CHARACTERISTICS OF MUTATIONS APPEARING SPONTANEOUSLY IN EXTRACELLULAR PARTICLES OF BACTERIOPHAGE T4[1]

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SPONTANEOUS mutations slowly accumulate in bacteriophage T4 at 0°C and at 20°C (DRAKE 1966a). When revertants of *rII* mutants were scored, it was found that among those tested only transition mutants containing GC[2] base pairs at the mutant site revert spontaneously in the absence of DNA replication. (Transitions consist of base pair substitutions in which the purine-pyrimidine orientation is preserved, in contrast to transversions, where this orientation is reversed.) Known transversions were not available for study, but transitions containing AT base pairs at the mutant site did not revert in the absence of replication. Sign mutants (containing additions or deletions of small numbers of base pairs) also failed to revert without replication. Thus the GC base pair or one of its component bases was implicated as the mutagenic target for spontaneous, replication-independent mutation, but the chemical nature of the process was not revealed.

A study of mutations accumulating in the forward ($r^+ \rightarrow r$) direction should establish the types of mutational lesions resulting from spontaneous alterations of GC base pairs. It may then become possible to infer the original causative chemical change. The numbers of *r* mutants accumulating over the original background at 0°C was small even after more than a year, whereas the numbers of *r* mutants accumulating at 20°C was much larger. A collection of mutants accumulated at the higher temperature was therefore chosen for analysis.

MATERIALS AND METHODS

Bacteriophage T4B and *Escherichia coli* were used throughout. General methods have been presented previously (ADAMS 1959; BENZER 1961; DRAKE 1966a). We have not knowingly altered the conditions for assaying wild-type and *r* phages during the course of this study.

A T4 wild-type stock was diluted 18,000-fold in L broth at pH 7.0, and samples were maintained at 0° and at 20° in rubber-stoppered Pyrex tubes. Mutant plaques exhibiting the *r* morphology were picked from *E. coli* B cells and were purified by replating and repicking. They were then spot tested for their ability to grow on KB cells. Most of the mutants growing well on KB cells are *rI* mutants, which appear to result exclusively from base pair substitutions, since they are induced by base analogues but not by proflavin (DRAKE, unpublished). Mutants growing poorly or not at all on KB cells were classified as *rII*. Many of these either revert at high frequencies, or else are very leaky, and are therefore unsuitable for detailed study.

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[2] Abbreviations: A, adenine; T, thymine; G, guanine; C, cytosine, or glucosylated 5-hydroxymethylated cytosine in the specific case of bacteriophage T4; 2AP, 2-aminopurine; 5BU, 5-bromouracil; HA, hydroxylamine; SM mutants, mutants accumulated during storage.

Stocks of *rII* mutants were grown in BB cells, and were mapped with spot tests, first into segments defined by deletions, and finally into sites (BENZER 1961).

Transition mutations (and possibly some transversion mutations) were identified by their positive responses in base analogue spot tests (FREESE 1959a; DRAKE 1966b). Plates containing a mixture of the *rII* mutant phage, KB cells, and a small number of B cells were spotted with 2-aminopurine (2AP) and 5-bromouracil (5BU) solutions, and incubated overnight. Only revertants grow into visible plaques. Unspotted regions of the plate constitute the background control, while a grouping of plaques around one or both of the spots indicates induced reversion.

Quantitative base analogue and proflavin reversion tests were carried out on all mutants which appeared not to respond to the base analogue spot tests. These tests have been described previously (FREESE 1959a; DRAKE 1963), and consist of growth in liquid culture, with or without the mutagen, followed by platings on KB cells (to detect r^+ revertants) and on B or BB cells (to detect total phages).

Mutants which responded to base analogues were also tested with hydroxylamine (HA). The procedure of FREESE, BAUTZ and BAUTZ-FREESE (1961) was used, except that the exposure to HA was 4 hours at 44°C, and the corresponding survival frequencies were around 10%. A group of mutants which reverted spontaneously, but which did not respond to any of the above agents, was also treated with hot acid (45°C, pH 4.5 acetate buffer) for 24 hours (FREESE 1959b). Survival frequencies were generally from 0.1% to 1%.

