STUDIES ON THE ESTABLISHMENT OF LYSOGENY BY BACTERIOPHAGE P2

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Abstract

Some aspects of the processes leading to lysogenization of cells of *Escherichia coli* or *Shigella dysenteriae* by phage P2 have been studied with the following results. Treatment of infected cells with chloromycetin, amino acid analogues, or 5-OH-uridine, or starvation for a required amino acid, all increase the frequency of lysogenization, whereas treatment with cyanide, azide, dinitrophenol, or pretreatment with ultraviolet light have no effect. Treatment of infected cells with proflavine also increases the frequency of lysogenization, and chloromycetin and proflavine are most effective in this respect when added about halfway through the latent period. It is suggested that the primary action of these substances is to block processes, beginning about that time, that lead to the maturation of the phage. At about the same time, the infected cells that become lysogenic show an apparent resistance to ultraviolet light, higher than that of either phage P2 or established lysogenic cells.

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STUDIES ON THE ESTABLISHMENT OF LYSOGENY BY BACTERIOPHAGE P2

I. Introduction

1. The lysogenic condition.

The first bacteriophages to be studied intensively were selected for their ability to multiply rapidly in and to lyse all the sensitive bacterial cells they infect (*virulent* phages). More recently, however, much attention has been given to *temperate* bacteriophages, which may multiply in a manner that leads to lysis of the infected host, but which also have the alternative possibility of establishing a symbiotic relationship (*lysogeny*) with the bacterium. Bacteria that have become lysogenic acquire and transmit to their progeny the potentiality of producing phage lytically in the absence of further infection from the outside. The entity (*prophage*) that gives the cell this potentiality is non-infectious and is associated with the genetic material of the bacterium (see reviews in 1,2). The physical nature of the association is not known.

2. The immunity of lysogenic cells.

An established lysogenic cell is not susceptible to lysis upon superinfection with phage of the same genetic constitution as that carried in the prophage state; it is said to be *immune* to superinfection. It was first thought

that an established prophage produced immunity by physically obstructing some preliminary interaction between the bacterial chromosome and the superinfecting phage, necessary for the multiplication of the latter (see 1). More recent evidence (3), however, suggests that immunity may be the result of a physiological response of the lysogenic cell, such as the production of a specific substance that neutralizes the superinfecting phage.

3. The decision between lysis and lysogeny.

A bacterial cell infected with a temperate phage is a system that must make a decision between two alternative outcomes--lysis or lysogeny. It is known that the proportion of infected cells that become lysogenic (frequency of lysogenization) may be changed by the multiplicity of infection (4), temperature (5,6), anaerobiosis, and treatment with various substances (7). In the cases studied in most detail, it was found that the treatment must be applied before or during some critical time in the early part of the latent period to be effective. This fact suggests that the infecting phage remains in an undecided state during part of its life cycle inside the host cell.

The establishment of lysogeny has also been studied by a genetic approach (8,9). It is possible to isolate mutants of temperate phages that are unable to establish lysogeny. The mutants can be divided into different classes by observing the result of the simultaneous infection of bacteria

with two different mutant isolates; infection with mutants of the same class always results in lysis of the cell, whereas infection with mutants of different classes may result in lysogenization of the cell. Each class of mutants corresponds to a cluster of closely-linked, genetic loci in the phage genome. This work suggests that there are several genetically controlled steps involved in the process of lysogenization and that this sequence of steps can be performed by cooperation between mutants that alone are unable to complete the sequence.

The sequence of steps that leads to the establishment of lysogeny can also be studied using techniques that have already been applied to the investigation of virulent phages. In an effort to elucidate the mechanism of intracellular multiplication of phage, Luria and Latarjet (10) and later Benzer (11) followed the changes in sensitivity (measured as the survival of the ability to give origin to a plaque) of infected cells (complexes) to ultraviolet light (UV) (L-L curves). They found that the complexes, which immediately after infection have about the same sensitivity as the phage used for infection, later become more resistant to irradiation. Although no exact interpretation of the changes in sensitivity can be given, they do serve to indicate the time at which the complexes pass through a certain stage of development, characterized by

an increased resistance to UV. This technique has also been applied to the lytic multiplication of some temperate phages (12).

More recent work of Tomizawa (13) suggests that the increase in UV resistance observed in the L-L curves is dependent on the synthesis of some protein or proteins by the complexes. He found that treatment of the complexes with the antibiotic chloromycetin, which is known to inhibit protein synthesis selectively (14,15), fixes them at the level of UV resistance that they had attained at the time the drug was added.

In the experiments to be reported here, the technique of the L-L curves was applied to the study of the establishment of lysogeny by following the changes in sensitivity to UV of cells infected with a temperate phage, as measured by the survival of the ability of the complexes to give rise to lysogenic colonies. If the UV sensitivity of a phage is of the same order of magnitude or greater than the sensitivity of both its host cell and the established lysogenic strain derived from the host cell, the establishment of lysogeny by an infecting phage particle might be expected to be reflected in a transition of the lysogenizing complexes from one level of UV sensitivity to another; i.e., from a sensitivity, immediately after infection, equal to the product of the sensitivity of the phage used for the infection by the sensitivity of the uninfected cells, to a

later sensitivity equal to that of the lysogenic strain. The original aims of the work to be reported here were (a) to characterize the changes in UV sensitivity of cells infected with a temperate phage, and (b) to determine, by the use of chloromycetin, whether the change in resistance of lysogenizing complexes is also, as Tomizawa found for lytic complexes, dependent on protein synthesis.

Shortly after the experiments were begun, however, Christensen (16) found that the frequency of lysogenization of phage P1 was increased by treating the infected cells with chloromycetin. Subsequently we found that the drug also increased the frequency of lysogenization of phage P2. Since chloromycetin is known to have a selective effect on protein synthesis, the experiments were extended to a study of the effect of the antibiotic on the frequency of lysogenization, as compared to the effect of other chemical agents known to interfere with specific cellular processes. These studied were undertaken in the hope of elucidating the nature of the processes that lead to the final decision between lysis and lysogeny and of obtaining from the time dependence of the effectiveness of the agents, some indication of the stage in the life cycle of the infecting phage that is important in making the decision.

The L-L curves obtained and the experiments on the effect of various chemical substances on the frequency of lysogenization by phage P2 are presented here. The possible contributions of the results to the study of the establishment of lysogeny are discussed.

II. Materials and Methods.

1. Phages and bacteria.

Temperate phage P2 and its hosts *Escherichia coli*, strain *C* (abbreviated as *C*) and *Shigella dysenteriae* (abbreviated as *Sh*) (17) were used. Two derivatives of *Sh* were used: a streptomycin resistant derivative, abbreviated as *Sh/s*, the standard plating indicator for P2, and an isolate made lysogenic for P2, abbreviated as *Sh(P2)*. Two derivatives of *C* were also used: an isolate made lysogenic for P2, abbreviated as *C(P2)*; and an arginine-requiring mutant, abbreviated as *C Arg*$^-$.

Three spontaneous virulent mutants of P2 were used: P2 vir^1, a *weak* virulent (does not form plaques on and does not kill bacteria made lysogenic for P2); P2 vir^6, an *intermediate* virulent (forms plaques on bacteria singly lysogenic for P2, but not on bacteria doubly lysogenic for P2); and P2 vir^3, a *strong* virulent (forms plaques also on bacteria doubly lysogenic for P2) (see 18).

Temperate phage P22, its host *Salmonella typhi-murium* (abbreviated here as *T*), and a multiple mutant of the latter which, among other things, is unable to ferment galactose (abbreviated as *T Gal*$^-$) were also used. These strains were obtained from Dr. N. D. Zinder.

2. Media and general techniques.

All cultures of C and T were grown in Davis medium (7 g K_2HPO_4, 2 g KH_2PO_4, 0.5 g Na citrate·5 H_2O 1 g $(NH_4)_2SO_4$, and 0.01% $MgSO_4$ in 1 liter distilled H_2O; 0.1% glucose is added after autoclaving; for experiments with phage P2, 5 X 10^{-4} M $CaCl_2$ is added). All cultures of Sh were grown in LB medium (10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl, 1 g glucose in 1 liter of distilled H_2O; after autoclaving the pH is adjusted to 7.4 with NaOH). Plating cultures of Sh/s, were grown in TB medium (10 g Bacto-tryptone, 5 g NaCl in 1 liter distilled H_2O). Plating for phage was done using the soft agar layer technique, on LB agar medium (LB medium containing 10 g agar per liter; after autoclaving 5 ml of a 0.5 M solution of $CaCl_2$ is added and the pH is adjusted to 7.4 with NaOH), for P2, and on EMB agar medium (10 g Bacto-tryptone, 1 g yeast extract, 5 g NaCl, 2.5 g eosin powder, and 15 g agar in 1 liter of distilled water; eosin powder consists of 0.65 g methylene blue, 4 g eosin Y, and 20 g K_2HPO_4; after autoclaving, 1% galactose is added) for P22. Bacterial assays of C or Sh were done by spreading on hard LB agar plates (LB agar containing 1.5% agar).

Lysates of P2 and P22 were prepared in a complete nutrient medium. The P2 lysates were purified by centrifuging at high and low speeds alternatively, and finally resuspend-

ing the phage in a 1:10 dilution of buffered saline (7 g Na_2HPO_4, 3 g KH_2PO_4, 4 g NaCl in 1 liter distilled H_2O; after autoclaving 10^{-3} M $MgSO_4$ is added).

The general phage techniques used were those described by Adams (19).

3. Special materials.

The chloromycetin (chloramphenicol) used in these experiments was a gift of Parke, Davis and Co., and the proflavine (2,8 diamino acridinium sulfate) was purchased from the K and K Laboratories. A stock solution of chloromycetin was made in phosphate buffer (pH 8) and stored in the cold. Stock solutions of proflavine were made in distilled water and stored in the cold in the dark; fresh solutions were made up every two weeks. No special attempt to control illumination was made in the course of the experiments with proflavine, unless specifically stated.

4. Assay for lysogenic bacteria.

To measure the number of lysogenic bacteria which arise as a consequence of the infection of sensitive cells by temperate phage, special methods must be used which either differentiate lysogenic from sensitive colonies on the same plate, or select lysogenic colonies by killing sensitive cells and their progeny.

By plating T cells infected with P22 on EMB agar con-

taining 1% galactose and using T Gal⁻ as the indicator strain (a technique developed in the laboratory of Dr. S. E. Luria at the University of Illinois), one can assay for lysing cells, lysogenic cells, and sensitive cells on the same plate. Lysing cells form plaques, the lysogenic cells form purple, galactose-fermenting colonies, which are surrounded by a clear halo of lysis and can be easily distinguished from the pink, non-fermenting indicator, and the sensitive cells form purple colonies without halos.

The number of infected cells that give origin to cells lysogenic for P2 is measured by spreading the infected cells on hard LB agar plates, incubating the plates at 37°C for two hours, and then spraying them with an aerosol of a lysate of P2 vir^1 (equivalent to ca. 10^8 P2 vir^1 particles per plate). It can be shown by reconstruction experiments with mixtures of sensitive and lysogenic cells that under the conditions used, all sensitive cells are lysed by this treatment, whereas the lysogenic cells give origin to colonies (table 1a). The incubation for two hours before spraying is sufficient to allow the newly originating lysogenic cells to develop full immunity to P2 vir^1 (table 1b). If the frequency of lysogenization is 10%, 1000 infected cells must be spread on a plate in order to obtain 100 lysogenic colonies. If there are also many uninfected cells on the plate, these may be infected with phage liberated by the 900 lysing cells and may give rise to additional lysogenic

colonies. Assuming that each lysing cell liberates about 100 phage particles, 10^5 free phage particles would be present on the plate. Reconstruction experiments with mixtures of 10^5 phage particles and varying numbers of uninfected cells (table 1c) show that up to 10^4 uninfected cells may also be present on the plate without contributing any significant number of secondarily lysogenized colonies. This ratio of phage to uninfected cells corresponds to a ratio of about 1 infected cell to 10 uninfected cells; i.e., to a multiplicity of infection of 0.1. At higher multiplicities of infection, of course, the error due to secondary lysogenization becomes even less. The experiments presented here were all done at multiplicities of infection of 0.1 or greater. It should be noted that the actual number of phage particles liberated per cell is probably less than 100 (certainly when the cells are grown in Davis medium), that phage particles liberated by lysing cells are distributed in clusters rather than dispersed homogeneously over the plate, that infected cells may not lyse immediately after plating so that the uninfected cells are not actually exposed to free phage during the entire incubation time preceding spraying, and finally that in many cases the frequency of lysogenization in the experiments to be reported was higher than 10%. All these factors tend to minimize any error due to secondary lysogenization.

