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Properties of *r* Mutants of Bacteriophage T4 Photodynamically Induced in the Presence of Thiopyronin and Psoralen

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About 4×10^{-4} *r* mutants were induced per lethal hit, a frequency characteristic of weak mutagens. Collections of mutants produced in the presence of either dye were indistinguishable in most of their properties. The *r*II mutants differed sharply from spontaneous mutants in their mutational spectra (fine scale map distribution) and their reversion responses to specific mutagens. Few or none of the induced mutants were induced to revert with proflavine (sign mutants; reading frame shift mutants). A majority were induced to revert with base analogues (base pair substitution mutants), and about half of these also responded to the hydroxymethylcytosine-specific agent hydroxylamine. A large minority of the mutants reverted spontaneously but failed to respond either to proflavine or to base analogues. We believe these mutants, as well as some of the mutants which did respond to base analogues, to be transversions (base pair substitutions which reverse the purine-pyrimidine orientation).

White light irradiation of viruses in the presence of any of a number of dyes may produce both inactivation and mutagenesis. In the presence of methylene blue or lumichrome, the predominant photochemical reaction with deoxyribonucleic acid (DNA) produces guanine degradation (2, 17-21). Mutations arising at the site of a degraded or missing guanine residue might comprise a variety of types of lesions. The elementary kinds of mutational lesions are transitions (single base pair substitutions in which the purine-pyrimidine orientation is preserved), transversions (single base pair substitutions in which this orientation is reversed), and sign mutations (additions and deletions of small numbers of base pairs). Analysis of the revertibility of T4*r*II mutants by various specific mutagens often identifies mutation lesions as one of these kinds.

We have photodynamically induced T4*r*II mutations in the presence of the dyes thiopyronin and psoralen (Fig. 1), and have studied their properties. These mutants superficially resemble T4*r* mutants photodynamically induced in the presence of proflavine (15, 16). Surprisingly, however, many of the mutants more nearly resemble transversions than transitions.

MATERIALS AND METHODS

Strains of phages and bacteria, and standard methods, have been described previously (1, 4, 6, 8). *Escherichia coli* and bacteriophage T4B were used throughout. T4*r* mutants were recognized by their plaque morphology on *E. coli* strain B. Phage stocks were grown on strain BB, which does not discriminate between *r*II and r^+ phages. Recombinant or revertant r^+ phages among an excess of *r*II phages were selectively plated on strain KB. The mutant T4*o*C2, resistant to osmotic shock, was isolated in this laboratory by C. Lundeen and N. Lancaster.

Psoralen and thiopyronin were gifts from A. Wacker. Their structures are illustrated in Fig. 1; methylene blue differs from thiopyronin in possessing a nitrogen atom instead of a CH group *para* to the S atom. They were dissolved in buffer (0.01 M phosphate plus 0.01 M magnesium, *p*H 7.0) and used at final concentrations of 50 μg/ml of psoralen and 2 μg/ml of thiopyronin. Sensitization of both T4 and its osmotic shock-resistant mutant T4*o* occurred within a few minutes, and reproducible single-hit inactivation curves were obtained. Samples (2 ml) in covered 60-mm plastic petri dishes were irradiated at room temperature 15 cm beneath a pair of 15-w white fluorescent bulbs (Champion F15 T8 CW) backed by a white reflector.

Mutants induced in the presence of psoralen and thiopyronin carry the prefixes P and T, respectively.

Spot tests were employed both for mapping (4) and for base analogue reversion tests (7, 9). For mapping, a drop each of both parental phages at 10^9 to 10^{10} per ml was mixed with about 0.5 ml of BB cells and incubated for 10 to 30 min at room temperature. The mixture was then spotted with sterile paper strips onto plates previously poured with KB cells. An amount of lysis exceeding that observed in single-parent control

