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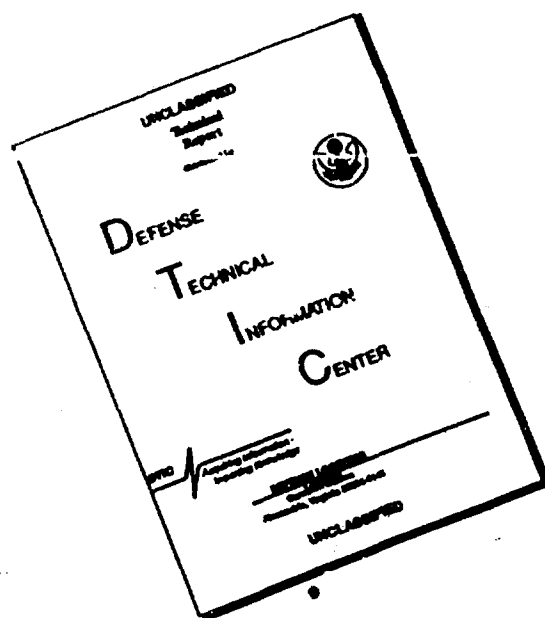
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ACRIDINE ORANGE RESISTANT MUTANT OF BACTERIOPHAGE λ

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It is known that acridine dyes can suppress intracellular development of many viruses [1-4]. Although the detailed mechanism of this suppression is known, it may be assumed that some late stages of viral particle synthesis are blocked [4]. Resistant mutants [4, 5] have been found of many bacteriophages sensitive to acridine dyes during intracellular reproduction. Genetic analysis reveals that resistance to various dyes is related to the function of different genes [5].

Viruses whose protein membranes are permeable to small molecules are inactivated when the virus-dye mixture is illuminated with visible light, but they retain their viability in the dark [6, 7]. Under specific experimental conditions we were able to observe inactivation of extracellular bacteriophage λ by acridine orange, and it occurred not only in the light but also in the dark. Evidence was obtained that dark inactivation is the result of interaction between the dye and the bacteriophage DNA.

In the present work we describe isolation and some properties of a phage λ mutant resistant extracellularly to acridine orange. An effort was made to determine to what the mutant's dye resistance could be related.

Material and Method

In this study we used the following strains of *Escherichia coli*: *E. coli* K12 λ -- the source of phage, *E. coli* K12S -- a variant of *E. coli* K12 nonlysogenic with respect to λ , *E. coli* C -- an indicator strain for phage λ . All of the strains were obtained from the collection of the Laboratory of Genetics of Bacteria and Bacteriophage, Institute of

Epidemiology and Microbiology imeni N.F. Gumaleva, USSR Academy of Medical Sciences. Bacteriophage $\lambda(k)$ was obtained by the method of ultraviolet induction of *E. coli* K12(λ). Phage $\lambda(c)$ was obtained by virulent replication of $\lambda(k)$ on *E. coli* C. The acridine orange resistant mutant of phage λ , $\lambda(a)$, was isolated from a population of $\lambda(k)$ as described below. Concentrates of phage $\lambda(a)$ and $\lambda(c)$ were obtained by the same method and under identical conditions. The method of obtaining pure preparations of phage $\lambda(c)$ was described by one of the present authors [8]. The phage (a) preparations used for spectral readings were obtained in the same manner.

For the experiments on inactivation of extracellular bacteriophage by the dye a phage suspension in medium M9 was diluted with 0.01 M phosphate buffer (pH -- 7.6) until E_{260} was under 0.02. Then 0.2 ml [milliliters] of such a dilution as added to 1.8 ml of the same buffer but with the required dosage of dye. The mixture was incubated in the dark for ten minutes at 20-22°, after which it was diluted with a medium binding free dye (0.14M of NaCl solution + 10% broth), and determination was made of the number of spot-forming particles by the agar layer method. Other methods used are described with the corresponding experiments.

Results

The kinetics of inactivation of phage $\lambda(k)$ by acridine orange is shown in Figure 1. The same result is obtained when extracellular phage $\lambda(c)$ is treated with dye. Some of the phage $\lambda(k)$ population which remained viable after treatment with doses of over 2-4 $\mu\text{g/ml}$ [micrograms per milliliter] consists of mutant forms but not particles that have this property phenotypically. In order to test this stabs were made in several of the plaques in the dishes, corresponding to doses in excess of 4 $\mu\text{g/ml}$, and the washings from the needle were resuspended in separate tubes. Treatment with 20 $\mu\text{g/ml}$ of acridine orange revealed that the suspension in each test tube consists of resistant particles.