RESULTS

Mutation rates: Frequencies of *r* mutants in a wild-type stock maintained in L broth, measured at intervals over more than three years, are shown in Figure 1. At 0°C about 90% of the phages were still alive after three years. At 20°C the phages were inactivated with 1.35-hit kinetics, and reached the 10% survival

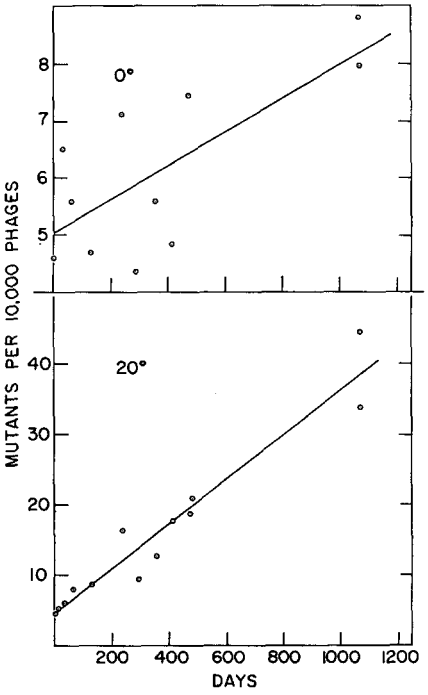

FIGURE 1.—Frequencies of *r* mutants in wild-type T4 stocks maintained in broth at 0°C (top) and at 20°C (bottom). The curves were calculated by the method of least squares, using all of the data.

TABLE 1

Mutation rates ± standard deviations determined from the data of Figure 1 by the method of least squares

Temperature (°C)	Background: mutants per 10^4 phages	Slope: mutants per 10^7 phages per day
0	5.04 ± 0.48	2.95 ± 0.93
20	4.58 ± 1.26	31.7 ± 0.8

level in about 800 days. This high viability made further tests for selection of preexisting mutants unnecessary.

Mutations are clearly accumulating at a linear rate at 20°, and probably also at 0°. The linear regression curves and their standard deviations were calculated by the method of least squares; the parameters are listed in Table 1. Although the values obtained from the samples held at 0° show considerable scatter, this appears to be random, under which assumption the slope of the curve differs from zero by more than three standard deviations.

The *r* mutants to be described were collected from the sample held at 20° at 474 and at 481 days. From the constants listed in Table 1, it is calculated that this (pooled) collection of mutants contained about 24% of preexisting background mutants.

Phenotypes: Repeated examinations of collections of spontaneous mutants arising primarily during the replication of bacteriophage T4 have shown that about one-third are *rI* mutations, about two thirds are *rII* mutations, and a tenth or less are a mixture of *rIII* mutations plus leaky or rapidly reverting *rII* mutations (BENZER 1957; DRAKE 1966a, b). Of the 221 *r* mutants initially isolated in the present study, 89 (40%) grew very well on KB cells, and were probably mostly *rI* mutants; 101 (46%) were ordinary *rII* mutants; and 31 (14%) were either very leaky or else reverted at high rates.

Both induced *r* mutations, and spontaneous *r* mutations newly arisen during T4 replication, commonly first appear as mottled plaques. These are mutational heterozygotes, which segregate both *r* and r^+ genotypes. Among the mutant plaques obtained from the 0°C samples during the first 64 days (when the increase of mutants over the background was negligible), 19/82 or 23% were mottled. Among the mutant plaques obtained from the 20°C samples at 474 and 481 days, 110/190 or 58% were mottled. It is likely that both percentages are underestimates, since under the conditions employed for scoring mutants, some originally mottled plaques would have cleared. However, most of the mutants clearly first appeared in the form of heterozygotes.

