EFFECT OF ANTIPHAGE SERUM ON THE VIRULENCE OF *CORYNEBACTERIUM DIPHTHERIAE*

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There is little doubt that the toxigenicity of diphtheria cultures is associated with the lysogenic state. Blair (1924) noted that filtrates from toxigenic cultures lysed atoxic cultures of the diptheria bacillus but not the toxigenic cultures, but later investigators were able to isolate certain phages which could lyse toxigenic strains. We now know that, in general, lysogenic cultures are resistant to their own and to related phages, and that susceptibility to a phage does not necessarily indicate that a culture is nonlysogenic and nontoxigenic.

Freeman (1951) and Freeman and Morse (1952) produced toxigenic cultures from nontoxigenic strains by incubating the latter with certain phages. Although many of the cells lysed, some were resistant and these yielded cultures which were both lysogenic and toxigenic. However, lysogenization does not necessarily impart virulence in this system. Freeman repeated his experiments, using the same avirulent strain but a different phage filtrate. In this experiment the lysogenic cultures obtained were avirulent.

Hewitt (1952*a*, *b*; 1954*a*, *b*, *c*), Groman (1953*a*, *b*; 1955), Parsons and Frobisher (1951, 1953) and Parsons (1955) have studied this problem most extensively and have confirmed the dependence of virulence upon lysogenicity and the conversion of the nontoxigenic cultures to toxigenic ones by the incorporation of a specific bacteriophage.

In contrast to the previous studies, the present investigation is concerned with the conversion from toxigenicity to nontoxigenicity rather than with the conversion from avirulence to virulence.

In the past, when diphtheria was rampant, it was not uncommon to find avirulent, nontoxigenic cultures in convalescent patients. These patients had been admitted with clinical diphtheria confirmed by culture and virulence tests. Thus the patient had harbored virulent diphtheria organisms at the time of admission. However, during the convalescent period, cultures obtained from the throat, while morphologically and culturally *C. diphtheriae*, proved to be avirulent. If these were descendants of the original infecting strain, they had in some way lost the ability to produce toxin.

Attempts to alter the toxigenicity of *C. diphtheriae* by cultivation of the organism in diphtheria antitoxin, at best, have been equivocal (Becker, 1927 and Jungeblut, 1928).

The work reported here deals with another way in which the virulence of *C. diphtheriae* may be lost, namely, by the action of phage antibodies.

Clarke (1952) demonstrated that in a lysogenic culture of *Bacillus megaterium* there is a failure in the transmission of prophage during cell division, so that nonlysogenic susceptible cells are produced. However, this inhomogeneity is not allowed to manifest itself *in vitro* since, due to spontaneous induction, there is always lysis of a small number of cells with liberation of free phage into the medium. This phage is able to infect the susceptible cells, and cause either lysis or lysogenization. By growing the lysogenic culture in citrate or oxalate, which ties up the free calcium ions necessary for adsorption to susceptible cells, Clarke was able to prevent the readsorption and, after numerous transfers, to obtain a nonlysogenic culture. This same procedure might well be applicable to the phage-host system under consideration here, since Barksdale and Pappenheimer (1954) confirmed the calcium ion requirement for adsorption of bacteria phage by *C. diphtheriae*.

Crowell (1926) obtained an avirulent strain of *C. diptheriae* from a highly virulent culture by subculturing single cell isolates. Cowan (1927) confirmed this observation, and one may postu-

[1] Based on studies conducted in preparation of the senior author's Doctorate thesis, in partial fulfillment for the Ph.D. degree.

late that these were instances of the failure on the part of the parent cell to transmit prophage to all of its descendants.

While *in vitro*, the calcium-free environment may be responsible for the conversion to nontoxigenicity, it is highly improbable that this condition can be responsible for the conversion of the virulent diphtheria culture in the human body where calcium ions are available. Therefore, another mechanism must be active for the conversion *in vivo*. Theoretically, antibodies to the phage, by binding the free phage, may have the same effect as the calcium-free environment, namely, the prevention of virus adsorption to the susceptible cells.

