PHAGE-HOST RELATIONSHIPS IN NONTOXIGENIC AND TOXIGENIC DIPHTHERIA BACILLI[1]

W. LANE BARKSDALE[2, 3] AND A. M. PAPPENHEIMER, JR.

WITH THE TECHNICAL ASSISTANCE OF EDELMIRA D. HENDEE

Department of Microbiology, New York University College of Medicine, New York, New York

Received for publication August 17, 1953

The taxonomic position of corynebacteria that possess all of the characteristic properties of *Corynebacterium diphtheriae* save only the ability to produce diphtherial toxin has been in dispute ever since the discovery of the diphtheria bacillus. The more conservative view has been that the nontoxin producing strains are "avirulent" diphtheria bacilli (Wilson and Miles, 1946). Their relationship to *C. diphtheriae* has been of particular importance from the epidemiological point of view since nontoxigenic strains have been found associated with epidemic diphtheria (Okell, 1929; Frobisher *et al.*, 1947; Edward and Allison, 1951). Until recently the only acceptable evidence which demonstrated a relationship between nontoxigenic and toxigenic strains was the finding by Crowell (1926) and by Cowan (1927) that nontoxigenic strains can be derived from toxigenic ones. In a carefully controlled series of experiments in which pure line descendants of a single cell of toxigenic *C. diphtheriae* were selected and tested for toxin production, Crowell derived one daughter cell line which produced no detectable toxin. Efforts to restore the toxigenicity of this line by guinea pig passage were unsuccessful. Cowan (1927) reported the derivation of nontoxigenic variants from the well known "Park Williams no. 8" strain.

While there seems little doubt that nontoxigenic strains have been obtained from toxigenic stocks, there was not, until the report of Freeman (1951), any evidence that toxigenic *C. diphtheriae* can be derived from nontoxigenic strains. Freeman treated populations of known nontoxigenic cultures on agar plates with a bacteriophage, B, and incubated the plates long enough to permit resistant colonies to grow out. When certain of these surviving colonies were tested in guinea pigs, they were found to produce toxin; when they were tested with phage B, they were found to be resistant and lysogenic. Subsequently, all strains converted from the nontoxigenic to the toxigenic state by Freeman were found to be lysogenic. Parson and Frobisher (1951), Groman (1953*a*), and Hewitt (1952) have reported similar findings on several strains of diphtheria bacilli including those with which Freeman worked.

It has been suggested that diphtherial toxin is related to the protein moiety of diphtherial cytochrome *b* (Pappenheimer and Hendee, 1947). The evidence for this hypothesis has been obtained from studies of the iron metabolism of the diphtheria bacillus. Freeman's discovery of the close relationship between phage infected, toxigenic diphtheria bacilli and uninfected, nontoxigenic diphtheria bacilli indicates the need for an investigation of the comparative metabolism of pairs of these strains, particularly with reference to their iron containing enzymes. Before undertaking such a study, we have felt that some knowledge of the diphtheria phage-host cell systems was needed. In the present paper are reported the results of a study of specific pairs of toxin producing and nontoxin producing bacilli in relation to the diphtherial bacteriophage, B, and certain of its temperate mutants.

MATERIALS AND METHODS

Cultures of bacteria and bacteriophage. Strains of C4 and C7 were received from Dr. Victor J. Freeman[4] as strains C444 and C770, respectively.

[1] These studies were supported in part by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army, Contract No. DA-49 and by a grant from the United States Public Health Service.

[2] William Hallock Park Fellow, New York University, 1949–1951.

[3] Submitted by W. L. Barksdale in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology, New York University.

[4] We are indebted to Dr. Victor J. Freeman for cultures of strains C444 and C770 and for a stock of phage B.

Both strains are nontoxigenic, *mitis*-like organisms and both are sensitive to lysis by virulent bacteriophage B also obtained from Dr. Freeman. St-5 is an avirulent *mitis*-like strain which has been maintained in this laboratory for some years. It is resistant to phage B but sensitive to a bacteriophage carried by the Park-Williams no. 8 strain.

Strains C4(β_1), C4(β_2), C7(β_1), and C7(β_2) are resistant to phage B and are lysogenic, toxin producing strains derived by treatment of C4 and C7 with the temperate phages β_1 and β_2. These phages, as will be shown later, are mutants of phage B which produce turbid plaques on the indicator strains. The P.W.8 strain used was originally obtained from the Connaught Laboratories in Toronto and has been maintained for some years in this laboratory.

To provide uniform inocula for use in the experiments to be described, cultures in the logarithmic phase of growth were subdivided and stored at −15 C in the deep freeze.

Culture media. 1. *PGT*[5] *liquid medium.* Mueller and Miller's (1941) medium was modified so as to support growth of the C4 and C7 strains by supplementing it with 0.5 mg calcium pantothenate, 0.5 g L-glutamic acid, and 100 mg DL-tryptophan per liter. The final pH was adjusted to 6.7 to 6.8, and the medium was dispensed in 30 ml amounts in 300 ml Erlenmeyer flasks and autoclaved at a pressure of 10 lb for 10 minutes.

2. *Solid medium for plaque counts.* Most of the phage titrations were carried out by plating on the PGT medium containing 1.5 per cent washed agar (Difco) as base. Thirty ml of base agar were used per petri dish, giving a depth of about 5 mm. The soft agar used in the agar-layer technique for phage assay contained 0.7 per cent agar.

3. *Tryptose-whey agar for plaque counts.* While the PGT medium best reveals differences in the host ranges of the β phages, we have recently found that the following simple medium gives larger and more uniform plaques and somewhat more reproducible counts.

A. *Base agar.* Five g NaCl, 10 g tryptose (Difco), and 15 g agar are made up to one liter with distilled water and sterilized by autoclaving.

B. *Soft agar.* Five g NaCl, 10 g tryptose, and 7 g agar are made up to one liter with distilled water. Just before use one part each of Supplements 1 and 2 are added to 10 parts of the melted soft agar.

Supplement 1: Fifty g glucose, 0.1 ml $FeSO_4 \cdot 7H_2O$ (1 per cent in 0.1 N HCl), 1.5 ml 0.1 per cent calcium pantothenate, and 7.5 ml 10 per cent $CaCl_2 \cdot 2H_2O$ are made up to 250 ml and sterilized by autoclaving.

Supplement 2: Dissolve 48 g dried skim milk in 500 ml distilled water. Bring to pH 3 with dilute HCl and centrifuge. The supernatant is adjusted to pH 6.5 to 6.8 with KOH and sterilized by filtration through a Seitz filter.

