*

Thornton William Sargent III

(Thesis)

February 27, 1959

Printed for the U. S. Atomic Energy Commission

Printed in USA. Price $1.50. Available from the
Office of Technical Services
U. S. Department of Commerce
Washington 25, D. C.

EFFECTS OF pH AND ANOXIA ON THE CELL MORPHOLOGY AND RADIATION SENSITIVITY OF *Escherichia coli*

Contents

EFFECTS OF pH AND ANOXIA ON THE CELL MORPHOLOGY AND RADIATION SENSITIVITY OF *Escherichia coli*

Thornton William Sargent III

Donner Laboratory of Biophysics and Medical Physics
University of California, Berkeley, California

February 27, 1959

ABSTRACT

The effects of varying the hydrogen ion concentration and oxygen tension in the environment of *Escherichia coli* during growth, irradiation, and post-irradiation incubation have been studied. Previous workers had found that when these cells are grown anaerobically, their resistance to x-rays is greatly increased. This resistance was attributed to the production of an anoxic condition within the cell during growth, in the same way as and additive to resistance conferred by anoxic conditions during irradiation.

It is shown here that protection conferred by anaerobic growth is independent of anoxia during irradiation, and can be abolished by causing growth to occur in alkaline rather than the usual acid conditions. The resistance resulting from anaerobic-acid growth is attributed to the multinucleate nature of the cells, as shown by cytological observations, DNA analysis, and the shape of survival curves.

INTRODUCTION

Although various ionizing radiations had previously been shown to have bactericidal action, Chambers and Russ (1912) were the first to obtain accurately curves in which survival was a negative exponential function of dose. These workers also observed motile bacteria after irradiation which, however, were unable to form colonies. Colony formation is the usual and most convenient criterion of survival even today, although it is widely recognized as not being a true criterion of "death." Thus the reproductive apparatus of the cell was implicated as the primary site of damage.

I. Interpretation of Survival Curves

1. Nature of Target.

Following the application of target theory to survival curves (Crowther, 1924, 1926) many investigators obtained exponential survival curves for many species of bacteria. According to target theory, such survival curves can be interpreted on the basis of a uniform population of cells, each cell containing a critical volume. The occurrence of a single ionization (or absorption of a uv quantum) within this volume produces an injury which leads to inability of the cell to divide. Lea (1947) postulated that the vulnerable structure within this volume was the genetic apparatus. He concluded, after analysis of experiments in which E. coli were exposed to radiations of different ionization densities, that killing could be accounted for by lethal mutations among 250 genes with an average diameter of 12 mμ. Evidence that inactivation of yeast cells occurs as a result of lethal mutations has been obtained by Mortimer (1956). There is also evidence for nongenetic damage; Gaulden and Perry (1958) have shown permanent blockage of mitosis by microspot ultraviolet irradiation of the nucleolus in grasshopper neuroblasts at critical stages of mitosis. Similar effects with ionizing radiations, however, have not been reported,

although St. Amand (1956) using the same material found unexplainable differences in chromosome breakage and viability with x-irradiation at different stages of mitosis.

2. Multiple-Hit-Type Curves.

Sigmoid survival curves can be interpreted by target theory to mean that a cell or a colony-forming unit contains more than one sensitive site, each of which must be inactivated to prevent the growth of a colony from the unit. Such interpretation has been successfully applied to a number of experiments. Wykoff and Rivers (1930) found clumps of cells to be responsible for sigmoid curves. Brownell (1955) and later Stapleton (1955) found in E. coli, B/r, a correlation between multiplicity, as calculated by target theory from x-ray survival curves, and the average number of nuclei per cell, observed microscopically. These two workers obtained different results, however, with regard to the slopes of the curves at different stages of growth.

Using yeast of ploidies ranging from haploid to hexaploid, Mortimer (1958) has shown that multiplicity of genetic material affects the shape of x-ray survival curves. The change of shape is at least qualitatively that which would be expected from target theory, although the results are complicated by dominant and recessive lethal effects (Owen and Mortimer, 1956). Using whole plants with ploidies up to 22 in Chrysanthemum, Sparrow and Schairer (1958) obtained data consistent with the assumption that there is a linear relationship between radiosensitivity and chromosome number.

Atwood and Mukai (1951) showed very neatly the effect of heterokaryosis on the shape of survival curves. They used a strain of binucleate Neurospora, one nucleus containing a mutation to a nutritional requirement and the other being prototrophic. X-ray survival curves were obtained which were exponential when the irradiated cells were grown on a minimal medium on which only the prototroph would grow, and sigmoid when grown on an enriched medium which allowed both nuclei to grow.

Hollaender, Stapleton, and Martin (1951) obtained sigmoidal survival curves for E. coli, B/r, grown in glucose broth under anaerobic conditions, when irradiated under either aerobic or anoxic conditions. Apparently, however, they did not consider the possibility that the sigmoidal shape could be due to multiplicity of nuclear material, as had been shown in previous cases of sigmoidal curves. They state: "The extreme resistance of the anaerobic glucose cells can then be explained on the basis of the complete additivity of these two protective systems; 1) presence of glucose and 2) absence of oxygen during growth." Apparently the effect was considered part of the oxygen effect by Hollaender, Baker, and Anderson (1951, p. 324). They write: "Saturating the suspension with nitrogen, however, will not necessarily remove the oxygen from inside the cell. A bacterium which is grown anaerobically and irradiated anaerobically may be considered to be a relatively oxygen-free cell. These experiments indicate that the oxygen inside the cell, as well as that in the surrounding suspension, is a very important factor."

It is apparently fairly well known that anaerobically grown E. coli are multinucleate (Latarjet, 1956). Howard-Flanders and Alper (1957) did find, however, that it was indeed more difficult to remove all oxygen from the interior of the cells than had been found by Hollaender and Stapleton (1953).

Hughes (1953) found that he could produce "L forms" in Bacterium coli, faecal type 1, L forms being forms in which cell growth (and presumably nuclear growth and division) continues but separation into daughter cells is inhibited. These forms were produced by anaerobic growth and had no connection with penicillin sensitivity, pH of the medium, nor with the agar itself. As soon as oxygen was admitted, the L forms resumed division and produced only small forms. Birge and Tobias (1954) suggested "These changes, when better understood, may explain the findings of Hollaender et al." (1951); they had found no change in the exponential form of the survival curve of anaerobically grown haploid yeast.

The experimental results of this paper confirm the suggestion of Birge and Tobias that anaerobic growth of E. coli confers protection by virtue of producing a target multiplicity.

II. Modification of Damage During Irradiation

One of the basic postulates of target theory is that ions act directly on some sensitive site in the cell, and this made it possible to calculate the volume of the site. Target theory began to be considered of less value when a wide variety of conditions during irradiation were found which altered the radiosensitivity of organisms, such as the presence of various protective agents (cysteine, ethanol, sodium hydrosulfite, etc.), concentration of cells, and oxygen tension. In order to explain these effects it seemed necessary to assume an indirect action. This required an intermediate molecule or free radical which was produced in the cell or in the water layer immediately around it by an ionization. The radical then diffused to the sensitive site or molecule and inactivated it. For an excellent review see Zelle and Hollaender (1955).

An attempt was made by Zirkle and Tobias (1953) to extend the target theory to account for these effects by including diffusion of radicals. The equations, however, contained so many parameters of unknown or arbitrary values particularly the diffusion constants of the radicals, that it was not possible to check the theory against experiment. Wijsman (1956), by assuming that the diffusion length of the intermediary radical is long compared to the cell nucleus, has derived a relatively simple equation which agrees with experimental data on yeast of different ploidies using radiations of various ionization densities. Hutchinson (1958) calculates from experiment and from the equations of Zirkle and Tobias that the diffusion length of OH radicals is 30 to 60 A, a result inconsistent with the diffusion length assumed by Wijsman.

1. Concentration Effects

A reduction in radiosensitivity when E. coli were irradiated in very dense suspensions was found by Hollaender, Stapleton, and Martin (1951) and Biagini (1955), and the results have been interpreted as evidence for an indirect effect. However, Gunter, and Kohn (1956) and Goucher et al (1956) showed that when such concentrated suspensions were saturated with oxygen the radiosensitivity was the same as with dilute suspensions. They concluded that the apparent protection due to concentration was simply a result of partial anaerobiosis caused by endogenous utilization of oxygen by the cells.

2. Chemical Protectors

The protection afforded by many compounds with which the cells may be incubated before irradiation (formate, succinate, pyruvate, serine, α-alanine, and ethanol) was shown by Stapleton, Billen, and Hollaender (1952) to be due to enzymatic removal of oxygen by endogenous respiration utilizing these compounds. They also found that dimercaptopropanol (BAL) and sodium hydrosulfite ($Na_2S_2O_4$), which do not require preincubation to give protection, remove oxygen independently of enzyme action and concluded that this protection was also due to anoxia.

In the majority of experiments, protective compounds have been found to have no additional protective effect when irradiation was carried out under anoxia, with the exception of β-mercaptoethanol and cysteamine (Hollaender and Doudney, 1955). The survival curves of bacteria protected by these compounds show a striking similarity in shape and slope, especially with cysteamine, to those obtained by Hollaender, Stapleton and Martin (1951) for the same bacteria grown anaerobically and irradiated in nitrogen. Howard-Flanders (in press) has suggested that these compounds may protect by inducing a physiological state similar to that caused by anaerobic growth and also by producing anoxia within the cell. This will be discussed later in light of the experiments reported in this paper.

Although chemical protectors do seem to act by removing free radicals from surrounding water in the case of enzymes and bacteriophage, Howard-Flanders (in press) concludes that there is at present no clear evidence for such a mode of protection in the case of bacteria and higher cells. Hollaender and Doudney (1955) conclude that the cysteamine enters or is adsorbed to the bacteria, because protection is not removed by centrifuging the cells out of the cysteamine solution and resuspending in saline. Also, certain recovery factors must be present during postirradiation incubation to realize the full protective effect indicating a possible metabolic role for cysteamine, as opposed to free-radical scavenging.

3. The Oxygen Effect

The increase in sensitivity to ionizing radiations caused by the presence of oxygen is one of the most universal of radiobiological effects. It was not extensively studied, however, until the discovery by Thoday and Read (1947) that the presence of oxygen during irradiation greatly increases the number of chromosome aberrations. Practically every effect of ionizing radiations on biological material has since been shown to be enhanced by the presence of oxygen, with the exception of certain cases of bacteriophage and transforming principle (Latarjet and Ephrati, 1948; Hewitt and Read 1950; Ephrussi-Taylor and Latarjet 1955).

When two survival curves obtained under different conditions, in particular in oxygen and under anoxic conditions, differ from each other at all survival levels by a constant multiple of the dose, the difference in the curves is usually expressed as this multiple, the dose modification factor or DMF. Where this factor is not constant over the range of survival studied, the radio-sensitivity or reciprocal of the dose at a stated survival is usually used to express differences in curves.

The DMF of oxygen is relatively constant for a wide range of biological effects studied with a radiation of a given ionization density, with a few exceptions, especially genetic effects (Anderson, 1951).

The DMF for most effects is close to 3 for 250-kv x-rays, about 2 for 50-kv x-rays, and becomes smaller for fast neutrons and still less for more densely ionizing radiations (see Howard-Flanders, in press, for compilation of data).

The mechanisms by which oxygen exerts its sensitizing effect is still unknown, although there are two main schools of thought, (a) free-radical action and (b) modifiable direct action. An additional hypothesis has been presented by Laser (1955) who interprets his data to mean that oxygen sensitizes by affecting the oxidation state of the respiratory-enzyme chain. This hypothesis has been thrown in considerable doubt by the finding of Howard-Flanders (1957) that nitric oxide is equivalent to oxygen in its sensitizing ability, yet is not respired by cells. Also Moustacchi (1958) found that bacteria which contained hemin and hence a cytochrome system showed survival curves in air and nitrogen almost identical with those of a hemin-deficient mutant of the same bacterium. Normal yeast and cytochrome-less petites also showed the same survival curves, both in air and nitrogen.