METHODS AND MATERIALS[2]

Indicator strain. The bacterial strain employed as the indicator throughout this investigation was a nontoxigenic, nonlysogenic strain of *C. diphtheriae*, designated as C_4, which is sensitive to the virulent B phage and to the temperate β and γ phages.

Bacteriophages. Two bacteriophages were used throughout this study. The virulent B phage, which gives clear plaques and which can be obtained in high titer, was used for the production of antiserum in the experimental animals. The temperate β phage, which is carried symbiotically by the toxigenic culture, $C_4(\beta)$, was employed in the *in vitro* and *in vivo* studies as well as in the neutralization experiments. This phage cannot be obtained in as high a concentration as the B phage and it produces cloudy plaques which at times are rather small and difficult to count when present in large numbers.

Preparation of phage filtrates. (1) Preparation of the virulent B phage:—The virulent phage was incubated with C_4 indicator cells in beef heart infusion broth for 2.5 hr at 30 C in a shaker. At this time sodium citrate was added, to give a final concentration of 0.07 M, in order to prevent virus adsorption. The mixture was returned to the shaker and incubated for 2 hr to insure an additional burst of all infected cells. It was then centrifuged for 20 to 30 min and the supernatant filtered through a Chamberland L5 filter. The filtrate was then assayed to determine the titer.

(2) Preparation of the temperate β phage:—Sodium citrate was added to beef heart infusion broth to give a final concentration of 0.07 M. The lysogenic strain, $C_4(\beta)$, was added and incubated for 6 to 7 hr in a shaker at 30 C. The mixture was then centrifuged, filtered through a Chamberland L5 filter and the filtrate assayed.

Media. At the beginning of the study the pantothenate-glutamate-tryptophan media of Barksdale and Pappenheimer (1954) were employed; however, these were abandoned in favor of the beef heart infusion media.

(1) Beef heart infusion broth:—1000 ml of beef infusion, 10 g of neopeptone, 5 g of NaCl, pH 7.3.

(2) Beef heart infusion agar:—The plating medium was the beef heart infusion broth with the addition of either 17 or 8 g of agar depending upon whether a hard or a soft agar was desired.

Phage assay. Appropriate 10-fold and 100-fold dilutions of phage filtrates were prepared in broth. To assay the phage filtrates, plaque counts were carried out using the agar overlay method described by Adams (1950). Soft agar was distributed in 2.0 ml amounts and held at 45 C in a water bath. Each tube received 0.2 ml of the indicator culture, and 0.1 ml of the appropriate dilution of phage. Thorough mixing was accomplished by means of a pipette. The mixture was immediately poured into a warmed petri plate containing 20 to 25 ml of hard agar. The plate was then rotated before the soft agar hardened to insure an even distribution over the area. The plates were allowed to harden, inverted, and incubated at 37 C for 18 to 24 hr.

Preparation of the indicator for the background growth and phage counts. In the diphtheria phage-host system the optimal conditions for each set of indicators and phages must be determined. For optimal results the indicator strain should be a relatively young, actively growing, well shaken culture. In this study the best results were obtained when the indicator was from 2.5 to 3.5 hr old. Use of indicators incubated for a longer or shorter time resulted in a poor background growth or greatly reduced plaque size. It was found that the most satisfactory results were obtained by adding 0.2 ml of a 3 hr indicator culture to 2.0 ml of soft agar used for the overlay.

[2] The culture and bacteriophages employed in this investigation were obtained from Drs. W. L. Barksdale, New York University College of Medicine, New York, New York, and N. B. Groman, University of Washington, School of Medicine, Seattle, Washington.

RESULTS AND ANALYSIS

Initially, difficulty was experienced in obtaining distinct plaques as well as in securing sufficiently high titers of phage for immunization purposes.