Mapping: It proved possible to map 100 of the *rII* mutants into 78 distinct sites; one additional mutant (SM 89) was an extended deletion. A few leaky mutants were sufficiently temperature-sensitive to be mapped at 42°C. A few rapidly reverting mutants could be mapped, but could not be used in subsequent reversion studies. The fine-scale map appears in Figure 2. The notations under the line segments indicate cistron segments defined by deletion mutants (BENZER

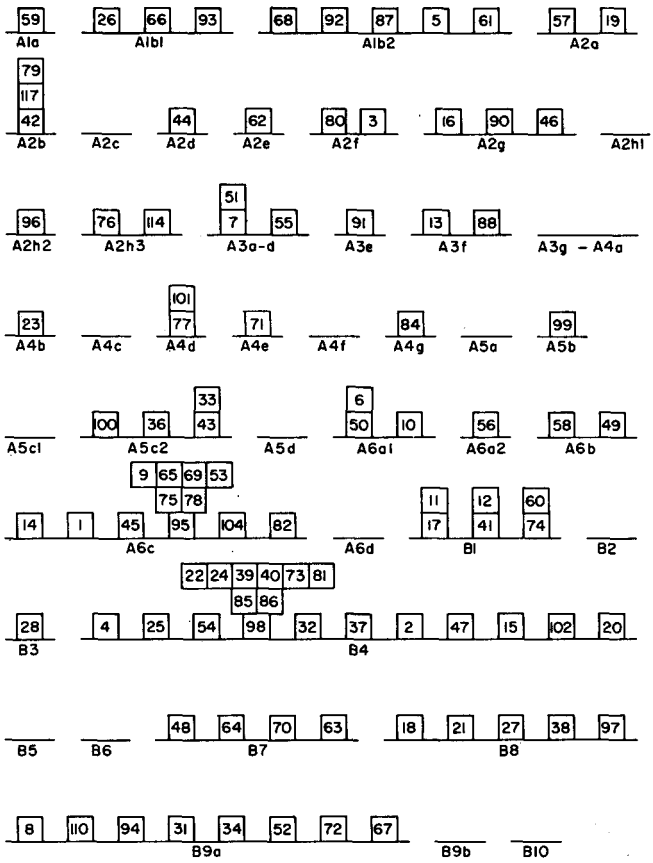

FIGURE 2.—Fine scale mapping of replication-independent *rII* mutants. Horizontal lines represent cistron segments defined by deletions (BENZER 1961), and are ordered. Boxes represent independently isolated mutants, whose sites within a segment are unordered. Numbers within boxes identify individual mutants.

1961). While the segments are ordered, the sites within a given segment have not been ordered. Multiple occurrences of independently arising mutations at the same site ("hot spots") are indicated by stacking boxes, and numbers within boxes designate individual mutants.

Comparison of Figure 2 with the map of more than 1600 spontaneous mutants compiled by BENZER (1961) reveals several minor and two extremely striking differences. Minor differences, sometimes of doubtful statistical significance, include a hot spot in A2b of Figure 2, missing from the BENZER map; a hot spot in A4c missing from Figure 2 but present in the BENZER map; and a considerably larger number of sites in B8 and B9 of Figure 2 than expected from the BENZER map. The major differences concern the two hot spots in A6c and in B4, which contain over 50% of all of the spontaneous mutants isolated by BENZER, but which are proportionately underrepresented in the present collection. In the A6c

hot spot, 19 occurrences would have been expected for an ordinary collection of the size of the present one, but only seven were found; in the B4 hot spot, 32 would have been expected, but only nine were found. In fact, the 16 mutants which did map at these two sites in Figure 2 are essentially completely attributable to the background of original, replication-dependent spontaneous mutants: 24% background × 50.7% mutants in the two hot spots = 12%, or 12 mutants expected, compared to the 16 found. Representatives of these two hot spots have in general proved to be sign mutants (e.g., they are inducible to revert with proflavin, but not with base analogues) (DRAKE 1963, 1966a). The absence of these two hot spots is therefore consistent with the earlier observation, that sign mutants did not *revert* during storage (DRAKE 1966a).

Reversion analysis: The chemical nature of a mutational lesion may sometimes be deduced by examining the susceptibility of the mutant to reversion induction by specific mutagens. The scheme which we used to classify the reversion responses of *rII* mutants is shown in Figure 3, where (+) indicates that induced reversion was detected, and (−) that it was not detected. The significance of the tests will be discussed later.

