One of the fundamental problems of genetics is the nature of differences between spontaneous and induced mutation. In higher organisms, mutations induced by ionizing radiations are known to include a relatively large number of chromosomal aberrations. Yet there is no doubt that spontaneously occurring chromosomal abnormalities have been important phylogenetically, and may themselves be the secondary consequences of spontaneous genetic changes.

Bonnier and Luning\(^1\) observe that the regression line expressing a linear relationship between intermediate doses of x-ray and mutation frequency in Drosophila fails to intercept the point derived from untreated and slightly irradiated controls. Similar findings have been obtained by Spencer and Stern\(^2\) with departures from linearity established as significant by Boag\(^3\) using a probit diagram. It is at least possible that the deficit


\(^{2}\) Spencer and Stern, J. Gen. Physiol., 13, 781 (1930).

of mutations observed following extrapolation of the regression line to control material is due to the inclusion, within any group of induced mutations, of types phenotypically similar but not necessarily identical to the entire potential array of spontaneous variants. An alternative interpretation would be that radiation, even at moderate dosage levels, accelerates unselectively the spontaneous mutation rate. This would lead to a more rapid production of mutants capable of spontaneous occurrence in the same relative proportions. No qualitatively new types would be anticipated, other than forms with spontaneous rates low enough to exclude detection in the majority of unirradiated populations.

Pending the development of an adequate system of microbial cyto- genetics, comparisons of mutagenic effects on bacteria must depend on biochemical or morphological criteria, with no precise knowledge of the cytological consequences. Nevertheless, the known effect of x-rays on other biological material has prompted the choice of ultra-violet radiation in the present study as an agent with effects more likely analogous to spontaneous mutation. X-rays, nitrogen mustard and other chemical mutagens will be analyzed in supplementary investigations.

Most mutations occur at rates that are too low to afford quantitative information without considerable difficulty in obtaining samples of adequate size. Available data are therefore largely confined to the best studied genetic materials—maize\textsuperscript{4,8} and Drosophila.\textsuperscript{6} The use of a screening technique allowing elimination of all non-mutant individuals automatically from the test population as employed in plant studies by Lewis\textsuperscript{7} and in bacteriological investigation by Luria and Delbruck\textsuperscript{8} and Demerec\textsuperscript{9} offers the best solution to the problem of obtaining samples of sufficient numerical size to permit comparisons between spontaneous and induced mutation. A disadvantage of ordinary bacterial strains is the inability to localize precisely genetic differences as specific gene mutations on the linkage map. The recombining strain of \textit{Escherichia coli} is not suitable for analysis of ultra-violet-induced mutations to phage resistance because of the lysogenic phage that it carries.\textsuperscript{10} We have performed our comparison of ultra-violet-induced and spontaneous mutation with strain B/r of \textit{E. coli}. This strain is resistant to radiation and penicillin,\textsuperscript{11} nitrogen mustard,\textsuperscript{12} oxidizing agents,\textsuperscript{13} proflavine, crystal violet, potassium tellurite, and safranin. Most of these agents may be used selectively to obtain strain B/r from strain B.\textsuperscript{12,14}

The method employed has been to use bacteriophage T1 as the selective agent.\textsuperscript{15} Mutations to T1 resistance in strain B/r are known to consist almost exclusively of two main types: B/r/1, resistant to T1 and sensitive to T5; and B/r/1, 5, resistant to both T1 and T5. The collective rate of mutation to T1 resistance is approximately $0.7 \times 10^{-8}$ per cell per generation.\textsuperscript{16} It is therefore possible by plating cell populations in excess of
this number on nutrient agar in the presence of phage to obtain considerable numbers of mutant colonies. All sensitive cells are lysed and provide no nutritional competition. By starting numerous independent cultures with small inocula of phage-sensitive cells, and isolating only a single mutant from one culture, it is possible to exclude the possibility that mutants will be related by descent from a mutant parent cell.

