RECOVERY FROM ULTRAVIOLET IRRADIATION IN ESCHERICHIA COLI

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"During the past fifty years, many investigators have studied the bactericidal activity of ultraviolet light but a review of their reports shows that little has been learned about many essential factors in the reaction" (Gates, 1929). Since that paper was written several different mechanisms have been proposed to account for the bactericidal action of radiation. Among these are (1) lethal mutations (Lea and Haines, 1940; Lea, 1946), (2) inactivation of enzymes (Dale, 1940, 1942), (3) production of poisons by decomposition of cellular material, and (4) indirect effects of the medium (Coblentz, 1924). All of these mechanisms are reasonable and have been demonstrated under special conditions. The problem yet to be solved in the study of the action of ultraviolet light on bacteria is to determine the relative importance of these processes and to gain further insight into the details of the mechanisms.

One or another process may predominate depending on the experimental conditions. Lea describes methods for distinguishing between direct effects on the organism and indirect effects of the medium. In the case of bacteria the direct effect is the primary one as shown by experiments in which the concentration of the bacteria is varied. Although Lea mentions the possibility of cell poisons and enzyme inactivation, he stresses the mechanism of lethal mutation.

However, the work of Witkin (1947) on radiation-resistant strains of *E. coli* and that of Demerec and Latarjet (1947) on radiation-induced mutations indicate that whereas lethal mutation may account for some of the bactericidal action, other mechanisms must also be involved. The evidence for this is as follows: (1) The resistant strain, B/r, shows a multiple-hit survival curve. (2) At a given dosage, the induced mutation rates to a particular bacteriophage-resistant variant are the same for strains B and B/r, but the bactericidal effects are greatly different. (3) The age of the culture has a slight effect on mutation rates but a large effect on the survival.

In the present investigation it was found that the survival of bacteria was greatly influenced by the treatment after irradiation. Under certain conditions the bacteria recovered from the effects of radiation, and it appeared that a study of the conditions for recovery might yield information on the nature of radiation damage. The findings also give more reliable techniques for radiation experiments, and reveal new differences between X-ray and ultraviolet effects and between resistant and nonresistant strains of bacteria. Finally, since bactericidal action is closely associated with the inhibition of division, this work may lead to new knowledge of the mechanism of division.

MATERIAL AND METHODS

Strain B and strain B/r of *Escherichia coli* obtained from Dr. Witkin were used throughout these experiments. The bacteria for each experiment were grown in liquid media with aeration. Difco nutrient broth and M-9 synthetic media were used unless otherwise stated. M-9 is a synthetic medium developed by Anderson (1946) that is particularly useful as it is transparent to ultraviolet in the spectral region used. It consists of ammonium chloride, phosphate buffer, and magnesium sulfate (solution A) and glucose (solution B). M-9A is used to indicate the salt solution only, but M-9 indicates the complete medium. Assays were made by diluting in M-9A, adding 0.1 ml of the final dilution to 2.5 ml of 0.7 per cent agar (kept liquid at 45 C), and pouring on a plate of 1.5 per cent agar. Difco nutrient broth and medium M-9 were used for the plates, and, unless otherwise noted, gentian violet (4×10^{-6} g per ml) was added to the agar to reduce the number of contaminants. Broth plates were incubated overnight and M-9 plates for 36 hours at 37 C.

As a general procedure the bacteria were centrifuged down from the medium in which they were grown, rinsed, and resuspended in M-9A. This suspension was diluted to give an estimated 10^8 bacteria per ml, a portion was kept as control, and 10-ml samples were used for irradiation. These were irradiated in petri dishes following the technique of Demerec and Latarjet, using a General Electric germicidal lamp at a distance of 92 cm. The radiation is effectively monochromatic at a wavelength of 2,537 A. In a few of the experiments the bacteria were diluted and spread on the surface of agar plates for irradiation.

