A STUDY OF THE EFFECT OF BACTERIOPHAGE ON PHAGOCYTOSIS

THESIS FOR THE DEGREE OF M. S.

John Richard Knudsen

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Introduction

The discovery of bacteriophage in 1915 has opened up hitherto unknown and unsuspected avenues of approach to the problems of infection and immunity. The importance of these problems cannot be over estimated for with their solution we may look for material advances in our efforts to control infectious diseases. Nothing is at present known regarding the identity of the principle and very little as to its mode of operation. Nevertheless, there is an enormous amount of experimental work from which will eventually come a solution of the problem of the nature of bacteriophage. Meanwhile it has found a few practical uses among which clinical applicatioh is outstanding. So striking have been the results in numerous instances that practice has greatly outstripped theory and we are now faced with the problem of providing an explanation for some of the occurrences reported and of building a foundation upon which advancements may rest.

Among other factors, stimulation of phagocytosis is of considerable interest in connection with bacteriophage therapy, and although a minor amount of work has been reported, there are many phases of the problem in need of confirmation and further study. Since phagocytosis is highly considered as a factor in immunity, it seemed that any contribution showing the relationship between bacteriophage and this phenomenon would be of considerable value. Although aware that the work to be reported in this paper by no means disposes of the problem, it is felt that the results

obtained constitute a real and useful contribution in this field of endeavor. The work of which this paper is a report was undertaken with the hope of adding something to our present knowledge of one phase of the question, namely, the effect of bacteriophage upon the phenomenon of phagocytosis.

In order properly to evaluate the results and to interpret their relationship to what has already been accomplished, it is necessary to summarize what the literature now contains on this question. Likewise in a subject as yet none too familiar to many biologists, it is advisable to present a brief description of the fundamental phenomenon involved.

Review of Literature

From time to time bacteriologists have observed that broth cultures of bacteria, after having undergone an apparent initial period of growth, suddenly for some reason became sterile. The explanation of this occurrence was furnished by Twort (1) who discovered a principle capable of producing this reaction at will. He was stopped, however, from continuing his researches by the war. It remained for d'Herelle (2) to demonstrate the serial passage of the principle responsible for the clearing of these cultures and to designate a source from which it could easily be obtained. This principle d'Herelle named Bacteriophage, more specifically, Bacteriophagus intestinale. While it is possible to conceive of a chemical substance capable of having just such an effect, the potency of any chemical decreases with dilution and upon serial passage the quantity necessary to produce the reaction would be diluted beyond its effective quantity.

Bacteriophage on the other hand is characterized by the fact that it remains effective far beyond the point of dilution at which chemicals would have any action. Also, the bacteriophage, transferred serially for an indefinite period, instead of decreasing in potency, actually increases both with respect to potency and to quantity. Hence the conclusion that this principle is living matter.

In addition to the lytic action of bacteriophage, numerous other evidences of its activity can be demonstrated, among these the formation of plaques. These transformations in colony appearance are characterized by clear, glassy and smooth areas containing the principle and become manifest when cultures of bacteria containing bacteriophage are cultured on solid media.

The phenomenon of bacteriophagy has suggested far-reaching possibilities in the study of biological problems and in the treatment of disease. Countless investigators have studied the subject from every possible angle but at present the exact identity of the active substance and its mode of operation have eluded search and although numerous physicians have made extensive use of bacteriophage in the treatment of disease, its value has not yetbeen generally accepted.

The literature bearing on the subject of bacteriophage has become so voluminous as to preclude the possibility of making an attempt at a summary in the present work. A recent article states that over two thousand references on this subject exist at the present time and several reviews, among which are those by d'Herelle (3) and Hadley (4), include excellent bibliographies to which the reader is referred in regard to questions not

related directly to the problem here presented.

Since we are concerned in this paper with bacteriophage and its effect upon phagocytosis, it may be well to review briefly our present knowledge of this subject.

The role of the leucocyte as a scavanger for the body and in immunity has long been a subject of vital interest and inquiry and methods of stimulating phagocytic activity have been investigated for over forty years. The power of the phagocyte to ingest foreign material including bacteria was known long before the time of Metchnikoff; but it was he who demonstrated that subsequent to ingestion, the process of digestion took place. For Metchinkoff the process of phagocytosis comprised the sole protection of man against the invasion of pathogenic organisms, a point of view which gave rise to the classic battle between the cellular and humoral theories of immunity. The keen observations of this man, however, on the phagocytic activity of the polymorphonuclear leucocyte cast considerable light on this subject and laid the foundation upon which the later significance of phagocytosis was based.

Subsequent discovery of other phagocytic cells of the body, particularly those of the reticulo-endothelial system, have served to impart considerable impetus to the idea that the ingestion of invading bacteria by various cells of the body is of major importance and should be taken into consideration when dealing with any infectious disease.

