# THE NATURE OF PROPHAGE IN LYSOGENIC BACILLUS MEGATHERIUM

By ELIZABETH M. MILLER and WALTHER F. GOEBEL, Ph.D.

*(From the Laboratories of The Rockefeller Institute for Medical Research, New York)*

(Received for publication, July 16, 1954)

A lysogenic bacterium is one which possesses the ability to yield bacteriophage and to transmit this property to its progeny through innumerable generations. The nature of the specific structure which perpetuates lysogenicity poses one of the most fundamental problems in virology. There has been considerable controversy concerning this agent; it has been thought to be a nonpathogenic bacteriophage or an infectious virus masked by a component of the cell it invades. These hypotheses, however, are not entirely compatible with the hypothesis proposed in recent years by bacterial geneticists who consider this structure, now termed prophage, to be comparable to a genetic unit which is perpetuated by cell division, and which endows every microorganism in a lysogenic culture with the potentiality of producing infectious virus. The experimental evidence in support of this concept has been extensively reviewed by Lwoff (1) and will not be further discussed here.

It is the purpose of this research to ascertain by immunological means whether the infectious virus elaborated by the lysogenic strain of *Bacillus megatherium* bears any serological relationship to the structure from which it arises, or whether prophage is immunologically impotent. The success of this project is dependent upon procuring cells free of extracellular phage, for it is obvious that if the lysogenic microorganisms are grown under conditions such that appreciable quantities of infectious virus are liberated, its readsorption on the mother cell would introduce a difficulty which would render valueless the interpretation of any subsequent serological data. This difficulty can be overcome by cultivating the lysogenic *B. megatherium* in a synthetic medium deficient in magnesium ions (2). In this medium the cells liberate minimal amounts of extracellular phage, quantities too small to function antigenically and hence to offer substantial interference with any immunological response that might be elicited by the prophage of the cells under investigation.

From that which is to follow, it will be seen that the lysogenic strain of *B. megatherium* is devoid of ability to incite in experimental animals specific antibodies which react with mature *megatherium* T phage, a fact which materially substantiates the hypothesis that prophage is a genetic structure and not an immature or temperate virus particle.

## *Methods and Materials*

*Bacterial Cultures.*—The lysogenic strain of *B. megatherium* 899a used in these experiments was originally obtained from Gratia and is descended from the lysogenic strain 899a originally described by den Dooren de Jong (3). Transplants of the culture were made weekly on 2 per cent bacto-peptone agar slants. The sensitive strain KM of *B. megatherium* was obtained from Professor Krueger of the University of California and was transferred daily on the same medium. The lysogenic strain 899a produces a number of different types of bacteriophage, in addition to the principal "T" phage (4).

*Media.*—A number of different media were used in the work which will be described, each having its own specific purpose (2, 5).

(*a*) The broth used for the production of phage stock and employed as a diluent for phage assays was prepared by dissolving 50 gm. of Difco bacto-peptone in 1 liter of distilled water. The medium was sterilized by autoclaving.

(*b*) The plating agar was obtained by adding 20 gm. of bacto-agar to 1 liter of the medium described under (*a*); the soft agar for phage assay was prepared by adding 12.5 gm. of bacto-agar to 1 liter of medium described under (*a*).

(*c*) The tryptose phosphate broth (T.P.B.) for cultivation of *B. megatherium* 899a was prepared by dissolving 29.5 gm. of desiccated Difco tryptose phosphate broth in 1 liter of distilled water and adjusting the pH to 7.2.

(*d*) The magnesium-deficient synthetic medium (SM) in which the lysogenic bacilli (strain 899a) used for immunization purposes were grown, was prepared by dissolving 10 gm. of l-asparagine and 7.099 gm. $Na_2HPO_4$ in 900 ml. of triple distilled water, followed by the addition of a solution of 0.392 gm. Fe $(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$ in 100 ml. of triple distilled $H_2O$. The pH was adjusted to 8.5, the solution autoclaved at 15 pounds for 10 minutes and filtered through a sintered glass funnel. The pH was readjusted to 7.5 and the solution was again autoclaved at 15 pounds for 20 minutes.

(*e*) Dialyzed peptone-yeast extract medium was employed for cultivating the sensitive strain KM used in the production of purified *megatherium* T phage. It was prepared as follows: 100 gm. of Difco dehydrated bacto-yeast extract was dissolved in 200 ml. of water and the solution was dialyzed against successive changes of distilled water. The dialysate, about 3 liters, was concentrated *in vacuo* to 500 ml. and preserved in the frozen state. A solution of 100 gm. of Difco bacto-peptone in 200 ml. of water was also dialyzed. The dialysate was concentrated to small volume *in vacuo* and then frozen and dried. Approximately 70 gm. of desiccated diffusible substance was obtained. The culture medium itself was prepared by dissolving 20 gm. of the dialyzed desiccated peptone in 975 ml. of distilled water; to this was added 25 ml. of the dialyzed yeast extract. The solution was adjusted to pH 7.0 and was sterilized by filtration.

*Cultivation of B. megatherium Strain KM for Phage Assay.*—*B. megatherium* strain KM which served as the host for viral assays, was cultivated on 2 per cent bacto-peptone agar slants. After incubating for 18 hours at 35°C., the bacteria were washed off with 5 ml. of 5 per cent bacto-peptone. 0.3 ml. of this suspension was used for each plating. The viral assays were made by the agar layer technique (6).

*Bacterial Count.*—The estimation of bacterial populations was made by counting the microorganisms in a hemocytometer, after staining the cells with a 5 per cent solution of safranine.

