ACRIFLAVINE RESISTANCE: A BACTERIOPHAGE MUTATION AFFECTING THE UPTAKE OF DYE BY THE INFECTED BACTERIAL CELLS

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The T-even bacteriophages T2, T4, and T6 are unusual among the bacteriophages of *Escherichia coli* in that their growth is considerably more sensitive to acridine dyes than that of the host bacteria.[1, 2] This increased sensitivity is not a property of the free, extracellular, phage particles but of the phage-infected bacterial cells, which in the presence of the dyes produce phage-specific deoxyribonucleic acid (DNA) and proteins but no intact progeny phage.[3–5] The first purpose of this paper is to report a greatly increased uptake of the acridine dye acriflavine by the bacterial cells immediately upon infection with bacteriophages T2, T4, or T6 but not after infection with an acridine-resistant phage, T1.

Acridine-resistant mutants have been isolated from the sensitive wild types of T2,[3, 6] T4,[2, 7] and T6.[2, 8] The growth of these mutants is still sensitive to acridine dye concentrations which do not affect the growth of the uninfected bacteria. There are two unlinked phage genes each conferring partial resistance to acridines[6, 7] and the doubly mutant phage are more resistant than either of the singly mutant phage. In bacterial cells mixedly infected with both sensitive and resistance phage, the entire complex is sensitive to acridines.[6, 9] The second purpose of this paper is to show that the resistant phenotype of mutations in one of the two genes, the *pr* gene in T2[6] and the *ac* gene in T4,[7] derives from a reduced uptake of acridines relative to cells infected with sensitive phage.

Materials and Methods.—Bacterial strains: *E. coli* strain B and its variant BBr (Brenner, personal communication) which is slightly more acridine-resistant than B.

Bacteriophage: T1 strain from this laboratory. T2H wild type and its mutants T2H *pr6*, T2H *q1*, and T2H *pr6q1*.[6] *pr* denotes resistance to the acridine dye proflavine. *q* denotes resistance to the acridine dye quinicrine (see Hessler[6] for the relative sensitivities of the various phage genotypes). T4D and its mutants T4D *ac41* and T4D *q41*.[7] *ac* denotes resistance to acriflavine. T6H[10] and its mutants[8] T6H *ac3*, T6H *ac4*, and T6H *ac5*. T6S[11] is far more acridine-resistant than T6H.

Media: Although the relative order of phage sensitivities is the same on all media tested, the actual level of acridine dyes which infected cells can tolerate depends not only on the genotype of the phage but also on the genotype of the host bacteria[12] and on the medium used. *M9:* Na_2HPO_4 6 gm, KH_2PO_4 15 gm, NaCl 5 gm, NH_4Cl 1 gm, $MgSO_4$ 0.001 *M*, $CaCl_2$ 0.0001 *M*, Difco Bacto gelatin 0.001%, glucose 4 gm, glutamic acid 0.02 gm, water 1 l. *Buffer:* KH_2PO_4 3 gm, Na_2HPO_4 7 gm, NaCl 4 gm, $MgSO_4 \cdot 7HOH$ 0.2 gm, water 1 l.

Chloramphenicol was acquired from Allen and Hanburys, Ltd., London. Acriflavine was from the British Drug Houses, London, and generally used at a concentration of 0.75 μg/ml (see Hessler[6]).

The standard bacteriophage techniques and terms are as described by Adams.[1]

Fluorescence: Acriflavine distribution and concentration were measured by fluorescence in a Locarte Fluorimeter (Locarte Co., London) with 6 mm (internal diameter) cylindrical cells through the courtesy of Dr. Payne of the Child Health Department at Hammersmith Hospital. The exciting light was the 436 mμ line of mercury. The fluorescence was observed through a Chance OGR1 (green) and a Chance OY3 (yellow) filter in combination (Chance-Pilkington

Optical Works, Flintshire, England). This system has a transmission peak about 540 mμ and excludes light below 510 mμ (see Oster[13]).

The basic experimental procedure was to grow *E. coli* BBr in M9 medium to a concentration of about 5×10^8 cells/ml at 37° with aeration by shaking. The cells were infected with phage at a multiplicity of about 5 phage/bacterium (P/B) and acriflavine (0.75 μg/ml) was added. The infected and uninfected cells were then incubated with aeration at room temperature (*ca.* 25°).

From time to time duplicate 10-ml samples were centrifuged at room temperature and the pellets resuspended in 10 ml buffer.

