THE BACTERIOPHAGE

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In six previous articles which have recently appeared in this publication under the title of "Filterable Virus and Rickettsia Diseases in the Tropics" we have described the various diseases affecting man which fall into these two groups. It has been thought fitting to continue this series with two other papers, one dealing with the bacteriophage and the other with the intracellular inclusion bodies which are associated with several virus diseases. The present paper deals with the bacteriophage, its properties, nature and observations concerning the clinical aspects of its use in various diseases.

DEFINITION

The word bacteriophage means "bacteria eater" and represents the term applied to a bacterial lytic principle by d'Herelle(1) in 1917. At present the bacteriophage can only be defined as a "substance", "agent", or "principle" which is filterable through the finest porcelain filters and which is capable of bringing about the dissolution of certain bacteria. Some investigators regard the bacteriophage as a living filterable virus; others believe the lytic principle to be of the nature of an enzyme; while there are those who think of this "agent" as a product of bacterial dissociation, autolysis, or as an hereditary by-product of the bacteria. Since opinion has been so divided during the past decade over which this agent has been studied, it is impossible to define accurately its nature. Of its properties and activity we know more. At present we consider the bacteriophage a filterable lytic agent, active in extremely high dilutions, capable of increasing in quantity at the expense of the lysed bacterium, and as a "substance" almost constantly present in the intestines of man and animals.

HISTORY AND DISTRIBUTION

In 1915 Twort(2) published a paper entitled "An investigation on the nature of ultramicroscopic viruses". In this paper Twort described certain transparent areas in a culture of staphylococci in which no cocci grew. Touching one of these transparent areas with

* This is the seventh of a series of articles taken from a monograph on Filterable Virus and Rickettsia diseases by the author. (Philippine Journal of Science May–June, 1929.)

a sterile platinum loop and then drawing the loop across the surface of a twenty-four hour agar culture of staphylococci, he found after a few hours, a streak marking the track of the loop which had become clear and transparent. Filtering the material from these transparent areas through a Berkefeld filter, he found that the filtrate would dissolve and kill most of the organisms in fresh staphylococcus cultures even in dilutions of one to a million. It is interesting to note that the first description of a lytic principle for bacteria, which is transmissible in series, was discovered in a culture of an organism which is Gram-positive. Except for the observation of Hankin in 1896 this is the first printed record concerning the bacteriophage. Hankin [3], nearly twenty years before, described the bactericidal action of the water of the Ganges and Jumna rivers in India for the cholera vibrio but the idea of bacteriophage was far beyond his imagination. To Twort then belongs the honor for being the first to bring this phenomenon to the attention of other investigators.

Previous to 1916 d'Herelle was interested in a peculiar disease affecting locusts which he believed to be caused by a filterable virus although a cocobacillus was easily cultivated from infected locusts and frequently presented cultural irregularities which puzzled him. Some colonies of this bacillus possessed idented irregular contours and at times there were areas entirely free from growth. d'Herelle was inclined to consider this disease as being caused by a filterable virus but having an "associated" organism, the cocobacillus, such as exists in hog cholera. Experiments carried out upon this disease of locusts led up to his observation of the bacteriophage. In one of his books on the subject of the bacteriophage d'Herelle describes his first experiments which led to the discovery of the bacteriophage in the stools of dysentery patients. He states "In August, 1916, an adult with a severe bacillary dysentery (Shiga) was under treatment in the Pasteur Hospital. Each day about ten drops of the stool were collected and placed in a tube of bouillon. After incubation over night the suspension was filtered through a Chamberland candle. Into some bouillon, previously inoculated with Shiga bacilli, about 10 drops of this filtrate were placed, and the material was returned to the incubator at 37° C. . . . Throughout the duration of the disease, all of the tubes, prepared each day in the same manner, gave normal cultures of *B. dysenteriæ*. One day, the tube prepared the day before remained sterile. Investigation showed that the patient gave evidence of notable improvement, and, as appeared later, this was shortly followed by definite convalescence. . . To the bouillon thus inoculated and containing filtrate, and which had remained to all appearances sterile, a suspension of Shiga bacilli derived from a fresh agar culture was added to yield a marked turbidity. This tube was placed in the incubator. After about ten hours it was again clear. . . This, of course, made it at once apparent that my first hypothesis was of necessity false, the truth of the matter being that the fecal material used in preparing the filtrate contained something which dissolved the dysentery bacilli. Nevertheless, my first hypothesis had one virtue, since, as it had led me for such a long time to consider the question of a virus pathogenic for the man or the animal it offered the suggestion that the dissolving principle might be a virus pathogenic for the bacterium."

