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Mutagenesis with light and proflavine in phage T4

II. PROPERTIES OF THE MUTANTS

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The adsorption by phage particles of a variety of dyes, particularly of the acridine and thiazine classes, renders them sensitive to inactivation by visible light (Welsh & Adams, 1954; Yamomoto, 1958; Kaufman & Hiatt, 1959). In an earlier paper evidence was presented showing that the photodynamic action of the acridine dye proflavine was mutagenic in phage T4 (Ritchie, 1964). Among the phage particles surviving photodynamic inactivation there was an enhanced frequency of the plaque-type mutation from wild-type (r^+) to rapid lysis (r) when compared with the frequency arising spontaneously. Surprisingly, control experiments, in which phage particles were treated with *either* proflavine or visible light, also showed an increased frequency of this mutation. The total mutation frequency from these control experiments was about half of that obtained among the survivors of photodynamic inactivation. Thus not all of the mutants observed among the survivors of photodynamic inactivation could be accounted for by the separate effects of light and proflavine. On this evidence it was concluded that the photodynamic action of proflavine was a mutagenic process. Supporting this conclusion is the evidence that the photodynamic action of acridine orange can induce mutations in *Escherichia coli* (Webb & Kubitschek, 1963; Nakai & Saeki, 1964).

Since the treatment of phage particles with either light or proflavine causes no inactivation there is little question of selection causing the enhanced mutation frequency. In the case of photodynamic inactivation selection has been ruled out by showing that r^+ and r phages have the same photodynamic sensitivity (Ritchie, unpublished results).

A preliminary study of 12 *r*II mutants isolated from the survivors of photodynamic inactivation revealed that half reverted to wild-type when exposed to the base analogue mutagen 5-bromodeoxyuridine, the remainder were reverted by growth in the presence of acridine (Ritchie, 1964). Usually, however, mutagens induce specifically either base analogue-revertible or acridine-revertible mutations and the two types are thought to arise by distinct and mutually exclusive mechanisms (Brenner *et al.*, 1961). Thus it seems unlikely that photodynamic action is responsible for the induction of both mutational types. One possible explanation is that mutants arising from the survivors of photodynamic inactivation consist not only of those induced by the photodynamic action of proflavine but also of those arising from the separate effects of light and proflavine, both of which have been shown to be mutagenic.

The results presented here tend to support such an explanation for the mixed nature of r mutants isolated from the survivors of the photodynamic action of proflavine.

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MATERIALS AND METHODS

Induction of mutants

A suspension of phage T4r^+ (Benzer, 1955) at 10^{10} particles/ml. in buffer was divided into two aliquots, one of which received 25 μg./ml. of proflavine hemisulphate (British Drug Houses). Both samples were incubated in darkness at 37°C. for 1 hour and then diluted 100-fold into buffer. One aliquot of the proflavine-treated phage was immediately plated on *E. coli* strain B. Mutants arising from this treatment are called P mutants. A second aliquot was irradiated for 5 min. with visible light and the surviving phage, about 1%, were assayed on *E. coli* B. Mutants arising from the photodynamic action of proflavine are designated as PL mutants. The phage incubated without proflavine were also exposed to visible light for 5 min. and plated on *E. coli* B. Mutants induced by light alone are given the prefix L.

The phage samples were irradiated at a distance of 51 cm. from the light source. The light source and conditions of irradiation have been described previously (Symonds & McCloy, 1958; Ritchie, 1964). The buffer used is the adsorption medium described by Hershey & Chase (1952).

RESULTS AND DISCUSSION

The P, L and PL *r* mutants were first classified into the three phenotypic groups, *r*I, *r*II, and *r*III (Benzer, 1955). The result is shown in Table 1. Of the P and L *r* mutants the great majority are *r*II mutants, about 75% and 90% respectively. Of the PL *r* mutants about half are *r*II, the remainder being equally divided between the *r*I and *r*III groups.

Table 1. *Distribution of P, L, and PL* r *mutants among the* r *groups*

	P mutants	L mutants	PL mutants
Number tested	16	18	71
Number of *r*I mutants	1	1	16
Number of *r*II mutants	12	16	35
Number of *r*III mutants	3	1	20

The *r* mutants were isolated by stabbing the plaque with a needle and purified from any contaminating r^+ phage by replating on *E. coli* strain B and selecting a well isolated *r* plaque. The mutants were classified by their phenotypes on *E. coli* strains B, BB (McFall & Stent, 1958) and K(λ) according to the scheme of Benzer (1955).