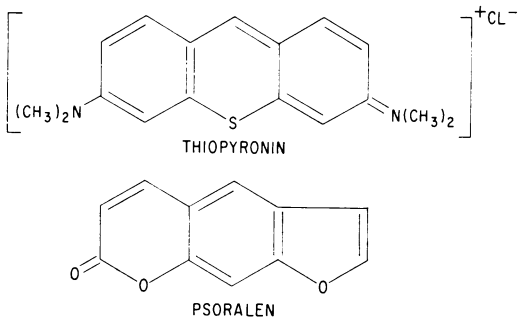

FIG. 1. *Structural formulas of thiopyronin and psoralen. Substitution by nitrogen of the CH group para to the sulfur in thiopyronin yields methylene blue.*

spots indicated recombination. The mutants were first located in segments defined by deletion mutations, and then at sites not further resolved by recombination spot tests. For reversion spot tests, plates were seeded with 2×10^7 to 2×10^8 mutant phages, 1.5×10^8 log-phase KB cells, and 5×10^6 log-phase B cells. After 5 to 10 min at room temperature, the plates were spotted with drops of 2-aminopurine at 10 mg/ml and 5-bromouracil at 2.5 mg/ml. After about 1 hr, when the drops were fully absorbed into the agar, the plates were incubated overnight at 37 C. Unspotted regions of the plates constitute the background controls, and a grouping of plaques around any of the spots indicates induced reversion.

The quantitative base analogue and proflavine reversion tests have been described previously (5, 9), and consist of growth in liquid culture, with or without the mutagen, followed by platings on KB and BB cells. The hydroxylamine reversion test of Freese, Bautz, and Freese (12) was used, except that the exposure was for 4 hr at 44 C, and the corresponding survival frequencies were about 10%.

RESULTS

Kinetics. Under the conditions of irradiation, T4B was inactivated with approximately single-hit kinetics at 0.36 hits per minute in the presence of thiopyronin, and at 0.14 hits per minute in the presence of psoralen. Thiopyronin was therefore approximately 64 times more effective than psoralen per weight unit of concentration, or approximately 110 times more effective on a molar basis. Irradiation in the absence of dyes was not detectably lethal or mutagenic within 60 min. The dyes themselves were neither mutagenic nor lethal in the dark. The mutational background (*r* plaques/total plaques) of the stock was 6.4×10^{-4} (5/7,803), a value very close to several other determinations on the same stock at about the same time. After 33 min of irradiation in the presence of thiopyronin, the mutant frequency was 44.9×10^{-4} (67/14,923). After 60 min of irradiation in the presence of psoralen, the mutant frequency was 45.2×10^{-4} (191/42,189). Assuming linear mutation kinetics, therefore, the mutation rates were 3.3×10^{-4} mutations per lethal hit with thiopyronin, and 4.6×10^{-4} mutations per lethal hit with psoralen.

T4B and T4*o*C2, an osmotically resistant mutant, were inactivated in the presence of either dye at the same rate. Multiplicity reactivation was often observed when assaying samples which had received large doses of irradiation, but was not further investigated.

Collection and phenotypic classification of mutants. T4*r* mutants identified from their plaque morphology on B cells consist of: *r*I mutants, which exhibit the *r* phenotype on all host cells; *r*II mutants, which are unable to grow on KB cells and other λ-lysogens, but which produce r^+-type plaques on BB cells; and a heterogeneous collection of mutants sometimes defined as *r*III (3), which produce r^+-type plaques on BB cells, and r^+ or *minute* plaques on KB cells. Most mutants with the *r*III phenotype have been found in this laboratory to be either very leaky or else rapidly reverting *r*II mutants. Many of these leaky *r*II mutants, as well as (partial) revertants from rapidly reverting *r*II mutants, are also temperature sensitive, and no longer plate on KB cells at 42 C. A few of the phenotypically *r*III mutants map at a locus distant both from the *r*I and from the *r*II regions.

The phenotypic classification of the mutants is summarized in Table 1. From the kinetic data, it is estimated that about 14% of the mutants were of spontaneous origin. Considerable numbers of *r*I and temperature-sensitive *r*II mutants are observed; all of the thiopyronin (T) mutants and at least 21 of 30 of the psoralen (P) mutants in the "*ts* or HR" category were, in fact, temperature-sensitive.