*Preparation of Megatherium T Virus.* Fresh stocks of *megatherium* T phage were prepared as follows. The bacilli from fresh slants of strain KM were suspended in 5 per cent bacto-peptone and diluted so as to contain $1 \times 10^8$ cells per ml. Tubes containing 20 ml. of the suspensions were shaken at the rate of 200 oscillations per minute for 3 hours at 35°C. The cultures, which now contained about $5 \times 10^8$ B/ml., were infected with $2 \times 10^9$ viral particles per ml. and incubated an additional 3½ hours with shaking. The microorganisms were

separated by centrifugation and the supernatant liquid was filtered through a Cours P3 candle. The solution was then assayed for its viral content; usually about $5 \times 10^{10}$ P/ml. were obtained.

*Preparation of Antisera.*—(*a*) *Antiphage sera.* In the work which is to be described the testing of various phage preparations for their ability to incite specific immune bodies in rabbits was performed as follows. The viral antigen under investigation, at the particular concentration described in the text, was injected intravenously each day in amounts of 0.2, 0.2, 0.5, 0.5, 1.0, 1.0 ml. per animal. After 1 week of rest a second course of injections of 1.0 ml. per animal was given on 4 successive days. When a third and fourth course of immunization were given, the schedule was the same as that prescribed for the second course. In all instances bleedings were made 1 week after the final injection.

Viral neutralization tests were performed as follows: The antiserum was diluted in bacto-peptone broth in increments of 10. 0.1 ml. of each dilution was added to a series of tubes containing 0.9 ml. of *megatherium* T phage ($1 \times 10^6$ P/ml.). After 30 minutes' incubation at 37°C., an aliquot was removed and diluted $10^{-2}$ in broth. 0.05 ml. of the latter was then plated in duplicate using *B. megatherium* KM as host. As a rule, serum dilutions of $10^0$, $10^{-1}$, $10^{-2}$, and $10^{-3}$ were tested. After scoring the plates, the percentage of virus which had been inactivated by each dilution was determined. From these values virus titration end-points ($EI_{50}$) were calculated by the 50 per cent end-point method of Reed and Muench (7). Normal sera of the animals were likewise tested for phage-neutralizing antibodies. Although considerable variation was observed, the normal serum as a rule failed to neutralize more than a few per cent of virus.

(*b*) *Antibacterial sera.* The antibacterial sera were obtained from rabbits which had been immunized with living cultures of the two strains of *B. megatherium*, strains 899a and KM. In each instance the vaccine was prepared by inoculating 150 ml. of 5 per cent bacto-peptone with the microorganism in question, followed by incubation, with aeration, for 18 hours at 35°C. The bacteria were centrifuged and resuspended in fresh 5 per cent bacto-peptone. The vaccine, containing approximately $5 \times 10^8$ cells per ml., was subdivided in appropriate amounts and stored at −17°C. The rabbits received four courses of injections. During the first course doses of 0.1, 0.1, 0.2, 0.5, 0.5, and 1.0 ml. were administered intravenously on successive days. After a week of rest, a second course of inoculations was given; at this time 1.0 ml. was given on 4 successive days. The remaining courses were identical with the second.

*Complement-Fixation Tests.*—Complement-fixation tests were performed by the procedure described by Casals, Olitsky, and Anslow (8). Commercially available sheep erythrocytes, antisheep hemolysin, and lyophilized complement were used in the hemolytic system. Prior to carrying out the complement-fixation reactions, both the antisera and the antigens were tested for their anticomplementary and hemolytic properties. The normal sera of the various animals were also tested for their ability to fix complement with the antigen used in the specific test. In no instance was it found that the normal sera would fix complement.

In carrying out the titrations, twofold dilutions of the sera were tested beginning with a 1:2 dilution. The highest dilution of serum giving a 4+ reaction (*i.e.* no hemolysis) was considered the end-point. In the tables which follow, the reciprocal of the end-point dilutions, rather than the complete titration, is tabulated.

## EXPERIMENTAL

*The Serological Relationship between the Sensitive (KM) and Lysogenic (899a) Strains of B. megatherium.*—Before pursuing any serological analysis designed to establish whether the prophage of lysogenic *B. megatherium* (strain 899a) bears an immunological relationship to the infectious T virus which this organism elaborates, it is of prime imprortance to learn whether the lysogenic

and sensitive (KM) strains contain a common antigenic component. Since the *megatherium* T phage used in testing the sera of animals immunized with the lysogenic strain (899a) is obtained by infecting the sensitive strain KM, it is apparent that any positive serological reactions which might be observed need not necessarily arise from the interaction between T virus and prophage immune body. The reaction might well result from the interaction of a bacterial antigen present in the KM lysate and an antibody, evoked by the same or similar antigen present in the lysogenic strain 899a.

It is unfortunate that cross-agglutination reactions are unsatisfactory for studying the serological relationship of these two strains of *B. megatherium*, for both agglutinate spontaneously. Gratia (9) has presented evidence showing that the lysogenic strain of *B. megatherium* with which he worked (and of which ours is a descendant) cross-agglutinated with his sensitive strain. In our hands, however, cross-agglutination tests have not been satisfactory and for this reason we have resorted to the use of the complement-fixation reaction. The suspensions of *B. megatherium* strains KM and 899a used in the complement-fixation tests were prepared as follows:—

Cultures of the mocroorganisms were grown with shaking for 18 hours at 35°C. in Todd-Hewitt broth (10)[1] and were killed with 0.5 per cent formalin at 37°C. for 8 hours. The bacilli were centrifuged, washed three times with sterile saline, and finally diluted to their original volume in saline. The bacterial antigens were then tested by complement fixation in homologous and heterologous antisera at the dilution indicated in Table I. The antibacterial sera were obtained from rabbits which had been subjected to four courses of immunization according to the schedule previously outlined.

