CULTURAL CONDITIONS AS DETERMINANTS OF SENSITIVITY OF *ESCHERICHIA COLI* TO DAMAGING AGENTS

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An extremely wide range of radiation sensitivities is displayed by various bacterial genera; for example, *Pseudomonas aeruginosa* has an LD_{50}[2] of about 1000 r (Goldblith et al., 1955), whereas *Sarcina rubens* has an LD_{50} in excess of 500,000 r (Kilburn, Bellamy, and Terni, 1958). It would seem that any explanation of the wide range of radiosensitivities should take into account the structural and physiological differences in the various types of organisms. The role of cell physiology in radiation sensitivity can be appreciated from experiments with a single species, *Escherichia coli*. Hollaender, Stapleton, and Martin (1951) reported that addition of glucose to a nutrient broth culture during growth of *E. coli* strain B/r affected the lethal response of the cell population to X rays. Cells harvested from nutrient broth cultures to which glucose had been added were more resistant than cells cultured on nutrient broth without this energy source. Anaerobiosis seemed to accentuate the effect of glucose on sensitivity to X-ray inactivation. These investigators also demonstrated that the desensitizing effect of cultural conditions was dissociated from the well-established modifying effect of gaseous environment during irradiation. Howard-Flanders and Alper (1957) confirmed the earlier findings and suggested that the sigmoid survival curves for the more resistant cells might be accounted for by structural changes in the cells (e.g., multicellular or multinuclear). Sargent (1958) also found that cultural conditions alter the sensitivity of *E. coli* and stressed the necessity for anaerobiosis during growth in development of radioresistance. Structural abnormalities were used to explain the difference in shapes of X-ray inactivation curves.

[1] Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

[2] LD_{50} and LD_{90} are used throughout this paper as the radiation dose required to inactivate 50 and 90 per cent of the population, respectively.

The purpose of the present paper is to (1) report a more critical examination of effect of culture conditions on the X-ray sensitivity of *E. coli* as well as the sensitivity to other agents, (2) evaluate the suggested explanations, and (3) lay firm groundwork for future studies on determinants of radiosensitivity of living cells.

EXPERIMENTAL METHODS

Three strains of *E. coli* were used in these investigations: B and B/r obtained from Carnegie Institution, Cold Spring Harbor, New York; kindly supplied by Dr. M. Demerec, denoted as B(CSH) and B/r(CSH); and the porphyrin-negative strain H_7 isolated by Dr. M. Beljanski (1955) and supplied by Dr. H. I. Adler. A strain of *P. aeruginosa* from the culture collection of the University of Tennessee, Department of Bacteriology, was used in some preliminary experiments.

The B and B/r strains were stocked on slants of nutrient agar (Difco), the H_7 strain was stocked on slants comprised of 1 per cent buffered peptone-glucose agar containing 8 mg of hemin per liter.

The cells to be used for radiation experiments were from 16- to 20-hr (stationary phase) liquid cultures. The standard liquid medium was 1 per cent peptone (Difco), to which various concentrations of glucose and KH_2PO_4-K_2HPO_4 buffer were added. Unless otherwise specified, the initial pH of the liquid media was 6.8. Aerated cultures used in most experiments were obtained by vigorous agitation of 20 ml of inoculated medium in 125-ml Erlenmeyer flasks on a gyrotory incubator shaker (New Brunswick Scientific Company) at about 240 cycles per min. Anaerobic cultures were grown in Pyrex tubes, 18 by 150 mm (o.d.), provided with thin glass tubes for bubbling 95 per cent N_2-5 per cent CO_2 continuously during the growth cycle. All cultures were incubated at 37 C.

The radiation effect investigated was inactiva-

tion or loss of ability of the exposed cells to produce a macroscopically visible colony after 24 to 48 hr incubation at 37 C. The composition of the plating medium for all experiments was as follows (per liter): peptone, 10 g; glucose, 2 g; KH_2PO_4, 4.5 g; $K_2HPO_4 \cdot 3H_2O$, 7.6 g; and agar, 20 g. In the present studies, as in those of Gunter and Kohn (1956), no more colonies developed with incubation for longer periods of time. Stationary-phase cultures were diluted 100-fold in 0.066 M PO_4 buffer (pH 6.8) at room temperature and irradiated immediately at this temperature.