One hundred and sixty-four B/r/1 strains resistant to phage T1 and of independent spontaneous origin have been obtained. Each mutant was isolated by the following procedure: A stock broth culture of B/r, aerated during growth, was assayed for titer and found to contain a background not exceeding 200 B/r/1 per 10⁸ cells when tested by three different T1 lysates. In eight separate experiments, a small initial population of phage-sensitive cells was obtained by diluting the stock culture and setting up independent subcultures containing broth volumes of 0.2 to 1 ml. and inocula of 2.5 cells to 1.2 × 10³. The small independent cultures were incubated at 37° for two days. Following incubation, between 0.05 and 0.2 ml. of each subculture was spread on nutrient agar plates that had previously been covered with 2 to 5 × 10⁸ lytic units of bacteriophage T1. After 48 hours of incubation, the isolated colony nearest the center of each plate was numbered, inoculated into a nutrient agar slant and streaked on a Petri plate containing nutrient agar. (Consecutive streaking serves to free the resistant strain from phage T1.) After incubation a single colony was therefore restreaked from the Petri plate. Part of a single colony from the second streaking procedure was later suspended in 2 ml. of broth, diluted 10⁻² in saline and tested for phage resistance and for ability to grow on minimal media containing agar, water, glucose and inorganic salts. In determining the pattern of phage resistance the standard experimental series of seven phages (T1–T7) was reduced by the omission of T4 and T7, except in testing strains resistant to T3. Among spontaneous mutations, at least, testing with T3 is the practical antecedent of a test with T4 or T7, since one-step bacterial mutants sensitive to T3 have never been found to show resistance to T4 or T7.

Isolation of induced mutants for comparative purposes could have been performed with relative ease by irradiating bacteria, allowing them to grow through several divisions, and then selecting delayed mutants by the phage aerosol technique. The attendant disadvantages of using phage aerosol in a crowded laboratory caused us to employ an experimental method more closely related to the technique of isolating spontaneous mutants. By means of the same T1 lysates, 114 ultra-violet-induced mutants were isolated. The dose of ultra-violet energy, obtained from a GE T-15 mercury vapor lamp, was about 720 ergs per millimeter squared, sufficient in the various separate experiments to sterilize 90–99.6% of the treated cells. Bacteria were irradiated at a saline dilution of 10⁻¹ in
Petri plates, shaken to minimize screening effects. The cells were then spread in 0.1-ml. quantities on nutrient agar plates and allowed to pass through from 2 to 9 divisions. The seeded plates were spread with from \(2 \times 10^5\) to \(10^9\) lytic units of phage T1, incubated for 48 hours and a single mutant selected and purified in the same manner as described previously for spontaneous mutants.

Validity of Method Used in Isolating Induced Mutants.—Populations attaining a size equivalent to the reciprocal of the mutation rate will on the average contain one T1 resistant mutant. The wide statistical fluctuations in the random process of mutation make it possible that mutations will be found in rare instances within growing sensitive populations.\(^8\)\(^9\) There is always a possibility, therefore, that experiments designed for the isolation of induced mutants will include a few spontaneous mutants. The number of spontaneous mutants can be kept to a minimum by isolating only one mutant from any specific experimental population. Control experiments show the small probability that spontaneous T1-resistant mutants will arise in the absence of irradiation in sensitive bacterial populations at less than \(10^7\) viable cells (table 1).

The failure of treated suspensions to show a direct correlation between mean titer and mutant population is presumably due to the occasional presence of an early mutation which produced a clone of phage-resistant cells within a single colony during the period after ultra-violet treatment and plating, but before addition of phage and resprreading of cells on the agar. When bacteriophage is spread over the agar following incubation of irradiated cells, individual clones are broken and distributed at random, contributing to a high variance in any analysis of the average number of mutants per plate. Table 1 shows that individual populations of less

\[\begin{array}{cccc}
\text{TITER/PLATE} & \text{PROPORTION OF POSITIVE CULTURES} & \text{AVERAGE NUMBER MUTANTS PER POSITIVE CULTURE} & \text{TITER/PLATE} \\
3 \times 10^3 & 0/2 & \ldots & 1.5 \times 10^3 & 0/5 & \ldots \\
6 \times 10^4 & 0/6 & \ldots & 5 \times 10^4 & 1/5 & 4.0 \\
1 \times 10^5 & 0/2 & \ldots & 1 \times 10^5 & 1/2 & 1.5 \\
5 \times 10^5 & 0/6 & \ldots & 5 \times 10^5 & 10/10 & 63 \\
2 \times 10^5 & 1/6 & 1 & 1 \times 10^5 & 10/10 & 15 \\
3 \times 10^5 & 2/6 & 1.5 & 4 \times 10^5 & 27/27 & 342 \\
7 \times 10^6 & 0/7 & \ldots & 6 \times 10^5 & 30/30 & 208 \\
2 \times 10^7 & 0/2 & \ldots & 1 \times 10^7 & 10/10 & 313 \\
\end{array}\]
than $2 \times 10^7$ viable cells are unlikely to contain mutants unless the cells have previously been irradiated. In isolating induced mutants, independent origin is assured by selecting from only one colony on a plate. A survey of all spontaneous and induced mutants describing resistance patterns to $T_1, T_2, T_3, T_5$ and $T_6$, together with nutritional deficiencies, is given in table 2. Only strains resistant to $T_3$ have been tested with $T_4$ and $T_7$.