EXPERIMENTAL RESULTS

In conducting any radiation experiments the following factors should be considered as parameters and carefully controlled: (a) organism; (b) conditions of growth (media, temperature, aeration); (c) phase of growth cycle; (d) conditions during radiation (media, temperature, surface or liquid); (e) radiation (wavelength, time, intensity); (f) conditions after irradiation (media, temperature, time before plating); and (g) conditions for growth of colonies (media, temperature, time).

Most of the factors investigated had an important effect on the survival. Also, it became apparent that no factor could be considered unimportant even though it had no obvious effect in one particular experiment. Some of the factors which were important under certain conditions had no effect in others. The rather large variations in results produced by apparently minor differences in technique may account for some of the discrepancies among different experiments that appear in the literature.

The conditions for growth of colonies and conditions after irradiation were the factors most intensively investigated, the others being treated as parameters. It was found that under most conditions the survival was greater if (a) the colonies were grown on plates made with medium M-9 rather than broth, and (b) following irradiation the bacteria were left in a liquid medium for a "recovery period" before being plated.

Effect of conditions during incubation of colonies. The first indication of increased survival on M-9 plates came from some observations on "snake" formation. It is well known that ultraviolet radiation causes a failure of the division mechanism and that long filaments or snakes are formed by continuation of growth without cell division. It was noticed that this snake formation is most prominent on the surface of broth agar plates, liquid broth giving shorter forms. In synthetic medium (M-9) snake formation was greatly reduced, even though growth measured turbidimetrically was proceeding at a normal rate. Since growth without snake formation implies normal division and possibly normal colony formation, comparative survival curves were taken using broth and M-9 plates. The increased survival on the M-9 plates is apparent in figure 1.

Two points concerning the procedure should be stressed: (1) The only difference between the two curves is in the plating medium and incubation time, i.e., samples for broth plates and M-9 plates were taken from the same final dilution tube. (2) The bacteria were kept chilled (5 C) during irradiation and dilution up to the time of plating. The importance of this point will be apparent later. The difference in the plating medium had little or no effect on the controls yet the survival curves differ in slope and shape.

A marked difference in survival after irradiation between plating on broth and synthetic plates having thus been established, exploratory experiments were conducted to determine what factors were responsible.

(1) *Temperature.* One possibility was that the more rapid growth on the broth plate was injurious. Also, it was learned from Dr. Kelner (1948) that the temperature of incubation was important in the survival of actinomycetes after irradiation. Consequently some broth plates were incubated at 27 C, and others were kept 12 to 24 hours at 15 C before incubation at 37 C. However, these plates did not show any increased survival.

(2) *Composition of plates.* It was found that the addition of 1 per cent broth to the M-9 medium of the plates was sufficient to give a marked decrease in the survival. This result is consistent with an earlier observation that 1 per cent broth would give a noticeable increase in snake formation in synthetic media after irradiation. It thus appears that the presence of some component of the broth rather than the increased rate of growth in the broth medium is responsible for the failure to survive.

(3) *Solidity of plates.* In one experiment a set of broth plates were used for the assay of survivors in which only the percentage of agar was varied. The count obtained doubled as the amount of agar dropped from 1.5 per cent to 0.3 per cent. This effect is consistent with the results obtained for recovery in liquid media (see below).

(4) *Enzymatic constitution of bacteria.* The actual enzymatic constitution of the bacteria depends markedly on the conditions during growth. In particular the enzyme systems of growing bacteria and resting bacteria are quite different, as are the enzymes of bacteria grown in broth, broth plus glucose, and synthetic media (Gale, 1947). Corresponding to these changes in the amounts of the various enzymes present, large differences in sensitivity to radiation are found.

Resting bacteria grown in broth (final pH 8) are much less sensitive than bacteria grown in broth plus glucose (final pH 5.5), or bacteria growing in broth (pH 7).