The results of the complement-fixation tests are shown in Table I. It is apparent that both the sensitive and lysogenic strains of *B. megatherium* elicit complement-fixing antibodies in rabbits. It will also be observed that both KM antibacterial sera contained complement-fixing antibodies reactive with the heterologous strain of *B. megatherium* 899a. In addition, sera produced by the lysogenic strain 899a cross-reacted with the sensitive strain KM. The results of these tests indicate clearly that these two strains of microorganisms possess similarities in their antigenic components, and that they are indeed serologically related.

In order to learn whether the serological cross-reactions of strains 899a and KM of *B. megatherium* were species-specific, a strain of streptococcus T 22/83/2 (obtained from Dr. Maclyn McCarthy) was grown in Todd-Hewitt broth, and tested in its homologous antiserum and in the antisera to the two *megatherium* strains. It was found that the homologous system fixed complement in dilutions

[1] In order to eliminate the possibility of fixation by non-specific antibodies evoked by constituents of the medium it was necessary to grow the microorganisms used in the serological tests in a different medium. The two strains of *B. megatherium* used as test antigens were grown in Todd-Hewitt broth rather than in 5 per cent bacto-peptone.

of serum as high as 1:64. When the streptococcus antigen was tested with the two *megatherium* antisera, however, there was no fixation whatsoever.

*Purification of Megatherium T Phage.*—From the foregoing it is apparent that the two strains of *megatherium* have antigenic components which are either identical or closely related serologically. Before testing the antisera against the lysogenic strain 899a for the presence of antibodies which might react with *megatherium* T phage it is essential that the virus used in the complement-

TABLE I

*Complement-Fixation Reactions of Sera of Rabbits Immunized with B. megatherium (Strains 899a and KM)*

| Rabbit serum No. | Immunized with | Test antigen | Complement-fixation titer* |
|---|---|---|---|
| 2 | KM | KM(control) | 64 |
| 3 | | | 128 |
| 4 | | | 64 |
| 2 | " | 899a | 8 |
| 3 | | | 32 |
| 11 | 899a | 899a(control) | 256 |
| 12 | | | 512 |
| 13 | | | 128 |
| 11 | " | KM | 16 |
| 12 | | | 32 |
| 13 | | | 32 |

* In this and in subsequent tables the values in this column represent the reciprocal dilution of serum giving complete fixation of complement (see text).

fixation test be purified so that it be free of all cross-reacting bacterial antigens. This was accomplished as follows:—

Three Roux bottles, each containing 100 ml. of 2 per cent dialyzed bacto-peptone–2 per cent agar medium, were seeded with 3 ml. of an aqueous suspension of KM, previously grown on the same medium for 18 hours at 35°C. The cultures were incubated at 35°C. for 18 hours, and the bacilli were washed from the bottles with 60 ml. of sterile distilled water. 40 ml. of this suspension ($2.5 \times 10^9$ B/ml.) was added to 1000 ml. of dialyzed peptone–yeast extract medium. The culture was shaken for 3 hours at 35°C. The bacterial population, initially $1 \times 10^8$ B/ml., increased during this interval to $4 \times 10^8$ B/ml. At this point 75 ml. of *megatherium* T phage containing $3 \times 10^{10}$ P/ml. was now added. The virus stock had been prepared previously by infecting KM bacilli grown in a smaller quantity of the same medium.

The flask containing the large volume of infected bacilli was shaken for 3½ hours at 35°C. and the bacilli were then removed by spinning at 1500 R.P.M. for 1 hour at 0°C. The supernate containing the virus ($4 \times 10^{10}$ P/ml.) was treated with filteraid and the clear filtrate was passed through a Cours P5 candle. After filtration there were $2.4 \times 10^{10}$ P/ml.

The viral suspension was next sedimented in a Spinco centrifuge at 0°C. and at 25,000 *G* for 2¾ hours. The viral pellets were resuspended in 7 ml. of sterile 1 M NaCl solution, and, after standing overnight, the solution was again spun for 20 minutes at 8000 *G*. The supernate containing the virus ($1.7 \times 10^{12}$) was resedimented for 1 hour at 26,000 *G*. A third cycle of differential centrifugation yielded 6 ml. of a suspension of virus ($6 \times 10^{11}$ P/ml.) which was used in the complement-fixation tests.

Electron microscopy revealed that the *megatherium* T phage prepared in the manner described was essentially free of extraneous matter. The photographs were taken by Dr. M. A. Jesaitis, using an R.C.A. electron microscope. When tested with *B. megatherium* KM antiserum the solution was found to be free of all complement-fixing antigens which might have come from the parent host cell; in addition, the viral suspension itself was not anticomplementary (*cf.* Table II). It should be said that when the *megatherium* T virus was concentrated

TABLE II

*Complement-Fixation Reactions of Purified megatherium T Phage in Homologous Antiviral and in Antibacterial (Strain KM) Sera*

| Rabbit serum No. | Immunized with | Test antigen | Complement-fixation titer |
|---|---|---|---|
| 182 | Megatherium T phage | Purified T phage $1 \times 10^{10}$ P/ml. | 256 |
| 3 | *B. megatherium* KM | " " | <2 |

by differential centrifugation from lysates of KM bacilli grown in undialyzed bacto-peptone medium, the virus was so highly anticomplementary that it could not be used.