5. Standard infection procedure.

The experiments are, for the most part, done in the following way: a fully grown, aerated, overnight culture, grown either in Davis medium or LB medium, is diluted into fresh medium of the same kind, and aerated at 37°C until the cells reach a titer of about 5×10^7 per ml. They are then centrifuged twice to wash them, and resuspended at 2 to 4×10^8 per ml in a non-nutrient adsorption medium, which for P2 and bacteria grown in Davis medium consists of Davis salts (the phosphate salts of the Davis medium, 0.01% $MgSO_4$, 5×10^{-4} M $CaCl_2$, and 1% NaCl), for P2 and bacteria grown in LB medium consists of saline (1% NaCl, 5×10^{-3} M $CaCl_2$, and 10^{-4} M $MgSO_4$), and for P22 consists of the (undiluted)buffered saline already described. Phage is then added to the concentrated cells at a phage to cell ratio of about 1, and allowed to adsorb for 10 minutes at 37°C with aeration. At the end of this time usually about 90% or more of the phage has adsorbed to the cells. The adsorption mixture is then diluted by at least a factor of 10^3 into aeration tubes containing Davis or LB medium and the substances to be tested, time zero of the experiment being the time of this dilution.

The total number of infected cells is calculated as the sum of the number of plaques obtained, corrected by subtracting the contribution from unadsorbed phage, plus the number of lysogenic colony formers.

6. Ultraviolet irradiation.

Free phage is irradiated by diluting the lysate (at least 100-fold, if it is not purified) into chilled Davis salts or saline, and irradiating an aliquot of the dilution in a watchglass.

Cells are irradiated by diluting a culture, which has grown to about 5×10^7 per ml in Davis or LB medium at least 100-fold into chilled Davis salts or saline, and irradiating an aliquot of the dilution in a watchglass.

If infected cells are to be irradiated, the procedure for infection is the same as described above except that the cells are concentrated to about 10^9 per ml in saline adsorption medium and aerated for 30 minutes at 37 C before infection. Phage is then added to the concentrated cells at a phage to cell ratio of about 0.1. After adsorption, the phage-cell mixture is diluted 10-fold into an aeration tube containing LB medium and 5×10^{-3} M $CaCl_2$ at 37 C, time zero of the experiment being the time of this dilution. At various times during the latent period, 100-fold dilutions are made from the aeration tube into chilled saline and aliquots of the dilution are irradiated in watchglasses.

If cells are to be infected after irradiation, a culture, which has grown to about 5×10^7 per ml in Davis or LB medium, is centrifuged and resuspended at about 2×10^8 per ml in Davis salts or saline. A 5 ml aliquot is irradiated in a Petri dish, which is continuously rocked by hand

during the exposure. After the desired dose has been given, an aliquot is removed and infected by the standard procedure. The cell survival curves obtained under these conditions do not differ significantly from those obtained from cells irradiated at more dilute concentrations in watchglasses.

A watchglass containing a sample is set into a half of a Petri dish containing ice and is thus kept chilled during the irradiation. All samples, either in watchglasses or Petri dishes, are irradiated at a distance of 35 cm from a 15 watt, G.E. "germicidal" lamp. The intensity at the surface of the sample is of the order of 1.5×10^3 ergs per sec per cm^2, or such that a dose of 20 seconds inactivates 99% of phage T2. All irradiation experiments are done under such conditions that photoreactivation can be considered negligible.

III. Experimental Results

1. The effect of chloromycetin.

If one infects cells of E. coli strain C with P2 in the manner described in Materials and Methods, and transfers the infected cells to Davis medium at time zero, 15-20% of the infected cells become lysogenic. The rest lyse after about 30 minutes, and liberate an average of 20 phages per cell. If, however, the infected cells are exposed to bacteriostatic concentrations of the antibiotic chloromycetin, lysis is inhibited, and the frequency of lysogenization increases with a concomitant decrease in the number of lytic centers. The final resultant frequency of lysogenization depends both on the time during the latent period at which the drug is added (figure 1) and on the length of exposure to the drug (figure 2). For an exposure of one hour, one observes a frequency of lysogenization of (30-40%) when the chloromycetin is added time zero. However, the maximum frequency of lysogenization of 70-80% is obtained when the drug is added at 10 minutes. When chloromycetin is added at later times, its effect on the frequency of lysogenization decreases and finally disappears. The effect of chloromycetin increases with increasing lengths of exposure to the drug up to one hour, after which the number of lysogenic cells remains constant.

If one assays for both lytic centers and lysogenic cells at various times after the addition of the chloromycetin, one finds (figure 2) that at first there is a rough correspondence between the loss of lytic centers and the increase of lysogenic cells. Later, however, lytic centers are lost without being recovered as lysogenic cells. An experiment was done to see whether these missing lytic centers could possibly be accounted for by an increase in the number of non-lysogenic colony-formers. Cells were infected with various multiplicities of P2 and treated with chloromycetin for an hour, beginning at time zero. As can be seen from table 2, the fraction of non-lysogenic colony-formers observed was always about equal to the number of uninfected cells expected for that multiplicity, and very much less than the number to be expected if the missing lytic centers gave rise to sensitive cells.

Concentrations of chloromycetin of 20 μg/ml to 200 μg/ml are bacteriostatic for strain C in Davis medium, and uninfected cells remain fully viable for at least one hour in the presence of these concentrations of the drug. There is no inactivation of free phage exposed for one hour to 25 μg/ml of the drug. Variations in the concentration of chloromycetin between 20 μg/ml and 200 μg/ml, added either at time zero or at 10 minutes have little effect on the maximum frequency of lysogenization obtained or on how rapidly it is attained.

If one infects cells of strain C with any one of the virulent mutants of P2, and adds 25 μg/ml of chloromycetin to the infected cells either at time zero or at 10 minutes, one finds that after one hour of exposure to the drug, 80-90% of the infected cells survive as lytic centers.

Chloromycetin also increases the frequency of lysogenization of strain Sh by P2, and of T by phage P22. If cells of strain Sh, growing in LB medium, are infected with P2, 5-10% of them become lysogenic and the rest begin to lyse at about 30 minutes, liberating 80-100 phages per cell. If the infected cells are exposed to 5 μg/ml of chloromycetin at 10 minutes, the frequency of lysogenization increases to about 30% of the infected cells after 20 minutes of exposure (figure 3). The viability of Sh cells exposed to 5 μg/ml of chloromycetin in LB medium, decreases rather rapidly with time, so that a correction must be made for the loss of lysogenic cells by inactivation.

If cells of strain T are infected with P22, and transferred to Davis medium, about 50% of them become lysogenic, and the rest begin to lyse at 30-35 minutes, liberating about 200 phages per cell. If the infected cells are exposed to chloromycetin either at time zero or at time 5 minutes, the number of lysogenic cells increases with a corresponding decrease in the number of lytic centers (table 3). A frequency of lysogenization of 100% is obtained when the chloromycetin is added 5 minutes after infection.

Although chloromycetin also increases the frequency of lysogenization of cells of Sh by P2, strain C, which grows more easily in synthetic media, was used as the host in experiments in which the action of chloromycetin was compared with that of various other chemical agents.

2. The effect of cyanide, dinitrophenol, and azide.

To see if the effect produced by chloromycetin could also be produced by other bacteriostatic agents, cells of strain C were infected with P2 and exposed to potassium cyanide, 2,4-dinitrophenol, and sodium azide at concentrations which inhibit the growth of uninfected cells and lysis of infected ones. At the concentrations tested, these agents did not affect the frequency of lysogenization when added at time zero (table 4). Experiments were also done in which the cyanide and dinitrophenol were added at 10 minutes, and these also showed no effect.

3. The effect of amino acid analogues, an amino acid requirement, and 5-hydroxy-uridine.

To see if the effect produced by chloromycetin could also be produced by other treatments that inhibit protein synthesis (see Discussion), three other sets of experiments were done. In the first set, cells of strain C infected with P2 were exposed to concentrations of the amino acid analogues 5-methyltryptophan and allyl-glycine which

inhibited the growth of uninfected cells. These analogues were effective in increasing the frequency of lysogenization by about a factor of two (table 4). It was possible to reverse the effect of 5-methyl-tryptophan by the addition of tryptophan.

In the second set of experiments, cells of strain C Arg^-, grown in Davis medium supplemented with arginine (20 μg/ml), were starved for an hour in Davis medium without the arginine supplement, infected with P2, and then placed again in Davis medium without the essential amino acid. Again the frequency of lysogenization was increased by about a factor of two (table 4).

In the third set of experiments, cells of strain C infected with P2 were exposed to 5-OH-uridine. Concentrations of either 10 μg/ml or 50 μg/ml of this substance increased the frequency of lysogenization by at least a factor of two, and the effect could be reversed by adding equal amounts of uridine (table 4). The concentrations of 5-OH-uridine used did not inhibit the growth of uninfected cells, and also allowed lysis of some of the infected ones. An attempt to eliminate this residual lysis by growing the cells in the presence of 10 μg/ml of 5-OH-uridine for 30 minutes before infection was ineffective, as is shown in table 4.

4. <u>The effect of proflavine</u>.

A 10 μg/ml concentration of proflavine somewhat inhibits the growth of cultures of strain C in Davis medium, but is not completely bacteriostatic. Under similar conditions, it inactivates free phage P2, leaving approximately 10% survivors in 15 minutes at 37°C. If cells of strain C are infected with P2 by the standard procedure and exposed to a 10 μg/ml concentration of proflavine, one finds that the dye has very marked effects on the frequency of lysogenization and on the survival of the lytic centers (figures 4 and 5). When the dye is added during the first 15 minutes, the lytic centers disappear rapidly; when it is added at later times, some of the infected cells succeed in completing the lytic cycle and liberate some phage even in the presence of the dye. The frequency of lysogenization is only slightly affected by proflavine when the dye is added immediately after infection. If the dye is added at later times, however, the frequency of lysogenization is increased very greatly, approaching 100% with treatment beginning at 15 minutes.

If one infects cells of strain C with the vir^1 mutant of P2 by the standard procedure, adds 10 μg/ml of proflavine to the infected cells at various times during the latent period, and measures lytic centers after one hour of exposure, about 60% of the infected cells survive as lytic

centers, if the substance is added at time zero, about 75% survive, if it is added at 5 minutes, and perhaps 90% survive, if it is added at 15 minutes. Some phage is liberated by the infected cells during their exposure to proflavine if the dye is added at 5 or 15 minutes.

An attempt was made to reverse the effect of proflavine added at zero minutes or 15 minutes after infection by simultaneously adding 10 μg/ml of riboflavine or a mixture containing 10 μg/ml of each of five nucleotides, but these substances produced no striking reversal of the effect at the concentrations used (table 5). Neither was the effect of the dye reversed by performing the experiment under conditions of darkness that reduce the inactivation of free phage by proflavine to 50% in 15 minutes (table 5).

From a comparison of figures 1 and 4 it would appear that the maximum effect of chloromycetin on lysogenization occurs when it is added 10 minutes after infection, while that of proflavine occurs when it is added at 15 minutes. It is possible either that these two substances affect two different reactions or that they affect the same reaction, but that the chloromycetin either (a) penetrates the cells more slowly or (b) has an inhibitory effect on lysogenization at later times in addition to its enhancing effect so that the frequency of lysogenization measured is

the resultant of these two effects. Possibility (a) was tested by adding an increased concentration of chloromycetin (200 μg/ml) at 15 minutes instead of the usual 25 μg/ml, under the assumption that an increase in the external concentration of the drug would decrease the time required by the cells to take up enough to affect the frequency of lysogenization. Possibility (b) was tested by adding 25 μg/ml of chloromycetin and 10 μg/ml of proflavine simultaneously at 15 minutes to see if it would also inhibit the effect of proflavine at this time. The results of these two experiments (table 6) are consistent with possibility (b), and inconsistent with (a).

Proflavine also increases the frequency of lysogenization of Sh by P2. If cells of strain Sh, growing in LB medium, are infected with P2 and exposed to 10 μg/ml of proflavine at 15 minutes, the frequency of lysogenization increases to about 60% after 15 minutes of exposure (figure 3). This concentration of proflavine is not quite bacteriostatic for Sh in LB medium.

5. The effect of ultraviolet irradiation.

The irradiation of the bacteria before infection with small doses of ultraviolet light was studied for its effect on lysogenization. If uninfected C is irradiated with doses of ultraviolet light that leave anywhere from 10% to 80% survivors and is then diluted into Davis medium at

$37^{\circ}C$, the cells do not divide for at least one hour. If they are examined microscopically, however, they can clearly be seen to increase in size during this time. If the cells are infected with P2 immediately after irradiation, the fraction of infected cells which become lysogenic is always a constant proportion of the surviving cells; in other words, the frequency of lysogenization among the survivors is constant. This is shown in figure 6 where the survival curve for those cells which become lysogenic is seen to parallel closely that for uninfected cells. The capacity of these irradiated cells to produce phage lytically is only slightly reduced over the range of doses used and the length of the latent period and the burst size of the phages are about the same as on unirradiated C.

It was also of interest to know if chloromycetin still could increase the frequency of lysogenization of cells which had been irradiated and then infected. Cells of strain C, which had been irradiated to leave 60% survivors, were either infected with P2 immediately after the irradiation, and then exposed to chloromycetin for one hour, or first diluted into Davis medium, and allowed to grow for 30 or 60 minutes, and then infected and exposed to the drug. In each case (table 7) exposure to chloromycetin increased the frequency of lysogenization by about the

same factor as for unirradiated cells. A similar experiment was also done in which the dose of ultraviolet light was such as to leave 36% bacterial survivors. This experiment gave the same results as the one just described, but in this case a correction had to be introduced because the cells became more sensitive to killing by chloromycetin after the irradiation

6. Irradiation of bacterial strains and of free phage.

Strains *Sh* and *Sh(P2)*, grown in LB medium, were irradiated by the procedure described in Materials and Methods, and the logarithm of the fraction of cells that survived as colony-formers was plotted against the dose of UV (figure 7). The survival curves for the two bacterial strains are not detectably different.