(a) Free-radical action. Following the discovery of the oxygen effect in E. coli by Hollaender, Stapleton, and Martin (1951), a variety of known and postulated radiochemical reactions were cited as evidence that oxygen was involved in the formation of various biologically active free radicals, particularly HO_2, or even hydrogen peroxide. Hence, the action of oxygen was considered to be via the indirect effect. [For an extended discussion and references see Bacq and Alexander, (1955)].

Hollaender and Stapleton (1953) published a curve showing the concentration of oxygen required to produce various survivals at a single dose of x-rays. A more recent determination of radiosensitivity as a function of oxygen tension by Howard-Flanders and Alper (1957), performed under conditions of much higher precision, produced a curve of rather different form. The oxygen tension that doubled radiosensitivity was 10-20 times lower than found previously.

(b) Modifiable direct action. On the basis of this and other experiments, there has developed another school of thought on the oxygen effect, the theory of modifiable direct action. For a thorough exposition of the ideas of this school and references to work supporting them see Howard-Flanders (in press). Two types of radiation damage are distinguished--one which occurs only in the presence of oxygen, and a second which is not influenced by the presence of oxygen and appears to result only from multiple ionization.

In order to produce the oxygen-sensitive damage, it is envisaged that an ionizing particle reacts directly with the target molecule, leaving it in a highly reactive state; there is some experimental evidence that this may be in the form of a carbon radical (Jayko and Garrison, 1958). If oxygen is present, it will react with the molecule probably within 1 μsec and produce a lesion that inactivates the cell. If oxygen is not present, the molecule will undergo other reactions which do not lead to inactivation, possibly by a return to its initial state. An equation has been derived, appropriate to a competitive reaction, which fits very well the data obtained.

A second kind of damage to a target molecule is postulated which is not dependent on the presence of oxygen. In order for this damage to lead to inactivation of the cell, there must be one or more other ionizations near it which produce radicals that then react with the target molecule. Howard-Flanders (in press) has extensively developed the "track-segment" method for interpretation of RBE vs LET effects, consistent with the ideas developed previously, and discusses its application to and agreement with various experimental data.

4. Hydrogen Ion Concentration

The effect of varying the hydrogen-ion concentration during irradiation seems to have been studied very little. Zirkle (1936, 1940, 1941) exposed fern spores and Drosophila eggs to x-rays at various concentrations of NH_4OH and CO_2. He assumed that both of these compounds penetrated the cell and altered the intracellular pH,

although he could not actually measure the pH inside the cell. He found a variety of maxima and minima in radiosensitivity at various concentrations. The relative heights of these maxima and minima, but not the concentrations at which they occurred, varied with the dose. He postulated that the peaks represented pH optima of different radiochemical reactions, and suggested that these reactions were precipitation of nucleoproteins at their respective isoelectric points.

Marshak (1938) exposed Allium cepa and Vicia faba seedlings to x-rays at various concentrations of NH_4OH and studied the number of chromosome abnormalities at anaphase. He found a six-fold reduction in the number of abnormalities as the NH_4OH concentration was increased from 0 to 6×10^{-3} N. He found no effect with CO_2.

These two studies are difficult to interpret because of the uncertainty in the intracellular pH, the toxicity of NH_4OH, for which corrections had to be made, the possibility of effects other than pH change of these compounds, and the total lack of effect of CO_2 in the case of A. cepa and V. faba. Thoday and Read (1947) suggest that the findings of Marshak may be partly due to anaerobiosis.

Alper and Ebert (1954) studied the effect of pH on the inactivation of bacteriophage, finding increasing sensitivity with increasing alkalinity. They had been led to the investigation by previous work which showed that phage inactivation was greatest under gas treatments that caused the least production of hydrogen peroxide. Bonet-Maury and Lefort (1950) had shown that hydrogen peroxide production in water is highest at acid pH, falling by 50% as the pH is increased from 2 to 3, remaining almost constant from 3 to 8, falling by another 30% between pH 8 and 9. The pH dependence of phage inactivation was attributed by Alper and Ebert to the step

$$HO_2 \rightleftharpoons H^+ + O_2^- .$$

Since Alper (1952) had shown previously that oxygen protected phage and hydrogen enhanced the damage of radiation, the indirect damage to phage was considered to be due to a reductive process, presumably

by O_2^-. At acid pH the yield of O_2^- would be less, resulting in less phage inactivation; they found indeed an increased resistance as the pH was decreased from 8.9 to 7.0 to 4.8. The effect of pH should occur only in oxygen, as the formation of HO_2 must occur first. In this report they found a much reduced effect of pH in hydrogen.

Dainton (1956) states that in anoxic neutral solution, the H radical may be assumed to be reducing, but in acid solution may be converted to H_2^+, an oxidizing agent. If oxygen is present, he considers it reasonable to assume

$$H + O_2 \longrightarrow HO_2$$

where HO_2 is also an oxidizing agent. However, the dissociation constant of HO_2 is 10^{-2} moles liter^{-1}, hence at biological pH's, i.e. pH 3 and higher, HO_2 would be entirely dissociated. Because the lowest pH used by Alper and Ebert was 4.8, there is thus a question as to whether the effect can be attributed to dissociation of HO_2. In a later paper by Alper and Howard-Flanders (1956), in which the equation mentioned under the oxygen effect was first presented, it was considered unlikely that the effect of oxygen is via the HO_2 radical, on the basis of the value of K obtained for the equation. It was postulated instead that the oxygen acts directly upon the target molecule which was first damaged by the ionization; no explanation was offered for the pH effect observed earlier.

Proctor et al. (1958) found that the sensitivity of Bacillus thermoacidurans to 3-Mev electrons was greater at pH 2.2 and 10.0 than at pH 7.0. Littman, Carr, and Brady (1957) used atomic hydrogen from an arc to reduce cysteine, and found that the production of H_2S was increased above pH 8, while the production of cystine was maximum at pH 7. Various radiochemical reactions were proposed as being responsible.

It is surprising that more work has not been done on the effect of pH, as there are many radiochemical reactions which are pH dependent (Dewhurst, Samuel and Magee, 1954). Many regions of

large pH effect lie outside the range of biological material, however, and many radiochemical reactions are not well established or are greatly affected by small amounts of solute.

Another difficulty in experiments involving pH is the difficulty in determining intracellular pH. There are relatively few vital dyes useful as pH indicators, and they may be misleading. For instance, neutral red is selectively absorbed by fat globules. It seems very likely, moreover, that because living cells contain a wealth of organic molecules as well as salts, they buffer their internal pH to a relatively constant value regardless of the external environment. It is known, for example, that isolated cell enzymes have pH optima often quite different from the pH of the intracellular fluid, and these optimum pH's are probably maintained locally in the cell by mechanisms such as Donnan equilibrium at membrane surfaces. Hence "intracellular pH" is probably not only difficult to alter but quite likely not a useful concept.

Nevertheless, it should be possible by varying the pH of the suspending medium to obtain some information as to whether or not radicals produced in the medium by radiation, presumably by reactions whose rates are pH dependent, can diffuse into the cell and damage it. Some studies of this nature are reported in this paper.

III. Post-irradiation modification

The survival of microorganisms after exposure to ionizing radiation can be altered by a variety of factors, although the number and complexity is not as great as with ultraviolet. Among such factors are temperature of postirradiation incubation (Stapleton, Billen and Hollaender, 1953), injection of certain cell fractions of unirradiated amoebae into irradiated amoebae (Daniels, 1956), presence of certain nutrients in the incubation medium (Stapleton, Sbarra and Hollaender, 1955), and presence of certain inhibitors in the incubation medium (Alper and Gillies, 1958a).

After ultraviolet irradiation of E. coli., Roberts and Aldous (1949) found no variation in recovery when the pH of liquid in which

irradiated bacteria were held after irradiation was varied between 5 and 9. Weatherwax (1956), however, found that the pH of the agar medium on which the cells were incubated after uv irradiation exerted a very large effect on recovery, being greatest at pH 4.8 and declining up to pH 8. He also found that this effect was exerted entirely before the first postirradiation division. No study of the effect of the pH of the postirradiation medium has been reported for ionizing radiation; such a study is reported in this paper.

Most attention has been centered on the nutritional aspects of recovery. Stapleton, Sbarra, and Hollaender (1955) found that a minimal medium of salts and glucose allowed the least survival with E. coli., but when the medium was supplemented with yeast extract or spleen extract, recovery was manifested. It was found that a combination of either glutamic acid, glutamine, or aspartic acid with guanine, uracil, and certain salts gave about the same recovery as spleen extract. Growth on a salts medium before irradiation gave highest recovery on all incubation media, growth in broth the poorest. They considered this to be evidence that new enzyme synthesis is necessary for recovery, since broth-grown cells were supplied with synthetic intermediates and presumably had not built up a stock of synthesizing enzymes. This did not explain the requirement for guanine and uracil, which are not used as building blocks in synthesis of enzymes.

Doudney (1956) found that E. coli which were protected by cysteamine during irradiation recovered radiation-blocked DNA synthesis if they were given certain amino acids plus guanine and uracil after irradiation. (It was not clear whether the block was on DNA synthesis itself or at some other point in the division process). He proposed that new protein and RNA synthesis compete with DNA synthesis, and that imbalance growth is a cause of cell death. Alper and Gillies (1958 b and c) support this view, having found that many "recovery" treatments act by the common mechanism of imposing suboptimal growth conditions, preventing an imbalance of synthetic

processes. Some further studies of nutritional recovery are reported in this paper.

By control of pH during growth, irradiation, and postirradiation incubation, combined with anoxic and aerobic conditions, additional information might be obtained on some of the aspects of radiobiology discussed above. Results of experiments of this nature are discussed in this paper.

MATERIALS AND METHODS

I. Organism Used

The stock of E. coli, B/r, used was originally obtained from Dr. Evelyn Witkin through Dr. R. Lowry Dobson of this laboratory. Lyophilized samples of this stock were prepared by Dr. Robert S. Weatherwax. Nutrient agar slants were prepared from cultures of these samples for experiment. Subculturing of stocks was avoided as much as possible, one slant being used for many experiments in order to avoid the possibility of mutation and selection especially of revertants to Strain B.

II. Culturing Methods

The culture medium used was nutrient broth (Difco). Cultures were incubated at 37° C for approximately 17 to 20 hours. The culture was agitated continuously by a stream of bubbles of either air or nitrogen (Linde water-pumped) which first had been passed through sterile water. The gas was admitted to the bottom of the culture tube by a glass tube that passed down the outside of the culture tube and entered at the bottom. This made the culture easily accessible to pipettes or pH-meter electrodes without interrupting the flow of gas.

III. pH Control

All growth and plating media that were controlled as to hydrogen-ion concentration were adjusted by addition of 10/15 M tribasic, dibasic, or monobasic phosphates or phosphoric acid. The final concentration of phosphate ion was always less than or equal to 1/15 M, and the molar sodium: potassium ratio always approximately 3:1. Buffer solutions were similarly 1/15M with sodium: potassium ratio of 3:1, and also contained 0.2 gm $MgSO_4$ per liter.

