

FACTORS RESPONSIBLE FOR THE DELAYED APPEARANCE OF RADIATION-INDUCED MUTANTS IN *ESCHERICHIA COLI*

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Received October 20, 1948

THAT mutations are produced in bacteria following treatment with X-rays and ultra-violet light has been shown by a number of workers (GRAY and TATUM 1944; GOWEN 1941; ROEPKE, LIBBY, and SMALL 1944; and TATUM 1945, 1946). However, where irradiated bacteria have been tested for the presence of induced mutants immediately after treatment few have been found, and detailed studies show that appreciable growth must take place in the treated population before the bulk of the induced changes appear (DEMEREK 1946; DEMEREK and LATARJET 1946).

The proportion of the total mutations which are delayed in this manner amounts to 99 percent or more in the one case studied (mutation of *E. coli* strain *B/r* to resistance to bacteriophage *T1*); and the population increase required in order for all of them to appear is approximately 2^{12} to 2^{13} times.

This "delayed effect" of irradiation is similar in both X-ray and ultra-violet treated material, and parallel phenomena have recently been demonstrated following exposure to nitrogen mustard (BRYSON 1947), sodium desoxycholate and acriflavine (WITKIN, personal communication).

The underlying cause of the delayed appearance of mutants, could be either (a) induced gene instability causing an increase in the spontaneous mutation rate over a number of cell generations, or (b) induced gene mutation requiring one or more cell divisions in order to affect the phenotype of the organism.

The first of these two interpretations (gene instability) assumes an effect of radiation which has not been observed in higher organisms, and thus implies a fundamental peculiarity of bacterial genes. However, gene instability lasting over many cell generations has been produced in *Drosophila* by the action of mustard-gas (AUERBACH 1947) and it is conceivable that a similar effect is produced in bacteria by irradiation (see PONTECORVO 1946).

The alternative interpretation assumes delayed phenotypic expression, an effect already demonstrated in a class of spontaneous mutations of *E. coli* (resistance to phage *T1*, NEWCOMBE 1948). However, spontaneous mutations require only two or three generations in order to become detectable, and this would seem inadequate to explain more than a part of the prolonged delay associated with induced mutation.

The two interpretations raise a number of points of fundamental importance:

(a) If there is a delay in the phenotypic expression of gene mutation, the evidence is relevant to the general problems of gene action and cell physiology.

(b) If radiation induces gene instability in the bacteria, there must be a fundamental difference between the genes of this organism and those of higher forms. This would be of evolutionary significance.

(c) On the other hand, if bacterial genes are identical to those of higher forms the general knowledge of the mutation process can be advanced, since this organism promises to yield quantitative information which would be unobtainable with larger and more slowly breeding materials (See LURIA 1946 for a discussion of the point).

For these reasons an attempt was made in the present study to discriminate between the two interpretations of the "delayed effect"

DELAYED APPEARANCE OF INDUCED MUTANTS

The available information concerning the delayed effect of irradiation and the further data required in order to discriminate between the alternative interpretations will now be considered.

The effect was first demonstrated by DEMEREC (1946) who adopted the following procedure: suitable numbers of irradiated cells were spread on the surface of nutrient agar and incubated for varying periods of time; at the end of this they were sprayed with phage *T1* to eliminate all susceptible bacteria, and incubated further until colonies of visible size appeared. Estimates of population increase during the various periods of growth prior to spraying were made by dilution and plate count, using samples from a broth culture of the treated bacteria which had been incubated at the same time.

Since the descendants of a treated bacterium are confined to one locality on the agar, a single colony will result from the presence of a resistant cell in a clone. Each resistant colony indicates that a mutation has taken place.

However, the method does not indicate the proportions of resistant and susceptible cells descended from individual treated bacteria, and this information is necessary if we are to decide whether the induced gene mutations took place prior to growth or after one or more cell divisions.

Furthermore, it is conceivable that the treated cells giving rise to resistant mutants do not start to divide until some time after the population as a whole has entered the exponential growth phase. If this is the case one can obtain only a maximum estimate of the number of cell generations required for the appearance of the induced mutants.

Further information is therefore necessary, and its nature will be evident from a consideration of the genetic consequences of the two possible causes, either acting alone or in conjunction with a delay in the onset of division. These are illustrated in figure 1 as follows:

- (A) delayed mutation,
- (B) delayed mutation plus delayed onset of division,
- (C) delayed phenotypic expression, and
- (D) delayed phenotypic expression plus delayed onset of division.

In this figure the mutation is represented as appearing after a population increase of 2^4 times. Also, it has been assumed that there is only one gene complement in the cell and that each gene behaves as a single unit toward the

irradiation. Other assumptions could have been chosen, but a consideration of them will be left until the discussion.

Experiments to discriminate between these interpretations will be considered in the subsequent sections.

MATERIALS AND METHODS

Escherichia coli strain *B/r* (WITKIN 1946, 1947) and phage *T1* (DEMEREK and FANO 1945) were used throughout these experiments. The mutations studied were those resulting in the development of resistance to phage *T1*, that is from *B/r* to *B/r/1* and *B/r/1,5*. No attempt was made to distinguish between these two categories of mutation, and throughout the paper all *T1* resistant mutants will be referred to collectively as *B/r/1*.