*Antigenicity of Megatherium T Phage.*—As was suggested earlier, immunological procedures might be of value in elucidating the nature of prophage as it occurs in the lysogenic strain of *B. megatherium*; only if the cells themselves are free of adsorbed extracellular phage. It has been well established that if the bacilli are grown in conventional media there is a liberation of free virus which is readily reabsorbed (11). Any immunological experiments conducted with such cells would be valueless for it could not be ascertained with certainty whether the antiviral response they might elicit is attributable to intracellular prophage or to readsorbed virus. It has been shown, however, that when the lysogenic strain of *B. megatherium* is grown in a synthetic medium deficient in magnesium ions, little detectable extracellular virus is produced (2). Cells grown in such a medium should therefore contain little or no adsorbed phage and any antiviral response which they might elicit in experimental animals could be attributed directly to their content of prophage.

Before attempting such experiments, however, it is desirable to learn some-

thing concerning the antigenic behavior of the T phage itself, of the minimal dosage necessary to evoke a specific immune response, whether formalized phage is still antigenically potent and whether intracellular phage, formed prior to the burst, is capable of evoking specific antiviral immune bodies.

*Minimal Amount of T Megatherium Phage Necessary to Elicit a Specific Immune Response.*—The minimum concentration of *megatherium* T phage necessary to evoke antiviral neutralizing and complement-fixing antibodies was determined by immunizing three groups of rabbits with viable and formalized virus at three different concentration levels, using the dosage previously described. The first group of animals received a suspension containing $2 \times 10^6$ viable phage per ml.; the second and third groups were injected with $1.1 \times 10^7$

TABLE III

*Complement-Fixing and Neutralizing Antibodies in Sera of Rabbits Immunized with Various Concentrations of megatherium T Phage*

| Rabbit serum No. | Immunized with | Neutralization titer* | Complement-fixation titer |
|---|---|---|---|
| | *P/ml.* | | |
| 15 | $2 \times 10^6$ | <5 | <2 |
| 16 | | <5 | <2 |
| 17 | | <5 | <2 |
| 76 | $1.1 \times 10^7$ | 159 | 8 |
| 77 | | 132 | — |
| 50 | $9.6 \times 10^7$ | 894 | 64 |
| 51 | | 621 | — |

* In this and in subsequent tables the values in this column represent the reciprocal dilution of serum which will neutralize 50 per cent of the virus tested.

and with $9.6 \times 10^7$ of formalized phage per ml. respectively. The virus used to inject the latter two groups of animals was prepared by treating viable phage ($1 \times 10^9$ P/ml.) with formalin (0.5 per cent) and incubating the mixture for 12 hours at 37°C. The suspension, which contained no viable virus, was then diluted to the appropriate concentration. In all instances two courses of injections were given. The results of the immunization experiment are presented in Table III.

It can be seen from Table III that only those animals which had received $1.1 \times 10^7$ formalized particles per ml. or more gave a significant antiviral response as measured by neutralization and complement-fixation tests. Rabbits which had been injected with $2 \times 10^6$ viable phage particles per ml. showed no significant increase either in neutralizing or complement-fixing antibodies. Although it is not indicated in the table it should be said that the normal sera of all the animals studied were tested for their ability to neutralize phage. In

no instance was more than 4 per cent of the total virus used in the test inactivated by the normal sera. These same sera when tested for their ability to fix complement, showed no positive fixation.

*Antigenicity of Intracellular T Phage.*—Although formalized extracellular *megatherium* T virus at a concentration level of approximately $1 \times 10^7$ particles per ml. of higher will evoke specific neutralizing and complement-fixing antibodies in rabbits, it is not known whether intracellular phage is capable of so doing. It is conceivable that under these conditions the antigenicity of the virus particle might remain masked. Before embarking upon a study of the antigenicity of intracellular prophage, it seemed desirable to ascertain whether mature and viable *megatherium* T virus within infected cells is capable of eliciting viral neutralizing antibodies in experimental animals. Consequently a culture of the sensitive strain (KM) of *B. megatherium* was singly infected with T virus, and the virus permitted to multiply to a point just prior to the burst. The cells were then killed with formalin, and their antigenicity was studied. The dosage of infected bacilli injected was such that the concentration of added virus was below the level of antigenic efficacy.

20 ml. of 5 per cent bacto-peptone was seeded with KM cells so that the initial concentration was $1 \times 10^6$ B/ml. The tube was shaken for 3 hours at 35°C. At this time the bacterial population, as determined by microscopic count, had increased to $1.1 \times 10^7$ B/ml. To this was added *megatherium* T phage so that the culture contained $1.04 \times 10^7$ P/ml. A determination of free virus in a centrifuged sample removed at the end of 33 minutes revealed that 94 per cent of the virus had been adsorbed. 15 ml. of the culture was removed at the end of 33½ minutes. The virus growth was stopped by addition of formalin to a concentration of 0.5 per cent. The tube was then chilled to 0°C. for 30 minutes and was finally permitted to stand at 37°C. for 12 hours.

The multiplication of virus was followed in the remaining portion of the viable culture. It was found that the burst began 50 minutes after the initial infection, when a slight increase in phage was observed in the supernate ($1.8 \times 10^7$ P/ml.). At intervals thereafter phage production was followed until 120 minutes had elapsed. The final viral assay revealed that the phage concentration was $1.6 \times 10^9$ P/ml. Thus, each infected organism produced approximately 150 new viral particles.