Different irradiation techniques were used, depending on the type of experiment: (1) For experiments in which complete survival curves were to be obtained, 7 ml of the diluted culture was placed in a 50-mm petri dish and stirred vigorously with a magnetic stirrer at 14.2 cm from the target of the X-ray tube; (2) for experiments in which a number of diluted cultures were to be subjected to a constant X-ray dose, 1 ml of the cultures was placed in individual 2-ml glass-stoppered pyrex volumetric flasks. The conditions of these experiments insured that the oxygen concentration in suspensions could not be reduced sufficiently to alter the radiation response. Eight such samples could be irradiated in a predetermined uniform field in a lucite ring-type holder at 14.2 cm from the target of the X-ray tube. The holder and tubes were rotated horizontally under the beam to further ensure uniform irradiation of all samples. A General Electric Maxitron 250 X-ray machine, operating at 250 kvp and 30 ma, served as the ionizing radiation source in all experiments. With 2 mm of added aluminum filtration (hvl, 0.34 mm of Cu) the dose rate to all samples was 4400 r per min.

In experiments involving ultraviolet irradiation, 7-ml portions of dilute buffer suspensions (0.066 M PO_4 buffer; pH 6.8) containing about 10^6 organisms per ml were irradiated in open sterile petri dishes (o.d., 50 mm), at 60 cm from a G. E. 15-watt germicidal lamp (intensity, 20 ergs:mm^2:sec). The suspensions were stirred with a magnetic stirrer during exposure; samples were removed at various times, diluted, and plated under nonphotorestorative yellow light. The plates were incubated in the dark at 37 C for 48 hr.

Heat inactivation studies involved inoculating 0.1 ml of various cultures into 10-ml tubes of 0.066 M PO_4 buffer preheated to 54 C in a constant temperature bath. One-tenth-ml samples were removed at various times, diluted immediately, and plated at room temperature.

Total cell counts were made microscopically by use of a Petroff-Hausser bacterial counting chamber. Wet mounts of cell suspensions were observed under a phase-contrast microscope.

Dry weights were determined by placing 1 or 2 ml of 10- to 20-fold concentrated suspensions in stainless steel planchets and drying them to constant weight at room temperature in a desiccator over $CaCl_2$.

Packed-cell volumes were determined by centrifuging concentrated suspensions in hematocrit tubes at 3500 rpm for 15 min.

Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) analyses were made on trichloroacetic acid extracts of washed cell suspensions, as described by Schneider (1945). Protein was estimated by turbidity or packed volume of the insoluble residue.

RESULTS

Most of the media used routinely for cultivation of *E. coli* (e.g., nutrient broth) are poorly buffered, and, even though the initial or reaction pH is about 6.8, the final pH for aerobic cultures increases to 8.3 or above as a result of the metabolic activity of the growing cells. On the other hand, if an energy source such as glucose is added to such a medium at a concentration of 0.2 per cent or higher, the final pH is reduced to 4.8 or lower as a result of acid production by the cells. In most, if not all, previous investigations of the effect of cultural conditions on radiosensitivity, relatively large changes in the hydrogen-ion concentration of the medium during growth were possible. In the experiments of Hollaender et al. (1951) and Howard-Flanders and Alper (1957), anaerobiosis was also imposed on cells in addition to supplementation of the medium with glucose. In the medium used by both groups of investigators (nutrient broth), growth of *E. coli* to a high titer (10^9 cells per ml or higher) would inevitably lead to changes in the final pH. Cells from anaerobic glucose-broth cultures were somewhat more resistant than aerobic glucose-broth cultures. In the present studies, we found that anaerobiosis is not required for development of resistant cultures. Figure 1 shows survival curves of *E. coli* strain B/r (CSH)

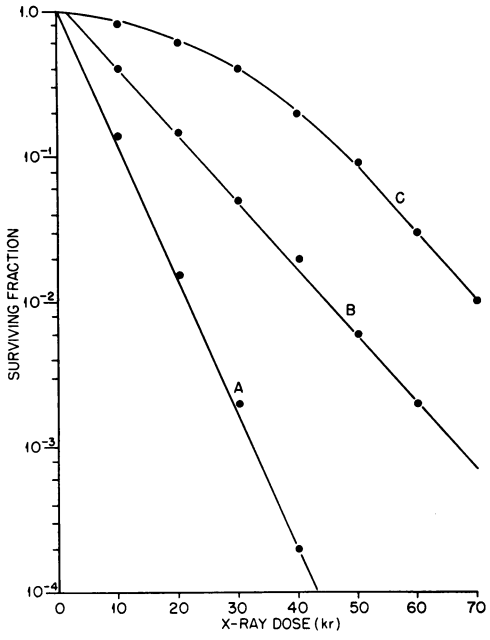

Figure 1. Survival curves for *Escherichia coli* strain B/r(CSH) grown aerobically under different conditions. Initial pH of all media was 6.8.

