BACTERIOPHAGE PHENOMENA[1]

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INTRODUCTION

During the past four years an increasing number of articles have appeared in the bacteriological literature on the so-called "bacteriophage phenomena." It seems advisable, therefore, to give a brief historical review of the data which have accumulated to date and the various theories advanced in regard to this subject, before reporting our own experiments. The fundamental discoveries will be stated first and then the more recent detailed experiments will be discussed as they bear upon the particular point under consideration.

General interest was first attracted to this subject by the work of d'Herelle which was published towards the latter part of 1917. It is now, however, conceded by the majority of workers in this field, that the first observations of this phenomenon were made by Twort in 1915.

Twort (1915) was working on the problem of cultivating filtrable viruses. In some of his experiments he used glycerinated calf vaccinia. He inoculated agar tubes with the vaccinia before the glycerine had sterilized it completely, and found that the colonies of yellow and white micrococci that grew out, showed what he described as "watery" areas. These areas could not be sub-cultured. He found, furthermore, that if the water of condensation from these tubes was plated, colonies of the micrococci developed which also became transparent. If he touched a normal colony of the micrococci with some of this "transparent material," the normal colony would become "watery." The

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"transparent material" was still active in high dilution (1:1,000,000), and remained so after passage through a Pasteur Chamberland filter. All attempts to sub-culture the filtrate proved negative. If the filtrate of the transparent material was added to an agar slant which was then inoculated with a normal culture of the micrococcus, the organism would start to grow, but the growth soon become translucent. The filtrate used in higher dilution, produced only a small number of transparent spots which appeared at various points of the culture. Twort found that the transparent material could be transmitted from one young culture to another indefinitely. He also observed that these clear areas appearing on slant would in time be overgrown, but that the transparent material was still active when added to a new, growing culture of the micrococci. Thus, Twort, in 1915, stated all the fundamental characteristics of this phenomenon which have been confirmed in practically every detail in connection with all the different "bacteriophages," active against a wide variety of organisms, that have been discovered since.

To summarize briefly the characteristics of the "transparent material" isolated by Twort from micrococci growing out from calf vaccinia:

1. It can be separated from the organisms from which it is derived by filtration.
2. It can be transmitted indefinitely from one culture to another.
3. It is most active against young actively growing organisms.
4. It has no action on dead organisms.
5. It cannot be transmitted without the presence of organisms in any media.
6. It is active in high dilution, 1:1,000,000.
7. It is fairly heat resistant, 60° for one hour being necessary to destroy it.
8. The action is not hastened by anaerobiosis.
9. It increases in quantity when allowed to act on a culture.
10. In old cultures the activity of the transparent material is diminished or impeded.
11. It acts to a less degree on closely related organisms, such as Gram positive cocci isolated from boils.
12. It does not act on organisms unrelated to the micrococci—such as *Bact. coli*, streptococci, tubercle bacilli, yeasts.

Twort in concluding his article considers most of the hypotheses as to the nature of this substance, that are still under discussion today. Twort assumes that if derived from the organism itself, it may be either

a stage in the life history of the organism which is filtrable and will not grow on any media and stimulates other cultures to pass to the same stage; or it may possibly be an enzyme which is secreted by the bacteria and leads to their own destruction, a process during which the amount of the enzyme is increased. Twort also discusses the possibility that this material might be a filtrable virus which is present as a contamination. Animal inoculation was absolutely negative, this definitely ruling out the vaccinia, and Twort found no very good reason for considering it a non-pathogenic virus. He concluded that it might best be regarded as "an acute infectious disease of micrococci."

Twort also mentions a bacillus not related to the *Bact. coli* group, isolated from cases of infantile diarrhea which had the property of "dissolving itself."

Twort's work did not attract very much attention, and it was not until d'Herelle published a series of papers on what is now generally considered the same phenomenon, that Twort's contributions to the subject were seriously studied.

D'Herelle (1917) was studying the effect of the addition of stool filtrates obtained from dysentery cases on the growth of the Shiga dysentery bacillus. In his original experiment, he obtained daily specimens of feces from a dysentery patient and filtered them, adding the filtrate to fresh Shiga cultures. He reported this experiment until one day when the patient had reached the convalescent stage, he observed that the turbid Shiga culture had after an interval become clear, subsequent to the addition of the last stool filtrate. He next found that the addition of a small quantity of this cleared culture to another turbid young Shiga culture would dissolve the second culture, and that he could repeat this process indefinitely from one dissolved culture to another. In one instance, he transmitted the lytic principle originally obtained from a stool filtrate, through 935 culture generations, in each case adding only a minute quantity of the dissolved culture to the next tube, so that the original stool filtrate which started the lysis was soon eliminated in the successive dilutions. The dissolved cultures were not always completely sterilized. If sub-cultured, a small number of colonies would be obtained, the character of which will be described in detail below.

The lytic material isolated by d'Herelle has all the characteristics of Twort's "transparent material" outlined above. The

fact that the stool filtrates and their derivatives were active only against living bacteria and could be carried on indefinitely from one culture to another convinced d'Herelle that he was dealing with an ultramicroscopic filtrable virus which was parasitic on bacteria, to which he gave the name "*Bacteriophagum intestinale.*" This name "bacteriophage" has now come into general use for phenomena of this type, irrespective of the source of the material which first starts the lytic process or the particular organism against which it acts, and in spite of the fact that the theory which the name implies, is not accepted by the majority of investigators at the present time.

D'Herelle does not believe that phenomena described by him relating to the typhoid-dysentery-coli group are the same as those described by Twort, and since repeated by Gratia and Callow with staphylococci and vaccine virus. D'Herelle states that the two phenomena cannot be the same because of the differences in heat resistance. Twort in his original article says that his "transparent material" resists a temperature of 52° for one hour, but is destroyed at 60° for one hour. D'Herelle considers that one of the essential criteria of true bacteriophage action is the fact that the bacteria which persist after the dissolving action of the bacteriophage has taken place, are killed at a temperature which does not destroy the bacteriophage. For instance, the Shiga bacillus is killed at a temperature of 60°C. for thirty minutes, whereas the particular bacteriophage which d'Herelle tested was only destroyed at 65°C. But, as Gratia (1921) has pointed out, the heat resistance of the bacteriophage is not a fixed property and depends upon the particular culture against which the bacteriophage is tested. Thus, in Gratia's experiment, a bacteriophage heated at 56°C. for thirty minutes lost its activity against staphylococcus A, but retained it for staphylococcus B, and regained it for strain A. Callow working in our laboratory has isolated a bacteriophage from vaccine virus active against staphylococcus which is diminished but not completely destroyed at 75° for thirty minutes. Thus, the heat resistance of bacteriophages is a variable property which depends not only on methods of testing, but also on the

way the bacteriophage is obtained. Any one who has compared the action of a staphylococcus bacteriophage isolated from vaccine virus and a dysentery or typhoid bacteriophage cannot doubt that the same phenomenon is involved in both cases. Certain minor differences do exist, but they can probably be attributed to the biological differences between staphylococci and members of the typhoid-colon-dysentery groups, since these organisms serve as the indicators upon which we depend for evidence of bacteriophage action.

D'Herelle has recently published a book on "Le Bacteriophage" which contains the results of four years of experimental work on this subject. Before going on to the theoretical aspects of the subject, it may be well to enumerate a few of the most important observations made by him, in addition to the fundamental principles which apply to all bacteriophage phenomena outlined above, from Twort's work.

(1) Filtrates from the stools of typhoid and dysentery convalescents in a large percentage of cases contain a lytic principle which has the power of dissolving or inhibiting the growth of typhoid or dysentery bacilli, and which can be transmitted in series indefinitely.

(2) The lytic principle isolated from stool filtrates is usually not absolutely specific, but is active in most cases against two or more members of the dysentery, typhoid or colon group.

(3) The activity of the lytic agent is often feeble on first isolation from the stool filtrates, but rapidly becomes more active when transmitted in series. Not only in subsequent generations, is the lysis more rapid and the amount necessary to produce lysis from one tube to the next much smaller, but also the range of activity may be extended, so that a filtrate which on first isolation is active only against Shiga and Y dysentery bacilli may after three or four passages with Shiga bacilli show activity against typhoid bacilli as well. It is also possible in some instances to make the lytic principle active against organisms which are not attacked at first, by successive contacts with these bacteria.

(4) Lysis occurs most readily with young cultures of bacteria which are multiplying rapidly. Antiseptics or temperatures which in anyway retard the growth of the bacteria, although they do not in any way lessen the activity of the lytic principle itself, interfere with the lytic action.

(5) It is impossible to transmit the bacteriophage in series in filtrates of broth cultures of susceptible organisms. The products of bacterial growth are not sufficient, the living multiplying bacteria are absolutely necessary.

These, briefly, were the most important observations made by d'Herelle at the time that we began working on this subject in November, 1920. His book which reached this country during November, 1921, contains many new facts which will be discussed below. D'Herelle in his first article published in September, 1917, claimed that the lytic principle isolated by him from dysentery stools and called by him bacteriophage, was an ultramicroscopic living organism parasitic on bacteria, and has maintained this point of view. He considers that the bacteriophage is a normal inhabitant of the intestinal tract, where it is parasitic on *Bact. coli.* During the course of intestinal disease, the bacteriophage becomes parasitic on the invading organism. D'Herelle believes that there exists only one bacteriophage which by processes of adaptation is capable of attacking different organisms with which it comes in contact.

The first investigator to question the living nature of the bacteriophage was Kabeshima (1920). He advanced the following reasons for thinking it a ferment.

(1) An extremely small amount of lytic filtrate is sufficient to dissolve a fairly large number of bacteria.

(2) The lytic filtrate is still active after standing for four years in a sealed tube.

(3) It resists heating (moist) at 70°C. for one hour.

(4) It resists the action of chloroform, toluene, alcohol, ether, carbolic acid, and acetone.

Kabeshima originated the following method of isolating bacteriophage: to 1 volume of lytic filtrate add 3 volumes of acetone. Let the mixture stand at room temperature for forty-eight hours, shaking at intervals. Evaporate the acetone. A yellowish powder remains. The lytic action of this powder according to Kabeshima, is stronger than that of the original lytic ferment.

Kabeshima explained the increase of bacteriophage which occurred when the lytic principle was brought in contact with a susceptible

organism in the following way: In the course of disease, a certain gland in the digestive tract in an effort to digest the invading bacteria, produces a catalyst. This catalyst activates a proferment contained in the bacteria, liberating a ferment which causes the organisms to autolyze. In the next generation this ferment acts as the catalyst and activates the proferment in the new bacteria, again causing them to autolyze, etc. Kabeshima does not state whether he considers the original catalyst and the liberated ferment identical, but their mode of origin is so different that this does not appear likely. In any case, the catalyst and the ferment have both the power of activating the proferment present in bacteria.

The next most important contribution to the subject was made by Bordet and Ciuca in a paper published October, 1920. Bordet was the first worker to demonstrate that a lytic principle such as that described by d'Herelle could be obtained without starting from a stool filtrate.

By injecting a certain strain of *Bact. coli* into a guinea-pig intraperitoneally, three or four times at intervals of a few days, Bordet and Ciuca obtained an exudate containing a large number of leucocytes and a few living bacilli. He added several volumes of broth to this filtrate, allowed it to stand at room temperature a few hours or a day and then filtered. This filtrate when added to a normal culture of the colon bacillus produced lysis transmittable in series.