A lysate of phage *P2* was irradiated as described in Materials and Methods and the logarithm of the fraction of phages that survived and gave origin either to plaques or to lysogenic colonies in strain *Sh* was plotted against the dose of UV (figure 8). The survival curves are not simple exponential curves and may be described as the sum of two exponential curves, the more resistant one extrapolating to 0.3 to 0.5 on the ordinate. If phages that have survived a dose of UV of 200 seconds, are grown into a stock and irradiated again, a survival curve of the same

shape is obtained. It is unlikely, therefore, that a curve of this shape is caused by a genetic inhomogeneity in the phage population. The curve for the survival of the lysogenizing function in strain Sh seems to follow that for the lytic function.

A survival curve for a virulent mutant of P2, P2 vir[1], is also given in figure 8. This curve is similar in all respects to that obtained for the lytic function of P2.

The irradiation of cells before infection somewhat decreases their capacity to produce phage. The irradiation of the cells also reduces to about the same extent as they are killed, their capacity to produce lysogenic colonies. Curves showing how the capacities of the cells decrease with UV dose are given in figure 9.

7. The irradiation of infected cells.

Because irradiation of the cells decreases their capacity, it is to be expected that complexes, irradiated immediately after infection, are more sensitive to UV than free phage irradiated before infection. More precisely, one would expect that the survival curve for complexes irradiated at time zero is the product of the curve for the survival of free phage by the curve for the capacity of the cells to produce phage, for the lytic function of the phage, or the product of the curve for the survival of free

phage by the curve for the capacity of the cells to produce lysogenic colonies, for the lysogenizing function of the phage.

In figures 10, 11, and 12, are given three sets of curves showing the progressive increase, during the latent period, in the resistance to UV of cells of strain Sh infected with a virulent mutant of P2, P2 vir[1], and those infected with temperate P2, showing either a lytic or a lysogenic response. The resistance of complexes of P2 vir[1] begins to increase shortly after infection and about 15 minutes after infection reaches a maximum that is maintained throughout the remainder of the latent period. The same pattern of increasing resistance is also obtained for another non-lysogenizing mutant of P2, P2 vir[3]. The resistance of complexes of temperate P2 that show the lytic response, begins to increase about 15 minutes after infection and reaches a maximum shortly before the end of the latent period. In both cases, the maximum resistance attained is about the same and corresponds to the capacity of the cells.

The resistance of complexes that become lysogenic begins to increase shortly after infection, and about 15 minutes after infection reaches a maximum that appears to be greater than the resistance of established lysogenic cells. Toward the end of the latent period, the resistance

decreases to a level that is comparable to that for established lysogenic cells.

IV. Discussion

The results concerning the effect on the frequency of lysogenization of various chemical agents will be discussed first and a simple interpretation of these results will be proposed. Then the UV data will be discussed.

Christensen (16) has reported that the antibiotic chloromycetin increases the frequency of lysogenization by phage P1. It has now been shown that this drug also increases the frequency of lysogenization by phages P2 and P22. It seems therefore that this effect of chloromycetin is rather general for temperate phages. It should perhaps be noted here that this result for P22 is just the opposite of that found by Lwoff, Kaplan, and Ritz (7). The reason for the discrepancy between these experiments is not known, but may be due to a difference in the techniques used to measure the frequency of lysogenization or in the multiplicities of infection used.

The inhibition of bacterial growth by chloromycetin has been studied in several systems (14,15), and it is generally agreed that bacteriostatic concentrations of the drug (5-50 μg/ml) inhibit the synthesis of proteins by the bacteria, but have little or no effect on the synthesis of either kind of nucleic acid, on respiration, or on phosphorylation reactions. It has also been reported that when bacteria are treated with certain amino acid analogues

or when mutant bacterial strains which require an amino acid for growth are deprived of the required amino acid, the synthesis of proteins by these cells is greatly inhibited relative to the synthesis of nucleic acids (20,21). Since all of these treatments increase the frequency of lysogenization by phage P2, one can postulate that it is the inhibition of the synthesis of some protein which causes this increase.

The frequency of lysogenization of strain C by P2 can also be increased two to three times above the controls by the addition of 5-hydroxy uridine to the infected cells. It has been suggested that this substance interferes with the incorporation of uracil into ribose nucleic acid (22). Since the concentrations used are not bacteriostatic, obviously protein synthesis is not generally inhibited, but it has been shown (23) that these concentrations inhibit the formation of an adaptive enzyme, β-galactosidase, by a strain of *E. coli*. One may thus infer that the increase in frequency of lysogenization is caused by the inhibition of the synthesis of a *new* protein, one necessary only for phage production and not for general bacterial metabolism.

Since the frequency of lysogenization is not affected by bacteriostatic concentrations of cyanide, azide, or dinitrophenol, which will also inhibit protein synthesis

by interfering with the energy supply of the cells, one can further conclude that the inhibition of protein synthesis alone is not sufficient to produce an increased frequency of lysogenization, but that a differential must be created between the synthesis of a new protein and some other process in the cell. One way to identify such a process is to look for specific inhibitors which cause a decrease in the frequency of lysogenization or counteract the effect of chloromycetin.

Kelner (24) has shown that DNA (desoxyribose nucleic acid) synthesis can be selectively inhibited by irradiating bacteria with small doses of ultraviolet light. From the experiments described in this paper we have seen that such doses of ultraviolet light have no effect on the frequency of lysogenization by P2 or on the ability of chloromycetin to increase the frequency of lysogenization. These experiments, however, deal only with the inhibition of bacterial DNA synthesis before infection, and say nothing about what the effect of the inhibition of phage DNA synthesis would be. Since P2, like most other phages, is able to multiply lytically on irradiated cells, the synthesis of phage DNA is clearly unaffected by the irradiation of the cells before infection.

In connection with the experiments just mentioned, one should note the difference in behavior between the two phages

P2 and λ. With the latter, Lieb (5) found that the frequency of lysogenization is strikingly reduced by pre-irradiation of the host cells. Since λ is an inducible phage (i.e., cells lysogenic for λ can be induced to lyse by irradiation with UV; see 1 for details), whereas P2 is not, this difference supports Lieb's supposition of an induction preceding infection in the case of λ.

Unfortunately the evidence available concerning the effect of proflavine on the synthesis of protein and nucleic acids is still rather scanty. The dye is known to form complexes with nucleic acids, and its bacteriostatic action may be overcome by the addition of nucleic acids and of certain amino acids (25). Foster (26) has shown that cells of *E. coli* strain *B*, infected with phage T2, lyse in the presence of proflavine, but do not yield infective phage particles. DeMars *et al.*(27) later showed that lysates of T2, prepared in the presence of proflavine, contain a large proportion of flattened, tailless particles ("doughnuts") and few, if any normal particles. The doughnuts contain little or no DNA and the inference is that proflavine interferes with the formation of infective phage particles by preventing the normal association of phage DNA and protein at maturation. A preliminary note by Manson (28) states that, although proflavine may inhibit the synthesis of DNA in T2 infected cells, it inhibits the

production of infective phage under conditions where it does not inhibit DNA synthesis. DeMars (29) has confirmed the finding that phage DNA may be produced in the presence of proflavine.

Whether proflavine has the same effect on the lytic multiplication of phage P2 as it has on T2, has not been determined. The concentration of the dye used in the experiments presented here was not sufficient to inhibit completely the formation of infective phage particles. The mechanism of action by which proflavine increases the frequency of lysogenization by phage P2 may be the same as that by which it prevents the formation of infective phage particles or may be an effect on some other reaction in the infected cell.

Both chloromycetin and proflavine are useful as tools to measure that part of the latent period during which the decision between lysis and lysogeny can be influenced. According to the experiments presented here, both substances are most effective in increasing the frequency of lysogenization if they are added about halfway through the latent period--at 10 minutes or 15 minutes. Similarly Christensen (16) found that chloromycetin was effective in increasing the frequency of lysogenization by phage P1 as late as 20-25 minutes after infection. (P1 has a latent period of about 45 minutes.) The fact that lytic

centers may be converted to lysogenic cells as late as halfway through the latent period suggests that there may be extensive multiplication of phage DNA before lysogenization and that the integrity of the infected cell is not irreversibly destroyed by this multiplication.

It was suggested earlier that the difference of 5 minutes between the times of maximum effectiveness of chloromycetin and proflavine could be explained by assuming that chloromycetin also has an inhibitory effect on lysogenization, so that the maximum effect obtained at 10 minutes is really the resultant of two reactions, one increasing and one decreasing the number of lysogenic cells. The relative depression of the frequency of lysogenization caused by the addition of both proflavine and chloromycetin at 15 minutes, as compared with the effect of proflavine alone, supports such a notion. It is reasonable to believe that both these substances affect, directly or indirectly, many reactions in bacteria, and this may explain why the frequency of lysogenization is not increased more when the treatment is started before 10 minutes, or why some of the potentially lysogenic cells are not recovered at all when exposed to the substances for too long a time.

In trying to determine the stage in the life cycle of the phage that is affected by chloromycetin and pro-

flavine, the following information may be taken into account. Hershey and Melechen (30,31) showed that at least two proteins must be synthesized during the multiplication of phage T2; a protein, produced in the first few minutes of the latent period (early protein) that is necessary to initiate the synthesis of phage DNA, and one, produced in the second half of the latent period (late protein), that is antigenically phage-specific (presumably precursors of the phage coat). The effect of chloromycetin on the synthesis of the early protein of T2 is maximal at the beginning of the latent period. The late protein of T2 is synthesized only immediately before and during the appearance of mature phage particles (32,33). Bozeman et al. (34) treated cells infected with phage T1 with chloromycetin at various times in the course of the latent period and compared normal lysis and premature lysis of the cells. They found that infected cells treated in the first half of the latent period do not lyse and form no mature phage particles as long as they remain in the presence of the drug. Cells treated at later times, however, liberate a number of phage particles equal to the number of mature phages that were present in the cell before the drug was added. Foster (26) found that proflavine prevents the formation of infective T2 particles if added any time during the first half of the latent period. If proflavine is added in the second half of the latent period, the infected cells liberate whatever mature particles were

formed before the addition of the substance.

The time during which chloromycetin and proflavine are effective in increasing the frequency of lysogenization by phage *P2* is comparable to the time during which chloromycetin prevents the synthesis of the late protein of *T2* (and thereby the maturation of *T2* particles), or the maturation of *T1* particles, and to the time during which proflavine prevents the maturation of *T2*. It is possible, therefore, that the suppression of phage maturation is part of the mechanism by which these two substances increase the frequency of lysogenization by *P2*. It cannot be the whole mechanism because, as we have seen, an increase in the frequency of lysogenization requires not only that protein synthesis be inhibited, but also that some other process (or processes) continue in the cell. According to the experiments with *T2*, neither chloromycetin nor proflavine inhibits the synthesis of phage DNA, once it has been initiated. If an increase in the frequency of lysogenization depends in some way on the synthesis of phage DNA, then the following qualitative model of the mechanism of action of chloromycetin and proflavine on the frequency of lysogenization by phage *P2* could be suggested as a useful working hypothesis. An infecting phage particle injects its DNA into a cell and the DNA begins to replicate itself by some unknown mechanism. We

assume (a) that the maturation of a phage particle is sufficient to commit irreversibly the infected cell to lyse and liberate phage, (b) that the probability of lysogenization of an infected cell increases with time and with increasing numbers of phage DNA replicas, (c) that the establishment of lysogeny involves the formation of some association between a replica of the genetic material of the infecting phage and the bacterial nucleus, and (d) that once the association has been formed, the lysogenized cell is able to prevent the maturation of any of the other phage DNA replicas, possibly by elaborating a substance that neutralizes them. Under normal conditions, the association is established in 10-20% of the infected cells before the appearance of the first mature phage particle. The addition of chloromycetin or proflavine to the cells delays maturation, thereby allowing more replication of the phage DNA and more time for the association to be established.

All the information discussed so far suggests that the middle of the latent period is the critical time in the decision between lysis and lysogeny. The L-L curves for the lysogenizing complexes of P2 also show a remarkable feature at this time, in that they show a greater resistance of the complexes to UV than would be expected from the capacity of cells irradiated before infection to

produce lysogenic colonies. One could offer a number of speculative explanations for this unexpectedly high level of resistance: for example, the DNA of the infecting phage may multiply in or around the nuclei of the cells, producing many replicas that serve as physical protection to the DNA of the cells; or that lysogenizing cells temporarily produce abnormally large amounts of RNA that protect the nuclei; or that phage material can repair, by some mechanism, UV damage to the cells.