In order to obtain a pure strain of phage $\lambda(a)$, seven phage passages were made from morphologically typical plaques. Exactly the same passage of phage $\lambda(c)$ served as a control. The kinetics of inactivation of phage (c) after seven passages was the same as that shown on Figure 1; phage $\lambda(a)$ retained its resistance to the dye. In order to determine the mechanism of mutation of resistance a comparative study was made of biological and physicochemical properties of the mutant and wild type phage.

After infection of *E. coli* bacteria, both phages yield under like conditions an equal proportion of infectious centers and lysogenic cells. Both phages are resistant to osmotic shock in a 5M solution of NaCl. In this experiment we used the Anderson method [9]. Phages $\lambda(a)$ and $\lambda(c)$ are equally inactivated by ultraviolet irradiation.

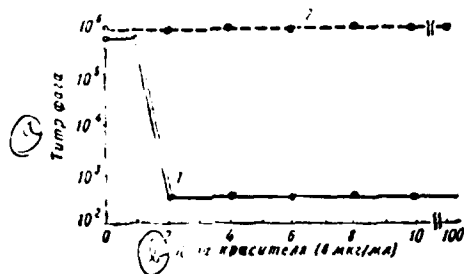


Figure 1. "Dark" inactivation of bacteriophage $\lambda(k)$ by acridine orange (1); 2 -- the same for phage $\lambda(a)$

Legend:

a) phage titer

b) dye dosage (in $\mu\text{g/ml}$)

Within the range of accuracy of the experiment no differences were detected between the curves of one cycle of reproduction of phages $\lambda(a)$ and $\lambda(c)$ on *E. coli* C. Bacteria that were in the middle of the logarithmic phase of growth were infected by phages $\lambda(a)$ and $\lambda(c)$ with a low multiplicity of infection (0.02). After five minutes of adsorption at 37° the suspension was diluted ten-fold with nutrient medium containing phage $\lambda(c)$ antiserum. Fifteen minutes later the antiserum was removed with 100-fold dilution of the mixture. Samples were collected every 5-10 minutes and determination was immediately made of number of spot forming particles. The results of this experiment are shown on Figure 2.

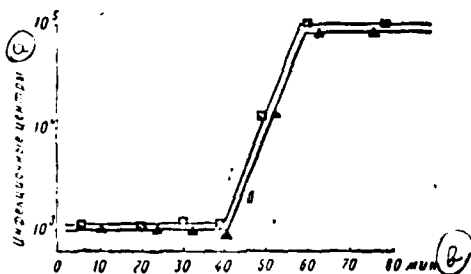


Figure 2. Curves of a single reproductive cycle of $\lambda(a)$ and $\lambda(c)$ on *E. coli* C

Legend:

I) infection with phage $\lambda(a)$

a) infectious centers

II) infection with phage $\lambda(c)$

b) ... minutes

The experiment is described in the text.

The kinetics of neutralization of $\lambda(a)$ and $\lambda(c)$ by phage $\lambda(c)$ antiserum is somewhat different, neutralization with $\lambda(k)$ antiserum is the same for the mutant and phage $\lambda(c)$ (see Table). The values for constants of rate of inactivation in this table are estimated by the initial segment of the inactivation curve, the curves proper are complex in shape.

Kinetics of neutralization of wild type phage and mutant
by antisera

(λ) Φαγ	ΑΝΤΙΣΕΡΟΥΜ κλ	
	κ λ (κ)	κ λ (c)
λ (a)	320	300
λ (c)	310	250

Legend:
a) phage

b) antiserum

Some differences were discovered between the phages, unrelated directly to sensitivity to the dye. Thus, the phage λ(a) plaques are larger and show more visible secondary growth than phage λ(c) plaques. Heterogeneity of plaque morphology typical for phage λ is retained by the mutant.

Some differences were noted between the adsorption properties of phages λ(a) and λ(c). Phage λ(a) in contrast to phage λ(c) is not adsorbed on E. coli K 12S. In addition a negligible and inconsistent difference is observed in rate of phage adsorption on E. coli C.