As is commonly observed in bacteriophage

TABLE 1. *Classification of photodynamically induced r mutants*

Type of mutant[a]	Thiopyronin		Psoralen	
	No.	Per cent	No.	Per cent
*r*I	26	27	96	47
*r*II	36	38	73	36
*r*II (ts or HR)	29	31	35	17
*r*III	4	4	0	0
Total	95		204	

[a] The *r*III mutants were defined phenotypically, but were not mapped; *ts* = temperature-sensitive, and HR = rapidly reverting.

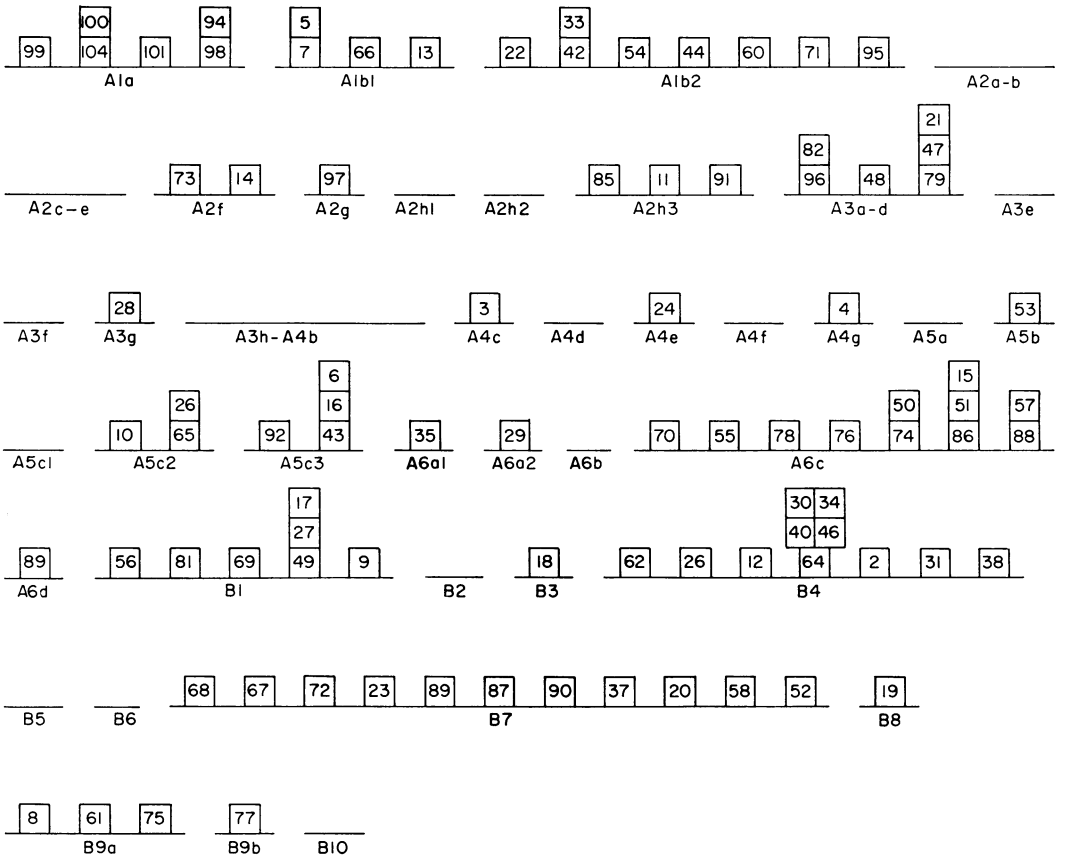

FIG. 2. *Map of rII mutants photodynamically induced in the presence of psoralen. Horizontal lines represent cistron segments defined by deletions (4). Boxes represent independently isolated mutants, whose sites within a segment are unordered. Numbers within boxes identify individual mutants.*