Certain quantitative differences are at once apparent in comparing spontaneous and induced mutants. The proportion of mutants resistant to $T_1$ but sensitive to $T_5$ is 44% in the spontaneous group, and only 8% in the induced group. Two remarkable qualitative differences also appear. All mutants requiring a supplementary growth factor other than tryptophane are in the induced series. Within this group are found single mutants requiring thiamine, tryptophane + tyrosine + phenylalanine,

<table>
<thead>
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<tr>
<td>PHAGE RESISTANCE PATTERN AND NUTRITIONAL REQUIREMENTS OF SPONTANEOUS AND ULTRA-VIOLET-INDUCED, T1-RESISTANT MUTANTS OF E. coli</td>
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<th>POLARITY</th>
<th>SPONTANEOUS Total</th>
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<th>TOTAL</th>
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<td>79</td>
<td>8</td>
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<td>B/r/1, 3, 4, 7</td>
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<tr>
<td>Total</td>
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<td>99</td>
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<tr>
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<td>100</td>
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<tr>
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<tr>
<td>Total</td>
<td>114</td>
<td>107</td>
<td>2</td>
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</table>

a Methionineless.

b Thiamineless, aromaticless, purineless and serineless.

purines, serine and methionine. In contrast all patterns of phage resistance excluding the common patterns $B/r/1$ and $B/r/1, 5$ have arisen spontaneously. The comparative rarity of mutations leading to requirements for supplementary growth factors other than tryptophane, or to phage resistance other than $B/r/1$ and $B/r/1, 5$ types, makes a definite conclusion as to significance difficult. If distribution were truly random, the non-random occurrence of all the five rare phage mutants in one class, and the five aberrant deficient mutants (auxotrophs) in another would seldom occur on the basis of chance ($P = <0.002$).

Related to the low proportion of $B/r/1$ in the irradiated group is a comparatively small number of mutants requiring tryptophane. Of 72 spontaneous $B/r/1, 52$ required tryptophane. In the induced series only 1 of 9 required this amino acid. Requirement for tryptophane is less likely to occur in the mutation $B/r \rightarrow B/r/1, 5$ than in $B/r \rightarrow B/r/1$.
In the former class (B/τ/1, 5) only 8 of 87 spontaneous mutants required tryptophane, and only 1 of 105 induced B/τ/1, 5 had a tryptophane requirement. The greater proportion of tryptophaneless in the spontaneous population is probably a significant departure from random distribution. Some investigators regard the appearance of tryptophane requiring B/τ/1, 5 mutants as evidence of a two-step process: B/τ/1 tryp—→ B/τ/1 tryp−/1, 5. In the second step, sensitivity to T1h host range mutants in T1 phage would simultaneously be lost. Although known as an experimental possibility, there is no evidence that two-step origin is required. The deficit of B/τ/1 mutants in the induced class (table 2) is not due to differential elimination of these individuals by host range phage mutants (T1h) in the lysates employed for isolation, since the same lysates were used in obtaining virus-resistant bacteria of both spontaneous and induced origin.

Another difference in the spontaneous and induced populations arises in the proportion of mutants that show partial resistance to phage T1. Such partial resistance is exhibited by 26 of the induced B/τ/1, 5 mutants, and by one of 3 induced B/τ/1 mutants, including a methionineless strain. By comparison only 3 strains partially resistant to T1 were found in the entire group of 164 spontaneous mutants. Partially resistant strains are called B/1p, B/1p5p, B/1p5, etc.

Partial resistance is characterized by reduced growth rate in broth containing over $5 \times 10^8$ lytic units per milliliter, but normal growth rate if the phage concentration is reduced to about $10^8$ lytic units per milliliter. In phage concentrations of $10^8$ per cubic centimeter, the composition of the B/1p culture remains unchanged, but in phage concentrations over $5 \times 10^8$ a culture grown from a small inoculum contains primarily B/τ/1. A similar effect of phage concentration is observed on nutrient agar, where colonies of B/τ/1p do not grow to normal size with 24 hours' incubation at 37°C, if high concentrations of T1 are present. Colonies isolated from heavily phaged plates are B/τ/1.