In cultures in which the pH was controlled during growth, the medium was first brought to the desired pH as measured by a Beckman

model-H2 pH meter, by addition of the appropriate phosphate. When wiped with sterile gauze, the electrodes did not introduce any contaminants. The culture medium was then inoculated with approximately one hundredth of the volume of a previous stationary culture at the same pH. Frequent additions of phosphate were found necessary to maintain constant pH when growing cultures at the opposite extreme of their normal pH. When the final titer was attained the culture was centrifuged, the plug rinsed, and resuspended to one-quarter volume in buffer of the same pH. To exhaust all nutrients the washed culture was bubbled at 37° C for 1 to 2 hours with the same gas with which it was cultured.

IV. Irradiation Procedure

For X-irradiation, 10λ (0.01 ml) of the full-titre suspension, 10^8 to 10^9 cells per ml was pipetted onto blocks of pure 3% agar (Difco) approximately 7 mm. in diameter. Since water absorbs strongly the x-rays of the energy used here the liquid was allowed to be completely absorbed by the agar before irradiation. In this way the bacteria remained on the surface of the agar and were exposed directly to the x-rays. From one to three blocks were placed inside a cylindrical Lucite chamber 22 mm in diameter and 27 mm high, with a top 0.25 mm thick. The same thickness of water (average atomic number similar to Lucite) absorbs about 13% of 50-kv x-rays. Survival curves with and without the top on the chamber showed, for any particular survival, that the dose without the top was about 13% higher than with the top. Measured doses are corrected for this absorption throughout this paper.

V. Gases During Irradiation

For irradiation in air, the blocks were placed in the chamber and irradiated without gas flushing. For irradiation in a nitrogen atmosphere, tank nitrogen was passed over red-hot copper to remove all traces of oxygen [as described by Howard-Flanders and Alper

(1957)], washed in sterile water. The chamber was flushed with this nitrogen for at least 10 min prior to, and during, irradiation. Flushing for 2 minutes was found to cause no further increase in survival.

VI. X-ray Source

The x-rays used in these experiments were generated by a Machlett OEG-60 x-ray tube with a beryllium end window 1-in. diam by 1-mm thick. No additional filtration was employed. The water-cooled target was 2 cm from the outer surface of the window. Adjustable shelves at various distances from the target made possible selection of different dose rates. The output of this machine was calibrated by Mortimer (1953). With the machine operating at 50 kv and 25 ma, the dose rate at the fifth shelf (used in these experiments) was 660 roentgens per second (r per sec). The dose rate under the Lucite top was thus 574 r per sec.

VII. Plating Methods and Media

After irradiation the blocks were placed in 1.0 ml of pH 7 buffer in a test tube and shaken; experiments showed that essentially all bacteria on the blocks were resuspended. Further dilutions with buffer of pH the same as that of the plate to be used reduced the viable titer to an appropriate value for plating.

Aliquots of from 0.05 to 1.0 ml were spread on the agar by means of a bent glass rod and incubated for 24 hr at 37° C. The criterion of cell survival was ability to form macrocolonies on agar. Plates were always brought to incubation temperature before plating and quickly returned to the incubator.

The agar medium was nutrient agar (Difco) or a synthetic minimal salts medium of the following composition:

$Na_2HPO_4 \cdot 2H_2O$	5.94 gms
KH_2PO_4	4.54
NaCl	0.5
$MgSO_4$	0.2
NH_4Cl	1.0
Glucose	4.0
Bacto-Agar (Difco)	15.0
H_2O	1 liter

Unless noted otherwise, the pH of the nutrient-agar plates was 6.8, and that of the synthetic-medium agar 7.0.

When the hydrogen-ion concentration of the agar plates was different than noted above, the desired pH was established by adding to the agar medium after autoclaving, sterile phosphate buffers described previously. The pH of the agar was checked by placing the electrodes of the Beckman pH meter in a 5-ml aliquot of the molten agar at about 45° C; the pH did not change appreciably upon solidification. The pH of the agar was always within 0.1 pH unit of the values specified.

In experiments using plates having different values of pH, survivals are expressed as fractions of controls plated without irradiation onto agar of the same pH. The dilution from which these platings were made were always in buffer of the same pH as the plate, so that the buffer would not change the pH at the surface of the agar.

VIII. DNA Determinations

The determinations of deoxyribose nucleic acid were made under the direction of Dr. Lola S. Kelly. The method used was essentially that of Schneider (1945). The indole reaction of Cerioti (1955) was used for determination of DNA, by the use of a Beckman DU spectrophotometer at a wavelength of 486 mμ. All samples were done in duplicate and compared to a DNA standard that was run in triplicate. All readings were compared to a blank of the solvent used for the DNA solutions.

IX. Statistics

Each point on the survival curves represents an average of colony counts on three or more plates. The range of error indicated is the standard error, with the standard error of the control plates (titer of unirradiated culture) included as a compounded error. Most of the curves represent two or more separate experiments.

RESULTS AND DISCUSSION

I. Growth and Recovery at Controlled pH.

1. Physiological effects of pH.

In the course of considering what effect the pH of the suspending medium during irradiation might have on E. coli, it was considered possible that suspending cells at a pH different from that at which they were grown might cause a shock to the cell, which would also affect survival. In order to eliminate this possibility cells were grown at constant pH as described under Materials and Methods, and irradiated at the same pH at which they were grown. If differences in survival result from growth at different pH values, then further experiments must be performed to distinguish between the effect of pH during growth and during irradiation.

Differences in the cell physiology might well be expected after growth at different pH values. Gale (1947) lists thirteen enzymes of E. coli, the activities of which are different if the bacteria are grown in casein digest at pH 5 or 7, or in 2% glucose with a final pH of 5.2. Blackwood et al. (1956) found that glucose fermentation at pH 5 produces ethanol, acetic and lactic acids, CO_2, and H_2, while at basic pH the major product is formic acid. It is unlikely that the presence of different exogenous metabolic end products would affect the survival, because they are removed by centrifugation. Conceivably, end products might remain intracellularly, and Hollaender, Baker, and Anderson (1951) found that formate, when present during irradiation protects E. coli. It will be seen, however, that growth conditions where formate is produced, namely growth at pH 8.5, cause much reduced survival. Production of different end products implies a difference in enzyme activities, an effect more likely to be a basis for differences in radiosensitivity.

It is generally thought that inactivation of a cell by ionizing radiation results from a change in a single or at most only a few vital

molecules in the cell. If a cell contained only one or a few molecules of any particular enzyme which if destroyed would inactivate the cell, the cell should be able to synthesize new enzyme unless the time at which the enzyme acted were of vital importance. Sufficient time might not then be available for new synthesis to replace the enzyme, for instance before the onset of the next division. The various recovery processes discussed earlier would then presumably act upon the synthesis of new enzyme being made to replace the destroyed one. Ability to synthesize new enzyme may depend not only on nutritional elements and temperature at time of synthesis but also upon the already existing complement of synthetic enzymes, which in turn would depend on the conditions of growth (Stapleton, Sbarra and Hollaender, 1955).

It might be expected that since growth at different pH values favors the synthesis of different enzymes, the ability to recover might be similarly affected. It also seems reasonable to suppose that the recovery process itself might have synthetic processes with pH optima, since growth after irradiation would likewise produce different enzyme complements varying with pH. However, since it is well known [Witkin (1956), Weatherwax, (1956)] that the recovery process occurs only in the relatively short time before the first postirradiation division, the pH might be expected to affect not so much the buildup of a particular enzyme complement as the rate of some specific reaction involved in recovery.

Survival might, moreover, involve interedependence of pH during growth both before and after irradiation. An enzyme produced optimally at one pH before irradiation may be required for a recovery process occurring optimally at another pH.

In order to test these possibilities, E. coli, B/r, were grown at pH 5, 7, and 8.5 both aerobically and anaerobically, irradiated at the same pH, and plated on media of pH 5, 6, 7, 8 and 9.

2. Aerobic growth

It can be seen from Fig. 1a and b and 2a that there is very little effect of plate pH when the cells are aerobically grown and plated

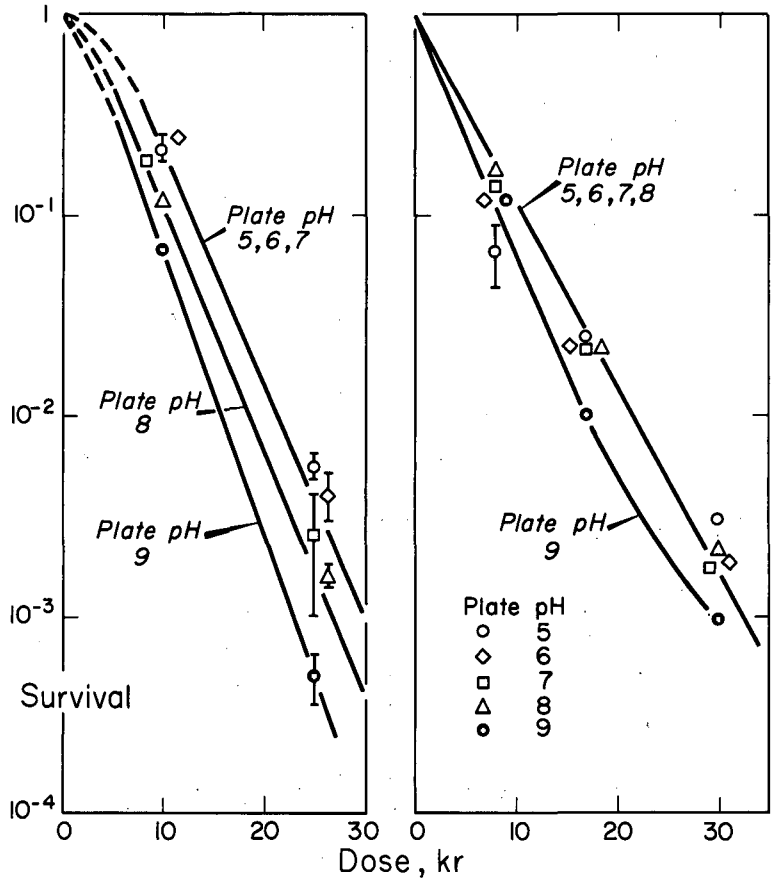


Fig. 1. X-ray survival curves of E. coli, B/r, grown aerobically in nutrient broth at controlled pH. Irradiated in air at growth pH, incubated on nutrient agar at various pH values. (a) Grown at pH 5; (b) grown at pH 7.

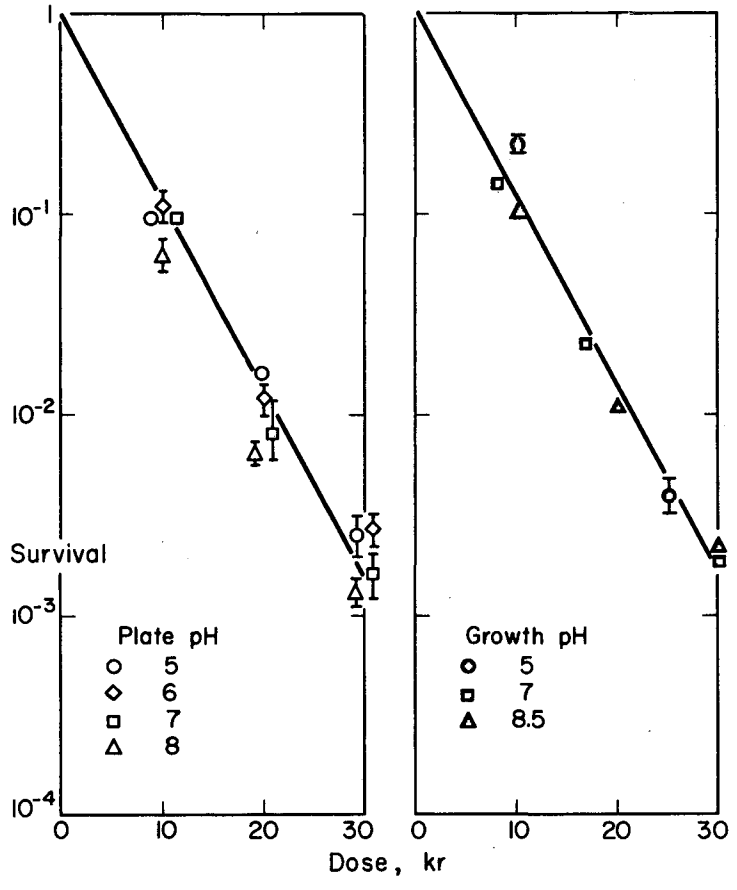


Fig. 2. X-ray survival curves of E. coli, B/r, grown aerobically in nutrient broth at controlled pH. Irradiated in air at growth pH, incubated on nutrient agar at various pH. (a) Grown at pH 8.5; (b) averages of survival on plates of pH 5, 6, and 7 at each growth pH.

on nutrient agar, with the exception of a slight tendency to lower survival on alkaline medium when the cells were grown at acid pH. It seems likely that this is a synergistic effect of irradiation and pH shock.