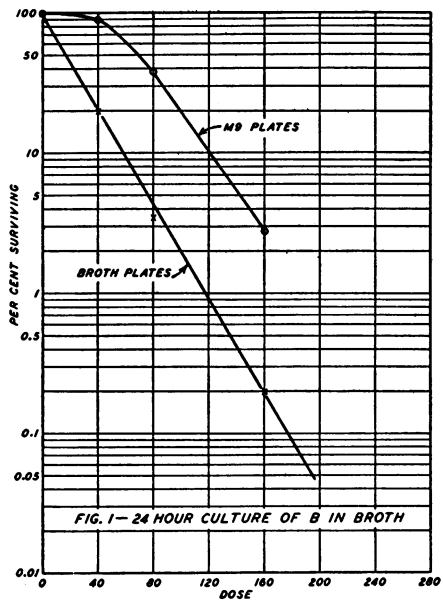

Figure 1

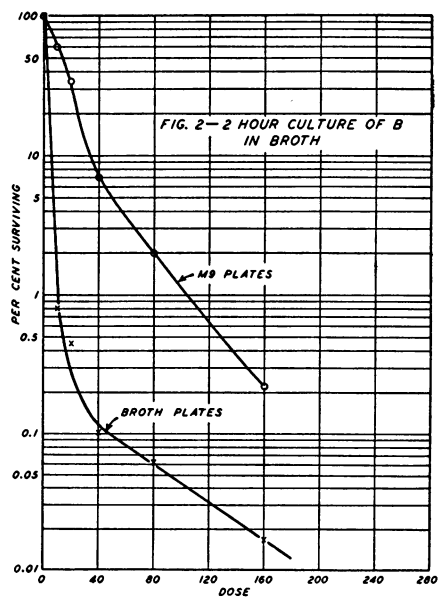

Figure 2

However, under these different conditions there is always a marked increase in survival when M-9 plates are used instead of broth plates (figures 1, 2, 3).

On the other hand, the mutation B to B/r probably affects only a few of the

bacterial enzymes as most of the properties of the resistant mutant are normal. With B/r, however, no difference in survival is observed with broth and synthetic

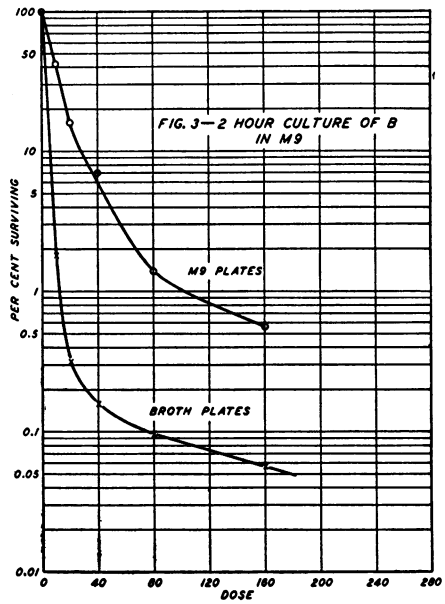

Figure 3

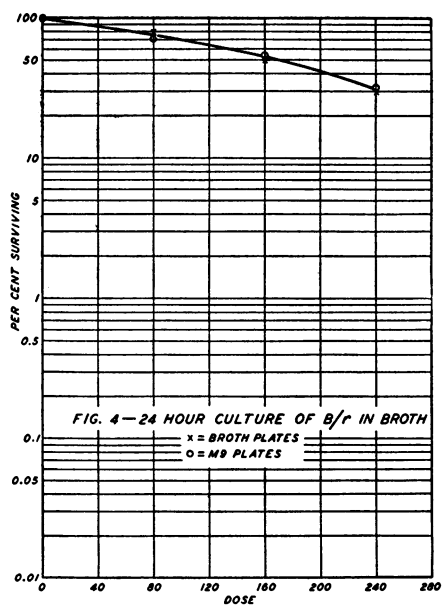

Figure 4

plates (figure 4). With very high doses of radiation B/r actually shows decreased survival on synthetic plates.

