BASE ANALOGUE INDUCED ARABINOSE-NEGATIVE MUTANTS OF *ESCHERICHIA COLI*[1]

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DEOXYRIBONUCLEIC acid codes information as a specific linear sequence of the base pairs adenine-thymine (AT) and guanine-cytosine (GC) (WATSON and CRICK 1953). Replacement of an AT pair by a GC pair, or a GC pair by an AT pair may cause a visible mutation. A transition is a base-pair change as written above in which a purine replaces a purine, and a pyrimidine replaces a pyrimidine (FREESE 1963).

Consideration of possible hydrogen-bonding led FREESE (1959) to suggest that 2-aminopurine (AP) should cause both AT to GC and GC to AT transitions. The chemical specificity of hydroxylamine (HA) for cytosine and the revertibility of HA-induced mutants by AP suggest that HA causes only GC to AT transitions (FREESE, BAUTZ-FREESE and BAUTZ 1961a).

Those mutants which are AT to GC changes should show increased reversion rates back to wild type under the influence of HA, while GC to AT changes should not revert under the influence of HA. These two classes of AP induced mutants, found for the bacterial virus T4 (FREESE *et al.*, 1961a,b) will be described for bacteria.

5-bromodeoxyuridine (BUDR) should cause AT to GC and GC to AT transitions, depending on whether the transition occurs during incorporation of the BUDR into DNA, or during replication of BUDR labeled DNA (FREESE 1963). Evidence supporting this hypothesis has been obtained from bacterial viruses (FREESE 1963; TERZAGHI, STREISINGER and STAHL 1962) and bacteria (RUDNER 1961; STRELZOFF 1961, 1962), although *in vitro* experiments indicate that BUDR causes nontransition as well as transition DNA changes (TRAUTNER, SWARTZ, and KORNBERG 1962). We will give evidence for the existence of BUDR induced transition and nontransition bacterial mutants. For discussions of specific mutagens see FREESE (1963), CLOWES (1964), and HAYES (1964).

MATERIALS AND METHODS

In establishing a mutation pattern, one wishes to select mutants from wild-type populations, and wild-type revertants from mutant populations. Such two way selection was developed for bacteria by BOYER, ENGLESBERG and WEINBERG (1962). There exist arabinose negative mutants (e.g. *ara*-139$^-$) of *Escherichia coli* which are sensitive to arabinose. Addition of a second arabinose

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negative mutation (*ara*$^-$) to the arabinose sensitive strain alleviates the sensitivity and enables the double mutant to grow on medium containing arabinose and an alternate carbon and energy source. Plating large numbers of arabinose sensitive cells on medium containing arabinose and an alternate carbon and energy source thus permits selection for *ara*$^-$ mutants. Selection for wild-type revertants is accomplished by plating large numbers of *ara*$^-$ mutant cells on a medium in which arabinose is the only carbon and energy source.

Abbreviations: DNA = deoxyribonucleic acid; A = adenine; G = guanine; C = cytosine; T = thymine; ara$^-$ = inability to utilize arabinose as a carbon and energy source; leu$^-$ = leucine dependence; thy$^-$ = thymine dependence; + = wild-type allele; AP = 2-aminopurine; HA = hydroxylamine; BUDR = 5-bromodeoxyuridine. Mutant alleles are written as *ara*-139$^-$, *ara*-1$^-$, etc. Allele numbers prefixed by A (e.g. *ara*-A3) represent mutations induced by AP; those prefixed by B were induced by BUDR.

Strains: Escherichia coli strains B/r and K-12 were used. The K-12 strain, Hfr KH600 *leu*$^-$ contained the arabinose region of strain B/r (HELLING and WEINBERG 1963). This strain is a derivative of *E. coli* K-12 Hfr P 4X-6. The arabinose sensitive mutant (*ara*-139$^-$) was described by ENGLESBERG *et al.* (1962). Arabinose negative mutants were induced in the arabinose sensitive strain and isolated as colonies capable of growing on a medium containing arabinose and supplemented with an alternate carbon and energy source. These strains were designated *ara*-139$^-$ *ara*-x$^-$, etc., as they were isolated.

The induced *ara*$^-$ mutation was separated from *ara*-139$^-$ by transduction with phage P1bt. Phage grown on B/r *ara*-139$^-$ *ara*-x$^-$ strains were used to transduce the *leu*+ marker into the Hfr *ara*+ *leu*$^-$ K-12 strain. Those *leu*+ transductants which were *ara*$^-$ were then crossed to an F$^-$ B/r strain which contained the *ara*-139$^-$ allele and the *ara*-H9$^-$ allele, the latter having been mapped close to the *ara*-139$^-$ alelle. The presence of *ara*+recombinants from these crosses indicated the absence of the *ara*-139$^-$ allele in the Hfr *ara*$^-$ strain; these strains could then be used in studies of reversion from *ara*$^-$ to *ara*+. In experiments involving BUDR, the *thy*$^-$ mutation was present in addition to other markers, necessitating addition of thymine to all media at a concentration of 100 μg per ml except where otherwise stated.