The adequate provision made through the discovery by Wright and Douglass (5) of a method for determining accurately the phagocytic activity of cells has enabled investigators to evaluate more carefully the role

of the phagocyte in immunity and has assured the acceptance of the phenomenon as a satisfactory explanation for certain types of immunity. The work of these and subsequent investigators is so well-known that it is unnecessary to give the details of their thousands of experiments. Briefly, these experiments indicate a definite relationship between opsonins and bacteriotropins on the one hand and the phenomenon of phagocytosis on the other. These former two substances are classed as antibodies and are, supposedly, increased by artificial immunization. Their effect upon the bacterial cell is to render it more susceptible to the phagocytic cell.

Among numerous incidental observations accompanying the study of bacteriophage was a report by d'Herelle and Eliava of having observed a marked increase in the number of bacteria taken up by the phagocytic cell in the presence of bacteriophage. The details of their work, however, were not given. These workers obtained opsonic indices as high as forty and came to the conclusion that the increase was due to an effect of the bacteriophage upon the bacterial cell.

This work was subsequently confirmed by Weiss and Arnold (6) who also failed to give details of their work. They obtained opsonic indices as high as 9.3 and likewise concluded that the increase was dependent upon the action of bacteriophage on the bacteria.

Smith (7) and Nelson (8) are the only recent contributors to the subject. Smith made an extensive study of this problem and reports having obtained indices with a maximum value of 32.48. Smith states also that the increased index is due to an action of the bacteriophage upon the

bacterium. He also includes in the same report results of opsonic work in which bacteriophage susceptible and bacteriophage resistant strains of the same race of staphylococcus were first subjected to the action of the bacteriophage for varying periods of time and then brought into contact with phagocytes and the difference in phagocytosis noted. He observed that phagocytosis of the bacteriophage susceptible organisms increased with the time of their exposure to the bacteriophage but that there was no such increase with the bacteriophage resistant strain. He also found that while dilution greatly lowered the index, there was still the same correlation as in undiluted bacteriophage. Smith believes the susceptible organism is capable of adsorbing bacteriophage whereas the resistant strain does not; therefore, the latter are not ingested to the extent that those susceptible are.

Nelson's results also indicate a marked increase in opsonic index in the presence of bacteriophage, obtaining a maximum value of 13.8 with susceptible cocci. He also noted that a much smaller number of the resistant strain were ingested; but it is his contention that the bacteriophage exerts its influence upon the phagocytic rather than upon the bacterial cell.

Gohs and Jacobson (9) and (10)in their investigation of the phagocytic activity of the leucocyte did not employ bacteriophage but endeavored to study the spontaneous ingestion of bacteria by the leucocyte. They used bacteriophage susceptible and bacteriophage resistant strains of the Shiga dysentery bacillus in their work, and observed a much smaller number of resistant organisms ingested, as compared to the susceptible

strain of the same race. Similar results have been obtained by other workers.

A study of the literature leads to some interesting comparisons and reveals certain significant disagreements but d'Herelle, Weiss and Arnold and Smith agree on one point at least, - that an increased index is observed in the presence of bacteriophage.

Significant as these observations may be, the problem is by no means solved and many questions remain which have a very important bearing on the ultimate use of bacteriophage in clinical medicine.

In the first place it is well known that individual races of bacteriophage have marked differences and it is still unknown whether the results so far reported are due to peculiarities, characteristic of certain bacteriophage races, or whether they represent a general phenomenon common to all bacteriophages. This problem can only be solved by extending the number of bacteriophage races used in experiments of this kind.

All the work so far reported involved the use of impure bacteriophage, that is, bacteriophage in combination with numerous other substances such as those composing the medium for growth, metabolic byproducts, endotoxins, exotoxins and proteins derived from the lysed bacterial cells. Smith observed this fact and stated that he was unable to determine whether his results were due to bacteriophage as such or to some other of the various constituents of the filtrates he employed. Although numerous methods for preparing pure bacteriophage have been suggested, it was not until the publication of Krueger's and Tamada's method that purified bacteriophage suitable for such work could be prepared.

Although there is some unanimity regarding the role of the bacterio-

phage in this phenomenon, it has by no means been determined that the increased ingestion of bacteria by leucocytes is dependent upon a preliminary action by the bacteriophage on the bacterium and furthermore, a matter of considerable significance from the standpoint of therapy, it has not been shown that the phenomenon repeats itself in vivo.

The knowledge of these unsolved problems and the recognition of the importance of attempting to clarify somewhat the present status of bacteriophage and phagocytosis, led to the undertaking of experiments which are to follow. They by no means cover the problem nor can it be said that the work is in any sense completed. Nevertheless, they do constitute a significant and valuable addition to our knowledge of this subject and represent an advance over what was previously known.

Experimental

For convenience in the presentation of this part of the investigation, each experiment has been separately listed and its specific purpose clearly stated, although, of course, they are all more or less related. The procedure in each operation is also stated, except in cases of identical repetition. Discussion of the results and their interpretation will be found in a subsequent section of the paper.