The cells in which viral reduplication had been arrested after 33½ minutes by the addition of formalin were separated by centrifugation. The supernate was decanted and the cells were resuspended in fresh medium so as to contain $1 \times 10^6$ bacteria per ml.

A group of 6 rabbits was now injected with the bacterial vaccine containing intracellular phage. The schedule of immunization was the same as that previously described. In all, three courses of injections were given, and each serum was assayed for its ability to neutralize *megatherium* T phage, using the procedure previously described. Tests were made after each course of immunization.

From the results presented in Table IV it can be seen that the bacteria in which the *megatherium* T virus had been permitted to reduplicate were fully capable of eliciting viral neutralizing antibodies in the experimental animals. Since the microorganisms used in the immunization experiments had been

singly infected, the concentration of absorbed virus could not have been greater than $10^6$ P/ml. This amount of virus is too small to elicit an immune response in experimental animals, as has just been shown. The viral neutralizing antibody which appeared must therefore have been evoked by the newly formed intracellular phage. Unfortunately we have no adequate means to determine the concentration of the latter at the 33½ minute interval.

In order to demonstrate that the intracellular virus was also capable of eliciting complement-fixing antibodies, the serum of one of the animals (No. 95) in the group was tested after the second and third course of injections. Both bleedings showed a 4+ reaction at a serum dilution of 1:16. Thus it is seen that intracellular phage is fully capable of stimulating both viral neutralizing and complement-fixing antibodies.

TABLE IV

*Neutralizing Antibodies in Sera of Rabbits Immunized with B. megatherium (Strain KM) Containing Intracellular Phage*

| Rabbit serum No. | Course of immunization | Neutralization titer |
|---|---|---|
| 92 | 2 | 24 |
| | 3 | 241 |
| 93 | 2 | 23 |
| | 3 | 200 |
| 95 | 2 | 153 |
| | 3 | 735 |
| 96 | 2 | 38 |
| | 3 | 553 |

These experiments portended well for those which are to be described later; for if intracellular phage can function antigenically, one would not anticipate any masking of its counterpart, prophage, in the lysogenic strain of *B. megatherium*.

*Antiviral Response Induced by B. megatherium 899a Grown in Tryptose Phosphate Broth.*—It has previously been reported that the lysogenic strain 899a of *B. megatherium* will give rise to antiviral immune bodies when injected into rabbits (9, 11, 12). It is unfortunate, however, that no experimental details were given in regard to the preparation of the immunizing antigen used in these important experiments. In the following account it will be seen that when the lysogenic strain of *B. megatherium* is grown in a medium in which certain of the cells undergo spontaneous lysis resulting in the release of phage, these microorganisms, even though carefully washed free of extracellular virus, are still capable of evoking a marked antiviral response.

20 ml. of tryptose phosphate broth was inoculated with a culture of *B. megatherium* 899a grown on yeast extract agar so that the initial concentration was $2 \times 10^7$ B/ml. The culture was incubated with shaking for 3½ hours at 35°C. At the end of this interval the bacterial population had increased to $5 \times 10^8$ cells per ml. The culture was then chilled and centrifuged at 0°C. for 10 minutes. The supernatant liquid, after filtration, contained $8.5 \times 10^7$ virus particles per ml. The chilled microorganisms were washed 5 times with 20 ml. portions of 5 per cent bacto-peptone at 0°C. The fifth washing contained only $2.8 \times 10^4$ virus particles per ml. The microorganisms were then killed by suspending them in 20 ml. of 5 per cent bacto-peptone containing 0.5 per cent formalin. The tube was incubated at 37°C. for 8 hours and then held for 6 hours at 4°C. before testing for sterility. The formalized bacilli, now containing $5 \times 10^8$ B/ml., were again centrifuged and the killed bacteria were resuspended in 20 ml. of fresh 5 per cent bacto-peptone. The suspension of bacteria was now divided into two parts and diluted so as to contain $1 \times 10^8$ and $1 \times 10^7$ B/ml. respectively. Rabbits were then immunized with these two vaccines. Each group of animals received 4 courses of injections using the quantities described under Methods. The sera were then tested for their content of viral neutralizing and complement-fixing antibodies.

The results of these experiments are presented in Table V in which it can be seen that the lysogenic microorganisms grown in tryptose phosphate broth were fully capable of inducing an antiviral response in rabbits. Even though the bacterial cells had been repeatedly washed and were essentially free of active virus prior to their being killed with formalin, nevertheless they still contained sufficient virus to evoke both neutralizing and complement-fixing antibodies. Whether this can be attributed to virus adsorbed on the surface of the bacilli, or to antigenically mature virus present within certain cells cannot yet be said. It should be pointed out, however, that when the lysogenic strain of *B. megatherium* is grown in tryptose phosphate broth, considerable amounts of extracellular phage are produced because of the spontaneous lysis of some of the cells in the culture. Gratia (11) has found that *megatherium* T phage is adsorbed by the lysogenic strain, 899a, and in this laboratory we have confirmed these observations.[2] In this connection it should also be said that *megatherium* T virus, when absorbed on heat-killed cells of the sensitive strain KM, is fully capable of eliciting viral antibodies.