It will be noted that no pH adjustment is required except in the case of Supplement 2. The final pH after autoclaving is usually about 6.8 to 7.0.

Growth curves. Well dispersed bacterial cultures are essential for quantitative studies involving the action of diphtherial phages. The tendency of diphtheria bacilli to grow in clumps may be virtually eliminated by constant shaking. Starter cultures were prepared by inoculating PGT medium with thawed stocks and incubating the fresh cultures overnight at 35 C on a shaking shelf making 120 strokes per minute. All experiments were made using cells in the logarithmic phase of growth shaken continuously in a water bath at 36.4 C. Growth was followed by determining the optical density at 590 mμ in the Beckman spectrophotometer and by viable counts in neopeptone agar. In figure 1 are plotted growth curves of the C4, C4(β_1), and P.W.8 strains. Both C4 and C7 strains and their lysogenic derivatives have generation times close to 70 minutes at 36.4 C. The P.W.8 strain grows more slowly, and its division time is about 155 minutes. Table 1 shows the relation between optical density, viable count, and bacterial nitrogen.

As a protective agent against surface denaturation of phage and of toxin, 0.1 per cent Eastman's purified gelatin was added to the PGT medium.

Phage titrations. Plaque counts were carried out using the modification of the agar layer technique of Gratia described by Adams (1950). Plaques 1 to 1.5 mm in diameter may be obtained with B and β phages providing an actively growing, *well shaken* culture of a suitable indicator strain is used. The optimal bacterial concentration lies between 2 and 4 $\times$ 10^8 bacteria per plate. In order to insure optimal adsorption and plaque formation, free calcium ions must be present. Plates are incubated at 30 C.

[5] Code letters for medium

Preparation of high titer stocks of phage B. Phage extracted from a single clear plaque was mixed with a small amount of actively growing C4 culture in melted PGT soft agar and poured over the surface of PGT agar plates. Plates showing confluent lysis were extracted with broth. Even when agar layers from a considerable number of plates showing confluent lysis are extracted by the method of Swanstrom and Adams (1951), it is not possible to obtain titers exceeding about 6×10^8 per ml. Higher titers may be obtained by adsorbing phage of titer 2 to 6×10^8 to the C4 strain in liquid medium containing free calcium ions and after one complete growth cycle, adding either phosphate or citrate to prevent further adsorption of phage. After the second burst has occurred, the culture is centrifuged and the supernatant passed through a Selas porcelain filter. This method has yielded regularly 10^{10} or more particles of phage B per ml.

Preparation of β_1 stocks. It is possible to prepare high titer β_1 phage by propagation in the C7 strain using a method similar to that described for preparing high titer B phage. This method is not to be recommended, however, because it leads to contamination with the virulent variant which, once it has appeared as a result of mutation, has a selective advantage over the temperate β phage.

The most satisfactory method for production of pure β stocks is by ultraviolet irradiation of the lysogenic strain (see below) followed by adsorption of the released phage to the C7 strain. After one burst of the latter in the presence of added phosphate or citrate to prevent further adsorption, harvests are made. Titers from 0.5 to 1×10^9 β_1 particles per ml have been obtained by this method.

For conversion experiments, the phage was further concentrated. For example, 100 ml β_1 phage (5.5×10^8 per ml) obtained as above were dialyzed against 5 liters M/50 Na_2HPO_4 containing two grams NaCl per liter. The pH after dialysis was 8.0. The dialyzed phage was concentrated then in a cellophane bag before an electric fan to a volume of about 6 ml and a titer of 5.1×10^9 particles per ml. At lower pH in the absence of added NaCl considerable loss

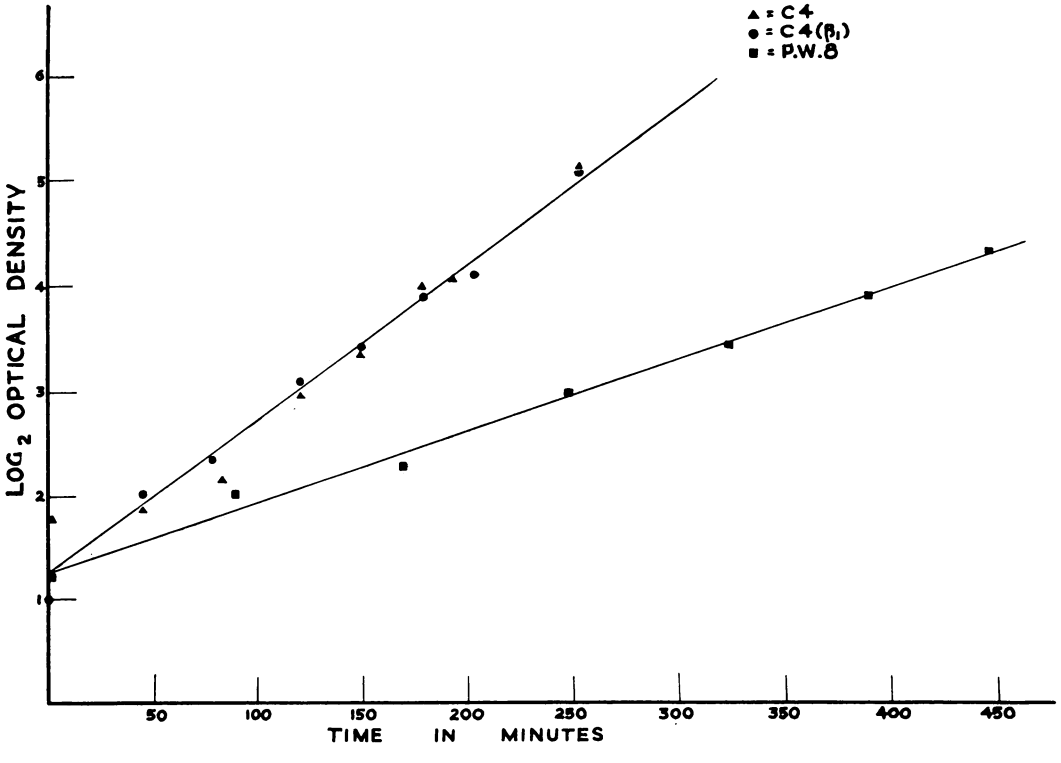

Figure 1. Growth curves of strains of *Corynebacterium diphtheriae*

TABLE 1

Growth of strain C4

TIME	OPTICAL DENSITY* AT 590 mμ	VIABLE COUNT (CELLS PER ML × 10^8)		KJELDAHL NITROGEN IN MICROGRAM PER ML
min				
0	0.121	1.76	1.8	6.56
		1.8		6.36
25	0.162			
45	0.185			
60	(0.215)	4.6	4.3	12.51
		4.0		11.52
85	0.258			
95	0.289			
105	0.338			
115	0.352			
120	(0.370)	7.1	6.75	16.4
		6.4		19.5

* Figures in parenthesis determined from growth curve.