The spot tests detected nearly all of the mutants which were capable of responding to base analogues. Of the 101 mutants tested, 43 were positive, one was negative but was shown to be a responder in the liquid culture tests, and eight were doubtful. The doubtful responders divided equally into responders and into nonresponders (SM30, SM58, SM60, SM74) in the quantitative tests. The responders are considered to contain base pair substitutions, and the base pair at the mutant site may be identified in the hydroxylamine test: hydroxylamine is a cytosine-specific mutagen. However, since some mutants "revert" by the appearance of partial revertants, an attempt must be made to distinguish them from complete revertants. Partial revertants may be detected by backcrosses to wild type, but in practice it has been found with mutants revertible by base analogues that

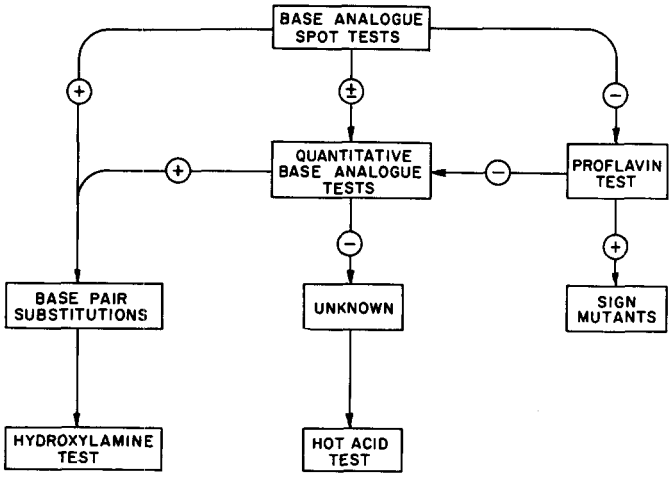

FIGURE 3.—Flow diagram for reversion analysis of *rII* mutants.

TABLE 2

Mutants revertible by base analogues

Mutant	Spot tests		Quantitative tests*				HA-FR	Mutant base pair
	2AP	5BU	Spon.	2AP	5BU	HA		
SM4	+	+	1.6	...	...	7,800.	6/10	GC
SM5	+	—	0.2	...	...	4.1	0/7	GC
SM6	+	±	480.	...	...	180,000.	0/20	GC
SM7	+	+	51.	...	...	1,000.	0/10	GC
SM8	+	+	1.1	...	...	1,370.	7/10	GC
SM10	+	—	0.7	...	...	12.	0/3	GC
SM11	+	+	2.5	...	...	13.	1/10	GC
SM16	+	+	3.4	...	...	8,200.	13/20	GC
SM17	+	+	2.3	...	...	10.	3/12	GC
SM19	+	—	170.	...	...	11,600.	5/10	GC
SM21	+	—	0.14	...	...	12.	0/4	GC
SM26	+	+	320.	...	...	360,000.	0/9	GC
SM31	+	+	6.8	...	...	3,400.	1/11	GC
SM34	+	±	0.5	...	...	2,900.	6/20	GC
SM36	+	—	150.	...	...	4,200.	0/8	GC
SM41	+	—	0.09	...	...	1.0	1/12	GC
SM42	+	+	4,250.	...	...	1,400,000.	2/10	GC
SM44	+	±	40.	...	...	2,400.	6/10	GC
SM48	+	—	3.3	...	...	58.	0/10	GC
SM51	+	+	130.	...	...	780.	0/8	GC
SM52	+	—	11.	...	...	1,370.	1/10	GC
SM54	±	±	860.	1,000.	2,100.	340,000.	1/6	GC
SM55	+	+	490.	...	...	40,500.	1/11	GC
SM64	+	—	1,280.	...	...	13,000.	3/9	GC
SM68	+	+	14.	...	...	1,300.	2/10	GC
SM70	+	—	230.	...	...	300,000.	0/11	GC
SM72	+	+	2.3	...	...	590.	0/10	GC
SM77	—	—	1.0	120.	390.	910.	6/11	GC
SM79	+	+	1,130.	...	...	2,000,000.	....	GC
SM87	+	—	0.3	...	...	2.1	0/1	GC
SM91	+	±	300.	...	...	300,000.	1/10	GC
SM92	+	+	1.4	...	...	65,000.	0/10	GC
SM101	±	—	1.2	6,600.	270.	1,400.	2/9	GC
SM117	+	+	4,300.	...	...	1,900,000.	1/10	GC
SM38	+	±	0.05	...	...	$\leq$ 1.7	....	?
SM80	+	—	0.05	...	...	$\leq$ 3.	....	?
SM88	±	—	0.1	12.	4.0	$\leq$ 6.	....	?
SM93	+	—	0.03	...	...	$\leq$ 3.	....	?
SM97	+	—	0.1	...	...	$\leq$ 2.	....	?
SM12	+	—	0.6	...	...	$\leq$ 0.2	....	AT
SM13	+	—	0.1	...	...	$\leq$ 0.2	....	AT
SM25	+	—	0.2	...	...	$\leq$ 0.2	....	AT
SM27	+	—	1.3	...	...	$\leq$ 1.	....	AT
SM61	+	—	4.7	...	...	$\leq$ 2.2	....	AT
SM63	+	—	25.	...	...	6.6	....	AT
SM66	±	—	0.06	...	...	$\leq$ 0.07	....	AT
SM96	+	±	35.	...	...	51.	....	AT
SM110	+	+	0.1	...	...	$\leq$ 0.7	....	AT