Thus, the cells in a suspension of *B. megatherium* KM ($1 \times 10^9$ B/ml.) were killed by heating for 2 minutes at 100°C. To these was then added *megatherium* T phage so that the concentration was $1.9 \times 10^9$ P/ml. After 18 hours at 35°C. 94 per cent of the virus had been adsorbed. After repeated washing to remove free virus, the cells ($1 \times 10^9$ B/ml.) were used

[2] *Megatherium* T phage is readily inactivated in tryptose phosphate broth. Thus when the virus ($7.3 \times 10^6$ P/ml.) is shaken at 35°C. for 30 minutes the titer drops 63 per cent ($2.7 \times 10^6$). If to this same virus one adds living lysogenic bacilli ($2.5 \times 10^8$ B/ml.), the titer drops 73 per cent in this same material ($1.8 \times 10^6$ P/ml.). It would appear, therefore, that some of the virus is adsorbed. The exact amount is undoubtedly greater than the figures would indicate because the culture itself is constantly producing virus, and the bacterial cells exert a stabilizing effect upon the phage. If this same experiment is performed in bacto-peptone (5 per cent) rather than T.P.B., only 4 per cent of virus is inactivated and 94.1 per cent is adsorbed.

to immunize a group of rabbits. It was found that all animals in the group, after two courses of injections, produced potent phage-neutralizing antisera.

TABLE V

*Neutralizing and Complement-Fixing Antibodies in Sera of Rabbits Immunized with B. megatherium (Strain 899a) Grown in Tryptose Phosphate Broth*

| Rabbit serum No. | Immunized with | Course of immunization | Titer | |
|---|---|---|---|---|
| | | | Neutralization | Complement fixation |
| | *B/ml.* | | | |
| 121 | $1 \times 10^7$ | 1 | <5 | — |
| | | 2 | <10 | 2 |
| | | 3 | 23 | 8 |
| | | 4 | 44 | 8 |
| 111 | " " | 1 | 9 | — |
| | | 2 | <5 | <2 |
| | | 3 | <5 | <2 |
| | | 4 | 29 | <2 |
| 112 | " " | 1 | <10 | — |
| | | 2 | <10 | <2 |
| | | 3 | <5 | <2 |
| | | 4 | 82 | <5 |
| 122 | $1 \times 10^8$ | 1 | <10 | <2 |
| | | 2 | 24 | <2 |
| | | 3 | 113 | 32 |
| | | 4 | 1635 | 32 |
| 117 | " " | 1 | <5 | <2 |
| | | 2 | 37 | 16 |
| | | 3 | 254 | 32 |
| | | 4 | 479 | 32 |
| 119 | " " | 1 | <5 | 4 |
| | | 2 | 64 | 16 |
| | | 3 | 100 | 8 |
| | | 4 | 247 | 16 |

*Antiviral Response Induced by B. megatherium 899a Grown in Magnesium-Deficient Synthetic Medium.*—When the lysogenic strain of *B. megatherium* 899a is grown in a medium containing magnesium ions, the majority of cells do not undergo lysis, yet they retain the ability to transmit prophage to their progeny. A certain small proportion of the cells, however, do undergo lysis and liberate free phage. If the microorganisms are cultivated in the medium

which is deficient in magnesium ions there is no apparent liberation of phage even on repeated transfer (2). Nevertheless, it can be shown that each cell in the culture retains its ability to produce virus, for when the cells are plated on a medium containing magnesium ions the colonies which arise all contain free phage. Whether the lysogenic cells grown in a magnesium-deficient medium embody an intracellular virus, or merely a genetic unit or prophage, capable of being transmitted to the progeny, has not yet been fully ascertained by immunological means. If virus is present the cells should elicit antiphage immune bodies. If, however, they merely carry the genetic unit or prophage, then one would not expect them to be capable of eliciting an antiviral immune response, because of the chemical differences of the components in question.

A culture of *B. megatherium* 899a grown on a bacto-peptone slant was taken up in 10 ml. of magnesium-deficient synthetic medium (SM). The bacteria were spun, washed three times with 10 ml. portions of SM, and finally resuspended in 10 ml. of the same medium. The culture was diluted to contain $1 \times 10^7$ bacteria per ml., and incubated with shaking at 35°C. for 4 hours. Two additional transfers were made using 20 ml. of the previous culture plus 80 ml. of fresh SM per flask. A fourth transfer of the culture, using 240 ml. of fresh medium, was finally seeded with 60 ml. of the third transfer. The flask, containing initially $1 \times 10^6$ B/ml., was placed in an incubator and shaken for 8 hours at 35°C. The bacterial population increased during this interval to $1.7 \times 10^7$ B/ml.[8] The suspension of bacilli was chilled and centrifuged at 0°C. for 15 minutes. The supernatant liquid was removed and filtered through a Cours P3 candle; it was found to contain $8 \times 10^4$ P/ml.

The chilled microorganisms were suspended in 30 ml. of cold SM and again spun at 0°C. The second supernatant, after filtration, contained less than $10^2$ P/ml. The bacteria were now resuspended in 30 ml. of SM containing 0.5 per cent formalin, and incubated at 37°C. for 12 hours. The formalin-killed microorganisms, at a concentration of $4 \times 10^8$ bacteria per ml., served as vaccine for the immunization of animals. In order to determine if the supernate in which the microorganisms were grown contained viral antigen capable of stimulating specific antiviral bodies, rabbits were immunized with this material also.

Since the synthetic medium itself rapidly inactivates *megatherium* T phage, an additional important control was included in this experiment. *Megatherium* T phage in bacto-peptone ($5 \times 10^{10}$ particles per ml.) was sedimented in the ultracentrifuge. The tube was inverted and carefully washed with sterile SM; the viral pellet was then suspended in this medium so as to contain $3 \times 10^9$ particles per ml. The phage was now shaken for 8 hours at 35°C. At this point the titer of infectious particles had dropped to $3 \times 10^3$ per ml. The content of the tube was treated with formalin at a concentration of 0.5 per cent and after incubation for 12 hours at 37°C. was diluted 1:10 in SM. The inactivated virus was then used for the immunization of an additional group of rabbits.