It should be noted, however, that the 15 minute L-L curve for lysogenizing complexes could also be interpreted as the result of an increase, produced by the UV treatment, in the absolute number of lysogenic cells, rather than as an unusually high resistance of lysogenizing complexes to UV. Such an interpretation would also account for the difference in the time at which complexes of P2 vir^1 and lysing complexes of P2 attain a maximum resistance to UV. Since both temperate P2 and its virulent mutant P2 vir^1 have the same latent period and about the same burst size, there is no *a priori* reason to expect that the phages would undergo similar stages of lytic development at different times. We could assume then, that the difference in the time at which complexes of P2 and of P2 vir^1 attain a maximum resistance to UV is only apparent and this apparent difference could be attributed to a loss of lysing complexes of

P2 by their conversion to lysogenizing complexes. In the second half of the latent period, the UV would be less effective in changing the frequency of lysogenization, and only then would the lysing complexes of P2 show the same high resistance to UV as complexes of P2 vir[1], and the lysogenizing complexes a UV resistance characteristic of lysogenic cells.

It should be possible to test experimentally whether UV irradiation actually increases the frequency of lysogenization by phage P2. If this interpretation proves correct, the action of UV in this respect should be characterized and compared with the action of other agents known to have the same effect. If UV irradiation does not affect the frequency of lysogenization, however, then the new phenomenon of the increased resistance to UV of lysogenizing complexes at a particular time in the latent period should be studied to determine its significance in the establishment of lysogeny.

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Figure 1. Variation in the susceptibility of P2 infected cells to the effect of chloromycetin on lysogenization in the course of the latent period. Cells of strain C were infected with P2 by the standard procedure. Chloromycetin (25 μg/ml) was added at various times during the latent period. Lysing and lysogenizing cells were assayed after one hour exposure to the drug. The frequencies on the ordinate are calculated on the basis of the total infected cells (set equal to 100%) in the untreated control. The number of lysing cells has been corrected for plaques produced by unadsorbed phage. The frequency of lysogenization in the untreated control was 19%.

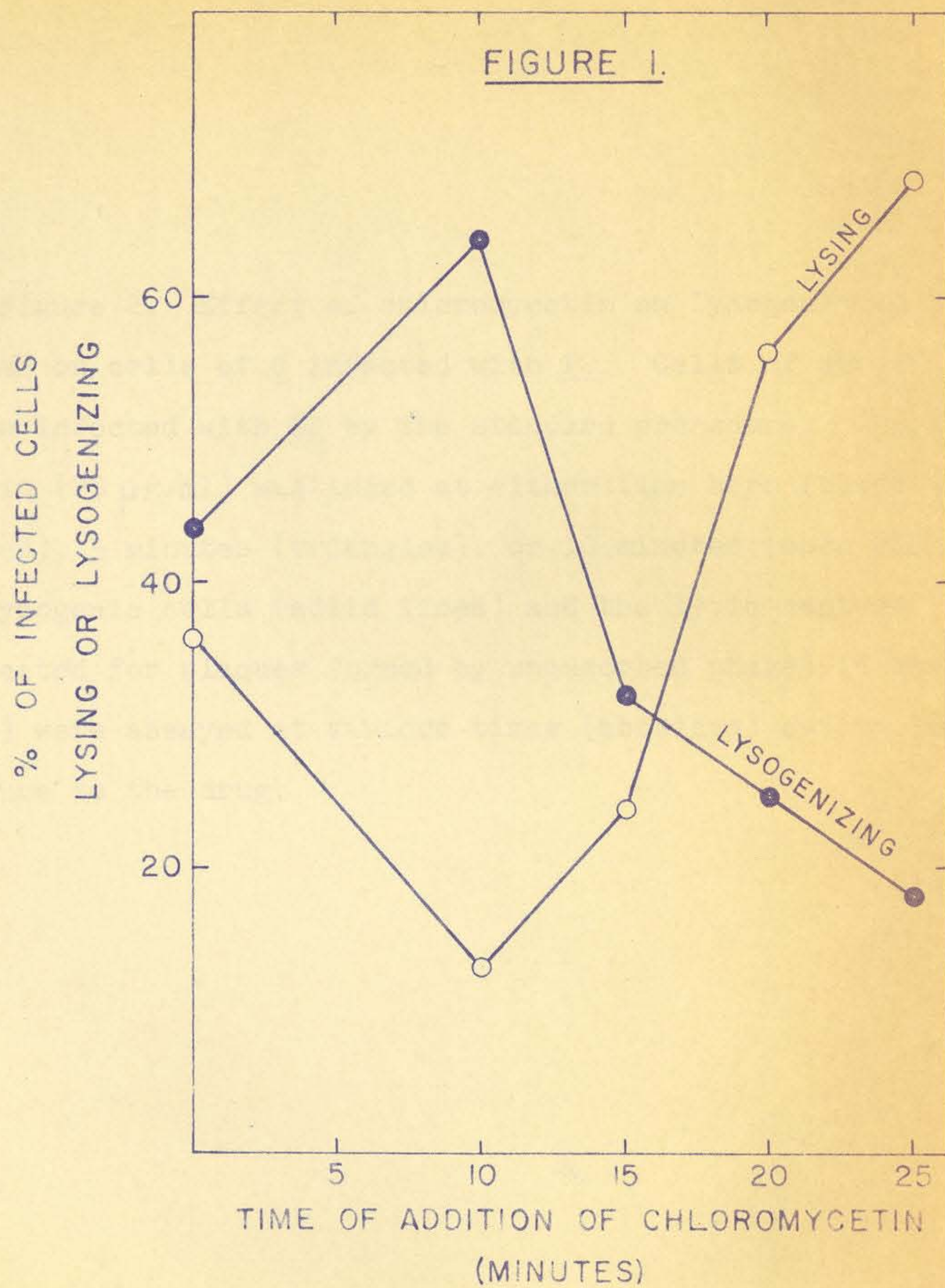

FIGURE I.

Figure 2. Effect of chloromycetin on lysogenization in time on cells of C infected with P2. Cells of strain C were infected with P2 by the standard procedure. Chloromycetin (25 μg/ml) was added at either time zero (black circles), 5 minutes (triangles), or 10 minutes (open circles). The lysogenic cells (solid lines) and the lytic centers (corrected for plaques formed by unadsorbed phage) (dashed lines) were assayed at various times (abscissa) during their exposure to the drug.

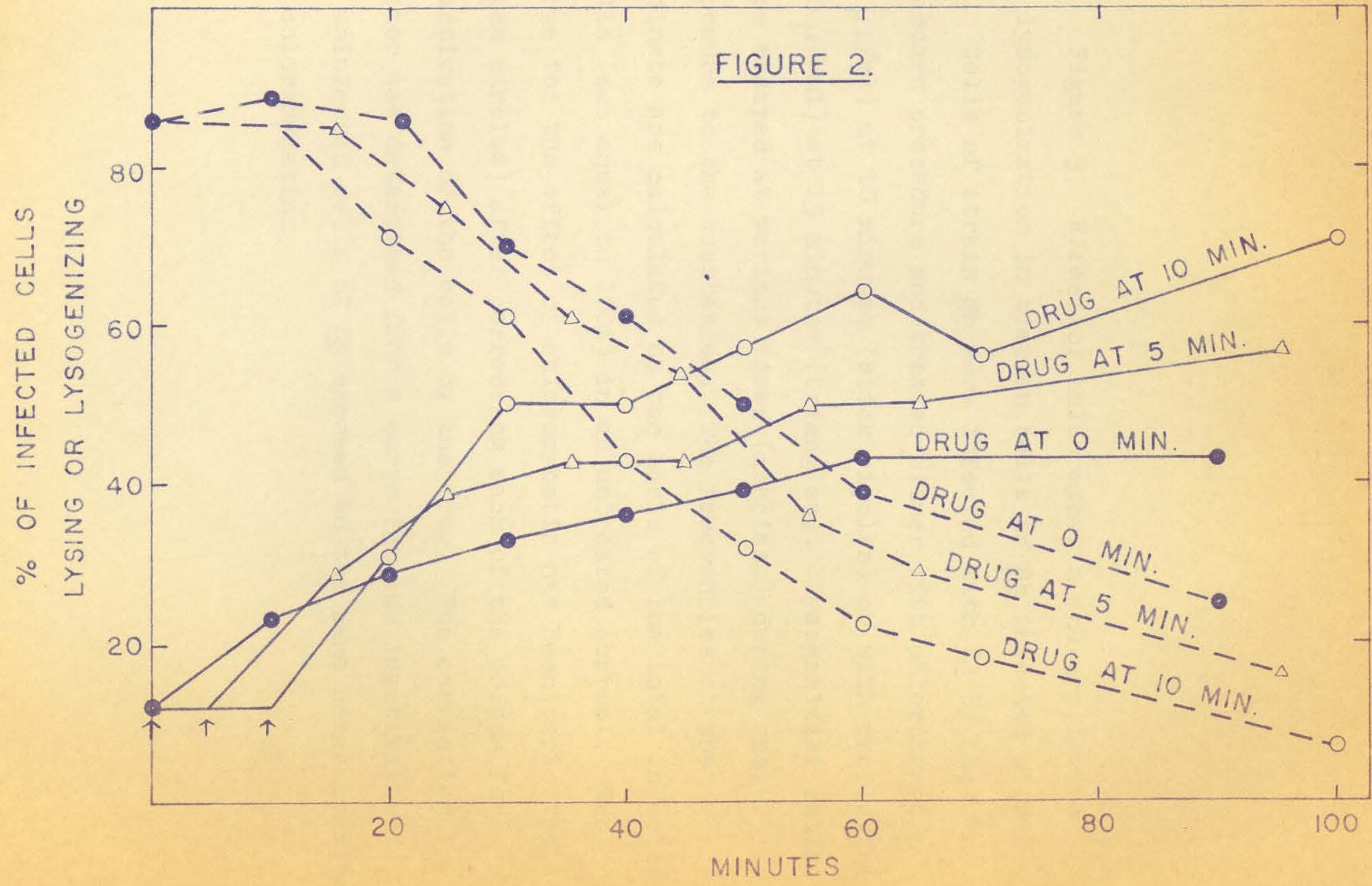
FIGURE 2.
% OF INFECTED CELLS
LYSING OR LYSOGENIZING
80
60
40
20
DRUG AT 10 MIN.
DRUG AT 5 MIN.
DRUG AT 0 MIN.
DRUG AT 0 MIN.
DRUG AT 5 MIN.
DRUG AT 10 MIN.
20
40
60
80
100
MINUTES

Figure 3. Effect of chloromycetin and proflavine on lysogenization in time on cells of Sh infected with P2. Cells of strain Sh were infected with P2 by the standard procedure and treated either with chloromycetin (5 μg/ml) at 10 minutes (black circles) or with proflavine (10 μg/ml) at 15 minutes (triangles). Lysogenizing cells were assayed at various times (abscissa) during their exposure to the substances. The frequencies on the ordinate are calculated on the basis of the total infected cells (set equal to 100%) in an untreated control. The curve for the effect of chloromycetin has been replotted (open circles) after correcting each of the points for inactivation of the cells by the drug. The correction factor was determined from a curve for the inactivation of uninfected cells of Sh exposed to the same concentration of chloromycetin.

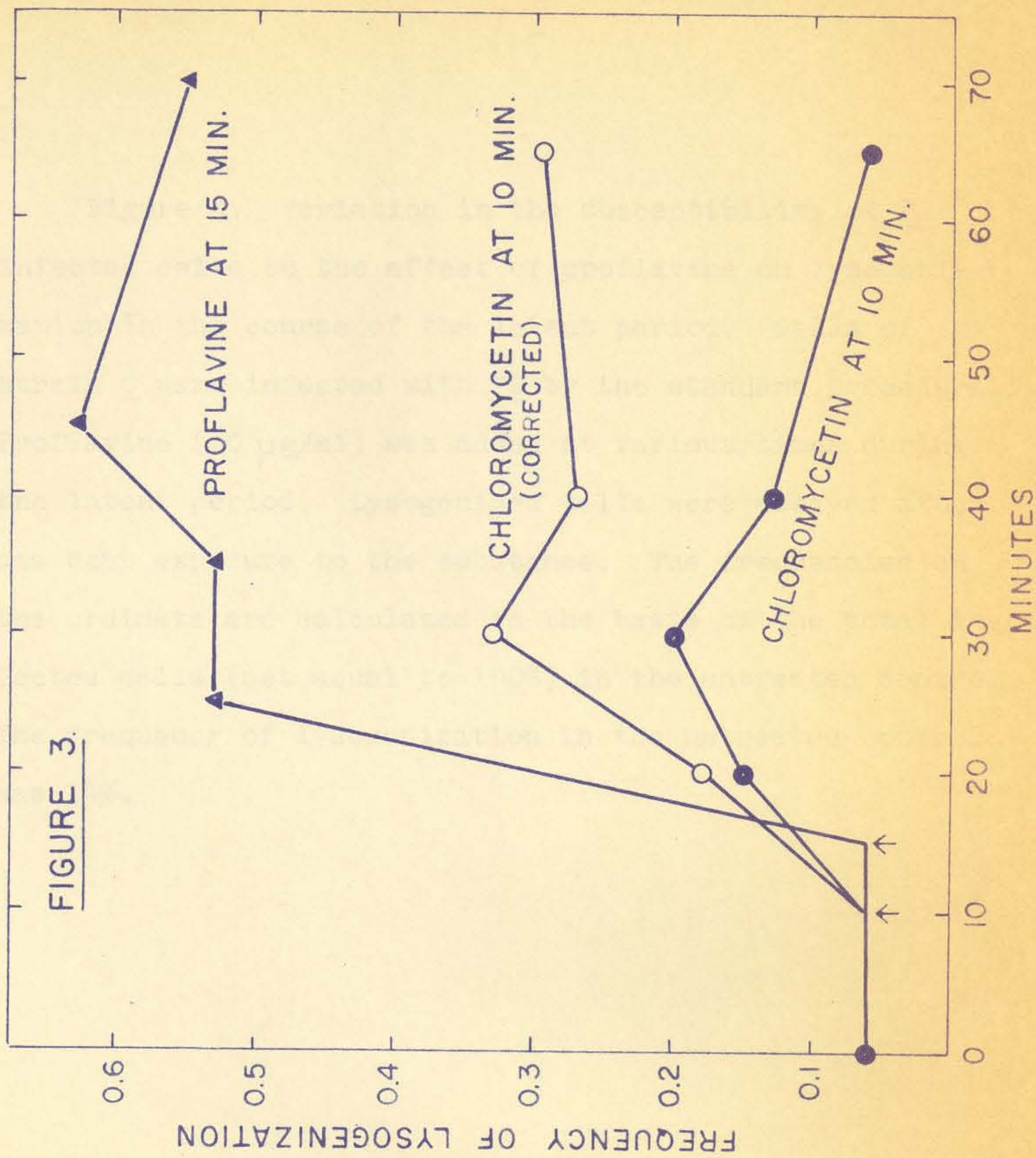
FIGURE 3.
PROFLAVINE AT 15 MIN.
CHLOROMYCETIN AT 10 MIN.
(CORRECTED)
CHLOROMYCETIN AT 10 MIN.
FREQUENCY OF LYSOGENIZATION
0.1
0.2
0.3
0.4
0.5
0.6
MINUTES
0
10
20
30
40
50
60
70