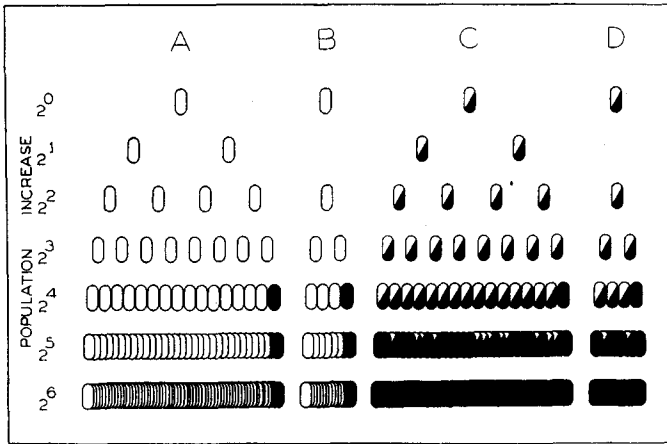


FIGURE 1.—Illustrating the genetic consequence of:
 (A) delayed mutation (due to induced gene instability)
 (B) delayed mutation plus delayed onset of division
 (C) delayed phenotypic expression
 (D) delayed phenotypic expression plus delayed onset of division.

Note: White figures represent non-mutants, white-black figures represent mutants which have not yet developed phenotypic phage resistance, and black figures represent phage resistant mutants.

Cultures of *B/r* were grown to saturation in a medium transparent to ultra-violet light (the M9 synthetic medium of ANDERSON 1946). Undiluted suspensions containing approximately 15×10^8 bacteria per cubic centimeter were irradiated with a General Electric Germicidal Lamp, this being estimated to deliver 95 percent of the ultra-violet energy in the 2,537 Ångstrom line. For irradiation, 10 cc quantities of the suspension were placed in Petri dishes of 100 mm diameter and shaken mechanically in order to ensure uniform exposure of all cells. The frequency and amplitude were such as to produce standing waves which did not quite reach the point of splashing.

A series of exposures was made ranging from one minute upward at a distance of 41 centimeters, the intensity being $1,600 \text{ ergs} \times \text{millimeter}^{-2} \times \text{min}$

utes⁻¹ as measured by a Westinghouse ultra-violet meter.

Numbers of viable bacteria were determined throughout the experiments by dilution and plate count. Numbers of phage resistant mutants were determined in a similar manner using agar plates which had previously been spread with phage *T1*.

EVIDENCE FROM THE ESTIMATED TIMES OF APPEARANCE OF
ACTIVELY DIVIDING MUTANT CLONES

Of the interpretations discussed earlier, C (figure 1) is the one most readily tested. This assumes that mutant clones are present as actively dividing entities from the time when the treated population first begins to multiply, and that the delay is a matter of the number of cell generations required for phenotypic expression, this ranging from zero up to 12 generations.

Information on this point can be obtained from the sizes of individual mutant clones in fully grown cultures provided that mutants and non-mutants divide at equal rates, an assumption which will be tested later. Thus, where n_1 is the number of viable bacteria in the inoculum, n_2 the number in the fully grown culture, and m_2 the number of cells from a single mutant clone at the end of growth, the time at which the mutant clone appears as an actively dividing entity can be determined from the formula

$$g = \log(n_2/n_1 \cdot m_2) \log 2$$

Where all the cells of the inoculum have started to divide at approximately the same time, g will represent the number of cell generations occurring prior to the appearance of the dividing mutant clone. Where, on the other hand, the cells of the inoculum have started to divide at widely different times one cannot, of course, speak in terms of cell generations in the culture as a whole, but the population of the culture at the time when the mutant clone appeared will have been approximately 2^g . This latter estimate of the population will not be strictly accurate since it takes no account of the numbers of viable cells which start to divide after the appearance of the mutant clone. It will, however provide a close approximation except in the very early stages of the growth of the culture before the numbers of cells in the actively dividing part of the population appreciably exceed the number which are still in the lag phase.

In interpretation C, which we are considering in this section, the value of g should be zero in the case of all mutant clones, whereas in interpretations A, B, and D it would be expected to vary for different mutations from zero up to approximately 12.

To test this, series of cultures were grown from treated bacteria, the numbers of cells in the inocula being adjusted so that induced mutant clones would occur in only one out of every eight to ten cultures. Thus, the cultures in which an induced mutant clone developed would rarely contain more than one.

Material from the one minute and two minute exposures were used in these experiments, and 480 cultures were grown from each. Forty-six cultures from the one minute treatment and 57 from the two minute treatment contained

induced mutant clones in which the value of g was 10.5 or less. Those with values of g higher than 10.5 were ignored since an appreciable number would be expected due to spontaneous mutation alone.

The data from these experiments are presented in tables 1 and 2, and in histogram form in figure 2. The results of a similar control experiment, using a sample of the same bacterial culture which had not been irradiated, are given in table 3, and are also included in the histograms of figure 2.