---

[8] We have observed considerable variation in the growth of *B. megatherium* in the magnesium-deficient medium described here. This is probably due to variations in the amounts of contaminating magnesium salts present in the chemicals used in the preparation of the medium. Some batches of media have failed almost entirely to support growth. By adding $MgCl_2$ ($10^{-4}$ M) it was found that the bacteria would reproduce to the extent of approximately 4 generations in 8 hours, without producing much detectable extracellular virus (about $6 \times 10^4$ P/ml.). In the work which is described we employed only those batches of media which supported growth without the addition of magnesium chloride.

As can be seen from Table VI, the sera of rabbits injected with *B. megatherium* 899a grown in magnesium-deficient medium, after two or more courses of immunization, did not show any significant neutralizing action against *megatherium* T phage. Furthermore the supernatant liquid in which these microorganisms had been grown was incapable of inducing either complement-fixing or viral neutralizing bodies in rabbits. Still more significant is the fact that infectious phage, inactivated by shaking in the synthetic medium, retained its antigenicity. Protocols need not be given.

TABLE VI

*Neutralizing and Complement-Fixing Antibodies in Sera of Rabbits Immunized with B. megatherium (Strain 899a) Grown in Magnesium-Deficient Synthetic Medium*

| Rabbit serum No. | Courses of immunization | Neutralization titer | Complement-fixation titer using | | | |
|---|---|---|---|---|---|---|
| | | | $10 \times 10^9$ P/ml. | $5 \times 10^9$ P/ml. | $1 \times 10^9$ P/ml. | $0.25 \times 10^9$ P/ml. |
| 47 | 2 | <10 | 0 | — | — | — |
| 49 | 2 | <10 | 0 | 0 | 0 | 0 |
| 90 | 2 | <5 | 0 | — | — | — |
| 92 | 2 | <5 | 0 | — | — | — |
| 99 | 2 | <5 | 0 | — | — | — |
| 120 | 2 | <5 | 0 | — | — | — |
| 105 | 2 | 0 | 0 | — | — | — |
| | 3 | <10 | 0 | 0 | 0 | 0 |
| | 4 | <5 | 0 | 0 | 0 | 0 |
| 109 | 2 | <5 | 0 | 0 | 0 | 0 |
| | 3 | 0 | 0 | 0 | 0 | 0 |
| | 4 | <5 | 0 | 0 | 0 | 0 |
| 182* | 2 | >12,800 | 128 | 256 | 256 | <2 |

* Serum of a rabbit immunized with *megatherium* T phage. This control titration demonstrates the minimum amount of purified phage necessary to bind complement when tested in homologous antiviral serum.

From these results it appears that *B. megatherium* 899a grown in a magnesium-deficient, synthetic medium neither liberates phage nor harbors antigenically active intracellular virus.

*Complement-Fixation Reaction of Sera of Rabbits Immunized with B. megatherium 899a Grown in Magnesium-Deficient Synthetic Medium.*—Although cells of the lysogenic strain of *B. megatherium*, when grown in synthetic magnesium-deficient medium fail to give rise to viral neutralizing antibodies, it is conceivable that these cells might contain an intracellular antigenic component related to mature infectious virus which could be demonstrated only by complement-fixation reactions. This concept has some basis in fact, for it has been shown by Schlesinger (13) that the brain tissue of infected mice contains non-infectious

complement-fixing material which is serologically related to influenza virus A. Furthermore Lanni (14) has shown that the non-infectious tailless particles, or "doughnuts" present in premature lysates of phage-infected *Escherichia coli*, give complement fixation when tested in antiphage serum.

Because the lysogenic and sensitive strains of *B. megatherium* contain serologically related antigenic components, it was necessary to use purified *megatherium* T virus, free of bacterial components, in the complement-fixation tests. The tests were performed by the procedure referred to, using the sera of 8 rabbits which had been subjected to two courses of immunization with the lysogenic cells grown in the synthetic medium. Two of these animals were given two additional courses of injections and their sera were likewise tested. The results of these tests are given in Table VI in which it can be seen that in no instance was there any demonstrable complement-fixing antibody in the sera of the various animals which had been repeatedly injected with the lysogenic strain of *B. megatherium* grown in the synthetic, magnesium-deficient medium. Not only is this indicative of the fact that the cells were free of significant amounts of adsorbed extracellular virus, but it is pertinent evidence that when lysogenic cells are grown under conditions in which prophage cannot be converted to mature infectious virus, the prophage itself fails to elicit any demonstrable antibody, either neutralizing or complement-fixing, which is related to mature virus. The serological evidence lends noteworthy support to the concept that prophage is not a particulate substance antigenically related to infectious virus.

## DISCUSSION

The history of the development of our knowledge concerning lysogenicity need not be reviewed here save to say that from the beginning it was believed by many working in this field that the capacity to perpetuate phage is hereditary. The earlier work of Bordet (15), or Burnet (16), and of the Wollmans (17) all supported this concept. In recent years experimental evidence of a more direct nature has been presented in the brilliant research of Bertani (18), Lwoff (19), and Lederberg (20), which adds additional support to the genetic hypothesis.

