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*INDUCTION OF SPECIFIC MUTATIONS WITH 5-BROMOURACIL**

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Communicated by M. Delbrück, December 6, 1957

INTRODUCTION

The hereditary characteristics of an organism occasionally undergo abrupt changes (mutations), and genetic techniques have traced these to alterations at definite locations in the genetic structure. Recently, the fineness of this genetic mapping has been extended to the level where the finite molecular units (nucleotides) of the hereditary material limit further subdivision. At this level, local details of the hereditary material should exert their influence; the frequency of mutation at a particular point should depend upon the local molecular configuration. It is therefore feasible to try to correlate genetic observations with precise molecular models, such as the one proposed by Watson and Crick[1] for the structure of DNA.

In a fine-structure study of spontaneous mutations in phage T4, the mutability at different points in the genetic structure was, in fact, found to be strikingly varied.[2] To relate mutability to actual chemical structure, it would seem promising to employ mutagenic agents of specific types, to act selectively on particular configurations. Since the initial discovery by Muller[3] and Stadler[4] on induction of mutations with X-rays and the discovery of chemical mutagenesis by Auerbach and Robson[5] and by Oehlkers,[6] many physical agents and chemical substances have been found to be mutagenic in many organisms. Some mutagens act selectively; in particular the induced reversion from biochemically dependent to independent strains has been shown to depend upon the mutant and the mutagen used. (For chemical mutagens in bacteria see Demerec.[7]) A recent comprehensive review of this subject has been published by Westergaard.[8] Mutagens in some cases produce gross chromosomal aberrations; in others the alterations are so small as to

be beyond the limited resolving power of genetic techniques for the organism used.

The absence of this limitation makes phage a suitable organism for our purposes. There have been reports of induction of mutations in phage by ultraviolet light,[9,10] nitrogen mustard,[11] streptomycin,[12] and proflavine.[13] A very provocative discovery is that analogues of the normal bases may be built into DNA in place of the usual ones and also raise the mutation rate. In particular, one such analogue, 5-bromouracil, has been proven by Dunn and Smith[14] to be incorporated into the DNA of phage (in place of thymine), and Litman and Pardee[15] have shown that it greatly increases the frequency with which phage mutants of various types arise.

In the present work, this possibility of directly affecting the DNA structure is combined with a genetic analysis of high resolving power, to make a fine-structure study of mutagenesis. Our attention will be restricted to the *r*II region of the genome of phage T4. The mutational alterations arising by 5-bromouracil induction are compared to, and shown to differ from, those which occur spontaneously.

METHODS AND MATERIALS

Strains: *phage* T4B; *bacterium* B (*E. coli* B) for the isolation of mutants and as plating bacterium for the determination of phage titers; S (*E. coli* K12S) for the preparation of phage stocks; K (*E. coli* K12S lysogenic for prophage lambda) as the selective strain for genetic tests.

Media: *broth* 1 per cent bacto-tryptone (Difco) plus 0.5 per cent NaCl; *glucose-salts* medium;[16] *sulfanilamide medium* same as used by Litman and Pardee,[15] except for higher sulfanilamide concentration (2 mg/ml) and addition of 1 μg/ml calcium pantothenate, 1 μg/ml pyridoxine, 1 μg/ml thiamine, 1 μg/ml uracil, and 20 μg/ml L-tryptophane (tryptophane required for adsorption of phage T4B to B in synthetic medium). Plates contain *broth* plus 1.3 per cent agar (Difco) with a top layer of broth plus 0.7 per cent agar.

A sample of *5-bromouracil* purified by ion-exchange column, was kindly supplied by Dr. Rose Litman.

Isolation of the Mutants.—Spontaneous mutants: Details on the isolation of spontaneously arising *r* mutants of phage T4, their properties, and the methods used in mapping them genetically are given in earlier publications.[2, 17] In brief, a stock of standard ("wild") type phage T4 (derived from a single T4 particle) is plated on B. Each phage particle produces a plaque containing around 10^7 progeny. The progeny include occasional *r* mutants, which can be found by picking the plaque and replating its contents. In order to assure that each mutant arises by an independent mutational event, no more than one *r* mutant is isolated from any one plaque of the standard type. Each *r* mutant is replated (to free it from any contaminating particles of standard type), an isolated *r*-type plaque is picked, and a stock of the mutant grown on bacteriums in broth.

