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INDUCED MUTATIONS AND POSSIBLE MECHANISMS OF THE TRANSMISSION OF HEREDITY IN ESCHERICHIA COLI

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One of the most remarkable facts brought to light by genetics is the fundamental similarity in the most diverse organisms of the basic mechanisms of the transmission of heredity from parents to offspring. The principal carriers of heredity appear generally to be discrete corpuscles, genes. Only in bacteria and in other asexual lower organisms is the nature of the hereditary transmission still unclear. The most efficient method in use for the detection of genes—namely, the crossing of different strains and observation of segregations in the offspring of the hybrids—is not applicable to asexual forms. The problem must be approached, then, with the aid of the less direct, yet in the final analysis reliable, method of studying mutations, particularly those induced by irradiation. As materials for investigations on the induction of mutations by radiation, bacteria have, in fact, certain advantages not found in higher organisms.

Notwithstanding the large amount of work that has been done in radiation genetics, the events that lead to the manifestation of changes in genes in irradiated cells are not well understood. One important reason for this situation is that in the materials most studied (Drosophila, maize) the effects of irradiation cannot be determined until after a considerable number of cell divisions have gone by following the treatment. Since in bacteria it is possible to observe induced changes soon after they occur, the work to be described here was undertaken in the hope that the results might contribute toward a better understanding of the origin of these changes.

It is known from the work of Luria and Delbrück[1] and of Demerec and Fano[2] that in the *B* strain of *Escherichia coli* spontaneous mutations to resistance to the bacteriophage now known as T1 occur with a frequency of about 1×10^{-8} per generation, and that at least two types of such mutants can be recognized. The more common type forms large colonies,

while the other forms tiny colonies. In this study the over-all mutability of *B* to resistance to T1 (*B/1*) was investigated without separating the two types of mutants.

Materials.—The mutant strain known as *B/r* (*B* resistant to radiations), which came from the *B* strain of *E. coli*, was used in these experiments. This mutant, found and studied by Mrs. Witkin,[3] was selected for our work because it is more resistant than the original *B* strain to both ultraviolet and x-ray irradiation. Since it can stand treatment with higher dosages of both radiations than the *B* strain, it was hoped that increases in the mutation rate would be more easily detectable by irradiating *B/r*. This strain was used in all experiments mentioned in this paper. For reasons of convenience, the abbreviated symbol, *B*, instead of the full symbol *B/r* will be used here.

Bacteriophage T1, known also as alpha and as P28, was used to isolate mutants resistant to that phage.

Methods.—The standard method used in this laboratory for determining how many bacteria resistant to a certain phage are present in a population of sensitive bacteria is, first, to plate on nutrient agar in a Petri dish about 5×10^8 phage particles and, a few minutes later, to plate on the same dish the bacteria to be tested. When this procedure is followed the phage comes into contact with the bacteria and lyses all those that are sensitive to the particular phage used, while the resistant ones survive to form colonies. By counting these colonies, the number of resistant bacteria among the total number plated is determined.

In our experiments it proved necessary to detect not only the number of *B/1* mutants (bacteria resistant to T1 phage) that showed up before the bacteria had had a chance to divide, but also the number that appeared after the bacteria had passed through a certain number of divisions. It was not satisfactory to let the bacteria divide in broth and then determine the number of *B/1* individuals by the standard method, because after several divisions it would have been impossible to distinguish between *B/1* bacteria that had originated as mutations and those that had resulted from division of *B/1* mutants present in the culture as a result of mutations occurring during one of the earlier divisions.

An effective method was developed, which satisfies the requirements of our experiments. A known number of bacteria is plated on nutrient agar medium on Petri dishes. These are incubated for the period of time necessary for the bacteria to pass through the desired number of divisions, and then the phage is sprayed over the surface of the plates as a fine aerosol. For this purpose we use a De Vilbiss glass nebulizer number 44. A stock suspension, containing about 5×10^9 phage particles per milliliter, is placed in the chamber of the nebulizer and the aerosol is generated by an air flow of six liters per minute, regulated by a Linde Oxygen Therapy