Thus the discovery of the bacteriophage was made by d'Herelle in the stools of dysentery patients and the filterable virus theory of the nature of the bacteriophage was conceived. During the past ten years, as will appear in subsequent discussion, d'Herelle has attempted to prove his theory of the virus nature of the bacteriophage and much experimental work has been presented upon the part of other investigators in opposing this view which, to the conservative mind, has appeared fantastic and a conclusion unwarranted by the facts which have been presented in its favor.

That there exists diverse strains of bacteriophage is now well recognized. Sewage universally contains bacteriophage. Consequently rivers receiving sewage from towns and cities frequently contain different strains of bacteriophage. We have isolated such strains from various streams and sewage supplies in America, Porto Rico and the Philippines. Isolation of bacteriophage active against the colon bacillus is a very simple matter with sewage as a source of material. Strains of bacteriophage have also been isolated from the stools of man and animals, from the urine and blood of patients, from old laboratory cultures, from the nodules of plants, from the tissues of man and animals, and from the intestines of silk worms. From the Pasig river in the Philippines we have isolated bacteriophage active against *B. coli*, *B. typhosus*, *B. dysenteriæ Shiga*, and *B. dysenteriæ Flexner*. In this stream untold numbers of carabaos wallow during every hour of the day. Excreta from such animals have been found by Basaca to be loaded with bacteriophage. Bacteriophage has been isolated from the water of the Seine by Dumas(4) and Collins(5) has reported its presence in the Huron river in Michigan. It can be stated definitely that all streams receiving sewage will be found to contain bacteriophage lytic for some organism, practically always for some strains of *B. coli*. The bacteriophage then originates in the intestines of man and animals and perhaps other forms of life such as insects, etc. Since the intestinal tract of man and animals is universally contaminated with *B. coli*, a commensal micro-organism, it is suggested that the bacteriophage is in many instances associated with this bacterium or some other bacterial form. Experimental evidence to support this suggestion will be presented.

THEORIES CONCERNING THE NATURE OF THE BACTERIOPHAGE

Several theories or hypotheses have been suggested to explain the phenomenon of bacteriophagy. d'Herelle, since his first work with the lytic principle, has considered the bacteriophage as a living ultramicroscopic and filterable virus, foreign and parasitic to bacteria. Kabeshima([6]) has suggested that the bacteriophage is a chemical principle foreign to the bacterium. This author suggests the possibility of a catalytic substance in the intestinal tract of animals which brings about the dissolution of bacteria by activating some pro-ferment present in the bacteria. Bordet and Cuica([7]) suggested the idea that bacteria undergo a nutritive vitiation under the influence of some product manufactured by leucocytes, and further that this vitiation is hereditary since the phenomenon is transmissible indefinitely in series. d'Herelle speaks of this concept as the hypothesis of an "abnormal inert principle". Kuttner([8]) explains the nutritive vitiation of the bacterium upon the basis of some ferment present in the intestinal tract of animals. A similar idea to that of Bordet and Cuica has been suggested in one of our own publications on this subject([9]). Lisbonne and Carrére([10]) have suggested the theory that the bacteriophage is the result of a bacterial antagonism Seiffert([11]) has suggested an exogenous autolysis as the cause of the phenomenon. Doerr([12]) thinks of the bacteriophage as a toxin which affects the bacterial metabolism. This author would place other agents such as the viruses of rabies, vaccinia, encephalitis lethargica, and sarcoma also in this category. A large number of investigators favor the idea that the bacteriophage is simply a product of normal autolysis. d'Herelle replies to the proponents of this theory that it is strange that the bacteriophage phenomenon occurs with young bacteria having no natural autolytic tendency, and does not take place with old bacteria which autolyse spontaneously. Bail([13]) believes that the lytic principle is normally present in the bacteria and is a living substance. There is also the possibility that the lytic principle may be abnormal to the cell, yet living and derived from the bacteria.

