INDUCED RESISTANCE TO GAMMA IRRADIATION IN *ESCHERICHIA COLI*

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The ability of microorganisms to become adapted to environmental conditions which have been shown previously to be fatal to the parent strain is a problem with practical as well as fundamental scientific implications. Just as thermophilic organisms present a major problem to the food processor, and antibiotic resistant strains cause difficulties in treatment of infections, so radiation resistant strains well may be a factor in radiation sterilization processes.

While the lethal and mutagenic nature of the interaction of radiations with microorganisms is well established, the mechanisms of this action are still a matter of speculation. For many years a "target theory", based on the "one hit-one effect" interpretation of Dessauer (1923), was accepted commonly, but recent experiments have shaken the basic tenets of this theory. In its place an explanation of radiation effects is being evolved based on physical and chemical changes taking place in the environment of the cells during irradiation. Following this view, it is not improbable that certain cells in any mass population may become adjusted to these previously fatal surroundings. Strains of radio resistant organisms then can be developed and selected. Cosmic, short wave, visible, and ultraviolet radiation, to which most organisms naturally are subjected, however, may have maximized already the radio resistance of most strains. An organism such as *Escherichia coli* which, by virtue of its normal habitats and mode of existence, is not subject much to radiation is therefore an ideal subject for this kind of study.

The development of strains of *E. coli* resistant to viruses and to penicillin has been reported by Demerec (1945) and Luria and Delbrück (1943). These investigators applied the statistical methods of variance analysis evolved by Luria and Delbrück (1943) to resolve the fundamental question: do these agents induce mutants, resistant to the inducing agent, in an initially homogenous population, or do they select spontaneously produced mutants by eliminating

nonresistant organisms? In both the cases of penicillin and virus resistance the close approximation of the variance to the mean of the number of resistant organisms, when the organisms all come from a single culture, is taken as sufficient evidence to indicate that mutations are spontaneous and the agent selective.

More recently, Witkin (1946), by a double irradiation technique, isolated a strain of radiation resistant *E. coli* from strain B which was designated as B/r. The method used is contingent upon the observation of Lea *et al.* (1937) that *E. coli*, on incubation for three hours at 37 C, produces a characteristic "long" form that then may be distinguished from the radio resistant B/r which develops normal microcolonies. In these experiments, 50 ergs/mm² of ultraviolet radiation were used before incubation, and a second dose of 700 ergs/mm² was used three hours later to kill the long forms. A comparison of the survival curves shows that one erg/mm² is, in effect, equivalent approximately to 40 roentgens of X-rays. An analysis of the data by the statistical method of Delbrück indicated that the radiation acted as a selective agent for mutations which were occurring spontaneously at the rate of 10⁻⁵ per organism per generation.

Further experiments by Witkin (1947), in which strain B was exposed to 3,800 ergs/mm² of ultraviolet, showed an increase in the "spontaneous" frequency of radio resistant mutants from 0.003 per cent (average) in the original culture to 4.1 per cent (average) in the survivors from high dose irradiations. These later experiments then can be interpreted only to mean that the selecting agent is also the inducing agent and that spontaneous mutations, when they occur, are negligible in comparison to the number of induced mutations.

Witkin's experiments are unique in the successful isolation of a radio resistant strain. Other observers report instead increased susceptibility to radiation injury of vegetative bacterial cells (Huber, 1952), *Triticum monococcum* (linkorn

wheat) (Smith, 1942), and *E. coli*, strain B/r (Witkin, 1952).

A number of radiation experiments have been carried out on "coliform" organisms. The more comprehensive of these, for example those of Lea *et al.* (1937), Hollaender (1950), and Wyckoff (1930), report "single-hit" destruction curves for conditions similar to those used in the present work and survival curves which are dependent upon environmental conditions during irradiation and the type and wavelength of the radiation. Increased radio sensitivity, such as is reported later in this paper for strain 15, has been observed generally at low survival ratios and has been ascribed to the accumulation of "nonlethal" hits which render the organisms more sensitive (Roepke and Mercer, 1947).