Station Flow Meter. Each plate is exposed to the phage aerosol for 1 minute by holding it close to the mouth of the nebulizer and moving it around during the exposure so that all regions of the plate are uniformly covered. Tests have shown that when this procedure is followed a sufficient number of phage particles adhere to the plate to produce complete lysis of sensitive bacteria, while resistant bacteria continue to divide and form colonies. The main advantage of this method is that the positions of the bacteria in a growing colony are not disturbed by application of the phage, and that therefore all mutations occurring within a certain period are represented by single colonies, no matter how many mutant bacteria have been formed by division of the original mutant individual. During the development of this method, tests were made of 201 colonies that appeared on a number of plates after exposure to T1 aerosol. All were found to be resistant to T1 and sensitive to another phage active on *B* (T2), indicating that the exposure to the phage had been sufficient to eliminate all sensitive bacteria without introducing any contaminant.

When this aerosol method for applying phage is used, it is important to take precautions against contaminating the laboratory with the phage. If spraying is done in the open the aerosol spreads rapidly from room to room and the phage may persist for several days. To prevent this, spraying is done in a box, measuring 25 × 25 × 23 cm., with a glass top, an opening on one end for the nebulizer, an opening on the other end with a cloth sleeve through which the arm holding a Petri dish is inserted, and an opening on one of the two sides with a tube through which air is sucked out of the box. During the operation, air containing the phage aerosol is drawn out from the box at a slightly higher rate than it is blown in through the nebulizer, and is passed through a heated copper coil to destroy the phage.

For ultraviolet treatment we used a General Electric germicidal lamp, about 80 per cent of whose output is radiation of wave length 2537 A. In all the experiments, the material was irradiated at a distance of 92 cm. from the source, where the intensity was approximately 4.2 ergs per second per square millimeter. In a majority of the experiments, 0.05 milliliter of broth containing bacteria was spread over a surface about 8 cm. in diameter in a flat Petri dish 10 cm. in diameter, so that the walls of the dish would not shade the bacteria. After exposure, the material was washed out with a known quantity of broth and used in the experiments.

X-ray treatment was done at the Memorial Hospital in New York City, under the supervision of Miss E. Focht, through the courtesy of Mr. L. D. Marinelli. A bacterial suspension in broth was irradiated in small glass tubes, with unfiltered rays generated at 180 kv. and 25 Ma. at an intensity of 2050 roentgens per minute.

Experimental Results.—Experiments with ultraviolet: In the first set of

experiments, we used a 24-hour-old culture of bacteria, which was concentrated by centrifuging so as to contain about 10^9 bacteria per milliliter. Samples of this suspension were irradiated in thin layers, then resuspended in suitable amounts of broth to give comparable concentrations of living bacteria in all samples.

The numbers of living bacteria in each sample were determined by plating and colony counts. The original suspension was also tested for number of *B/1* mutants. When exceptionally high numbers of mutants were present in the original culture, they would mask any increase in mutation rate produced by the radiation; and therefore experiments in which this happened were not taken into account.

From each of the irradiated and control samples, twelve portions of 0.1 ml. were plated on different plates. Three plates from each sample were phage treated immediately, three were treated after 2 hours of incubation, three after 3 hours, and the last three after 4 hours. These plates were then incubated, and counts of phage-resistant colonies were made after 48 hours.

TABLE 1

SUMMARY OF 7 EXPERIMENTS, SHOWING THE NUMBER OF *B/1* MUTANTS AMONG *B* BACTERIA TREATED WITH VARIOUS DOSAGES OF ULTRAVIOLET AND INCUBATED FOR VARIOUS LENGTHS OF TIME BEFORE APPLICATION OF PHAGE

INCUBATED BEFORE PHAGING	NUMBER OF *B/1* COLONIES AFTER IRRADIATION OF 4 MIN.	2 MIN.	1 MIN.	CONTROL
0	19	6	4	0
2 hrs.	118	95	24	4
3 hrs.	548	414	226	55
4 hrs.	2081	1234	486	295
No. of bacteria plated	1.12×10^7	1.23×10^7	8.61×10^6	2.42×10^7

To determine the growth rate of bacteria after irradiation, dilutions from each sample were also incubated in broth and plated at intervals. From these platings, the number of bacteria present on the plates at various times was calculated, on the assumption of similar rates of growth on agar and in broth.