The hypotheses concerning the nature of the bacteriophage are many and in fairness one must state that d'Herelle has presented a theory of the nature of bacteriophage which no one has, as yet, been able to refute completely and by experimental evidence prove that he is wrong. Arguments based upon careful experimental work have been presented for most of these opposing theories but no single clear-cut experiment has as yet been performed which will prove any one of these hypotheses and disprove d'Herelle's idea of the living filterable virus nature of the lytic principle. We do not advocate the virus theory of the bacteriophage because we feel that the experimental evidence so far advanced does not prove its living nature but in all justice towards d'Herelle it must be admitted that he has presented a thesis in his living virus theory of the bacteriophage which is almost convincing and one which is exceedingly difficult to discredit. For a detailed discussion and analysis of the various hypotheses which have been advanced to explain the phenomenon of bacteriophage, the reader is referred to d'Herelle's books on the subject.

BACTERIA FOR WHICH LYTIC PRINRIPLES HAVE BEEN DESCRIBED.

A wide variety of bacteria have been found susceptible to the phenomenon of bacteriophagy. The list includes B. *dysenteriæ* Shiga, Hiss, and Flexner; B. *gallinarum; Pasteurella bovis;* B. *pestis;* B. *typhosus, Para A and B; B. suipestifer*; *B. enteriditis; B. typhimurium; B. coli; Friedlander bacillus; Bacillus of Flacherie; B. proteus; Bacillus* of Swine fever; *B. diphtheriæ;* Nodule bacteria of leguminosæ; *B. subtilis; Vibrio choleræ;* the *Staphylococcus, Enterococcus,* and *Streptococcus; B. pyocyaneus;* pseudo-bacteriophage for *B. Anthracis; Thermophilic bacillus;* T 60 and *Psychrophilic bacteria.*

PROPERTIES OF THE BACTERIOPHAGE

It is well recognized that there exist many different strains or races of bacteriophage. It has also been well established that all strains of bacteriophage are filterable through ultra-filters. It has further been demonstrated by d'Herelle, Bronfenbrenner and Korb ([14]), ourselves([15]) and others that the lytic principle is particulate. According to d'Herelle these particles are about the size of the micella of serum globulin. Prausnitz([16]) states that the diameter of the bacteriophage particle is about the size of the micella of collargol used in his experiments, or about 20 milli-microns. von Angerer([17]) gives a figure of 30 milli-microns for its diameter; Jotten([18]) and Arnold([19]) have described experiments which demonstrate beyond doubt that the bacteriophage is diffusible through the agar upon which is planted the bacteriophage with its susceptible microorganism. The bacteriophage is evidentally non-volatile although it may be carried over in distillates by droplets if care is not taken to prevent this. By titration it has been shown by d'Herelle and others that particles of bacteriophage tend to sediment to the bottom of a vessel on standing and to some degree by centrifugation. That the particles are certainly unevenly distributed in a liquid suspension has been indicated in our work. The bacteriophage is not soluble and exists only in suspension, perhaps in a colloidal state. Wollman([20]) thought that he was able to digest the lytic principle with trypsin but further work indicated that this destruction is only partial. The possibility exists that in bouillon the bacteriophage particle is adsorbed on to protein molecules. This is indicated by the fact that the precipitate formed from bouillon by the addition of acetone or alcohol is found to contain the lytic principal in a very high concentration. d'Herelle states that one strain of bacteriophage has remained alive in a sealed ampoule for nine years. He says "When first prepared there were over 2000 million corpuscles per cubic centimeter, after four years the number was reduced to only 100 million, after nine years to but 40 millions." The bacteriophage "multiplies" in an alkaline medium though a few strains have been described which "multiply" in an acid medium. In general the bacteriophage particles flocculate under the influence of acids (Da Costa Cruz) a phenomenon which also occurs with the majority of bacteria. Since these bacteria carry a negative electric charge it follows that the bacteriophage particle is also charged negatively. d'Herelle terms it a "negative colloid". The bacteriophage is also precipitated by saturation with ammonium sulphate according to Maison([21]) but it may be recovered still virulent from the precipitate as in the case of an acetone or alcohol precipitate. Magnesium apparently acts in the same way according to de Poorter and Maisin([22]). In acetone or alcohol precipitates the bacteriophage is destroyed if contact is prolonged for several days.