D'Herelle in his early experiments described bacteriophages of such great activity that they sterilized the cultures completely, and no growth was obtained when the dissolved cultures were plated. Bordet and Ciuca were the first investigators to show that in most cases bacteriophage action did not completely sterilize the culture that had been dissolved, but that if such a culture were plated a small number of colonies would develop that presented peculiar characteristics. They develop more slowly than normal colonies and have a tendency to be irregular in outline, and grow discreetly. If these colonies are transplanted to broth, the broth never becomes definitely turbid, and the supernatant fluid of this broth culture, if added to a normal colon culture, has lytic power. In other works, certain colonies that grow out after lysis has taken place, carry the lytic power. Border and Ciuca found that if these cultures were sub-cultured on agar for several generations, they would grow luxuriantly, and often present a glassy mucoid type of growth. They found that these mucoid coli were more virulent

and less easily phagocyted than normal *Bact. coli*. They also reported that if a guinea pig received an M.L.D. of *Bact. coli* and then received an injection of dissolved culture heated at 58° for thirty minutes, this animal survived, whereas the control died in eight hours.

Bordet and Ciuca like Kabeshima, did not agree with d'Herelle that bacteriophage action was due to a living virus. They interpreted this phenomenon as a hereditary tendency for a given culture to autolyze, acquired during exposure to unfavorable circumstances, such as are produced by the leucocytic response in the peritoneal cavity of the guinea pig.

Bordet and Ciuca (1921 a) were the first workers to produce an antilytic serum by inoculating rabbits with increasing amounts of dissolved culture filtrate. The serum of a rabbit treated in this way, was able, according to Bordet and Ciuca, to neutralize the lytic action of their bacteriophage completely. If equal quantities of the lytic agent and the lytic immune serum were added to a culture of *Bact. coli*, normal growth would be obtained, whereas, if in a parallel tube the lytic agent alone was added, no growth occurred. Bordet and Ciuca also showed that the antilytic serum could change a lytic irregular colony such as has been described above, back to a normal colony in the following way. Two agar slants were inoculated, respectively, with 7 drops of the antilytic serum and 7 drops of normal rabbit serum, and incubated overnight. The following day these two slants and one agar slant to which nothing had been added, were inoculated with a lytic colon culture. The slant which had been in contact with the antilytic serum showed confluent, normal growth after eighteen hours; the other two slants, sparse growth of irregular colonies. If these three slants were transplanted to broth, the *Bact. coli* that had grown in the presence of the antilytic serum would cloud the broth uniformly, whereas the broth transplants from the other two slants would remain clear. Thus, the antilytic serum could inhibit or prevent the inheritance of the lytic quality.

The important contributions made by Bordet and Ciuca are:

(1) That a lytic agent transmittable in series could be demonstrated in the leucocytic exudate obtained by repeated injections of *Bact. coli* into the peritoneal cavity of a guinea pig.

(2) That after lytic action had taken place on a given culture, certain colonies developed on subculture which carried the lytic agent in subsequent generations.

(3) That the lytic agent was antigenic; the antilytic serum was able to prevent the action of the lytic agent, and also convert a lytic-bearing colony to a normal colony.

Thus, three fundamentally differing theories had been advanced when we began working on the subject of bacteriophage. D'Herelle attributed these phenomena to an ultramicroscopic virus parasitic on bacteria, a normal inhabitant of the intestinal tract of man and animals; Kabeshima, to a new type of enzyme which attacked living cells and was liberated in increased quantity from the substrate on which it acted, and lastly, Bordet and Ciuca, to an acquired hereditary tendency to autolyze, induced by the action of leucocytes on bacteria.

I. OBSERVATIONS ON THE ISOLATION OF THE LYTIC AGENT BY THE METHODS OF BORDET AND CIUCA, AND OF D'HERELLE

Bordet and Ciuca's experiment in which they produced *Bact. coli* bacteriophage experimentally rather than isolating it from stool filtrate in the course of a pathological condition, seemed of the utmost importance from the point of view of explaining the nature of the lytic agent. Our first attempt was, therefore, to duplicate the result of these workers.

Ten guinea-pigs were injected with 10 different strains of *Bact. coli* and the exudate treated exactly according to the directions of Bordet and Ciuca. In no instance, however, was any lytic activity of the exudate demonstrable. Wollstein (1921) repeated Bordet and Ciuca's original experiment, and recommended injecting the guinea-pig once instead of three or four times. We have been able in 2 guinea-pigs to produce a lytic exudate by a single intraperitoneal injection of typhoid bacilli.

It is the opinion of all workers, however, that this method of obtaining the bacteriophage principle is extremely unreliable, and that the factors that determine its development are not understood. Bail has recently reported that he has been able to obtain lytic exudate by the injection of Shiga bacilli, using a single dose of such a size that the pig would require two or three days to die. Gratia has also used this method successfully with staphylococci, but states that he cannot control the various factors in the experiment and that he cannot be sure of obtaining a lytic exudate every time.

D'Herelle has never been able to repeat this experiment of Bordet and Ciuca. He considers the development of lytic exudates as due to the permeation of the intestinal wall by a bacteriophage which preëxisted in the intestine of the pig, as a result of the irritation due to infection in the peritoneal cavity. He attributes the irregularity of these experiments to the fact that the presence of a bacteriophage in the intestine of a particular guinea-pig active against, or at least capable of adaptation against the strain injected, is purely accidental. D'Herelle does not include any guinea-pigs in his series investigating the presence of bacteriophage in the feces of normal animals. Recently, however, Lisbon and Carrère (1922) have attempted to show that the origin of the bacteriophage is in leucocytes rather than in the intestine, but their experiment is not clear cut. They produced sterile abscesses in a series of 5 animals by the injection of sterile petroleum. They were able to isolate a lytic agent active against Shiga dysentery bacilli from the pus thus obtained, in every case. They state, however, that the feces of 4 out of 5 animals tried contained a bacteriophage active against Shiga bacilli. The feces of these animals were not examined before the abscesses were induced, so that it is difficult, since we are dealing with an extremely diffusible substance, to say whether or not the bacteriophage originated in the leucocytes or in the intestine. Since this method of obtaining a bacteriophage principle was first described by Bordet and Ciuca, so many simpler methods of starting the lytic process have been discovered that give greater promise of solving the nature of these phenomena, that it has not seemed worth while to analyze the conditions obtained by peritoneal injections more closely. The fact that leucocytes may play some rôle in starting the lytic process is also suggested by the fact that staphylococcus bacteriophage is so easily isolated from green calf vaccine virus and also, as shown by Callow, from boils.

We may state, however, that while we were unable to obtain a lytic exudate by the method of Bordet and Ciuca with 11 strains of *Bact. coli*, we succeeded in two instances when typhoid bacilli were injected.

Isolation of a typhoid bacteriophage by the d'Herelle technique

A bacteriophage principle was isolated by the technique discovered by d'Herelle, from the stool of a typhoid convalescent, Ida Olsen, sent to me by the courtesy of Dr. Krumwiede of the Research Laboratory of the Health Department.

The stool was plated and typhoid bacilli isolated. Nothing abnormal was noted in the appearance of the typhoid colonies. They agglutinated readily on the slide in a typhoid immune serum in a dilution of 1:100. A small particle of feces was emulsified in broth and incubated overnight. On the following day twice the volume of broth was added, the emulsion was centrifuged, and filtered through a Berkefeld filter. This filtrate was tested out in the following way, against the homologous typhoid strain, no. 18.

(a) 2 cc. extract broth + 1 loop of four hour broth culture no. 18 + 0.5 cc. stool filtrate.

(b) 2 cc. extract broth + 1 loop of four hour broth culture no. 18 + 0.5 cc. sterile salt.

These tubes were incubated for three hours and then left standing overnight at room temperature. At the end of this time the control tube (b) was definitely cloudy, and the tube with the filtrate (a) still clear. One half of the tube (a) was now added to 2 cc. extract broth + 1 loop of a young broth culture of typhoid no. 18. This second tube also failed to show growth as compared with the control on the following day. The inhibitory property of tube (a) could be carried on from one generation to another indefinitely. It was also found that one of these inhibited tubes could dissolve a young typhoid culture which was definitely turbid, clarifying it completely. The lytic and inhibitory action was stronger after a few generations in contact with typhoid bacilli than it was in the original stool filtrate.

This lytic principle which has been isolated for over a year and a half at the present writing and with which most of the work described in this paper was done, differs in no essential from the many bacteriophages described by d'Herelle. The Olsen bacteriophage acts non-specifically within the typhoid, colon, dysentery group. It is lytic and inhibitory for Shiga, Mt. Desert and Flexner dysentery bacilli. In the original experiments when the Olsen bacteriophage was not far removed from

the original stool filtrate, it showed no action on paratyphoid A or B. But when tried again recently against these cultures, it was active against both of these organisms. The Olsen bacteriophage has no lytic action against laboratory stock strains of *Bact. coli-communis* and *communior*. It does, however, act against a recently isolated *Bact. coli* which occurred in the stool culture of a typhoid carrier referred to below. Typhoid strains show the greatest variation in susceptibility to lytic agents. The Olsen bacteriophage has acted on most of the typhoid cultures tried, whether a stock culture (Mt. Sinai) or recently isolated strains, with the exception of the Rawlings strain. It has recently been possible to obtain a susceptible variant of the Rawlings culture (see below). D'Herelle states that from the point of view of susceptibility to lytic agent, both the typhoid group and the colon group must be regarded as heterologous, whereas the members of dysentery groups are all fairly susceptible. The Olsen bacteriophage has no action against a stock strain of cholera or against any Gram positive organism that has been tried, such as the pneumococcus or staphylococcus. No systematic attempts to acclimatize the Olsen bacteriophage to organisms against which it was not active have been made. The fact that the range of activity of a particular bacteriophage could be extended was first shown by d'Herelle and has since been repeated by other workers, notably Bordet and Ciuca (1921b). This adaptation of the bacteriophage is of theoretical importance and must be taken into account in any theory that is advanced.

Types of colonies developing after lysis

The Olsen bacteriophage never completely sterilized the cultures which it dissolved or inhibited. On subculture it was found that a small number of colonies developed which were in most cases of two types; one a typical round typhoid colony, the other an irregular jagged colony. The latter type if fished into broth, produced little or no clouding and the supernatant fluid could be shown to contain the lytic agent in the same way that Bordet and Ciuca had demonstrated in connection with

the lytic-bearing colonies of *Bact. coli*. The round colony when fished to broth grew normally and the supernatant fluid had no lytic action.

These two types of colonies, one a normal colony, the other the lytic carrying colony were obtained with every culture against which the Olsen bacteriophage was active, dysentery as well as typhoid, and had not been described by d'Herelle at the date of our first communication, February 16, 1921 (Kuttner, 1921). If one of the normal colonies is restreaked, nothing but typical round typhoid colonies develop which apparently do not carry the lytic agent. If one of the irregular lytic bearing colonies is restreaked, both normal and irregular colonies are obtained. In one experiment a lytic colony and a normal colony obtained by plating a dissolved culture (Olsen bacteriophage acting on the homologous typhoid culture) was restreaked daily for 15 successive generations. In no case did the restreaking of a normal colony yield anything but normal colonies. The restreaking of the lytic colonies regularly produced both types: lytic colonies and normal colonies. The relative proportion of the two types varied, depending upon what part of the lytic colony had been touched with the platinum needle.

Twort in his original article states that the degenerative changes he described with micrococci characteristically started at the edge of the colony. In working with typhoid and coli cultures we have observed a great variation in the lytic colonies which appears to be a fairly definite characteristic of certain strains. Thus, if the Olsen bacteriophage acts on a Mt. Sinai strain of typhoid, the lytic action is most intense at the center of the colony. Similarly, with another bacteriophage (Newton) acting on a *Bact. coli* culture, lysis also begins at the center and has done so consistently with this strain. In restreaking a lytic colony, the greatest proportion of lytic colonies are obtained if the original lytic colony is touched at the point where the lytic process appears to be most active, whether this happens to be at the center of the colony or at the periphery. In our experience, in most cases, even if the lytic area of the colony was touched with platinum wire as delicately as possible, a certain

number of what appeared to be absolutely normal colonies developed. On the other hand, we do not agree with Bail's (1921) statement that after a few generations, the colonies carrying the lytic agent revert to normal. This depends as pointed out above, upon the manner of restreaking. Bordet and Ciuca report that the culture of *Bact. coli* derived from a lytic colony was still lytic after 150 transplants.