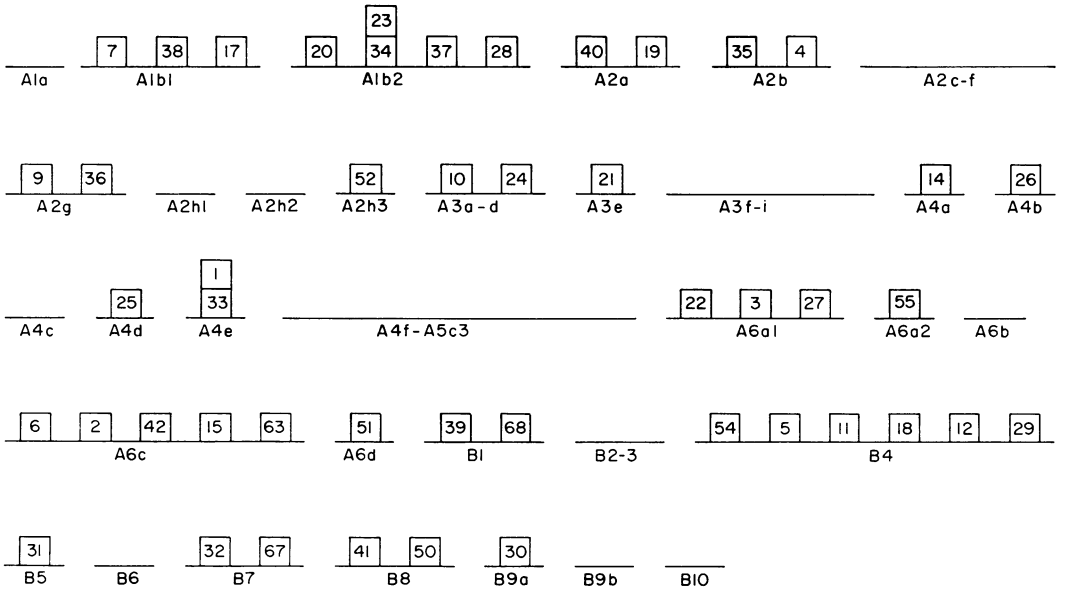

FIG. 3. *Map of rII mutants photodynamically induced in the presence of thiopyronin.*

T4, many of the *r* mutants were first observed in mottled plaques. These contain both *r* and r^+ phages in approximately equal numbers, and arise from mutational heterozygotes. Approximately 55% of the thiopyronin mutants and 54% of the psoralen mutants arose in observably mottled plaques. These percentages are underestimates, however, since assay conditions were chosen to maximize the detection of *r* mutants, and the same conditions also tend to obscure mottling in plaques.

Genetic analyses. All of the nonleaky *r*II mutants, as well as a few temperature-sensitive mutants whose growth in KB cells was nearly completely blocked at 42 C, were mapped with spot tests. Deletions were rare: only four were observed in the set of mapped mutants, and these may have arisen from the spontaneous background; about 8% of spontaneous mutants may be deletions (3). The maps of point mutants are shown in Fig. 2 and 3. These maps may be compared with Benzer's map of spontaneous *r*II mutants (4). He observed that fully half of the spontaneous mutants occupied two prominent "hot spots" (highly mutable sites), located in segments A6c and B4, respectively. Representatives of these sites have in general proved to be sign mutants, e.g., they are reverted by proflavine (Drake, *unpublished data*). These hot spots do not appear in the map of the thiopyronin mutants. They appear in the map of the psoralen mutants, but probably only as representatives of the spontaneous background (expected number = 14% background × 91 mapped *r*II mutants × 50% in the two hot spots = 6.4; observed number = 7 or 8). In general, the mutants do not show clustering to the same extent as do spontaneous and base analogue-induced mutants (4).

Mapped *r*II mutants with sufficiently low reversion frequencies were then subjected to reversion analysis, following the scheme outlined in Fig. 4. The logical basis for this scheme will be summarized in the Discussion. The mutants fell into three categories: those induced to revert by base analogues, those induced to revert by proflavine, and those reverted by neither agent.