With minor variations, the procedure outlined above was used in the seven experiments summarized in table 1. This table shows the total number of bacteria plated in each series and the numbers of *B/1* colonies found on Petri dishes where the phage had been added immediately after irradiation or after incubation periods of 2, 3 or 4 hours. It is evident that in all tests made with treated series the numbers of *B/1* colonies were considerably higher than in the control series.

The larger number of *B/1* in the series where the phage was applied immediately after irradiation indicates that the ultraviolet treatment was effective in inducing *B/1* mutants which appeared before the first bacterial division had been completed.

TABLE 2

ANALYSIS OF DATA FROM TABLE 1, GIVING ESTIMATES OF THE MUTATION RATES

INCUBATED BEFORE PHAGING	4 MIN.			2 MIN.			1 MIN.			CONTROL		
	NO. OF BACTERIA	B/1	RATE × 10^8	NO. OF BACTERIA	B/1	RATE × 10^8	NO. OF BACTERIA	B/1	RATE × 10^8	NO. OF BACTERIA	B/1	RATE × 10^8
0	1.12×10^7	19	170	1.23×10^7	6	49	8.61×10^6	4	46	2.42×10^7	0	...
2 hrs.	1.79×10^7	118	...	2.71×10^7	95	...	3.79×10^7	4	..	2.4×10^8	4	...
Increment 0–2 hrs.	0.67×10^7	99	1478	1.48×10^7	89	601	2.93×10^7	20	68	2.2×10^8	4	1.8
3 hrs.	1.43×10^8	548	...	2.17×10^8	414	...	3.03×10^8	226	..	19.2×10^8	55	...
Increment 2–3 hrs.	1.25×10^8	430	344	1.90×10^8	319	168	2.65×10^8	202	76	16.8×10^8	51	3.0
4 hrs.	11.44×10^8	2081	...	17.36×10^8	1234	...	24.24×10^8	486	..	153.6×10^8	295	...
Increment 3–4 hrs.	10.01×10^8	1533	153	15.19×10^8	820	54	21.21×10^8	260	12	134.4×10^8	240	1.8

Growth tests made in broth showed that the initial period of lag in the growth (lag period) is lengthened in treated bacteria, an observation which has already been made by Hollaender and Duggar.[4] After an incubation period of 2 hours, the number of bacteria in the control series increased on the average by a factor of 10, the number in material treated for one minute increased 4.4 times, that in material treated 2 minutes 2.2 times, and that in material treated 4 minutes 1.6 times. After the lag period had passed, both treated and control bacteria multiplied regularly, doubling their number once in about every 20 minutes. No study was made of the multiplication of bacteria on agar, but it is unlikely that there is any considerable difference between the rates of growth on agar and in broth during the several early divisions before the number is large enough to produce crowding on agar. Indeed, observations made under the microscope indicate that the length of the lag phase on agar is about the same as that in broth.

Thus when the phage was applied to cultures that had been incubated for 2 hours in the series that received 4-minute irradiation the bacteria were just passing through the first division, while in the other three series they had already passed through one, two, and more than three divisions, respectively, by the time the phage was applied. Table 1 shows that the number of *B/1* mutants, where plates were incubated for 2 hours before adding phage, was very much greater than in the controls. A similar situation obtained in the case of cultures that were incubated for 3 and for 4 hours before phage treatment. It is evident that new *B/1* mutants continued to appear at a higher

rate in the treated series than in the controls, even after the bacteria had passed through several divisions.

From the data given in table 1, and from the calculated number of bacteria present at each time, the rate of mutation of *B* to *B/1* may be estimated. Such an analysis of the data is presented in table 2. For the cultures where phage was applied immediately after treatment, the mutation rate was calculated directly from the number of bacteria plated and the number of *B/1* colonies observed. In order to calculate the mutation rate among bacteria that were formed by division of plated individuals during the 2 hours of incubation, the total number of bacteria present after that time had to be estimated. This was done by multiplying the number of plated bacteria by 1.6, 2.2, 4.4 and 10 for the 4-, 2- and 1-minute series and the controls, respectively. As has already been pointed out, these values represent average increases in the number of bacteria and were determined by parallel experiments made in broth.