TABLE 1

Delayed appearance of actively dividing mutant (B/r/1) clones following one minute irradiation with ultra-violet light. ($m_2/50$ =number of resistant bacteria in a 0.1 cc sample of a fully grown test culture. g =estimated number of cell generations between irradiation and the appearance of an actively dividing mutant clone.)

| EXPERIMENT | A | | B | | C | | D | |
|------------------------|--------------------|--|--------------------|--|--------------------|--|--------------------|--|
| No. of test cult. | 120 | | 120 | | 120 | | 120 | |
| Vol. of test cult., cc | 5 | | 5 | | 5 | | 5 | |
| Av. bact. in inoc. | 98 | | 71 | | 25 | | 45 | |
| Av. end no. of bact. | 11.3×10^8 | | 11.7×10^8 | | 11.6×10^8 | | 13.5×10^8 | |
| Cult. with $g < 10.5$ | 11 | | 14 | | 12 | | 9 | |

| CULTURES | $m_2/50$ | g | $m_2/50$ | g | $m_2/50$ | g | $m_2/50$ | g |
|----------|------------|-----|----------|-----|----------|------|----------|-----|
| 1 | 1,270,000* | — | 790,000* | — | 410,000 | 1.2 | 200,000 | 1.3 |
| 2 | 140,000 | 0.7 | 730,000* | — | 228,000 | 2.2 | 140,000 | 1.9 |
| 3 | 85,600 | 1.4 | 410,000 | 0.0 | 160,000 | 2.5 | 87,100 | 2.6 |
| 4 | 18,120 | 3.7 | 114,700 | 1.5 | 116,500 | 3.0 | 28,300 | 4.2 |
| 5 | 8,400 | 4.8 | 29,800 | 3.5 | 102,800 | 3.2 | 24,100 | 4.4 |
| 6 | 7,200 | 5.0 | 24,800 | 3.7 | 56,500 | 4.0 | 15,200 | 5.1 |
| 7 | 4,510 | 5.7 | 15,300 | 4.4 | 52,100 | 4.2 | 1,161 | 8.7 |
| 8 | 3,730 | 6.0 | 12,200 | 4.8 | 37,500 | 4.8 | 1,100 | 8.9 |
| 9 | 3,230 | 6.2 | 9,100 | 5.2 | 12,000 | 6.3 | 757 | 9.1 |
| 10 | 3,120 | 6.3 | 8,900 | 5.2 | 9,400 | 6.6 | — | — |
| 11 | 498 | 9.3 | 2,170 | 7.3 | 947 | 9.9 | — | — |
| 12 | — | — | 1,090 | 8.2 | 877 | 10.1 | — | — |
| 13 | — | — | 724 | 8.8 | — | — | — | — |
| 14 | — | — | 713 | 8.8 | — | — | — | — |

* Numbers of resistant bacteria larger than required to give a value of g equal to zero.

Values of g were obtained which ranged from zero upward, and which might well exceed the value of ten if induced mutations could be detected against the rising background of spontaneous mutations.

The very small number of genotypically mutant clones which appear at the onset of growth suggest that delayed phenotypic expression alone cannot account for the "delayed effect." However, in order to estimate the times at which mutant clones appear it has been assumed that mutants and non-mutants increase at the same rate. Experiments to test this assumption are reported in the next section.

TABLE 2

Delayed appearance of actively dividing mutant (B/r/1) clones following two minutes irradiation with ultra-violet light. ($m_2/50$ =number of resistant bacteria in a 0.1 cc sample of a fully grown test culture. g =estimated number of cell generations between irradiation and the appearance of an actively dividing mutant clone.)

| EXPERIMENT | A | | B | | C | | D | |
|------------------------|--------------------|--|-------------------|--|--------------------|--|-------------------|--|
| No. of test cult. | 120 | | 120 | | 120 | | 120 | |
| Vol. of test cult., cc | 5 | | 5 | | 5 | | 5 | |
| Av. bact. in inoc. | 311 | | 247 | | 205 | | 193 | |
| Av. end no. of bact. | 12.0×10^8 | | 9.5×10^8 | | 10.5×10^8 | | 9.3×10^8 | |
| Cult. with $g < 10.5$ | 22 | | 8 | | 12 | | 15 | |

| CULTURES | $m_2/50$ | g | $m_2/50$ | g | $m_2/50$ | g | $m_2/50$ | g |
|----------|----------|------|----------|------|----------|-----|----------|------|
| 1 | 45,300 | 0.8 | 36,700 | 1.1 | 24,200 | 2.1 | 100,000 | 0.0 |
| 2 | 28,500 | 1.4 | 4,400 | 4.1 | 11,200 | 3.2 | 23,600 | 2.8 |
| 3 | 26,800 | 1.5 | 1,613 | 5.6 | 5,000 | 4.4 | 3,200 | 4.9 |
| 4 | 17,400 | 2.2 | 1,502 | 5.7 | 4,300 | 4.6 | 3,000 | 5.0 |
| 5 | 13,700 | 2.5 | 1,311 | 5.8 | 2,037 | 5.6 | 2,800 | 5.1 |
| 6 | 10,200 | 2.9 | 776 | 6.6 | 1,750 | 6.8 | 1,680 | 5.8 |
| 7 | 8,300 | 3.2 | 78 | 9.9 | 656 | 7.3 | 1,200 | 6.3 |
| 8 | 5,400 | 3.8 | 70 | 10.1 | 449 | 7.8 | 1,184 | 6.3 |
| 9 | 4,300 | 4.2 | — | — | 353 | 8.2 | 826 | 6.9 |
| 10 | 3,350 | 4.6 | — | — | 254 | 8.6 | 499 | 7.6 |
| 11 | 2,100 | 5.2 | — | — | 232 | 8.8 | 385 | 8.0 |
| 12 | 1,039 | 6.2 | — | — | 122 | 9.2 | 252 | 8.6 |
| 13 | 609 | 7.0 | — | — | — | — | 217 | 8.8 |
| 14 | 594 | 7.1 | — | — | — | — | 120 | 9.6 |
| 15 | 574 | 7.1 | — | — | — | — | 88 | 10.2 |
| 16 | 338 | 7.8 | — | — | — | — | — | — |
| 17 | 297 | 8.0 | — | — | — | — | — | — |
| 18 | 91 | 9.7 | — | — | — | — | — | — |
| 19 | 76 | 10.0 | — | — | — | — | — | — |
| 20 | 62 | 10.0 | — | — | — | — | — | — |
| 21 | 54 | 10.5 | — | — | — | — | — | — |
| 22 | 54 | 10.5 | — | — | — | — | — | — |

Competitive growth of mutant and non-mutant strains

The results presented in the previous section could arise from wide differences in the rates of increase of mutant and non-mutant strains when in competition. It is therefore necessary to determine whether differences of the required magnitude do in fact exist.