For each dose point at each growth pH the survivals on plates of pH 5, 6, and 7 were averaged, and the average survival at each growth pH is plotted in Fig. 2b. It can be seen that a single straight line adequately fits all the points and that there is no effect of growth pH on survival. There is thus also no effect of the pH of the medium in which the cells are irradiated, except in the unlikely event that the effects of growth and irradiation pH are equal and opposite.

3. Anaerobic growth

When B/r is grown anaerobically in glucose nutrient broth at different pH values there are more marked effects on x-ray survival as shown in Fig. 3, 4, and 5. The effect of pH of nutrient agar plates is similar to but greater than that with aerobically grown cells, showing increased sensitivity when cells grown at pH 5 are incubated on alkaline plates, a similar but smaller effect when grown at pH 7, but no effect of plate pH when grown at pH 8.5. If pH shock alone were responsible for the decreased survival of acid-grown cells on alkaline plates, it might be expected that the reverse shock of plating alkaline-grown cells on acid media would show a similar sensitivity, but this is not observed. This does not exclude the possibility that acid-grown cells, both aerobically and anaerobically grown, are sensitive to pH shock while alkaline grown cells are not. It seems more likely that anaerobic acid growth produces some condition in the cell which alters the susceptibility of the recovery process to pH change.

The nature of this condition may be suggested by examining survival on minimal-salts medium in Figs. 3, 6 and 7. When grown at pH 7 or 8.5 there is no effect of plate pH when minimal medium is used, but when grown at pH 5 there is a marked optimum of survival using pH 6 plates. Moreover, growth at pH 5 and 7 produces sigmoidal survival curves on nutrient agar, less so at pH 7, but on minimal

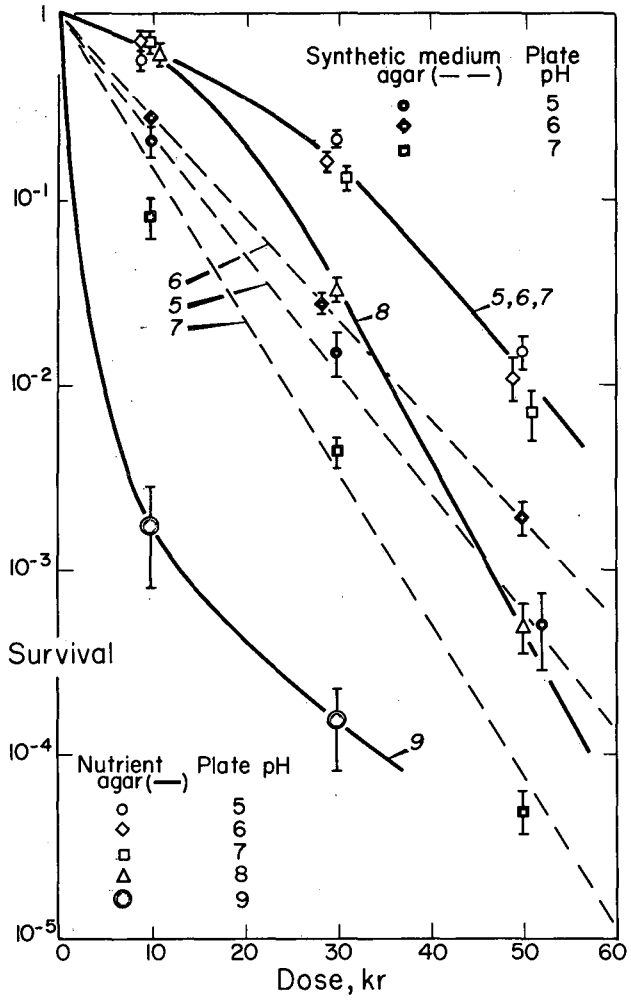


Fig. 3. X-ray survival curves of E. coli, B/r, grown anaerobically in glucose nutrient broth at pH 5. Irradiated in air at pH 5, incubated on nutrient agar and on synthetic-medium agar at various pH.

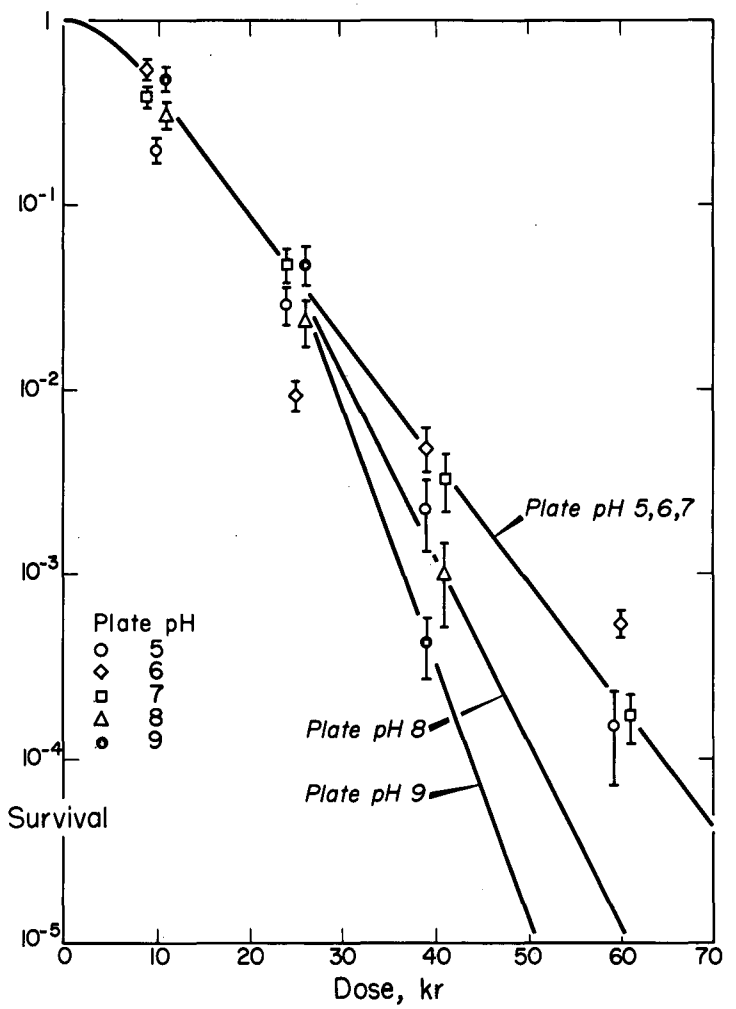


Fig. 4. X-ray survival curves of E. coli, B/r, grown anaerobically in glucose nutrient broth at pH 7. Irradiated in air at pH 7, incubated on nutrient agar at various pH.

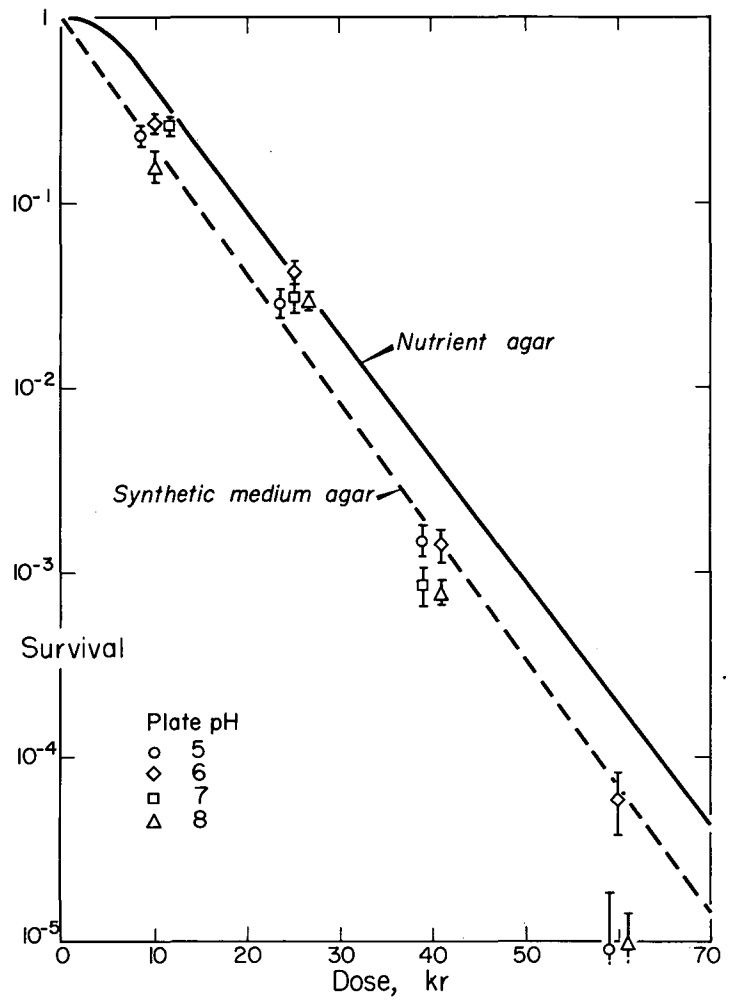
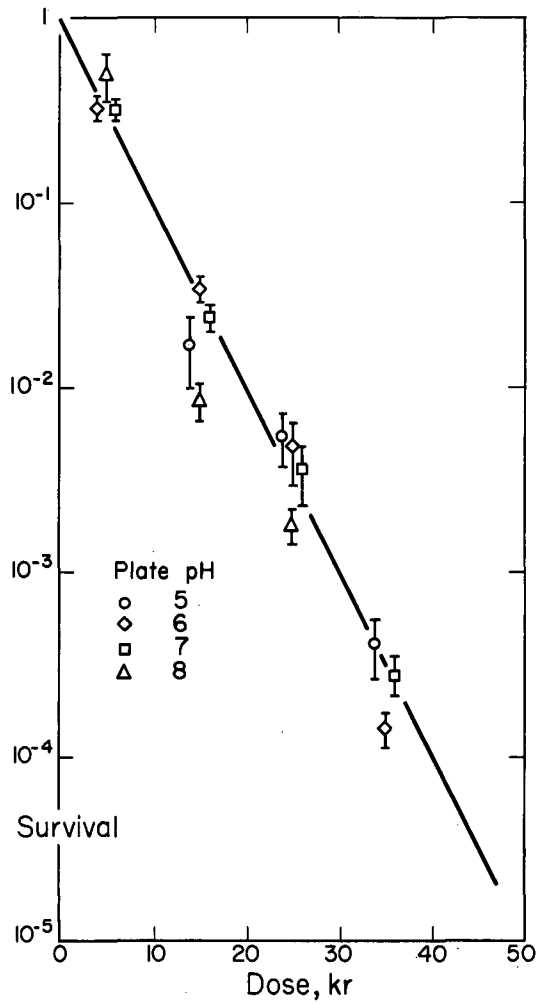


Fig. 5. X-ray survival curves of E. coli, B/r, grown anaerobically in glucose nutrient broth at pH 8.5. Irradiated in air at pH 8.5. Irradiated in air at pH 8.5, incubated on nutrient agar at various pH values.