If a series of lytic typhoid or dysentery colonies are examined under the microscope, it will be found that there are often between the lytic colonies, minute transparent granular masses that have been called "appearances" by previous workers. They are structureless deposits which fail to grow when transplanted. It will also be found that the lytic colonies owe their irregular shape to the fact that their edges or centers, as the case may be, have faded into these "appearances." All variations between a fairly large lytic colony with only a little transparent material and the entirely translucent "appearances" which can only be seen with the microscope, occur. These transparent masses probably represent the débris left when a colony composed entirely of "sensitive" bacilli is dissolved and all the organisms killed.

"Susceptible" and "resistant" individuals in a single culture

Thus it is apparent that the lytic agent separates any susceptible culture into three types of individuals: The most susceptible bacillus whose descendents are all represented by the "appearance;" the susceptible bacillus whose descendants are both sensitive and resistant, represented by the lytic colony; and the bacillus which is itself resistant, and gives rise to nothing but resistant descendents, represented by the normal colony. These three types of individuals must preëxist in the culture as is indicated in the following experiment:

A young typhoid culture is washed off in salt solution and the emulsion is divided into three tubes and placed in a freezing mixture for thirty minutes. In the same way bacteriophage filtrates, undiluted and diluted 1:1000 are brought to a temperature of between 5° and

6°C., and just prevented from freezing solid. Some sterile salt solution and some centrifuge cups are also cooled. After half an hour, the undiluted bacteriophage is added to one tube of emulsion and the diluted bacteriophage to another, and the tubes are shaken. The ice salt solution is added to the control tube. The mixtures are kept in the freezing mixture and plated with the least possible loss of time. In both cases with the undiluted and diluted bacteriophage, "appearances," lytic colonies and normal colonies develop, whereas the salt control shows only normal colonies.

This experiment showed that the three types of bacilli preexisted in the emulsion. The bacilli in the normal colony had been exposed to the same amount of lytic agent as the other organisms, and had apparently failed to unite or adsorb any bacteriophage, or if they had united or adsorbed the lytic agent, were not affected by it. The question that now arose was to find out the nature of the union between the susceptible bacilli and the lytic agent. Was it a definite union or merely an adsorption? The next step was also carried out at as low a temperature as possible, so as to prevent the growth of the bacilli.

The three tubes of the above experiment were placed in the cooled centrifuge tubes, and centrifuged to throw down the organisms. The supernatant fluid was poured off, the centrifuge cups and the tubes containing the sediment of bacteria recooled. Some of the cold salt solution was added and the sediment thoroughly emulsified by means of a capillary pipette which had been cooled. This process of washing the bacilli was repeated twice, and the emulsion then streaked.

The tube in which the bacilli had been in contact with concentrated bacteriophage yielded "appearances," lytic colonies and normal colonies. The tube to which the diluted bacteriophage had been added showed only normal colonies, as did the salt control.

Did the concentrated bacteriophage divide the culture into three types of bacilli after one single short contact, or was it a question of the concentrated lytic agent not having been completely removed by the washings? In the latter case it was the mechanical adhesion of the bacteriophage to the organisms

that had separated the culture into the three individuals of different degrees of susceptibility as they grew out in the agar streak. But in any case, it is possible to state that all susceptible cultures contain individuals of three different potentialities in respect to lytic agents. Whether the so-called resistant individuals that make up the normal colony represent, as has been claimed, the descendants of an individual that became immune to the small amount of bacteriophage that adhered to it, and has conferred this immunity to his descendants; or whether the physical surface of different bacteria vary so that the lytic agent adheres more readily to some individuals than to others, remains an open question.

Bacteriophage action, therefore, seems to depend on two main factors: the concentration of the lytic agent and the number of susceptible bacteria. This is well illustrated by the action of the lytic agent on cultures on solid media. If the lytic agent is added undiluted to an agar slant which is subsequently inoculated with a susceptible culture, practically no growth occurs. If high dilutions of the lytic agent are added, growth is normal except for certain transparent areas that occur at various points of the culture. The number and size of these transparent areas diminishes with the increasing dilution of the lytic principle. D'Herelle considers that each clear spot represents a colony of the virus. He believes that the concentration of the lytic agent at definite points in this way is characteristic of living things. Gratia's (1921) interpretation of these facts seems equally plausible at the present stage of our knowledge: each culture is made up of a whole scale of individuals ranging from the most susceptible to the most resistant. As the lytic agent is diluted, it is capable of dissolving only the most sensitive organisms which occur at various points of the culture.

It seemed of interest to determine whether the normal colonies that developed after lysis were totally and permanently refractive to further exposure to the lytic agent.

A normal resistant colony obtained by the action of the Olsen bacteriophage on strain 18 was isolated and to this culture the lytic agent

was again added in a dilution of 1:10. Lysis occurred more slowly than with a normal culture, but was definite. The tube was streaked and a resistant colony isolated and this second culture again exposed to the lytic agent. This was repeated four times and in the last case, definite clearing with the resistant culture did not take place, although on streaking the turbid culture a small number of lytic colonies were obtained. The resistant culture obtained after four successive exposures to the lytic agent was subcultured for five generations on agar and then again exposed to the lytic agent. It has lost a great part of its resistance and was nearly as susceptible as the stock culture.

Bordet and Ciuca (1921b) fished a lytic colony of *Bact. coli* to broth and kept it in the incubator for eight days and for twenty-two days at room temperature. At the end of this time they reisolated the culture and found it entirely resistant to lysis. They do not state, however, whether it again became susceptible on subculture. More recently, Eliava and Pozerski (1921) reported that they isolated a Shiga culture that had resisted lysis. It was resistant to bacteriophage action, but after 8 transplants it became more susceptible again.

Variations in typhoid strains—can a non-susceptible strain become susceptible?

The proportion of sensitive and resistant bacilli in a given culture could, therefore, be varied by exposure to a lytic agent. A culture which had become definitely resistant could be made susceptible again by transplantation. It seemed probable that certain strains of typhoid which were naturally resistant to lysis might be rendered susceptible by altering the cultural conditions under which they were growing. As stated above, the Rawlings strain of typhoid had consistently been resistant to lysis by the Olsen bacteriophage. This strain was known to ferment xylose slowly, whereas all the other strains that were susceptible to this particular lytic agent, fermented xylose rapidly.

It seemed, therefore, worthwhile to obtain a variant of Rawlings which fermented xylose rapidly, and see if it was more susceptible to lysis than the stock strain.

The Rawlings strain was streaked out on agar plates containing 1 per cent xylose according to the method described by Morishima for obtaining daughter colonies. The plate was sealed with plasticene and incubated four days. At the end of this time typical daughter colonies had developed which when fished to xylose broth, fermented this sugar in twenty-four hours, whereas the stock strain usually required seven days to ferment. The xylose rapid fermenting variant of Rawlings and the slow fermenting stock culture were now exposed to the Olsen bacteriophage, but no lysis was obtained and no lytic colonies developed on subculture.

Since Rawlings was a very old stock culture, it was thought possible that it had become resistant, due to the fact that it had been held as a stock culture and transplanted at long intervals from dried cultures. It was, therefore, transplanted daily for eight generations on moderately alkaline (pH 7.6) and very alkaline agar (pH 9); since it had been shown by Gratia that an alkaline reaction favors lysis, it was thought that possibly susceptible bacilli might develop as the result of growth in alkaline media. This did not prove to be the case and no evidence of lysis was obtained when these cultures were exposed to the Olsen bacteriophage. We have now, however, succeeded in obtaining a susceptible variant of the Rawlings strain. At the same time that the above experiments were being carried out, the stock Rawlings strain was transplanted to 100 cc. broth and allowed to remain in the incubator for four months and then left at room temperature for two months. The organism remained in the broth from June, 1921, until November, 1921. It was then isolated and identified as typhoid by fishing to Russell double sugar medium, on which it gave a typical reaction, and by the agglutination test. This strain reisolated from the broth proved susceptible to the Olsen bacteriophage and subsequent transplants from this culture have remained so for a period of three months.

Isolation of a resistant variant from an originally susceptible strain without exposure to bacteriophage

In April, 1921, Gratia reported that he had obtained a resistant variant from his susceptible *Bact. coli* culture by reisolating it

from the pellicle formed in an old broth culture. Pinhead, glassy colonies protruded from this pellicle, and by subculturing one of these, he obtained a resistant culture. The resistant culture was more motile and also more virulent than the stock culture. The resistance of this resistant variant was not, however, absolute. It was much more marked in acid than in alkaline media.

During May, 1921, it was suddenly noticed that the stock strain of typhoid, no. 18, which was transplanted almost daily, had become resistant to various agents (see below) which had formerly caused it to undergo lysis. It was then tested against the Olsen bacteriophage and it was found that no lysis was obtained with any of the transplants of no. 18 made after May 14. By going back to a culture of April 23, a susceptible transplant of no. 18 was again obtained. The sudden development of resistance on the part of this strain cannot be attributed to aging in this case, since the culture was in daily use. It was found that the stock agar we had been using for transplanting this culture had been somewhat more acid (pH 7) than we had used before, and we thought that perhaps the predominance of the resistant individuals over the susceptible might be attributed to this.

We, therefore, transplanted a susceptible and a resistant variant of no. 18 twice daily on acid, pH 6.8, agar and on alkalin pH 8.2 agar for twelve generations. We transplanted as rapidly as possible because we knew that the maximum number of susceptible individuals occurred in young cultures. We then tested these four different cultures (18 susceptible, twelfth generation, agar pH 6.8; 18 susceptible, twelfth generation, agar pH 8.2; 18 resistant, twelfth generation, agar pH 6.8; 18 resistant, twelfth generation, agar pH 8.2) against the Olsen bacteriophage.

The susceptible strain was still susceptible from both the acid and the alkaline medium, and the resistant strain still resistant from both media. At the same time, June, 1921, that the above experiment was done, the two variants of no. 18 were inoculated into 100 cc. of broth each, and allowed to age in the same way as the Rawlings culture described above.

In summing up our results with various strains of typhoid, we may state that we do not understand the factors that determine the susceptibility or resistance of a culture. The reaction of the medium and the rapidity of transplantation do not seem to affect the relation of susceptible and resistant individuals in a given culture. A culture that had been isolated for six months suddenly became resistant without being exposed to bacteriophage, and a different transplant of the same original culture has remained susceptible for a year and a half, and has been used constantly in various tests. In the case of the Rawlings culture, originally resistant, a susceptible variant has been obtained by reisolating the strain from an old broth culture. In conclusion, we may say that in typhoid cultures the relation between susceptible and resistant organisms is not a very stable one, and that for reasons of which we are ignorant, one of the other type may predominate.

II. ISOLATION OF THE LYTIC AGENT FROM THE TISSUES AND BLOOD OF NORMAL ANIMALS

The action of the Olsen bacteriophage on typhoid and dysentery cultures was studied in detail in order to familiarize ourselves with the lytic process. It was fairly obvious, however, that unless one was willing to accept d'Herelle's theory of a living virus, no progress in finding out the nature of the phenomena could be made, unless it was possible to isolate a lytic principle without the interaction of the living animal body. The fact that the lytic agent could be transmitted indefinitely in series and was active only against vigorously growing bacilli, suggested that the bacteriophage principle might be derived from the organisms themselves.