* Revertants per 10^7 phages. Spon. = spontaneous. HA-FR = fraction of false revertants in HA test.

TABLE 3

Mutants revertible by proflavin

Mutant	Spontaneous*	Proflavin*	Mutant	Spontaneous	Proflavin
SM9	8.2	350.	SM69	7.7	54.
SM15	15.	560.	SM73	15.	200.
SM20	0.2	29.	SM75	12.	380.
SM22	15.	510.	SM76	0.2	5.1
SM24	13.	210.	SM78	12.	190.
SM28	6.7	130.	SM81	15.	320.
SM32	3.3	62.	SM82	6.7	130.
SM39	17.	340.	SM84	0.5	3.8
SM40	14.	370.	SM85	9.7	270.
SM46	0.02	0.13	SM86	15.	270.
SM50	0.06	2.4	SM95	5.3	140.
SM53	4.8	80.	SM98	12.	320.
SM59	4.6	52.	SM99	1.7	10.
SM65	7.1	410.	SM100	0.8	6.5
SM67	$\leq$ 0.01	0.53			

* Revertants per 10^7 phages.

examination of the phenotype of the revertant on B cells detects almost all of the partial revertants detectable in backcrosses (DRAKE 1963). A routine sampling was therefore made of hydroxylamine-induced revertants. Any deviation in plaque morphology from the wild phenotype was taken as evidence of reversion to some state other than the original wild type. All of the mutants responding to base analogues are described in Table 2.

All mutants which failed to show a definite response in the base analogue spot tests were further tested for the induction of reversions by proflavin. The responding mutants are considered to contain sign mutations. These are described in

TABLE 4

Mutants refractory to base analogues and to proflavin

Mutant	Spontaneous*	Mutant	Spontaneous
SM1	3,300.	SM49	4,100.
SM2	880.	SM56	240.
SM3	0.01	SM57	2.7
SM14	3,700.	SM58	470.
SM18	6.1	SM60	650.
SM23	24,000.	SM62	210.
SM30	10,000.	SM71	0.01
SM33	200.	SM74	720.
SM37	5,700.	SM90	0.2
SM43	150.	SM94	0.8
SM45	0.3	SM102	980.
SM47	1,600.		

* Revertants per 10^7 phages.

TABLE 5

Reversion responses of mutants

Base analogues	Proflavin	Type of mutant	HA	Hot acid	Number and percent
+	(—)*	Base pair substitution	+	..	34
			—	..	9
			?	..	5
—	+	Sign mutation	..	..	29
—	—	Unknown	..	+	1
			..	—	16
			..	±	6

* Mostly not tested.

Table 3. As indicated below, these mutants probably comprise most of the original background of replication-dependent spontaneous mutations.