The first of these problems was solved by the finding that the age of the indicator culture C_4 was extremely important. Best results were obtained when broth was inoculated with enough cells to give barely visible turbidity with an overnight culture, and then shaken at 32 C for 2.5 to 3.5 hr. Two-tenths ml of such a culture per plate gave good background growth and permitted satisfactory plaque formation.

At times, however, the indicator culture seemed to acquire some resistance to the phage, and it became necessary to plate it out and select a suitable sensitive colony for a new indicator.

In efforts to obtain preparations of phage of high titer several methods were employed. Induction with ultraviolet light (Barksdale and Pappenheimer, 1954) and hydrogen peroxide (Lwoff, 1953) were tried, as was penicillin (Price, 1947), but none of these expedients yielded titers of temperate phage higher than 10^6.

Preparation of B phage filtrates of high titer. Since the highest titers obtained up to this point with the temperate phage were about 10^6, and since titers of approximately 10^{10} were desired, it was decided to abandon the temperate β phage and to experiment with the virulent B phage. The virulent phage was a mutant of the temperate phage, and it was hoped that the antigenic relationship between the two would be sufficiently close that an antiserum produced by injections of the former would neutralize the latter.

The age of the susceptible cells and the addition of sodium citrate to the medium had proved to be important in producing the best β phage filtrates and, therefore, these points were observed in the procedures employed to obtain high B phage titers. The indicator cells were incubated in beef heart infusion broth for 2 hr, B phage was then added and incubation was continued for an additional 2.5 hr. Sodium citrate was added to prevent further virus adsorption and the mixture was returned to the shaker for 2-hr after which it was centrifuged, filtered and assayed. The titers obtained, 10^9 and 10^{10}, indicated that the preparations could be used advantageously for immunizing rabbits.

Production of immune serum in rabbits. The procedure for the production of immune sera consisted of subcutaneous injections into each of three rabbits, at three day intervals, of 1.0 ml of the B phage. The animals tolerated the first injection poorly and were dead within a week. Presumably the filtrates contained diphtheria toxin.

TABLE 1

Phage neutralizing ability of sera from 3 rabbits following 6 injections of B phage

Serum Dilutions	Final Serum Dilutions	Plaque Counts of B Phage with Antiserum*			Plaque Counts of β Phage with Antiserum†		
		1	2	3	1	2	3
Undiluted	1:2	0	0	0	0	0	0
1:10	1:20	36	20	35	4	0	4
1:20	1:40	118	20	55	29	3	1
1:40	1:80	114	120	48	60	5	4
1:80	1:160	162	172	61	76	15	8

* Control plates averaged 155 plaques.
† Control plates averaged 69 plaques.

A new set of rabbits was obtained and immunized with diphtheria toxoid by giving three 0.1 ml subcutaneous injections at 10 day intervals. Following this series of injections the animals were allowed to rest for 10 days, at which time all three were Schick negative. Samples of blood were collected from each rabbit by means of an ear vein puncture, and the serum was tested against the B phage to ascertain the presence or absence of antiphage activity in as much as 0.3 ml of the undiluted serum.

The rabbits were then given six injections of B phage as described above. Four days after the sixth injection the rabbits were bled and the titers of the sera were determined against both B and β phages. Various dilutions of the sera were prepared and equal volumes of phage dilutions were added. After incubation at 37 C for 30 min samples were plated in duplicate in the usual way. The results of this test are given in table 1; all the sera contained antibodies against both phages.

Injections of 1.0 ml of B phage were continued at three day intervals. Following the 14th injection, samples of blood were collected, and neutralization tests showed that the antibody titers had increased. The results of these tests are given in table 2.