Media: Media were as described by ENGLESBERG (1961) unless otherwise specified.

Induction of arabinose negative mutations by AP: The arabinose sensitive strain was inoculated into nutrient broth, aerated overnight at 37°C, and diluted 1 to 400 into casein hydrolysate glucose liquid medium. Ten 0.2 ml samples were distributed to ten small tubes. AP was added to the remaining mixture to a concentration of 1 mg per ml, and 0.2 ml samples were distributed to small tubes. The small tubes were incubated overnight without aeration, and then assayed for the frequency of new *ara*$^-$ mutants, which would have the genotype *ara*-139$^-$ *ara*-x$^-$, by use of appropriate selective media (BOYER, ENGLESBERG and WEINBERG 1962). Viable counts were also made on nutrient agar. Only one *ara*$^-$ mutant was purified from each tube to assure that each mutant studied was an independent occurrence.

The AP induced *ara*$^-$ mutants studied were selected subclass of the *ara*$^-$ mutants isolated. Their mutational sites were restricted to a small region of the L-ribulokinase structural gene (BOYER 1963).

Induction of arabinose positive revertants by AP: Each *ara*$^-$ mutant was treated as above except that in the final step *ara*+ revertants were assayed on medium containing arabinose as a sole carbon source.

Induction of ara$^-$ *mutants by HA:* The arabinose sensitive strain was inoculated into 10 ml of tris glucose liquid medium, incubated for 17 hr at 37°C with aeration, centrifuged, and resuspended in 10 ml of HA reaction mixture which was 1.0 M for HA and at a pH of 7.0 (FREESE *et al.*, 1961). The reaction mixture containing the cells was aerated 30 min at 37°C, and then added to 20 ml of L-broth to protect cells from hydroxylamine during the rest of the procedure. The cells were centrifuged twice, and the pellet was resuspended in 20 ml of L-broth. After incubation overnight at 37°C with aeration, samples were removed for viable counts on nutrient agar, and for assays of *ara*-139$^-$ *ara*-x$^-$ mutants on the selective medium previously described. Controls were treated in the same way except that no HA was used.

Induction of ara+ *revertants with HA:* The transduction procedures described above were

used to eliminate the *ara*-139$^-$ allele. The ara$^-$ strains were treated with HA as above, with the final assay made for *ara*+ reversion frequency.

Induction of ara$^-$ *mutants by BUDR; Procedure I:* The arabinose sensitive thy$^-$ strain was aerated in nutrient broth overnight at 37°C; 0.5 ml of this was added to 50 ml of casein hydrolysate liquid medium containing 10 μg/ml thymine and aerated at 37°C to a turbidity of 13 Fisher Units (ENGLESBERG 1961). A Fisher unit is 1 OD unit × 100. The culture was centrifuged, and resuspended in 1.0 ml of saline. Of the suspension 0.5 ml was added to each of two 80 ml aliquots of casein hydrolysate medium lacking thymine and aerated ½ hr at 37°C. BUDR was then added to one aliquot to 10 μg per ml, and thymine to the other aliquot to 10 μg per ml. Both aliquots were aerated 1 hr at 37°C, and thymine was added to 100 μg per ml. After overnight growth at 37°C, viable counts were made on nutrient agar, and *ara*$^-$ mutant counts on selective medium containing 100 μg thymine per ml.

Induction of ara$^-$ *mutants by BUDR, Procedure II:* Cells were treated as in Procedure I until the time for BUDR addition. In Procedure II BUDR was added to 30 μg per ml, the cells were aerated ½ hr, and the cells were plated directly on medium selective for *ara*$^-$ mutants. Controls were similarly plated directly. The *ara*-139$^-$ allele was removed as described above. The BUDR-induced mutants have not been subjected to extensive mapping. Presumably the mutational sites of these mutants are scattered throughout the arabinose genes.

Induction of ara+ *revertants by BUDR, Procedure II:* Only Procedure II was used to induce *ara*+ revertants by BUDR. An ara$^-$ thy$^-$ strain was treated with BUDR as above, with the final assay being for frequency of *ara*+ revertants by use of minimal medium supplemented with thymine, and containing arabinose as a sole carbon and energy source.