Prior to the onset of visible lysis and the increase in infective centers with resistant phage, the pellets changed in appearance from that of normal bacterial pellets to that of threads of DNA, brightly stained by absorbed acriflavine. Therefore the pellets were resuspended with the aid of a few drops of a deoxyribonuclease (British Drug Houses, London) solution. The acriflavine dye in both the supernatants and the pellets was measured by fluorescence. The total fluorescence from both infected and uninfected cultures decreases with time due to quenching of the acridine dye, presumedly in the pellet.[13, 14] For this reason the fluorescence of the resuspended pellets does not increase as much as the fluorescence of the supernatants decreases. Nevertheless, the phenomena reported here can be seen in both the pellets and the supernatants.

Results.—Effect of phage infection on the uptake of acriflavine by E. coli: Figure 1 shows the uptake of acriflavine by uninfected *E. coli* and by cells infected with T1, T2H, T4D, and T6H. Although there is very little effect on dye uptake of T1 infection, there is a sharp decrease in the fluorescence of the supernatants following infection with T-even bacteriophage paralleled by an increase in the fluorescence of the resuspended pellets. This uptake slows down after 10 min and has essentially stopped after 20 min at 25°.

In this experiment the acridine dye was added to the cells 5 min after the phage. It can be asked whether this rapid uptake of dye can only take place during the first stages of phage infection—that is, is there an early "leaky" stage during which

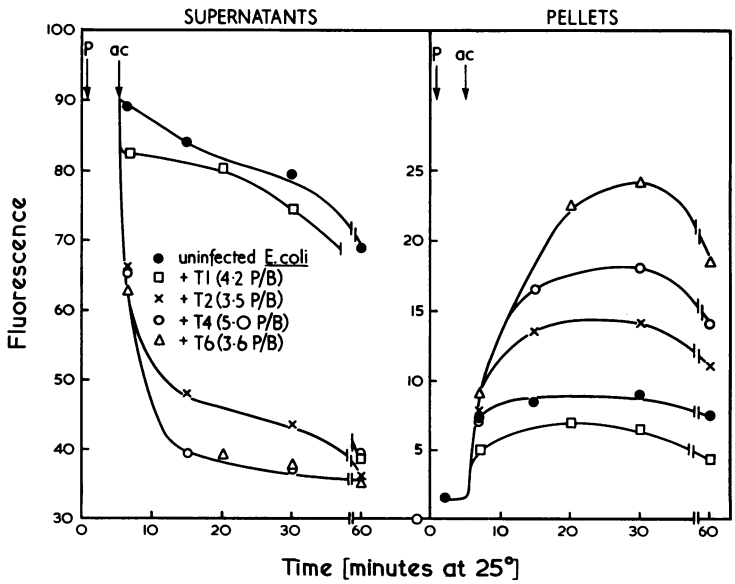

FIG. 1.—Uptake of acriflavine by uninfected *E. coli* and by cells infected with T1, T2H, T4D, and T6H. Tryptophan (10 μg/ml) as an adsorption cofactor and phage added at 0 min; acriflavine added at 5 min at 25°. Bacterial concentration 6.5×10^8/ml. Fluorescence: average of two samples each centrifuged at times indicated. Scale of fluorescence such that 0.75 μg/ml acriflavine in M9 gives 100 units.

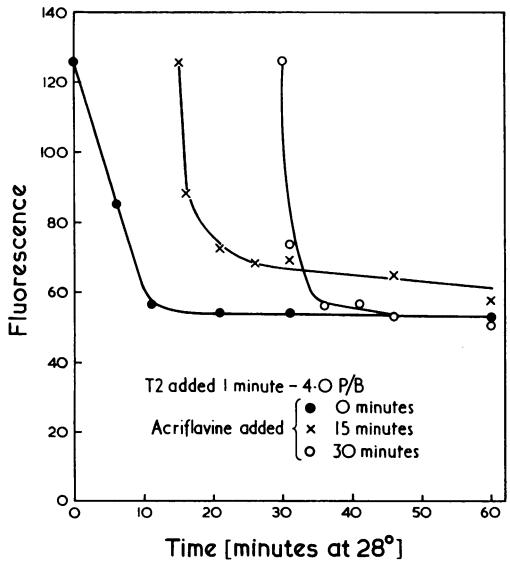

FIG. 2.—Effect of adding acriflavine at various times after the addition of phage. T2 wild-type phage added at 1 min to 8×10^8 bacteria/ml. Acriflavine added at times indicated, and samples removed and centrifuged at room temperature. Fluorescence of the supernatants in arbitrary units.

dye can get into the cells followed by a stage lasting most of the lytic cycle during which the cells are relatively impermeable to acriflavine. Such an early "leaky" stage was found by Doermann[15] and studied by Puck and Lee[16, 17] who followed the release of P^{32} from cells infected with bacteriophage T2. The results of an experiment to test this question are shown in Figure 2. A delay of up to 30 min between the time of adding phage and the time of adding acriflavine has no effect on the uptake of dye. In fact, the kinetics of uptake are more rapid with the late addition of acriflavine, because the cells are already infected and the kinetics of the 0-min curve reflect those of phage adsorption.