Conversion tests in vitro. As mentioned previously, Clarke had demonstrated that it was

TABLE 2

Phage neutralizing ability of sera obtained by 14 injections of B phage

Serum Dilution	Final Serum Dilution	Plaque Counts of B Phage with Antiserum*	
		1	2
1:10	1:20	9	5
1:20	1:40	12	14
1:40	1:80	25	40
1:80	1:160	30	18
1:160	1:320	55	31
1:320	1:640	69	72

* Control plates averaged 117 plaques.

possible for a lysogenic culture to lose its prophage by serial broth passages if the readsorption of free phage in the medium was prevented. Theoretically, successive transfers of a lysogenic culture through broth containing phage antibodies should accomplish the same result. Accordingly, antiphage serum which was active in at least a 1:80 dilution was diluted 1:20 with broth, passed through a Chamberland L5 filter and stored in the refrigerator. Four ml of this medium were inoculated with the toxigenic C_4 (β) strain and incubated for 24 hr at 37 C. Each day a loopful of the previous day's culture was transferred to 4.0 ml of fresh serum-broth medium and incubated. Two controls were run simultaneously; one containing 4.0 ml of a 1:20 dilution of normal rabbit serum in broth, the second containing 4.0 ml of broth only. These were inoculated with the toxigenic culture and transferred daily as described above. Every seven days the antiphage serum culture and the controls were tested for lysogenicity in the following way. One ml of the experimental and control cultures was transferred to broth containing sodium citrate to prevent virus adsorption. The tubes were incubated in the shaker for 6 to 7 hr and filtered. The filtrates were then titrated for phage content.

At the start of the experiment, all three cultures produced phage titers of 10^4 and 10^5. By the 20th passage the titers of phage in the antiserum cultures were approximately 10^3, and by the 30th passage, 10^2 or less, whereas the controls continued to produce titers of 10^4 and 10^5 as at the beginning. By the 50th passage the antiserum culture showed no evidence of phage, while the controls continued to produce normal titers. A loopful of this antiserum culture was streaked on an agar plate and 20 isolated colonies were transferred to broth and incubated as described previously, to confirm the loss of lysogenicity. When the filtrates of these isolates were tested, each one gave plaques when plated on the indicator. Similar results were obtained with the antiserum cultures of later passages. The titers produced in subcultures of individual colonies ranged from 10^5 down to only 5, with most about 10^2. Such results might be explained by decreased rates of spontaneous induction.

After 107 passages, the original lysogenic culture which had been grown in the presence of phage antibody had apparently converted completely, and using the methods described above, lysogenicity could not be demonstrated in the individual colonies that were isolated.

A similar experiment was conducted using a 1:10 immune serum broth mixture in place of the 1:20 serum dilution in the previous test. The results were similar to those obtained previously, except that the changes in the characteristics of the cultures were accelerated. In this experiment conversion was accomplished by 92 passages.

Both of the converted cultures were tested for virulence by intracutaneous inoculation into guinea pigs, and the loss of toxigenicity demonstrated. Thus by serial passages of the toxigenic strain in immune serum nontoxigenic, nonlysogenic cultures resulted.

Conversion tests in vivo. The procedure for the *in vivo* tests was to give the animals a large enough dose of toxigenic organisms to establish an infection, yet, by subsequent administration of antitoxin, to prevent the early death due to the toxin liberated. In a preliminary experiment, an overnight agar slant of $C_4(\beta)$ toxigenic cells was washed off with 5.0 ml of broth. A normal guinea pig was given 0.1 ml of the cell suspension intracutaneously, followed 4 hr later by the intraperitoneal injection of 1000 units of antitoxin (which had been shown to contain no antiphage activity). It was expected that this antitoxin would neutralize the toxin produced by the injected cells, thus permitting the bacterial infection to continue in the skin. The free phage liberated by the culture would be available for the stimulation of antibody production, and, if the animal survived for a sufficient period, an avirulent culture would result.

The reaction at the site of injection was very

slight after 24 hr but increased in size and intensity until the 5th day at which time the center of the infected area was necrotic. This reaction lasted for 3 days without noticeable change and then began to fade. On the 12th day the site of infection was quite dry and the scab which had formed appeared ready to slough off. On the 20th day the guinea pig died.