EXPERIMENTAL METHODS

Cultures of *E. coli*, strains 15 (ATCC 9723), B, and B/r, used in these experiments, were grown throughout in standard nutrient broth (Difco). Samples in test tubes were exposed to gamma radiation from a tubular Co⁶⁰ source essentially the same as that described by Manowitz (1951). The dose rate in the irradiation chamber was 80,000 roentgens (r) per hour, measured in air by the polyvinyl chloride film dosimeter techniques of Henley and Miller (1951). All samples in each group were introduced at the same time, individual tubes being withdrawn at intervals up to 180 minutes, depending on the dose they were to receive. Source chamber temperatures during all irradiations were 15 ± 3 C.

Uniform procedures for the preparation of irradiation samples were established and strictly maintained, for pretreatment and environmental conditions markedly influence the results of irradiation. For experiments with strain 15, the following routine was followed:

(a) Transfer from stock culture (nutrient agar) to nutrient broth followed by overnight (16 to 20 hours) growth at 37 C.

(b) Transfer of 0.1 ml of this broth culture to each of 8 tubes containing 5 ml of broth, followed by incubation for 17 hours at 37 C.

(c) Irradiation of these samples for periods up to 180 minutes (240,000 r) as previously described.

(d) Seventeen hours before the next irradiation, 8 tubes containing 5 ml of broth were inoculated with 0.1 ml of the sample (3 hours) from

the previous group that had been exposed to 240,000 r. These tubes were incubated again for 17 hours and irradiated as before.

This procedure was continued for 6 consecutive days, using the maximum dose tube from each group as inoculum for the following set.

With strains B and B/r the techniques just outlined were modified in the following manner to permit irradiation on alternate days:

(a) At the end of each exposure, 1 ml of the 180 minute tube was transferred to 10 ml of broth and incubated.

(b) Approximately 17 hours before the next irradiation, 1 ml from this tube was transferred to 25 ml of broth again, followed by incubation at 37 C.

(c) Immediately prior to irradiation, 2 ml of this last broth culture were placed in each of 8 tubes. These samples were exposed then as before and, following irradiation, the 180 minute tube again is used as inoculum for the next group.

At regular intervals the species purity of the test culture was checked. Samples were streaked on eosin methylene blue and McConkey agar plates which had been dried in the incubator for 2 days at 37 C. Characteristic reactions on these differential media together with microscopic examination were used as indications of purity. No contamination was observed in any of the experiments.

Populations were counted by plating appropriate dilutions. To minimize the spreading of colonies, a layering technique was used. First 5 ml of nutrient agar were poured and allowed to solidify. Next the sample dilution mixed with a second portion of agar was added, followed after solidification by a third, covering layer of agar.

RESULTS

Plots of the log of the per cent surviving organisms versus radiation dose (figures 1 to 3) have been selected as the most convenient method for presenting the results of these experiments. It should be pointed out, however, that the use of "per cent" survival values at high total doses may be misleading, particularly in cultures having large initial populations, for even though the fraction killed closely approaches unity, the absolute number of viable organisms remaining is appreciable.

In the strain 15 irradiation (figure 1) each transfer group appears to become more radiation

Figure 1. Increase in radiation sensitivity of transfers of *Escherichia coli*, strain 15.

Figure 2. Increase in radiation resistance of transfers of *Escherichia coli*, strain B.

sensitive with increasing doses, indicated by the increasingly negative slope of each individual survival curve. Subcultures from the samples receiving 240,000 r show increasing susceptibility to radiation injury with each transfer, corroborating the general observations of other observers (Smith, 1942; Huber, 1952). The trend toward increased radio sensitivity after the second transfer, however, is not pronounced enough to warrant separate lines for each set of data. The overlapping of points makes further distinctions both difficult and indefensible.

Figure 3. Increase in radiation resistance of transfers of *Escherichia coli*, strain B/r.

In the strain B irradiations (figure 2) an increasing sensitivity to radiation during the course of exposure again was observed. The general shape and characteristics of all these curves are explainable on the assumption that a number of "near hits" make the organisms more radiation labile. On this basis it is indicated that, rather than selecting a spontaneously produced radio resistant mutant, the agent itself is inducing mutations, and phenotypic reproduction to these mutants is not taking place during irradiation. If the opposite were true, one would expect survival

curves which slope concavely upwards (decreasing sensitivity) at low survival fractions.