MU-16270

Fig. 6. X-ray survival curves of E. coli, B/r, grown anaerobically in glucose nutrient broth at pH 7. Irradiated in air at pH 7, incubated on synthetic-medium agar at various pH values. Nutrient-agar curve from Fig. 4.

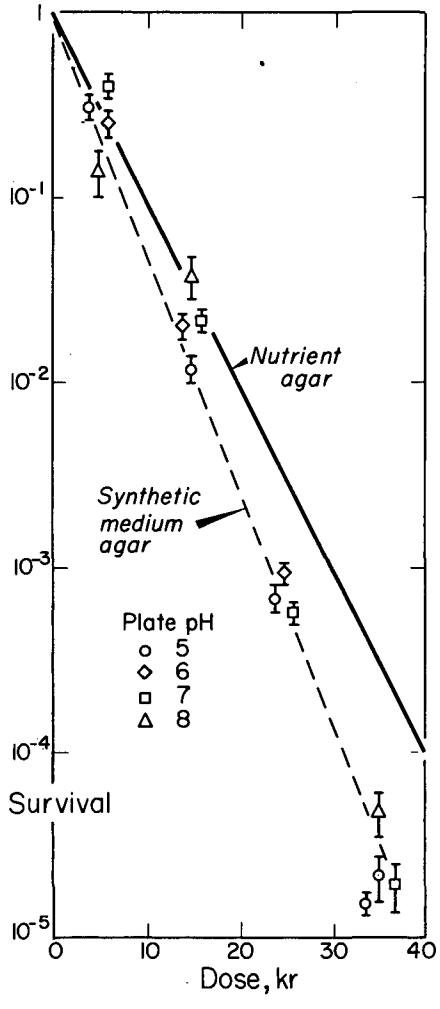


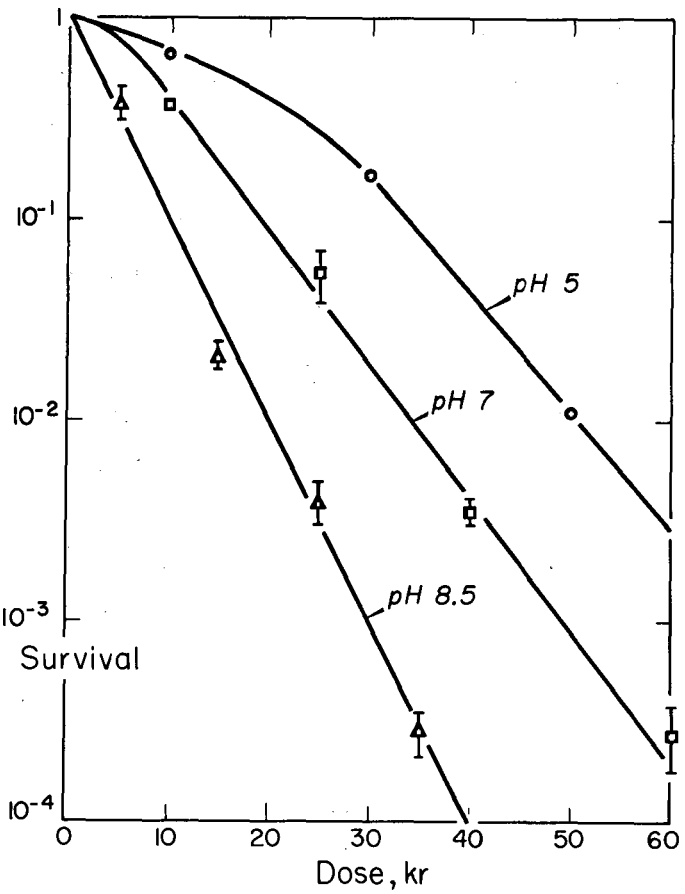
Fig. 7. X-ray survival curves of E. coli, B/r, grown anaerobically in glucose nutrient broth at pH 8.5. Irradiated in air at pH 8.5, incubated on synthetic-medium agar at various pH values. Nutrient agar curve from Fig. 5.

medium the curves are always exponential. This recalls the results of Atwood and Mukai (1951) mentioned earlier, where survival of one compared to two nuclei changed the shape of the survival curve from exponential to sigmoid.

It might be postulated that anaerobic growth at pH 5 produces cells with multiple targets, and that two types of damage occur in these cells. One type of damage involves one target only, and this target differs from the others in that when it is inactivated the entire cell is inactivated. This target might be one involved in cytoplasmic as distinguished from nuclear division. Furthermore, to fit the experimental data, damage to this target must be recoverable on nutrient agar but not on synthetic-medium agar. The other type of damage would be of the classical multiple-target type, in which all of the targets must be inactivated to inactivate the cell. Such cells would yield a sigmoidal curve when plated on nutrient agar, because the single-hit damage would recover. Plated on synthetic-medium agar, cells with surviving targets of the multiple-hit type would nevertheless not survive if the single-hit target were inactivated, hence the survival curves would be essentially exponential.

The largest effect of pH, however, is that caused by growth in anaerobic glucose broth. The points of the curves of Figs. 3, 4, and 5 are averaged from nutrient-agar plates of pH 5, 6, and 7 which gave identical survival curves, and are plotted in Fig. 8. It can be seen that growth at pH 5, the normal condition for such cells without pH control, produces cells which show a survival curve similar to that found by Hollaender, Stapleton, and Martin (1951). However, at pH 7 the curve becomes less sigmoidal, and the slope becomes a little steeper. At pH 8.5 the curve is strictly exponential and the slope much steeper; it is in fact, indistinguishable from the curve for aerobically grown cells in Fig. 2. The nature of the mechanism responsible for this change will be discussed in Section IV.

There is also the question of whether the observed effect is dependent upon the pH during anaerobic growth or during irradiation.



MU-16272

Fig. 8. X-ray survival curves of E. coli, B/r, grown anaerobically in glucose nutrient broth at pH 5, 7, and 8.5. Irradiated in air at same pH, respectively. Each point is average survival on nutrient agar at pH 5, 6, and 7 at each growth pH, from Figs. 3, 4, and 5.

Examination of this point will be deferred until Section III after the oxygen effect on cells grown at different pH values has been discussed.

II. The Oxygen Effect and Growth at Controlled pH

Hollaender, Baker, and Anderson (1951, p. 324) attributed the very considerable protection afforded E. coli, B/r, by growth in glucose broth under anaerobic conditions to an extreme reduction of intracellular oxygen. This protection occurs whether oxygen is present or absent during irradiation and is additive to the protection due to the lack of extracellular oxygen. Because the protection afforded by anaerobic growth was found to vanish when the growth pH was kept the same as that during aerobic growth, as shown in the previous section, the next question was whether the protection also vanished when the cells were irradiated anoxically.

1. Growth at pH 5

Cultures grown at pH 5 in glucose broth with continuous nitrogen bubbling at 37° (the usual conditions of anaerobic growth) were first irradiated in air and in purified nitrogen. The survival curves are shown in Fig. 9. Considerable variation was found from one culture to another as evidenced by the scatter of points. When the dose-modification factor is not constant at all doses, the ratio of doses at a particular survival is usually used. This ratio is not constant with dose in Fig. 9; the ratio of dose in nitrogen to dose in air is approximately 2.8 at 10^{-1} survival and 2.3 at 10^{-2} survival. Both of these are somewhat higher than usually reported for 50-kv x-rays, as mentioned previously.

Extrapolation of the linear portions of these curves to zero dose yields a number that can be interpreted on the basis of target theory as the average number of sensitive sites per cell. The intercept for cells irradiated in air is approximately 2.5, and irradiated in nitrogen approximately 18. A similar extrapolation applied to the data of Hollaender et al. (1951) yields approximately 100 and 24, respectively, agreeing fairly well for the latter but differing greatly for the former.

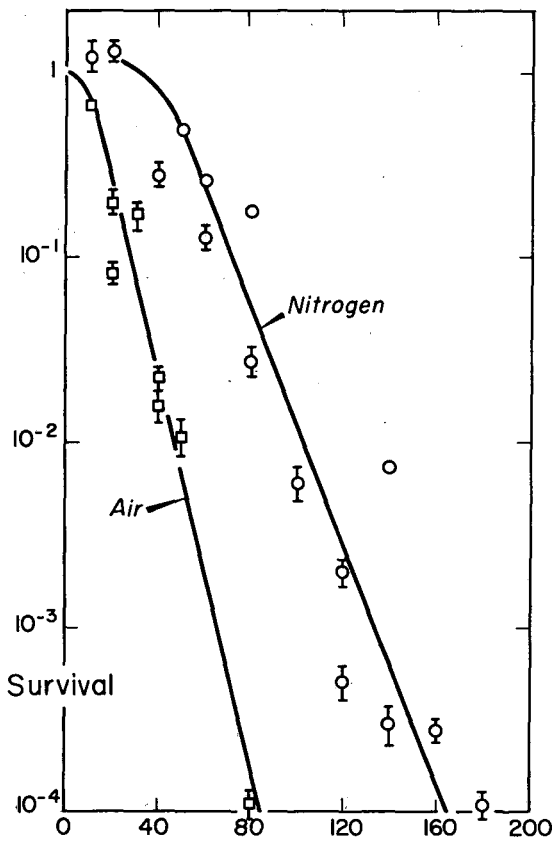


Fig. 9. X-ray survival curves of E. coli, B/r, grown anaerobically in glucose nutrient broth at pH 5. Irradiated at pH 5 in air and in nitrogen. Incubated on unbuffered nutrient agar at pH 6.8.

To have cells from the same culture with apparently different numbers of sensitive sites depending on the presence or absence of oxygen during irradiation seems difficult to explain. It might be assumed that some of these sites are sensitive to radiation damage in the presence of oxygen and are not sensitive in its absence. A smaller extrapolated target number would then be expected in the presence of oxygen, as obtained here, but the reverse would not be expected as found by Hollaender et al.

2. Growth at pH 8.5

Cultures grown anaerobically at a constant pH of 8.5 were irradiated in air and in purified nitrogen; the survival curves are shown in Fig. 10. The curves of Figs. 8 and 9 are redrawn without points in Fig. 10 for comparison.

The ratio of dose in nitrogen to dose in air is quite close to 3 at all survivals for cells grown anaerobically at pH 8.5. This is very much the same ratio as that for cells grown anaerobically at pH 5, and it thus seems that the oxygen effect during irradiation is relatively independent of the conditions during growth. Depletion of intracellular oxygen by anaerobic growth is not ruled out, although independence from the oxygen effect makes it very unlikely. Anaerobic growth at pH 8.5, might conceivably cause failure to synthesize, or prevent the action of, an enzyme synthesized during growth at pH 5.0 which removes intracellular oxygen.

Growth anaerobically at pH 8.5 produces a survival curve with a slope approximately the same as that for growth at pH 5, but the apparent target number when irradiated in nitrogen falls from 24 for pH-5 cells to about 1.5 for pH-8.5 cells. When the two cultures are irradiated in air, the apparent target number falls from 2.5 to 1, i.e. an exponential curve, but in air the slope becomes more steep at pH 7 and 8.5 rather than remaining parallel. Because of the wide scatter of points for the anaerobic pH-5 cells in nitrogen, it is not sufficiently certain that the curve is parallel to the pH-8.5 nitrogen curve to speculate on the difference in behavior of the slopes in air and in nitrogen.