Before taking up the experimental work, it may be advisable to define such terms as "opsonins", "opsonic index" and the method used in the determination of the index. The term "opsonin" literally means "to make ready for food" and "phagocytosis", "to eat", - so that these antibodies are substances effective in increasing the natural tendency of the leucocytes

for eating or ingesting bacteria by producing a change in the bacterial cell, rendering it more desirable as food for the leucocyte. The method used for making the opsonic tests is that outlined by Wright and Douglass and consists, with minor changes, briefly in obtaining leucocytes from rabbits by bleeding from the marginal ear vein and collecting in 1% sodium citrate solution. The blood is centrifuged slowly to allow the red cells to settle, then at increased speed the leucocytes form a white layer covering the red cells. The clear supernatant fluid is discarded and the leucocytes removed with a pipette and washed by resuspending in saline and centrifuging. The supernatant fluid is removed as before, and the white cells again resuspended in saline. They are then ready for use. A twenty-four hour agar slant culture of the organism is washed off with saline and a suspension of approximately 1000 millions per cubic centimeter prepared. Equal portions of the leucocytic suspension, bacterial suspension and serum or bacteriophage are taken up with a pipette, well mixed and incubated for 15 minutes at 37° C. At the end of the incubation period and following a second thorough mixing, smears are prepared and opsonic counts made. The smears are stained by the methylene blue method. The bacteriophage used throughout the present work is different in race from that used in the literature cited. The method of Wright and Douglass was also followed in making the opsonic counts, and consists briefly in finding the average number of bacteria ingested by the leucocytes, a definite number of the latter being counted. In the present work an attempt was made to find the number of organisms in two hundred polymorphonuclear leucocytes and the average number obtained for one leucocyte is known as

the "phagocytic index". The opsonic index (the opsonic power of the fluid being tested) is obtained by dividing the figure representing the phagocytic index of the material being tested, the opsonic power of which is unknown, by the phagocytic index of normal phagocytes, that is, spontaneous ingestion of the same organism by the phagocyte, or phagocytosis in the absence of any stimulating substances such as opsonins.

Although d'Herelle obtained his high indices by incubation of leucocytes with bacteria and bacteriophage, several investigators have been unable to confirm his results and as the belief is held by many that the increased index is due to an action of the bacteriophage upon the bacterial cell rather than upon the phagocytic cell or upon the process of phagocytosis itself, it seemed advisable to repeat part of d'Herelle's work, using his method.

Experimental Work

EXPERIMENT I.

Object:

This experiment was designed to confirm and to extend d'Herelle's observations on opsonic indices approximating forty obtained by incubation of leucocytes, bacteriophage and susceptible bacteria.

Procedure:

The test organisms used were B. typhosus and Staphylococcus aureus. Eighteen hour agar slants were prepared and the growth washed off with normal saline to give a suspension of approximately 1000 million bacteria per cubic centimeter as determined by nephelometer.

Bacteriophage active against these bacteria was employed. It should

be noted that d'Herelle made use of a Shiga bacteriophage and B. dysenteriae Shiga. Thus while this experiment is in part intended to repeat d'Herelle's work, it is also intended to extend his observations through the use of different races of bacteriophage and different bacterial cultures.

Leucocytes were obtained by the method described on page nine.

Normal serum was procured from human bloods sent to the laboratory for diagnostic purposes. These sera when pooled contain a very small amount of opsonin. Immune serum against B. typhosus was a portion of a stock supply prepared at the State Biological Plant and used in the State Laboratory for routine diagnostic work. No antistaphylococcus serum was available.

The various combinations of leucocytes, bacteria and opsonizing substances are shown in Table I.

The method of making the determinations is identical throughout all the experiments. Capillary tubes of about .5 mm. outside diameter and 20 cm. in length were used. A mark was placed on the capillary about 2 cm. above the tip and the leucocyte suspension drawn up into the tube by gentle suction as far as the mark. A column of air about .5 cm. was then admitted to the tube and next the opsonizing substance, (bacteriophage or serum), was drawn up to the same mark. Last the organisms were similarly measured and the ingredients mixed by blowing the contents of the tube onto a glass slide. By drawing the mixture several times into the tube and forcing it out again, a thorough mixing was accomplished. The fluid was then drawn into the tube several

centimeters above the tip which was then sealed in a flame. Incubation in a water bath at 37 degrees for fifteen minutes followed immediately after sealing the tube. The seal was then broken, the contents of the tube ejected onto a glass slide and a film prepared by smearing the slide. These films were then allowed to dry at room temperature.

The method of staining resulted from a number of experiments designed to determine which was most satisfactory. In view of the fact that in other hands other methods might prove better, it does not seem advisable to draw any conclusion as a result of these trials or to recommend the method selected as superior to some others. Under the conditions prevailing, the use of the Wright triple stain proved best adapted to these particular experiments. This gave a blue color to all organisms used as well as to the nuclei of the cells. The cytoplasm stained a light pink which gave good contrast.

A Leitz research microscope with a mechanical stage, oil imersion objective and a fifteen X occular giving a calculated magnification of 1200 diameters was used for all counts. As a rule two hundred leucocytes were examined including mononuclear cells but not lymphocytes and the number of organisms within the cytoplasm of each cell was recorded. From this a phagocytic index was obtained by dividing the number of organisms by the number of leucocytes counted.