The bacteriophage particle is adsorbed by a number of substances. The protein in bouillon has already been referred to. Various authors have demonstrated that the bacteriophage is adsorbed by infusorial earth and kaolin. (Seiffert([23]), Glidemeister and Herzberg([24]) and others). Much of the work done on adsorption however has not taken into account the hydrogen ion concentration of bacteriophage suspension and opinion is at variance upon this property of the bacteriophage. d'Herelle believes there is relatively little adsorption of bacteriophage by these substances.

The bacteriophage is also affected by irradiation. Appelmans([25]) has inactivated bacteriophage by exposure to ultraviolet rays for ten minutes. Gildemeister([26]) states that the bacteriophage is sensitive to ultraviolet light as are bacteria. Gerretsen, Gryns, Sack and Söhngen

[27]) found that ultraviolet light destroyed B. *radicicola* but not its lytic principle after exposure for fifteen minutes. McKinley, Fisher and Holden([28]), however, have found that a strain of bacteriophage lytic for B. coli is destroyed by ultraviolet light much in the same way as are two strains of known filterable viruses, i, e., herpes and Levaditi's so-called encephalitis virus. Exposure to ultraviolet light at a distance of one foot for forty minutes is sufficient to attenuate or destroy both the bacteriophage and the two filterable viruses employed. B. *coli* was not completely destroyed at this exposure. Later experiments by these authors showed that much shorter periods of exposure were effective in destroying both the lytic principle and the filterable viruses mentioned. Fisher and McKinley([29]) exposed various concentrations of bacteriophage to the action of ultraviolet light and concluded that the resistance to ultraviolet rays of the lytic principle is directly proportional to its concentration and appears to be a logarithmic function thereof. The effect produced by ultraviolet light is not a photosensitization to heat. Brutsært([30]) found that radium emanations did not destroy the bacteriophage. Baker and Nanavutty([31]) have recently made a quantitative study of the effects of ultraviolet rays upon the bacteriophage and state that the susceptibility of the bacteriophage is of the same order as that of bacteria and that the relative lethal efficiencies of different regions of the spectum are the same for the bacteriophage as for B. *coli.* The work of these authors lends confirmation to that of Rivers and Gates([32]) who have demonstrated that the susceptibility of vaccinia virus to irradiation is of the same order as that of bacteria.

As has been mentioned there are many different strains of the bacteriophage. This is indicated by several properties which they possess. Some strains of bacteriophage always produce very minute pinhead plaques on solid cultures while others produce plaques many times as large. In a way the size of the plaques may be controlled by increasing the concentration of the agar. This has been demonstrated by Bronfenbrenner and Korb. However on a uniform agar concentration different strains of lytic principle will exhibit different sized plaques. Differences between strains of bacteriophage are also noted in their response to heat. One strain may be destroyed at a temperature of 65° C. while another will require 70° or 75° C. when heated for thirty minutes. d'Herelle states that heat destruction is preceded by attenuation and that 75° C. may, in general, be regarded as the thermal death point of the bacteriophage. Bacteria sensitive to the action of such a lytic principle are of course destroyed at much lower temperatures. The multiplicity of bacteriophage strains is also indicated by the specificity of the various strains. Within certain limits adaptation of the bacteriophage to more than one organism is possible but not always. Some races are apparently markedly specific.