From the above table it may be calculated that optical density (590 mμ) = 0.100 ≏ 5.6 μg bacterial N/ml ≏ 1.8 × 10^8 bacteria per ml.

may occur, presumably, because of adsorption to the cellophane. Finally, the concentrated phage was dialyzed overnight against one liter sterile PGT medium and filtered, giving a sterile stock containing 2.5 × 10^9 β_1 particles per ml.

Irradiation with ultraviolet light. A 15 watt "germicidal" lamp (G.E.), emitting 80 per cent of its energy in the wavelength 2537 A, was used as a source of ultraviolet light. Samples were irradiated in a layer of medium two mm thick at a distance of 76 cm from the center of the lamp.

Toxigenicity tests. The intracutaneous test of Fraser and Weld (1926) was employed in rabbits. Twenty to 25 cultures (40 to 50 sites, including controls) could be tested in a single rabbit. Organisms to be tested were grown overnight in neopeptone meat infusion broth. Two-tenths ml of each culture was injected intracutaneously at the various sites from tuberculin syringes. The remaining cultures in the syringes were stored then for 5 hours in the cold. At this time 2,000 units of commercial diphtheria antitoxin were administered intraperitoneally, and the corresponding control sites were injected with the stored organisms. By this means each test culture served as its own control. A positive lesion consists of a central necrotic zone surrounded by an area of erythema. These lesions are readily distinguishable from the localized, purulent reactions produced by the nontoxin producing indicator strains, C4 and C7. In order to recover diphtheria bacilli from the lesions, the rabbits were killed and the reaction sites wiped with acetone. The interior of each lesion was abraded with a sterile cotton swab, and the organisms obtained in this way were streaked on chocolate agar plates. After 48 hours' incubation at 37 C colonies were selected for subculture.

EXPERIMENTAL RESULTS

Properties of B and β_1 phages. When a drop of phage B stock is placed on an agar plate, the surface of which has been spread uniformly with cells of the C4 strain, a clear zone of lysis appears after incubation and only a few "resistant" colonies arise within the lysed area. Some of these resistant colonies are lysogenic and toxigenic. The C4 (β_1) strain was isolated from a lesion produced in the skin of a guinea pig inoculated with a culture picked from such a B resistant colony. The phage, β_1, carried by this derived strain differs in certain important respects from phage B.

Plaque morphology. Phage B produces clear, transparent plaques on both indicator strains C4 and C7. On the other hand, *none* of the lysogenic strains derived from C4 or from C7 has been found which carries a phage capable of producing clear plaques on these indicator strains. Phage β_1 usually produces no plaques on C4 although under certain conditions cloudy areas of barely perceptible outline may be distinguished. Fortunately, on strain C7, β_1 phage produces turbid plaques which may be counted without difficulty.

It would appear, therefore, that β_1 is a "temperate" (Jacob *et al.*, 1953) mutant of phage B, and as will be demonstrated below, β_1 and not B is the agent responsible for bringing about the conversion of C4 to C4(β_1). One other temperate variant of phage B, β_2, has been encountered. β_2 produces turbid plaques on the C4

TABLE 2

Plaque morphology of B and β phages

INDICATOR STRAIN	PHAGE		
	B	β_1	β_2
C4	Clear	None	Cloudy
C7	Clear	Cloudy	None

strain but no visible plaques on C7. The plaque characteristics of these three phages when applied to the two indicator strains are summarized in table 2.

The reverse mutation of β_1 to B can be demonstrated by propagation of β_1 on C4 or C7. The occasional clear B plaques which appear on plating are distinguished readily from the large number of cloudy β_1 plaques. The phage extracted from such clear plaques produces clear plaques on both indicator strains.

Stability. High titer stocks of both B and β_1 phages slowly lose their potency even when stored in the cold in broth (Groman, 1953*b*). It is necessary therefore to prepare fresh stocks at frequent intervals.

In broth at 56 C B and β_1 are inactivated at the same rate according to a first order process. At this temperature the titer of both mutants falls to half the initial value within approximately 6 minutes.

Adsorption. Adsorption of both B and β_1 phages to the C4 and C7 strains is inhibited to the same extent by citrate and by phosphate ions. No plaques are formed on media from which calcium ions have been removed as com-

