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*THE MECHANISM OF 5-BROMOURACIL MUTAGENESIS IN THE BACTERIOPHAGE T4**

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The pyrimidine 5BU[1] can be incorporated into DNA in place of thymine.[2] Conditions of growth which promote 5BU-incorporation are mutagenic to phage.[3] The mutagenicity of 5BU is attractively explained by the hypothesis of Watson and Crick.[4] According to this hypothesis, (some) mutations result from base-pairing mistakes at the time of DNA duplication. These mistakes are a consequence of tautomerization of either an incorporated base in an "old" chain or an incoming base on the growing end of a "new" chain. They result in "base-pair transitions."[5] Within this framework, 5BU is thought to be mutagenic because it has a rate of tautomerization[6] which is higher than that of thymine.

This hypothesis makes the distinctive prediction that phage particles containing 5BU should show a high rate of mutation when permitted to duplicate in the absence of 5BU. Mutations arising under these "clean-growth" conditions must be presumed to represent transitions of the type AT → GC. The experiments described here demonstrate the existence of "clean-growth" mutagenesis in phage T4.

This demonstration lends support to the hypothesis for mutation proposed by Watson and Crick and permits the identification of the base pairs present at a number of mutational sites. Previous authors[7] have interpreted within a similar framework their results on base-analogue-induced mutations.

Materials and Methods.—The phage strain used was T4B.

The bacterial strains used were *Escherichia coli* B and B3. The latter, a thymine-requiring strain, was obtained from S. Brenner.

Media: Broth: H_2O, 1 liter; bacto-tryptone, 10 gm; NaCl, 5 gm. Bottom agar: broth with 1.1 per cent bacto-agar. Top agar: broth with 0.7 per cent bacto-agar. pH 8 agar: bottom or top agar containing 0.1 *M* tris(hydroxymethyl)-

aminomethane adjusted to pH 8.0 with HCl. M9: H_2O, 1 liter; Na_2HPO_4, 7 gm; KH_2PO_4, 3 gm; NH_4Cl, 1 gm; supplemented after autoclaving with glucose, 4 gm; $MgSO_4$, $10^{-3}M$; $CaCl_2$, $10^{-4}M$.

Assay of phage: 0.05 ml amounts of appropriate dilutions of phage were added to 0.5 ml amounts of B grown to a concentration of 2×10^8 per ml. After 7 min incubation at 37°, 2.5 ml of melted top agar and 200 μg of egg white lysozyme (Worthington) were added, and the entire contents of the tubes were poured into plates containing 36 ml bottom agar. The plates were incubated overnight at room temperature.

Selective assay of e^+ phage: 0.05 ml amounts of appropriate dilutions of phage and 0.5 ml of B grown to a concentration of 2×10^8 per ml were added to 2.5 ml of melted pH 8 top agar and the contents of the tube were immediately poured onto plates containing 36 ml of pH 8 bottom agar; 15 min later, the plates were placed into a 37° incubator and incubated at this temperature overnight. One ml of $CHCl_3$ was placed in the lids of the inverted plates, after which they were vented with a strip of aluminum foil and incubated at room temperature for three to six hr. Plaques with characteristic large halos were scored as e^+.

Induction of mutations: (1) 2AP. Cells of strain B grown to a concentration of 2×10^8 per ml in aerated broth were added to an equal volume of broth containing 1 mg 2AP per ml at 37°. Two min later, four T4 particles were added for every bacterium present, and the infected cells were incubated at 37°, with aeration, for an hr. One tenth volume of $CHCl_3$ was then added, and the tube was shaken vigorously.

(2) 5BU. Cells of strain B3 were grown to a concentration of 10^8 per ml in aerated M9 supplemented with 2×10^{-5} M thymine. The bacteria were then centrifuged and resuspended at the same concentration in M9 supplemented with 1.15×10^{-4} M 5BU and grown, with aeration, to a concentration of 2×10^8 per ml. Four T4 particles were added for each cell present, and ten min later an additional seven particles per cell were added. The culture was lysed at 45 min after infection by shaking with chloroform.