Induced mutants: These were isolated from the yield of bacteria infected and allowed to burst in the presence of sulfanilamide and 5-bromouracil. A culture of B was prepared by inoculation of 0.4 ml. of overnight culture (grown in glucose-salts medium) into 20 ml. of sulfanilamide medium and aeration for 3.5 hours to reach a cell concentration of 7×10^8 per ml. At this time, 1 mg. of 5-bromouracil and 4×10^4 particles of T4 standard-type phage[18] were introduced simultaneously. Drops

of the mixture were rapidly distributed—one drop into each of 200 tubes. After incubation at 37° C. (for 30 minutes) to allow the infected cells to burst, the content of each tube was plated on B. As is typical in this sulfanilamide medium, the average yield per cell was very small, of the order of one viable progeny particle per infected cell. Each plate contained (after incubation) from 30 to 100 plaques, in most cases including one or more *r*-type plaques. The over-all proportion of *r*-type particles among the progeny was about 2 per cent. To assure the independent origin of each mutant, no more than one *r* plaque was picked from a plate. Each such mutant was purified by replating, and a stock prepared on S in broth.

Genetic Mapping of the Mutants.—Different *r* mutants of T4, although producing similar plaques on B, fall into groups distinguished by their behavior on a second host, K. Those mutants with which we are here concerned, of the *r*II group, do not produce plaques on K. This property is the key to the high resolution with which they can be mapped genetically. When two *r*II mutants are crossed, the appearance of any standard-type recombinants among the progeny is readily detected by plating on K. If standard-type progeny *are* produced in a cross (above the background rate due to spontaneous reversion of the mutants), it is concluded that the two mutants contain alterations at different locations in their genetic structures.

Our objective is to compare these locations for spontaneously arising and for 5-bromouracil-induced mutants. The task of crossing a large number of mutants, two by two, to see which pairs yield recombinants is enormous. However, this process can be shortened by making use of a set of mutants having large alterations, as shown in Figure 1. Each new isolated *r*II mutant is crossed with each mutant of this set (by means of simple spot tests). By noting with which mutants of the set it does or does not produce standard-type recombinants, the mutation can be assigned to a particular *segment* of the map. Thereafter, only mutants belonging to the *same* segment need be crossed with each other. Thus the number of crosses required for analyzing a batch of mutants is greatly reduced.

The genetic procedure has therefore been to (1) isolate many independently arising *r*-type mutants; (2) choose those of the *r*II group; (3) test each *r*II mutant against the mutants of Figure 1, thereby locating its mutational alteration in a particular segment of the map; and (4) cross the mutants belonging to the same segment with each other to determine which mutations share common locations. For the present purposes, no attempt was made to determine the *order* of these locations *within* a map segment.

Reversion Rates of Mutants.—The different *r*II are characterized not only by the positions of their mutational alterations in the map but also by differences in their frequency of reversion to particles that resemble the standard type in their plating behavior on K and B. These revertants arise spontaneously during the growth of a given *r*-type phage, and their typical frequency, in a stock grown up from an inoculum of 100 *r* phages, is called the "reversion index."

RESULTS

Over-all Mutation Frequencies.—The ratio of the induced to the spontaneous rate of mutation cannot be given accurately, since different procedures of isolation were used. A rough estimate may be made as follows: In a broth lysate of T4B grown (on S) from an inoculum of a few particles up to a population of 10^{10}, the proportion

of spontaneously appearing r particles is typically 2×10^{-4}. Under the conditions used for induction, on the other hand, r mutants appeared in a proportion of around 2 per cent in the progeny from a single growth cycle. To obtain the probability of mutation *per phage replication*, these values must in each case be adjusted to account for the accumulation of mutants during the growth of the population. This effect, which is roughly proportional to the logarithm of the ratio of final to initial phage titer, should be several fold (perhaps four times) larger for a lysate than for one cycle of growth. Therefore, the rate of induced over-all r mutations can be estimated as several hundred times the spontaneous rate.