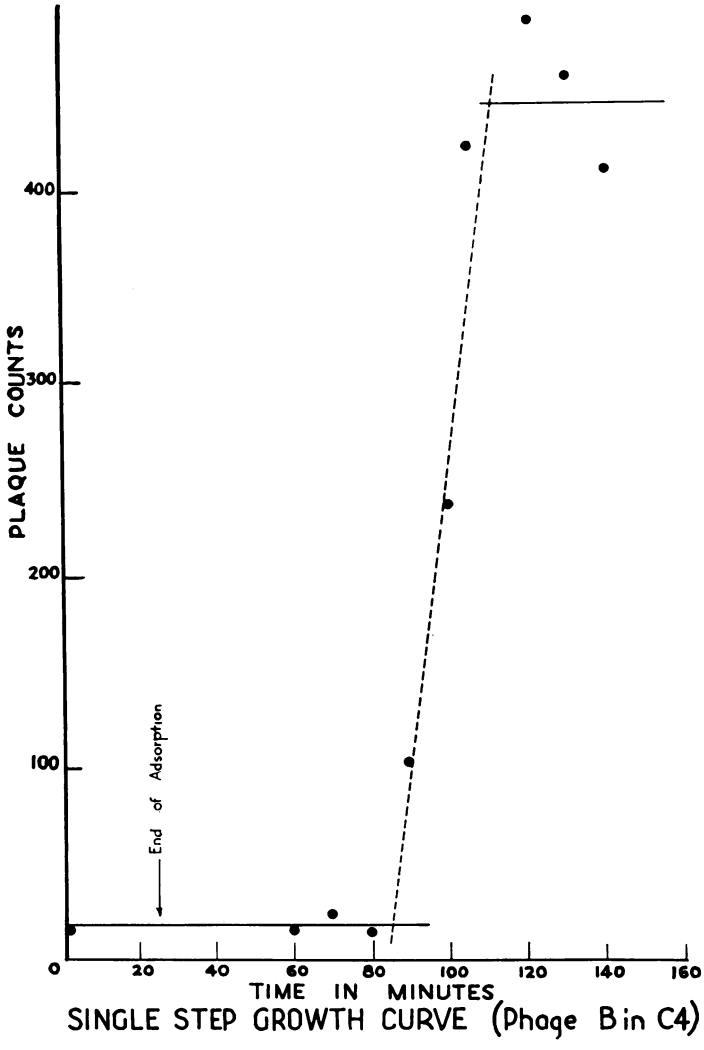

Figure 2. Growth of phage B in strain C4 at 36.4 C. One-tenth ml phage B (3.6×10^8 particles per ml) added to 1.9 ml C4 (1.1×10^8 cells per ml) in PGT medium containing 0.1 per cent gelatin and M/500 $CaCl_2 \cdot 2H_2O$. Diluted 10^5 times in broth at 25 minutes.

pletely as possible. In general, addition of $CaCl_2 \cdot 2H_2O$ to a final concentration of 0.001 M results in satisfactory plaques on solid medium and a satisfactory rate of adsorption in liquid medium, but the quantity of calcium necessary for most efficient adsorption will, of course, vary with the different media and different bacterial populations. Moreover, it is probable that other factors besides calcium ions are involved in adsorption to the bacteria. In any case, providing conditions are comparable, the rate of adsorption of phage B and its temperate mutant β_1 to strains C4 and C7 is the same.

Burst size and latent period. The technique of Ellis and Delbrück as described by Adams (1950) for the one step growth curve was employed for studying the growth of B and β_1 in C4 and C7. In the experiment illustrated in figure 2, phage B and cells of C4 were incubated together for 25 minutes at 36.4 C before dilution. Adsorption was 82 per cent complete at this time. The multiplicity corrected for adsorption was 0.15. As seen from figure 2, the burst occurred after an 86 minute latent period, liberating close to 30 phage B particles per infected bacterium.

A similar experiment, in which the growth of β_1 phage in C7 was followed, gave a latent period of 77 minutes and a burst size of 24.

Spontaneous release of bacteriophage by lysogenic strains. With the indicator strains and temperate phages mentioned thus far, four lysogenic strains have been obtained: C4(β_1), C4(β_2), C7(β_1), and C7(β_2). As a result of numerous titrations of supernatants of one of these, C4(β_1), it appears that in actively multiplying populations about one cell in 50,000 lyses with the release of β_1 phage. The number of cells undergoing similar lysis in C7(β_1) populations has always been found to be higher than in C4(β_1); the difference has ranged from about 2 to 5-fold.

Induction of lysis by irradiation with ultraviolet light. Lwoff *et al.* (1950) have shown that cultures of lysogenic *Bacillus megaterium*, strain 899, may be induced to lyse with release of phage following exposure to ultraviolet light. It has been demonstrated since that lysis of many other lysogenic bacteria, although not all, may be induced by ultraviolet irradiation (Lwoff, 1953).

When populations of C4(β_1) are irradiated with a dose of ultraviolet light optimal for induction of lysis, the growth curve (figure 3) is indistinguishable from that of the unirradiated control culture for a period slightly exceeding one division time. At this time the turbidity of the irradiated culture stops increasing and the culture may show partial clearing. Examination of stained smears from the irradiated culture before the optical density has reached its maximum reveals that, although the cells continue to increase in size, apparently they do not divide. In fact, as can be seen from table 3, the viable count actually falls. The behavior of the C7(β_1) strain following irradiation is similar to that of C4(β_1).

Both C4 and C7 indicator strains and their lysogenic derivatives carrying β_1 prophage are quite sensitive to killing by ultraviolet light. The survival curves shown in figures 4 and 5 indicate that there is little difference in sensitivity between the lysogenic strains and the uninfected cells from which they were derived. The optimal irradiation time for production of the greatest number of infectious centers from strain C4(β_1) has its peak close to the time required to kill 50 per cent of the bacterial population (figure 4).

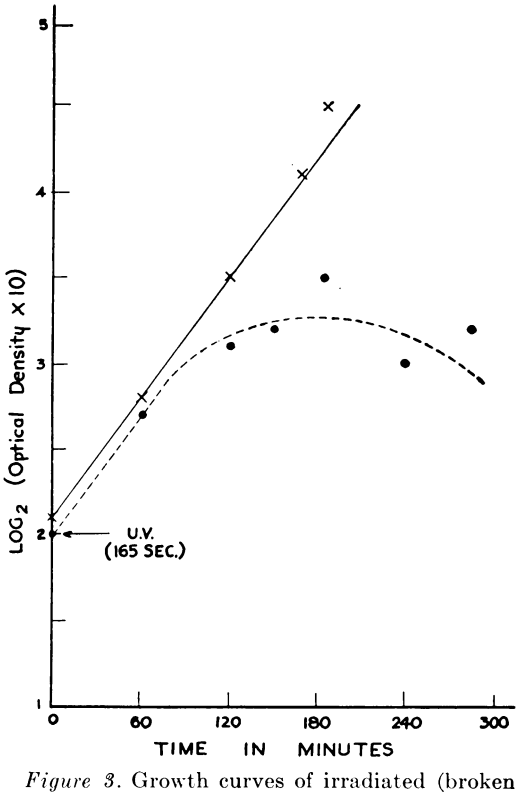

Figure 3. Growth curves of irradiated (broken line) and unirradiated (solid line) C4(β_1).

TABLE 3

Viable counts and free phage titers of C4(β_1) at various times following 135 seconds irradiation in PGT medium containing gelatin

TIME	IRRADIATED C4(β_1)		UNIRRADIATED C4(β_1)	
	Viable count per ml × 10^7	Free phage per ml × 10^8	Viable count per ml × 10^7	Free phage per ml × 10^5
min				
0	3.15	—*	7.4	1.7
90	2.5	0.00048		
120	2.9	0.46		
150	2.0	4.5	16.4	—
180	1.6	4.0		
240	—	2.4	36	—

* The infectious center count was 5.9 × 10^6 immediately following irradiation.