For such a test to be critical it is necessary (a) that the conditions of growth be identical to those in the original experiment, (b) that the mutant strains will not have undergone further change which would alter their ability to compete with non-mutants, and (c) that a number of independent mutant strains be tested.

(a) In the present tests particular attention was paid to the following conditions: (1) culture size, (2) size of the inoculum (and thus the period of

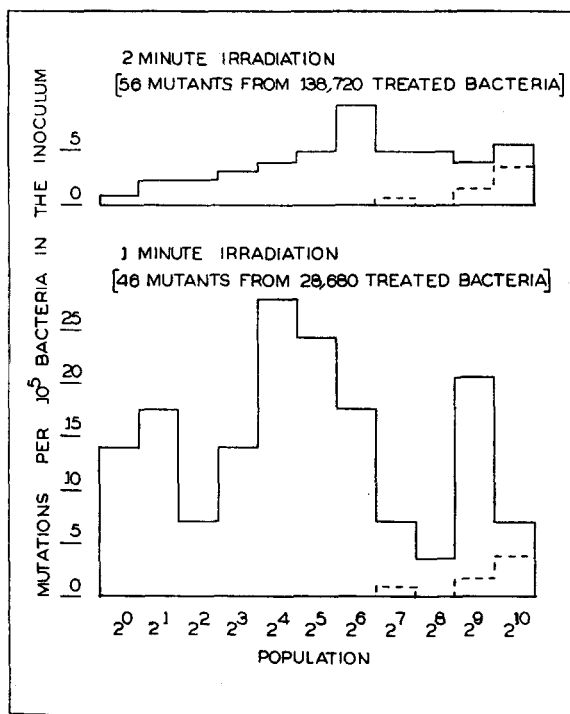


FIGURE 2.—Estimated times of appearance of ultra-violet induced mutations as actively dividing mutant clones. Time is expressed in terms of general population increase. Mutant clones from control experiment are indicated by broken lines. (From tables 1, 2, and 3.)

TABLE 3

Control experiment. Time of appearance of actively dividing mutant (*B/r/1*) clones in cultures from unirradiated cells. ($m_2/50$ = number of resistant bacteria in a 0.1 cc sample of a fully grown test culture. g = estimated number of cell generations between resting stage, and the appearance of an actively dividing mutant clone.)

| EXPERIMENT | A | |
|------------------------|--------------------|-----|
| No. of test cult. | 120 | |
| Vol. of test cult., cc | 5 | |
| Av. bact. in inoc. | 1154 | |
| Av. end no. of bact. | 11.5×10^8 | |
| Cult. with $g < 10.5$ | 8 | |
| CULTURES | $m_2/50$ | g |
| 1 | 131 | 6.6 |
| 2 | 40 | 8.1 |
| 3 | 33 | 8.4 |
| 4 | 18 | 9.0 |
| 5 | 18 | 9.0 |
| 6 | 16 | 9.3 |
| 7 | 15 | 9.4 |
| 8 | 15 | 9.4 |

growth required to reach saturation), and (3) ratio of mutants to non-mutants in the inoculum.

(b) Inocula for the tests were taken directly from experimental cultures which contained induced mutant clones. This avoided the possibilities of change and selection inherent in isolating the mutant, freeing it from phage, and re-mixing with a non-mutant strain, a procedure which has been necessary when making similar tests with spontaneously occurring mutants.

(c) Resistant clones from six independent mutations were tested. Two of these were estimated to have appeared early in the growth of the cultures ($g=0$), two at an intermediate time ($g=6$), and two late in the growth of the cultures ($g=9$).

Two separate procedures were used. In the case of the early appearing mutant clones, ten or more cultures were grown from inocula containing approximately 700 bacteria, of which about 40 were resistant mutants. Any change in the proportion of mutants resulting from differences in the rate of increase was expressed as

$$m_2/n_2 \times n_1/m_1$$

where, n_1 and n_2 are the total numbers of bacteria, and m_1 and m_2 the numbers of resistant cells in the inoculum and in the fully grown test cultures. Ten or more independent single-plate estimates were made of each of these four values, those of n_2 and m_2 being each from a different test culture. Independent

TABLE 4

Change in the proportion of mutants ($B/r/I$) to non-mutants (B/r) as a result of competitive growth using induced mutant clones in which the estimated value of g is zero.

| EXPERIMENT | A | B |
|--------------------------------|--------------------|--------------------|
| No. of test cult. | 17 | 10 |
| Vol. of cult., cc | 5 | 5 |
| Av. bact. in inoc. (n_1) | 707 | 719 |
| Av. end no. bact. (n_2) | 12.7×10^8 | 14.2×10^8 |
| Av. mutants in inoc. (m_1) | 47 | 40 |
| Av. end no. mutants (m_2) | 7.5×10^7 | 9.3×10^7 |
| Generations growth | 20.4 | 20.6 |
| Av. $m_2/n_2 \times n_1/m_1$ | $0.94 \pm .26$ | $1.20 \pm .40$ |

estimates of the value $m_2/n_2 \times n_1/m_1$ were thus obtained for each test culture. Averages of these and standard deviations are given in table 4 together with other experimental data.