We proceeded on a theory first advanced by d'Herelle, but discarded by him in favor of the living virus. According to this theory, bacteriophage action was due either to the activation of the natural autolysin contained in all bacteria, or to the removal of an autolysin-inhibiting substance. Once this natural autolysin was liberated it could in turn liberate more of the autol-

ysin from the next generation of bacteria and so on indefinitely. This hypothesis will be again discussed at the end of the paper.

The work of Cantacuzene and Marie (1919) on the action of intestinal mucosa on cholera vibrioes and that of Turro (1921) on the action of tissue extracts on a variety of bacteria, suggested that a bacteriophage principle might have played some part in the experiments reported by these workers.

With the work of Cantacuzene and Marie, and Turro in mind, we prepared various tissue extracts as has been already reported in a brief note.

Bacteriophage action started with extracts of intestinal mucosa

Experiment. March 18 the small intestine, large intestine, and a part of the abdominal wall of 3 normal guinea-pigs which had been bled to death for complement, were removed. The tissues were thoroughly washed in running water and cut into small pieces with a scissors. Without any preliminary drying each of the three types of tissue was divided roughly into 4 parts and placed in 12 bottles. Each type of tissue was extracted in 50 per cent glycerol, 25 per cent glycerol, 5 per cent glycerol and salt solution, 50 cc. of fluid being added to each bottle. The bottles were put on ice from March 18 until March 21 and then plated to see to what extent they were contaminated. The tissue to which the 50 per cent glycerol had been added showed no growth. The plates from the other bottles developed a small number of colonies, mainly staphylococci.

All the bottles were then incubated from March 22 to March 29. On March 29 a small amount of the supernatant fluid from the bottles of small intestine, large intestine and abdominal wall extracting in 50 per cent glycerol were centrifuged and filtered through a Berkefeld filter to obtain a clear fluid. These filtrates were titrated to make sure that the reaction was neither sufficiently acid nor alkaline to interfere with the growth of the typhoid bacillus. The filtrates were tested in the following way against the typhoid strain 18, 0.1 cc. of a fairly heavy saline emulsion from one eighteen hour agar slant being added to 2 cc. extract broth, pH 7.8. The amount of emulsion added was sufficient to make the tubes slightly but definitely turbid.

March 30

TUBE	AMOUNT OF BROTH	CULTURE 18	PREPARATIONS TESTED	4 HOURS	24 HOURS	
	cc.	cc.				
1	2	0.5	0.5 cc. 50 per cent glycerol, small intestine, pH 6.8	++	+1	Normal growth
2	2	0.5	0.25 cc. 50 per cent glycerol, small intestine, pH 6.8	++	+	Few lytic colonies
3	2	0.5	0.50 cc. 50 per cent glycerol, large intestine, pH 7.0	++	++	Normal growth
4	2	0.5	0.25 cc. 50 per cent glycerol, large intestine, pH 7.0	++	++	Normal growth
5	2	0.5	0.50 cc. 50 per cent glycerol, abdominal wall, pH 6.8	++	+	Normal growth
6	2	0.5	0.25 cc. 50 per cent glycerol, abdominal wall, pH 6.8	++	+1	Normal growth
7	2	0.5	0.50 cc. 50 per cent glycerol, solution	++	++	Normal growth
8	2	0.5	0.25 cc. 50 per cent glycerol, solution	++	++	Normal growth
9	2	0.5	0.50 cc., Olsen bacteriophage	±	±	Normal growth
10	2	0.5	0.25 cc., Olsen bacteriophage	±	±	Lytic colonies
11	2	0.5	0.50 cc., salt solution	++	++	Normal growth
12	2	0.5	0.25 cc., salt solution	++	++	Normal growth

++ = turbidity equal to control.
− = complete clearing.

The tubes were placed in the incubator for four hours and examined, and then put back in the incubator for twenty hours more, and again examined for clearing. The tubes were all plated at this time and then heated at 56°C. for thirty minutes to prevent the overgrowth of resistant types.

The twenty-four hour plate of tube 2 showed lytic colonies identical with those of tube 10, the lytic control tube. The platings from none of the other tubes showed any lytic colonies. Tube 1 where a greater amount of active intestinal extract was used showed no lytic colonies, but this is often the case, since tubes to which a greater amount of the lytic agent whatever its origin is added, usually clear faster and are then often more rapidly overgrown with the resistant organisms. The same thing occurred in this particular experiment in the control tube 9. Glycerol alone failed to give rise to lytic colonies. The degree of clearing is a very unreliable criterion, especially on first isolation of a lytic

agent. The development of lytic colonies is the most definite proof of the presence of a lytic agent. Clearing alone is never sufficient evidence for bacteriophage action.

The lytic colonies obtained from tube 2 were studied in detail and found to have all the characteristics of lytic colonies obtained by action of the Olsen bacteriophage.

April (1) The supernatant fluid of the bottle with the small intestine extracting in 25 per cent glycerol was centrifuged and filtered through a Berkefeld. Another lot of the bottle with small intestine and 50 per cent glycerol was filtered, and then these filtrates small intestine 25 per cent glycerol incubating March 22 to April 1, small intestine 50 per cent glycerol incubating March 22 to April 1, together with the first extract found active small intestine March 22 to March 29 (tested March 30) were set up on April 1. Tube 2 in the previous protocol was also tested to see whether the lytic agent isolated from the mucosa was transmissible in series. The test was set up in the same number as the previous one.

April 1

TUBE	AMOUNT OF BROTH	CULTURE 18	PREPARATIONS TESTED	PLATES AFTER TWENTY-FOUR HOURS
	cc.	cc.		
1	2	0.5	0.50 cc. 50 per cent glycerol, small intestine, March 22 to March 29	Normal growth
2	2	0.5	0.25 cc. 50 per cent glycerol, small intestine, March 22 to March 29	Many lytic colonies
3	2	0.5	0.50 cc. 50 per cent glycerol, small intestine, March 22 to April 1	Normal growth
4	2	0.5	0.25 cc. 50 per cent glycerol, small intestine, March 22 to April 1	Many lytic colonies
5	2	0.5	0.50 cc. 25 per cent glycerol, small intestine, March 22 to April 1	Normal growth
6	2	0.5	0.25 cc. 25 per cent glycerol, small intestine, March 22 to April 1	Many lytic colonies
7	2	0.5	0.50 cc. tube 2, March 30	Many lytic colonies
8	2	0.5	0.25 cc. tube 2, March 30	Many lytic colonies
9	2	0.5	0.50 cc. Olsen bacteriophage	Many lytic colonies
10	2	0.5	0.50 cc. salt	Normal growth

The 25 per cent glycerol extraction of the pooled small intestine tissue was also found active; the result obtained with the 50 per cent glycerol on March 30 was repeated. The lytic agent derived from the mucosa was found to be active in the second generation (tubes 7 and 8).

The lytic process started with these extracts in no way differed from the bacteriophage action started with Olsen bacteriophage. It could be transmitted in series and, the speed of lysis and number of lytic colonies obtained increased markedly in the first three or four generations. Extracts of the large intestine with 50 per cent glycerol also proved active after a longer period of extraction March 22 to April 25. Extracts of the abdominal wall were never found to be lytic. The preparations with 50 per cent glycerol and salt in spite of the addition of 5 per cent chloroform, became so contaminated that they were discarded without being tested.

It was thus shown that lysis transmittable in series could be started with glycerol extracts of large and small guinea-pig intestinal mucosa in certain instances. We also tried to see if this property was confined to the intestinal mucous membrane, or whether it could also be found in other organs. We, therefore, prepared a liver extract exactly according to the directions of Turro (1921).

Bacteriophage action started with an extract of liver tissue

March 31, 1921, a liver taken from a normal guinea pig was minced, shaken up in about 6 volumes of acetone, dried in vacuo and pulverized. To approximately 1 gram of liver powder, 20 cc. of sterile salt solution were added. To one tube 40 drops of chloroform were added, to the other a small amount of dry sodium fluoride. Both tubes were incubated fifteen hours. At the end of this time the tube to which the sodium fluoride had been added was contaminated with a large Gram positive bacillus. The tube to which the chloroform had been added was apparently sterile. The former was centrifuged and filtered through a Berkefeld, the other was merely centrifuged. Both these preparations were tested against typhoid no. 18 in the same way as the intestinal extracts. The liver extract to which the chloroform had been added was not as active in the first generation as the one to which sodium fluoride had been added. The following protocol where the specificity of the liver extract is tested is of interest.

The liver extract (sodium fluoride March 31) was tested against the following 6 strains: typhoid no. 18, typhoid Rawlings, Shiga dysentery, Mt. Desert, cholera and the *Bact. coli* obtained from Dr. Bordet. In this case, the extract was tested against each culture in two ways by lysis and by inhibition; that is, in the former case the 2 cc. of broth, the amount used consistently throughout these tests, was inoculated with a sufficient amount of the culture emulsion taken from an eighteen hours slant to produce definite clouding. In the latter case the tube was merely heavily inoculated without producing visible turbidity. This method of setting up the tests in two ways is to be recommended where a new and feeble lytic agent is being tested, because the danger of overgrowth with resistant types always exists. By inoculating a large and small number of organisms, the chances of finding lytic colonies or transparent areas, which is the surest indication of bacteriophage action, at one or the other intervals of plating, is increased. In the majority of our more recent tests we have adopted the following routine: The tubes are plated after two hours and then left standing at room temperature, and then plated again.

April 12, 1921

TUBE	AMOUNT OF BROTH	CULTURE		LIVER (NaFl) EXTRACT APRIL 1	PLATED AFTER FOUR HOURS
	cc.			cc.	
1	2	Typhoid no. 18	0.50 cc.	0.2	Few lytic colonies
2	2	Typhoid	0.05 cc.	0.2	Little growth, few lytic colonies
3	2	Rawlings	0.50 cc.	0.2	Normal growth
4	2	Rawlings	0.05 cc.	0.2	Normal growth
5	2	Shiga	0.50 cc.	0.2	Transparent areas
6	2	Shiga	0.05 cc.	0.2	Very delicate growth, no lytic colonies
7	2	Mt. Desert	0.50 cc.	0.2	Normal growth
8	2	Mt. Desert	0.05 cc.	0.2	Transparent areas
9	2	Cholera	0.50 cc.	0.2	Normal growth
10	2	Cholera	0.05 cc.	0.2	Normal growth
11	2	Bordet *B. coli*		0.2	Normal growth
12	2	Bordet *B. coli*		0.2	Normal growth

A similar series of tubes was set up at the same time in which sterile salt solution was substituted for the liver extract. The plates from these tubes all showed normal growth. The action of sodium fluoride alone was also tried, and in those concentrations which did not interfere with growth, normal colonies were obtained. It is interesting to note that the Rawling strain is resistant to the liver extract in the same way that it is to the Olsen bacteriophage. The range of activity of this liver extract was about the same as that of the Olsen bacteriophage. Cholera and the Bordet *Bact. coli* were not affected by either.

The lytic process started with the liver extract was shown to be transmissible in series. In order to demonstrate even more clearly that the same substance was at work in the liver extract and in the Olsen bacteriophage, 2 rabbits were immunized, one with dissolved typhoid culture filtrate (Olsen bacteriophage), the other with the active liver extract. The rabbits were injected intravenously at intervals of four or five days. The rabbit 1098 injected with the Olsen dissolved culture filtrate, was bled the 4th day after the sixth injection. The rabbit 335 injected with active liver extract, was bled one day after the fourth injection.

The normal and immune sera of these two rabbits were then tested as to their ability to convert lytic colonies to normal by the method described by Bordet (1921c).

Experiment. June 13, 1921, 4 agar slants were inoculated in the following way:

(1) with 7 drops of the normal serum of rabbit 1098.
(2) with 7 drops of the immune serum of rabbit 1098.
(3) with 7 drops of the normal serum of rabbit 335.
(4) with 7 drops of the immune serum of rabbit 335.