Revertant assays: The mutagen treated cells were incubated 24 hr at 37°C. With each procedure a reconstruction experiment was performed to make the assay more objective; about 100 wild-type cells were plated with a background of the *ara*$^-$ strain in question and incubated with the other plates. A comparison of the reverted colonies could then be made with known wild-type colonies. Tabulated data were based on counts of all colonies which appeared after 24 hours incubation, since small and intermediate sized colonies were observed in mutagen treated populations after 48 hours incubation.

RESULTS

The frequency of spontaneous *ara*$^-$ mutants in a population of arabinose sensitive cells was usually 2 to 3×10^{-5} (Figure 1). Treatment with AP increased this frequency 100-fold in small tubes, as well as in aerated cultures (Figure 1). Out of 30 AP induced *ara*$^-$ mutants, 28 showed increased reversion rates when treated with AP, and in most cases the reversion rate was increased more than 100-fold (Table 1). One of the nonreverting mutants (*ara*-A103$^-$) was a deletion as determined by crosses (BOYER 1963), while the other nonreverting mutant (*ara*-A85$^-$) did not revert with BUDR or HA, nor did it revert spontaneously (Table 1).

Not all of the *ara*$^-$ mutants represent individual mutational sites. Of the mutants used here, four mutational sites are represented more than once. The *ara*-A15, *ara*-A3, *ara*-A74, *ara*-A71 and *ara*-A82 alleles represent one mutational site; *ara*-A34, *ara*-A91 and *ara*-A92 represent one mutational site; *ara*-A26 and *ara*-A79 represent one mutational site; and *ara*-A32 and *ara*-A90 represent one mutational site (BOYER 1963). It should be noticed that the mutants having identical mutational sites have similar reversion patterns. All AP induced mutants studied were determined to be L-ribulokinase negative mutants, including those whose mutational sites have not been mapped.

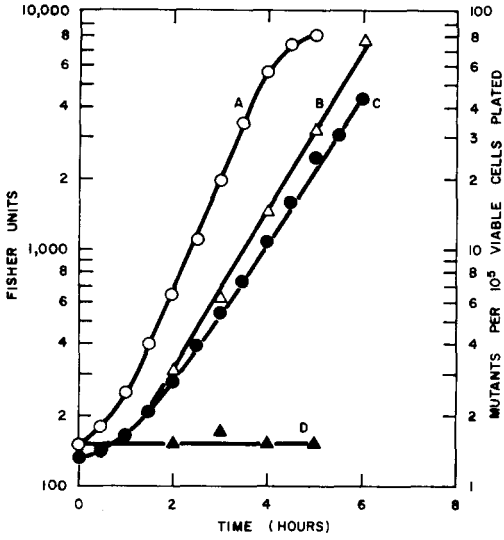

FIGURE 1.—Effect of 2-aminopurine on the growth rate and mutation rate of the arabinose sensitive strain (*ara*-139$^-$) in aerated medium. Curve A: growth rate, as measured by turbidity measurements, in casein hydrolysate-glucose medium. Curve B: growth rate, as measured by turbidity measurements, in casein hydrolysate-glucose medium plus 2-aminopurine, 1 mg/ml. Curve C: *ara*$^-$ mutation rate as a function of time in the presence of 2-aminopurine (1 mg/ml). Curve D: spontaneous mutation frequency as a function of time in the absence of 2-aminopurine.

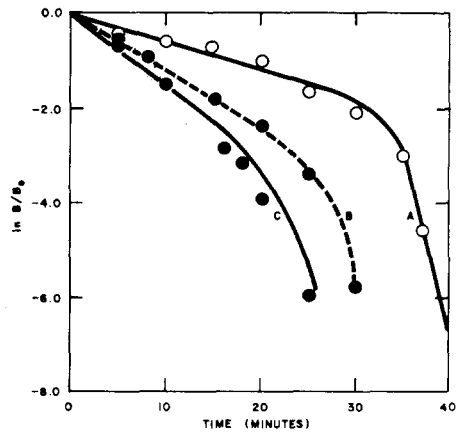

FIGURE 2.—Inactivation of the arabinose sensitive (*ara* 139$^-$) strain by hydroxylamine. Early stationary phase (curve A), log phase (curve B) and late stationary phase (curve C) cultures of the arabinose sensitive strain, were resuspended in the HA solution, pH 7.0, and incubated at 37°C. Samples were withdrawn at various time intervals and immediately diluted 10^{-3} into L-broth. Viable counts were then determined. B_0 = viable cells per ml of culture at $t = 0$. B = viable cells per ml of culture at time t.