Conditions after irradiation. It was observed that when bacteria were ir-

radiated and diluted in M-9A, successive samples taken at intervals from the same dilution tube showed a rapid increase in the number of organisms capable of forming colonies on broth plates. This was not due to ordinary growth and division as (1) there was no energy source available; (2) the count from non-irradiated controls remained constant; and (3) strain B/r does not show this effect. The bacteria apparently recover from the effects of radiation.

It was found that the recovery did not depend to any extent on the presence or absence of any components of the fluid. Distilled water, saline, M-9, M-9A, and broth all gave approximately the same results. Changes of pH from 5 to 9 had no significant effect. The concentration of bacteria in the recovery tube was also without effect in the range 10^8 per ml to 10^3 per ml. Further experi-

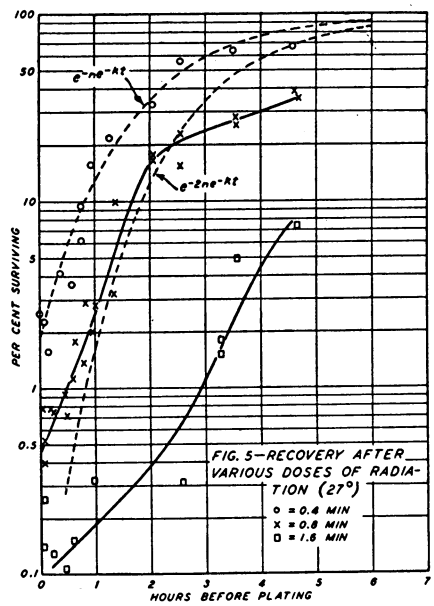

Figure 5

ments are being conducted to determine the effect of various chemicals on the recovery process. At present it does not appear that recovery is influenced by reducing or oxidizing agents.

Factors that did affect the recovery curve were (1) radiation dose (figure 5), and (2) temperature of the recovery tube (figure 6). In connection with the temperature effect it was found that the irradiated bacteria could be kept for 1 hour at 5 C, during which time no recovery occurred, and that they subsequently showed normal recovery on being brought up to 37 C.

In the particular experiment in which broth was used as a recovery fluid, it appeared that the important factor was the time spent in the fluid medium before transfer to solid medium. However, there were other minor differences as the plates contained agar and gentian violet in addition to the broth. Consequently, further experiments were made to test the individual effects of these factors.

Comparing recovery curves taken with and without gentian violet in the plates we find that there is a small effect. The count on the gentian violet plates is approximately one-half that of the plain plates immediately after irradiation, but the difference diminishes as recovery proceeds. After 2 hours no difference is found; neither is there any difference in the bacteria that had not been irradiated. Evidently irradiation does sensitize the cells to the action of gentian violet, but during recovery the sensitivity to gentian violet disappears. Furthermore, the main features of the recovery phenomenon cannot be explained in terms of a gentian violet effect as they remain even when gentian violet is not used.

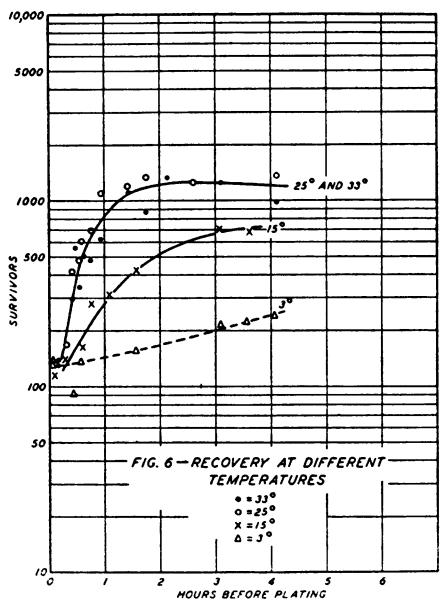

Figure 6

The effect of the agar was also tested. The presence of 0.07 per cent agar in the recovery tube had little effect, but variation of agar concentration of the plates from 1.5 per cent to 0.3 per cent doubled the count obtained immediately after irradiation. The critical concentration for bacterial survival is therefore approximately the same as the critical concentration for the change from liquid to solid.