Curve	Growth Medium	Final pH	Final Titer
			× 10^9 cells/ml
A	1% peptone (unbuffered)	8.4	1.8
B	1% peptone, 0.066 M PO_4 buffer	6.8	4.1
C	1% peptone-0.2% glucose, 0.008 M PO_4 buffer	4.8	2.4

grown under three different conditions. All were cultured aerobically and irradiated in 0.066 M PO_4 buffer (pH 6.8) at room temperature (25 C). The difference in sensitivity of types A and B cultures seems to involve a slope change, whereas types B and C cultures seem to show survival curves that differ chiefly in the magnitude of the shoulder. Sigmoid survival curves of the type obtained for the type C culture are often referred to as multihit, multitarget, or multiunit curves and suggest a multiplicity of events required to bring about inactivation (Atwood and Norman, 1949; Gunter and Kohn, 1956; Tobias, 1952). The best fit of the final slope of curve C as shown in figure 1 extrapolates at zero dose to a hit or target number of between 10 and 100.

TABLE 1

Chemical content of resistant and sensitive cells of Escherichia coli strain B/r(CSH)

Determination	Ratios of Resistant/ Sensitive Cells*
DNA	1.4
RNA	1.6
Protein	1.5
Packed-cell volume†	2.0
Dry weight	1.5

* The average of four determinations is expressed on a per cell basis, based on total cell counts.

† Wet cells.

Several investigators have suggested that a multicellular or multinuclear state might explain the type of survival curves obtained for resistant cultures (Birge and Tobias, 1954; Howard-Flanders and Alper, 1957; and Sargent, 1958). Microscopic examination and comparative chemical analysis of resistant (C) and sensitive (A) cultures should test the applicability of such an explanation based on gross structural differences. Typical results of chemical analyses of resistant and sensitive cells are given in table 1. Since only relative compositions are needed for comparison, absolute concentrations are not shown. These data show that in spite of minor variations in the relative concentrations of DNA, RNA, and protein, the relative concentrations of none of the components measured are indicative of the high multiplicity required to explain the shape of the survival curves on the basis of the multitarget analysis. Microscopic observation of wet mounts of cells from the undiluted cultures, diluted cultures, or irradiated diluted cultures fails to give any evidence of clumping. These preparations do, however, show that the resistant cells (C) are about three times as large as sensitive cells (A), an observation that is supported by measurements of packed-cell volumes (table 1). Cell wall stains (Webb, 1954) of dried preparations show only occasional septa in the resistant as well as in the sensitive cells making it appear unlikely that multicellularity can be invoked as an explanation of the difference in radiosensitivity.

Several strains of *E. coli* were grown and irradiated under conditions similar to those described for B/r(CSH). All strains showed qualitatively similar but not quantitatively identical

TABLE 2

Comparison of LD_{90} values for various bacteria

Bacteria	LD_{90} Dose for Culture at Final pH Indicated		
	8.3	6.8	4.8
	kr	*kr*	*kr*
Escherichia coli strain:			
B/r(CSH)	10	24	50
B(CSH)	8	—	18
B/r(ORNL)*	29	—	50
H_7 (+ hemin)	10	15	20
Pseudomonas aeruginosa	4	4	4

* A culture originally obtained from Carnegie Institution, Cold Spring Harbor, about 12 years ago and subcultured in our laboratory.

changes in sensitivity as a function of the final pH (table 2). Since the curves for resistant and sensitive cells of any particular strain are of different shape, LD_{90} values are expressed. Some readers may question the differences in sensitivity of different B/r strains shown in table 2; these differences are real and caution against assuming that all strains bearing this nomenclature from various laboratories are identical (Adler and Haskins, 1960). The variation found among strains may be useful in elucidation of the processes involved in development of radioresistance in *E. coli*.

Effect of final pH, substrate, and substrate concentration. It was mentioned above that changes in pH that occur in growing cultures of *E. coli* seem to be well correlated with changes in radiosensitivity of the stationary-phase population. By altering the ratios of buffer concentration and glucose concentration, it was possible to obtain stationary-phase cultures at several final pH levels over the range 4.8 to 8.4. Figure 2 is a plot of the surviving fraction of cells at 30 kr, as a function of the final pH of such cultures if irradiated in 0.066 M PO_4 buffer at 25 C. All data reported are for *E. coli* strain B/r(CSH). The decrease in resistance as a function of pH includes not only slope changes but also changes in the magnitude of the shoulder of the survival curves (*see* figure 1).