The reason for the grossly unequal distribution of the P and L *r* mutants is unknown. From the available data it appears that for a variety of mutagens the proportion of *r*II mutants ranges from 35% to 60% with no apparent correlation between the types of mutagen (Benzer, 1955, 1957; Benzer & Freese, 1958; Brenner, Benzer & Barnett, 1958; Freese, 1959*a*). As will be shown below, exposure of phage particles to either light or proflavine results in mutations primarily of the acridine-revertible type. There is good reason to believe that such mutations are often lethal (Brenner *et al.*, 1961). Thus one might imagine that the *r*I and *r*III genes are more susceptible than the *r*II region to lethal mutagenesis with P and L mutagens. However, this is difficult to reconcile with the fact that *r* mutants arising spontaneously or from growth of phage-infected cells in acridine, which are mainly acridine-revertible, contain *r*I and *r*III mutants to the extent of 35% to 50% (Benzer, 1955, 1957; Benzer & Freese, 1958; Brenner, Benzer & Barnett, 1958). At present the results are insufficient to warrant a conclusion.

Information concerning the mutagenic processes inducing P, L and PL mutations was obtained from a study of the revertibility of the respective *r*II mutants to wild-type.

The detection of the reversion depends on the fact that r^+ phage are able to grow on strains of *E. coli* lysogenic for phage λ, whereas *r*II mutants are characteristically inhibited (Benzer, 1955).

Of the 16 L, 12 P and 30 PL *r*II mutants tested all reverted spontaneously to wild-type generally with a frequency of $1/10^7$ to $1/10^8$. However, a small number of P and L mutants had very low rates, of the order $1/10^9$ or less and a few from all three classes were very unstable with up to 1% of the phages plating on K(λ). Thus all three processes induce primarily point mutations.

To afford some insight into the molecular nature of the mutational events responsible for the P, L and PL mutants the respective *r*II mutants were examined for their ability to be reverted by the base analogue mutagens 5-bromodeoxyuridine (BD) and 2-aminopurine (AP) and by the acridine, proflavine (PF). BD and AP are thought to induce base pair transitions (Freese, 1959*b*). Acridine mutagenesis probably results from base pair additions and deletions which displace the reading of the DNA code (Crick *et al.*, 1961). It should be pointed out that this type of acridine mutagenesis occurs when the acridine is present during the growth of phage-infected cells and should not be confused with the *in vitro* P and PL mechanisms involving the treatment of free phage particles.

Table 2 shows the reversion properties of the P, L and PL *r*II mutants when grown in the presence of BD, AP, and PF.

Table 2. *Induced reversion properties of P, L and PL* r*II mutants*

P mutants				L mutants				PL mutants			
	Mutagen				Mutagen				Mutagen		
No. of mutants	BD	AP	PF	No. of mutants	BD	AP	PF	No. of mutants	BD	AP	PF
6	−	−	+	11	−	−	+	4	−	−	+
1	+	+	−	1	+	+	−	4	−	○	+
								2	+	+	−
								3	+	○	−

Induction of reverse mutations with proflavine. An aerated culture of *E. coli* B was grown at 37°C. to a titre of 2×10^8 cells/ml. in M9 medium supplemented with l-tryptophane (0·01 g./l.) and l-glutamic acid (0·02 g./l.). After centrifugation the cells were resuspended in an equal volume of fresh medium to which 4 μg./ml. proflavine (PF) was added. About 2 min. later the bacteria were infected with 1–2 phage/cell of the *r*II mutant and incubated at 37°C. After 30 min. the infected cells were assayed on *E. coli* strains B and K(λ). A duplicate control culture, infected in the absence of PF, was incubated for 10 min. before plating on the same indicator strains. The titre on K(λ) divided by the titre on B determines the fraction of infected cells containing r^+ phage. The K/B ratio after PF treatment divided by the K/B ratio of the control is the induction ratio. An induction ratio of 3 or higher was scored as positive induction. Increases of up to 500-fold were observed. The decision to plate infected cells rather than phage progeny was based on the finding that, for a given mutant, the induction ratio was the same in both cases. Incubation of the PF-treated complexes for 30 min., which is beyond the normal latent period, was made possible by the reversible inhibition of lysis produced by acridines such as PF (Ritchie, unpublished results) and 9-amino acridine (Susman, Piechowski & Ritchie, 1965).

