THE SOURCE OF PHOSPHORUS IN BACTERIOPHAGE*

ROBERT A. GOLDWASSER

Until recent years, very little was known concerning virus metabolism or the source of materials used by viruses for metabolic purposes, and physical and chemical methods were used mainly for the characterization of virus particles. These methods have contributed a great deal to our knowledge of viruses. However, some later investigations on influenza virus, mouse pneumonitis virus, and Rous sarcoma virus have shown that purified preparations of these agents may contain antigens and chemical substances of the normal host tissue, bound, perhaps chemically, to other substances characteristic of the virus alone.[2, 9, 14, 19, 20] These results suggest a need for re-interpretation of earlier chemical analyses of the various viruses.

Recently, certain investigators have turned their attention to the study of the metabolism of viruses. Thus, for example, studies of the enzyme content of various virus preparations have shown that the viruses themselves probably are incapable of carrying out the necessary metabolic reactions for production and utilization of energy. This leads to the conclusion that the host cells must furnish such enzymes.[28]

Price,[21, 22, 24] working with *Staphylococcus muscae* and a bacteriophage to which this strain was susceptible, has attempted to demonstrate growth requirements of bacteriophage through additions of specific medium components to penicillin-inhibited bacteria. Specific enzyme poisons have also been used by some investigators, such as Price,[23] Fitzgerald and Lee,[12] and Fitzgerald and Babbit,[11] in attempts to clarify problems of bacteriophage metabolism. Cohen and Fowler[7] have concluded as a result of their investigations that the synthesis of tryptophane is necessary for the growth of T_2 bacteriophage. They further concluded that tryptophane need not be present as a component of the host's external environment but must be present in the environment to which the virus is exposed. These investigators have

* From the Department of Bacteriology, Yale University. The material here presented constitutes a portion of a dissertation submitted in 1948 for the degree of Doctor of Philosophy. This work has been aided by a grant from the James Hudson Brown Memorial Fund of the Yale University School of Medicine.

Received for publication May 13, 1949.

also shown[8, 13] that addition of other amino acids as well as purines and pyrimidines to a minimal medium stimulates the production of virus and that the rate of synthesis of these compounds from simpler ones in the minimal medium determines the rate and extent of growth of the virus.

In investigations on the phosphorus metabolism of bacterial viruses, Cohen, [3, 4, 5] using P^{32} as a tracer element, found that the main source of phosphorus used in the formation of T_2r^+ and T_4r^+ bacteriophage was to be found in components of the medium in which the bacteria and viruses were suspended rather than in compounds synthesized by the bacteria prior to their infection with virus. However, a certain percentage of the phosphorus found in the bacteriophage seems to have been derived from the latter source. These results were confirmed by Putnam and Kozloff,[25] who found that approximately 23% of the phosphorus of virus T_6r^+ was derived from bacterial phosphorus, and the rest was derived from the medium.

Cohen,[3, 4, 5] as well as Putnam and Kozloff,[25] purified their virus preparations mainly by means of differential centrifugation followed by dialysis. The specific activity of the total virus phosphorus as well as that of the desoxyribonucleic acid phosphorus was then determined and compared with specific activities of the host cells and of the media.

In view of the fact that only lysis inhibiting strains of bacterial virus were used by the abovementioned investigators, it was felt that similar investigations on a non-lysis inhibiting strain of bacteriophage might furnish valuable additional information as to some of the host-parasite metabolic relationships. It was with this purpose in mind that the present investigation was undertaken.

It was also felt that differential centrifugation and dialysis alone might not be sufficient to eliminate contaminating phosphorus-containing compounds from bacteriophage preparations and that these might introduce a considerable source of error into the determination of the phosphorus content of the bacteriophage particles themselves. Therefore, a biological method was used, in combination with the usual physical methods of detection of radioactive tracer elements, which eliminated, to a considerable degree, this source of error. The biological method involved the adsorption of bacteriophage to phage-sensitive cells. These cells were subsequently washed, and the radioactivity of the adsorbed material was measured with a Geiger-Mueller counting apparatus.