Since the rate of division of irradiated bacteria becomes normal once they pass the lag phase, and since the lag phase is completed in all series after 2 hours of incubation, it is assumed that from then on the number of individuals in each of the series doubles every 20 minutes. For each one-hour period, then, the number increases by a factor of 8. Consequently, values for the total number of bacteria present after 3 and 4 hours of incubation were obtained by multiplying by 8 the estimated values at the end of the previous hour.

It is realized that these estimates are only approximate. However, it is felt that the approximation is sufficiently close for the purpose at hand.

Examination of table 2 reveals that the calculated rate of mutation of *B* to *B/1*, per number of bacteria, increases in the treated series until the bacteria have passed through one or two divisions. Thereafter the rate begins to fall.

In order to determine if or when the rates reach the normal level of about 1×10^{-8}, a set of six experiments was performed in which bacteria were irradiated for 4 minutes and phage applied either immediately after the treatment or after incubation of 4, 5 or 6 hours. A summary of these experiments is presented in table 3, in the same form as the data shown in table 2. To obtain the estimate of the total number of bacteria present after 4 hours of incubation, the number of plated bacteria was multiplied by 102.4, which is equal to the $1.6 \times 8 \times 8$ used in table 2. Values for 5 and 6 hours were obtained by multiplying by 8 for each 1-hour period. It is evident from table 3 that the mutation rate continued to decrease with subsequent bacterial division and that at 6 hours, when the bacteria had passed through approximately 13 divisions, it had reached normal level.

Experiments with x-rays: A 48-hour culture of bacteria in broth was used as the source of material. About 1.3 cc. of bacterial suspension was

placed in each of three small glass tubes, which were then irradiated with 10,000, 20,000 and 50,000 roentgens, respectively. After irradiation, 15 platings of 0.05 cc. of the bacterial suspension were made on Petri dishes from each of the three tubes. Three Petri dishes from each set were exposed to phage aerosol immediately after plating, and others were exposed in groups of three after incubation of 1, 2, 3 and 4 hours. A summary of two experiments is given in table 4. The data are arranged as in table 2.

Tests made in broth indicated that the lag period of the bacteria was not materially affected by x-ray treatment. Under the conditions of these experiments, the number of bacteria doubled after 1 hour in broth at 37°C.

It is evident from table 4 that, as in the ultraviolet experiments, only a fraction of the induced mutants showed up immediately after treatment, while the appearance of a large proportion of them was delayed until after the treated bacteria had passed through several divisions. Comparisons of the data of tables 2 and 4 indicate obvious differences between the ultraviolet and the x-ray results. These are now being investigated further, and an analysis of this work will be presented in another paper.

TABLE 3

SUMMARY OF 2 EXPERIMENTS, WITH ESTIMATES OF THE RATES OF MUTATION OF *B* TO *B/1* AFTER ULTRAVIOLET TREATMENT OF 4 MINUTES FOLLOWED BY INCUBATION FOR VARIOUS LENTHS OF TIME BEFORE APPLICATION PHAGE

INCUBATED BEFORE PHAGING	NUMBER OF BACTERIA	*B/1*	RATE $\times 10^8$
0	2.25×10^6	7	311.1
4 hrs.	2.3×10^8	234	...
Increment 0–4 hrs.	.....	227	101.7
5 hrs.	18.4×10^8	297	...
Increment 4–5 hrs.	16.1×10^8	63	3.9
6 hrs.	147.2×10^8	543	...
Increment 5–6 hrs.	128.8×10^8	246	1.9

Discussion.—Changes induced by irradiations cannot be distinguished from those which occur spontaneously and which have already been extensively studied.[1, 2] In the course of these irradiation experiments several hundred *B/1* strains were grown in broth and tested for resistance to T1 and T2. They were found to be resistant to the first-mentioned phage and sensitive to the second, just as were the spontaneously originating strains described in previous experiments. Similar morphological types are found among both spontaneous and radiation-induced mutants—namely, a type that forms normal, large colonies and one that forms tiny colonies.