This procedure could not be used in the case of experimental cultures containing smaller proportions of resistant bacteria (those in which the mutant clones were estimated to have appeared later in the growth of the cultures). Inocula from these, in order to have a sufficient number of resistant cells to minimize statistical fluctuations in the value of m_1 , would have in addition

very large total numbers of bacteria. This in turn would decrease the period of growth required to reach saturation, and thus might tend to obscure differences in the rates of growth of mutant and non-mutant strains. To overcome the difficulty small inocula were used, containing on the average one resistant mutant in every eight to ten instances. Large numbers of cultures (60 to 120) were grown, and the value $n_1/m_1 \times m_2/n_2$ determined for those which had received a resistant mutant in the inoculum, m_1 being assumed to be unity in all cases. No correction was made to compensate for the occasional occurrence of

TABLE 5

Change in the proportion of mutants (B/r/I) to non-mutants (B/r) as a result of competitive growth, using induced mutant clones in which the estimated value of g is 6 in experiments A and B, and 9 in experiments C and D.

| EXPERIMENT | A | B | C | D |
|---|-------------------|--------------------|-------------------|--------------------|
| No. of test cult. | 120 | 120 | 60 | 60 |
| No. with mutant in inoc. | 15 | 27 | 7 | 9 |
| Vol. of cult., cc | 5 | 5 | 5 | 5 |
| Av. bact. in inoc. (n_1) | 463 | 1280 | 2030 | 1770 |
| Av. end no. bact. (n_2) | 8.5×10^8 | 13.2×10^8 | 9.2×10^8 | 10.6×10^8 |
| Assumed no. of mutants in inoc. (m_1) | 1 | 1 | 1 | 1 |
| Av. end no. of mutants (m_2) | 3.0×10^6 | 2.8×10^6 | 3.2×10^6 | 1.7×10^6 |
| Generations of growth | 20.5 | 19.7 | 18.6 | 19.0 |
| Av. $m_2/n_2 \times n_1/m_1$ | $.16 \pm .05$ | $.32 \pm .19$ | $.78 \pm .44$ | $.28 \pm .09$ |

two resistant mutants in an inoculum, since the magnitude of any bias from this source is negligible for present purposes.

The four mutant clones estimated to have appeared later in the growth of the experimental cultures ($g=6$ and $g=9$), have all been treated in this manner. Average values of $n_1/m_1 \times m_2/n_2$ are given in table 5, together with standard deviations.

If one is to interpret the varying numbers of resistant cells in cultures from treated bacteria (tables 1 and 2) as due merely to variations in the growth rates of the mutant clones, these being present from the beginning as actively dividing entities, then mutant clones in which $g=9$ should in these tests give values for $n_1/m_1 \times m_2/n_2$ of approximately 0.0019, those in which $g=6$ values of approximately 0.016, and those in which $g=0$ values of approximately unity. That there are some significant differences in growth rate is indicated in tables 4 and 5, but these are obviously too slight to account for the data presented in the previous section.

Thus, the histograms in figure 2 may be said to represent with a fair degree of accuracy the times of appearance of induced mutants as actively dividing mutant clones. These histograms provide information which is quite parallel to that of DEMEREC (1946) on phenotypic expression. And, combining the evidence from both sources it can be said that in the early stages of the growth of irradiated bacteria most of the induced mutations are neither phenotypically

detectable nor are they present as actively dividing mutant clones which will later develop phenotypic expression (interpretation C of figure 1).

However, whether they are present in a non-dividing state (interpretation D) or whether the treatment has merely acted to raise the spontaneous rate of mutation (interpretations A and B) can be determined only from a study of the progeny of single treated cells.

EVIDENCE FROM THE PROPORTIONS OF RESISTANT BACTERIA IN
THE PROGENY OF SINGLE TREATED INDIVIDUALS

To discriminate between the remaining interpretations (A, B, and D of figure 1) it is necessary to determine whether or not the descendants of single treated bacteria may include mixtures of resistant and susceptible cells. Because of the possibility of more than one nucleus in the bacteria any data from mixed clones would have to be considered statistically, but observations of a relatively small number of wholly resistant clones would constitute critical evidence.

To secure cultures from single treated bacteria, colonies were first obtained by growing irradiated suspensions on agar. Broth cultures were then inoculated with single whole colonies picked from the plate by means of a loop, and were incubated until they had reached saturation. (The possibility of a colony having arisen from more than one treated bacterium will be considered later.) The approximate number of resistant cells in each culture was then determined by spreading a loopful (approximately 0.005 cc) of each on agar previously spread with phage *T1*, and incubating. Where the number of surviving colonies indicated a high proportion of resistant bacteria (that is, more than one per 2^{10}), careful tests were made to determine more precisely the numbers of resistant and susceptible cells.

The most probable number of cultures required to obtain one induced mutant clone may be estimated from table 1. For the one minute treatment it appears that approximately 600 would be needed. In practice 3,480 were grown from this material and two containing induced mutant clones were obtained (table 6).