These tubes were then incubated in the inclined position overnight. On the following day they were found to be sterile. They were then inoculated with equal amounts of a broth fishing of a lytic colony obtained by the action of the Olsen bacteriophage on no. 18.

(5) A plain agar slant was also inoculated with the same material. After eighteen hours the following result was obtained:

Tube 1 showed a small number of lytic and normal colonies.

Tube 2 showed heavy confluent growth.

Tube 3 showed a small number of lytic and normal colonies.

Tube 4 showed a fair amount of confluent growth, no lytic colonies.

Tube 5 showed a small number of lytic and normal colonies.

This experiment was repeated two days later, and the same result was obtained. The antilytic serum produced by injecting the active extract (liver) had the same power to neutralize the growth of lytic colonies as the antilytic serum produced by the inoculation of Olsen bacteriophage. The normal serum of neither of the rabbits interfered with the development of the lytic colonies.

Bacteriophage action started with normal rabbit serum

Bordet in describing the production of antilytic sera, mentioned the fact that the lytic agent persisted in the circulation for a period of forty-eight hours. In starting to immunize 2 rabbits, one with Olsen dissolved culture filtrate no. 1052, the other with active liver extract no. 335, we took normal bleedings and also bled again eighteen hours after the first injection to see whether the lytic agent could still be demonstrated in the circulation. We then tested the four bleedings obtained in this way as follows:

TUBE	AMOUNT OF BROTH	CULTURE 18	SERA TESTED	SIX HOUR PLATES
	cc.	cc.		
1	2	0.3	0.2 cc. normal rabbit serum no. 335	No lytic colonies
2	2	0.3	0.2 cc. rabbit serum no. 335 after first injection	Some lytic colonies and transparent areas
3	2	0.3	0.2 cc. normal rabbit serum no. 1052	Some lytic colonies
4	2	0.3	0.2 cc. rabbit serum no. 1052 after first injection	No lytic colonies
5	2	0.3	0.2 cc. salt solution	No lytic colonies

It was thus found that normal rabbit serum in some instances contained a lytic agent. Transmission of the lytic agent in series from tube 3 offered no difficulties. This experiment was repeated three times. The suspicion that the two tubes of no. 1052 had been accidentally mixed could be definitely eliminated because, in taking the normal bleeding, a large quantity of blood had been taken, since the normal serum would be required as

a control for future tests, whereas, only a small amount of blood was necessary for the demonstration of the persistence of the lytic agent in the circulation. It is also to be noted that the serum of the rabbit 335 injected with one dose of active liver extract, was active after eighteen hours.

During the past year out of 50 normal rabbits bled, the serum of 6 contained a lytic agent active against typhoid bacilli. No data were obtained as to what determined the presence of the bacteriophage principle in the circulation of these particular animals. The serum of 2 of these rabbits when bled again, failed to show any lytic activity, indicating that the condition was transitory. An active serum remained so for an indefinite period when stored on ice. The lytic agent in the serum was not related to the complement since the activity was not decreased by inactivation at 56° for thirty minutes, and in two instances seemed definitely increased after inactivation. The activity of two immune sera, one rabbit and one horse, was also tested, but they were found negative.

Possible connection between bactericidal titer of normal rabbit sera and bacteriophage action

It is of interest in this connection to recall the observations of Teague and McWilliams (1917) who found a great variation in the bactericidal titer of different rabbit sera, and observed that the bactericidal titer of immune rabbit serum was much lower than that of normal rabbit serum. It seemed possible that the explanation of the exceptionally high bactericidal titer of certain rabbits might be attributed to bacteriophage action, but had this been the case, it would have been extraordinary if the extremely characteristic lytic colonies had not been observed before. We consequently duplicated as closely as possible the tests made by Teague and McWilliams with one of our lytic sera. We were short of active serum and could use 0.7 cc. only instead of 1 cc. Seven tenths cubic centimeter of serum was inoculated with a fairly light suspension of typhoid bacilli and plated after five hours incubation and again after eighteen hours.

In both five-hour and eighteen-hour plates a small number of lytic colonies were obtained. We did not count the typhoid suspension accurately, and it is quite possible that with the use of such a large amount of serum and inoculating a relatively small dose of bacteria, only the resistant colonies might develop, giving a small number of perfectly typical colonies. Teague and McWilliams used streak plates in the same way that we do in determining lytic action. It is customary in most bactericidal tests to make "pour" plates, in which the recognition of a small number of lytic colonies would be extremely difficult. Whether or not bacteriophage action played a part in the results obtained by previous workers cannot be definitely stated, but in the future any one working on the bactericidal titer of normal rabbit sera will have to test for bacteriophage action. The differentiation of bactericidal action and lytic action can easily be made because of the heat resistance of the lytic agent. A small number of normal guinea-pig sera were also tested against typhoid, but none of them showed any lytic activity. A few isolated experiments with normal human sera were negative.

The lytic agents found in serum, like those described from other sources, were resistant to drying. One of the active sera which had dried completely in the bottom of a tube, when redissolved in a little salt solution, still proved active. We found that we were unable to absorb out the lytic agent from a serum by a single absorption with typhoid bacilli at 0°C.

Absorption of lytic agent from serum

One cubic centimeter of active rabbit serum no. 1052 and the saline emulsion of a fresh agar slant of the Mt. Sinai typhoid strain were both placed in a freezing mixture for thirty minutes. The serum and the bacteria were then combined and kept at a freezing temperature for four hours. The experiment was carried out at the low temperature to prevent the growth of the bacteria since we know that the amount of the lytic agent is increased with the growth of the organisms. At the end of this time, the mixture was centrifuged to throw down the organisms. The serum was pipetted off and heated at 58° for thirty minutes to kill any remaining bacteria. In order to prove that some of the lytic agent

had actually united with the bacteria, the organisms were washed three times (to eliminate the serum which had merely adhered) and then streaked out on an agar plate. At the same time, 1 cc. of sterile broth was added and the organisms incubated overnight, to see if the growth thus obtained would be normal. On the following day, this tube was also plated.

The plate made immediately after washing the bacteria showed no lytic colonies, but the plate from the broth tube showed definite evidence of lysis. The serum after being tested for sterility, was set up with the Mt. Sinai culture to see if the lytic agent had been absorbed out. Typical lytic colonies were obtained after the serum and culture had been in contact for three hours. Thus, four hours absorption was not sufficient to remove the lytic agent from the active normal rabbit serum.

The specificity of one of the active sera was tried out in one experiment. We were always very much handicapped by not being able to get a very large quantity of active normal serum, since we had no way of judging when a serum would be active. In this instance, the serum which was lytic for typhoid strain no. 18, was not active for Mt. Desert, but too little work has been done on this point to draw any conclusions.

Is the presence of the lytic agent in the circulation secondary to its presence in the intestinal canal?

At the same time that most of these experiments were carried out, d'Herelle's findings on the occurrence of bacteriophage in the feces of various normal animals were not known to us, so that no attempt was made to correlate the active rabbit sera with the presence of bacteriophage in the feces. In his book, d'Herelle states that he examined the feces of 2 rabbits and found that the filtrate obtained was active in one case against Shiga, Mt. Desert and Flexner dysentery bacilli, while the filtrate from the other rabbit showed only a very slight activity against the Shiga bacillus. When we first discovered the lytic agent in normal rabbit sera, we thought that the lytic activity could be attributed to some ferment-like activity which activitated the bacteria to autolyze, since normal rabbits do not in any way come in contact with members of the typhoid-dysentery group.

But if a Shiga bacteriophage can be isolated from the feces of a normal rabbit, it may be argued that the sera of certain rabbits which happen to contain a bacteriophage against typhoid or Shiga in their intestinal tract, may be active because under certain circumstances the bacteriophage penetrates into the circulation from the intestine. Since normal rabbits do not have either typhoid or Shiga bacilli in their feces, it may be assumed that feces of these animals must contain organisms closely related to these bacteria in their fecal flora, since the bacteriophage is only known to exist in the presence of susceptible bacilli.

We devised the following experiment in our attempt to obtain some information on this point.

We bled 6 normal rabbits, and at the same time plated the feces of each on Endo plates. The fecal flora of rabbits does not seem to be very varied, since, in most cases, only two types of organisms were obtained, and in some, only one type. The plates were examined carefully under the microscope to see if any small lytic colonies of organisms more or less completely dissolved, could be detected in the direct plates, but none were found. The organisms obtained were isolated on Russell's medium. The fecal emulsion was incubated overnight and then heated at 60° for one hour, since we planned to test the fecal suspension for evidence of bacteriophage if any of the sera proved active. The sera were tested against the homologous strains or strain of *Bact. coli*, and also against other organisms. The protocol of only one rabbit serum will be given to show the method, since the experiment was entirely negative.

Rabbit serum no. 530 inactivated 56° for thirty minutes

TUBE	AMOUNT OF BROTH	CULTURE	SERUM	TWO HOUR PLATES	EIGHTEEN HOUR PLATES
	cc.		*cc.*		
1	2	No. 530 homologous *Bact. coli* (1)	0.3	No lytic colonies	No lytic colonies
2	2	No. 530 homologous *Bact. coli* (2)	0.3		
3	2	Typhoid no. 18	0.3		
4	2	Typhoid Mt. Sinai	0.3		
5	2	Typhoid Mallon	0.3		
6	2	Mt. Desert	0.3		
7	2	Newton *Bact. coli*	0.3		

The sera were thus tried against 7 different strains, 2 of the organisms being derived from the same rabbit's intestinal tract, but none of them proved active against either the homologous *Bact. coli* or the other cultures tried. Most of the workers who have studied stool filtrates from normal as well as pathological human cases, state that bacteriophage action could probably be demonstrated in every stool if it were tested against the right organisms. But the problem of finding the right organisms is not an easy one. This line of research has not been pursued further because of the discovery made by several different workers, including ourselves, that the bacteriophage principle can be obtained from the bacteria alone under certain circumstances without the action of any external agent.

We have thus been able to start lytic action with 2 different types of tissues derived from guinea-pigs, and also with normal rabbit sera. The intestinal mucosa of certain guinea-pigs extracted with glycerol when added to normal typhoid cultures produced lysis transmittable in series. In the same way liver extracts from guinea-pigs can in certain instances produce the the same result. The presence of lytic agents in these tissues active against typhoid bacilli is, however, extremely rare. Normal rabbit sera (6 out of 50) also occasionally contain the lytic agent for typhoid bacilli, but we do not understand the conditions that determine the lytic activity of the blood of these animals. The experiments in which the bacteriophage action of these normal tissues or normal sera was shown, were always carefully controlled and the culture without the addition of the particular extract or serum, never gave any evidence of lysis.

III. EXPERIMENTS ON THE ORIGIN OF THE LYTIC AGENT IN THE BACTERIA THEMSELVES

Bacteriophage action may thus be started by a wide variety of agents from normal as well as from diseased animals, but whatever agent starts the process, it is clear that eventually in a series, the lytic agent must be derived from the bacteria themselves, unless we are willing to accept the parasitic nature of the lytic agent.

If the lytic agent is really an activated autolysin, it ought to be possible to isolate it from old, spontaneously autolyzing cultures.

On June 13, 1921, two bottles of 100 cc. of broth were inoculated respectively with the strain of the stock typhoid strain of typhoid no. 18 which had spontaneously become resistant to bacteriophage action and with the susceptible variant of no. 18. These broth cultures had remained in the incubator for a period of four months and had then been left at room temperature for two more months. On November 9, some of the supernatant fluid from these two bottles was removed, centrifuged and heated at 58°C. for thirty minutes. These heated supernatants were then tested against the susceptible variant of no. 18, which had been in constant use as a test culture. Large amounts were added because it was thought that if present at all, the lytic agent would be feeble. The test was set up as follows: 1 cc. amounts of the heated supernatants were inoculated directly with 0.1 cc. of no. 18, and at the same time, the test was carried out in the usual way, 2 cc. of broth being inoculated with 0.1 cc. of culture, and then 0.5 cc. of the material to be tested, added.