The results of these experiments are found in Table I. No attempt to analyze or interpret these results will be made in this section. All results will be presented in Part IV.

TABLE I.

A.	Cells Counted	Organisms Ingested	Phagocytic Index	Opsonic Index
1.B.typhosus + W.B.C.(control)	200	108	.54	1.00
2. " + W.B.C.+ normal serum	200	199	.90	1.66
3. " + " + immune serum	200	217	1.06	1.96
4. " + " + bacteriophage	200	307	1.53	2.83
5. " + " + " dil. 1-1,000,000	200	173	.86	1.60
6. " + " + " heated	200	156	.78	1.44

B.	Cells Counted	Organisms Ingested	Phagocytic Index	Opsonic Index
1.Staph.aureus +W.B.C.(control)	200	120	.60	1.00
2. " aureus +W.B.C.+ normal serum	200	178	.89	1.48
3. " " +W.B.C.+bacteriophage	200	155	.77	1.28
4. " " + " + " dil. 1-1,000,000	200	188	.94	1.56
5. " " + " + " heated	200	120	.60	1.00

EXPERIMENT II.

EXPERIMENT II.

Object:

The following experiment was undertaken in an attempt to determine the effect of time or of preliminary incubation of bacteria with bacteriophage upon phagocytosis. This seemed particularly advisable in view of the fact that it was by such a method that Smith and Weiss and Arnold obtained their exceptionally high indices.

Procedure:

Leucocytes were obtained as described on page nine. The bacterial suspension was prepared from an eighteen hour agar slant culture of B. typhosus as in Experiment I. Equal quantities of the bacteriophage or serum and the bacterial suspension were well mixed, placed in small Wasserman tubes and incubated at 37° C. At the end of fifteen minutes a portion of each mixture was removed and the bacteria washed twice with saline to remove all bacteriophage and finally resuspended in saline. The tubes were returned to the incubator for additional

incubation. To one part of the suspension of washed bacteria was added an equal part of the leucocytic suspension, as described on page eleven, and the mixture incubated for fifteen minutes at 37° C. Following this incubation smears were prepared and stained and opsonic counts made. In the same way a test was made after the one hour incubation of bacteria and bacteriophage. The results of this test follow.

TABLE II.

	Cells Counted	Organisms Ingested	Phagocytic Index	Opsonic Index
A.	Fifteen minutes preliminary incubation			
1. B. typhosus + W.B.C. (control)	200	127	.63	1.00
2. " + W.B.C. + normal serum	100	115	1.50	2.38
3. " + " + immune serum	10	8	.80	1.26
4. " + " + bacteriophage	67	80	1.20	1.90
B.	One hour preliminary incubation			
1. B. Typhosus + W.B.C. + normal serum	150	215	1.43	2.26
2. " + W.B.C. + immune serum	100	230	2.30	3.65
3. " + " + bacteriophage	100	176	1.76	2.79

EXPERIMENT III.

EXPERIMENT III.

Object:

The following set of experiments was performed in order to study the effect of purified bacteriophage upon phagocytosis and to extent the work involving preliminary incubation of bacteria and bacteriophage upon phagocytosis.

Procedure:

The method for the purification of bacteriophage as outlined by Krueger and Tamada (11) is fundamentally one of cataphoresis. Bacteriophage being supposedly negatively charged should migrate towards the anode and protein impurities, on the other hand, at the hydrogen ion concentrations employed are neutral in charge and do not migrate. A

medium free of protein and from which bacteriophage can be easily removed has been discovered in pure agar.

The apparatus devised by Krueger and Tamada and used in this experiment is drawn in Plate I, page sixteen. Purified agar used in the process is prepared according to the method of Dominickiwics (12), which follows: From thirty to forty grams of agar are dissolved in 100 cc. to 200 cc. of water, enough water being added so that the solution will flow in a thin stream when poured. While still hot, it is poured slowly into 800 cc. to 1000 cc. of 95% alcohol strongly acidified with acetic acid. The alcoholic solution is stirred vigorously while the agar is added. The result is a white precipitate which upon standing settles to the bottom. The clear supernatant fluid is then decanted off and the precipitate freed of all liquid by means of suction. The precipitate is now washed repeatedly with pure 95% alcohol until all traces of the acetic acid have been removed. This step is extremely important for the presence of acid will inhibit solidification of the pure agar, especially when a low percentage concentration is desired. Then precipitate is dried at 37° C. for several days or until the odor of alcohol is removed. At this time the coarsest particles are rendered more usable by grinding in a mortar.