The mutants induced to revert with base analogues are described in Table 2. They are considered to contain single base pair substitutions. They may be further subdivided by their responses to hydroxylamine. The responding mutants are considered to contain guanine-hydroxymethylcytosine base pairs at the reactive site. However, the reactive site need not be the originally mutated site, but may instead be a suppressor site. If the revertant arises by intracistronic suppression, its plaque morphology on

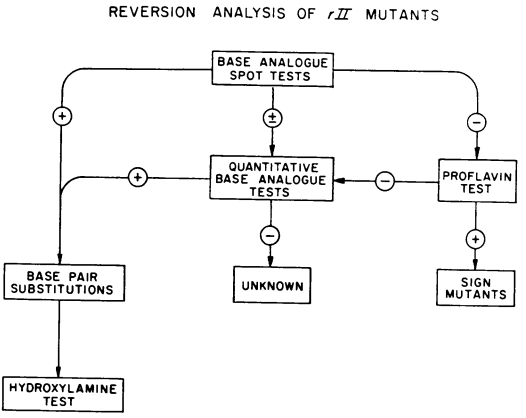

FIG. 4. *Flow diagram for reversion analysis of rII mutants.*

B cells is very often different from the wild type (5). Whenever a hydroxylamine-induced reversion was detected, therefore, a number of revertants were picked from KB cells and restreaked onto B cells. The fraction producing plaques distinguishable from the wild type is tabulated in the "HA-FR"column of Table 2. Since these phages were very heavily mutagenized by the hydroxylamine treatment (several per cent, for instance, containing secondary *r*I mutations), the frequency of false revertants may be overestimated.

The *r*II mutants which were induced to revert with proflavine are described in Table 3. These mutants uniformly failed to respond to base analogues. They are considered to contain additions or deletions of small numbers of base pairs.

The mutants which were reverted neither with base analogues nor with proflavine are described in Table 4. These mutants all reverted spontaneously.

Table 5 summarizes the classification of the mutants on the basis of reversion analyses. The distributions of the P and the T mutants among the reversion categories were homogeneous after grouping the HA(−) and the HA(?) responses (chi square = 0.63, $P \approx 0.9$), and the two collections were therefore pooled. Half (51%) of the mutants contain base pair substitutions, 22% contain sign mutations, and 27% remain enigmatic.

DISCUSSION

Mutation rates. Despite their considerably different molar efficiencies, the specific mutagenicities of thiopyronin and psoralen (expressed as mutational events per lethal event) are essentially identical. Approximate values have also been reported for the survival and the corresponding induced mutant frequency of phage T4 irradiated

TABLE 2. *Mutants revertible by base analogues*

Mutant	Spot tests		Quantitative tests[a]					Mutant base pair
	2AP	5BU	SPON	2AP	5BU	HA	HA-FR	
T7	+	−	180			6,200	0/10	GC
T15	+	−	330			11,000	0/10	GC
T26	−	+	3,200			3,900,000	7/12	GC
T27	+	+	370			12,500	4/14	GC
T28	+	+	9.9			1,600,000		GC
T36	+	+	7.3			930	5/10	GC
T37	+	+	0.9			365	4/20	GC
T42	+	−	100			9,800	2/7	GC
T1	+	+	1.1			3.4		AT
T4	+	−	6.5			≤0.1		AT
T9	+	−	0.03					AT
T10	−	−	0.1	310	0.02	≤0.3		AT
T14	+	±	0.6			3.0		AT
T18	+	±	1.2			≤0.4		AT
T19	+	±	2.2			≤0.5		AT
T24	+	−	1.0			0.3		AT
T25	+	+	2.1			4.1		AT
T38	+	+	4.7			1.1		AT
T41	+	+	8.4			1.0		AT
P4	+	−	0.2			1.6	1/5	GC
P5	+	+	44			5,600	7/20	GC
P6	+	+	0.2			580	2/10	GC
P9	+	+	3.6			39	3/10	GC
P10	−	−	1.2	380	1.3	160	2/10	GC
P16	+	+	0.6			13		GC
P21	+	−	345			76,000	0/8	GC
P23	+	+	2.8			9,700	3/10	GC
P28	+	+	17			770	5/16	GC
P42	−	−	55	6,400	575	2,500	6/10	GC
P44	+	−	960			103,000	1/10	GC
P48	+	+	2.6			1,400	1/10	GC
P52	+	−	4,900			30,000	15/20	GC
P61	−	−	0.7	35	0.2	40	0/7	GC
P66	+	+	175			5,000	2/10	GC
P71	−	−	1.5	170	550	9,200	2/10	GC
P72	−	−	2.0	7.0	250	1,500	0/10	GC
P79	+	±	0.6			130	5/10	GC
P54	−	−	2.5	450	600			?
P60	−	−	0.2	210	150	2.0	4/5	?
P73	−	+	≤0.03			≤0.1		?
P12	+	−	0.6			1.2		AT
P13	+	−	0.1			≤0.7		AT
P17	+	+	3.0			4.4		AT
P22	+	±	0.16			≤0.13		AT
P24	+	−	0.1			0.1		AT
P27	+	+	5.6			9.7		AT
P43	+	±	11			15		AT
P47	+	+	0.1			≤0.03		AT
P49	+	−	4.3			≤6.7		AT
P74	+	+	6,700			6,300		AT
P75	+	+	0.1			≤0.15		AT
P82	+	±	310			360		AT
P85	+	−	10			37		AT
P89	+	+	770			590		AT