Dilution assays of B/1p cultures were made on unphaged plates, and on plates with sufficient phage to delay the appearance of visible colonies by about 10 hours. The phaged plates contained more than enough T1 to eliminate all fully sensitive B/τ bacteria before B/τ/1 mutations were likely to occur. The number of cells initiating colonies on phaged and unphaged plates was most frequently equal, showing that the non-B/τ/1 cells in the B/τ/1p culture cannot be B/τ. From these facts we conclude that the predominating type of cell in the B/1p culture is in itself responsible for many of the peculiarities of the culture, and that this cell is different from B/τ and B/τ/1.

Many strains with partial resistance produce plaques when exposed to concentrations of phage that would give confluent lysis of a sensitive
strain. Thus nutrient agar plates spread with three independent B/r/1_p strains and \(3 \times 10^6\) lytic units of T1 gave 10, 38 and 107 cloudy plaques respectively. At high phage concentrations the cloudy plaques become confluent and large clear plaques appear on two specific B/r/1_p strains as a type of host range variant capable of completely lysing the partially resistant bacteria. All B/r/1_p strains were tested for efficiency of plating. This expresses the ratio of cloudy plaques found on B/r/1_p to clear plaques observed on the parent strain, B/r. Where plaques were found on B/r/1_p the correlated low efficiency of plating was not due to poor adsorption of phage in a test of one strain.

Exposure of irradiated bacteria to mixtures of phages is a more efficient method of detecting multiple patterns of phage resistance and may reveal complex types as noted in the spontaneous group of table 2. Occurrence of unique auxotrophs in the treated sample is thought to be of greater significance, particularly since other investigators with considerable experience in the nutritional requirements of spontaneously arising phage-resistant bacteria have found no auxotrophs that did not require tryptophane\(^{18}\) or a known biochemical precursor. Anderson obtained 27 independent strains requiring tryptophane and resistant to T1. One of these also had a deficiency for proline.

Tryptophane-requiring mutants not resistant to T1 may easily be obtained by the penicillin method.\(^{19}\) The association of resistance to T1 and tryptophane deficiency can nevertheless occur as the result of a single mutation or complex of mutations producing the related phenotypic changes either at once or in sequence too rapid to be detected by our present methods. Resistance to T1 and T5, or to T3, T4 and T7 also is associated.\(^{18}\) Anderson has approached this problem by assuming that a series of catenary reactions exists, governed by specific enzymes and leading to phage sensitivity. The dichotomous nature of these processes implies that genetic blocks occurring early in a series of branching events would influence ability of the cell to act as host for several viruses, leading to familiar mutants such as B/1, 5 or B/3, 4, 7. Inability of the cell to synthesize tryptophane interferes with T1 reproduction, and also prevents cell multiplication on minimal media.

By Anderson's interpretation, reverse mutation from a mutational block interfering with a common precursor for phage reproduction and cell growth on minimal media should restore the original wild-type phenotype. Tests to be reported show that in specific instances it is possible for nutritionally deficient, phage-resistant strains to yield prototrophs without loss of the resistance pattern to phage. If we adopt the scheme of Anderson, a loss of nutritional deficiency without change of phage resistance pattern could be interpreted, not as a reverse mutation affecting the site of the original enzymatic block, but as an additional mutation influencing another position.
in the chain of biochemical events and restoring prototrophy (non-deficiency) without relation to phage sensitivity. It would then be probable that the "reverted" types obtained from ultra-violet-induced phage-resistant auxotrophs would differ genetically from spontaneous prototrophs of the same resistance pattern. Differences between parental strains and prototrophs arising by reverse mutation are often observed. The Anderson hypothesis has served a useful purpose, but becomes increasingly forced as new varieties of T1-resistant mutants are found.

Auxotrophic strains requiring growth supplements other than tryptophane, or strains resistant to more than one complex of phages (e.g., B/r/1, 3, 4, 7, 6, table 2) occur far more frequently than expected on the basis of simultaneous origin of independent mutations in a single individual. In explanation it could be assumed that mutation causing one class of phenotypic changes greatly increases the probability of a simultaneous or rapidly ensuing mutation of another type capable of independene occurrence at a lower rate. Another interpretation is that the phenotypant $a + b + c$ is not due to the simultaneous genetic events $a' + b' + c'$, but arises from the independent mutation $d'$. The ultimate solution of these problems will not alter the fact that in this experiment induced mutations differ from spontaneous.

* This paper is based upon work done for the Biological Department, Chemical Corps, Camp Detrick, Frederick, Maryland, under Contract No. DA-18-064-CML-449 with the Long Island Biological Association.

8. Luria, S. E., and Delbruck, M., Genetics, 28, 491–511 (1943).