The immunologists have not fared so well. To our knowledge no truly pertinent immunological evidence concerning the nature of prophage has been presented. There is one exception, however, which must be mentioned and which has been quoted as a personal communication in a review by Lwoff (1). He states that Lanni attempted to detect antigens of a phage $P_2$ in lysates obtained by the sonic disintegration of a lysogenic strain of *Shigella dysenteriae*, but to no avail. Although in our hands this procedure for disrupting bacteria has proved highly unpredictable in so far as the preservation of sensitive intracellular constituents is concerned, it would appear from the results of Lanni's experiments that neither virus itself nor viral components are present in his lysogenic strain of *Shigella dysenteriae*.

In this connection it should be pointed out that Barry (21) was unable to demonstrate by immunological means the appearance of a viral neutralizing antigen in *E. coli* B infected with the lytic coli-dysentery phage $T_3$ prior to the appearance of intracellular virus.

It has now been well established that bacteriophage can give rise in experimental animals to at least two types of antibodies,—those which fix complement when tested with the virus, and those which neutralize the infectivity of the virus. Recently Rountree (22) has reported that both the coli phage $T_5$ and the staphylococcal phage 3A elicit complement-fixing antibodies in rabbits and that these appear prior to the viral neutralizing antibodies.

The experiments which have been reported in this communication were designed to ascertain whether the cells of the lysogenic strain of *B. megatherium* can elicit specific antibodies which bear any serological relationship to the infectious virus to which these cells give rise. It has been seen that when the lysogenic organism is cultivated under conditions in which little extracellular phage is produced, no significant amount of viral antibodies is evoked, either complement-fixing or viral neutralizing. The slight increase in the latter which was observed to occur in some animals was too small to be of significance; it undoubtedly arose from the stimulus furnished by small amounts of adsorbed extracellular virus. It must be remembered that each cell in the vaccine was a potential producer of virus at the time the microorganisms were killed and that each cell must have contained at least one unit of prophage. In terms of mature virus, the concentration of bacilli (and hence prophage) which was injected was well in excess of the minimal amount necessary to evoke antibody. Yet none was formed, and so it must be concluded either that prophage is not antigenic, or, if it is, that its antibody bears no relationship to the antigens of mature infectious *megatherium* virus.

The observations reported here indicate that the prophage of *B. megatherium* is not related antigenically to the lytic virus which on occasion these cells elaborate. The immunological evidence lends support to the hypothesis that prophage is a genetic structure which is transmitted on cell division to each new generation of the microorganism.

The authors wish to express their thanks to Drs. Peter Olitsky and Jordi Casals for their interest and counsel, and to Dr. John Northrop, who has followed the course of this work throughout.

## SUMMARY

1. By complement-fixation tests it has been shown that the lysogenic and sensitive strains of *B. megatherium* share one or more antigenic components which are serologically related but not identical.

2. Bacilli of the lysogenic strain of *B. megatherium*, when grown under conditions such that little extracellular phage is produced, fail to evoke antibodies in rabbits which react either in complement-fixation or neutralization tests with

purified *megatherium* T phage. From this it must be concluded either that prophage is not antigenic or that any antibody which it might elicit does not react with the antigens of the mature virus.

The observations reported in this communication accord with the hypothesis that prophage is a genetic structure.

## BIBLIOGRAPHY


1. Lwoff, A., *Bact. Rev.*, 1953, **17,** 269.
2. Northrop, J. H., *J. Gen. Physiol.*, 1951, **34,** 715.
3. den Dooren de Jong, L. E., *Zentr. Bakt.*, 1931, **120,** 1.
4. Gratia, A., *Compt. rend. Soc. biol.*, 1936, **123,** 1018. Murphy, J. S., *J. Exp. Med.*, 1953, **98,** 659, 640.
5. Northrop, J. H., *J. Gen. Physiol.*, 1952, **35,** 471.
6. Comroe, J. H., Jr., *Methods Med. Research*, 1950, **2,** 7.
7. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27,** 493.
8. Casals, J., Olitsky, P. K., and Anslow, R. O., *J. Exp. Med.*, 1951, **94,** 123.
9. Gratia, A., *Compt. rend. Soc. biol.*, 1937, **124,** 577.
10. Todd, E. W., and Hewitt, L. F., *J. Path. and Bact.*, 1932, **35,** 973.
11. Gratia, A., *Ann. Inst. Pasteur*, 1936, **57,** 652.
12. Wollman, E., and Wollman, E., *Compt. rend. Soc. biol.*, 1939, **131,** 442.
13. Schlesinger, R. W., *Proc. Soc. Exp. Biol. and Med.*, 1950, **74,** 541.
14. Lanni, F., and Lanni, Y. T., *Cold Spring Harbor Symp. Quant. Biol.*, 1953, **18,** 159. Lanni, Y. T., *J. Bact.*, 1954, **67,** 640.
15. Bordet, J., *Ann. Inst. Pasteur*, 1925, **39,** 711.
16. Burnet, F. M., and McKie, M., *Australian J. Exp. Biol. and Med. Sc.*, 1929, **6,** 277.
17. Wollman, E., *Bull. Inst. Pasteur*, 1928, **26,** 1.
18. Bertani, G., *Cold Spring Harbor Symp. Quant. Biol.*, 1953, **18,** 65; *J. Bact.*, 1954, **67,** 696.
19. Lwoff, A., and Gutmann, A., *Ann. Inst. Pasteur*, 1950, **78,** 711.
20. Lederberg, E. M., *Genetics*, 1951, **36,** 560.
21. Barry, G. T., *J. Exp. Med.*, 1954, **100,** 163.
22. Rountree, P. M., *Brit. J. Exp. Path.*, 1951, **32,** 341. *Australian J. Exp. Biol. and Med. Sc.*, 1952, **30,** 567.