The bacteriophage is susceptible to the action of various chemicals and disinfectants. The response of the bacteriophage to these substances depends somewhat upon the race. The various races of bacteriophage respond differently. In general it may be said that in its resistance to chemicals and disinfectants the bacteriophage is comparable to the spores of bacteria. In some instances it appears to be more resistant than spores to the disinfectant employed. This resistance also depends somewhat upon the temperature at which the destructive agent is permitted to act. For example Bronfenbrenner and Korb found that the bacteriophage is rapidly destroyed by absolute alcohol at ordinary temperatures, but that it resists absolute alcohol for five or six days if kept at 0° C.

FACTS WHICH FAVOR THE LIVING NATURE OF THE BACTERIOPHAGE

The fact that bacteriophage increases in quantity at the expense of the lysed bacterium is the fundamental argument upon which d'Herelle bases his theory of the bacteriophage. This he regards as evidence of reproduction and multiplication. The theory of the living nature of the bacteriophage is also supported by the fact that, within certain limits, the bacteriophage possesses the powers of adaptation and assimilation. These are regarded as the combination of characters which constitute the criteria of life.

OPPOSING VIEW

Many investigators have been unwilling to accept these criteria as proven in the case of the bacteriophage. Increase in quantity of bacteriophage at the expense of the lysed bacterium is not regarded in the minds of many as representing reproduction in the sense in which this word is usually employed. It is argued for instance that the bacteriophage may represent merely a lytic principle elaborated by the bacterium itself and is enzymatic in nature. Bordet believes that the bacterium is vitiated by the action of the cells of the host and that this vitiation continues on through the subsequent generations of the strain. This is suggested in the strain of *B. coli* of Lisbonne and Carrére which manufactures a lytic principle active against a strain of *B. shiga dysentery*. d'Herelle regards this strain of *B. coli* as contaminated with the bacteriophage. Such a strain, however, it has been shown by us produces antilysins for the lytic principle when injected into normal rabbits and when primary cultures of this organism in broth show no evidence of the phenomenon of bacteriophagy. d'Herelle will refute this with the statement that the bacteriophage contaminant is not present in sufficient concentration in the primary culture to manifest itself but even in its weak concentration it is antigenic. This is of course possible. However it is not probable. *B. coli* Lisbonne in culture presents no evidence that it possesses the power of elaborating a lytic principle for *B. dysenteriæ Shiga.* In other words *B. coli* Lisbonne itself is not affected by the lytic principle. Therefore the lytic principle active against *B. dysenteriæ Shiga* is not increasing in quantity (in the d'Herelle sense) when it exists in a pure culture of *B. coli* Lisbonne for the simple reason that *B. coli* Lisbonne is not sensitive to it. In hundreds of subcultures of *B. coli* Lisbonne we have never seen any evidence of lytic plaques or any other indication that a bacteriophage is present in the culture. The bacteriophage must (according to d'Herelle) increase in quantity at the expense of the sensitive bacterium. In the *B. coli* Lisbonne culture the sensitive bacterium so necessary for the "multiplication" of the lytic principle is missing. And further is it found that subculture generation after generation of *B. coli* Lisbonne possesses the power of elaborating bacteriophage for *B. dysenteriæ Shiga.*

If we accept d'Herelle's contention that *B. coli* Lisbonne is contaminated with the bacteriophage and knowing that the bacteriophage does not lyse *B. coli* Lisbonne and hence, in d'Herelle's sense is unable to "multiply" at the expense of this organism, how then are we to explain the fact that each generation of *B. coli* Lisbonne is capable of elaborating lytic principle for *B. dysenteriæ Shiga?* The *B. coli* Lisbonne divides approximately every seventeen minutes. If each bacterium contains bacteriophage or possesses bacteriophage adsorbed upon its surface, this quantity of lytic principle will be divided with each division of the microbe. Or one new microbe may have no lytic principle. One hesitates to attempt to calculate the dilution of lytic principle which would take place after several subcultures of this organism. It is known however that a subculture prepared from one colony of *B. coli* Lisbonne after several generations, is still capable of elaborating lytic principle active against *B. dysenteriæ Shiga.* This is strong evidence against the assumption that the *B. coli* Lisbonne is contaminated with the bacteriophage and to our mind indicates definitely that *B. coli* Lisbonne possesses the inherent ability of elaborating lytic principle active for *B. dysentery Shiga.* In other words this property is functional and is perhaps a metabolic process upon the part of *B. coli* Lisbonne. This is strong indication that the lytic principle is an enzyme elaborated by *B. coli* Lisbonne in this particular instance.