After the apparatus has been sterilized, the bridges C and D are filled with sterile purified agar 3% containing .2% NaCl. This is done by inverting the bridge and resting the lower end on a flat surface until the agar has solidified. Then the glass tube I is filled half full with sterile purified .5% agar containing .2% NaCl. When this has become solidified, the remainder of the tube is filled nearly to the top with

APPARATUS FOR PREPARATION OF RELATIVELY PURE BACTERIOPHAGE

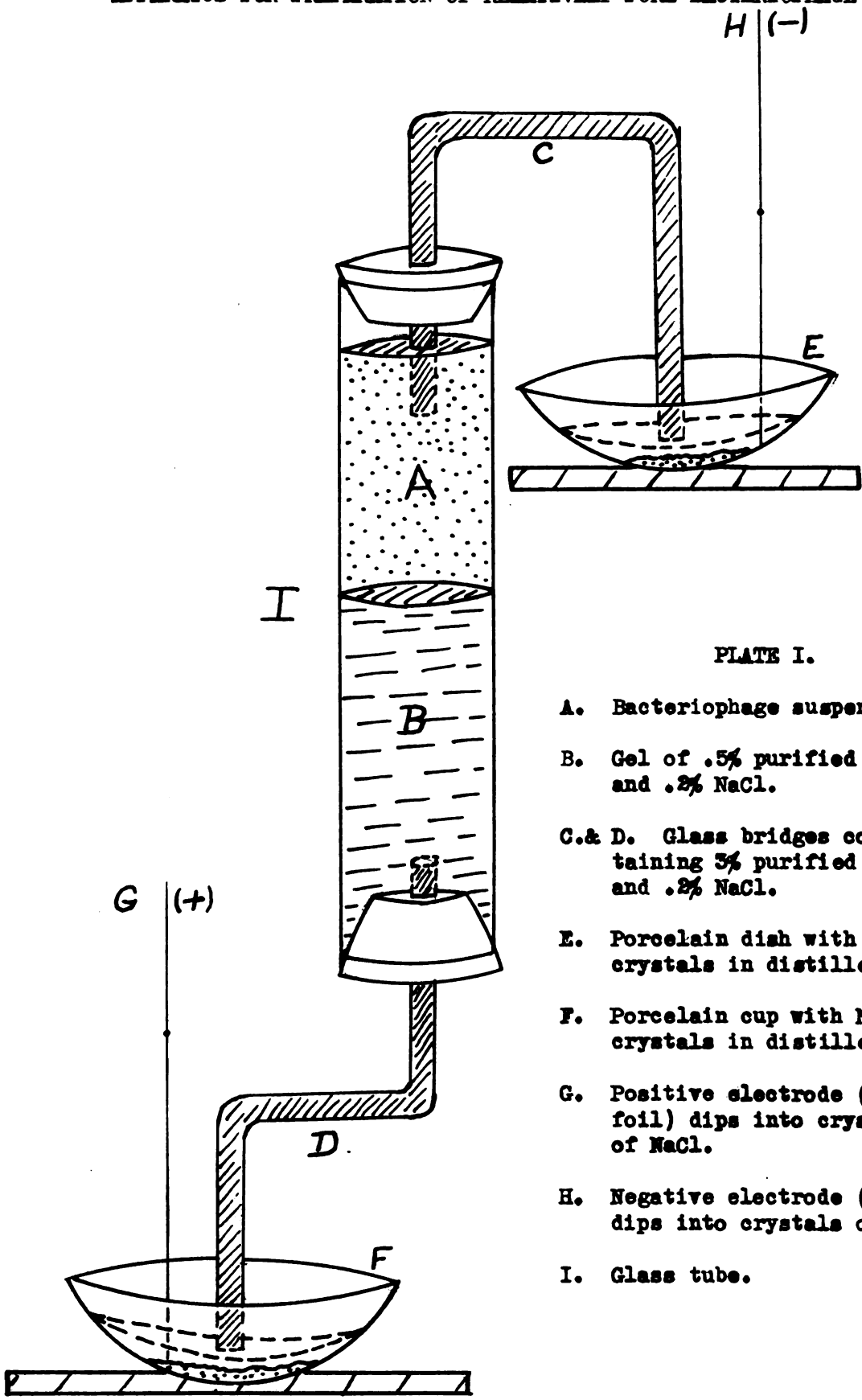

PLATE I.

A. Bacteriophage suspension.

B. Gel of .5% purified agar and .2% NaCl.

C.& D. Glass bridges containing 3% purified agar and .2% NaCl.

E. Porcelain dish with $CuCl_2$ crystals in distilled water.

F. Porcelain cup with NaCl crystals in distilled water.

G. Positive electrode (silver foil) dips into crystals of NaCl.

H. Negative electrode (copper) dips into crystals of $CuCl_2$.

I. Glass tube.

the bacteriophage, leaving only room for the stopper. The negative electrode H is composed of copper which dips into crystals of $CuCl_2$, distilled water being added to the desired level. The positive electrode G is composed of silver foil which dips into a solution containing crystals of NaCl.

A current of from 100 volts to 125 volts and from 5 milliamperes to 12 milliamperes is passed through the apparatus for 18 hours.to 20 hours. This does not necessarily have to be a continuous current. The bacteriophage above the agar is changed at least once during this time. At the end of this period, the supernatant fluid is removed with a pipette and discarded. The lower bridge and stopper are then removed and the agar allowed to slide out the upper end of the tube. A layer of the agar, one-half inch thick, which had been in contact with the bacteriophage is sliced off with a sharp sterile instrument, and the remainder sliced into a sterile grinding machine and a few cubic centimeters of sterile saline added. After being macerated for two to three hours, it is taken out and the coarse particles removed by filtering through a coarse filter. Since no information was available concerning the effects of filtration on purified agar, two different types of filters were used, Seitz and Mandler.