The frequency of HA induced *ara*$^-$ mutants was of the order of ten times the spontaneous mutant frequency in a typical experiment, i.e., treated cells contained 24 *ara*$^-$ mutants per 10^5 cells while untreated cells contained only 0.6 mutants per 10^5 cells. HA induced mutants appeared in cells treated with HA for 30 to 35 minutes. More prolonged treatment led to rapid killing (Figure 2) without any increase in mutant frequency. Cultures started from small inocula and aerated 14 hr at 37°C were less sensitive to killing than younger or older cultures. These 14 hr cultures were susceptible to the mutagenic effect of HA. HA increased the frequency of *ara*$^+$ revertants with many *ara*$^-$ mutants, and in most cases this increase in reversion frequency was greater than tenfold (Tables 1 and 4).

Induction of *ara*$^-$ mutants with BUDR using Procedure I produced a 50-fold increase in mutant frequency (Table 2), while Procedure II produced a 250-fold increase in mutant frequency (Table 3). Only 2 out of 10 of the *ara*$^-$ mutants induced by Procedure I were revertible by AP, while 7 out of 9 of the *ara*$^-$ mutants induced by Procedure II were revertible by AP (Table 4). Of the AP

TABLE 1

Reversion of aminopurine induced mutants

Mutant strain	Revertants per 10^8 viable cells plated*			
	2-aminopurine	Hydroxylamine	5-bromodeoxyuridine	Spontaneous
A6	20	8	3	<0.5
A38	25	12	0.1	<0.1
A51	40	8	10	<0.1
A87	7	18	1	<0.5
A93	15	8	5	1.0
A98	13	77	<0.2	<0.5
A100	54	36	<0.1	<0.5
A2	35	<0.5	50	<0.5
A3	13	..	190	<0.5
A7	12	..	50	0.1
A8	32	..	100	0.3
A10	115	..	100	<0.1
A15	29	<0.5	100	<0.1
A16	23	<0.5	100	<0.5
A17	28	..	100	0.1
A22	16	<0.3	100	<0.1
A26	41	<0.2	235	<0.5
A32	25	<0.5	100	0.1
A34	49	0.5	316	<0.5
A71	18	..	151	<0.5
A74	12	..	135	0.5
A76	23	..	151	0.5
A79	98	<0.5	350	<0.5
A82	30	0.3	125	<0.5
A90	14	..	51	<0.5
A91	35	..	315	<0.5
A92	33	..	425	<0.5
A95	53	4	60	<0.1
A85	<0.4	..	<0.5	<0.5
A103	<0.3	..	<0.1	<0.1

* < a frequency indicates no revertant colonies.

TABLE 2

Induction of arabinose negative mutations with 5-bromodeoxyuridine (I)

Step	Viable count per ml	Mutants per 10^5 viable cells plated
1. Initiation of thymine starvation	4.5×10^7	5
2. After ½ hr thymine starvation	4.5×10^7	5
3. (a) 1 hr with thymine (control)	1.6×10^8	4
(b) 1 hour with BUDR (10 μg/ml), thymine added here	1.2×10^8	100
4. (a) 14 hr residual growth of control	3.2×10^9	2
(b) 14 hr residual growth of BUDR treated cells	3.2×10^9	250

TABLE 3

Induction of arabinose negative mutations with 5-bromodeoxyuridine (II)

Step	Viable count per ml	Mutants per 10^5 viable cells plated
1. Initiation of thymine starvation	3.5×10^7	2
2. After ½ hr thymine starvation	3.0×10^7	3
3. (a) ½ hr with thymine	6.0×10^7	3
(b) ½ hr with BUDR (30 μg per ml)	3.3×10^7	500

TABLE 4

Reversion of 5-bromodeoxyuridine-induced mutants

Mutant strain	Revertants per 10^8 viable cells plated*			
	2-aminopurine	Hydroxylamine	5-bromodeoxyuridine	Spontaneous
Procedure I†				
B6	39	60	<0.5	<0.5
B7	25	51	<0.5	<0.5
B8	<0.5	..	<0.5	<0.1
B9	<0.5	..	<0.3	<0.1
B12	<0.5	..	<0.5	<0.5
B13	<0.5	..	<0.5	<0.5
B18	<0.5	..	<0.5	<0.5
B24	<0.5	..	..	<0.5
B26	<0.5	..	..	<0.5
B21	<0.5	..	..	<0.5
Procedure II‡				
B73	40	<0.2	1.0	<0.3
B74	30	<0.2	<0.5	<0.3
B76	30	22	<0.5	<0.4
B80	30	84	<0.5	<0.8
B83	<0.9	<0.4	0.5	<0.8
B84	<0.9	<0.4	<0.5	<0.5
B87	100	86	<0.5	<0.5
B88	90	78	0.5	<0.4
B91	60	100	<0.5	<0.4

* < a frequency indicates no mutant colonies.
† Mutants obtained after replication of BUDR labeled DNA overnight.
‡ Mutants obtained after a ½ hour BUDR pulse and immediate selection.

revertible mutants, 7 out of 9 were also revertible by HA, while none of the BUDR induced mutants were revertible by BUDR (Table 4).