Preliminary data were obtained on the relation between the type of substrate and the development of radioresistance (table 3) for *E. coli* strain B/r(CSH). There are differences in the resistance of cultures that arrive at the same

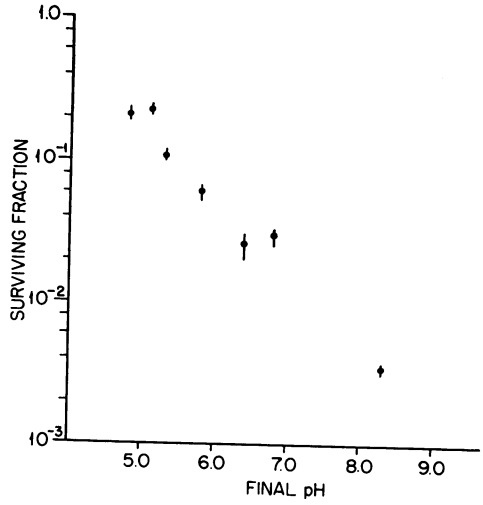

Figure 2. Surviving fractions of *Escherichia coli* strain B/r(CSH) at a constant X-ray dose of 30 kr plotted as a function of the final pH of the growth medium. The mean of surviving fractions and their standard deviations are based on five or more experiments. The pH values were obtained empirically by altering the concentrations of the buffer and glucose in the growth medium.

TABLE 3

Comparison of substrates for development of radioresistance in Escherichia coli strain B/r(CSH)

Growth Medium	Final pH	LD_{90} Dose
		kr
Peptone	8.4	10
Peptone + glucose*	4.8	50
Peptone + fructose*	4.8	27
Peptone + xylose*	4.9	35

* Present in 0.5 per cent concentrations.

final pH level on various substrates; however, more substrates will have to be tested before we can evaluate the role of substrate in development of resistance. The available data on glucose concentration indicate that, over a wide range, the relative resistance of cultures is not dependent on glucose concentration, but rather correlates better with the final pH obtained.

It might be suggested that intracellular pH determines the sensitivity of the irradiated cell. The effect of pH on production of free radicals or peroxides in pure aqueous systems has been studied by others (Dale and Davies, 1951; Fricke,

TABLE 4

Preirradiation incubation and effect on radiosensitivity of Escherichia coli strain B/r(CSH)

Type of Culture	Incubation Medium*	pH	Time	Surviving Fraction at 30 kr
			min	
Sensitive	—†	—	—	5.0×10^{-3}
cells; peptone; final pH, 8.4	KCl	7	60	3.8×10^{-3}
	KCl	7	240	6.5×10^{-3}
	Peptone adjusted to pH 5 with lactate	5	60	5.0×10^{-3}
Resistant	—†	—	—	1.2×10^{-1}
cells; peptone-glucose; final pH, 4.8	KCl	7	60	1.4×10^{-1}
	KCl	7	240	1.0×10^{-1}
	Peptone	7	60	1.8×10^{-1}
	Peptone	7	240	1.0×10^{-1}

* All incubations were static at 25 C.

† Resistant and sensitive cells were held or irradiated, or both, in 0.066 M PO_4 buffer, pH 6.8.

1934). The influence of pH on the reaction of *E. coli* with protective chemicals has been reported by Kohn and Gunter (1959). We have been able to show, however, that over the range of interest, the pH of the suspending medium during irradiation does not detectably affect the sensitivity of either resistant or sensitive cell populations. In fact, cells incubated for various times in saline or peptone medium adjusted to different hydrogen ion concentrations before irradiation show no increase or decrease in sensitivity (table 4). Moreover, *E. coli* B/r(CSH) cultured in peptone-glucose medium at constant pH (5.6) by automatic titration with NaOH continuously during the growth cycle do not show the radioresistance characteristic of that pH. Nevertheless, they do become more radioresistant if the pH is decreased by acid production upon removal of the automatic pH control device, suggesting that change in pH may trigger a process responsible for development of radioresistance.