TABLE 6

Proportions of induced mutants ($B/r/1$) in cultures from single treated bacteria.

| EXPERIMENT | A | B |
|-------------------------------|-------------------|-------------------|
| No. of test cultures | 1,800 | 1,680 |
| Vol. of cult., cc | 2 | 2 |
| Bact. in inoc. (n_1) | 1 | 1 |
| Av. end no. bact. (n_2) | 7.2×10^8 | 6.8×10^8 |
| No. of cult. with $g < 10.5$ | 1 | 1 |
| Cultures with $g < 10.5$ | | |
| No. of estimates of m_2/n_2 | 8 | 8 |
| Av. value of m_2/n_2 | $1.04 \pm .03$ | $0.97 \pm .19$ |
| Value of g | 0.0 | 0.0 |

It was found that these contained resistant bacteria only, and tests were made to ensure that there had been no contamination. First, the bacteria were tested against phage *T2* and found to be susceptible. This identified them as *E. coli* and rendered the possibility of bacterial contamination remote. Second, the cultures were tested for the presence of phage and a negative result indicated that the pure mutant clone had not arisen as the result of accidental contamination with *T1* and subsequent selection.

Further, these two cultures cannot have been derived from phenotypically resistant bacteria present prior to growth ("zero-point mutants") since no such resistant cells were found in samples containing 1.6×10^6 treated bacteria.

Also, it is extremely unlikely that they arose from cells which in cultures from large inocula would have given rise to actively dividing mutant clones at the beginning of growth since only four such clones are shown in table 1, out of a total of 46. From this, the chance that both are of such origin is $(4/46)^2$ which is equal to 0.008.

Thus, mixtures of mutant and non-mutant individuals among the descendants of a single treated bacterium are not characteristic of the "delayed effect." This fact rules out interpretations A and B of figure 1 which assume an induced gene instability.

It now appears that the mechanism outlined under D, in which a delay in the onset of division is combined with a delay in phenotypic expression, must be operative in causing the observed delay in appearance of induced mutations. It is of course conceivable that in addition there are individual instances of induced gene instability, but it would be difficult to establish their existence, and at the present time there is no reason to assume such a dual interpretation.

In the next section evidence concerning the assumed variable delay in onset of division will be considered.

DELAYED ONSET OF DIVISION IN IRRADIATED CELLS

In the previous section it was shown that to explain the "delayed effect" a delay in the onset of division in the induced mutants such that the bulk of them do not start to divide until the population has increased appreciably must be assumed.

This assumption involves two possible concepts. Either (a) the delay in the onset of division is associated with the presence of the mutation which we are considering or (b) there is a variability in the time of onset of division, not in any way associated with resistance to *T1*, such that the majority of the treated bacteria do not start to divide until the population has risen to many times its original number. The former of these two concepts would seem unlikely on *a priori* grounds, but it remains for the latter to be demonstrated.

Under normal conditions, bacteria from a saturated culture of *E. coli* strain *B/r* inoculated into fresh broth medium and incubated, do not divide until after an interval of approximately seventy minutes (at 37°C). Following this "lag period" the population doubles every 19 to 20 minutes until the saturation density is approached. As a result of irradiation, this lag period is considerably increased, usually by from one to two hours depending upon the dose, but once

divisions have begun (or at least very shortly thereafter) the population rises exponentially at the normal rate (DEMEREK 1946). This, of course, does not necessarily mean that division starts in all of the treated individuals at the same time, and even with wide differences in the time of onset the population increase would become approximately exponential as soon as the actively dividing individuals arising from the early divisions appreciably outnumbered those which had not yet started to divide. For this reason, evidence as to the existence of a cell to cell variation in the onset of division in treated bacteria cannot be obtained from the growth curves of liquid cultures.

In order to obtain a clearer picture of the times at which irradiated bacteria enter division, suitable dilutions of an irradiated suspension ($1,000 \text{ ergs} \times \text{millimeters}^{-2}$; survival = 24.1 percent) were spread on agar and incubated for vary-

TABLE 7

Time of onset of divisions in irradiated B/r, and numbers of induced mutations which become phenotypically detectable. (Dose = $1,000 \text{ ergs} \times \text{millimeters}^{-2}$; survival = 24.1 percent.)

| INCUB. (MIN.) | NUMBERS OF MICROCOLONIES | | | | | | | | | VIABLE CELLS (EST.)* | INDUCED MUTA- TIONS (/10 ⁸)† |
|------------------|--------------------------|-----|----|----|---|---|----|---|---|----------------------------|---|
| | CELL GENERATIONS | | | | | | | | | | |
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | | |
| 0 | 1,000 | — | — | — | — | — | — | — | — | 240 | 0 |
| 60 | 1,000 | — | — | — | — | — | — | — | — | 240 | 20 |
| 80 | 999 | 1 | — | — | — | — | — | — | — | 241 | 12 |
| 100 | 979 | 21 | — | — | — | — | — | — | — | 261 | 9 |
| 120 | 968 | 28 | 4 | — | — | — | — | — | — | 280 | 12 |
| 140 | 970 | 18 | 8 | 4 | — | — | — | — | — | 310 | 20 |
| 160 | 950 | 35 | 6 | 8 | 1 | — | — | — | — | 356 | 120 |
| 180 | 954 | 40 | 0 | 2 | 4 | — | — | — | — | 372 | 225 |
| 200 | 925 | 48 | 7 | 5 | 4 | 9 | 2 | — | — | 796 | 517 |
| 210 | 807 | 126 | 35 | 15 | 1 | 6 | 10 | — | — | 1,408 | 795 |
| 240 | 803 | 104 | 42 | 26 | 3 | 4 | 13 | 4 | 1 | 2,404 | 977 |

* As no allowance is made for the inviable cells which have undergone a single division, the early figures will represent a slight overestimate. This bias becomes negligible in the later figures.