TUBE	AMOUNT OF BROTH	CULTURE 18	OLD BROTH CULTURES TESTED	FIVE HOUR PLATES
	cc.	*cc.*		
1	2	0.1	1.0 cc. old broth 18D (resistant variant)	Transparent areas
2		0.1	0.5 cc. old broth 18D (resistant variant)	Lytic colonies
3	2	0.1	1.0 cc. old broth 18 (susceptible variant)	Normal growth
4		0.1	0.5 cc. old broth 18 (susceptible variant)	Normal growth
5	2	0.1	1.0 cc. sterile broth	Normal growth
6		0.1	0.5 cc. salt	Normal growth

This test was repeated using the filtrate of this old broth culture, together with the previously used heated supernatant fluid. The same result was obtained. The strain of typhoid was then reisolated from this culture. Only normal colonies were obtained on streaking. A fresh agar slant made by transplanting one of these colonies was tested against the Olsen bacteriophage. It was still wholly resistant.

Thus a bacteriophage, active against the susceptible variant of no. 18, had been extracted after prolonged incubation from the resistant variant. Under the heading, variations in typhoid strains, above, it will be noted that this strain had become resistant to lytic action without exposure to the lytic agent. The streak from the bottle of 18D showed that it had become contaminated with a *Staphylococcus albus* and a diphtheroid and it was thought that possibly the question of symbiosis might play a part in the production of bacteriophage from the organisms themselves. Carrère and Lisbon (1922) have recently had a similar idea, but this has now definitely been disproved, since pure cultures yield bacteriophage.

At the time we obtained the above result, we had not read Bail's (1921) article of September 15, 1921, in which he stated that he had isolated a bacteriophage from 3 old broth cultures active against Flexner dysentery bacilli. He does not state what organism was originally inoculated into the old broth. In a more recent article, Otto and Munter (1921) have succeeded in isolating bacteriophage in 9 instances, active against several organisms of the typhoid-dysentery group, obtained from old broth cultures of Flexner, Mt. Desert and Shiga dysentery and typhoid bacilli, respectively. The broths varied from three weeks old to six months. Unfortunately, the authors do not state whether they reisolated the strain from the broth to see whether it was resistant to the bacteriophage produced in the fluid in which it was growing, nor in case the bacteriophage was obtained in an old typhoid culture, for instance, whether the lytic fluid was active against the homologous typhoid strain, or only against other typhoid strains. It would also be of interest to know whether the broth was inoculated with old stock cultures, or recently isolated ones.

We have succeeded in obtaining another bacteriophage from a two months old typhoid culture (no. 18 susceptible) active against Shiga dysentery, but apparently against no other organism. We have not tried to date, to see whether by allowing it to act on Shiga dysentery for a few generations, we could obtain a bacteriophage active against typhoid. Culture no. 18 has been isolated for a year and a half.

The most interesting results in regard to obtaining bacteriophage directly from the bacteria themselves have been obtained by Callow in this laboratory, and are about to be published. She has found that the filtrates of certain strains of staphylococci prepared as described below, added to young broth cultures of certain other strains of staphylococci, proved lytic.

The filtrates were obtained by growing staphylococci on agar plates for eighteen hours, washing off the growth in sterile broth, or better, in distilled water containing 0.02 per cent sodium hydroxide, shaking the emulsion for thirty minutes to an hour, and then filtering through a Berkefeld filter. The filtrate thus obtained was active for 1 or more strains of staphylococci, but in no instance against the homologous strain. It was thus possible to wash the lytic agent directly off certain strains of staphylococci from comparatively young cultures active against other strains. Miss Callow has obtained the same results with filtrates of young broth cultures, but the results have been much less constant than with the washings from the agar cultures. In the broth cultures also the filtrate was only in very rare instances, active against the homologous strain originally inoculated into the broth, and this result was only obtained with older broth cultures. The strains used by Miss Callow in these experiments had been isolated from boils for a period of three or four months and had been transplanted frequently. Miss Callow had not tried the method of washing off agar growths with older stock cultures of staphylococci, so that we cannot state that old as well as recently isolated strains produce a bacteriophage principle by this method.

A small number of experiments have been done using the method originated by Miss Callow with strains of typhoid and dysentery bacilli, but to date without success. A variety of strains were tried. No. 18 susceptible and 18D, the resistant variant, were used, also the Shiga bacillus, which is generally agreed to be the most susceptible organism of the typhoid-colon-dysentery group. Another recently isolated strain of typhoid and a susceptible *Bact. coli* culture were also tried. Six hour, twenty-four hour and four day agar growths were shaken in alkaline solution, but none of the filtrates tested against a variety of cultures showed any lytic action. However, this work has been done very recently and most of the strians only tried once so that the results are not wholly conclusive.

Summary of isolation of bacteriophage from bacteria themselves

It can be stated that in the hands of four different workers, Bail, Otto and Munter, Callow and ourselves, it has been possible to isolate a bacteriophage from the bacteria themselves. With the typhoid-dysentery-colon group, this can most easily be done with old broth cultures. With staphylococci, the best results are obtained by washing off young agar cultures. The bacteriophages thus obtained are in the results obtained by Callow and ourselves, not usually active against the strain with which the broth or agar was originally inoculated. In the case of the bacteriophage, active against Shiga bacilli, obtained from an old typhoid culture, the typhoid culture had been isolated for a period of over a year, and been constantly used as a control and never given any signs of spontaneous lysis.

A recent paper by Lisbon and Carrère (1922) is of interest in this connection.

These authors state that they have been able to obtain a bacteriophage active against Shiga dysentery by inoculating a Shiga broth culture with a recently isolated *Bact. coli*. After incubating this mixed culture for a variable length of time, the broth is filtered. This filtrate is then added to a culture of Shiga bacilli and carried along for three or four generations. A bacteriophage active against the Shiga bacilli is finally obtained. They obtained a similar result by using a strain of Proteus, but they do not state whether it was recently isolated or not.

D'Herelle (1922) has answered Lisbon and Carrière by saying that since the strains of *Bact. coli* that they used were all recently isolated from stools and urine, the probability is that they were dealing with organisms that were carrying a bacteriophage, although apparently no evidence of lysis in the *Bact. coli* culture was observed. D'Herelle has described in his book (page 58) what he calls mixed cultures of bacteria and the ultramicroscopic virus in which the resistance of the bacteria is sufficient to prevent the formation of lytic colonies, and the virus is carried by what appear to be perfectly normal colonies.

Bordet and Ciuca were the first workers to show that the lytic agent could be carried with a certain type of *Bact. coli* colony ob-

tained after the culture was exposed to lysis. We have found that in the case of typhoid and dysentery cultures, bacteriophage action always divided the culture into lytic-bearing and what appeared to be non-lytic normal colonies. We streaked one of these normal colonies for fifteen successive generations, as reported above, on agar without ever obtaining anything but normal colonies. We also fished some of these normal colonies to broth and tried out the supernatant fluid comparison with the supernatant fluid of broth fishings of lytic colonies, without obtaining any evidence that the lytic agent was carried by these normal colonies. We, therefore, concluded that only the colonies in which we could definitely see evidence of lysis—i.e., lytic colonies—carried the virus.

In obtaining bacteriophage principles from old broth cultures and from agar washings of young growths of Staphylococcus (Callow) it seemed at first that we had definitely proved that the lytic material was derived from the bacteria themselves. But we realize now that the argument will not be conclusive until we are able to demonstrate lytic activity in old broth cultures of old laboratory strains, or perhaps by the aid of the Barber single cell isolation method. In both instances where we obtained lytic activity with old broth cultures, the culture used had in one instance been isolated six months and in the other over one year, but it was originally derived from a typhoid case in which the feces were shown to contain a potent bacteriophage. We have also described in detail how this culture fluctuated in resistance to the Olsen bacteriophage. The defenders of the filtrable virus theory would see proof in this for their claim that an apparently normal culture can carry a bacteriophage without giving any evidence of it, since this culture six months after isolation suddenly became resistant and when inoculated into broth, after prolonged incubation, produced a bacteriophage. The sudden resistance of this strain might, however, also be explained more simply by saying that in the course of the transplantation a series of resistant bacilli, which we know to exist in every culture, happened to be transferred for successive transplants until nothing but resistant bacilli were present.

The German writers do not give the history of the strains used by them. Miss Callow to date has only been successful with strains of recent isolation. We cannot, therefore, at the present time, completely exclude the possibility that apparently normal bacteria may carry an ultramicroscopic parasite.

IV. RESISTANCE OF THE LYTIC AGENT

Acetone resistance (Olsen bacteriophage)

A great many workers have tried to disprove the living virus theory in regard to bacteriophage action, notably Kabeshima, by pointing out the great resistance of the lytic agent. In our first attempt we were unable to confirm the method devised by Kabeshima for isolating the lytic material by precipitation with acetone. Recently, however, we have been able to demonstrate lytic activity in an acetone precipitate. Instead of starting with the simple dissolved culture filtrate obtained by the action of the Olsen bacteriophage on a typhoid culture, we concentrated the filtrate to one-tenth its volume by evaporation with a fan.

To 50 cc. of this concentrated lytic broth 150 cc. of acetone were added and the mixture was allowed to stand at room temperature and shaken from time to time. At the end of 48 hours the acetone was evaporated off until only a syrupy brown liquid remained. This was centrifuged, and a small amount of yellow precipitate obtained. The supernatant fluid was pipetted off and the precipitate partially redissolved in salt solution. Both the salt solution solute thus obtained and the supernatant fluid proved active.

It may be argued that the only reason that this concentrated lytic broth was able to withstand the forty-eight-hour exposure to acetone was because it was protected by the concentration of the proteins in the broth; but in any case, it shows a fairly high degree of resistance for the lytic agent. We concentrated the lytic precipitate in the first place to see if we could obtain a lytic agent of much greater potency. In one experiment the lytic titer was very much increased after concentration, but in subsequent tests the increase in titer was not so striking. The

Olsen bacteriophage is active in a dilution of 1:1,100,000, with most batches, without being concentrated.

We also precipitated 1 volume of the concentrated lytic broth with 9 volumes of acetone, and centrifuged the emulsion thus obtained, and redissolved the precipitate in salt solution. The precipitate dissolved very readily. The solution thus obtained, however, showed no lytic activity.

Typhoid dissolved culture filtrate (Olsen bacteriophage), if concentrated at one-tenth its volume, will resist exposure to 3 volumes of acetone for forty-eight hours. If, however, exposed to 9 volumes of acetone for a very short period, it is destroyed.

Resistance to alcohol precipitation

D'Herelle, in his book, states that by means of alcohol precipitation he has been able to separate the filtrable virus from the enzyme by which it acts.

If the lytic filtrate is exposed to 95 per cent alcohol for forty-eight hours the virus is destroyed, but the enzyme by which the virus does its work is still active. If, therefore, the redissolved precipitate obtained by adding 9 volumes of alcohol to one of lytic broth, is added to a turbid broth culture of a susceptible organism the clarification of the broth takes place, but the lytic agent is no longer transmissible in series, and no lytic colonies are obtained on streaking the dissolved cultures. The virus according to d'Herelle resists exposure shorter than forty-eight hours.

We have not to date been able to verify this particular experiment, but we have confirmed the extreme resistance of the lytic agent to alcohol precipitation. Exposure of 1 volume of concentrated lytic broth to 12 volumes of absolute alcohol for two hours did not destroy its activity. Miss Callow, using saline washings of agar cultures which contain a minimum of protective protein, has noted resistance to precipitation with alcohol for twenty-four hours.

The lytic agent, therefore, is more resistant than any form of life with which we are familiar, with exception of certain spores and an ultramicrobe parasitic on tobacco cited by d'Herelle.