(3) HA. Phage stocks were treated for eight hr according to the procedure described by Freese *et al.*[8]

5BU-substituted phage stocks were prepared according to Method I of Stahl *et al.*[9]

Results.—The selection of a set of useful 5BU-revertible mutants: In order to be useful for the experiments to be described below, *e* mutant strains must be able to produce plaques with a high efficiency, must be unable to produce plaques under unfavorable conditions suitable for the selective assay of e^+, must produce phage-cell complexes which are not subject to lysis with chloroform, and must have a relatively high rate of revertibility by 5BU.

A number of mutants of independent origin from a population of phage enriched for mutants[10] were screened for these properties. From among these, three 2AP-induced and three 5BU-induced mutants were chosen for further study. The mutants that were found to be suitable represented a small fraction only of all mutants screened.

The measurement of reversion frequencies in 5BU-substituted stocks growing in the absence of 5BU: 5BU-substituted stocks of suitable *e* mutants were prepared ac-

cording to the procedure of Stahl *et al.*[9] In order to obtain phage that were uniformly and highly substituted, phage grown in the presence of 5BU were centrifuged in a cesium chloride density gradient and fractions collected according to a method developed by Jean J. Weigle.[11] The most dense fractions were selected for study.

Such substituted stocks contain, in addition to *e* phage, a minority of e^+-like phage as well as phage heterozygous for *e* and e^+. In order to detect reversions induced in the substituted stocks growing in the absence of 5BU, the revertants and heterozygotes already present must be eliminated. The elimination is achieved by "anti-selection" during the "clean-growth" cycle itself as follows. B cells are infected with a low multiplicity of substituted *e* phage, unadsorbed phage are inactivated by the addition of anti-phage serum, and chloroform is added at various times later than 17 min after infection. Bacteria infected with e^+ or with e/e^+ heterozygotes lyse due to the presence of intracellular phage-evoked lysozyme, while those infected with *e* phage do not lyse.[12] The remaining infected bacteria are separated from free phage and antiserum by repeated cycles of centrifugation and are finally lysed by the addition of egg white lysozyme. Titration of this lysate yields an index of the frequency of e^+ revertants arising during the cycle of clean growth.

The frequency of e^+-like revertants in substituted stocks of various *e* mutants was compared with the frequency of revertants arising during a cycle of "clean growth." It was expected that a class of *e* mutants would be found capable of reverting during "clean growth." These would yield a frequency of revertants comparable to that observed in the original 5BU-substituted stock. Mutants that revert only when 5BU is in the environment ought, on the other hand, to yield a much lower frequency of revertants after "anti-selection" and "clean growth" than before. In fact, the frequency of revertants ought to resemble that in lysates obtained from unsubstituted stocks.

The results presented in Table 1 indicate that two of the six mutants tested re-

TABLE 1

REVERSION OF 5BU-GROWN *e* MUTANTS DURING GROWTH IN THE ABSENCE OF 5BU

		e^+ frequency $\times 10^6$					
		In 5BU Stocks		In Thymine Stocks			
		Before:	After:	Before:	After:	Ratio in 5BU stocks:	Ratio after anti-selection and clean growth:
		Anti-selection and clean growth		Anti-selection and clean growth			
Mutant	Origin					After/Before	5BU/Thymine
*e*C3	2AP	70	1.2	1.5	2.0	0.02	1
*e*C4	2AP	180	620	0.6	3.6	3	172
*e*C9	2AP	150	110	6.9	1.5	1	73
*e*D1	5BU	250	2.1	1.1	3.6	0.008	1
*e*D50	5BU	100	2.0	0.7	1.2	0.02	2
*e*D55	5BU	110	3.3	1.2	2.5	0.03	1

E. coli B were grown to a concentration of 2×10^8 per ml in aerated broth at 37°. An average of 0.05 phage particles was added for each bacterium present. Three min later, anti-T4 serum (to give a final "*k*" value[13] of 1.0) was added. One-tenth volume of chloroform was added to the cultures at 19 min after infection (5BU-grown phage) or 17 min after infection (thymine-grown phage),[14] and the cultures were vigorously mixed with the chloroform. After further 10 min incubation at 37°, the aqueous phase was centrifuged, the pellet resuspended in an equal volume of broth, and the centrifugation repeated twice more. Egg white lysozyme (10 μg per ml) was then added. The cultures were incubated for 10 min at 37° and then immediately assayed for total and for e^+ phage.

vert efficiently during "clean growth," whereas the other four revert only during growth in the presence of 5BU.