In one experiment, the results of which are recorded in table 3, an overnight shake-culture of C4(β_1) was diluted in PGT liquid medium containing 0.1 per cent gelatin to an optical density corresponding to 10^8 bacteria per ml. Twenty-five ml of this culture were irradiated under the usual conditions; the other 25 ml of culture served as a control. The flask containing the irradiated cells was protected from light, and all subsequent samplings were carried out under subdued illumination. The growth was followed by optical density determinations and by viable counts for a period corresponding to three division times. At intervals the total phage count was determined by titration on the C7 indicator strain. Sixty-five minutes after irradiation, Na citrate was added to both flasks in a final concentration of 0.07 M to prevent adsorption of subsequently released phage.

In table 3 are recorded the viable counts for the irradiated and the control cultures and the titers of free β_1 phage found at different times during the experiment. The actual count found for the unirradiated control at zero time was 7.4 × 10^7 bacteria per ml. The count for the same culture immediately following treatment with ultraviolet light was 3.2 × 10^7 bacteria per ml. The number of infectious centers per ml at this time as determined by plating on C7 was only 5.7 × 10^6 (8 per cent of the original population). Following 90 to 120 minutes shaking in the irradiated medium, induced liberation of β_1 phage commenced and the burst was complete at 150 minutes, at which time 4.5 × 10^8 plaque

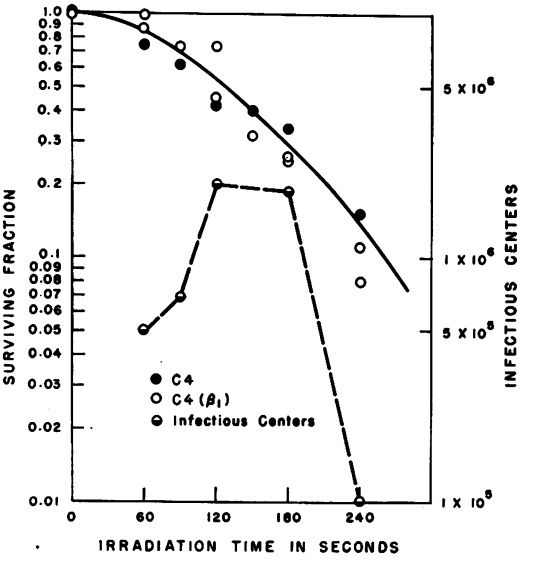

Figure 4. Survival curve of C4 and C4(β_1) following irradiation with ultraviolet light. The lower curve shows the number of infectious centers found when C4(β_1) was plated on C7 immediately after irradiation.

forming particles per ml were found. This corresponds to a burst size of at least 80 which is more than twice that found in one step growth curves using either B or β_1 phage. Bursts of this size have been obtained repeatedly. More recently we have observed that even in the presence of catalase (Wyss *et al.*, 1948; Lwoff and Jacob, 1952; Lwoff, 1953) the number of infectious centers increases during the 90 minutes following irradiation. An analogous increase in infectious centers following irradiation of other lysogenic bacteria has been discussed in some detail by Lwoff (1953).

It is of interest to note that the classic P.W.8 strain is lysogenic and its behavior following irradiation is similar to that of C4(β_1) and C7(β_1) strains as shown in figure 6. The phage released by the P.W.8 strain does not appear to be active against C4 and C7 but does form plaques on the St-5 strain.

Relation between lysogenicity and toxigenicity. Induced lysis and the release of toxin. Filtrates prepared from cultures of C4(β_1) and P.W.8 four hours after ultraviolet irradiation were inoculated into the skin of rabbits. The lesions produced by either filtrate were minimal and were actually less marked than those produced by filtrates of

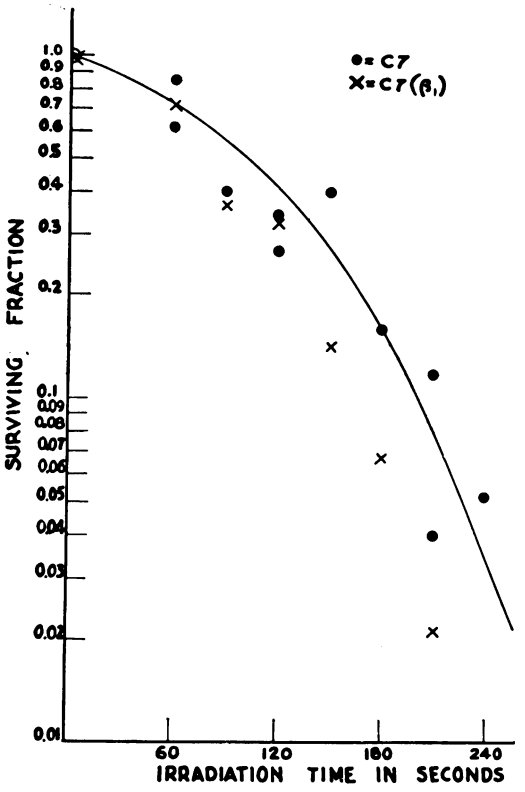

Figure 5. Survival curve of C7 and C7(β_1) following irradiation with ultraviolet light.

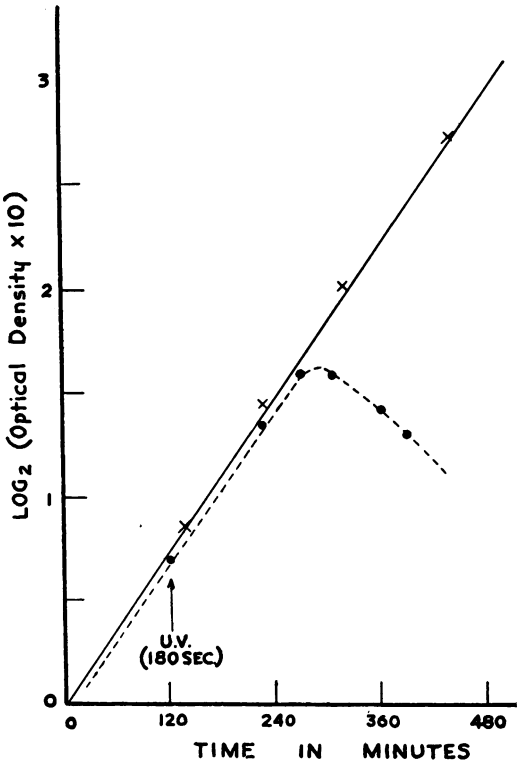

Figure 6. Growth curves of irradiated (broken line) and unirradiated (solid line) P.W.8 strain following irradiation.

unirradiated control cultures or the lesion produced by injection of a Schick test dose (1/50 guinea pig mld) of diphtherial toxin. These observations indicate that lysis *per se* does not result in the release of toxin.