Effect of mutations to acridine resistance on the uptake of acriflavine: If the increased acridine sensitivity of T-even infected cells is related to an increased uptake of the dye from the medium, we may ask what is the effect on uptake of the two mutant genes which confer resistance to acridine dyes. Cells infected with T2H phage carrying the mutation *pr* for acridine resistance take up less acriflavine from the medium than comparable cells infected with the wild-type phage or mutant phage T2H *q* (Table 1). The double mutant T2H *prq* is indistinguishable from the single mutant T2H *pr* with regard to acriflavine uptake although it is much more resistant to acriflavine with regard to the production of phage progeny.[6] Although they take up less dye than cells infected with the sensitive phage, cells infected with the *pr* mutant phage have an increased uptake over that of the uninfected cells.

Comparable results to those with wild-type T2H and T2H *pr6* were obtained with wild-type T4D and T4D *ac41* (Fig. 3), and infection with T6S causes less acriflavine uptake than infection with T6H.

There is a surprising result in Figure 3 (also seen in Table 1 and the T1 part of

TABLE 1

UPTAKE OF ACRIFLAVINE BY CELLS INFECTED WITH DIFFERENT T2 MUTANTS

	Fluorescence (arbitrary units) Supernatant	Pellet	Multiplicity, phage/bacterium
Uninfected *E. coli* BBr	112 ± 5	10 ± 0	0
T2H wild type	34 ± 2	24 ± 3	3.5
T2H *pr6*	62 ± 4	9 ± 0	5.3
T2H *q1*	26 ± 5	21 ± 3	4.7
T2H *pr6q1*	65 ± 6	8 ± 1	4.1

Phage added at 0 min to 5×10^8 bacteria/ml. Acriflavine added at 10 min. Temperature 22°. Fluorescence data: the average (± standard deviation) of four samples, two each centrifuged at 16 and 23 min.

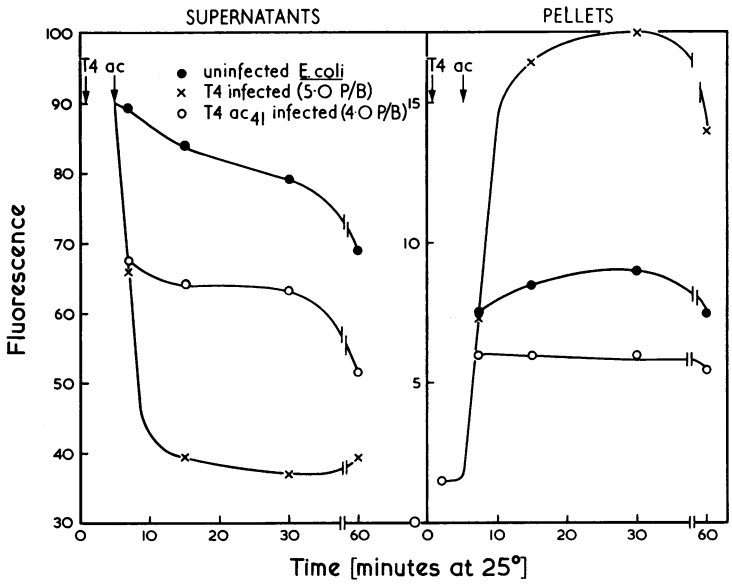

FIG. 3.—Uptake of acriflavine by uninfected *E. coli* and by cells infected with wild-type or mutant T4D. Tryptophan (10 μg/ml) as an adsorption cofactor and phage added at 0 min; acriflavine added at 5 min to 6.5 × 10^8 bacteria/ml. Fluorescence as in Fig. 1.

Fig. 1): the resuspended pellets from the cells infected with acridine-resistant phage had even less fluorescence (i.e., were more completely quenched) than those from the uninfected bacteria. The reason for this result is not known but may reflect a different mode of binding of the dye molecules.

The "physiology" of dye uptake: The rapid kinetics shown in Figures 1, 2, and 3 raise two questions: (1) whether the measured uptake is the result of the adsorption of the phage to the bacterial surface or of something that takes place within the infected cell (from without or from within?), and (2) whether dye uptake is dependent on physiological activities of the infected cell (active or passive?).