Reconstruction experiments with e^+-like mutants: It seemed possible that certain e^+-like revertants are not eliminated by our "anti-selection" procedure. Were

that the case, the high frequency of e^+ found after "clean growth" would not necessarily represent revertants arising in that growth cycle.

In order to test this possibility, reconstruction experiments were performed with e^+-like revertants from each of the two mutants that exhibited apparent "clean growth" reversion. Since a fraction of 5BU-induced reversions could be expected to be heterozygotes,[15] the reconstruction experiments were performed using bacteria infected with equal numbers of e^+-like revertants and mutants, which were then treated exactly as they had been in the course of the "anti-selection." The results presented in Table 2 indicate that the e^+-like revertants were efficiently eliminated

TABLE 2

THE ELIMINATION OF e^+-LIKE REVERTANTS FROM MIXED INFECTIONS WITH e PHAGE DURING ANTI-SELECTIVE GROWTH

	e^+ from e^+ and eC4 mixed infection	e^+ from e^+ and eC9 mixed infection	e from eC4 infection
Number of infected bacteria in final growth tube	2.0×10^6	2.0×10^6	2.0×10^6
Number of phage recovered from final growth tube	2.1×10^3	4.3×10^3	6.3×10^7
Number of phage recovered from final growth tube per infected bacterium	1.1×10^{-3}	2.2×10^{-3}	32

E. coli B were grown to a concentration of 2×10^8 per ml in aerated broth at 37°. An average of seven phage particles each of an e mutant and of an e^+-like revertant derived from it was added for each bacterium present. Four min later, anti-T4 serum (to give a final "k" value[18] of 10.0) was added, and after an additional two min the infected bacteria were diluted 100-fold into broth containing bacteria grown to 10^8 per ml. At 19 min after infection, one-tenth volume of chloroform was added and the cultures were treated as described in Table 1.

by the "anti-selection," even when present in cells together with mutants.

Patterns of reversion of the six mutants examined: The frequency of reversion of each of the six mutants was measured after treatment with a variety of mutagens in order to determine whether "clean growth" revertibility was associated with a definite pattern of response to other agents. The results presented in Table 3 do

TABLE 3

PATTERNS OF REVERSION OF THE VARIOUS e MUTANTS

Mutant	Origin	5BU revertible*	5BU clean-growth revertible*	HA revertible	Ratio of revertant frequencies with and without HA	2AP revertible	Ratio of frequency of reversions with and without 2AP
eC3	2AP	Yes	No	No	1.0	No	0.85
eC4	2AP	Yes	Yes	No	0.7	Yes	11.2
eC9	2AP	Yes	Yes	Yes	60	No	0.42
eD1	5BU	Yes	No	No	0.8	Yes	4.0
eD50	5BU	Yes	No	No	1.0	Yes	4.2
eD55	5BU	Yes	No	No	0.8	Yes	3.0

* See Table 1.

not suggest a consistent pattern of revertibility for the two classes of mutants here described. Three points must be emphasized, however. (1) The number of mutants examined (six) is very small. (2) The mutants are highly selected (as described above). (3) The significance of a failure to demonstrate induced mutagenesis is not clear since the sensitivity of our test for induced reversion has not been determined. These reservations refer only to the AP and HA results and do not detract from the primary conclusion of this paper.

Discussion.—The primary conclusion to be drawn from these experiments is that 5BU can exert its mutagenic action after it is incorporated into phage DNA. In

molecular terms, this probably means that during replication the incorporated 5BU at a particular site mistakenly pairs with guanine rather than adenine. If so, those reversions of 5BU-substituted phage that originate during a cycle of growth in the absence of externally supplied 5BU represent AT to GC base pair transitions. Conversely, 5BU-induced reversions that originate only in the presence of externally supplied 5BU should represent GC to AT transitions.