Conversion to lysogenicity by β_1 phage. In order to study conversion of sensitive bacteria to lysogenicity and toxigenicity by temperate phage, cultures of C4 and C7 were mixed with varying multiplicities of β_1 phage. After a suitable adsorption period, usually 50 minutes, the extent of lysis was determined and the culture was analyzed for resistant, toxigenic organisms, using lytic phage B to lyse the remaining sensitive cells. The following precautions were found to be essential:

(1) Experiments must be carried out with well dispersed suspensions of single cells. If clumps are present, mixed colonies will be formed on plating. These often present a nibbled appearance and may also be watery or mucoid.

(2) A pure stock of the temperate phage β must be available.

(3) A high titer stock of the lytic B phage is required. When sensitive bacteria that have been treated with β phage are plated on the surface of chocolate agar plates and the colonies arising from the surviving cells are picked and tested for toxigenicity, it is found that most of them are still sensitive to B phage. Even when sensitive cells are treated with a high multiplicity of β phage, 95 per cent or more of the surviving bacteria may remain unconverted to lysogenicity. If the surface of the plate had been spread previously with a high titer of virulent B phage, the sensitive survivors are almost entirely eliminated and 80 to 100 per cent of the colonies prove to be toxigenic when tested in the skin of rabbits. Removal of unconverted organisms by means of virulent phage also serves to eliminate errors in interpretation due to conversion taking place *on the plate itself.* In this regard it should be pointed out that even when small numbers of untreated cells of C4 and C7 (on the order of 400) are plated on the surface of agar previously spread

with 10^9 B phage particles, a few colonies of sensitive bacteria arise (0.1 to 0.30 per cent of those plated).

The following experiment illustrates the method by which the conversion rate was determined: To 4 ml dialyzed, concentrated β_1 phage, in PGT medium (2.5×10^9 particles per ml) were added one ml actively growing C4 (5.8×10^8 bacteria per ml) and 0.1 ml one per cent $CaCl_2 \cdot 2H_2O$. At the same time, 5 ml of the C4 culture were added to 20 ml PGT medium. Viable counts were made on the control and on the phage treated cultures immediately following mixing and after 50 minutes' shaking in the water bath at 36.4 C. Three sets of plates were used for the viable counts: (a) Neopeptone meat infusion agar pour plates, (b) dilutions spread on the surface of chocolate agar plates, and (c) on chocolate agar plates the surface of which had been spread uniformly a few minutes before inoculation with 10^9 particles of phage B. The growth curve of the control culture was followed by determinations of optical density made at 10 minute intervals over a 70 minute period. An aliquot of the phage treated culture was centrifuged at 50 minutes and the supernatant titrated for unadsorbed β_1 phage. Adsorption was found to be 97.5 per cent complete at this time. In addition, the total phage was titrated on the C7 indicator strain at 50 minutes. The number of

TABLE 4

Lysis and conversion of C4 to C4(β_1) with β_1 phage

	EXPERIMENT NO.					
	1	2	2a‖	3	4	Control
Time t in minutes	50	5	50	50	50	0
Initial β_1 input per ml	2×10^9	1.5×10^9	1.7×10^9	1.5×10^8	1.5×10^7	0
Initial C4 count per ml	1.4×10^8	1.4×10^8	(7×10^7)	1.4×10^8	1.4×10^8	1.4×10^8
Multiplicity*	14.3	10.7	24	0.55	0.083	0
Total survivors at time t	5×10^7	1.1×10^8	3.9×10^7	1.2×10^8	2.2×10^8¶	1.4×10^8
Resistant survivors at time t†	1.0×10^6	4.0×10^6	3.1×10^5	1.2×10^6	8.3×10^5	5×10^6
Infectious centers at time t	1.4×10^8	—	—	5.9×10^7	8.8×10^6	—
Fraction total survivors tested which were lysogenic‡ and toxigenic at time t	1/52	—	—	—	—	0
Fraction resistant survivors tested which were lysogenic and toxigenic at time t	10/12	15/15	10/10	18/21	8/24	0/5
Per cent original infected population lysed§	78%	22%	72%	77%**	76%**	0
Per cent original infected population converted to toxigenicity§	0.36%	2.85%	0.29%	0.93%	1.4%	0

* Multiplicities were corrected for adsorption and, also, by the Poisson distribution, for uninfected bacteria.

† "Resistant" survivors are those giving rise to colonies on plates uniformly spread with 10^9 lytic phage B particles.

‡ In this and other similar experiments, of 147 β treated C4 and C7 survivors, 4 proved to be toxigenic when tested in the rabbit skin. All toxigenic cultures so far tested for lysogenicity have produced phage.

§ In calculating the per cent of the original infected population lysed and converted to toxigenicity, it was assumed that the survivors had grown at the normal growth rate. The survival rate found was accordingly corrected for growth during the 50 minute period. Conversion rates were also corrected for the fraction of "resistant" survivors actually found to be toxigenic. It is realized that the method of calculation is subject to inherent error and conversion rates given are probably low. Figures for the per cent lysis, marked with two asterisks (**), were calculated by dividing the infectious centers found at the 50 minute plating by the original infected population and multiplying by 100.

‖ After the β_1-C4 mixture of Experiment 2 had been shaken for 25 minutes at 36.4 C, an equal volume of β_1 phage (1.9×10^9 per ml) was added and shaking was continued for a further 25 minutes.

¶ Calculated from growth curve of control.

infectious centers so obtained gave an indication of the extent of actual lysis due to infection with the β_1 phage.

Counts of the pour plates were made the following day. The chocolate plates were counted at 40 hours when all of the 12 colonies which appeared in the virulent phage treated chocolate plate (10^{-5} and 10^{-6} dilutions) were picked and transferred to tubes of neopeptone meat infusion broth. Fifty-two colonies were picked from the chocolate plates which had not been spread with B phage. The following day all of the cultures were tested for toxigenicity by intracutaneous injection into rabbits.

It can be seen from table 4 that, when mixtures of β_1 phage and C4 are shaken together for 50 minutes at 36.4 C and subsequently plated, between 72 and 78 per cent of the infected cells are lysed, even though the multiplicity of infection is varied between 0.08 and 24. On the other hand, the per cent of the infected population converted to toxigenicity appears to decrease with increasing multiplicity. It will aso be noted from table 4 that, in every case, 20 to 25 per cent of the original population which would have been expected to have absorbed one or more phage particles were neither lysed nor converted and gave rise to sensitive colonies when plated.