A culture was taken from the surface of the lesion at the 6th day, when the infection appeared to be at its peak. Upon plating and incubating, some diphtheria-like colonies could be identified. Three such colonies were examined microscopically and proved to be morphologically indistinguishable from *C. diphtheriae*. By the usual tests one of the cultures proved to be lysogenic; the other two were nonlysogenic. After making the culture from the surface of the lesion, the area was scarified and material from the deeper layers was plated. These cultures yielded many colonies of diphtheria organisms. Four were subcultured and tested for the presence of phage, two proving to be nonlysogenic.

More cultures were obtained on the 12th day, when the scab sloughed off, leaving a deep punched-out ulcer. In these cultures, preliminary tests failed to demonstrate the presence of phage. The growth of the organisms in all cultures became less luxuriant on transfer, and eventually they were lost for reasons not determined. Therefore, conclusive evidence as to conversion was not obtained.

Another attempt to demonstrate conversion *in vivo* was conducted with two rabbits; one was normal, the other was one of those immunized animals in which the antiphage serum had been produced. Samples of blood were collected from the rabbits prior to the injection of the toxigenic culture in order to establish the presence or absence of phage antibodies in the sera. The neutralization test was conducted as outlined above. Results of the titrations against the β phage for a 2 hr neutralization period indicated the absence of antiphage activity in the normal animal, and its presence in the immune rabbit.

An overnight agar slant of the toxigenic culture was washed off with 2.0 ml of broth and 0.1 ml injected intracutaneously into each animal. To prevent early death from the toxic effects, the normal rabbit received 1500 units of antitoxin intraperitoneally 4 hr later, whereas the immune rabbit, as a precaution, received 500 units. The normal rabbit developed a small raised area around the site of injection, however, the central area did not become necrotic, and by the seventh day the infection was obviously subsiding. Presumably this rabbit had received too much antitoxin. Therefore, in a further attempt to establish an infection, the rabbit received a second injection of toxigenic organisms adjacent to the site of the first but without additional antitoxin. The result was similar to that of the first experiment, although the intensity of the infection was more pronounced and eventually ulceration occurred. At this time the lesion was cultured but only a few colonies were obtained. Upon isolation of pure cultures, preliminary tests were conducted to determine the presence or absence of phage. These tests did not reveal phage and upon transfer the cultures were lost, as had happened to those in the previous experiment.

An interesting observation was made in the case of the immune rabbit in this experiment. Following the injection of the virulent culture the entire ventral surface of the animal became inflamed and edematous. The central area of necrosis was about 3 in. in diameter as compared with an area of about 1 in. in the normal animal. Following ulceration the reaction subsided and the rabbit appeared normal. The most logical interpretation of this reaction seemed to be that of hypersensitivity.

Since the cultures obtained from the normal rabbit had appeared to be nonlysogenic before they were lost, it was decided to test for the presence of phage antibodies in the rabbit. The test was conducted with a 1:4 serum dilution against the virulent B phage. The results of the test were negative even after a 2 hr neutralization period. Although it had been shown that the antibodies produced against the virulent B phage were capable of neutralizing the temperate β phage, the converse need not necessarily have been true. Therefore, the activity of the serum was tested against the β temperate phage with resulting neutralization of more than 50 per cent as shown in table 3. Preinoculation serum had no neutralizing action.

Although it had been shown that the toxigenic culture in the animal body could produce antibodies to the carried phage, and that apparently nontoxigenic cultures could be obtained from the lesions, rigorous confirmation of the conversion

TABLE 3

Neutralization of β phage by serum taken 3 weeks after injection of a rabbit with virulent Corynebacterium diphtheriae

Neutralization Time	Plaque Counts		Survivors	Neutralized
	Controls	Antiserum 1:4		
30 min...	148	155	100.0	0.0
60 min...	153	134	88.2	11.8
90 min...	147	57	38.7	61.3
120 min...	158	66	41.8	58.2

TABLE 4

Results of the neutralizing activity of sera from infected guinea pigs in which the virulent cultures had converted to the nonlysogenic state. Neutralization of β temperate phage