Changes in sensitivity to agents other than X rays. So far, we have discussed changes in X-ray sensitivity induced by cultural conditions. It seemed important to determine if these changes were specific for damage brought about by ionizing radiation. Cell suspensions similar to those used for X-ray studies, type A and C cultures, were

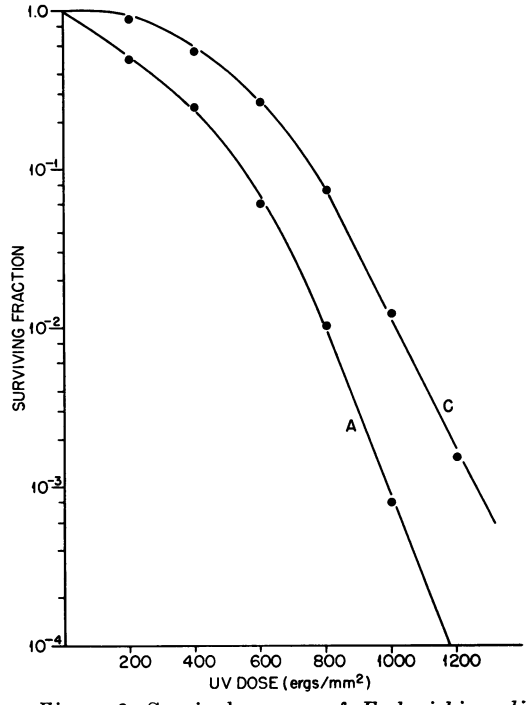

Figure 3. Survival curves of *Escherichia coli* strain B/r(CSH) as a function of ultraviolet dose (2537A). (A and C cultures are similar to those described in text and figure 1.)

subjected to ultraviolet and heat inactivation. Survival curves for ultraviolet radiation are shown in figure 3. Type C cultures resistant to X rays are likewise more resistant to ultraviolet than are type A cultures. The same is true for heat inactivation (figure 4). The change in radiosensitivity brought about by cultural conditions seems to be more pronounced for X rays and heat than for ultraviolet radiation. Data similar to those obtained for X-ray survival as a function of final pH of cultures are not yet available for heat and ultraviolet radiation. Also, no comparison of other strains or other genera has been made. On the basis of the evidence presented, it is clear that the resistance developed in *E. coli* is not specific for ionizing radiation. Detailed experimentation with a variety of other agents may supply important information about the nature of the processes involved in development of resistance in this species.

To test the generality of the phenomena, some experiments were made with cultures of *P. aeruginosa*. Although the work is incomplete, it is obvious from the data in table 2 that the

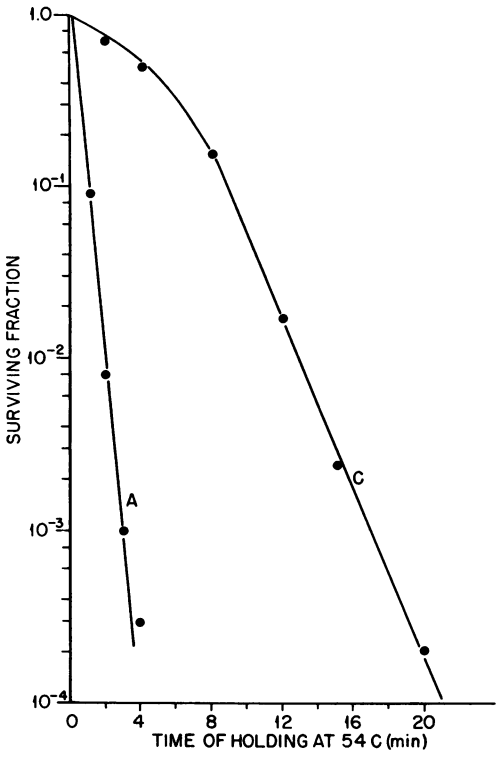

Figure 4. Heat inactivation curves of *Escherichia coli* strain B/r(CSH). A and C cultures are similar to those described in text and figure 1.

resistance of this bacterium changes little, if any, under conditions similar to those in which large changes are detected in *E. coli*. It seems safe to conclude from these data that development of pH changes per se is not responsible for the development of radioresistance. It should be noted, however, that Pseudomonas bears little resemblance to *E. coli* in its metabolism.