The simplest interpretation of the changes from *B* to *B/1* is that they are mutations comparable to gene mutations in higher organisms. However, since our knowledge of the mechanisms governing the transmission of heredity from parents to offspring in bacteria is still inadequate, other

TABLE 4

SUMMARY OF 2 EXPERIMENTS, WITH ESTIMATES OF THE RATES OF MUTATION OF *B* TO *B/1* AFTER X-RAY TREATMENT WITH VARIOUS DOSAGES AND INCUBATION FOR VARIOUS LENGTHS OF TIME BEFORE APPLICATION OF PHAGE

INCUBATED BEFORE PHAGING	10,000 r			20,000 r			50,000 r		
	NO. OF BACTERIA	*B/1*	RATE $\times 10^8$	NO. OF BACTERIA	*B/1*	RATE $\times 10^8$	NO. OF BACTERIA	*B/1*	RATE $\times 10^8$
0	7.8×10^7	21	26.9	6.2×10^7	44	71.0	7.2×10^6	57	791.7
1 hr.	1.56×10^8	57	...	1.24×10^8	51	...	1.44×10^7	88	...
Increment 0–1 hr.	0.78×10^8	36	46.2	0.62×10^8	7	11.3	0.72×10^7	31	430.6
2 hrs.	12.5×10^8	148	...	9.9×10^8	224	...	1.2×10^8	291	...
Increment 1–2 hrs.	10.9×10^8	91	8.3	8.7×10^8	173	19.9	1.06×10^8	203	191.5
3 hrs.	100.0×10^8	318	...	79.2×10^8	353	...	9.6×10^8	378	...
Increment 2–3 hrs.	87.5×10^8	170	1.9	69.3×10^8	129	1.9	8.4×10^8	87	10.3
4 hrs.	.....	...	...	.....	...	...	76.8×10^8	569	...
Increment 3–4 hrs.	.....	...	...	.....	...	...	67.2×10^8	191	2.8

possibilities should be considered. Only by eliminating such alternative explanations can one hope to determine whether the hereditary mechanisms in bacteria are fundamentally similar to those in higher forms.

For example, the genes in bacteria may not be organized into chromosomes, and several or many genes of the same kind may be present in each individual. Recessive mutations—which, as is well known, constitute a large proportion of all mutations—would then attain phenotypic expression only after the individual had passed through several divisions, and the mutant gene had been sorted out and had replaced its allele in some individuals.

Certain other interpretations should be considered. All changes from *B* to *B/1* affect reactions between bacteria and phages. Since sensitive bacteria adsorb phages but resistant ones do not, and since adsorption is a surface phenomenon, it is possible that changes in the surface bring about a condition which ultimately may be responsible for the origin of resistance. It might be supposed that resistance originates as a change in a small sector of bacterial surface, for example, a receptor, and that it spreads in successive divisions so that it finally affects one whole bacterium, which then reproduces as a resistant mutant.

However, the evidence presented here shows that some of the induced mutations become phenotypically effective at the time of the first bacterial division. This would indicate that the mechanism responsible for the transmission of resistance to phage is neither

one involving many genes of the same kind, as first suggested, nor a surface phenomenon which would require several cell divisions before being manifested.

Two points should be particularly emphasized in discussing the data presented in this paper: (1) that some of the induced mutants are phenotypically effective by the time the first bacterial division occurs, and (2) that new mutants continue to appear during several bacterial generations.

It is known from the work of Delbrück[5] that one sensitive bacterium may adsorb as many as 250 phages, while resistant bacteria do not adsorb any. Presumably an adsorbed phage enters a bacterium and multiplies therein until finally the bacterium is lysed—it bursts and liberates phage particles Therefore, there are two critical steps in the life of a bacterium which determine whether or not it will be lysed by phage if this is present. These steps are the adsorption of phage by the surface and the multiplication of phage within a bacterium. It is evident that if either of these two processes is prevented from occurring a bacterium will become resistant to phage, and that if a mutation in a bacterium is capable of making it resistant it must be capable of blocking one of these two processes. If the adsorption of phage were immediately blocked by a mutated gene, this would mean that such a gene exerted an immediate influence on the organism and affected the forces active in adsorption. On the other hand, if a mutation prevented the multiplication of phage, this might mean that the mutated gene became active during the bacterial division, since, as a rule, the multiplication of phage is intimately connected with the division of bacteria.