Effects on filament formation. The conditions which favor recovery are also found to reduce filament or snake formation. Snake growth is greater on broth agar than in liquid broth; also, it is greater in broth than in synthetic media.

Experiments were conducted to observe the effect on snake growth of the recovery treatment in liquid. In these experiments bacteria were irradiated for various intervals; samples were taken and divided into two groups. One group was kept (in M-9A) at 5 C to prevent recovery and the other at 37 C to permit recovery. After 1.5 hours both were plated on broth plates and observed

microscopically as snake formation proceeded. It was characteristic that the "recovered" bacteria showed snake formation typical of "nonrecovered" bacteria that had received roughly one-fourth the exposure to radiation. As gauged by the appearance of the filaments, three-fourths of the radiation effect was removed by the treatment.

Effects of other wavelengths. Single experiments were performed to test whether recovery occurred after exposure to X-rays or to the radiation from a sun lamp (Westinghouse type R S). In each case the exposure was chosen to give 2 per cent survival (without recovery). After X-ray treatment no recovery was observed; with the sun lamp the recovery was similar to that observed with 2,537 A.

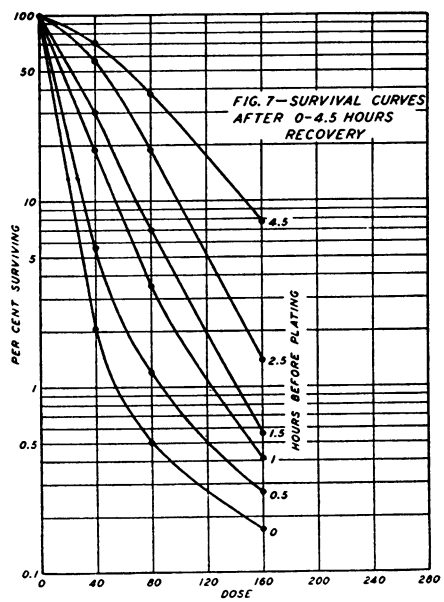

Figure 7

DISCUSSION

"Radiation sickness." The experiments described above show that recovery from radiation damage, which has been observed in other organisms, also occurs with bacteria. Perhaps it would be better to describe the situation as "radiation sickness," a condition in which bacteria will survive or not, depending on their subsequent treatment. For example, the bacteria that survive when plated on synthetic plates differ from nonirradiated bacteria as 90 per cent of them would have died if they had been plated on broth plates—a procedure that is completely nontoxic to ordinary bacteria. Also, the "sickness" is demonstrated by snake formation, which occurs at low doses from which most of the bacteria survive. Hollaender (1943) has also reported evidence of "radiation sickness" after exposure to ultraviolet in an experiment in which the radiated bacteria were unable to withstand prolonged exposure to saline solution.

Improvement in technique. In general, irradiated bacteria are much more sensitive to minor variations in procedures. Figure 1 shows that widely different results can be obtained by using different plating media. Figure 5 shows that erratic results may be easily obtained from variations in the time required to dilute samples before plating. The count may rise by a factor of two for each 5 minutes in the dilution tube. This source of error can be easily eliminated (once it has been recognized) by the use of chilled dilution tubes.

The colony count may vary by a factor of two depending on the consistency (consequently age) of the plates. Also, the use of gentian violet will reduce the count by a factor of two. Bacteria that have not been irradiated are not sensitive to any of these factors. In radiation experiments it is necessary to re-ex-