Induction of reverse mutations with BD and AP. This is the method described by Freese (1959*a*). A sample of the mutant was plated on a mixture of K(λ), 2×10^8 cells and B, 1×10^7 cells. When the top layer had set, 0·03 ml. samples of BD (5 mg./ml.) and AP (2 mg./ml.) were placed on either side of the plate. The plate was refrigerated at 4°C. for 2 hours to allow diffusion of the mutagen and then incubated at 37°C. An increase in the number of plaques in the region of the mutagen was scored as positive induction.

+ = positive induction; − = no induction; ○ = not tested.

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Of the 7 P mutants, 6 were reverted by PF but not by BD or AP. Thus exposure of T4 phage particles to PF induces mutations primarily of the acridine type. The one mutant reverted by BD and AP but not by PF could be of spontaneous origin since the frequency of P *r* mutants is only 10-fold higher than the spontaneous rate (Ritchie, 1964). During incubation of phage particles with PF the dye penetrates the phage head membrane and upon dilution from the PF medium there is a gradual release, extending for several hours, of the dye from the sites of photodynamic action (Ritchie, unpublished results). Since for the induction of P mutants, phage particles are plated immediately upon removal from PF it seems reasonable to assume that the phage will inject PF into the cells along with their DNA at infection. The situation in these infected cells is then somewhat analogous to phage-infected cells grown in the presence of PF, which is well known to induce mutations of the acridine type (De Mars, 1953; Brenner, Benzer & Barnett, 1958).

Of the 12 L *r*II mutants, 11 were reverted by PF but not by BD or AP. The one base analogue-revertible mutant could be of spontaneous origin for the same reason as the base analogue-revertible P mutant. Therefore, exposure of T4 phage particles to visible light is also a mutagenic process of the acridine type. The nature of this mechanism is unknown, nor is it known if visible light interacts directly with the DNA or induces some other change leading to genetic instability. The possibility that ultra-violet light (UV) and not visible light is the source of L mutations seems unlikely for several reasons; (*a*) the phage samples were irradiated through a 1 cm. thick layer of glass, (*b*) no inactivation results from the light doses used, and (*c*) UV induced *r*II mutants of T4 are equally divided between the base analogue and acridine types (Drake, 1963). However, we have not ruled out possible effects of long wavelength UV.

Approximately 60% of the PL *r*II mutants are of the acridine type, being reverted by PF but not by BD or AP. The other 40% are reverted by BD and, where tested, also by AP. Although Drake (1963) reported that UV induces mutations of both the acridine and base analogue types, generally mutagens specifically produce only one type. For this reason we are inclined to believe that PL mutants are of mixed origin. Specifically, we suggest that the photodynamic action of proflavine (PL) causes mutations of the base analogue type and that the acridine mutants arise by the separate effects of proflavine (P) and light (L). An earlier report (Ritchie, 1964) showed that the sum of the frequencies of P and L *r* mutants is about half of the frequency found among the survivors of photodynamic inactivation. The present work shows that both P and L mutants are of the acridine type and that half of the PL mutants are also of this type.

The conclusion that the photodynamic action of proflavine induces base analogue type mutations receives some support from the observations of Simon & Van Vunakis (1962) that the photodynamic action of methylene blue on free DNA bases selectively destroys guanine and, to a smaller extent, thymine. Assuming that proflavine behaves similarly, then the removal of guanine (or thymine) would allow the substitution of another base to produce a base analogue type mutation.

SUMMARY

In summary, *r*II mutations induced by exposure of T4 phage particles to either visible light or proflavine are acridine revertible. In both cases mutation occurs without inactivation. The *r*II mutations induced by the photodynamic action of proflavine on T4 phage particles are equally divided between the acridine revertible and base analogue revertible classes. It is proposed that photodynamic action induces base analogue mutations and that the acridine revertible category arises from the separate effects of light and proflavine.

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