The residue of mutants, responding neither to base analogues nor to proflavin, contain mutational lesions of unknown types (Table 4). Although further reversion tests on these mutants are possible with a variety of mutagens, only hot acid (24 hours at 45° at pH 4.5) seemed to hold sufficient promise of mutagenic specificity to be useful. Only one mutant responded strongly to hot acid, in the sense that revertants were induced many-fold over the background: SM94 reverted at the rate of 1.8×10^{-6} mutational hits per lethal hit. Several other mutants exhibited responses only 1.5- to 4-fold over the background, and are therefore doubtful responders: SM1, SM2, SM30, SM60, SM74, and SM90.

The reversion responses of the mutants are summarized in Table 5. Nearly half of the mutants (48%) were base analogue-revertible, and also mostly apparently revertible by HA. This is a much higher figure than the 14% reported by FREESE (1959b) in a study of spontaneous mutants which presumably arose mostly during bacteriophage replication; in similar studies, DRAKE (1966b, c) also reported 22% and 21% of ordinary spontaneous mutants to be base pair substitution types.

When the reversion responses of the mutants are compared with their map locations, recurrrences at a given site are generally seen to consist of the same mutational type. The two largest hot spots, for instance, contain exclusively sign mutations. Only the site containing SM6 and SM50 appears to be inhomogeneous.

DISCUSSION

Appearance and mapping of mutants: The data available at the time of the first report of spontaneous mutation in extracellular bacteriophage T4 particles (DRAKE 1966a) were inadequate to establish a significant measure of forward mutation at 0°, although reversion was clearly observable. Calculations including more recent additions to these data have provided a forward mutation rate more than three standard deviations from zero (Table 1), assuming a linear mutation rate and a random scatter of the values around the mean.

SM mutants differ strikingly from spontaneous mutants arising during the growth of phage stocks, both in the kinds of lesions produced, and in their map distribution. Comparison of Figure 2 with the data of BENZER (1961) reveals many differences, especially the absence of the two giant hot spots in segments A6c and B4.

At least 69% of the SM mutants were heterozygous (58% observed, corrected for background), and the actual figure is probably higher, since assay conditions were chosen to provide the best detection of mutants, but not necessarily the best detection of mottling in the mutant plaques. The heterozygotes are presumably heteroduplexes resulting from alterations in only one of the members of a base pair.

Theory of reversion analysis: The significance of data on the induced reversion of T4*rII* mutants depends upon the mutagenic specificities attributed to proflavin, the base analogues, and hydroxylamine. Recent reviews have discussed several aspects of this subject (FREESE 1963; KRIEG 1963; ORGEL 1965). Briefly, the base analogues and proflavin separate mutants into three distinct classes, those responding specifically to only one agent, or to neither. (A rare class of *rII* mutants responds to both agents, but at least some members of this group clearly respond by the induction of extracistronic suppressors (BRENNER, personal communication).) If reversion is induced by proflavin, the mutant is considered to contain an addition or a deletion of a small number of base pairs, regardless of whether reversion occurs at the same or at a nearby site. Mutants responding to base analogues are considered to contain a base pair substitution. If the induced reversion can be shown to occur at the same site, then the mutation is identified as a transition (AT $\longleftrightarrow$ GC). If reversion frequently occurs by intracistronic suppression, the originally mutated site may contain either a transversion, such as GC $\longleftrightarrow$ CG, or a transition. Hydroxylamine specifically induces GC $\rightarrow$ AT transitions, and to the extent that reversion is induced at the originally mutated site, it therefore distinguishes between the two possible transitions.

Partial reversion may be detected by a backcross to wild type, or by direct phenotypic examination of revertants. In an earlier study of *rII* base pair substitution mutants induced by ultraviolet irradiation, essentially no more partial revertants were detected by backcrosses than by examining phenotypes (DRAKE 1963). However, a satisfactory disproof of the presence of a suppressor may be very difficult for several reasons. Mutations occupying nearly adjacent base pairs may recombine at much less than the expected frequency (TESSMAN 1965). Although amino acid substitutions are almost never restored to the original by two different base pair substitutions within the same codon (NIRENBERG *et al.* 1965), amino acid substitutions may occur at the same or adjacent sites to provide a fully functional polypeptide, as exemplified by the hemoglobins (PERUTZ, KENDREW, and WATSON 1965). Thus reversion analysis in any single instance is not rigorous, although the analysis of many mutants is usually very informative.