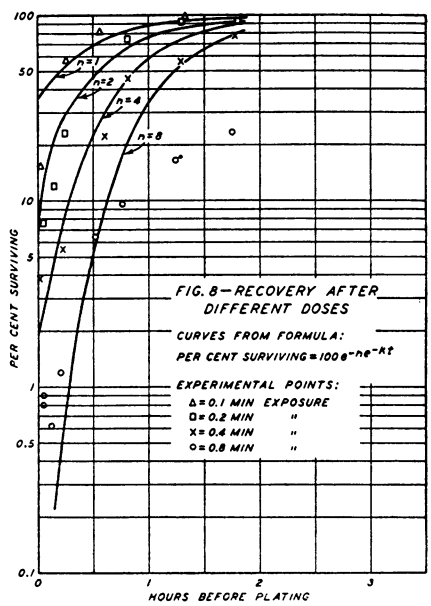

Figure 8

amine even those details of technique that are ordinarily inconsequential. The recovery process might also be used to advantage to increase the yield of ultraviolet-induced mutants.

Interpretation of the forms of survival curves. Figure 7 shows survival curves obtained by plating after increasing intervals of the recovery treatment. The form of the curve shifts from concave to convex, passing through a straight line condition. It would obviously be inadvisable in this case to interpret the straight line condition as evidence for "single hits" in a homogeneous population.

Some of the survival curves obtained by plating on M-9 agar (figure 1, for example) show multiple-hit characteristics; others show single-hit behavior. In view of the findings with recovery in liquid, it would seem that these may be variations in the extent of recovery on the M-9 plates; when recovery is complete multiple-hit curves are obtained. The cause of the variation is not known.

However, it is certain that the shape of the survival curve depends considerably on the exact experimental conditions and any interpretations based on the shape of the survival curves should be made with due caution.

Mechanisms of radiation damage (ultraviolet). The evidence against the hypothesis that lethal mutations account for the bactericidal action of ultraviolet light has already been cited. The mechanism of indirect action has received renewed attention in the experiments of Wyss *et al.* (1948). Their experiments give further evidence that the main effect is direct; the doses of radiation required for appreciable action through the medium are much higher than those used here. This leaves for consideration (a) the inactivation of enzymes, (b) the production of cell poisons, and (c) other mechanisms.

It must be remembered that the mechanism of killing by small doses of the ultraviolet radiation is a subtle one. The processes leading to growth seem unaffected, as can be shown by turbidity measurements or microscopic observation. Virus growth is still possible (Anderson, 1948). Also, the phosphorus uptake and its distribution among the various fractions seem normal (Abelson and Roberts, 1948). The division mechanism, however, is impaired, and growth without division leads to the formation of snakes (Hinshelwood, 1946; Nickerson, 1948). If the balance between growth and division is not too severely disturbed, the snake eventually breaks up into normal viable cells. At higher doses the division mechanism does not come back into balance and the result is lethal. For still higher doses, growth is also affected and snakes are not formed.

It has been shown above that irradiated bacteria behave quite differently when plated on synthetic agar than when plated on broth agar. In the former case the snake formation is greatly reduced and the probability of survival is considerably higher. As the bacteria in both cases are damaged to the same extent when plated, either the recovery is more rapid on the synthetic plates or the effect of the damage is reduced. The experiments on recovery in liquids show that there is little difference between broth and synthetic media as recovery fluids, so it appears that the damage is less disturbing in synthetic media.

This might be the case if there are different rate-limiting factors in the two media. In broth the rates of growth and division are probably limited by enzyme systems involved in synthesis of new protoplasm. No additions to the media will increase the rates. In the synthetic media the rates of growth and division are limited by the supply of intermediate metabolic products as the rates can be increased by additions to the media. Thus in synthetic media damage to the enzyme system would have less effect because the supply of substrate rather than the enzyme is the rate-determining factor. This hypothesis then leads to a conclusion consistent with the observations. In synthetic medium the balance between growth and division is less disturbed; consequently, snake formation will be reduced and the bacteria have a higher probability of surviving.

The slower growth on the synthetic plates also allows more time for recovery processes. However, this is not the principal factor involved, as broth plates incubated at lower temperatures to give slower growth did not show higher survival. The recovery process should be effective at the lower temperatures,

as it was found to be independent of temperature in the range of 26 to 37 C. It would appear that the irradiated bacteria are able to survive and form colonies on synthetic plates in spite of the radiation damage. This seems qualitatively different from the recovery process in liquids in which the effects of the damage seem to be removed.