Experiment 2a shows that the proportion of sensitive survivors found at 50 minutes was not decreased even when a high multiplicity of phage was added in two stages, a second charge being added after 25 minutes' shaking. In this experiment lysis and conversion were determined after only 5 minutes of contact between bacteria and β_1 phage. Despite this short interval, the conversion to toxigenicity was nearly 3 per cent, the highest rate observed, while only 22 per cent of the total population were lysed.

Toxin production by converted strains. The P.W.8 strain is unique among the diphtheria bacilli that we have studied in that it is able to grow in iron deficient media until the cellular iron content falls to about one-sixth that of cells grown in excess iron (Pappenheimer, 1947). The C4(β_1) and C7(β_1) strains are unable to grow well in media of such low iron content, and it seems probable that this accounts for their inferior toxin producing capacity as compared to the P.W.8 strain. Nevertheless, 5 to 10 Lf per ml and between 2.5 and 5 L+ doses per ml of toxin have been obtained in C4(β_1) and C7(β_1) filtrates from cultures containing optimal concentrations of iron. The P.W.8 strain produced 60 to 80 Lf per ml under similar conditions.

DISCUSSION

The relationships between phage B, phages β_1 and β_2, and the indicator strains C4 and C7 are similar to those existing between "virulent" and "temperate" bacteriophages which act on other bacterial species. Burnet and Lush (1935, 1936) clearly showed that a temperate phage, C, capable of converting a strain of staphylococcus, SF, to lysogenicity, gave rise to a mutant, C^1, which completely lysed strain SF. Phage C gave turbid plaques on strain SF whereas those produced by C^1 were clear; the plaque diameters of the two phages were the same. Gratia (1936) and, later, Northrop (1953) obtained two types of phage, T and C, from *B. megaterium*, strain 899a. T phage formed cloudy plaques and gave rise to lysogenic strains when added to the sensitive strain 899. C phage, on the other hand, formed clear plaques and caused complete lysis of the indicator strain. A similar pattern of relationships between phages and host cells has been demonstrated for certain gram negative bacilli by Bertani (1953) who has investigated their "lysogenization" by temperate phages. Since virulent phages are incapable of inducing the lysogenic state, except through the agency of one of their temperate mutants, it is not surprising that the β_1 phage, rather than B, is the agent responsible for converting C4 and C7 to C4(β_1) and C7(β_1).

Both C4(β_1) and C7(β_1) are *mitis* strains and thus far appear to differ from C4 and C7 only in their ability to produce toxin. All lysogenic strains prepared by infecting the indicator strains with the β phages have been found to be toxigenic, and all experimentally prepared toxigenic *C. diphtheriae* as well as the few naturally occurring toxigenic strains which we have examined have been found to be lysogenic. Toxin production, then, appears to be a distinctive attribute of the lysogenic state.

Our experiments show that, under comparable conditions, the fraction of the C4 population lysed by β_1 phage remains constant (about 75 to 80 per cent of the initial population) over a wide range of multiplicities of β_1, ranging from 0.08 to 24, whereas the fraction of cells converted to toxigenicity and lysogenicity decreases with

increasing multiplicity. However, in one experiment with a high multiplicity where contact between β_1 phage and the bacteria was limited to 5 minutes, only 22 per cent of the population lysed on plating and the conversion rate rose to nearly 3 per cent. The rate of conversion to the lysogenic state is lower than that found in various other systems (Bertani, 1953). Nevertheless, it is too high to be the result of the selection of mutants. In this respect our results agree with those of Groman (1953*a*, *b*) who has shown that conversion to toxigenicity and lysogenicity does not represent a selection of toxigenic mutants through phage action.

At the present time very little can be said regarding the nature of the conversion process and the relation of prophage to toxin production. It has been suggested that conversion to toxigenicity might involve a phenomenon analogous to the "transduction" of hereditary characters described in lysogenic salmonella strains by Zinder and Lederberg (1952). Transformation mediated by phage (transduction) requires that the active phage lysate be prepared from cells carrying a gene for the property to be transduced. β_1 phage, on the other hand, will effect conversion whether it is obtained following propagation on C7, *a nontoxin producing strain*, as a mutant from B phage or as an induced lysate from irradiated C4(β_1) or C7(β_1). When a population of suitable salmonella cells is exposed to filtrates capable of effecting transductions and conversions, there is no fixed relationship between conversion to lysogenicity and transduction of a given genetic marker. In fact, while many cells may become infected with temperate phage, only about one in a million cells will show signs of genetic exchange. On the other hand, in the case of converted *C. diphtheriae*, every lysogenic cell is a toxigenic cell. For these reasons we feel that conversion to toxigenicity should not be considered as "transduction" until further evidence is forthcoming.

The possibility has been considered that development of phage β from prophage may interfere with the cellular metabolism in such a way that toxin is formed as an abnormal product and subsequently released as a result of lysis. As already noted, this possibility is virtually excluded by the fact that no toxin appears in lysates from irradiated cultures of either C4(β_1) or P.W.8 strains. It should be pointed out, however, that toxin production by the diphtheria bacillus normally occurs only when the supply of iron in the culture medium has become exhausted. The irradiation experiments were performed in the presence of excess iron. It is thus conceivable that toxin is only released as a result of phage lysis of iron deficient cells. Mitsuhashi *et al.* (1949) have studied growth and toxin production in shake cultures of the P.W.8 strain as a function of time. Toxin production only occurs during the final 6-fold increase in growth (corresponding to the last 3 divisions) when, as we have shown, the bacterial iron content falls to about one-sixth its maximum value (Pappenheimer, 1947). If toxin is produced by phage lysis of cells during this period, it may easily be calculated that the extent of lysis would have to be considerable. Yet, neither Mitsuhashi's data nor our own observations have shown the slightest indication that a significant degree of lysis occurs at the time toxin is produced.

Two other negative findings seem worthy of mention. We have obtained no evidence that diphtheria toxin is a "bacteriocine" (Jacob *et al.*, 1953), i.e., a specific antibiotic of the colicine type (Fredericq, 1953). This would, *a priori*, appear unlikely because a wide variety of diphtheria bacilli infected with a variety of different bacteriophages all produce the same toxin. The bacteriocines are characteristically endowed with host ranges fully as specific as those of phages.

Finally, it may be noted that horse antitoxin exhibits no antiphage B neutralizing activity. There is thus no serological evidence for any structural relationship between phage and toxin.