Ultraviolet light inactivates phage particles by several mechanisms.[18] Phage with a small number of UV "hits" are damaged mainly in their DNA and retain most of their physiological effects on infected bacteria. After a large number of UV hits, inactivated phage are somewhat similar in their properties to osmotically shocked, DNA-free, phage "ghosts." Figure 4 contains the results of an experiment which included both lightly irradiated and heavily irradiated T2H. The small number of UV hits has no effect on acriflavine uptake, as was expected from the results of Edgar and Epstein,[9] which show that cells infected with such phage are still sensitive to acridine dyes. However, after 98 phage lethal hits (PLH) when the ability of the damaged phage to destroy bacterial nuclei has been largely lost (see Fig. 5 of ref. 18) and when the acridine sensitivity has been largely inactivated,[9] there is a much reduced uptake of acriflavine by the infected cells.

An experiment with osmotically shocked ghosts of T2 showed essentially the same results as in Figure 4. There was a much reduced uptake of acriflavine by the ghost-killed cells but still significantly more uptake than with the uninfected cells.

To approach the question whether the uptake of dye after phage infection is

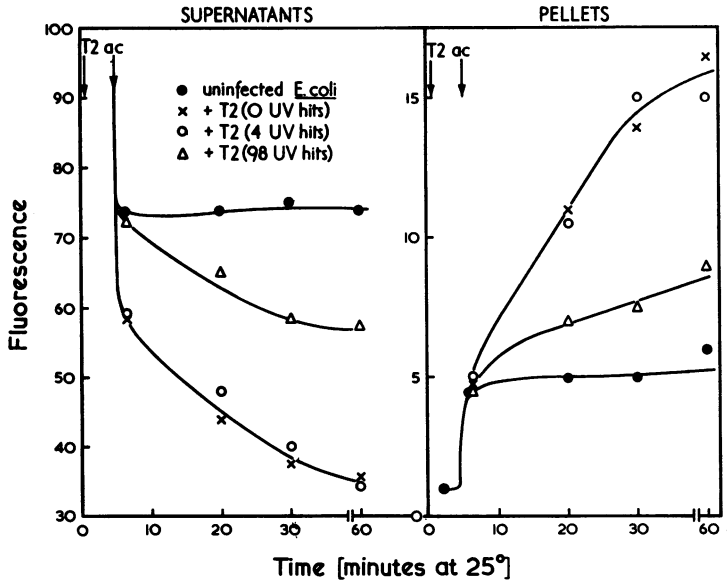

FIG. 4.—The effect of UV-inactivated phage on the uptake of acriflavine by the infected cells. T2 wild type either unirradiated or UV-irradiated (at 254 mμ with a germicidal lamp) were added at 0 min to 4 × 10^8 bacteria/ml. Acriflavine added at 5 min, and samples removed and centrifuged as indicated. The 98-hit phage, added at a total multiplicity of 3.4 P/B, left bacterial survivors indicating an average of 2.4 killing particles/bacterium. Fluorescence as in Fig. 1.

active or passive, a variety of metabolic inhibitors was used. These experiments are complicated by the finding that, in the uninfected bacteria, the *exclusion* of acriflavine is an energy-dependent process. For example, uninfected bacteria incubated with acriflavine at 4° or in the presence of *M*/100 KCN take up somewhat more dye than similar bacteria incubated at 23° or 37° without inhibitors.

Chloramphenicol when added prior to phage infection suppresses the production of both phage-related proteins and DNA.[19, 20] Chloramphenicol partially prevents the phage-directed uptake of acriflavine (Fig. 5). One function turned off by chloramphenicol is that of the *pr* gene, since in the presence of the antibiotic there is no difference in dye uptake between cells infected with the sensitive and resistant phage. These results were anticipated by Hessler (personal communication) who finds that chloramphenicol prevents the proflavine-photosensitization of vegetative T2.

Similar results to those with 25 μg/ml chloramphenicol were obtained with 200 μg/ml chloramphenicol or *M*/100 KCN. These latter experiments are not as unambiguous as that in Figure 5, since KCN or the greater concentration of chloramphenicol cause increases in dye uptake by uninfected *E. coli*. Fluorouracil (10 μg/ml 5-fluorouracil plus 20 μg/ml thymidine)[21] had little, if any, effect on acridine uptake.

Discussion.—The results of the experiments in this paper can be explained most easily in terms of phage-induced changes at the bacterial surface (wall and membrane).