DISCUSSION

Virtually all AP-induced mutants were revertible by AP, and these fell into two main classes: those not revertible by HA but strongly revertible by BUDR, and those revertible by HA but showing no, or relatively weak, BUDR-induced reversion.

Chemical evidence indicates that HA causes only GC to AT transitions (BROWN

and SCHELL 1961; FREESE *et al.*, 1961a). The following hypothesis explains this pattern. AP induced both AT to GC and GC to AT transitions, so that AP could increase reversion rates in AP induced mutants. This agrees with other data for bacteria (BALBINDER 1962; DEMEREC 1960; STRELZOFF 1961, 1962; MARGOLIN and MUKAI 1961). HA reverts only the AT to GC mutants, by increasing the rate of reverse transition back to AT in these mutants, as was found for the bacterial virus T4 (FREESE *et al.*, 1961b). This would imply that BUDR transitions were mainly AT to GC.

BUDR-induced mutants fell into two classes, AP revertible and AP nonrevertible. Nearly all the AP-revertible mutants, but none of the others tested were also HA revertible. The following hypothesis explains these results. The revertible mutants were AT to GC transitions, while the nonrevertible mutants were nontransition type mutants. Nontransition type DNA alterations have been induced by BUDR *in vitro* (TRAUTNER *et al.*, 1962). The revertibility of the majority of mutants in the transition class by HA but not by BUDR supports our earlier conclusion that the BUDR induced transitions were mainly AT to GC, an analogous conclusion to one drawn for single stranded phage S13 (HOWARD and TESSMAN 1964).

Since we could vary the frequency of transition and nontransition type BUDR induced mutants by varying our experimental technique, and RUDNER (1961) and STRELZOFF (1962) have observed different results with BUDR in response to different procedures, it is not surprising that different workers have found varying results in bacteria with BUDR. Both STRELZOFF working with *E. coli*, and RUDNER with Salmonella concluded that BUDR caused either GC to AT or AT to GC type transitions. KIRCHNER (1960), studying histidine negative mutants of Salmonella, found that 11 of 12 AP induced mutants were revertible by BUDR, while only 3 in 12 of these mutants were revertible by AP itself. ALLEN and YANOFSKY (1963) found that some tryptophan dependent mutants of *E. coli* were revertible by AP, some by BUDR and some by both mutagens.

The occurrence of various types of suppressor mutants has been discussed by ALLEN and YANOFSKY (1963), YANOFSKY, HENNING, HELINSKI and CARLTON (1963), YANOFSKY (1964), and HENNING and YANOFSKY (1962). The presence of suppressors might obscure a clear pattern of forward and reverse mutation, but would probably not cause the pattern we observed. Revertants of about one-half of these mutant strains tested were subjected to analyses for unlinked suppressors; in none of the cases tested were unlinked suppressors observed (BOYER 1963).

Although the molecular specificities which we suggest here are not the only ones that could be offered, they constitute a working hypothesis that fits most of our observations and which is reasonably compatible with the specificities proposed for most similar investigations. We have adopted the viewpoint of HAYES (1964) that even the occurrence of some anomalies "should not deter us from provisionally accepting the more obvious consistencies at their face value."

Since each of the mutagens used caused an increase in the number of new arabinose negative mutants in arabinose sensitive populations, and also increased

the frequency of arabinose positive mutants in arabinose negative populations, it seems certain that the effect of the mutagens was to increase mutation rate rather than to alter selective conditions.

SUMMARY

The patterns of induced revertibility were determined for a set of base analog-induced arabinose-negative mutants. Our results fit the following interpretations of mutagen specificity: (1) Hydroxylamine induced transition of guanine cytosine base pairs to adenine thymine base pairs in DNA. (2) 2-aminopurine caused both adenine thymine to guanine cytosine, and guanine cytosine to adenine thymine transitions. (3) 5-bromodeoxyuridine caused both transition and non-transition DNA changes, and transitions of adenine-thymine to guanine-cytosine were more frequent than guanine-cytosine to adenine-thymine transitions.

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