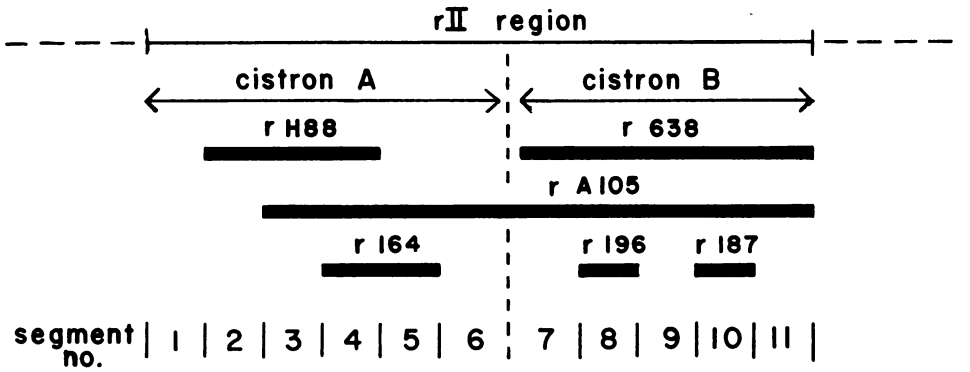

FIG. 1.—Genetic map of the rII region of phage T4, showing the division of the map into segments by a set of rII mutants having large alterations. Each of the two "cistrons" is a functional unit defined by the cis-trans test.

The relative positions of the alterations have been established by their overlapping relationships with a much larger set of mutants. To locate the defect of a new mutant, it is crossed with each member of the set, and standard-type recombinants are tested for. From the results of these crosses the defect can be assigned to one of the segments. For example, if a mutant gives standard-type recombinants with all except r H88, r A105, and r 164, its defect must be located in segment 4. One which gives recombinants with all must have its defect in segment 1. Segments 7, 9, and 11 are not distinguished by this set of mutants.

Genetic Analysis of the Mutants.—Spontaneous mutants: A series of 238 spontaneously arising mutants was isolated, numbered r 101 through r 338, and analyzed as described above. Of the mutants, 132 belonged to the rII group. Of these, 6 were not readily mappable because of high reversion rates or leakiness (ability to grow feebly on K). The results of mapping the remaining 126 mutants are shown in Figure 2.

The relative positions of the sites indicated *within* a map segment have no significance. Any two mutants that yield standard type progeny when crossed are sketched as horizontally separated, while those groups for which recombination could not be detected (at a level of about 0.01 per cent recombinant progeny or more) are assigned to the same or adjacent horizontal sites. Some mutants fail to give recombinants with (any of two or more) other mutants known to give recombinants with each other; the alterations of such mutants are represented as horizontal bars. A striking feature of the map is the existence of certain "hot spots," where mutations recur with high probability.

Induced mutants: A series of 170 r mutants, arising under the action of 5-bromouracil in sulfanilamide medium, was numbered N 1 through N 170. Of these, 89 were of the rII type. Twelve of the latter were not readily mappable because