Figure 4. Variation in the susceptibility of P2 infected cells to the effect of proflavine on lysogenization in the course of the latent period. Cells of strain C were infected with P2 by the standard procedure. Proflavine (10 μg/ml) was added at various times during the latent period. Lysogenized cells were assayed after one hour exposure to the substance. The frequencies on the ordinate are calculated on the basis of the total infected cells (set equal to 100%) in the untreated control. The frequency of lysogenization in the untreated control was 14%.

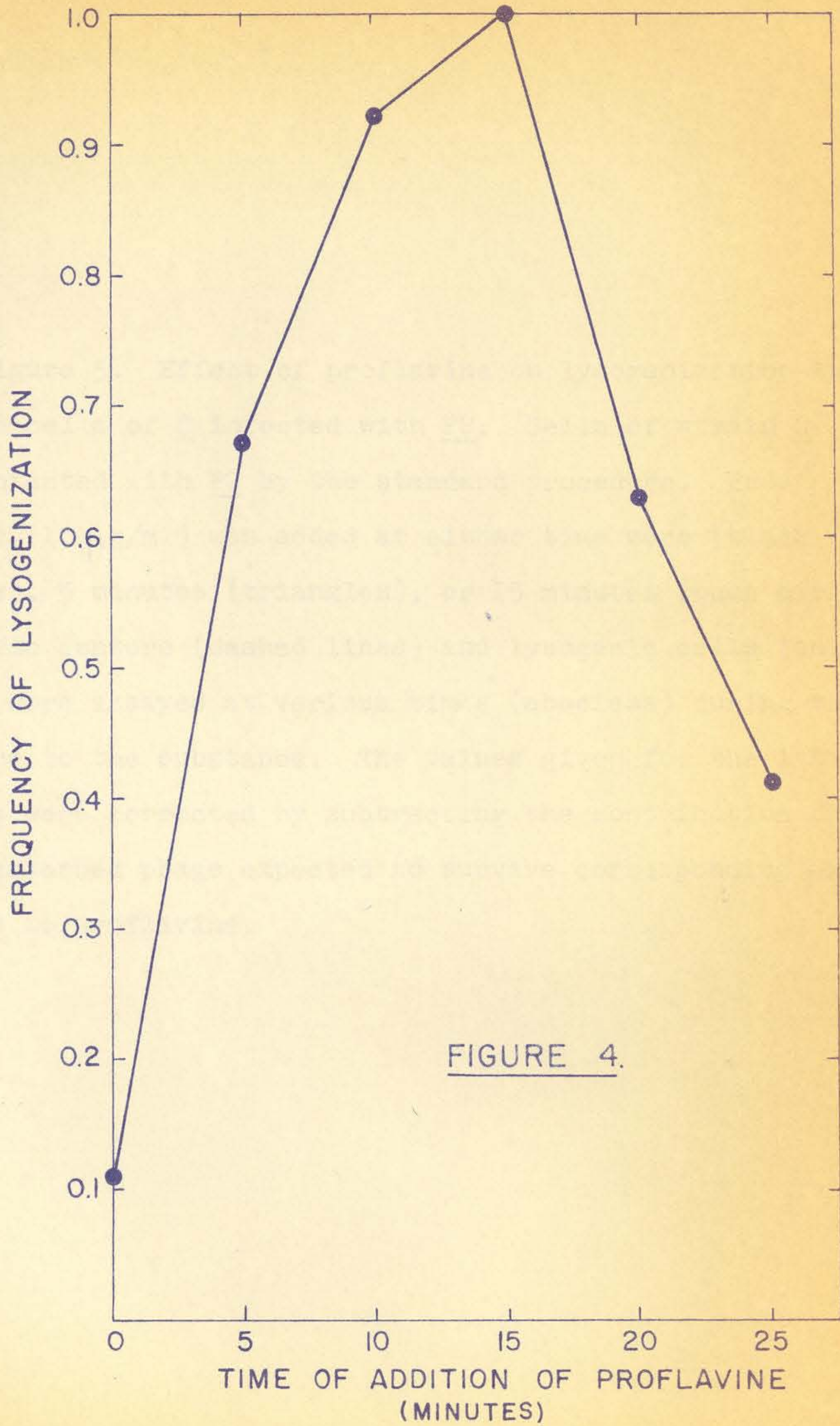

FIGURE 4.

Figure 5. Effect of proflavine on lysogenization in time on cells of _C_ infected with _P2_. Cells of strain _C_ were infected with _P2_ by the standard procedure. Proflavine (10 μg/ml) was added at either time zero (black circles), 5 minutes (triangles), or 15 minutes (open circles). The lytic centers (dashed lines) and lysogenic cells (solid lines) were assayed at various times (abscissa) during their exposure to the substance. The values given for the lytic centers were corrected by subtracting the contribution from the unadsorbed phage expected to survive corresponding exposures to proflavine.

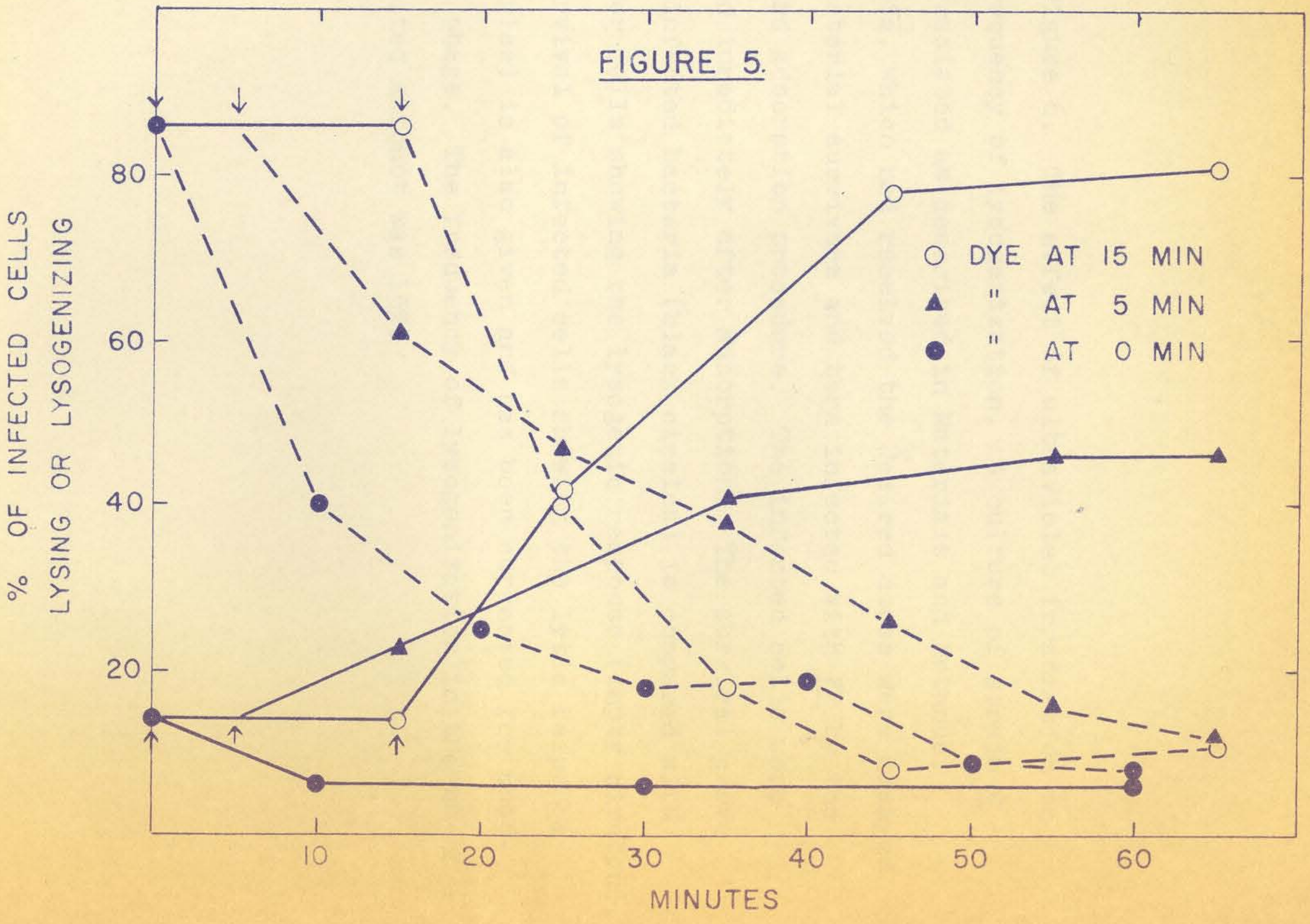
FIGURE 5.
○ DYE AT 15 MIN
▲ " AT 5 MIN
● " AT 0 MIN
% OF INFECTED CELLS
LYSING OR LYSOGENIZING
80
60
40
20
10
20
30
40
50
60
MINUTES

Figure 6. The effect of ultraviolet irradiation on the frequency of lysogenization. A culture of strain C was irradiated as described in Materials and Methods. Aliquots, which had received the desired doses were assayed for bacterial survivors and then infected with P2 by the standard adsorption procedure. The infected cells were assayed immediately after adsorption. The survival curve for uninfected bacteria (black circles) is compared with that for cells showing the lysogenic response (empty circles). The survival of infected cells showing the lytic response (triangles) is also given and has been corrected for unadsorbed phage. The frequency of lysogenization in the unirradiated aliquot was 15%.

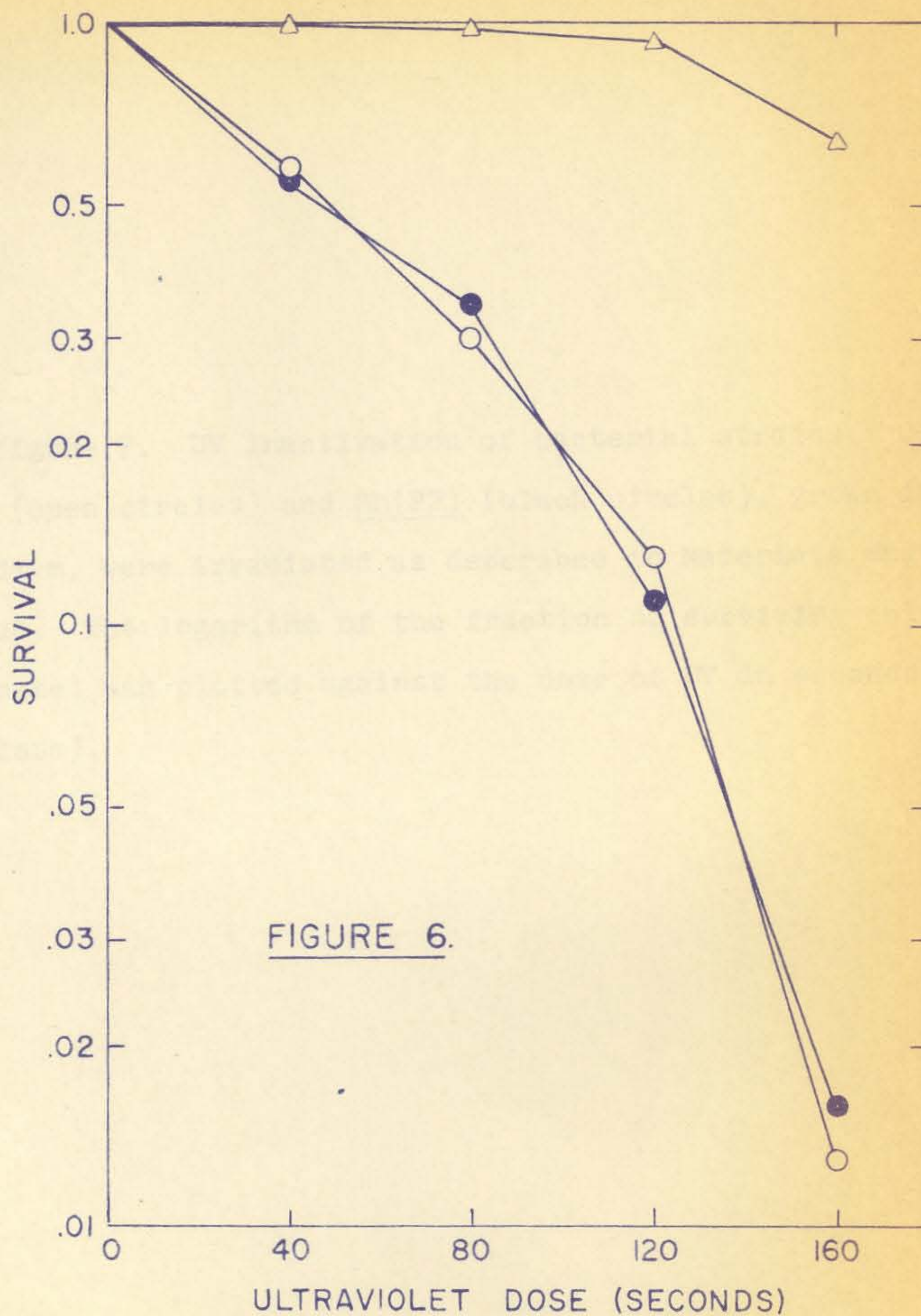
1.0
0.5
0.3
0.2
0.1
.05
.03
.02
.01
SURVIVAL
0
40
80
120
160
ULTRAVIOLET DOSE (SECONDS)
FIGURE 6.