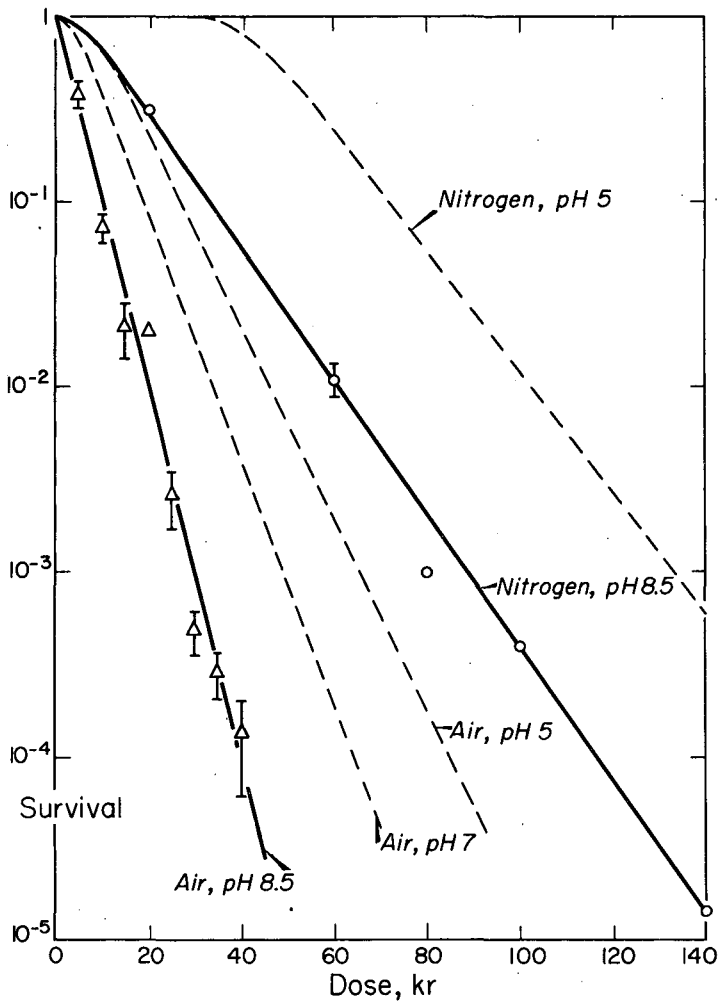


Fig. 10. X-ray survival curves of E. coli, B/r, grown anaerobically in glucose nutrient broth at pH 8.5. Irradiated at pH 8.5 in air and in nitrogen. Incubated on unbuffered nutrient agar, pH 6.8. Dashed-line curves for anaerobic growth at pH 5 and 7 are from Figs. 9 and 8, respectively.

However, the change in apparent target number is at least qualitatively significant and will be discussed after presentation of additional data in Section IV.

Survival curves of cells grown at pH 8.5 are compared in Fig. 11; the curves for irradiation in nitrogen are redrawn from Fig. 10. It can be seen that although anaerobic growth at pH 8.5 reduces the resistance of the cells to approximately that of aerobic growth at the same pH, there is still an additional protection afforded by anerobic growth. This protection can be represented by a dose-reduction factor which is very close to 1.6 for cells irradiated either in nitrogen or in air. Thus it appears that protection by anaerobic growth is not related to the effect of atmosphere during irradiation, as suggested by Hollaender, Baker, and Anderson (1951). The nature of the protection by anaerobic growth will be further discussed in Section IV.

There is an apparent contradiction of the above, which occurs at the end of Section I.3., in which it is stated that anaerobic pH-8.5 cells irradiated in air yield a survival curve (Fig. 8) indistinguishable from that for aerobic cells in Fig. 2b. Aerobically grown cells in Fig. 11 show a steeper slope than in Fig. 2b. The only difference between the cultures of Fig. 11 and 2b is that the cells of Fig. 2b were plated on nutrient agar containing 1/15M phosphate buffer, while the cells of Fig. 11 were plated on nutrient agar without phosphate. The aerobic-air survival curve of Fig. 11 is the same whether the cells are grown with phosphate added to the nutrient broth or not, but no experiment was performed in which a single culture was grown with phosphate and incubated on plates both with and without phosphate. In other words, it is possible that the presence of phosphate during growth and incubation produces the higher survival of Fig. 2b. This possibility was tested for anaerobically grown cells, and it was found that presence or absence of phosphate in the plates had no effect on survival, but it was not tested for aerobically grown cells.

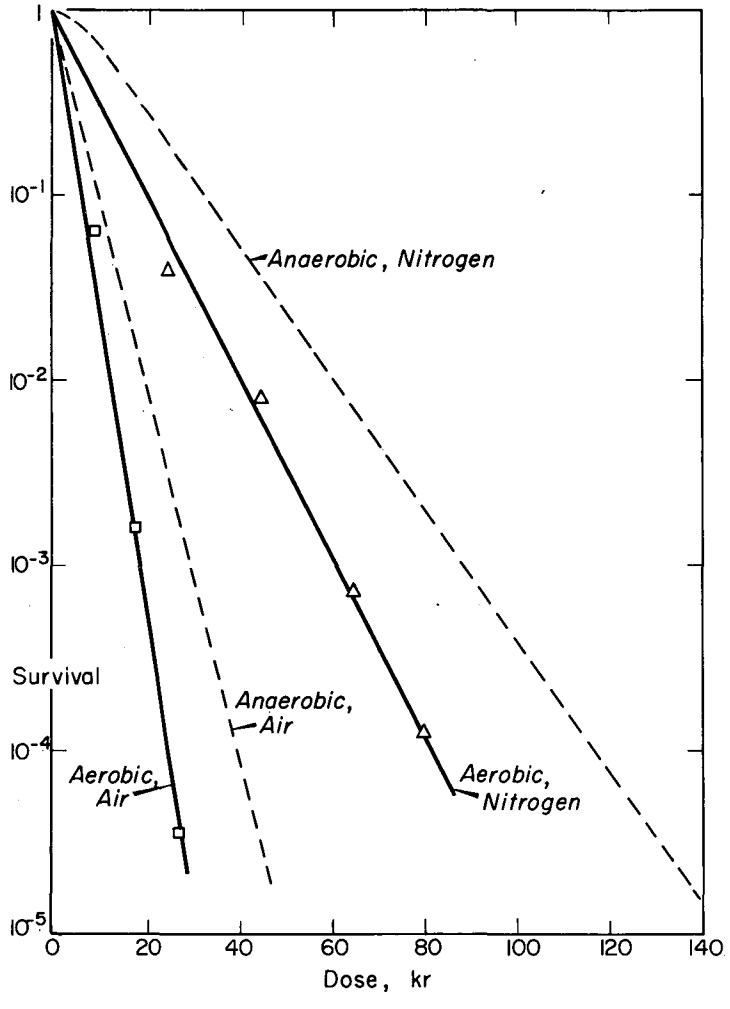


Fig. 11. X-ray survival curves of E. coli, B/r, grown aerobically and anerobically at pH 8.5. Irradiated in air and in nitrogen at pH 8.5. Incubated on unbuffered nutrient agar at pH 6.8. Dashed-line curves for anaerobic pH-8.5 growth are from Fig. 10.

III. The Effect of pH during Irradiation

1. Aerobic Growth

As shown in Fig. 2b there is no change in survival of aerobically grown cells when they are grown at different values of pH and irradiated at the growth pH. There is the possibility, as mentioned in Section I. 2., that the effects of pH during growth and irradiation are equal and opposite, but because of the unlikelihood of such additivity at three different pH values this possibility was not examined.

2. Anaerobic Growth

The large changes in sensitivity of cells grown anaerobically at different pH's made it necessary to determine whether the change was due to the pH during growth or during irradiation.

Cultures grown anaerobically at pH 5 and 8.5 were centrifuged and resuspended in buffer of the same pH and starved anaerobically for 2 hr. Two aliquots were then withdrawn from each culture and centrifuged. One was resuspended at pH 5.0 and the other at pH 8.5 as determined by pH meter. Irradiation was carried out on agar blocks buffered to the same pH, one at each pH being irradiated side by side for each dose point. Irradiations were done both in air and in purified nitrogen.

As seen in Fig. 12, when anaerobic pH-5 cells are irradiated at pH 8.5, the radiosensitivity is increased, the ratio of doses at survivals below 50% being about 1.3. This is much less than when the cells are grown at these two pH's. The ratio is the same for cells irradiated either in air or in nitrogen, indicating that the mechanism is independent of the oxygen effect. Irradiation at pH 7 produces survival about the same as at pH 5.

When anaerobic pH-8.5 cells are irradiated at pH 5, as shown in Fig. 13, there is no increase in resistance toward that of pH-5-grown cells, in either air or nitrogen, corresponding to the change shown in Fig. 12. If the change in radiosensitivity with pH of Fig. 12 were due to an effect of pH on chemical intermediates produced in the medium it would be expected that a similar change would occur in the

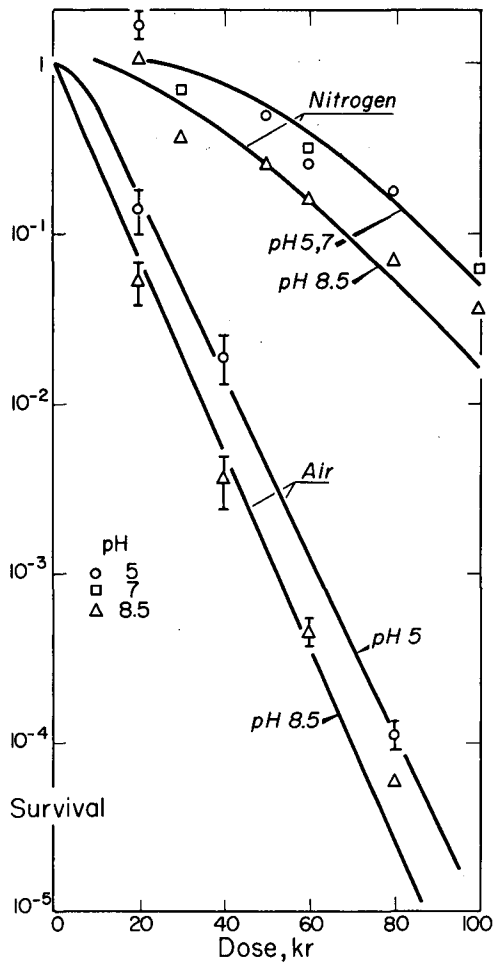


Fig. 12. X-ray survival curves of E. coli, B/r, grown anaerobically at pH 5. Irradiated in air and in nitrogen at pH 5, 7, and 8.5. Incubated on unbuffered nutrient agar pH 6.8.