Guinea Pig Sera	Plaque Counts		Neutralized
	Control	Serum	
			%
1	153	125	18.3
2	92	18	80.7

had not been accomplished because the cultures had been lost. Therefore, six guinea pigs, three normal and three immunized against the toxin, were injected with 0.1 ml of the diphtheria culture prepared as in the previous tests. Four hours after the injection the normal animals received 1000 units of antitoxin and the immunized animals 300 units. The latter group developed hard papules around the site of injection, but no ulceration occurred. The infection in the three normal animals progressed as described for the animals in the previous studies, with a central necrotic area about 1 in. in diameter, followed by ulceration on the 8th, 13th, and 15th days. Blood samples were obtained from the first two of these animals on the 20th day, but the third died unexpectedly so that a sample was not obtained. Neutralization tests were conducted with each serum and the results are given in table 4. The serum from "normal" guinea pig no. 1 neutralized 18 per cent of the temperate β phage in the 2 hr neutralization period, while that from no. 2 proved to be stronger, neutralizing approximately 80 per cent of the β temperate phage in the 2 hr period.

During the course of the infection numerous plate cultures were made from the ulcerated areas. Even in those obtained 8 days after infection, some 50 per cent of the colonies were nonlysogenic. Subcultures of these individual colonies were made and at least one culture from each group was tested for virulence in guinea pigs. In all instances the results confirmed the nontoxigenic nature of the cultures.

Thus, it was demonstrated that if the animal can overcome the toxic effects of the infection and survive for some weeks, the body will produce antibodies to the carried phage, and the lysogenic, virulent organisms will convert to a nonlysogenic, avirulent form. Judging from the *in vitro* experiments, phage antibodies may well have been responsible for establishing this conversion.

Lysogenization of the converted avirulent cultures. One culture from each of the various groups of avirulent cultures, converted during the course of the study, was subjected to the lysogenization procedure. A drop of each young actively growing avirulent culture was transferred to 2.0 ml of β phage. This mixture was shaken for 5 min by hand and placed at 4 C for 1 hr. Tenfold dilutions were made of each phage-bacterium mixture to a final dilution of 10^{-6} and 0.1 ml portions were spread over agar plates which had been previously seeded with virulent B phage. After an incubation period of 24 to 48 hr, 3 colonies were picked from those plates which contained 30 to 300 colonies, transferred to broth and incubated for 8 hr. Agar slants were made by transfer and the remaining mixture was tested for phage. At least one of each set of cultures tested showed the presence of phage. The result was double-checked by assaying the corresponding agar slant cultures prepared before filtration.

Each of the newly lysogenized cultures was then tested for toxigenicity by guinea pig inoculation, with the corresponding avirulent precursor serving as a control. The results confirmed that each of the avirulent cultures which had been lysogenized now produced toxin whereas the controls remained avirulent. Thus it was substantiated that the converted avirulent cultures obtained in the animal studies were in fact *C. diphtheriae* and not some diphtheroid, since the virulence could be restored by the incorporation of the specific bacteriophage.

DISCUSSION

The relationships between the virulent B phage, the temperate β phage and the susceptible indicator strain appear to be similar, in general, to the relationships of virulent and temperate phages in other systems, i. e., the B phage formed clear plaques, whereas those formed by the temperate β phage were cloudy, and the indicator did not acquire resistance to the virulent phage as readily as to the temperate phage. However, the conditions which are required in order to enable the virus to manifest itself seem to be considerably more rigid than with most other systems.

The data presented here have shown that the immune sera produced by injections of the virulent B phage are able to neutralize the temperate β phage as well as the virulent phage itself (table 1). This cross-neutralization of the temperate phage by antiserum to the B phage made it possible to use the virulent phage, which could be obtained in high titer, for the production of the antiphage serum to be used in the *in vitro* studies. In contrast, the antiserum produced during infection against the β phage did not display reciprocal cross-neutralization of the virulent phage.