Figure 7. UV inactivation of bacterial strains. Cells of Sh (open circles) and Sh(P2) (black circles), grown in LB medium, were irradiated as described in Materials and Methods. The logarithm of the fraction of surviving cells (ordinate) was plotted against the dose of UV in seconds (abscissa).

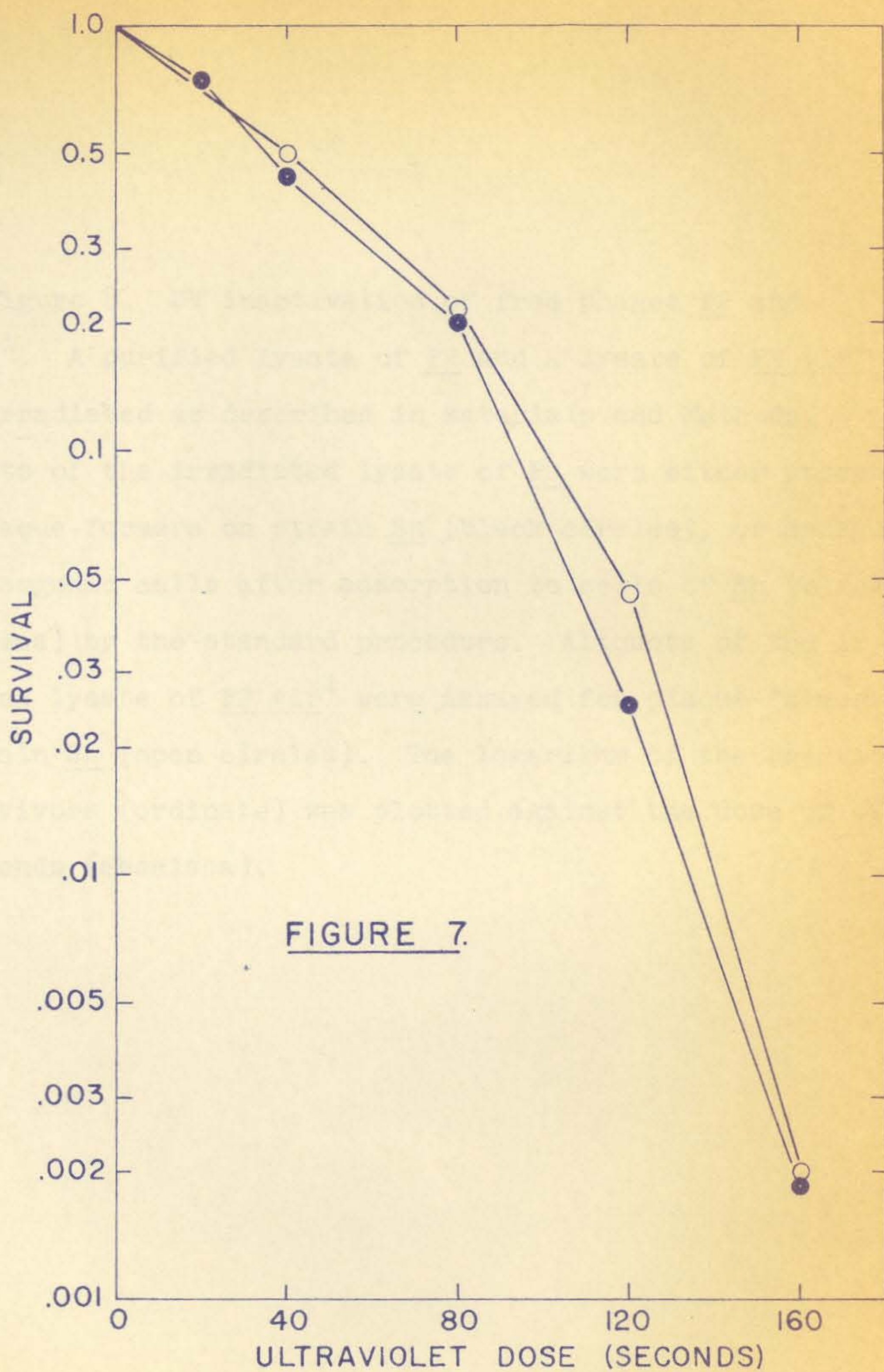

FIGURE 7.

Figure 8. UV inactivation of free phages P2 and P2 vir^1. A purified lysate of P2 and a lysate of P2 vir^1 were irradiated as described in Materials and Methods. Aliquots of the irradiated lysate of P2 were either assayed for plaque-formers on strain Sh (black circles), or assayed for lysogenic cells after adsorption to cells of Sh (black triangles) by the standard procedure. Aliquots of the irradiated lysate of P2 vir^1 were assayed for plaque-formers on strain Sh (open circles). The logarithm of the fraction of survivors (ordinate) was plotted against the dose of UV in seconds (abscissa).

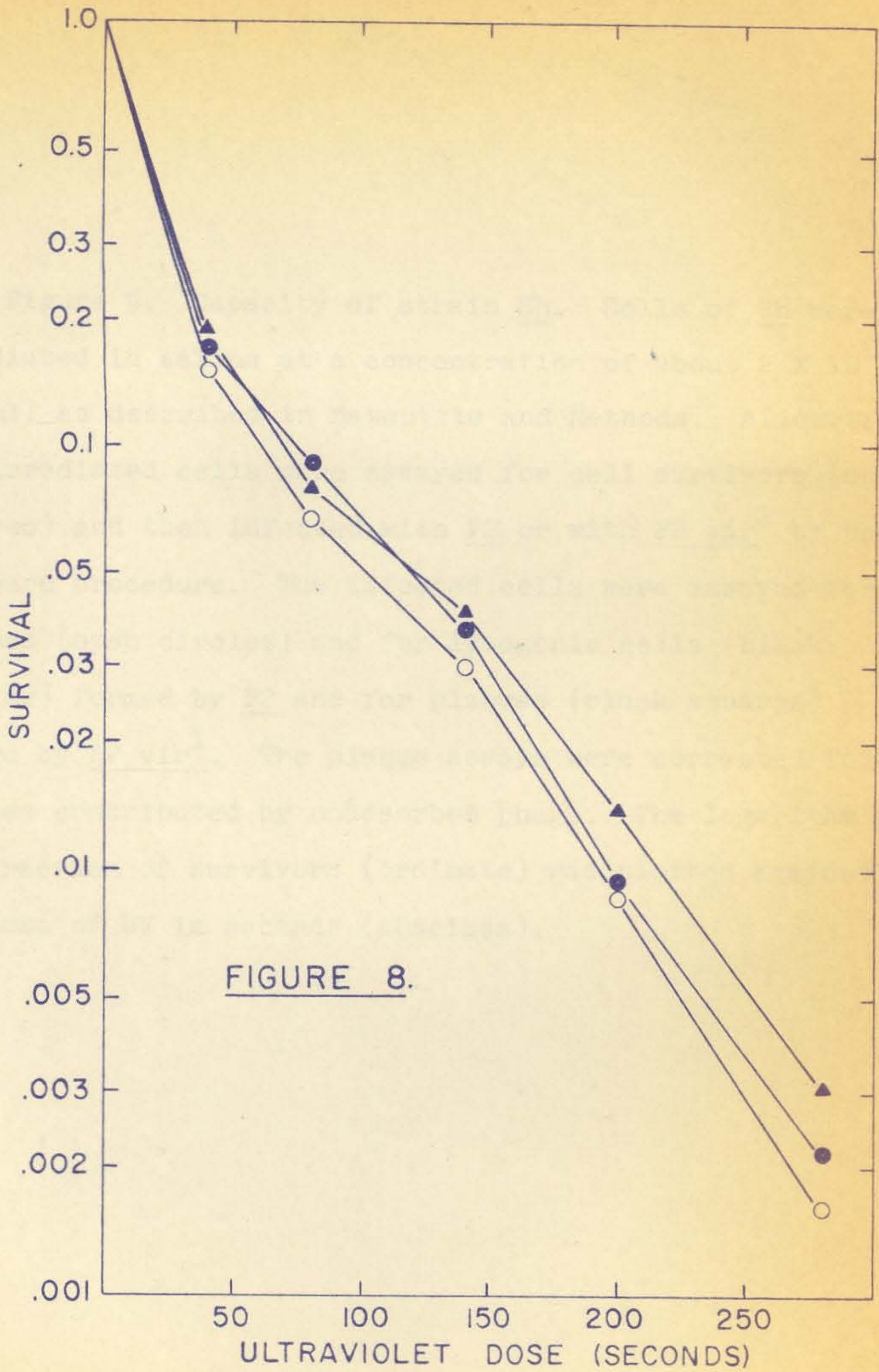
1.0
0.5
0.3
0.2
0.1
.05
.03
.02
.01
.005
.003
.002
.001
SURVIVAL
50
100
150
200
250
ULTRAVIOLET DOSE (SECONDS)
FIGURE 8.

Figure 9. Capacity of strain Sh. Cells of Sh were irradiated in saline at a concentration of about 2×10^8 per ml, as described in Materials and Methods. Aliquots of the irradiated cells were assayed for cell survivors (open squares) and then infected with P2 or with P2 vir^1 by the standard procedure. The infected cells were assayed for plaques (open circles) and for lysogenic cells (black circles) formed by P2 and for plaques (black squares) formed by P2 vir^1. The plaque assays were corrected for plaques contributed by unadsorbed phage. The logarithm of the fraction of survivors (ordinate) was plotted against the dose of UV in seconds (abscissa).

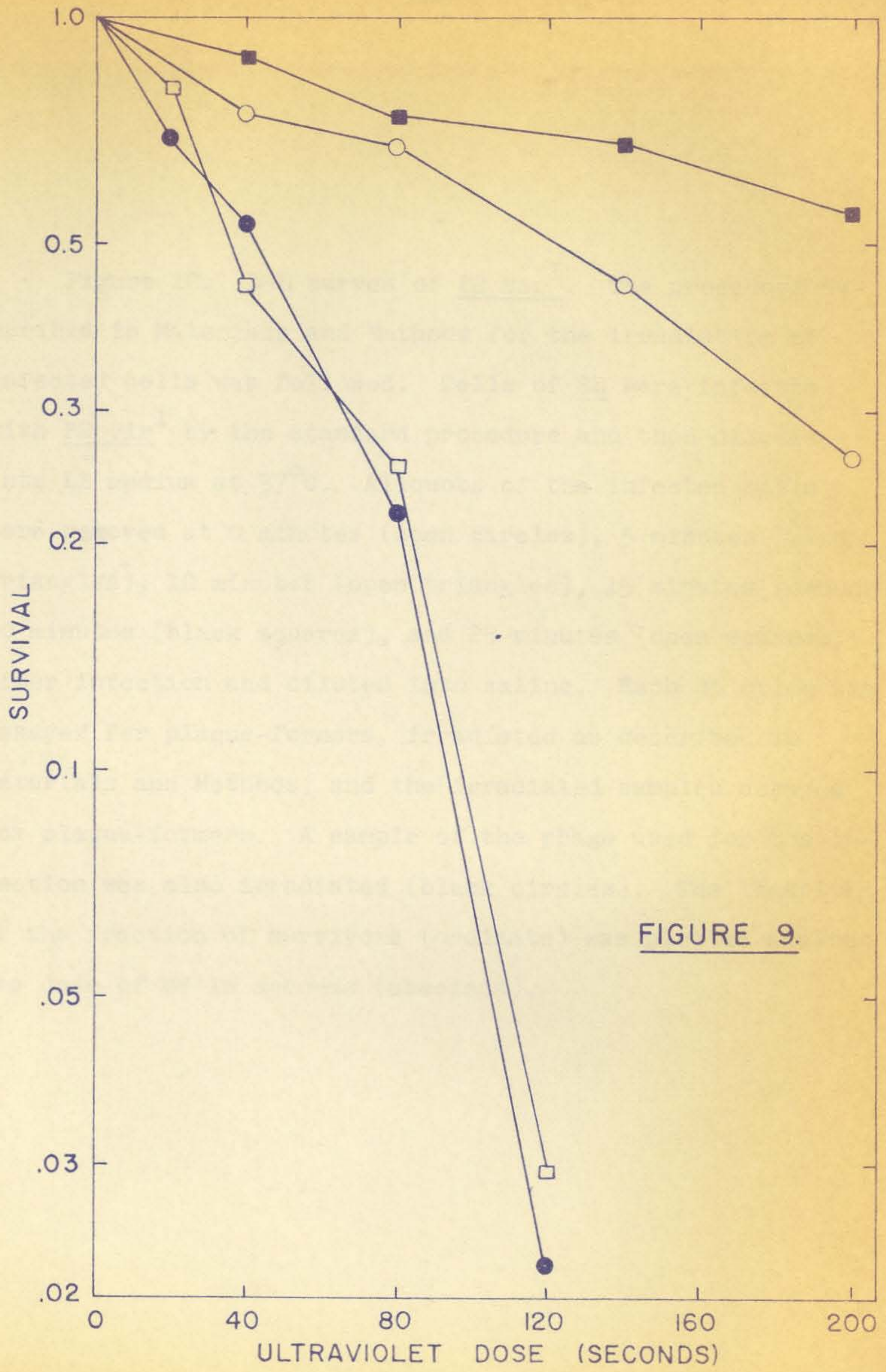
1.0
0.5
0.3
0.2
0.1
.05
.03
.02
SURVIVAL
0
40
80
120
160
200
ULTRAVIOLET DOSE (SECONDS)
FIGURE 9.