Phage λ(a) retains its resistance to acridine orange also, with intracellular development on E. coli C (Figure 3). However, since in this case suppression of phage λ(c) is mild, the difference between sensitivity of infected bacteria to acridine orange is considerably lesser than the corresponding difference between extracellular phages λ(a) and λ(c). The E. coli C in the middle of the logarithmic phase of growth in a complete medium were washed off and suspended in 0.01 M of MgSO₄ solution. The suspension was incubated for one hour at 37°, then phage was added so that the multiplicity of infection would equal 1.0. After 20 minutes of adsorption the culture was transferred into fresh nutrient medium (1% Bacto peptone water). It was verified that from this moment on development of phage in the infected bacteria occurs in the manner shown on Figure 2. Ten minutes later the infected bacteria were transferred into buffer with dye, similarly to what was done in the experiments for inactivation of extracellular phage. Incubation with the dye lasted 20-23 minutes at 37° after which immediate determination was made of the number of infectious centers (before lysis began).

Both the mutant and wild type phage are resistant to a similar dye, tryptaflavine. A 50 μg/ml dosage did not induce phage inactivation either in the dark or under the light of a 150 watt light bulb at a distance of 60 cm. It well may be that such illumination was insufficient for photodynamic inactivation.

When purified phage λ(c) preparations are mixed with dye solution a phage-dye complex is formed and this is associated with a change in

spectrum of dye absorption. Analogous changes in dye absorption occur in the presence of phage $\lambda(a)$, and when the concentration of phage in the mixture is altered the spectral absorption dye change identically in both the case of phage $\lambda(c)$ and phage $\lambda(a)$. These changes in dye spectrum (Figure 4) qualitatively correspond to those observed in the DNA-acridine orange system [10].

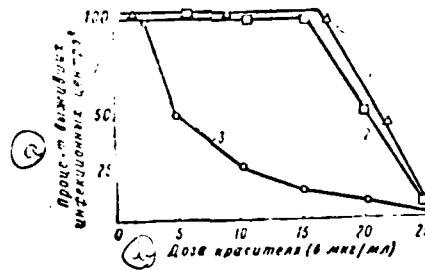


Figure 3. Effect of acridine orange on bacteria *E. coli* C infected with phages $\lambda(a)$ and $\lambda(c)$

Legend:

- | | |
|---------------------------------------|---|
| 1) noninfected culture | a) percentage of surviving infectious centers |
| 2) infectious centers of $\lambda(a)$ | b) dye dosage ($\mu\text{g/ml}$) |
| 3) infectious centers of $\lambda(c)$ | |

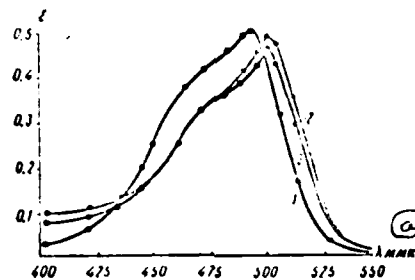


Figure 4. Absorption spectra of phage-dye complexes.
Dye concentration: $4 \mu\text{g/ml}$; phage titer: $1 \cdot 10^{12}$.
Solvent: phosphate buffer 0.01 M, pH: 7.6 +
0.005 M ethylenediaminetetraacetic acid sodium salt

Legend:

- | | |
|-----------------------------|-----------------------------|
| 1) dye | 3) dye + phage $\lambda(c)$ |
| 2) dye + phage $\lambda(a)$ | a) millimicrons |

During centrifugation of the phage-dye mixture some of the dye is precipitated along with phage. Evidently the dye that cannot be removed from the phage-dye complex with simple dilution of the mixture is precipitated. Within the range of accuracy of the experiment no difference

was observed between the ability of phages $\lambda(a)$ and $\lambda(c)$ to bind acridine orange. For these reasons it is improbable to assume that the mutant's resistance could be related to impermeability of its protein sheath for dye molecules. Physicochemical analysis of DNA isolated from phages $\lambda(a)$ and $\lambda(c)$ by the usual phenol method failed to reveal any reliable differences either in absorption spectra or nucleic acid melting curves (Figure 5). In order to determine the temperature contour of melting a DNA solution in 0.01 M phosphate buffer (pH -- 7.6) was heated in a sealed "thermostatized" cuvette. Optic density at $\lambda = 260$ millimicrons was measured 15 minutes after the temperature shown on the ordinate was reached.