The most glaring inadequacy of reversion analysis in the T4*rII* system at present is its inability specifically to identify transversions. Some transversions

are probably capable of responding to base analogues by intracistronic suppression, while others may fail to respond either to base analogues or to proflavin. The hot acid treatment, whose mutagenicity may be due to depurination, appears able to induce both transitions and transversions but unable to induce sign mutations (E. B. FREESE 1961). Hot acid might reveal transversions; however, it may also be guanine-specific, and therefore of limited sensitivity.

Mutants which fail to respond to base analogues, proflavin, or hot acid might be transversions, or might contain multiple lesions (such as two close base pair substitutions). Such mutants would have to be capable of reverting spontaneously at approximately normal rates, however. The nonresponders could also contain lesions which responded too weakly to the mutagens for the response to be detected above the spontaneous background; however, a previous study of about 100 mutants induced by UV (DRAKE 1963) revealed essentially no such ambiguous mutants.

The nature of SM mutants: Sign mutations were not detectably accumulated during the course of these experiments: neither the two largest T4*rII* hot spots, nor proflavin-revertible mutants generally, appeared above the background levels. Earlier studies under identical conditions had also failed to detect *reversion* among sign mutants (DRAKE 1966a).

Most (48/71) of the remaining mutants could be induced to revert by base analogues. We therefore conclude that spontaneous, replication-independent mutation specifically induces base pair substitutions.

A majority (34/48) of the mutants revertible by base analogues were also revertible by hydroxylamine, apparently to the original wild type. This result is surprising in view of the earlier finding that spontaneous *reversion* occurred only in mutants revertible by hydroxylamine. The two most obvious mutagenic processes capable of encompassing both results are: (1) Only GC → AT transitions are induced, but these AT-transition mutants form a special and previously unrecognized class whose members in some cases are nonrevertible by base analogues, or in other cases are revertible by hydroxylamine to a suppressed state that is not recognized by phenotypic tests. (2) Transversions are induced from GC base pairs, probably often proceeding in the direction GC → CG. Hydroxylamine-induced reversion would then occur at the same site, leading to an overall GC → TA change. This would generally result in a single amino acid difference between the original wild type and the revertant (NIRENBERG *et al.* 1965). The special hypothesis is also required, that these substitutions escape recognition by phenotypic tests; they would of course automatically escape detection in a backcross.

We favor the hypothesis that these mutations arise by transversions. All of the mutations induced by base analogues (FREESE 1959b), and all of the mutations induced by UV except for the sign mutations (DRAKE 1963), are revertible by base analogues; the totally nonresponding class such as we observe here is absent. Transitions induced by hydroxylamine are reverted by base analogues but not by hydroxylamine (FREESE, BAUTZ, and BAUTZ-FREESE 1961; FREESE, BAUTZ-FREESE, and BAUTZ 1961; CHAMPE and BENZER 1962). Furthermore, we can

imagine mechanisms capable of producing transversions. One of the most obvious and most frequently invoked is depurination, which proceeds rapidly at high temperatures (GREER and ZAMENHOF 1962), and may proceed slowly even at 0°. Evidence in favor of the mutagenicity of depurination is scarce, however; mild heating of alkylated cells strongly promotes mutagenesis (STRAUSS 1962; but see STRAUSS, 1964), but other depurinating treatments (heat, acid) deaminate cytosine, and thus presumably induce GC → AT transitions (SHAPIRO and KLEIN 1966). However, rearranged or partially degraded purines constitute a less obvious and presently poorly defined class of possible intermediates in the production of transversions. Some of these may pair with normal purines and be accepted by the DNA replicating system. A possible example is 3-isoadenosine (LEONARD and LAURSEN 1966).

SUMMARY

T4*rII* mutations accumulated linearly among extracellular bacteriophage particles maintained at 0° and at 20°. Sign mutants did not appear. Most of the mutants which did appear contained base pair substitutions, but about 25% could not be identified because of their failure to revert when treated with proflavin, base analogues, or hot acid. Although the mutations presumably arose from GC base pairs, many were apparently induced to revert by the C-specific mutagen hydroxylamine. It is suggested that many of these mutations are transversions arising from mispairing events involving altered purines, or following depurination.

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