ACKNOWLEDGMENT

We are grateful to Mr. Murray J. Seldeen for his valuable help in several of the experiments.

SUMMARY

Corynebacterium diphtheriae bacteriophage obtained from Freeman contained a *lytic* phage, B, and its temperate mutant, β. Phage B causes almost complete lysis of the indicator strains C4(C444) and C7(C770). Phage β, while causing partial lysis, is the active agent responsible for converting the indicator strains to their lysogenic, toxigenic derivatives, C4(β) and C7(β), which are resistant to lysis by phage B. In all other respects the behavior of B and β phages is iden-

tical, i.e., burst size and latent period on the indicator strains, rate of adsorption, stability, and rate of heat inactivation.

β phage development in the C4(β) and C7(β) strains may be induced by ultraviolet light irradiation. The classic P.W.8 strain is also lysogenic and may be induced by irradiation. The phage liberated by P.W.8 differs in its host range from B and β phages. The conversion rate of C4 to C4(β_1) has been determined at different β_1 phage multiplicities. Toxin is not released from the cells by phage lysis nor is toxin serologically related to phage B.

REFERENCES

Adams, M. H. 1950 Methods of study of bacterial viruses. In *Methods in medical research.* Vol. II. Yearbook Publishers, Inc., Chicago.

Bertani, G. 1953 Infections bactériophagiques secondaires des bacteries lysogènes. Ann. inst. Pasteur, **84,** 273–280.

Burnet, F. M., and Lush, D. 1935 The staphylococcal bacteriophages. J. Pathol. Bacteriol., **40,** 455–469.

Burnet, F. M., and Lush, D. 1936 Induced lysogenicity and mutation of bacteriophage within lysogenic bacteria. Australian J. Exptl. Biol. Med. Sci., **14,** 27–38.

Cowan, M. L. 1927 The separation of virulent cultures of *C. diphtheriae* into virulent and avirulent types. Brit. J. Exptl. Pathol., **8,** 6–11.

Crowell, M. J. 1926 Morphological and physiological variations in the descendants of a single diphtheria bacillus. J. Bact., **11,** 65–74.

Edward, D. G., and Allison, V. D. 1951 Diphtheria in the immunized, with observations on a diphtheria-like disease associated with nontoxigenic strains of *Corynebacterium diphtheriae.* J. Hyg., **49,** 205–214.

Fraser, D. T., and Weld, C. B. 1926 The intracutaneous "Virulence Test" for *Corynebacterium diphtheriae.* Trans. Roy. Soc. Can. V, **20,** 343.

Fredericq, P. 1953 Colicines et bactériophages. Ann. inst. Pasteur, **84,** 294–311.

Freeman, V. J. 1951 Studies on the virulence of bacteriophage-infected strains of *Corynebacterium diphtheriae.* J. Bact., **61,** 675–688.

Frobisher, M., Jr., Parson, E. I., and Updyke, E. 1947 The correlation of laboratory and clinical evidence of virulence of *C. diphtheriae.* Am. J. Public Health, **37,** 543–548.

Gratia, A. 1936 Dissociation du bactériophage du *Bacillus megatherium* lysogène 899 en deux varietés distinctes. Compt. rend. soc. biol., **123,** 1018–1020.

Groman, N. B. 1953*a* The relation of bacteriophage to the change of *Corynebacterium diphtheriae* from avirulence to virulence. Science, **117,** 297–299.

Groman, N. B. 1953*b* Evidence for the induced nature of the change from nontoxigenicity to toxigenicity in *Corynebacterium diphtheriae* as a result of exposure to specific bacteriophage. J. Bact., **66,** 184–191.

Hewitt, L. F. 1952 Diphtheria bacteriophages and their relation to the development of bacterial variants. J. Gen. Microbiol., **7,** 362–371.

Jacob, F., Lwoff, A., Siminovitch, L., and Wollman, E. 1953 Definition de quelques termes relatifs à la lysogenie. Ann. inst. Pasteur, **84,** 222–224.

Lwoff, A. 1953 L'Induction. Ann. inst. Pasteur, **84,** 225–241.

Lwoff, A., and Jacob, F. 1952 Induction de la production de bactériophages et d'une colicine par les peroxides, les ethyleneamines et les haloginoalcoylamines. Compt. rend., **234,** 2308–2310.

Lwoff, A., Siminovitch, L., and Kjeldgaard, N. 1950 Induction de production de phage dans une bacterie lysogene. Ann. inst. Pasteur, **79,** 815–858.

Mitsuhashi, S., Kurokawa, M., and Kojima, Y. 1949 Studies on the production of the toxin by *Corynebacterium diphtheriae.* Japan J. Exptl. Med., **20,** 261–269.

Mueller, J. H., and Miller, P. A. 1941 Production of diphtheric toxin of high potency (100 Lf) on a reproducible medium. J. Immunol., **40,** 21–32.

Northrop, J. H. 1953 Growth and phage production of *B. megatherium.* J. Gen. Physiol., **36,** 581–599.

Okell, C. C. 1929 The relationship of virulent to avirulent diphtheria bacilli. J. Hyg., **29,** 309–312.

Pappenheimer, A. M., Jr. 1947 Diphtheria toxin. III. A reinvestigation of the effect of iron on toxin and porphyrin production. J. Biol. Chem., **167,** 251–259.

Pappenheimer, A. M., Jr., and Hendee, E. D. 1947 Diphtheria toxin. IV. The iron enzymes of *Corynebacterium diphtheriae* and their possible relation to diphtheria toxin. J. Biol. Chem., **171,** 701–713.

Parson, E. I., and Frobisher, M. 1951 Effect of bacteriophage on virulence of *Coryne-*

bacterium diphtheriae. Proc. Soc. Exptl. Biol. Med., **78,** 746–747.

SWANSTROM, M., AND ADAMS, M. H. 1951 Agar layer method for production of high titer phage stocks. Proc. Soc. Exptl. Biol. Med., **78,** 372–375.

WILSON, G. S., AND MILES, A. A. 1946 In Topley and Wilson's *Principles of bacteriology and immunity*. 3rd Edition, Vol. I, p. 458. The Williams & Wilkins Company, Baltimore.

WYSS, O., CLARK, J. B., HAAS, FELIX, AND STONE, W. S. 1948 The role of peroxide in the biological effects of irradiated broth. J. Bact., **56,** 51–57.

ZINDER, N., AND LEDERBERG, J. 1952 Genetic exchange in *Salmonella*. J. Bact., **64,** 679–699.