[a] Revertants per 10^7 phage. SPON = spontaneous. HA-FR = fraction of false revertants in HA test.

TABLE 3. *Mutants revertible by proflavine*[a]

Mutant	SPON	Proflavine
T2	4.1	160
T3	3.0	110
T5	6.7	3,400
T6	4.5	270
T12	2.4	110
T31	0.3	4.0
T40	52	8,500
P3	5.6	43
P30	5.0	510
P34	8.4	420
P40	8.2	280
P46	4.8	125
P50	4.5	77
P51	3.2	220
P53	0.04	0.65
P57	2.9	110
P64	4.8	120
P65	0.1	100
P68	0.2	1.6
P69	0.1	95
P86	4.6	160
P87	0.4	1.4
P88	4.5	140

[a] Revertants per 10^7 phage. SPON = spontaneous.

TABLE 4. *Nonresponding mutants*

Mutant	SPON[a]
T11	240
T17	0.05
T20	1.4
T22	54
T23	150
T29	3,200
T30	0.07
T32	14
T34	1.5
P7	0.08
P8	0.1
P14	0.8
P15	2,700
P18	8
P19	0.1
P20	13
P26	2,200
P29	210
P33	0.8
P35	53
P37	0.1
P58	0.3
P62	1,700
P67	510
P76	96
P77	0.07
P81	180
P90	0.2

[a] Spontaneous revertants per 10^7 phage.

with white light in the presence of proflavine (15), from which a mutation rate of 4.3×10^{-4} mutations per lethal hit may be calculated. These dye-light combinations may therefore be classified as weak mutagens, together with ultraviolet and other agents which produce 10^{-4} to 4×10^{-4} mutations per lethal hit in T4 (see reference 7 for a comparison of several mutagens tested on page T4).

The T and P *r*II mutants arose by induction and not by selection. This is clear from a comparison, both of their map distributions and of their reversion responses, with collections of spontaneous mutants characterized by Freese (10) and by Benzer (4). Thus, at least half of the induced T and P mutants contain base pair substitutions, compared with 14% of spontaneous T4*r*II mutants, and the two largest "hot spots" in the map distribution of spontaneous mutants fail to appear in the map distribution of T and P mutants.

Although Table 1 revealed some possible differences between T and P mutants, the much more detailed analysis summarized in Table 5 did not reveal significant differences. The two groups of mutants will, therefore, be considered together in the following discussion.

Reversion analysis. The application and reliability of methods for determining the nature of mutational lesions by measuring the induced reversion of mutants by specific mutagens has been reviewed several times (8, 11, 13). With very rare exceptions, T4*r*II mutants are induced to revert by base analogues, by proflavine, or by neither agent, but not by both agents (Fig. 4).