DISCUSSION

There is considerable interest in the role of metabolism and cell physiology in determining radiation sensitivity of living cells. The data presented earlier by Hollaender et al. (1951) indicated that cell sensitivity to X-ray inactivation is dependent in some way on the conditions during growth of cultures of *E. coli* strain B/r. Since that report, a number of investigators have attempted to explain away the phenomenon by attributing the apparent resistance attained by anaerobic cultures, or cultures that develop a low final pH, to gross structural abnormalities such as clumping or to a genetic change (such as increased ploidy or a multinuclear state) resulting from the conditions of growth. Moreover, the conditions conducive to development of radioresistance are not abnormal for a facultative anaerobe that, by generic classification, has a predilection for acid production on a substrate such as glucose. It is undoubtedly true that, in a completely unbuffered medium, a lower pH than any obtained in these studies might influence the viability of the cultures.

The routinely higher final titers in the more radioresistant cultures, and their high ratio of viable cells to total cells, do not indicate any peculiarity of cultures arriving at final pH of 4.8. The foundation for suspecting this to be an abnormal condition probably lies in the frequent use of nutrient broth as a standard medium for culturing *E. coli*. The higher cell titer and high viability of peptone-glucose cultures suggest that nutrient broth may be a limiting medium for *E. coli*. We shall not attempt here to set standards for cultures, but rather to demonstrate that alterations in the medium can influence the sensitivity of *E. coli* to inactivation by deleterious agents.

We can propose that the sigmoid curves reflect the protection afforded by some heat- or radiolabile compounds, a product of metabolism present in resistant cells in low concentration. Since the cells were in general irradiated under conditions in which none of such a compound would be produced, radiation or thermal destruction of the compound could result in the inactivation kinetics displayed. This hypothesis is now being thoroughly tested. It is logical that not all microorganisms would produce the same metabolic products under the conditions described here for a single species.

Although no gross structural differences between resistant and sensitive cultures were found in the present experiments, it is important to consider the possible implications of differences in the distribution, activity, and the fine structure of the cell components, especially the genetic material in the resistant and sensitive cultures.

No mention has yet been made of the possible role of selection for resistant and sensitive mutants within normal populations of *E. coli*. Since inocula of resistant or sensitive cultures into fresh peptone or peptone-glucose media always give populations characteristic of the medium

rather than the inoculum, it seems unlikely that such selection is responsible for the effect seen. Nevertheless, it must be proved that mutations were not produced and selected by the very techniques used in the present experiments.

The growth conditions used here might have an influence on the recovery or repair of damage induced by radiation or the other agents studied. Preliminary experiments demonstrate that there is no difference in the growth rates of resistant and sensitive cells in a liquid medium of the same composition (except agar) as the routine plating medium. It is not known if the same is true of irradiated cells. The plausibility of the relative ability of resistant and sensitive cells to recover or repair damage is strengthened somewhat by Gale's (1940*a*, *b*) well-known demonstration that conditions similar to those studied here have a great influence on specific enzyme content and activity of cells of this genus.

ADDENDUM

Our attention has been drawn to the work of Schechtman, Plochoi, and Filipova (1958) and Schechtman (1960) in which they report that the presence of glucose during growth of *B. coli communis* causes the cells to be more resistant to inactivation by X rays and α-particles. The suggestions are made that the mode of inactivation is different for cells cultured with and without glucose, and that the greater resistance of the cells grown with glucose is related to development of increased ploidy. In the present study our chemical analyses of resistant and sensitive cells fail to give evidence for differences in ploidy.

SUMMARY

The sensitivity of several strains of *Escherichia coli* to inactivation by X rays can be modified by alterations in the medium in which the cells are grown before irradiation. Remarkable changes in the slopes, as well as the shapes of the X-ray survival curves are found; sensitive cells yield exponential survival curves with increasing dose of radiation, whereas the more resistant cells yield highly sigmoid curves. The sigmoid survival curves cannot be explained simply on the basis of a gross structural abnormality such as a multicellular or multinuclear condition, or on the basis of plating artifacts such as clumping. The relative amounts of deoxyribonucleic acid (DNA) per cell in resistant and sensitive cells do not accord with the suggestion that the cells differ in ploidy or numbers of nuclei. The relative resistance of cultures does, however, correlate well with the hydrogen ion concentrations that they attain in the stationary phase. The development of radioresistance is probably not an effect of pH since another genus, grown under similar culture conditions, does not develop resistance. The hydrogen ion concentration or composition of the irradiation medium, with or without prior incubation, has no detectable effect on either sensitive or resistant cells of *E. coli* strain B/r(CSH). In the same organism, the relative radioresistance parallels relative resistance to ultraviolet and thermal inactivation.

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