The *pr* gene in T2 and the *ac* gene in T4 control an early, energy-dependent change in the surface of the infected bacteria, which results in an increased uptake of

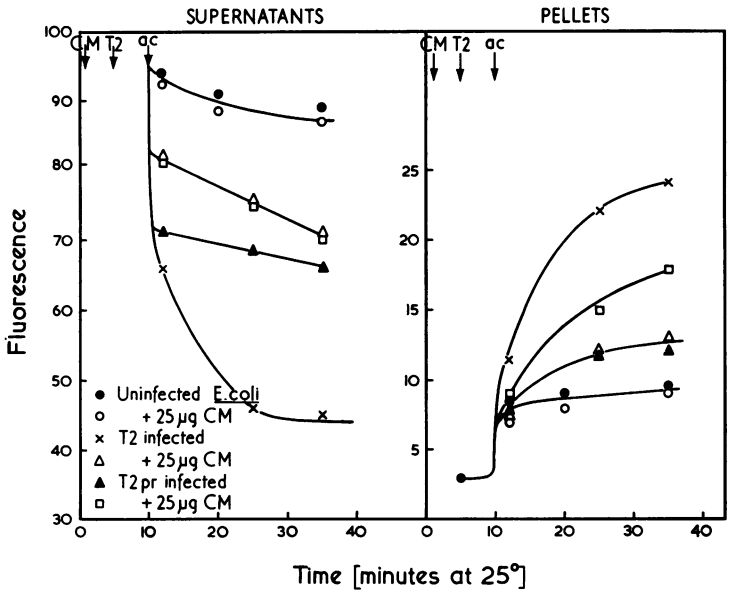

FIG. 5.—The effect of chloramphenicol on the uptake of acriflavine by uninfected and infected *E. coli*. Bacterial concentration 7×10^8. Chloramphenicol (25 μg/ml) added at 0 min; phage T2 (2.8 P/B) or T2 *pr6* (3.4 P/B) added at 5 min; acriflavine added at 10 min. Fluorescence as in Fig. 1.

acriflavine following infection with the wild-type (sensitive) phage. The functioning of these genes requires protein synthesis in the infected cells. The increased dye uptake may reflect either a change in a structural element of the bacterial surface which after phage infection has an increased binding capacity for acridines (see, e.g., refs. 22 and 23; this may be similar to the somatic antigens[24] which are induced by lysogenic phage even during lytic infections) or may be due to a phage-induced acridine "pump" which carries the dye into the cells where it is fixed by receptors which are similar in both sensitive and resistant infected cells. The acridine pump may be a phage-controlled alternative to an essential bacterial (cation) pump which is destroyed during infection. With the wild-type phage, the pump would accumulate acridines as well as its normal substance; the resistant *pr* mutant would induce a pump which can discriminate between the normal cation and the dye. That the *pr* mutant is still functional is suggested by the slight reversal by chloramphenicol of acridine uptake by T2 *pr* infected cells. The normal function of this hypothetical pump is under study.

In addition to the *pr* gene-controlled acriflavine uptake, there is an increased dye uptake caused by the adsorption of the phage particle onto the bacterial surface. It does not require protein synthesis and can equally well be brought about by DNA-free phage ghosts or UV-killed phage. This increased uptake is also seen with cells infected with the resistant *pr* mutant phage.

The inhibitory effect of acridines on phage-infected cells occurs late in the course of infection[2, 3, 5] since phage-related structures, DNA and proteins, are produced in the presence of acridine dyes, but mature phage particles are not assembled. The present experiments do not provide an answer to the site of acridine action

but show an early, phage-directed effect on the uptake of dye by infected cells before significant amounts of phage precursor materials have begun to appear. Therefore studies of the *pr* gene probably will not provide information about the assembly of phage particles (as hoped by Kellenberger[5] and Hessler[6]). The mode of action of the *q* gene remains unknown, as does the physical-chemical basis of the ghost-induced and *pr* gene-controlled changes.

Phenomena similar in many respects to those described in this paper were studied 20–30 years ago with protozoans. Hawking[25] measured the uptake of acriflavine by sensitive and resistant trypanosomes and was able to show that the sensitive cells absorbed far more dye than the resistant cells. Marshall and Dearborn[26] found much higher concentrations of quinicrine inside red blood cells infected with malarial parasites than in uninfected red blood cells.

Summary.—Experiments on the uptake of acriflavine by uninfected *E. coli* cells and by cells infected with acridine-sensitive or -resistant bacteriophage show that (1) sensitivity to acriflavine is related to a greatly increased uptake of dye by infected cells; (2) one of the two mutations to acridine resistance in bacteriophage T2 and T4 results in a reduced uptake of dye; and (3) the process responsible for increased acriflavine uptake is dependent on phage-directed protein synthesis in the infected cells.

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