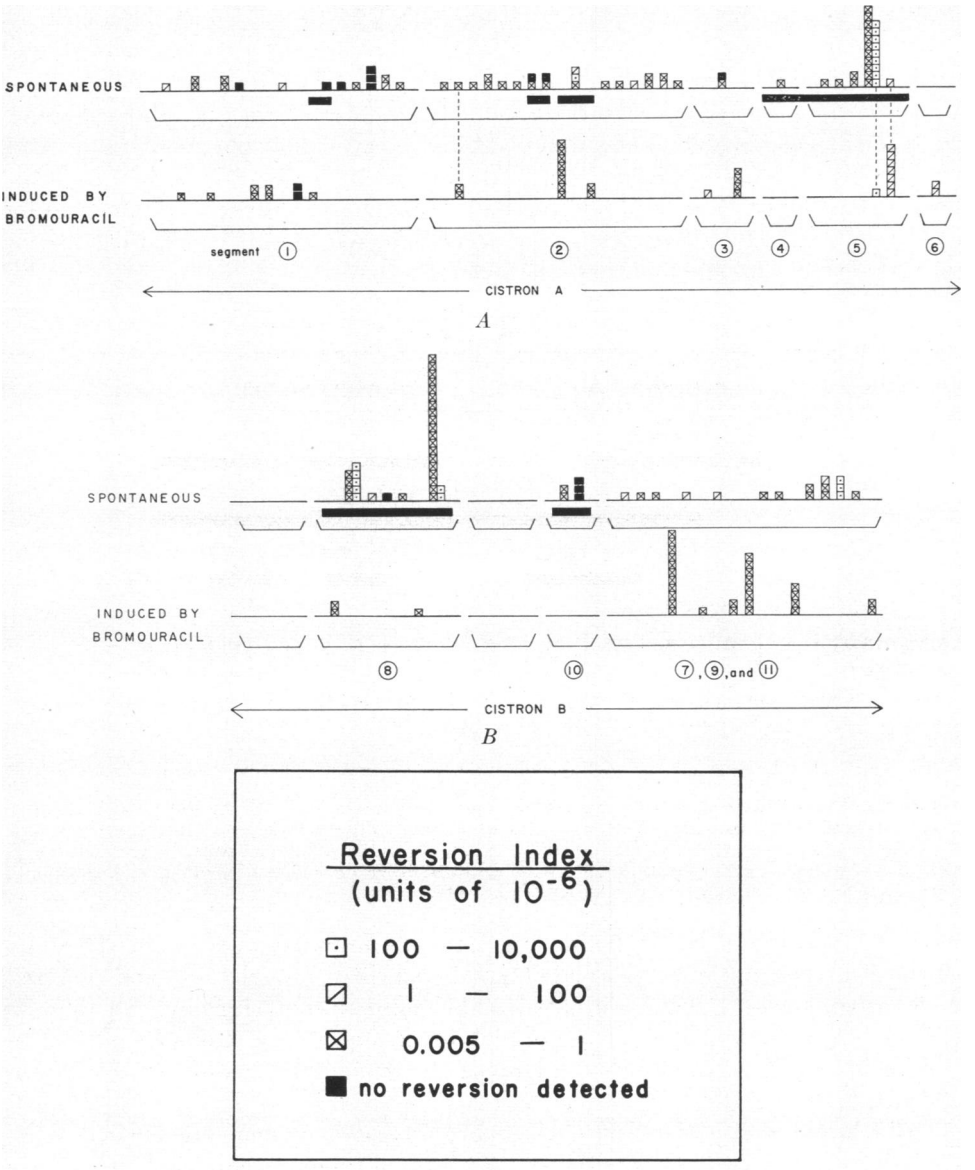

FIG. 2.—Genetic maps showing the locations of spontaneous and 5-bromouracil-induced mutational alterations in the *r*II region of phage T4 divided into cistron A and B. Each mutant is represented by a box, placed in the proper segment of the map and shaded to indicate its reversion index. The relative order (but not the lengths) of segments 1, 2, 3, 4, 5, 6, 8, and 10 have been established. Segments 7, 9, and 11, are lumped together and considered as one segment. The order and position of different mutations within one segment have not been determined.

they were leaky. The results of mapping the remaining 67 mutants are shown in Figure 2. This set of mutants reveals "5-bromouracil hot spots" that are located at different positions from the spontaneous ones.

It must be emphasized that the vertical scales in Figure 2 are very different for the two sets of mutants. The over-all induced mutation rate was several hundred-

fold higher, and only half as many induced mutants are mapped; therefore, the occurrence of an induced mutant once represented in the induced set corresponds to roughly 10^3 times higher mutability than the occurrence of a spontaneous mutant once represented in the spontaneous set. Hence, for those "hot spots" in the induced set for which no spontaneous mutation has been observed, the mutability with bromouracil is at least 10^4 times larger than is the spontaneous mutability.

Reverse Mutations.—Among the spontaneous *r*II mutants, not only are forward mutation frequencies different for different sites, but the reversion indexes as well cover an enormous range. Among the 132 spontaneous *r*II mutants, reversion indexes range as high as 5×10^{-2} down to less than 10^{-8}. For 19 of them, reversion has not been detected.

The induced mutants, on the other hand, are more homogeneous with respect to reversion index. There is only one mutant of very high reversion index (N 76) and two non-reverting mutants (N 101, N 32). Most of the remainder have reversion indexes of the order of 10^{-8}. Notably rare are mutants containing larger aberrations (i.e., ones that fail to give standard-type recombinants with two or more other mutants that do themselves show recombination).

Some preliminary experiments have been performed on the induction of reversion of various mutants with 5-bromouracil. For some mutants, in particular, chosen from some of the "5-bromouracil hot spots," increases over the spontaneous rate of reversion by factors as high as 10^4 have been observed. Some spontaneous mutants show no or only much smaller effects. These experiments are still in progress.

DISCUSSION

Clearly, the mutagenic effect of 5-bromouracil is not merely a general enhancement of spontaneously occurring mutations. Rather, it is a *specific* effect. In denoting the action of a mutagen as specific, one might be comparing the effects on different phenotypic characteristics in the whole organism, different cistrons, or different locations within a single cistron. Were it not for the high resolution of our genetic techniques, it might have been erroneously concluded that 5-bromouracil acts aspecifically, since, among all the induced mutants of the *r* phenotype, the proportion of *r*II mutants and even the ratio of A cistron to B cistron mutants are quite comparable to the spontaneous values. It is only at finer resolution that the different effects are revealed, and it is seen that mutations are, for the most part, induced at specific places in the genetic structure.