† Averages based on four plates each; inoculum 2.4×10^7 bacteria; using the method of DEMEREK (1946).

ing periods of time. At the end of incubation the plates were chilled to stop all divisions and were examined under the high power dry objective. From each plate the proportions of bacteria which had undergone 0, 1, 2, 3, 4, etc. divisions were determined. Ten different periods of incubation, differing by 20 minute intervals, were used; there were four replicate plates, and a thousand microcolonies were scored for each of the incubation periods. The results of this experiment are presented in table 7.

Since the intervals were arranged to coincide with the division period of the bacterium, it is possible from the table to determine what proportion of the viable cells enter the first division at a particular time, and what proportion

continue to divide after the first division. It will be seen that well over half of the bacteria which pass through one division do not divide further, but that practically all which pass through the second division go on dividing indefinitely. Thus, only those which pass through two divisions contribute to the effect which we are considering. Of these, it will be seen that only a small proportion take part in division when it first commences, that more than half are delayed until after the first have passed through their fifth division, and that a very appreciable proportion do not divide until after some have passed through the eighth division. Because of the difficulty of estimating the numbers of bacteria in very large microcolonies the observations could not be extended beyond this point.

This picture is qualitatively what is required to explain the "delayed effect" without assuming any correlation between the mutation which is being studied and the delay in onset of division. It does not, of course, eliminate all possibility of such a correlation, but it does show that it is not the major factor.

Thus the "delayed effect" of irradiation is due to a delay of one or more cell divisions between mutation and phenotypic expression (already observed in the case of spontaneous mutations), plus a variability in the time at which irradiated bacteria start to divide.

EXPRESSION OF DELAYED EFFECT

During the present study, two main conclusions have been reached regarding the "delayed effect" of irradiation: (1) that the mutations are present in the irradiated population prior to growth but fail to produce any phenotypic change until one or more cell divisions have taken place in the mutant bacteria, and (2) that many of the mutations occur in individuals which will not start to divide until the treated population has increased considerably.

A delay of two or three cell divisions between spontaneous mutation and phenotypic expression has already been demonstrated (NEWCOMBE 1948) and the simplest interpretation in the case of the induced mutations would be that the delay is of similar origin. There has been one obstacle to a common interpretation namely that induced mutations appeared to take longer to achieve phenotypic expression. Concerning this it has been shown in the present work that the increased time need not necessarily be due to a greater number of cell divisions before induced mutations are expressed, but may be the result of a variability in the time of onset of division of the treated bacteria such that many individuals fail to divide until early growth has produced an appreciable population rise. There remains then little reason to assume that the delayed phenotypic expression in spontaneous and induced mutations is due to different causal factors.

MECHANISM OF DELAYED EFFECT

The delay in phenotypic expression may be interpreted in terms of (a) genetic systems, or (b) enzyme systems (see DEMEREC 1946; DEMEREC and LATARJET 1946).

Genetic interpretations would assume that the effect of the mutated unit is

obscured by the presence of one or more normal homologous units and that expression occurs when individuals containing mutated units only are produced through division and segregation. The reduplicated structures might be:

- (1) gene lamellae
- (2) cytoplasmic genes
- (3) chromosomes
- (4) nuclei.

That the delay may be due to the presence of a number of gene lamellae or of cytoplasmic genes is rendered unlikely by the finding of resistant clones from single treated bacteria which are unmixed with susceptible cells. It is still conceivable however, that duplicate sets of chromosomes or duplicate nuclei are present at the time of treatment and that they have been rendered inviable through lethal mutation or structural change.

One of these possibilities, that of diploidy or polyloidy, may be eliminated since spontaneous mutants would be obscured for an indefinite period. The possible presence of more than one nucleus however deserves serious consideration.

There is no cytological evidence of the number of nuclei in resting cells, the stage at which our material was irradiated. However, ROBINOW (1945) and BOIVIN (1947) have observed that rapidly dividing cells contain either two or four staining bodies arranged in linear order within the cell. There is good reason to believe that these are nuclei. Further, they appear to be included in daughter bacteria at cell division in a regular manner merely by the formation of a wall separating sisters if there are only two, or pairs of sisters if there are four.

If this situation exists in the resting stage it could account for the delayed appearance of induced mutants. Thus, where two nuclei are present one cell division would be required for a mutation to be expressed, and where there are four nuclei two divisions would be required.

However, the delay observed in the case of spontaneous mutations (NEWCOMBE 1948) could not be explained in this manner for the following reason. In this interpretation the number of phenotypically resistant bacteria arising from a mutation would double with each cell generation following the appearance of the first one. The number of phenotypically resistant bacteria arising from a spontaneous mutation however more than doubles during each of the three divisions taking place after the first one has appeared, and at the end of this period there are, not eight, but something like six times this number. Thus the presence of more than one nucleus could not account for the observed delay in the case of spontaneous mutations although it might well produce an additional delay which could not be detected by the methods used. (The possibility of a mode of segregation of four nuclei other than that indicated above has been considered but will not be discussed in detail since one has to assume a highly improbable behavior in order to account for the spontaneous mutation data. Briefly, this would involve an almost regular exchange of place of the two inner nuclei.)

Thus it seems likely that non-genetic mechanisms are involved in the de-

layed expression of spontaneous mutations. And, it is reasonable to assume that induced mutations are likewise subject to a non-genetic delay, whether or not a segregation effect is also present.