The halos produced by the induced e^+-like revertants under various conditions are identical to those produced by the standard type e^+ phage. The halo size serves as a very delicate index of the temperature sensitivity of the lysozyme; it is thus likely that the lysozyme of e^+-like revertants resembles that produced by standard type phage. Whether the lysozyme produced by these revertants is identical to the standard one can be determined only by detailed analysis of the protein of the revertant. The identification of the nature of the mutant base pair must await this chemical analysis. It may not be possible, even given such analysis, to come to a definitive conclusion since any particular amino acid may be coded for by a number of different sets of bases.

Freese *et al.* suggest[8] that hydroxylamine (HA) induces primarily GC to AT transitions. It was therefore expected that the mutants that were observed to revert only in the presence of externally supplied 5BU would also be reverted by HA. No increase was observed in the frequency of revertants after treatment with HA. Our reservations regarding the significance of this observation have been detailed above.

HA induces reversions in the mutant *e*C4. The 5BU-induced reversions of this mutant must represent AT to GC transitions. The observation that HA induces reversions could be accounted for by assuming either that HA may sometimes cause AT to GC transitions or that the site of the HA-induced reversions is not identical to that of the 5BU clean growth reversions.

It would be expected that if HA-induced mutations represent primarily GC to AT transitions, they ought to be readily revertible by 5BU during clean growth. This prediction could not be tested since no hydroxylamine-induced mutants satisfying the requirements of our experimental method have been found.

Summary.—5-Bromouracil-revertible mutants in the lysozyme gene of phage T4 fall into two classes. Members of one class revert only when 5BU is present in the environment; those of the other class revert after 5BU is incorporated into the phage DNA. This observation supports the widely held notion that 5BU induces mutations via the mechanism of base-pairing mistakes proposed by Watson and Crick.

The importance of the prediction concerning 5BU-mutagenesis tested in this paper was brought to our attention by Matthew Meselson in 1956. We wish to acknowledge also the many assistants, students, and colleagues who have accompanied one of us (F. W. S.) in his six-year search for this Holy Grail.

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[1] The abbreviations used are: 5BU, 5-bromouracil; A, adenine; T, thymine; G, guanine; C, cytosine; 2AP, 2-aminopurine; HA, hydroxylamine; *e*, lysozyme-defective mutant; e^+, standard type.

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[13] $P/P_0 = e^{-kt}$, where P = plaque-forming units present after t min exposure to anti-phage serum and P_0 = plaque-forming units present at $t = 0$.

[14] The number of intracellular phage present at a time of t min after infection with 5BU-grown phage equalled that at $t - 2$ min after infection with thymine-grown phage. The fraction of revertants did not differ appreciably at 15, 17, and 19 min after infection (thymine-grown stocks) and 17, 19, and 21 min after infection (5BU-grown stocks).

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A COMPUTATIONAL PROCEDURE FOR OPTIMAL SYSTEM DESIGN AND UTILIZATION

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1. *Introduction.*—A problem in the optimal control or utilization of a specified system which has been the object of much study in recent years is the following:[1, 2] Determine a control vector $y(t)$ on the interval $0 \leq t \leq T$ in such a manner that the system s, which is in state $x(t)$ at time t and has as its dynamical equation

$$\dot{x} = f(x, y), \; x(0) = c, \tag{1}$$

is operated in the least costly fashion, where the cost of the process is given by the functional

$$J[y] = \int_0^T g(x, y)dt. \tag{2}$$

As a rule, though, the function f depends not merely on x and y but also on a vector of system parameters, a, which is to be chosen before the process begins and which remains constant during the process. Thus, a may be viewed as a vector of system design parameters. The usual procedure for determining the system design parameters is to guess several choices for a, determine the corresponding optimal modes of operation, and then select the most promising choice of a and mode of operation. Our aim in this note is to show how we may apply the quasilinearization technique[3, 4]