At the time of Smith's investigations no method of obtaining bacteriophage in a pure state was known, that is, bacteriophage from which a large part of the proteins contained in the medium such as products of bacterial metabolic activity and of disintegration, as well as toxins, etc., have been removed, and the later discovery of such a method by Krueger and Tamada offered an opportunity to study the properties of purified bacteriophage as regards opsonic work. It is a well known fact that the specific

effect of the various proteins cannot be predicted in every case and it is well within the limits of probability that these proteins might interfere with the action of bacteriophage. A purified bacteriophage might, therefore, reveal certain specific characteristics and powers which would not appear in opsonic testing carried out or performed with simple stock bacteriophage.

During the process of purifying the several bacteriophages used in the present work, a very interesting and perhaps valuable observation was made, namely, that while all other bacteriophages studied collected at the anode, thus carrying a negative charge, the staphylococcus bacteriophage collected at the cathode and is therefore the only positively charged bacteriophage at present known.

This experiment is identical in every respect with Experiment II with the exception of the addition of purified bacteriophage in the tests, the test organisms being B. coli, B. typhosus, and staphylococcus aureus. Following are the results of this experiment in tabulated form.

TABLE III.

A. (1)	Fifteen minutes preliminary incubation			
	Cells Counted	Organisms Ingested	Phagocytic Index	Opsonic Index
1. B. typhosus + W.B.C. (control)	122	232	1.90	1.00
2. " + W.B.C. + normal serum	200	380	1.90	1.00
3. " + " + immune serum	200	490	2.45	1.28
4. " + " + bacteriophage	200	530	2.60	1.36
5. " + " + " Seitz purified	200	555	2.70	1.42
6. " + " + " Mandler "	200	490	2.45	1.28
(2)	One hour preliminary incubation			
2. B. typhosus + W.B.C. + normal serum	200	257	1.28	.67
3. " + W.B.C. + immune serum	200	540	2.70	1.42
4. " + " + bacteriophage	200	542	2.71	1.42
5. " + " + " Seitz purified	200	550	2.75	1.44
6. " + " + " Mandler "	200	500	2.55	1.34

TABLE III. (cont'd.)

	Cells Counted	Organisms Ingested	Phagocytic Index	Opsonic Index
B.				
(1)		Fifteen minutes preliminary incubation		
1. B. typhosus + W.B.C. (control)	200	192	.96	1.00
2. " + W.B.C. + normal serum	200	281	1.40	1.45
3. " + " + immune serum	200	152	.76	.79
4. " + " + bacteriophage	200	382	1.96	2.04
5. " + " + " purified	200	260	1.30	1.35
(2)		One hour preliminary incubation		
4. B. typhosus + W.B.C. + bacteriophage	200	321	1.60	1.66
5. " + " + " purified	200	342	1.71	1.78
C.				
(1)		Fifteen minutes preliminary incubation		
1. B. typhosus + W.B.C. (control)	200	334	1.67	1.00
2. " + W.B.C. + normal serum	200	470	2.35	1.40
3. " + " + immune serum	200	374	1.87	1.11
4. " + " + bacteriophage	200	666	3.33	1.99
5. " + " " " purified	200	426	2.13	1.26
(2)		One hour preliminary incubation		
4. B. typhosus + W.B.C. + bacteriophage	200	848	4.24	2.54
5. " + " + " purified	200	740	3.70	2.21
D.				
(1)		Fifteen minutes preliminary incubation		
1. B. coli + W.B.C. (control)	200	254	1.27	1.00
2. " + W.B.C. + normal serum	200	410	2.05	1.61
3. " + " + bacteriophage	200	454	2.27	1.78
4. " + " + " purified	200	774	3.87	3.04
(2)		One hour preliminary incubation		
3. B. coli + W.B.C. + bacteriophage	200	1004	5.02	3.95
4. " + " + " purified	200	950	4.75	3.74
E.				
(1)		Fifteen minutes preliminary incubation		
1. Staph. aureus + W.B.C. (control)	200	252	1.26	1.00
2. " aureus + W.B.C. + normal serum	200	285	1.42	1.12
3. " " + " + bacteriophage	200	210	1.05	.83
4. " " + " + " purified	200	585	2.97	2.35
(2)		One hour preliminary incubation		
3. Staph. aureus + W.B.C. + bacteriophage	200	294	1.47	1.16
4. " " + " + " purified	200	427	2.13	1.69

EXPERIMENT IV.

EXPERIMENT IV.

Object:

Smith's report includes work in which susceptible and resistant strains of the same race of staphylococcus were used to determine the relative effect of bacteriophage upon the phagocytosis of these two strains. The following experiment was designed to verify Smith's results.