Figure 10. L-L curves of P2 vir[1]. The procedure described in Materials and Methods for the irradiation of infected cells was followed. Cells of Sh were infected with P2 vir[1] by the standard procedure and then diluted into LB medium at 37°C. Aliquots of the infected cells were removed at 0 minutes (open circles), 5 minutes (black triangles), 10 minutes (open triangles), 15 minutes (crosses), 20 minutes (black squares), and 25 minutes (open squares) after infection and diluted into saline. Each dilution was assayed for plaque-formers, irradiated as described in Materials and Methods, and the irradiated samples assayed for plaque-formers. A sample of the phage used for the infection was also irradiated (black circles). The logarithm of the fraction of survivors (ordinate) was plotted against the dose of UV in seconds (abscissa).

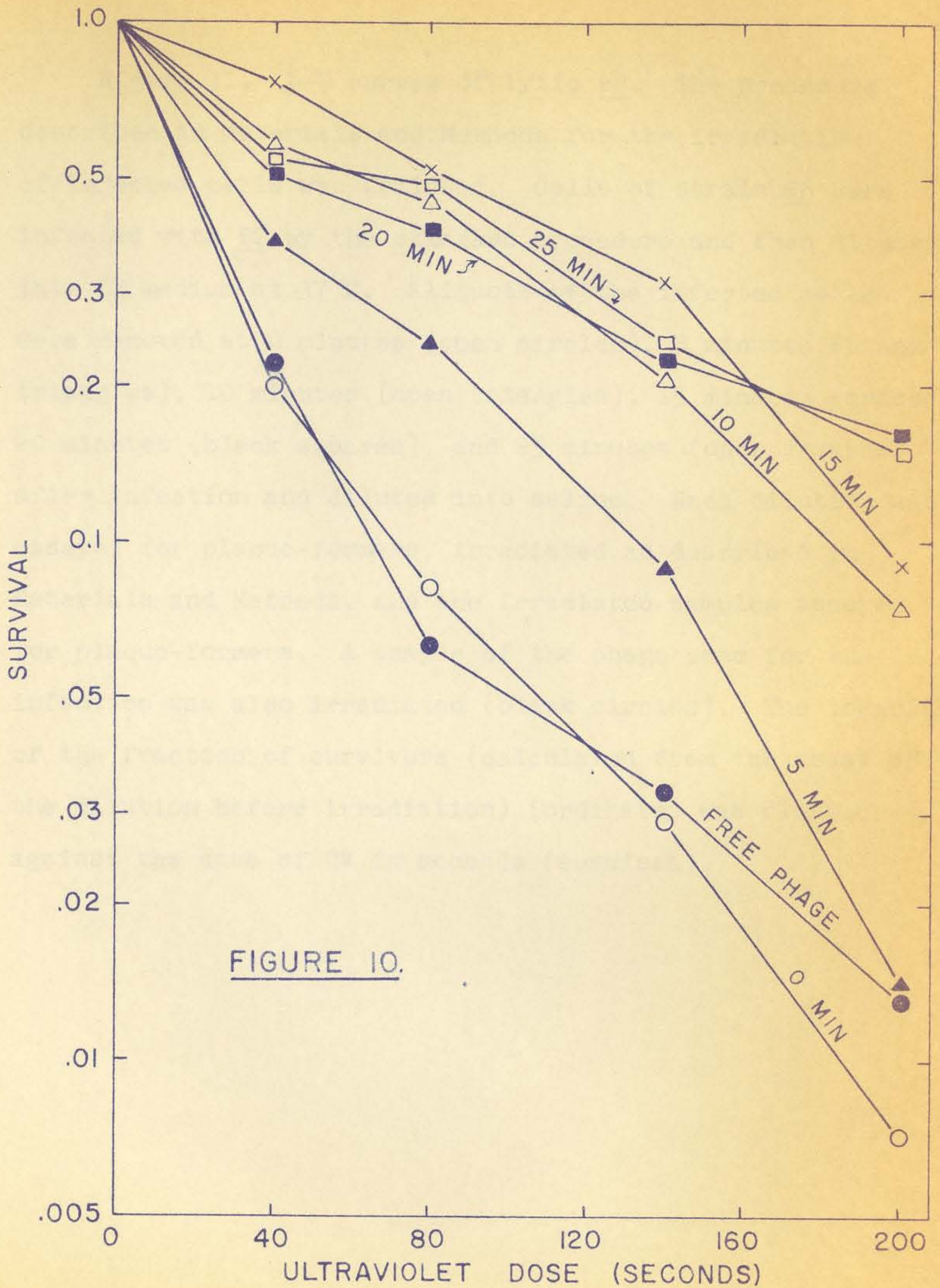

FIGURE 10.

Figure 11. L-L curves of lytic P2. The procedure described in Materials and Methods for the irradiation of infected cells was followed. Cells of strain Sh were infected with P2 by the standard procedure and then diluted into LB medium at 37°C. Aliquots of the infected cells were removed at 0 minutes (open circles), 5 minutes (black triangles), 10 minutes (open triangles), 15 minutes (crosses), 20 minutes (black squares), and 25 minutes (open squares) after infection and diluted into saline. Each dilution was assayed for plaque-formers, irradiated as described in Materials and Methods, and the irradiated samples assayed for plaque-formers. A sample of the phage used for the infection was also irradiated (black circles). The logarithm of the fraction of survivors (calculated from the assay of the dilution before irradiation) (ordinate) was plotted against the dose of UV in seconds (abscissa).

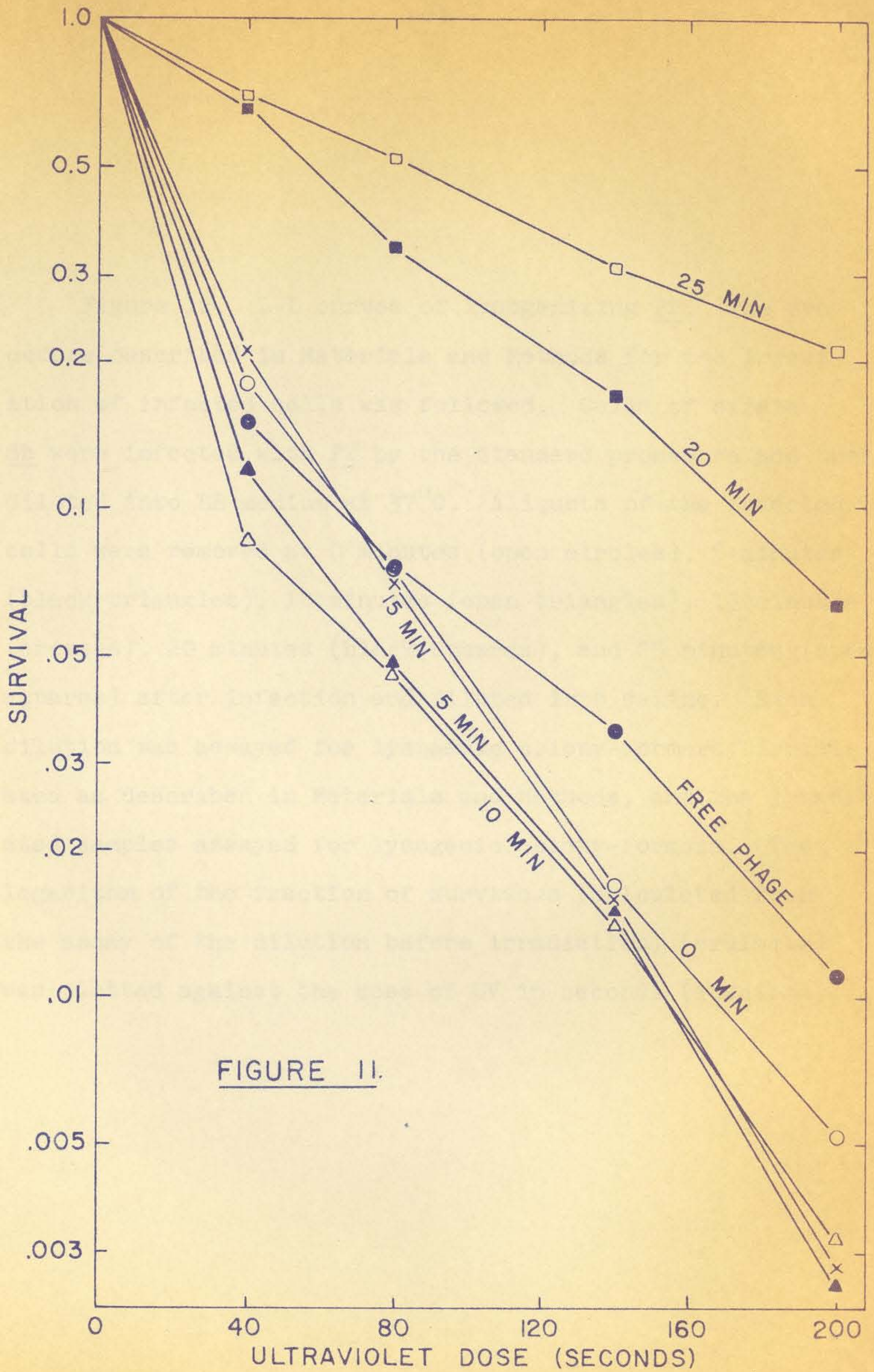

FIGURE 11.

Figure 12. L-L curves of lysogenizing P2. The procedure described in Materials and Methods for the irradiation of infected cells was followed. Cells of strain Sh were infected with P2 by the standard procedure and then diluted into LB medium at 37°C. Aliquots of the infected cells were removed at 0 minutes (open circles), 5 minutes (black triangles), 10 minutes (open triangles), 15 minutes (crosses), 20 minutes (black squares), and 25 minutes (open squares) after infection and diluted into saline. Each dilution was assayed for lysogenic colony-formers, irradiated as described in Materials and Methods, and the irradiated samples assayed for lysogenic colony-formers. The logarithm of the fraction of survivors (calculated from the assay of the dilution before irradiation) (ordinate) was plotted against the dose of UV in seconds (abscissa).