We have also verified the observation of other workers that the lytic agent is resistant to exposure to 50 per cent glycerol and chloroform. It is interesting to note that Rettger in 1905 had found that autolysins of the bacteria were more resistant to 10 per cent chloroform than to 10 per cent toluol. We exposed the Olsen bacteriophage to 10 per cent chloroform and 50 per cent glycerol for a period of thirteen days without loss of activity.

V. IS THE LYTIC AGENT ANTIGENIC?

We have immunized 4 rabbits with typhoid cultures dissolved by the action of the Olsen bacteriophage. All the rabbits were injected intravenously and stood the injections well. One rabbit received altogether ten injections, one six injections, the other two received four injections at intervals of about four days. We were never able to develop an antilytic serum of the potency described by Bordet and Ciuca.

In the following experiment rabbit 1052 was bled after three days after the eighth injection, and rabbit 1098 was bled four days after the sixth injection. The experiment was carried out as follows: Equal mixtures of the immune serum and the Olsen bacteriophage were made, and added immediately and after thirty minutes' incubation and after eighteen hours' incubation. The test was set up as follows:

TUBE	IMMEDIATE BROTH	CULTURE 18	SERUM-BACTERIOPHAGE MIXTURES	READINGS EIGHTEEN HOURS	PLATES AFTER EIGHTEEN HOURS
	cc.	*cc.*			
1	2	0.5	0.2 cc. mixture Olsen + serum 1052, bled May 23	++ clumped	Lytic colonies
2	2	0.5	0.2 cc. mixture Olsen + serum 1098, bled May 20	++ clumped	
3	2	0.5	0.2 cc. mixture Olsen + serum 1097 (normal)	++	
4	2	0.5	0.1 cc. mixture Olsen	+	
5	2	0.5	0.2 cc. saline	++	Normal growth

(Turbidity equal to control, indicated by ++.)

The same result was obtained after the mixtures of serum and Olsen bacteriophage were incubated for thirty minutes and for eighteen hours.

One other protocol is of interest in which the immune serum was tested against dilutions of the Olsen bacteriophage, and in which immune typhoid serum was used as a control in order to show that agglutination alone did not interfere with lytic action. In this case the immune serum was added to the typhoid broth first and then the dilutions of the Olsen bacteriophage added. Serum was used from Rabbit 1052, which at that date had received nine injections.

December 7, 1921

TUBE	BROTH	CULTURE 18	SERUM	OLSEN BACTERIOPHAGE	SIX HOUR PLATES
	cc.	*cc.*			
1	2	0.1	0.2 cc. 1052 bled July 1	0.1 cc. undiluted	Many lytic colonies
2	2	0.1	0.2 cc. 1052 bled July 1	0.1 cc. 1:100	Many lytic colonies
3	2	0.1	0.2 cc. 1052 bled July 1	0.1 cc. 1:1000	Few transparent areas
4	2	0.1	0.2 cc. immune typhoid horse serum	0.1 cc. undiluted	Many lytic colonies
5	2	0.1	0.2 cc. immune typhoid horse serum	0.1 cc. 1:100	Many lytic colonies
6	2	0.1	0.2 cc. immune typhoid horse serum	0.1 cc. 1:1000	Many lytic colonies
7	2	0.1		0.1 cc. 1:1000	Many lytic colonies
8	2	0.1		0.1 cc. 1:1000	Many lytic colonies
9	2	0.1		0.3 cc. salt	Normal growth
10	2	0.1	0.2 cc. immune typhoid horse serum		Normal growth

The bacteriophage immune serum did not prevent lysis even if the bacteriophage was diluted 1:1000, although it was diminished in tube 3. Typhoid immune serum did not inhibit lysis. The other rabbit gave similar results. In some cases the antilytic serum tended to prevent the clearing up of the bacterial emulsion, but on plating lytic colonies were obtained in every instance. We have not, therefore, been able, like Bordet and Ciuca, to produce an antilytic serum of sufficient potency to prevent lytic action

permanently. We must agree with d'Herelle and Bail that the inhibition of lysis with immune sera prepared by the inoculation of large amounts of dissolved culture filtrate into rabbits is not complete. We have not found that normal rabbit sera interfere with lysis.

Protection experiments

It has been reported that guinea-pigs can be protected against an M.L.D. of culture by the injection of the homologous dissolved culture filtrate, and many workers have used typhoid and staphylococci bacteriophages in treating human cases.

We have performed experiments in which we have injected bacteriophage before and after the infection had begun, and have not found that the injection of lytic filtrate had any very striking advantages over the injections of sterile broth. The M.L.D. of the typhoid culture used was large, the washings of one eighteen-hour agar slant of typhoid being necessary to kill a pig of between 200 and 300 grams. Smaller doses were tried, but failed to kill the control pigs regularly. In the final experiment two pigs were used for each step, a light pig and a heavy pig as far as possible in pairs, the heavier pair in each case being used for controls. Eight pigs were used altogether; 4 pigs were injected with the M. L. D. of culture at 10:30 a.m. After four hours 2 of these pigs were injected with 2 cc. of sterile broth each, the other 2 were injected with 2 cc. of the homologous typhoid dissolved culture filtrate (Olsen). In the other pigs the bacteriophage and the sterile broth were inoculated first, and after an interval of four hours the M. L. D. of culture was injected. The cultures for the animals which received the lytic agent and broth first were stored on ice during the interim, to prevent further growth. All the injections were made intraperitoneally.

January 19, 1922. Animals in which the culture was injected first

	C. P.	WEIGHT	M. L. D. TYPHOID CULTURE	TIME	BROTH OR BACTERIOPHAGE	TIME	RESULT
		gms.		*a.m.*		*p.m.*	
Controls	364	330	1 slant no. 18	10:30	2 cc. broth	2:30	Dead 1/20/22, 9 a.m.
	363	245	1 slant no. 18	10:30	2 cc. broth	2:30	Dead 1/20/22, 9 a.m.
Bacterio-phage	369	250	1 slant no. 18	10:32	2 cc. Olsen	2:32	Dead 1/20/22, 9 a.m.
	362	180	1 slant no. 18	10:32	2 cc. Olsen	2:32	Dead 1/19/22, 10 p.m.

Animals in which the bacteriophage was injected first

	C. P.	WEIGHT	BROTH OR BACTERIOPHAGE	TIME	M. L. D, TYPHOID CULTURE	TIME	RESULT
		gms.		*a.m.*		*p.m.*	
Controls	400	270	2 cc. broth	10:35	1 slant no. 18	3:00	Survived
	323	210	2 cc. broth	10:35	1 slant no. 18	3:00	Found dead 1/20/22 9 a.m.
Bacterio-phage	325	270	2 cc. Olsen	10:37	1 slant no. 18	3:05	Survived
	394	195	2 cc. Olsen	10:37	1 slant no. 18	3:05	Died in 48 hours

At 4:30 p.m., January 19, 1922, all the pigs were punctured and plates made of the exudate with the following result:

No. 364. Good growth; normal typhoid colonies.
No. 363. Good growth; normal typhoid colonies.
No. 369. Many lytic colonies.
No. 362. No growth.
No. 400. Growth obtained; normal.
No. 323. Growth obtained; normal.
No. 325. Many lytic colonies.
No. 394. No growth.

Lysis was, therefore, going on actively in the peritoneum of the pigs 325 and 369, which had received inoculations of bacteriophage. No growth was obtained from the other two bacteriophage pigs, 362 and 394. Every pig that died was autopsied to make sure that it had not died from any other cause, and typhoid bacilli were isolated from the peritoneal cavity of each. Only two pigs out of the eight survived: one that had been injected with 2 cc. bacteriophage and one that had been injected with 2 cc. of sterile broth four hours before the injection of the M.L.D. of typhoid bacilli.

Conclusions from animal experimentation

We have not been able to confirm the opinion of other workers that the protective action of dissolved culture filtrate is very striking. We were unable to show any very definite advantage in the case of the Olsen bacteriophage over the injection of

sterile extract broth. Bordet and Ciuca obtained definite protection in guinea-pigs against injections of *Bact. coli*. Since guinea-pigs are not susceptible to infections either with typhoid or *Bact. coli* and death in both cases is probably of toxic origin, conclusions as to the value of bacteriophage treatment for human beings cannot be drawn from these experiments.

Study of the leucocytic exudates obtained from guinea pigs in protection experiment

The peritoneal exudates from all the pigs that died were collected, twice the volume of sterile broth added, and the tubes left standing at room temperature for forty-eight hours. At the end of this time they were plated with the following results:

No. 364. Small number of lytic colonies.
No. 363. Many lytic colonies.
No. 369. No lytic colonies, pure typhoid.
No. 362. No lytic colonies, pure typhoid.
No. 323. No lytic colonies, pure typhoid.
No. 394. No lytic colonies, pure typhoid.

Thus the exudates from the two control pigs showed lytic colonies, whereas the exudates from the pigs that had been injected with bacteriophage which had previously shown lytic colonies (no. 369), after standing at room temperature showed nothing but resistant types.

The tubes were kept on ice from January 22 to January 24 and then heated at 59° for thirty minutes. and tested for sterility. These exudates were set up against the stock strain no. 18 as follows:

TUBE	AMOUNT OF BROTH	CULTURE 18	PERITONEAL EXUDATES	PLATES AFTER SEVEN HOURS
	cc.	*cc.*		
1	2	0.1	0.5 cc. no. 364	Few transparent areas
2	2	0.1	0.5 cc. no. 363	Transparent areas
3	2	0.1	0.5 cc. no. 369	Little growth, 1 lytic colony
4	2	0.1	0.5 cc. no. 362	Little growth, 2 normal colonies
5	2	0.1	0.5 cc. no. 323	Normal growth
6	2	0.1	0.5 cc. no. 394	No growth obtained
7	2	0.1	Salt solution	Normal growth

As already stated under the section on the production of bacteriophage by the method of Bordet and Ciuca, it was possible to produce a lytic exudate by a single injection of typhoid bacilli intraperitoneally into guinea-pigs.

VI. IS THE LYTIC PHENOMENON A FACTOR IN RECOVERY FROM INFECTION?

Occurrence of bacteriophage in carrier stools

It seemed of interest in view of the claims made for the beneficial results obtained by d'Herelle in the treatment of dysentery cases with bacteriophage, and by others in the treatment of boils with staphylococcus lytic agent, to determine the incidence of bacteriophage in carrier stools.

Through the courtesy of Dr. Krumwiede of the Research Laboratory of the Health Department, we were able to obtain carrier stools at frequent intervals, and also in one instance a specimen of blood from one of the carriers.

We obtained carrier stools from two typhoid carriers of long standing, Mary Mallon and Mary Newton, residing at the Riverside Hospital, at weekly intervals for a period of six weeks. The fecal specimens were plated on Endo's medium and the homologous strain of typhoid isolated. The proportion of typhoid colonies in the stool of Mallon was consistently less than with Newton. The plates were always carefully examined with the microscope but in no instance were any lytic or abnormal colonies observed.

The fecal suspensions were treated in two ways, the first time the specimen was received: in one case the suspension was thoroughly shaken and filtered immediately, in the other it was shaken and then incubated for four hours and filtered. The filtrates thus obtained from Newton and Mallon were tested against the homologous strain of typhoid and also against the stock strain of typhoid no. 18, since it was thought that the typhoid strain in these carrier stools might be resistant.