Relevant to this is McILWAIN'S (1946) speculation that enzyme production may be associated with gene reproduction. This speculation is based on an argument indicating that the number of molecules of certain enzymes in a cell is small, probably one or two.

However, before the evidence on delayed phenotypic expression of mutation in bacteria can be related to these general problems it will be necessary to know more of the relationship between mutation, delay, and phenotypic expression, and in particular whether this sequence is or is not a function of the mutagenic agent, the environmental factors at the time of the mutation, the particular gene mutation, or, finally, of the organism in which mutation is studied.

What is clear from the evidence so far obtained, is that the delayed appearance of mutations in bacteria following irradiation does not in any way constitute evidence of a fundamental genetic peculiarity of these organisms. Relevant to this general problem, although not to the specific question of delayed phenotypic expression, are the data on irradiation killing. These will be considered in the next section.

KILLING

Following lethal doses of irradiation, bacteria do not disintegrate as when treated with strong disinfectants; motile forms may remain motile, spores may germinate, and bacterial cells may pass through one or two divisions (BRUYNOGHE and MUND 1935; unpublished observations of ROBINOW and LEA reported in LEA 1946 page 325; LURIA 1939). The present experiments show that the majority of the "killed" cells of *E. coli* strain *B/r* undergo a single division (table 7). The possible implications of this behavior will now be considered.

Irradiation is known to kill the cells of higher organisms through:

- (a) non-genetic mechanisms
- (b) lethal mutation
- (c) chromosome unbalance following breakage and rearrangement.

Non-genetic mechanisms appear to act in the killing of the radiation susceptible strain *B* of *E. coli*. This is indicated by the fact that the sensitive strain *B* and the resistant mutant *B/r* do not differ in their response to the mutagenic action of irradiation (DEMEREK and LATARJET 1946). Also, strain *B* is more susceptible than *B/r* to such presumably non-mutagenic chemicals as penicillin and sodium sulphathiazole (WITKIN 1947). It may thus be concluded that the bulk of killing in strain *B* is not due to genetic effects. However, in strain *B/r* genetic effects may well be significant.

Lethal mutations have been suggested by LEA (1946) as a possible cause of death, and could conceivably account for the delayed killing of *B/r* (table 7) provided that they, like mutations to phage resistance, have a delayed phenotypic expression. This however is not the only possible interpretation.

Chromosome unbalance has long been considered the major cause of cell death in higher organisms following irradiation. Here the effects, in terms of

chromosome breakage and rearrangement, can be studied in detail at metaphase following treatment, and it can be seen that certain of the daughter cells will lack portions of the chromosome material and will contain replicates of other portions.

The expression of this mechanism of killing is complicated in diploid tissues by the presence of a duplicate set of chromosomes which enable the cells to survive considerable loss and reduplication. In haploid cells however the effect is more clear cut. Here it can be shown that irradiation and chromosome breakage has little effect on cell behavior until division occurs, but that after it has taken place normal function ceases. For example in *Tradescantia* 800 r given after pollen grain division has no detectable effect upon pollen germination, but if given prior to this division germination is inhibited in practically all of the treated grains (NEWCOMBE 1942).

That a chromosomal mechanism has not been suggested earlier for radiation induced killing of bacteria has in all probability been due to the absence in the past of any indication of the existence of bacterial chromosomes. But, in view of the cytological evidence of ROBINOW (1945) and BOIVIN (1947), and the recent genetic evidence of LEDERBERG (1947), this interpretation has become increasingly plausible.

ACKNOWLEDGMENTS

This investigation was originally contemplated while the senior author was working under DR. M. DEMEREC, but was postponed in favor of a study of spontaneous mutation. The experimental approach is quite different from that originally considered, but much is owed to the early discussions with DR. DEMEREC concerning possible interpretations of the data available at that time.

CONCLUSIONS

Ultra-violet induced mutations in the bacterium *E. coli* strain *B/r*, resulting in resistance to the bacteriophage *T1*, have been studied. It was known that the majority of these mutations do not appear until after the treated population is permitted to grow, some being delayed by as much as twelve generations (or a 2^{12} times increase) of the treated population (DEMEREC, 1946). The present study has yielded the following information regarding the underlying causes of this phenomenon:

(1) The delayed appearance of induced mutations is due (a) to the necessity for one or more cell divisions to take place before mutation can be expressed phenotypically, and (b) to a failure of many of the induced mutant cells to start dividing until the population has increased considerably.

(2) This delay in phenotypic expression appears to be similar to that previously demonstrated in the case of spontaneous mutations (NEWCOMBE, 1948) and it therefore seems probable that it is due to a non-genetic (such as enzyme) mechanism.

(3) There is a wide variation in the time at which individual treated cells start to divide. This accounts for the failure of many of the induced mutants

to commence division until the population has risen considerably, without assuming that the presence of the mutation is responsible for the delay.

Thus delayed appearance of radiation-induced mutants does not indicate any fundamental difference between the genic materials of bacteria and those of higher organisms. Also these experiments indicate a relationship between mutation and expression which would be difficult to demonstrate in multicellular tissues.

Relevant to the question of the genetic similarity of bacteria and higher organisms, it has been found that the majority of the *B/r* individuals "killed" by the ultra-violet irradiation are capable of a single cell division. This behavior is similar to that of the cells of higher organisms and the possibility of a chromosomal mechanism is suggested.

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