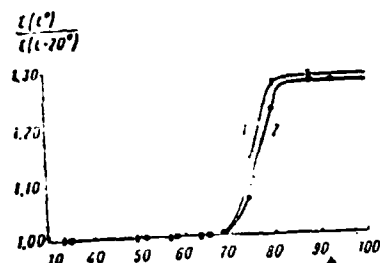


Figure 5. Melting curves of phage $\lambda(a)$ and $\lambda(c)$ DNA. The experiment is described in the text.

Legend:

1) $\lambda(a)$ DNA

2) $\lambda(c)$ DNA

We also failed to detect reliable differences in absorption spectra of DNA $\lambda(a)$ -dye and DNA $\lambda(c)$ -dye complexes (Figure 6).

There is unique coagulation of DNA with increase in dye concentration to a specific level in the DNA-dye mixture. Fibers form in the solution similar to those obtained when DNA is precipitated with alcohol. At equal concentrations of DNA these "critical concentrations" of dye are also the same for $\lambda(a)$ DNA and $\lambda(c)$ DNA. Evidently the structural disturbances of DNA in concentrated dye solutions are the same for $\lambda(a)$ DNA and $\lambda(c)$ DNA.

Discussion

In spite of the fact that the data submitted herein are insufficient to form a clear idea about the molecular mechanism of mutation in our case, some hypotheses can be ruled out. There is a correlation between viral sensitivity to photodynamic inactivation and permeability of their protein sheaths for small molecules [6, 7]. It may therefore be assumed that the resistance of phage $\lambda(a)$ to the dye may be related to impermeability of its protein sheath for the dye. True, all of the

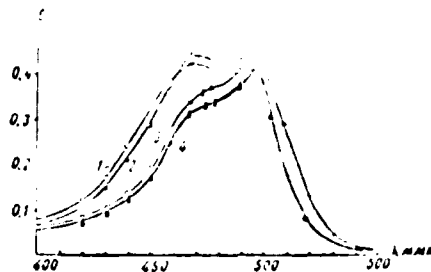


Figure 6. Spectra of phage $\lambda(a)$ or $\lambda(c)$ DNA-dye complexes

Legend:

- 1) 10 $\mu\text{g/ml}$ $\lambda(a)$ DNA + 4 $\mu\text{g/ml}$ acridine orange
 - 2) 10 $\mu\text{g/ml}$ $\lambda(c)$ DNA + 4 $\mu\text{g/ml}$ acridine orange
 - 3) 30 $\mu\text{g/ml}$ $\lambda(a)$ DNA + 3 $\mu\text{g/ml}$ acridine orange
 - 4) 30 $\mu\text{g/ml}$ $\lambda(c)$ DNA + 3 $\mu\text{g/ml}$ acridine orange
- Maximum of curves 3, 4 at $\lambda = 500 \pm 2$ millimicrons;
for curves 1, 2 at $\lambda = 467 \pm 2$ millimicrons.

above-described data are referable to inactivation in the dark, and in this case the existence of the above correlation can only be surmised. However, our results contradict the assumption that the protein membrane of phage $\lambda(a)$ is impermeable for acridine orange. Indeed, both phages are resistant to osmotic shock, consequently their protein membranes can be permeated by small molecules. In addition, both phages bind an equal amount of dye, and the bound dye absorption spectra are also the same in these cases, and they are similar to the spectra of DNA-dye complexes [9]. Since it is quite probable that dark inactivation of phage λ by acridine orange takes place due to interaction between the dye and phage DNA [8], it may be assumed that resistance of the mutant is related to some unknown distinctions of its nucleic acid. Although this hypothesis cannot be refuted, our results indicate that there are no visible differences between the nucleic acid structure of the mutant and wild type phage. Both DNAs are double-coiled, they are equally sensitive to ultraviolet light, they react the same with the dye. Further investigation of the molecular mechanism of mutation of resistance to the dye would be of interest in view of the prospect of using this type of mutation in genetic research.

Conclusions

1. During incubation of a mixture of phage λ and acridine orange in a solution of low ionic force at a pH of 7.6, dark inactivation of the phage takes place.
2. A phage mutant has been isolated and described; it is resistant

to acridine orange both within and outside the cell.

3. Acquisition of resistance to the dye cannot be the result of impermeability of the mutant's protein membrane for dye molecules.

4. No differences were detected between the absorption spectra and melting curves of nucleic acids in the mutant and wild type phage. Both DNAs modify identically the spectrum of acridine orange.

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