Procedure:

This consisted in obtaining leucocytes in the usual manner and preparing suspensions of the two strains of staphylococcus to be used in the test. With these suspensions two series of bacteria-bacteriophage mixtures were made, each mixture consisting of 1 cc. bacterial suspension, .5 cc. bacteriophage and 3.5 cc. of slightly alkaline broth. The various mixtures of each series were made at different times so as to incubate for periods varying from one-half hour to four hours. Following incubation, the organisms were washed twice with saline by centrifuging, resuspending in saline and re-centrifuging. The suspensions of washed bacteria were then mixed with leucocytes and incubated for fifteen minutes at 37°C. Smears, staining and opsonic counts were made as in other tests. Results of this experiment follow:

TABLE IV.

Period of Contact (hours)	Staphylococcus B-1122 Susceptible Strain		Staphylococcus 1631 Resistant Strain	
	Phagocytic Index	Opsonic Index	Phagocytic Index	Opsonic Index
1/2 Staph.+W.B.C.+bacteriophage	2.17	1.14	1.88	1.07
1 " + " + "	2.95	1.55	2.13	1.21
2 " + " + "	2.86	1.50	1.63	.93
4 " + " + "	4.86	2.55	1.12	.64
Control (not incubated)	1.90	1.00	1.75	1.00

TABLE IV. (cont'd.)

A second test identical with the preceding one except for a control follows:

Period of Contact (hours)	Staphylococcus B-1122 Susceptible Strain Phagocytic Index	Staphylococcus 1631 Resistant Strain Phagocytic Index
1/2 Staph. + W. B. C. + bacteriophage	2.37	1.16
1 " + " + "	3.10	1.60
2 " + " + "	2.70	1.33
4 " + " + "	4.21	1.32
No control		

EXPERIMENT V.

EXPERIMENT V.

Object:

Since results in vitro can never be taken to indicate what results will be in vivo, experiments similar to those above were performed on living rabbits.

Procedure:

To this end rabbits of approximately the same weight were selected and given an injection of a suspension of either the bacteriophage susceptible cocci or the resistant strain of cocci of the same race immediately followed by an injection of bacteriophage. The amount of bacteriophage and bacterial suspension injected was determined on the basis of 1 cc. of the material for each kilogram body weight. Control rabbits received no bacteriophage. Administration of these materials given by way of the marginal ear vein. At stated intervals bleedings were made from the marginal ear vein collecting several drops of blood in either 1% sodium citrate or saline solution. Leucocytes were obtained as described on page nine, smears prepared and stained and the phagocytic index determined.

In part A rabbit number 298 weighed 1690 grams, was brown in color

and received 1.7 cc. of the susceptible strain of cocci. This rabbit served as the control in the first test. Rabbit number K 285 weighed 1525 grams, was black in color and received 1.5 cc. each of the suspension of susceptible cocci and of the bacteriophage.

In part B, rabbit number K 300 served as the control; weighed 1760 grams, was brown in color and received 1.7 cc. of the susceptible cocci and 1.7 cc. sterile broth. Rabbit number K 295 weighed 1485 grams, was black in color and received 1.5 cc. each of bacteriophage and the bacterial suspension (susceptible cocci).

Parts A and B are identical except for the intervals of bleeding.

In part C, the resistant strains of cocci were used to determine the effect of bacteriophage on their phagocytosis in vivo. Otherwise, this test was identical with the other parts of the experiment. Rabbit number K 294 served as control, receiving no bacteriophage, weighed 2065 grams, was black in color and received 2 cc. of the suspension of resistant cocci. Rabbit number 299 weighed 1775 grams, was brown in color and received 1.8 cc. each of the bacterial suspension and of the bacteriophage. All rabbits used in this experiment died within twenty-four hours to one week.

The results of this experiment follow:

TABLE V.

A.

Time of test after injections (hours)	Rabbit # 298 Received staphylococcus No bacteriophage	Rabbit # 285 Received staphylococcus Received * bacteriophage
	Phagocytic Index	Phagocytic Index
1	1.67	2.50
3	2.38	2.59
6	1.18	1.88
18	2.81	2.41
24	2.17	2.67

TABLE V. (cont'd.)

B.

Time of test after injection	Rabbit # K 300 Received staphylococcus No bacteriophage	Rabbit # K 295 Received staphylococcus Received " bacteriophage
	Phagocytic Index	Phagocytic Index
10 minutes	.43	.96
20 "	.82	.84
45 "	1.00	1.09
1 hour	1.05	1.05
2 hours	.55	1.16
3 "	.96	.75
18 "	1.26	2.25
24 "	1.00	1.15

Another, the third experiment was designed to show the effect in vivo of bacteriophage on the phagocytosis of resistant organisms.

C.