While many investigators have been willing to accept the hypothesis of d'Herelle that the bacteriophage is a living ultramicroscopic virus there are still those who are not willing to accept this view as final. While d'Herelle's view offers much in its favor the evidence is not complete. For the present we prefer to consider the bacteriophage as an ultramicroscopic particulate substance, diastatic in its action, granular in form and inanimate, which is probably derived from the bacterial cell during a stage in its life cycle as a result of the influence of tissue cells of the host upon the bacterium. We have likened the bacteriophage to the zymogenic granules of the pancreas and its lytic action to that analogous to the action of zymogen, an enzyme elaborated by the zymogenic granules. The response of the lytic principle to external agents such as heat, chemicals and disinfectants, ultraviolet light, etc., may be considered analogous to that of various enzymes as well, as in some instances, to that of bacteria. The bacteriophage granules which are precursors of the lytic principle, may be elaborated by the bacterial cell, a phenomenon brought about by the cells of the host acting upon the bacterium.

Some other criterion is essential to establish the living nature of the bacteriophage. Bronfenbrenner(33) has been unable to detect respiration and we have reported similar conclusions(34). If this could be demonstrated it would be the strongest argument in favor of the living nature of the lytic principle.

d'Herelle has steadfastly maintained that since the lytic principle increases in quantity at the expense of the lysed bacterium it (the lytic principle) must be living in nature. It has been pointed out that no analogy exists in nature where a non-living substance will exhibit this property. While still in the experimental stages we would invite attention to the recent observations of Manwaring (see Science, n. s. Vol. LXIX, No. 1799, June, 1929, Supplement, page X) on the growth of serum. This author injected horse serum into the blood stream of rabbits and a few days later found not merely the presence of all the horse serum originally used, but an actual increase of from 200 to 400 per cent. This indicates the possibility of a non-living substance being capable of growing (increase in quantity) by itself in a suitable environment.

The scope of this paper is too limited to review the recent publication of Hadley(35) on the subject of the bacteriophage. This author has suggested in his "homogamic" theory a new line of thought and attack. The reader is referred to the exhaustive treatment of this subject, as published by Hadley, for a resumé of the literature and a critical analysis of the entire subject.

Since the discovery of the bacteriophage many attempts have been made in various parts of the world to employ this substance in the treatment of disease. As is the history of most new therapeutic agents the early reports of the use of the bacteriophage in the treatment of infection were overly enthusiastic. Time and further experimentation has tempered the opinions of most investigators and the present trend of thought on this subject may be said to be more conservative.

The bacteriophage has been employed in a wide range of infectious processes. It has been used in bacillary dysentery, in the typhoid fevers, in avian typhosis, in *B. coli* infections, in staphylococcus and streptococcus infections, etc. Reports on the efficacy of this substance in the disease processes indicated have differed somewhat in the hands of the various investigators who have worked with it. For detailed accounts of this work the reader is referred to d'Herelle's([1]) publications. We believe that it is fair to state, at present, the treatment of disease with the bacteriophage is not entirely upon a sound basis; that further well controlled work needs to be done. We have observed cases of bacillary dysentery improve markedly under treatment with the bacteriophage([36]) though we have seen others in which no benefit was derived from this therapy. We have reported the use of the bacteriophage in the treatment of certain staphylococcus and streptococcus infections([37]) though subsequent investigations have caused us to believe that much of the benefit which was derived from this treatment was due to non-specific protein and not to the lytic principle contained in the bouillon. Gratia([38]) has indicated to us a similar opinion. At present we believe that a conservative attitude should be shown toward this question and that more careful, well controlled studies should be made before coming to definite conclusions regarding the treatment of infections with this agent.

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