Experiments were designed in such a way as to leave very little doubt that *B/1* mutants that appeared after bacteria had passed through several divisions were due either to mutations that originated after irradiation or mutations that were induced at the time of irradiation but failed to manifest their effect until the organism had passed through several cell divisions.

The simplest way to explain the appearance of mutants immediately after treatment as well as after several bacterial divisions would be to assume that these bacteria are diploid, and that the immediate mutants are a result of coincidental changes of both alleles by two independent hits, while the delayed mutants appear as a result of segregation of heterozygotes. Two objections make this hypothesis improbable. One of them is the large number of immediate mutants found in the ultraviolet and, particularly, the x-ray experiments. The observed numbers of these mutants differed from the calculated values by a factor of about 10 in the ultraviolet experiments and by a factor of about 1000 in the x-ray experiments. These discrepancies could be accounted for by assuming that some of the homozygous mutants could be induced by a single hit—particularly in the case of x-rays. Such an assumption would not be unreasonable, since Lea and Catcheside[6] have estimated that the field of action of one x-ray

ionization is about 0.1 micron, so that any two alleles that happened to be located that close to each other would have a chance of being affected together.

The other, and more serious, objection against this hypothesis is the distribution of mutants. A few of them appear immediately after treatment, but the larger proportion do not express themselves until after several cell divisions have taken place. If delayed mutants were due either to a simple segregation of a heterozygote or to aberrant divisions of asexual cells resulting in segregations, it would be expected that the largest proportion of delayed mutants would appear during the second bacterial generation, and that the frequency would rapidly diminish thereafter.

Another possible explanation of the observed delay in the appearance of mutants assumes that the bacterial population used in experiments was not homogeneous—that is, that some bacteria were haploid, others diploid, and still others polyploid. By assuming certain proportions of the various types, a mixture could be postulated which would give the observed results. In all the experiments reported so far, resting bacteria were used; these were obtained by growing bacteria in broth, at 37°C., for either 24 or 48 hours. It is known that growing bacteria are physiologically very different from resting bacteria. Since the proportions of haploid, diploid and polyploid individuals—if these did occur at all—might be expected to be different in different physiological conditions, we compared data obtained from ultraviolet irradiation of resting and actively growing bacteria. The results were not significantly different.

Another possible hypothesis assumes that all mutations occur during irradiation but that some are delayed in their manifestation until the supply of the substrate manufactured by the gene and necessary for the production of material that makes the bacteria sensitive to the phage is exhausted. It might then be expected that changes in the environment might affect the rate of utilization of the substrate.

Still another hypothesis may be considered to explain the results obtained. It may be assumed that two types of change are induced by irradiation—one type producing gene changes, and the other inducing some change in the cell, either of the chromosomes or of the cytoplasm, which increases the mutability of the gene system. The latter type of change would decrease in effectiveness with each cell division, and after a number of divisions lose its potency. According to this hypothesis, the calculated mutation rate per number of bacteria would be highest after the early divisions, a situation observed in our experiments (table 2).

In irradiation work with Drosophila a similar delayed effect is observed after treatment of sperm. In that case mosaics, or "fractional" mutants, are obtained. Muller[7] and several other workers have interpreted this observation by assuming that the chromosomes in some of the sperms are

already split, and that in such cases the irradiation produces a change in only one of the two strands. Neuhaus,[8] however, after studying the distribution of the size of mosaic regions, reached the conclusion that these mosaics are due to new mutations occurring as a result of a delayed effect of the irradiation.

In the experiments described in this paper various doses of ultraviolet radiation and x-rays were used. The data indicate an increase in the effect with increase of the dosage. Specially designed experiments are now under way, however, to determine the relationship between the dosage and the number of induced mutants; this problem will be discussed elsewhere. An effort is being made also to devise experiments which will differentiate between the various possibilities for interpretation of the results of experiments discussed here.

Summary.—The rate of mutation from sensitivity to resistance to bacteriophage T1 in the *B* strain of *E. coli* was measured after treatment with ultraviolet radiation (2537 A) and with x-rays.

Both radiations produced a substantial increase in the mutation rate, which increase became greater with higher doses of radiation.

Some of the induced mutations manifest themselves by the time the treated bacteria begin to multiply, while the majority of them express themselves after the bacteria have passed through several divisions.

Several possibilities for interpretation of results are discussed.

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