February 7, 1922

TUBE	AMOUNT OF BROTH	TYPHOID CULTURE	STOOL FILTRATE	TWO HOUR PLATES
	cc.			
1	2	Mallon	1 cc. Mallon shaken filtrate, February 1, 1922	Many lytic colonies
2	2	No. 18	1 cc. Mallon shaken filtrate, February 1, 1922	Normal growth
3	2	Mallon	1 cc. Mallon shaken filtrate, incubated 4 hours, February 1	Many lytic colonies
4	2	No. 18	1 cc. Mallon shaken filtrate, incubated 4 hours, February 1	Normal growth
5	2	Newton	1 cc. Newton shaken filtrate, February 1	Normal growth
6	2	No. 18	1 cc. Newton shaken filtrate, February 1	
7	2	Newton	1 cc. Newton shaken filtrate, incubated 4 hours, February 1	
8	2	No. 18	1 cc. Newton shaken filtrate, incubated 4 hours, February 1	
9	2	Mallon	0.2 cc. Olsen bacteriophage	Many lytic colonies
10	2	Newton	0.2 cc. Olsen bacteriophage	Suspicious colonies but not definitely lytic
11	2	No. 18	0.2 cc. Olsen bacteriophage	No growth
12	2	Mallon	Salt solution	Normal growth
13	2	Newton		
14	2	No. 18		

The stool filtrate of Mallon was definitely lytic for the homologous strain of typhoid, but not for the stock strain no. 18. The Mallon strain of typhoid was not resistant to the Olsen bacteriophage. The filtrate filtered immediately after shaking was as good as the one filtered after four hours' incubation. The filtrate from Newton had no action on the homologous typhoid strain nor on the stock strain no. 18.

The specificity of the Mallon typhoid strain was tried out as shown in the table on page 95.

The tubes were incubated for one hour and then held at room temperature overnight, and plated. The filtrate was again active against the homologous strain of typhoid and against Shiga, but against none of the other cultures tried.

Specificity of the Mallon filtrate, February 1, 1922

TUBE	AMOUNT OF BROTH	CULTURE	FILTRATE	PLATED AFTER EIGHTEEN HOURS AT ROOM TEMPERATURE
	cc.			
1	2	0.1 cc. Mallon typhoid	0.5	Few lytic colonies
2	2	0.1 cc. no. 18 typhoid	0.5	Normal growth
3	2	0.1 cc. Newton typhoid	0.5	Normal growth
4	2	0.1 cc. Rawlings stock	0.5	Normal growth
5	2	0.1 cc. Shiga	0.5	Reduced growth, "appearances"
6	2	0.1 cc. Mt. Desert	0.5	No lytic colonies
7	2	0.1 cc. Typhi Murium	0.5	No lytic colonies

Tube 1 in the last experiment was heated at 56° for thirty minutes to kill the resistant forms, and then the supernatant fluid tried against four typhoid strains to see if the potency of the Mallon bacteriophage was increased in the second generation.

February 11, 1922

TUBE	AMOUNT OF BROTH	TYPHOID CULTURE	SUPERNATANT FLUID	TWO HOUR PLATES
	cc.			
1	2	Mallon	0.5 cc. tube 1, February 9	Lytic colonies
2	2	No. 18	0.5 cc. tube 1, February 9	Normal growth
3	2	Newton	0.5 cc. tube 1, February 9	Transparent areas
4	2	Rawlings stock	0.5 cc. tube 1, February 9	Normal growth

In the second generation the Mallon bacteriophage had extended its activity and was lytic for the Newton strain of typhoid, as well as for the homologous strain.

Bacteriophage in a carrier stool active against a homologous Bact. coli culture

Repeated tests showed that the filtrates from the stool of the carrier Newton had no action on the homologous or other typhoid strains. It seemed of interest, therefore, to determine whether or not the Newton stool might possibly contain a bacteriophage active against some other organism present in the fecal flora. We consequently isolated three different types of lactose-fermenting organisms occurring on Endo plates of the Newton stool, to

Russell's medium and tried the Newton filtrate against them with the following result:

February 12, 1922

TUBE	AMOUNT OF BROTH	CULTURE	STOOL FILTRATE AND CONTROLS	TWO HOUR PLATES	EIGHTEEN HOUR PLATES
	cc.				
1	2	Newton typhoid	1 cc. Newton filtrate, February 10	Normal growth	Normal growth
2	2	Newton typhoid	1 cc. salt	Normal growth	Normal growth
3	2	Newton coli (1)	1 cc. Newton filtrate, February 10	Normal growth	Normal growth
4	2	Newton coli (1)	1 cc. salt	Normal growth	Normal growth
5	2	Newton coli (2)	1 cc. Newton filtrate, February 10	Reduced growth ("appearances")	No growth
6	2	Newton coli (2)	1 cc. salt	Normal growth	Normal growth
7	2	Newton coli (3)	1 cc. Newton filtrate, February 10	Normal growth	Normal growth
8	2	Newton coli (3)	1 cc. salt	Normal growth	

By setting up a second generation from tube 5 definite lytic colonies were obtained with this particular strain. A bacteriophage active against *Bact. coli* (2) has been obtained from the stool of Newton 6 successive times from specimens obtained at 1 week intervals. The growth of this susceptible *Bact. coli* is very characteristic; it forms a large, coarse colony, and in broth has a tendency to sediment out, leaving the supernatant fluid clear. The Newton filtrates have not proved active against the other types of *Bact. coli* present in the Newton stool.

Each specimen of Newton received was plated and contained in every instance a large number of typhoid colonies. The colonies of

Bact. coli (2) were much less numerous, and in two out of the 6 specimens could not be found on the plates.

We have not studied the occurrence of bacteriophage consistently in a sufficient number of carriers to state in what proportion of carriers bacteriophages active against the homologous typhoid strain occur.

Conclusions on work with carrier stools

We have been able to demonstrate a bacteriophage active against the homologous strain of typhoid in the stool filtrates of a carrier of over ten year's standing and also in one that has been positive for nine months. In another typhoid carrier of long standing, we have shown a bacteriophage active against a certain strain of *Bact. coli* occurring in the fecal flora. Specimens from both these carriers consistently contained these particular bacteriophages over a period of six weeks. The proportion of the susceptible organisms in the stool containing bacteriophage seemed to be reduced, and in two instances the susceptible organism could not be found. The presence of bacteriophage in carrier stools may possibly explain the often observed fact, that carrier stools are sometimes negative.

A specimen of blood obtained from one of the carriers (Newton) was examined for the presence of bacteriophage in the circulation, but none was found.

It is worth reporting, furthermore, that the Ida Olsen from whom the Olsen bacteriophage used throughout this paper was isolated in November 1920 when she was in the convalescent stage after typhoid, has also developed into a chronic carrier. Her stools have been positive for typhoid for a period of one and one-half years. A specimen of feces has recently been obtained from her, but we have not so far been able to obtain a bacteriophage from it.

Discussion as to the nature of bacteriophage

Evidence as to the nature of bacteriophage is still inconclusive. D'Herelle and his co-workers are convinced that all lytic phenomena are due to an ultramicroscopic virus, normally

parasitic on the bacteria of the intestinal tract, but capable by adaptation of attacking a large number of organisms.

Kabeshima first brought forward the point of view that the resistance of the lytic agent was such as to rule out living protoplasm, and that the whole manner of action of the bacteriophage suggested enzyme activity. It is obvous that the lytic agent cannot be classified as an enzyme as commonly defined, since it characteristically acts on living rather than dead cells. Furthermore, there is no analogy in the literature of ferments for an enzyme which is quantitatively increased after acting on a substrate.

Bordet and Ciuca's observation that lytic activity could be demonstrated in the leucocytic exudate obtained in the peritoneal cavity of a guinea-pig by the injection of a normal bacterial culture, seemed to point away from the parasitic theory of bacteriophage, indicating that the source of the lytic agent was probably in the bacteria themselves. In the same way, our own experiments in which we produced lysis of typhoid bacilli, transmittable in series by the action of extracts of normal tissue and of normal serum seems to diminish the likelihood of an external parasite, and suggests that the bacteria themselves are the source of the lytic agent. But by far the most striking evidence that the bacterial cell itself secretes bacteriophage under certain circumstances, or liberates it when it disintegrates in a certain way, is the fact that the lytic agent can be demonstrated in old broth cultures in the case of typhoid and dysentery bacilli, and in alkaline washings of eighteen hour agar cultures, in the case of the staphylococcus.

The high resistance to heat and various chemical reagents also renders d'Herelle's theory less likely, but is in itself not sufficient evidence that the lytic agent is not a living thing, since as d'Herelle has pointed out, there are certain forms of life, such as the spores of *B. subtilis*, and an ultramicroscopic virus occurring on tobacco, that are equally resistant.

Again, as an important argument against the filtrable virus conception, we may cite our own experiments as well as those of others in which the lytic principle was developed in old broth

cultures of previously resistant strains, and has been obtained in salt solution washings of young agar cultures derived from normal colonies. These facts cannot be reconciled with the filtrable virus theory without assuming that apparently resistant bacteria may remain carriers for generations of the infecting parasite. From this point of view, since all the cultures used in these experiments were originally derived from the animal body where they may have been exposed to bacteriophage action, it is possible that what we assume to be a normal colony, is only one which is infected to a less degree with the lytic agent. Under certain circumstances the bacteriophage may become 'dissociated from the bacteria, and then be demonstrable. Therefore, until the strains of bacteria used to show that bacteriophage develops spontaneously in the process of bacterial growth can be definitely shown to be free of a contaminating parasite by the use of the Barber single cell technique, it will not be possible to disprove conclusively the ultramicroscopic virus theory of bacteriophage.

In the present state of our knowledge, however, taking all facts into consideration it seems to us more reasonable to assume the simpler hypothesis that the bacteriophage represents a secretion of the bacteria, produced under certain circumstances, and of the nature of an autolysin. This autolysin, usually liberated in old bacterial cultures, as a consequence of cell disintegration acts as a catalyst which destroys the delicately adjusted equilibrium occurring in actively growing cells between constructive forces and destructive forces, in favor of the latter. Solution of the bacterial cell consequently results and occurs in such a way that more of the autolysin is liberated.

What the significance of the bacteriophage may be from a therapeutic point of view is difficult to estimate at the present time. The fact that a bacteriophage isolated from boils as shown by Callow, is usually not active against the homologous strain of staphylococcus, is not very encouraging. Also the isolation of bacteriophages active against the homologous strain of typhoid from chronic carriers and the development into a carrier of a convalescent case in which there was a typhoid

bacteriophage present, are not easy to correlate with the supposed therapeutic value of the bacteriophage. The data presented are too limited to warrant any conclusions. Progress will probably be made by determining whether bacteriophage phenomena occur in other pathological conditions such as pneumonia and meningitis.

SUMMARY

It has been shown that bacteriophage phenomena with members of the typhoid group can be initiated by means of a variety of agents: normal tissue extracts from guinea-pigs, and normal rabbit sera, and that the lytic agent is, therefore, not necessarily due to any pathological condition. Bacteriophage action has also been obtained with old typhoid broth cultures in the same way as reported by Bail with dysentery. The bacteriophages produced in this way have usually not been active against the homologous strain, but have been active against other strains of the typhoid-dysentery group.

It is suggested that bacteriophage phenomena may possibly have played a part in the observations made by previous workers on the bactericidal titer of normal rabbit serum for typhoid.

High resistance reported by other observers of bacteriophage to acetone and to alcohol precipitation, and also to chloroform and glycerol have been confirmed.

Attempts to prepare antilytic sera of sufficient potency to prevent lytic action completely, were unsuccessful.

Intraperitoneal injections of bacteriophage into guinea pigs have not afforded any very definite protective action against one M.L.D. of typhoid bacilli.

A bacteriophage principle active against the homologous strain of typhoid in two typhoid carriers has been demonstrated. In another typhoid carrier it has been shown that there was no bacteriophage present active against typhoid, but that the filtrates consistently contained one active against a certain strain of *Bact. coli.* In one other case, where a bacteriophage active against the homologous strain of typhoid was isolated during the convalescent stage of the disease, the patient has developed into a chronic carrier.

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