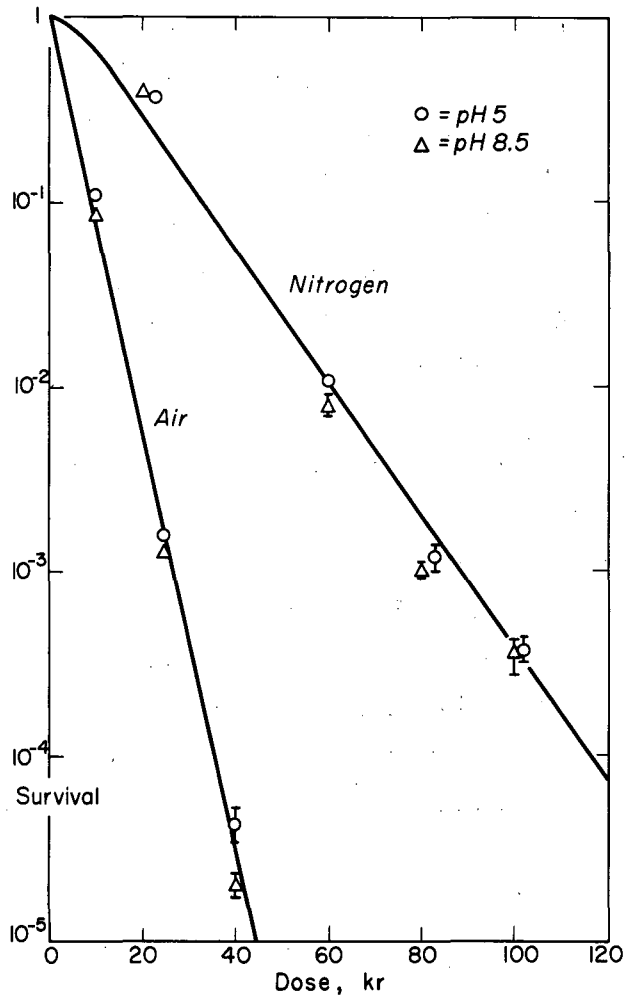


Fig. 13. X-ray survival curves of E. coli, B/r, grown anaerobically at pH 8.5. Irradiated in air and in nitrogen at pH 8.5 and at pH 5. Incubated on unbuffered nutrient agar pH 6.8.

curves of Fig. 13. Such a change does not occur, however; a mechanism for the change which occurs with anaerobically grown pH-5 cells will be proposed in Section IV.

The pH's examined here cover at least part of the range of pH-sensitive radiochemical reactions discussed in the Introduction, Section II. 4. In particular the fall in production of H_2O_2 found between pH 8 and 9 by Bonet-Maury and Lefort (1950) should have produced increased survival at pH 8.5 if H_2O_2 is involved; survival at this pH was found to be decreased, however, for anaerobically grown cells, and unchanged for aerobically grown cells. This is additional evidence for discarding the already unpopular hypothesis of damage due to intercellular H_2O_2 produced by radiation.

There is a total lack of effect of pH during irradiation which can be attributed to radiochemical intermediates produced in the medium. This lends support to the conclusions of Howard-Flanders, in press, and Kimball (1957) p. 213: "... intercellular indirect action is improbable except under special circumstances."

Because of the impossibility of changing or determining the intracellular pH, as discussed on page 13 of this work, nothing can be said about what effect pH might have on radiochemical intermediates which may be produced within the cell.

IV. Cell Morphology and Growth at Controlled pH.

1. Titer, Generation Time, Microscopic Appearance during Irradiation

Changes in final titer, generation time, and cell morphology were observed with variation in growth pH under aerobic and anaerobic conditions, as shown in Table I.

Table I

Culture conditions	Culture pH	Generation time (min)	Final titer	Cell morphology (stationary phase)
Aerobic broth	5	90	4.4×10^9	small, spherical
	8.5	20	1×10^9	small, spherical
Anaerobic glucose broth	5	120	2.8×10^8	elongated, multinucleate
	8.5	22	1.2×10^9	short rod

Under normal conditions, with pH not externally controlled, a culture of aerobic broth-grown cells reaches a final pH of 8.5 at stationary phase, while an anaerobic glucose culture produces a final pH of from 5.0 to 4.8. It can be seen that controlled pH has the general effect that growth at the abnormal extreme of pH, for both aerobic and anaerobic conditions, produces a final titer about four times higher than normal, and changes the generation time by a factor of about five.

The appearance of the anaerobically grown cells during controlled pH growth is interesting. During logarithmic growth at pH 5 they maintain their rod form, the average length of the rods increasing somewhat when stationary phase is reached. Under phase contrast these stationary phase rods can be seen to contain from 4 to 8 nuclei each, although some of the nuclei are much less distinct than others. These bodies seen with phase contrast have been identified with the genetic material in E. coli, by other authors [See Cortelyou, Amundson, and Mc Whinnie (1956) for references.]

During logarithmic growth at pH 8.5, tremendously long filaments are produced, with nodes marking off distances along the filament approximately the length of normal rod-shaped cells. As stationary phase is reached, these long filaments disappear very rapidly, and in their place are found short rods resembling those of normal aerobic

log-phase growth. Although not actually observed it was presumed that the long filaments divided into short rods when stationary phase was reached.

2. DNA Content

Because of the sigmoidal nature of the anaerobic pH-5 survival curves and the microscopic appearance of the cells, it was decided to do an analysis of DNA in the various cultures as described in Materials and Methods. The results are summarized in the Table II.

Table II

Method of culture	Average Optical Density 486 mμ	γ DNA in 1.0 ml of culture	Number of bacteria in 1 ml sample	γ DNA per bacterium	DNA ratio
Aerobic, broth, unbuffered, final pH 8.5	0.294	100.8	4.45×10^{9}	2.26×10^{-8}	1.0
Anaerobic, glucose, broth, unbuffered, final pH 5.0	0.217	74.3	6.5×10^{8}	11.4×10^{-8}	5.0
Anaerobic, glucose, broth, buffered pH 8.5	0.302	103.3	2.4×10^{9}	4.31×10^{-8}	1.9

The optical density of a 2 ml cuvette of pure DNA standard of 17.6 γ/cc was 0.257. Only 2 ml. from the 5 ml. of DNA solution obtained from each sample was measured in a 2 ml. cuvette, so γ DNA per sample was obtained by:

$$\frac{\text{OD sample}}{\text{OD standard}} \times 17.6 \times 5 = \gamma \text{ DNA in 1 ml of culture.}$$

The value of 2.26×10^{-8} γ per bacterium obtained for an aerobic culture of E. coli, B/r, is in reasonable agreement with that found by

Morse and Carter (1949), which was 3.5×10^{-8} γ per bacterium for resting cells. Their culture medium contained 0.5% NaCl, while that used here did not.

3. Survival Curves and Cell Morphology

The remarkably high radioresistance of anaerobically grown E. coli, B/r, can now be reasonably explained on the basis of the results obtained. The multinucleate nature of these cells, observed by phase contrast (Sec. IV. 1.) is confirmed by the DNA analysis of Sec IV. 2. These cells contain an average of five times as much DNA as aerobically grown cells. While the DNA multiplicity does not agree exactly with the extrapolated target number from survival curves, this is perhaps not unexpected. The extrapolated target number is very sensitive to small changes in slope of the linear portion of the curves; furthermore, it is different when irradiation occurs in air or nitrogen, being 2.5 and 24 respectively as shown in Sec. II. 2.

That the sigmoidal nature of the survival curves of anaerobically grown cells is due to nuclear multiplicity is further confirmed by the fact that by simply altering the growth pH, the multiplicity of the curves almost entirely vanishes. At the same time the average DNA per cell falls from 5 to 1.9 times the value of aerobically grown cells, and the microscopic appearance of the cells is that of short rods, compared to the long rods of anaerobic pH-5 cells. This is sufficient evidence to eliminate the unlikely possibility mentioned in Sec. II. 2., that growth at pH 8.5 prevents the endogenous metabolism of intracellular oxygen. The supposition of Hollaender, Baker, and Anderson (1951) that anaerobic growth protects by removing intracellular oxygen, is also eliminated. The suggestion of Birge and Tobias (1954) that the protection may be due to nuclear multiplicity, is confirmed.

The results are also in agreement with Howard-Flanders and Alper (1957): "The survival curves obtained (with E. coli, B/r) with cultures of different origin accorded with the view that the method of culture influenced the number of viable units per organism, while the inherent radiosensitivity of any unit was constant. "

Even at pH 8.5, however, anaerobically grown cells still are more resistant than aerobic cells, by a factor of 1.6, as mentioned in Sec. II. 2. These cells contain 1.9 times as much DNA as aerobic cells, and this may be the cause of the remaining protection. There may also be some merit in the explanation of Hollaender, Stapleton and Martin (1951), that anaerobic growth protection is due to the complete additivity of (1) presence of glucose, and (2) absence of oxygen during growth. Probably the presence of glucose in the absence of oxygen causes the pH during growth to go acid by producing lactic and acetic acid as end products. Glucose and (or) lack of oxygen may also be responsible for the protection remaining when the cells are grown at pH 8.5.

The mechanism involved in formation of long filaments by anaerobic glucose growth of E. coli may throw some light on the radiobiological problem of chemical protection. A possible mechanism is suggested by the experiments of the following workers.

The work of Hughes (1953) showed that such filaments or L forms could be produced by anaerobiosis. He found the production of such forms to be independent of pH. Here it was found that filaments were indeed formed at all pH's during anaerobic growth, but at stationary phase at pH 8.5 the filaments broke up, but did not at pH 5.

Fowler (1951), studying the fermentation of glucose by E. coli, found that changing the culture from aerobic to anaerobic produced growth changes very similar to those in adaptive enzyme formation. Lominski, Cameron, and Wyllie (1958) separated from cultures of Streptococcus faecalis a material which they believed to be an enzyme. This material caused cultures of S. faecalis which had been induced to grow in long chains to break up into single cells, a process they termed "unchaining." The activity was maximum at pH 7. Preliminary results indicated a similar cell-separating system in Gram-positive and Gram-negative rods.

The above results suggest strongly that the filamentous growth of E. coli, B/r, in anaerobic glucose culture at pH 5 is due to the inhibition of an enzyme system responsible for the separation of daughter

cells after nuclear reproduction and division has occurred. This inhibition is removed by growth at pH 8.5, either by producing the proper pH for the enzyme or by preventing an inhibitor from being formed.

This enzyme system may even account for the difference between strains B and B/r. Strain B can be induced to grow long filaments by very small doses of radiation, while B/r cannot. Morse and Carter (1949) found differences in DNA content of B and B/r during lag phase which may also be due to the action or failure of such an enzyme.

The results of Section III. 2., Fig. 12 may also be due to such a mechanism. Anaerobic pH-5 cells irradiated at pH 8.5 may have their radioresistance reduced by virtue of this enyzme system being activated at the favorable pH, and some of the pH-5 filaments are separated into cells typical of the more radiosensitive cells grown at pH 8.5. Cells grown at pH 8.5, as in Fig. 13, cannot show pH-5 resistance when irradiated at pH 5 because the enzyme cannot reassemble small units into filaments.

If such a relatively simple mechanism as the action of a single enzyme is responsible for the multinucleate protection of anaerobic growth, the suggestion of Howard-Flanders mentioned earlier may find support. He suggests that the chemical protectors β-mercaptoethanol and cysteamine, the only protective compounds which have not been shown to protect by virtue of oxygen removal, may exert their protection by inducing a physiological state similar to that due to anaerobic growth as well as by producing anoxia within the cell. As suggested in the previous paragraph it is possible that physiological conditions similar to those produced during growth can be induced by conditions present only during irradiation, although in this case the effect seems to be opposite to that required to explain the action of the chemical protectors. It would be interesting to study the action of these protectors on cells grown anaerobically at pH 5 and 8.5.

ACKNOWLEDGMENTS

Grateful acknowledgment is made to the following people for their assistance and support in this work: Dr. R. L. Dobson for guidance and discussion; Dr. Lola S. Kelly for assistance in the DNA determinations; Drs. Paul Howard-Flanders and Robert K. Mortimer for valuable discussion and encouragement. This work was supported in part by the U. S. Atomic Energy Commission.