Since the proportion of large aberrations and non-reverting mutants is notably smaller among the 5-bromouracil-induced mutations, the induced changes in the genetic structure appear to involve small molecular substitutions rather than large changes of the genome. Further, the more homogeneous properties of the induced mutants with respect to reversion rates indicate that a certain class of molecular transitions may be involved here.

Under the conditions used, the *total* frequency of occurrence of all *r*II mutants in 5-bromouracil was raised several hundred fold above the spontaneous rate. The increase in rate at specific locations in the genetic structure was much larger. For example, 11 occurrences of mutation at one site were observed out of 67 induced *r*II mutants, while none occurred at that site among twice as many spontaneous *r*II

mutants. Thus the probability of mutation at that point, per replication of the phage, was raised by a factor greater than twice eleven times several hundred, or of the order of 10^4.

Conversely, note the site at which there were 19 occurrences among 132 spontaneous *r*II mutants, while among the 67 induced mutants none were observed. Therefore, 5-bromouracil had little, if any, positive mutagenic effect at that point. The disappearance of the spontaneous "hot spot" does not, of course, mean that spontaneous mutations at that point were suppressed but simply that they were not increased in proportion to other mutations.

Only three sites (indicated by dotted lines in the figure) are represented in both sets of mutants. In each of those cases, there was only a single occurrence in one of the sets, so that the ratio of induced to spontaneous frequencies cannot be reliably computed. The mutation rate seems to have been truly increased by 5-bromouracil in 2 of the cases (2 and 7 occurrences in the induced set) because one occurrence among the induced series represents a thousandfold greater probability of mutation. In the third case, there is, in the induced set, a single occurrence of a highly revertable mutant that appearaed nine times in the spontaneous set. This single event probably belongs to the small background of spontaneous mutations among the induced ones (*ca* 1 or 2 in 100).

The methods used in the isolation of the two sets of mutants were quite different, and one cannot be sure whether factors other than the addition of 5-bromouracil could have affected the results. Although sulfanilamide alone does not appreciably increase the total mutation rate, the possibility that the distribution of mutations might change has not been excluded. Sulfanilamide, as an analogue of *p*-amino benzoic acid, inhibits the formation of folic acid, thereby inhibiting nearly all the methylation and hydroxymethylation steps (Cohen and Barner).[19] In our sulfanilamide medium, nearly all the major chemicals containing the methyl or hydroxymethyl group are added except the deoxyribonucleotides of thymine and 5-hydroxymethylcytosine, which thus are expected to be deficient. The deficiency in thymine facilitates the incorporation of 5-bromouracil into DNA. Whether the deficiency in 5-hydroxymethylcytosine enhances the probability of the false incorporation of 5-bromouracil into a hydroxymethylcytosine site of the phage DNA remains to be seen.

One is not in a position, from these experiments alone, to reach a clear conclusion as to the molecular mechanism of mutagenesis; thus, at this stage, it cannot be decided whether or not the tautomeric shift of the DNA bases from the *keto-* to the *enol-* form is mainly responsible for the production of point mutations, as suggested by Watson and Crick.[1] If it is assumed that only two kinds of nucleotide pairs are present in DNA (i.e., adenine—thymine, and guanine—5-hydroxymethylcytosine), the existance of hot spots of spontaneous mutation and the appearance of different hot spots with 5-bromouracil would suggest that not every nucleotide pair of a given type mutates with the same probability. Rather, the mutability of a nucleotide pair would have to depend upon its position.

In any case, the striking results of this preliminary investigation indicate that it would be fruitful to pursue this line of investigation, using a range of mutagenic substances in systems where the chemical events are under proper control.

SUMMARY

A set of spontaneously arising rII mutants of phage T4 is compared with a set of mutants induced by the action of 5-bromouracil. When analyzed by genetic mapping techniques of high resolution, the two sets of mutants are found to be quite different. The mutagen does not merely enhance the over-all mutation rate but acts at specific locations in the hereditary structure.

The induced mutants are mostly of the nature of small, revertible alterations rather than gross defects. The reversion rates of induced mutants are less varied than those of spontaneous mutants, indicating that a certain class of molecular transition is involved.

These preliminary results encourage the hope that this sort of genetic analysis can lead toward an understanding of the mechanism of mutation and the identification of the specific chemical configurations composing the genetic structure.

Note: The occurrence at a given location of one mutation in the induced set corresponds to *roughly 10^3 times higher mutability* of this spot in 5-bromouracil than is indicated by the occurrence of one mutation in the spontaneous set.

* Aided by grants from the National Science Foundation and the American Cancer Society

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