Time of test after injection (hours)	Rabbit # 294 Received resistant staphylococcus 1631. No bacteriophage.	Rabbit # 299 Received resistant staphylococcus 1631 Received bacteriophage
	Phagocytic Index	Phagocytic Index
1 hour	.55	.30
3 hours	.72	.99
6 "	.76	2.22
18 "	3.30	.46
24 "	Died	.30

Discussion

As pointed out in the beginning of this paper, much of the work is in the nature of an attempted confirmation of previous experiments with an extension of the work along the lines suggested by the omissions of previous investigators. Thus, for example, the obtaining of indices as high as forty by d'Herelle through a direct incubation of bacteriophage and bacteria in the presence of leucocytes has never obtained confirmation, although there have been numerous reports of high indices following a preliminary action of bacteriophage upon the bacterium. This obviously

is a very significant difference. The ultimate use of this apparent stimulation of phagocytosis in clinical medicine is, however, not dependent upon the mechanics of the reaction and while the observation that a preliminary contact of the bacteriophage and the bacterium is essential to an increased index may not limit the therapeutic properties, it certainly has its effect upon procedure. d'Herelle made use of only one organism and one bacteriophage race. It is obviously impossible to generalize on the bases of such an experiment, hence, there is a distinct obligation on the part of anyone attempting to repeat d'Herelle's experiment to employ, if possible, a diversity of bacteriophage races and susceptible organisms. This is exactly what was attempted in Experiment I, although the addition of two different bacteriophage races comes far short of the ideal. The results are very far from confirming d'Herelle's observations, although it is true that in the presence of undiluted bacteriophage in the case of B. typhosus the maximum opsonic index was obtained.

The results of the staphylococcus experiment, on the other hand, are absolutely negative and it will be found that in general throughout these experiments that staphylococcus bacteriophage gave similar results. This is particularly significant in view of the fact that the staphylococcus bacteriophage used was the polyvirulent strain, originally isolated by Gratia, which is in general use throughout the world wherever therapeutic work is carried on. This particular bacteriophage has repeatedly been demonstrated to be entirely different from any other well studied lytic principle. In connection with the work presented here in the presence

of purified bacteriophage, one very significant difference in this particular respect was very definitely determined. While the other known bacteriophages are negatively charged and collect at the anode in cataphoresis, the staphylococcus bacteriophage, on the other hand, was found to collect at the cathode, thus indicating a positive charge. The significance of this result is likely to become far reaching. It may be considered as one of the most important contributions of the entire study. It should be pointed out that the staphylococcus bacteriophage used by Smith and with which he obtained his high indices was not the polyvirulent race used in these experiments.

The effects of preliminary incubation of bacteriophage and bacteria are revealed in all of the experiments with the exception of the first and the last, but are best demonstrated in Table III, although even here no very striking increases are obtained. Two conclusions might be drawn from these results, either that the technique was inadequate or that the indices previously reported are peculiar only to certain organisms and certain bacteriophage races. Certainly, until further study of this problem is available, it cannot be stated that increased opsonic indices as a result of preliminary incubation of bacteriophage and bacterium are to be generally expected.

In view of these results, it is rather difficult to say very much about the effects of purified bacteriophage. Such increases as could be observed were in general equally good with the purified material and it would further appear, although on very weak evidence, that the Seitz filter is to be selected rather than the Mandler in preparing material

for this purpose.

That there is a difference in the ability of leucocytes to ingest different organisms has long been recognized and that this difference may in part be explained by the susceptibility or resistance to bacteriophage of the bacterium selected has been indicated by Gohs and Jacobson, by Smith and by Nelson. It is again shown in Experiment IV where a susceptible and a resistant strain of staphylococcus were employed. In the final analysis, the results of any observation of opsonic indices upon which application to therapeutic work is based should depend upon the ability to reproduce this phenomenon in vivo. The observations of the behavior of leucocytes long after they are removed from the body and after various manipulations is scarcely an indication of the true state of affairs within the body. Efforts to study the ability of circulating phagocytic cells to ingest bacteria have been largely confined to tissue cultures. There is no obvious reason why a living animal cound not serve as a test tube for the study of the ability of such cells to ingest bacteria. But it would appear that the experiments reported under V are the first attempts at a study of this nature. That the results are somewhat disappointing from the standpoint of indicating any effect of bacteriophage does not detract from the observation that such a method although entailing many difficulties may be practically applied.

A closer analysis of the results reported in the various experiments is possible, but in view of the fact of the very slight differences to be found in most instances, it is felt that such analysis would lead to entirely erroneous conclusions. It is realized that much further work along

this line is required. The results so far obtained indicate the direction in which subsequent investigations might be directed. Opsonic work is both tedious and time consuming and it is felt that the work presented represents about all that could be accomplished in the time available and while there may be some disappointment at the inability to report definite conclusions on some of the phases of the problem, it is felt that enough has been done to justify one or two statements which should have their effect on the development of this entire subject.

Conclusion.

1. The incubation of bacteriophage with susceptible bacteria and leucocytes leads to a small but definite increase in the opsonic indices, an increase measured in part by the character of the organisms used and by the selection of the race of the bacteriophage.

2. Such increases as are obtained are apparently materially affected by the preliminary incubation of bacteriophage and bacteria.

3. Purified bacteriophage is apparently equal to the unaltered material in its ability to stimulate phagocytosis.

4. The use of living animals for the determination of phagocytic activity of living cells appears feasable, but there is no indication that the presence of bacteriophage in such animals affects the degree of phagocytic activity.

5. Staphylococcus bacteriophage as opposed to all other known bacteriophage races carries a positive electrical charge.

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