The assumption that the cells do not reproduce during irradiation, which at first appears unsound since the generation time for these cells is about 20 minutes, seems reasonable in view of the observation of Gates (1933) who noted a delay of four hours after ultraviolet irradiation before cellular division takes place. Microscopic counts of total (viable and nonviable) cells, N , taken before and after three hours of irradiation make it possible to calculate a reproduction coefficient, R ; for the rate of change in N is equal to the reproduction coefficient times the number of viable cells. The number of viable cells is the total count, N , minus $N_0(1 - e^{-kt})$, the number of dead cells, so that:

$$\frac{dN}{dt} = R(N - N_0(1 - e^{-kt})) \quad (1)$$

The solution to this equation, with $N = N_0$ at $t = 0$, is:

$$N = \frac{N_0 R}{k + R} (e^{Rt} - e^{kt}) + N_0 \quad (2)$$

where

N = total count at time t

N_0 = initial count; $t = 0$

k = slope of the lethal curve

R = reproduction coefficient (cells per cell) between $t = 0$ and $t = t$

t = time

The reproduction coefficient was found to be 1.15 cells per cell over the 3 hour irradiation period, indicating that about one out of seven bacteria reproduce in this time.

It will be noted that the tendency to increased radio resistance diminishes with continued subculturing. While data from the last transfers of strain B are increasingly difficult to separate, it is not possible to say that any clear limit to the increased resistance has been reached in over twenty transfers.

Irradiation and subculturing of B/r (figure 3) gave substantially the same picture. Strain B/r, when received, was less radio resistant than the later transfers of B, shown by plotting the survival curve of the seventeenth B transfer on figure 3. After several transfers the resistance of B/r also was increased appreciably.

DISCUSSION

In addition to providing qualitative illustrations of developing radio resistance, the results of the strain B and B/r irradiations may be used to estimate the rate at which mutations are induced under the conditions employed. From the data of figure 2 it is possible to calculate a "ratio" of induced mutation, since:

$$\frac{dn}{dD} = -k_1 n - Mn \quad (3)$$

where

n = number of radio sensitive organisms

D = dose in roentgens

M = mutations produced per roentgen

k_1 = "inactivation coefficient" for the "pure" radio sensitive organism defined by the relation, $n = n_0 e^{-k_1 D}$ in the early, straight-line portion of the survival curve.

Similarly, for resistant organisms:

$$\frac{dx}{dD} = -k_2 x + Mn \quad (4)$$

where

x = number of radio resistant mutants

k_2 = coefficient defined as k_1 , by the relation $x = x_0 e^{-k_2 D}$

Solving (1) for n , substituting in (2), and solving the resultant differential equation give the relation:

$$x = \frac{Mn_0}{k_2 - (k_1 + M)} \left[e^{-(k_1 + M)D} - e^{-k_2 D} \right] \quad (5)$$

where n_0 = initial number of n (sensitive) cells.

The constants k_1 and k_2 may be evaluated from the slopes of the control and the subsequent survival curve since k_1 and k_2 are much greater than R , the reproduction coefficient. Equation (5) then may be employed to determine M by taking a very high dose and assuming that at this value we are counting essentially only radio resistant organisms on the plates. This initial mutation ratio has been found to be approximately 4×10^{-7} per roentgen for strain B and 5×10^{-8} for B/r.

This relationship may be used to predict the number of resistant cells present in any instance. Figure 4 is such a plot of equation (5) calculated from the data of the strain B control run (figure 2), using the 3 hour (240,000 r) point, noted on

the plot, to calculate M . Two independent runs were made to check the theoretical equation. The agreement between experimental and theoretical is good over a limited range. At doses under 240,000 the total number of cells is appreciably larger than predicted because of the presence of large numbers of nonresistant organisms.

Since these experiments were conducted, Saz *et al.* (1952) have reported similar effects in the development of bacterial resistance to antibiotics. They report no stepwise and discontinu-

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SUMMARY

Escherichia coli, strain 15, showed an increasing susceptibility to radiation injury when an irradiated culture was used as inoculum in a series of retransfers and irradiations. Using the same techniques it was possible to induce radio resistance in both strains B and B/r. The reproduction frequency over the three hour irradiation period was found to be 0.15, and the initial mutation ratio (mutants per roentgen) was estimated at 4×10^{-7} for strain B and 5×10^{-6} for strain B/r. A method for estimating the number of resistant cells present in a culture under radiation is developed and illustrated from the survival curve data.

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Figure 4. Population prediction in irradiated cultures (equation 5).

ous change in antibiotic resistance, the maximum resistance of colonies at each transfer step being a function of the concentration to which the organism has been exposed. It also may be that certain colonies are natural mutants.

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