The results of the *in vitro* studies seem to substantiate, in the diphtheria phage-host system, Clarke's (1952) hypothesis concerning the loss of prophage. In our *in vitro* conversion experiments, antiserum was employed to inactivate the free virus in the medium, whereas Clarke employed citrate or oxalate to bind the free calcium ions; however, in each case the final result was the prevention of readsorption of free phage. The manner in which the culture converted to the nonlysogenic state merits some comment. As expected, the phage titer of the culture after growth in the presence of immune serum decreased gradually during consecutive passages, the first assumption being that some of the cells had lost their prophage so that the titers decreased simply due to the presence of fewer lysogenic cells. Eventually, the immune serum culture appeared to become nonlysogenic when tested in the usual manner. However, when isolates from this apparently nonlysogenic culture were tested individually, each one gave evidence of phage production, although the majority of the titers were significantly lower than normal. A logical explanation seems to be that the rate of spontaneous induction is reduced in some way, and then, by a simple process of selection, influenced by the antiserum, the cells and their progeny which have a high rate of induction make up an ever-decreasing proportion of the culture and are supplanted by those cells which have a low rate of induction. Consequently, the majority of the cells still possess prophage, but the number of cells in which the prophage is activated to the vegetative state is greatly reduced, thus yielding cultures which produce low phage titers. Eventually, the prophage is lost in certain cells and the culture is converted in a manner similar to that just discussed to explain the conversion to cultures having a decreased rate of spontaneous induction. In retrospect it would have been interesting to have conducted virulence tests on those cultures which had a low rate of induction to determine whether the virulence had decreased also. Is there a relation between virulence (amount of toxin produced) and the spontaneous induction rate?

Since the effect of an altered rate of induction was not mentioned in Clarke's report, and since the difference in the number of passages in the presence of antiserum required to convert the culture is much greater than that when citrate or oxalate are used, the question arises as to whether antibody *in vitro* is as effective as citrate or oxalate in preventing adsorption of virus by cells.

The tests of lysogenicity of the cultures obtained from the animals were, in effect, done on the progeny of single cells, since material was plated directly from the lesions and isolated colonies tested. In contrast, the *in vitro* experiments involved testing 0.1 ml of the whole culture for the presence of phage.

In other words, the *in vivo* experiments showed that some nonlysogenic cells developed early, whereas those done *in vitro* demonstrated that some lysogenic cells were present in cultures even after many passages.

It was shown that prior to infection the sera of the animals did not have any activity against the phage carried by the toxigenic strain, but that following the injection of the toxigenic culture, conversion to nontoxigenicity occurred and antibodies against the carried phage were at that time present in the sera. Thus, *in vivo* and *in vitro* experiments suggest that the con-

version of the toxigenic culture was caused by the antiphage sera.

The present study has demonstrated that diphtheria phage is antigenic, that antiphage bodies are able to neutralize the phage, and that a nonlysogenic, nontoxigenic culture results after successive passages of the virulent culture in broth containing phage antibodies. In addition, the data show that the normal animal, i. e., an animal without serum activity against the carried phage, will, following infection, produce antibodies to the phage carried by the diphtheria organism injected, and, if the toxigenic effects are overcome so that an early death is prevented, conversion will take place. Thus, the results of the experiments suggest that antiphage bodies built up during the infection may be responsible for the appearance of avirulent diphtheria organisms during convalescence.

It is obvious that future studies of clinical cases of diphtheria are needed to substantiate the experimental findings here reported. The presence of antiphage bodies in convalescence after diphtheria should be investigated. The necessary material for such a study was obtained from the Detroit epidemic of 1956–1957, and will be examined in the future. A similar study could be conducted in cases of skin diphtheria, which the Army studied during World War II (Liebow *et al.*, 1946).

SUMMARY

In vitro conversion has been shown to take place during serial passages of a toxigenic strain of *Corynebacterium diphtheriae* in broth containing antiphage serum, and *in vivo* conversion occurs as phage antibodies are developed in the infected animal.

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