Damage to the division mechanism could be caused by (a) photochemical inactivation of some of the enzymes involved in division or (b) photochemical production of a poison that inhibits the enzyme system. The evidence of the earlier work favors the poison hypothesis to a certain extent (Witkin, 1947). The phenomena of recovery add more evidence in its favor although it is not possible to rule out alternative theories. The following characteristics of the recovery process should be considered: (1) recovery is not accelerated by external sources of nitrogen or energy; (2) recovery depends on temperature in a way that suggests an enzymatic reaction; (3) recovery is not influenced appreciably by the pH of the medium in the range 5 to 9; (4) recovery proceeds in liquids but is stopped or at least retarded on solid media; (5) no recovery is observed with strain B/r; and (6) no recovery is observed with strain B inactivated with X-rays.

In terms of the enzyme inactivation hypothesis, recovery must consist of the repair or resynthesis of damaged molecules essential to the organism. In this case, the reaction might be expected to depend on external energy sources in a fashion similar to the process of adaptive enzyme formation. Also, it is difficult to understand the effect of the solid medium in preventing recovery.

In terms of the poison hypothesis, recovery consists in the removal of the poison. The effect of the solid media indicates that diffusion may be important in the mechanism, although more than simple physical diffusion is involved because of the long time required and the temperature dependence. It is suggestive that strain B/r, which is not sensitive to this particular form of damage, is also apt to be resistant to penicillin and sulfathiazole. If the poison hypothesis is correct, we are dealing with an extremely toxic agent as one molecule per bacterium is effective. Perhaps such a poison could be produced by a minor photochemical modification of a normal metabolite.

The recovery curves can be calculated on the basis of simple assumptions: (1) the radiation produces a number of "hits" (poison molecules) in the bacteria; (2) these "hits" have a Poisson distribution; (3) the "hits" decay independently with a decay constant K; and (4) the bacteria will survive if no "hits" are present at the time of plating. Recovery should then proceed according to the equation:

$$R = e^{-ne^{-Kt}}$$

in which R is the fraction surviving, n is the average number of hits per bacterium, and t is time. Also, n is calculated from the survival at $t = 0$, and K is adjusted to fit with the experimental points after recovery. Thus one curve can be adjusted to fit the experimental points at two places, but the remainder of the curve is determined. The curves for other doses are completely specified, and no further adjustment is possible.

Figures 5 and 8 show a comparison of these curves with the experimental re-

sults. As this calculation does not take into account the presence of resistant organisms or the killing by other mechanisms, the agreement is more remarkable than the discrepancies.

Other mechanisms of radiation damage. Since there is no recovery with strain B/r, we must assume that other qualitatively different mechanisms are involved in this case. This confirms the indications obtained from the forms of the survival curves (Witkin, 1947). Also, the bactericidal action of X-rays on strain B is qualitatively different from ultraviolet action as there is no recovery from X-rays.

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SUMMARY

Escherichia coli, strain B, after ultraviolet irradiation, may survive to develop colonies or not, depending on certain minor changes in their subsequent treatment. Changes in survival level by as much as a factor of 100 have been observed. Transitions from "single-hit" to "multiple-hit" survival curves are also found. This effect should be recognized and controlled if errors in radiation experiments and their interpretation are to be avoided. A study of the conditions for recovery from this "radiation sickness" gives further evidence for a cell poison hypothesis. According to this hypothesis, the primary effect in the killing of strain B by ultraviolet is the production of a poison within the cell. Under certain conditions this poison can be inactivated or removed and the cell remains viable. Under other conditions the poison selectively inhibits the mechanism of cell division and unbalance is created between division and growth, a filament or "snake" is produced, and the cell finally dies. The killing of strain B by X-rays and B/r by ultraviolet is due to a different mechanism as no snakes are produced and no recovery is observed.

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