BIBLIOGRAPHY

1. T. Alper and M. Ebert, Influence of Hydrogen-ion Concentration on Radiation Effects. Science 120, 608-609, 1954.
2. T. Alper and N. E. Gillies, Dependence of the Observed Oxygen Effect on the Postirradiation Treatment of Micro-organisms. Nature 181, 961-963 (1958).
3. T. Alper and N. E. Gillies, "Restoration" of Escherichia coli Strain B after Irradiation; its Dependence on Suboptimal Growth Conditions. J. Gen. Microbiol. 18, 461–472 (1958).
4. T. Alper and N. E. Gillies, "Restoration" of Microorganisms and Suboptimal Growth Conditions. Rad. Research 9, 86 (1958).
5. T. Alper and P. Howard-Flanders, Role of Oxygen in Modifying the Radiosensitivity of E. coli B, Nature 178, 978-979 (1956).
6. E. H. Anderson, The Effect of Oxygen on Mutation Induction by x-rays. Proc. Nat. Acad. Sci. 37, 340-349 (1951).
7. K. C. Atwood and F. Mukai, Radiation Effects on Neurospora Conidia, Quarterly Progress Report for period ending Nov. 10, 1951. Biology Division, Oak Ridge National Laboratory, pp. 29-33, 1951.
8. Z. M. Bacq and P. Alexander, Fundamentals of Radiobiology (Butterworths Scientific Publications, London, 1955).
9. C. Biagini, Studies in the Mode of Action of Ionizing Radiations. Influence of Cell Concentration on Lethal Effects of X-rays on Escherichia coli, Arch. Biochem. Biophys. 56, 38-44 (1955).
10. Ann C. Birge and C. A. Tobias, Radiation Sensitivity of Yeast Cells Grown in Aerobic and Anaerobic Environments. Arch. Biochem. Biophys. 52, 388-393 (1954).
11. L. Blackwood, T. Neish, and C. Ledingham, Glucose Dissimilation at Controlled pH by E. coli. J. Bact. 72, 497-499 (1956).
12. P. Bonet-Maury and M. Lefort, Radiochemical Equilibria in Irradiated Water, Nature 166, 981-982 (1950).
13. A. S. Brownell, The Effect of Physiological and Morphological Changes on the Radiation Sensitivity of Escherichia coli. UCRL-3055, July 1955.

14. G. Cerioti, Determination of Nucleic Acids in Animal Tissues. J. Biol. Chem. 214, 59-70 (1955).

15. H. Chambers and S. Russ, The Bactericidal Action of Radium Emanation, Proc. Roy. Soc. Med. 5, 198-212 (1912).

16. J. R. Cortelyou, L. M. Amundson, and M. A. McWhinnie, A Phase Contrast Study of the Chromatinic Bodies of Escherichia coli subsequent to Ultraviolet Radiation. J. Bact. 71, 462-473 (1956).

17. J. A. Crowther, Some Considerations Relative to the Action of X-rays on Tissue Cells, Proc. Roy. Soc. (London) B 96, 207-211 (1924).

18. J. A. Crowther, The Action of x-rays on Colpidium colpoda. Proc. Roy. Soc. (London), B 100, 390-404 (1926).

19. F. S. Dainton, Some Aspects of Radiation Chemistry which are Relevant to some Radiobiological Problems, in Progress in Radiobiology, J. S. Mitchell, B. E. Holmes, and C. L. Smith, Eds., (Oliver and Boyd, London and Edinburgh, 1956), pp. xix-xli.

20. F. W. Daniels, Recovery from Lethal x-irradiation Injury in Amoebae after Injection of Centrifugal Portions of Nonirradiated Cells. Rad. Research 5, 604 (1956).

21. H. A. Dewhurst, A. H. Samuel, and J. L. Magee, A Theoretical Survey of the Radiation Chemistry of Water and Aqueous Solutions, Rad. Research 1, 62-84 (1954).

22. H. Ephrussi-Taylor and R. Latarjet, Inactivation par les rayons x d'un facteur transformant du pneumocoque. Biochim. Biophys. Acts 16, 183-197 (1955).

23. C. B. Fowler, The relationship between fermentation and enzymatic adaptation. Biochim. Biophys. Acta 7, 563-573 (1951).

24. E. F. Gale, The Chemical Activities of Bacteria, (University Tutorial Press, Ltd., London, 1947), p. 58.

25. R. Goucher, I. Kamei, and W. Kocholaty, Some Results and Interpretations of x-irradiation Studies with E. coli, Arch. Biochem. Biophys. 65, 522-533 (1956).

26. R. L. Gunther, Donner Laboratory, University of California, Berkeley, private communication, 1958.
27. M. Haissinsky, Mechanism of Radiochemical Transformations in Aqueous Dilute Solutions, Disc. Faraday Soc. 12, 133-143 (1952).
28. H. B. Hewitt and J. Read, Search for an Effect of Oxygen on the Direct x-ray Inactivation of Bacteriophage. Brit. J. Radiol. 23, 416-423 (1950).
29. A. Hollaender, W. K. Baker, and E. H. Anderson, Effect of Oxygen Tension and Certain Chemicals on the X-ray Sensitivity of Mutation Production and Survival, Cold Spring Harbor Symposia on Quantitative Biology 16, 315-326 (1951).
30. A. Hollaender and C. O. Doudney, Studies on the Mechanism of Radiation Protection and Recovery with Cysteamine and β-mercapto-ethanol, in Radiobiology Symposium, Z. M. Bacq and P. Alexander, Eds. (Butterworths Scientific Publications, London 1955).
31. A. Hollaender and G. E. Stapleton, Fundamental Aspects of Radiation Protection from a Microbiological Point of View. Physiol. Revs. 33, 77-84 (1953).
32. A. Hollaender, G. E. Stapleton, and F. L. Martin, X-ray Sensitivity of E. coli as Modified by Oxygen Tension. Nature 167, 103 (1951).
33. P. Howard-Flanders, Effect of Nitric Oxide on the Radiosensitivity of Bacteria. Nature 180, 1191-1192 (1957).
34. P. Howard-Flanders, chapter in Advances in Biological and Medical Physics (Academic Press, New York, in press).
35. P. Howard-Flanders and T. Alper, The Sensitivity of Micro-organisms to Irradiation under Controlled Gas Conditions, Rad. Research 7, 518-540 (1957).
36. F. Hutchinson, Reduced Yield in Dilute Solutions Irradiated at very High Dose Rates, Rad Research 9, 13-23 (1958).
37. M. E. Jayko and W. M. Garrison, Formation of $\geq C = O$ Bonds in the Radiation-induced Oxidation of Protein in Aqueous Systems, Nature 181, 413-414 (1958).

38. R. F. Kimball, Nongenetic Effects of Radiation on Microorganisms, Ann. Rev. Microbiol. 11, 199-220 (1957).

39. H. Laser, Influence of Oxygen on Damage to Micro-organisms by Ionizing Radiation, Symposium on Radiobiology, Z. M. Bacq and P. Alexander, Eds. (Butterworths Scientific Publications, London, 1955).

40. R. Latarjet, Institut du Radium, Paris, private communication, 1956.

41. R. Latarjet and E. Ephrati, Influence protectrice de certaines substances contre l'inactivation d'un bacteriophage par les rayons x. Compte Rend. Soc. Biol. 142, 497-499 (1948).

42. D. E. Lea, Actions of Radiation on Living Cells (Macmillan, New York, 1947).

43. I. Lominski, E. J. Cameron, and G. Wyllie, Chaining and Unchaining Streptococcus faecalis - a Hypothesis of the Mechanism of Bacterial Cell Separation, Nature 181, 1477 (1958).

44. A. Marshak, Alteration of Chromosome Sensitivity to x-rays with NH_4OH. Proc. Soc. Exp. Biol. Med. 38, 705-713 (1938).

45. R. K. Mortimer, Cytological and Environmental Factors Related to the Effects of Radiations on Yeast Cells, Ph. D. Thesis, University of California, Berkeley, 1953.

46. R. K. Mortimer, Evidence for Two Types of X-ray-induced Lethal Damage in Saccharomyces cerevisiae, Rad. Research 2, 361-368 (1955).

47. R. K. Mortimer, Radiobiological and Genetic Studies on a Polyploid Series (Haploid to Hexaploid) of Saccharomyces cerevisiae, Rad. Research. 9, 312-326 (1958).

48. M. L. Morse and C. F. Carter, The Synthesis of Nucleic Acids in Cultures of Escherichia coli, Strains B and B/r, J. Bacteriol. 58, 317-326 (1949).

49. E. Moustacchi, "L'effect oxygene" dans l'irradiation x de microorganismes deficient en enzyme de la respirations aerobie, Ann. Inst. Pasteur, 94, 89-94 (1958).

50. M. E. Owen, and R. K. Mortimer, Dominant Lethality Induced by X-rays in Haploid and Diploid Saccharomyces cerevisiae, Nature 177, 625-626 (1956).

51. R. F. Proctor, S. A. Goldblith, G. J. Fuld, and E. M. Oberle, Radiosensitivity of Bacillus thermoacidurans under Different Environmental Conditions, Rad. Research 8, 51-63 (1958).

52. R. R. Roberts, and E. Aldous, Recovery from Ultraviolet Irradiation in E. coli. J. Bacterial. 57, 363-575 (1949).

53. W. Schneider, Phosphorus Compounds in Animal Tissues. I. Extraction and Estimation of Deoxypentose Nucleic Acid and of Pentose Nucleic Acid. J. Biol. Chem. 161, 293-303 (1945).

54. A. H. Sparrow, and L. A. Schairer, The Radioresistance of High Polyploids, Rad. Research 9, 187 (1958).

55. G. E. Stapleton, Variations in the Sensitivity of Escherichia coli to Ionizing Radiations during the Growth Cycle. J. Bacteriol. 70, 357-362 (1955).

56. Stapleton, Billen, and Hollaender, The Role of Enzymatic Oxygen Removal in Chemical Protection against x-ray Inactivation of Bacteria, J. Bacteriol. 63, 805-811 (1952).

57. G. E. Stapleton, D. Billen and A. Hollaender, Recovery of x-irradiated Bacteria at Suboptimal Incubation Temperatures, J. Cellular Comp. Physiol. 41, 345-357 (1953).

58. G. E. Stapleton, A. J. Sbarra, and A. Hollaender, Some Nutritional Aspects of Bacterial Recovery from Ionizing Radiations, J. Bacteriol. 70, 7-14 (1955).

59. J. M. Thoday and J. Read, Effect of Oxygen on the Frequency of Chromosome Aberrations Produced by X-rays, Nature 160, 608 (1947).

60. R. S. Weatherwax, Reactivation of uv-irradiated E. coli, J. Bacteriol. 72, 329 (1956).

61. R. A. Wijsman, A New Approach to the Mathematical Theory of the Action of High-energy Radiation on Single Cells. I. Formulation of the Theory, Rad. Research 4, 257-269 (1956). II. Consequences of the Theory, ibid. 270-277.

62. E. Witkin, Time Temperature, and Protein Synthesis; A Study of uv-induced Mutation in Bacteria, Cold Spring Harbor Symposium on Quant. Biol. 21, pp. 123-140 (1956).

63. R. W. G. Wyckoff and T. M. Rivers, The Effect of Cathode Rays upon Certain Bacteria, J. Expt'l Med. 51, 921-932 (1930).

64. M. R. Zelle and A. Hollaender, Effects of Radiation on Bacteria, Radiation Biology, Vol. II, A. Hollaender, Ed., (McGraw-Hill, New York, 1955) pp. 365-430.

65. R. E. Zirkle, Modification of Radiosensitivity by Means of Readily Penetrating Acids and Bases; Am. J. Roentgenology Rad. Therapy Nucl. Med. 35, 230-237 (1936).

66. R. E. Zirkle, The Influence of Intracellular Acidity on the Radiosensitivity of Various Organisms, J. Cellular Comp. Physiol. 16, 301-311 (1940).

67. R. E. Zirkle, Combined Influence of x-ray Intensity and Intracellular Acidity on Radiosensitivity, Ibid. 17, 65-70 (1941).

68. R. E. Zirkle and C. A. Tobias, Effects of Ploidy and Linear Energy Transfer on Radiobiological Survival Curves, Arch. Biochem. Biophys. 47, 282-306 (1953).