TABLE 5. *Summary of reversion responses of mutants*

Base analogues	Proflavine	Type of mutant	HA	No.	Per cent
+	(−)[a]	Base pair substitution	+	26	25
			−	25	24
			?	3	3
−	+	Sign mutation		23	22
−	−	Unknown		28	27

[a] Mostly not tested.

Mutants induced to revert by proflavine are considered to contain deletions or additions of small numbers of base pairs, and are called sign mutants or reading frame shift mutants. Only

22% of the P and T mutants responded to proflavine. (Only a few of the mutants in Table 2 were also tested with proflavine, but none of those tested responded.) If most of the 86% of spontaneous *r*II mutants reported by Freese (10) *not* to respond to base analogues are in fact sign mutants, then the 14% of T and P mutants arising from the spontaneous background would have contributed about 12.6 sign mutants to the total. The observed number of 23 is significantly greater (chi square = 5.5, $P \approx 0.02$), but the numbers of mutants involved is small and depends further upon our estimate of the spontaneous background. We can therefore only state that sign mutations are induced either very weakly, or else not at all, under the chosen conditions.

Mutants induced to revert by base analogues are considered to contain single base pair substitutions. About half of the T and P *r*II mutants, or 58% when corrected for the spontaneous background as described above, did in fact respond to base analogues. Two other characteristics of the total collection of *r* mutants also indicate that many contain base pair substitutions. Many *r*I mutants were induced (Table 1); *r*I mutants are induced by base analogues, but not by proflavine (Drake, *unpublished data*). Many of the induced *r*II mutants could not be subjected to reversion analysis because they were too leaky; these also presumably contained base pair substitutions, since sign mutations have catastrophic effects upon polypeptide synthesis. Leaky *r*II mutants, on the other hand, commonly arise after base analogue and hydroxylamine mutagenesis (Drake, *unpublished data*).

Among the mutants which responded to base analogues, nearly half also responded to the cystosine-specific agent hydroxylamine. If our classification of revertants as wild type is correct, these mutants contain guanine-hydroxymethylcytosine base pairs at the mutated sites.

Mutants which are induced to revert neither with base analogues, nor with proflavine, cannot be satisfactorily classified. About 27% of the *r*II mutants studied here fell into this category. It is unlikely that these mutants are either sign mutations or transitions, since proflavine-induced mutants are invariably induced to revert with proflavine, and base analogue-induced (transition) mutants are uniformly induced to revert with base analogues. It also seems unlikely that they contain multiple mutational lesions, since they revert at normal rates. The only remaining elementary form of "point" mutational lesion is the transversion. Unfortunately, however, we do not have a specific test for transversions.

It is also probable that many of the mutants which did respond to base analogues also contain transversions. Thus, some of the base analogue-induced reversions probably failed to restore the wild-type DNA configuration. Amino acid exchanges frequently fail to cause loss of function [consider, for instance, hemoglobin (14)].

Ritchie (15, 16) described some T4*r*II mutants photodynamically induced in the presence of proflavine, which he concluded were base analogue types (transitions). Three, however, responded neither to a base analogue nor to proflavine. These mutants, therefore, closely resemble T and P *r*II mutants.

Photochemical mechanisms. A variety of dyes sensitize viruses to photodynamic inactivation and mutation, but chemical descriptions of their effects upon DNA are sparse and sometimes inconsistent. Methylene blue, thiopyronin, lumichrome, and acridines may catalyze the oxidative destruction of guanine, and to a much lesser extent the destruction of thymine and perhaps even cytosine (2, 17–21). Psoralen, on the other hand, is active in the absence of oxygen, but has not yet been shown to mediate the destruction either of purines or of pyrimidines (21).

Since we must look to reactions which are capable of generating transversions, two classes of photoproducts will be of greatest interest: those leading to complete depurination, with a resulting possibility of inserting a base at random during DNA replication or repair; and those leading to partial purine degradation, forming a product capable of associating with a purine rather than a pyrimidine during an ensuing round of DNA replication. Although a variety of guanine degradation products have been detected, most have not been identified. An interesting exception is parabanic acid (20).

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