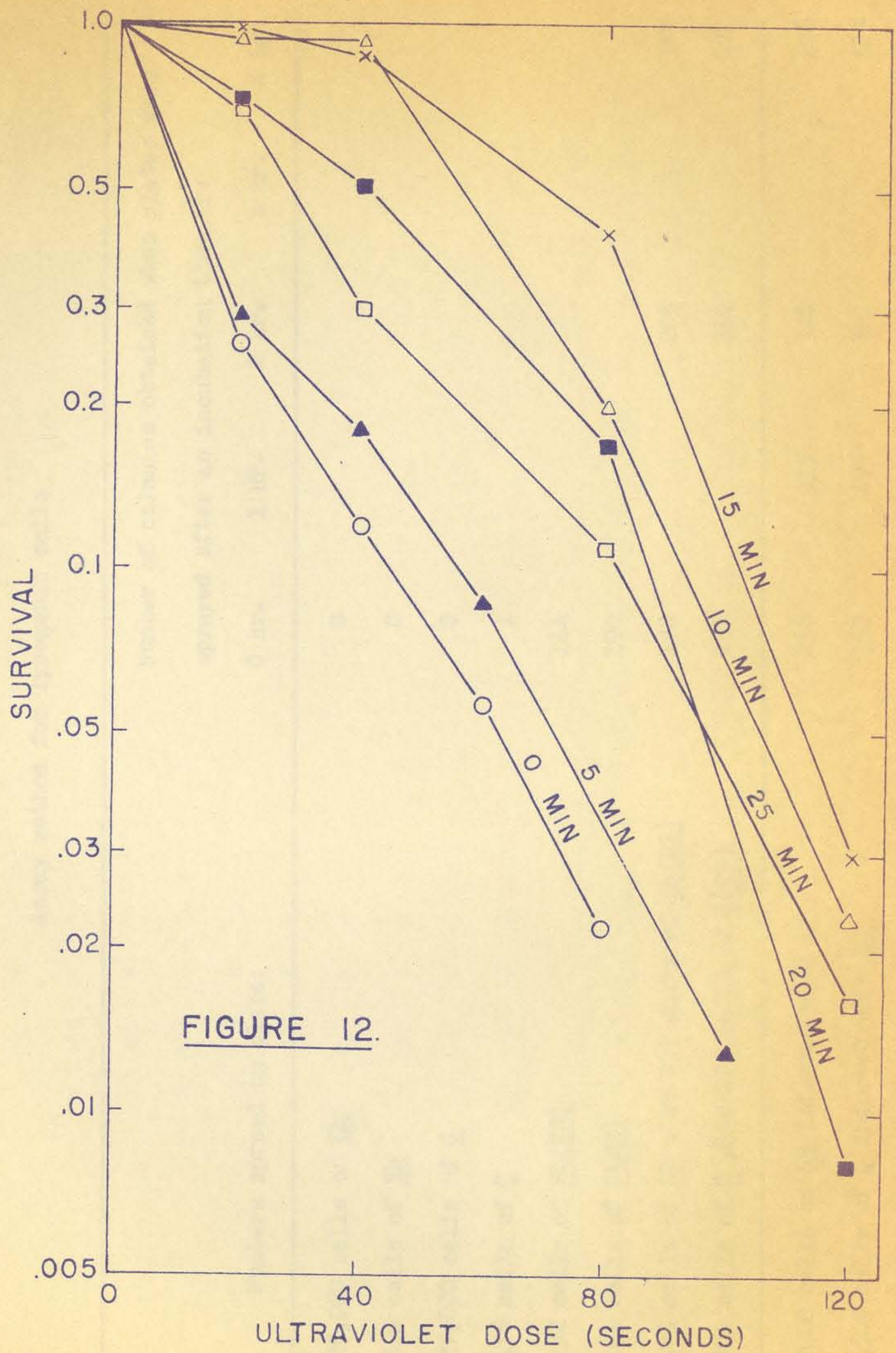

FIGURE 12.

Table 1

Assay method for lysogenic cells

	Number of colonies obtained when plates were sprayed after an incubation time of:				
Numbers spread on plate:	0 hr.	1 hr.	2 hr.	3 hr.	4 hr.
25,000 cells of Sh	0				
250 cells of Sh	0				
27,000 cells of C	0				
a) 135 cells of C	1				
132 cells of Sh(P2)	144				
194 cells of C(P2)	190				
193 cells of Sh plus 207 cells of Sh(P2)	188		206		197
204 cells of C plus 254 cells of C(P2)	253		240		272
b) 2000 cells of Sh infected with P2	236	315	328		296
b) 1500 cells of C infected with P2	357	497	501		519

	250 cells of Sh	0	0	0
	2000 " " "	1	3	1
	10,000 " " "	4	2	8
	50,000 " " "	12	37	125
c) 100,000 particles	500,000 " " "	91	184	320
of P2 plus:	250 " " C	0	1	2
	2000 " " "	2	2	18
	10,000 " " "	14	8	23
	50,000 " " "	18	72	154
	500,000 " " "	-	300	600

a) Cells of strains Sh, Sh(P2), C, or C(P2), grown in LB medium, were spread separately or mixed together in the numbers indicated, on hard LB agar plates. The plates were incubated at 37° C for the length of time indicated, sprayed with the standard amount of P2 vir[1] (about 10^8 particles per plate), and then returned to 37° C to allow colonies to develop.

b) Cells of strain Sh, grown in LB medium, or of strain C, grown in Davis medium, were infected with about 4 P2 phages per cell by the standard procedure. The adsorption mixtures were

spread at appropriate dilutions on hard LB agar plates. The plates were incubated at 37° C for various lengths of time, sprayed with the standard amount of P2 vir^1, and returned to 37° C to allow colonies to develop. The number of colonies given in the table is the sum of the numbers on a pair of equivalent plates.

c) Cells of strain Sh or of strain C, grown in LB medium, were spread on hard LB agar plates in the numbers indicated, together with particles of phage P2. The plates were incubated at 37° C for various lengths of time, sprayed with the standard amount of P2 vir^1, and returned to 37° C to allow colonies to develop.

The number of cells or of phage particles spread on the plates was estimated from independent assays.

Table 2

Attempt to recover lytic centers lost after treatment with chloromycetin as non-lysogenic colonies

m.o.i.	time of assay	lysing	lysogenizing	Fraction of total cells forming non-lysogenic colonies: observed	expected from m.o.i.	expected from assumption
0.9	0 min	47%	9%	38%	41%	
	60 min	20%	18%	31%	41%	56%
1.8	0 min	71%	13%	13%	17%	
	60 min	20%	24%	16%	17%	53%
3.6	0 min	93%	15%	3%	3%	
	60 min	20%	29%	4%	3%	62%

Cells of strain C were infected with various amounts of P2 by the standard procedure. Chloromycetin (25 μg/ml) was added at time zero, and assays were made at time zero, and after one hour of exposure to the drug. The multiplicity of infection (m.o.i.) was calculated as the average number of input phages adsorbed per cell. The fraction of total cells lysing or lysogenizing is calculated on the basis of the total input cells (set equal to 100%) assayed at the beginning of the experiment. The fraction of cells lysing is corrected for plaques formed by unadsorbed phage. The "observed" non-lysogenic colony-formers are calculated

Table 2 (continued)

by subtracting the number of lysogenic colony-formers from the total colony formers. The non-lysogenic colony formers "expected from m.o.i." are calculated on the basis of a Poisson distribution of the infecting phage. The non-lysogenic colony formers "expected from the assumption" are calculated on the assumption that all the lytic centers lost after one hour of chloromycetin treatment are recovered either as lysogenic or non-lysogenic colony formers, i.e. (non-lysogenic colony formers at time zero) + (lytic centers at time zero - lytic centers at 60 minutes) - (lysogenic colony formers at 60 minutes - lysogenic colony formers at time zero).

Table 3

Effect of chloromycetin on lysogenization by phage P22

	Titers/ml (adsorption tube)			
	Lytic centers	Lysogenic cells	Uninfected cells	Frequency of lysogenization
no chloromycetin	9.9×10^7	1.1×10^8	2.8×10^7	55%
chloromycetin added at t=0	5.0×10^7	1.5×10^8	4.0×10^7	75%
chloromycetin added at t=5 min	8.5×10^6	2.1×10^8	3.9×10^7	100%

Cells of strain T were infected with P22 by the standard procedure. Chloromycetin (25 μg/ml) was added either at time zero or at 5 minutes. Assays were made at 15 minutes for the untreated control, or after 15 minutes of exposure to the drug. The titer of input cells (adsorption tube) was 2.2×10^8/ml. No correction was introduced here for unadsorbed phage which was only 4% of the input phage.

Table 4

The effect of various treatments of cells infected with P2 on the frequency of lysogenization

Treatment	Experiment	Untreated control		Treated			
				Assays at t=30 min.		Assays at t=60 min.	
		Fraction of infected cells		Fraction of infected cells		Fraction of infected cells	
		lysing	lysogenizing	lysing	lysogenizing	lysing	lysogenizing
KCN (10^{-2} M)	a.	76%	24%	-	-	62%	15%
	b.	81%	19%	69%	19%	69%	14%
	c.	80%	20%	-	-	53%	19%
	d.	86%	14%	-	-	56%	9%
dinitrophenol ($3X10^{-3}$ M)	a.	76%	24%	-	-	81%	21%
	b.	81%	19%	80%	21%	76%	15%
	c.	80%	20%	-	-	68%	15%
NaN_3 (10^{-2} M)	a.	76%	24%	-	-	81%	15%
5-methyl tryptophan (500 μg/ml)	a.	76%	24%	-	-	58%	46%
(2 mg/ml)	b.	83%	17%	63%	21%	56%	36%

Table 4 (continued)

5-methyl tryptophan (2 mg/ml)	c.	84%	16%	78%	31%	52%	45%
5-methyl tryptophan (2 mg/ml and tryptophan (200 µg/ml)	a.	84%	16%	85%	14%	lysis	15%
allyl-glycine (500 µg/ml)	a.	83%	17%	71%	27%	52%	32%
C Arg⁻ starved for arginine	a.	79%	21%	50%	50%	39%	46%
5-OH-uridine (50 µg/ml)	a.	82%	18%	-	-	lysis	44%
(10 µg/ml)	b.	82%	18%	-	-	lysis	52%
"	c.	87%	13%	70%	27%	lysis	47%
5-OH-uridine (50 µg/ml) and uridine (50 µg/ml)	a.	82%	18%	-	-	lysis	23%
5-OH-uridine (10 µg/ml) and uridine (10 µg/ml)	a.	82%	18%	-	-	lysis	24%
C pretreated with 5-OH-uridine (10 µg/ml) for 30 minutes before infection	a.	87%	13%	-	-	lysis	45%

Table 4 (concluded)

Cells of strain C were infected with P2 by the standard procedure. The sum of the numbers of cells lysing and of cells lysogenizing in the untreated control is set equal to 100%. Titers of lysing cells are corrected for plaques formed by unadsorbed phage. The number of lysogenic cells in the untreated control is measured by making three assays - at time zero, 25 minutes and 60 minutes - and averaging these figures. In all cases the treatment started at time zero unless otherwise stated.

Table 5

Attempt to reverse the effect of proflavine by riboflavine, nucleotides, or reduced illumination

	Fraction of infected cells lysogenized	
Treatment started at	0 min.	15 min.
proflavine (10 μg/ml), normal light	4%	102%
proflavine (10 μg/ml), reduced light	13%	100%
proflavine (10 μg/ml) plus riboflavine (10 μg/ml), normal light	4%	70%
proflavine (10 μg/ml) plus nucleotides (10 μg/ml each), normal light	3%	100%
riboflavine (10 μg/ml), normal light	16%	23%
nucleotides (10 μg/ml each), normal light	13%	22%

Cells of strain C were infected with P2 by the standard procedure. Proflavine (10 μg/ml), riboflavine (10 μg/ml), a mixture containing 10 μg/ml of each of five nucleotides (adenylic, guanylic, cytidylic, thymidylic, and uridylic acids), or combinations of these substances, were added at time zero or time 15 minutes during the latent period, in normal light or reduced light, and the infected cells were assayed after one hour of exposure to the various substances. The fraction of infected cells lysogenized is calculated on the basis of the total infected cells (set equal to 100%) in the untreated control. The frequency of lysogenization in the untreated control was 17%.

Table 6

Effect of an increased concentration of chloromycetin or a combination of chloromycetin and proflavine on the frequency of lysogenization

Substance added at 15 minutes	Fraction of infected cells: lysing	lysogenizing
untreated control	83%	17%
chloromycetin (25 μg/ml)	43%	45%
chloromycetin (200 μg/ml)	29%	52%
proflavine (10 μg/ml)	10%	86%
chloromycetin (25 μg/ml) + proflavine (10 μg/ml)	19%	44%

Cells of strain C were infected with P2 by the standard procedure. Chloromycetin (25 μg/ml or 200 μg/ml) or proflavine (10 μg/ml) or a combination of the two was added 15 minutes after infection, and the infected cells were assayed for lysing and lysogenizing cells after one hour of exposure to the various substances. The fraction of infected cells lysing or lysogenizing is calculated on the basis of the total infected cells (set equal to 100%) in the untreated control. The fraction of lysing cells has been corrected for plaques produced by unadsorbed phage.

Table 7

The effect of chloromycetin on the lysogenization of irradiated cells

Treatment	Fraction of infected cells lysogenized	
	no chloromycetin	chloromycetin added at 10 minutes
unirradiated control	14%	60%
cells irradiated, and infected immediately	7%	30%
cells irradiated, and infected after 30 minutes growth in Davis medium	9%	30%
cells irradiated, and infected after 60 minutes growth in Davis medium	7%	25%

A culture of C was irradiated as described in Materials and Methods, and the cells were either infected with P2 immediately after irradiation (standard adsorption procedure), diluted into Davis medium at 37° C and exposed to chloromycetin (25 μg/ml) for one hour (the drug being added at 10 minutes), or the cells were first diluted into Davis medium at 37° C and allowed to grow for 30 minutes or 60 minutes, and then centrifuged, resuspended in Davis salts, infected with P2, diluted again into Davis medium and exposed to chloromycetin (25 μg/ml) for one hour (again the drug is added at 10 minutes). After the irradiated cells had been growing for some time in Davis medium, they became somewhat sensitive to

Table 7 (continued)

killing by centrifugation and a slight correction has been made to take care of this. The frequencies of lysogenization are calculated on the basis of the total infected cells (set equal to 100%) in the unirradiated control.