These experiments are presented in two sections. In Section I are control experiments, the main purpose of which was to show that the presence of

bacteriophage does not influence bacterial adsorption of phosphorus compounds which are not associated with the bacteriophage particles themselves. In Section II are presented the experiments dealing with the sources from which bacteriophage obtains phosphorus.

Materials and methods

Radioactive phosphorus in the form of KH_2PO_4 or H_3PO_4 was used in the experiments involving adsorption of inorganic phosphates. In other experiments, the phosphorus compounds were derived from bacteria lysed by bacteriophage or by mechanical means.

The medium used for the growth of bacteria and bacteriophage was beef heart infusion broth.

The bacteria used in the adsorption tests were: (1) a strain of *Shigella paradysenteriae* (Flexner 2), henceforth denoted as D43; (2) a variant of D43, denoted as D/Pα, which was resistant to the strain of bacteriophage used in this work; and (3) a strain of *Bacillus mesentericus.*

The bacteriophage, active against D43, was isolated from raw sewage obtained from a sewage disposal plant. This phage will henceforth be denoted as Pα.

The bacteria used in the adsorption tests were grown in bottles, each containing 50 ml. of infusion broth. The cultures were allowed to grow for twenty-four hours at 37° C. and were then sedimented by centrifugation. The packed cells were taken up in physiological saline in such volumes that the number of bacteria per ml. of the different strains of bacteria to be used in any given test was approximately equal, as measured by the turbidity of the suspensions. The turbidities were measured in a Klett-Summerson photoelectric colorimeter.

In some experiments where suspensions of living bacteria were used in the adsorption tests, these suspensions were kept cold (in ice water) in order to prevent growth of either bacteria or bacteriophage. In other experiments the bacterial suspensions were heated prior to their use in adsorption tests in order to attain the same result. Each method gave essentially the same results in subsequent adsorption tests, both as to adsorption of phosphates and of bacteriophage.

For the assay of radioactivity, a Geiger-Mueller counting tube and scaling circuit were used, the tubes being of the bell jar or the cylinder type.* Discs of filter paper, slightly smaller in diameter than the windows of the Geiger-Mueller tube, were placed on glass slides. One-tenth ml. of the material to be assayed, generally of a fluid nature, was spread as uniformly as possible on these discs, which were then allowed to dry. The filter paper discs were finally covered with scotch tape and the slides assayed. Each slide was assayed for a period of at least three minutes on a convenient scale. When the recorder indicated counts of less than double the background, assays were carried out for at least six minutes. For convenience in the comparison of results, all counts were corrected to the scale of 2 by multiplying by the proper factor.

---

* This apparatus was constructed at the Department of Physics of Yale University and made available through the courtesy of Dr. Ernest C. Pollard.

Previous to, as well as following, the assay of a sample, the background was determined, and a radioactive uranium glass standard was assayed. Since counts of the uranium glass standard were found to vary considerably from time to time, corrections were made for an arbitrary count of the standard of 360 cts./min. Corrections were also made for radioactive decay whenever experiments using the same source of radioactive phosphorus were performed but at various intervals of time. In addition, all counts as given in the tables were corrected for the background.

Assays were performed on all solutions containing radioactive phosphorus at the beginning of the experiments and at all intermediate steps.

Bacteriophage titrations were performed in the usual manner on solid media, and titers are expressed as the number of plaques times the dilution of the bacteriophage suspension, of which one ml. gave the corresponding number of plaques.

SECTION I

STUDIES ON THE ADSORPTION BY BACTERIA OF PHOSPHORUS COMPOUNDS OF VARIOUS TYPES IN THE PRESENCE AND ABSENCE OF BACTERIOPHAGE

Procedures

Experiment 1

To each of four tubes were added 0.5 ml. of radioactive KH_2PO_4 solution. To two of the tubes were added 4.5 ml. of infusion broth, and to the remaining two tubes were added 4.5 ml. of stock Pα grown in infusion broth. To one tube containing phage, and to one tube containing broth but no phage, were added 2.5 ml. of D/Pα suspension containing about 5×10^{10} cells/ml.,

TABLE 1

Material assayed	*Ct./min.*
Medium	18,100
Adsorbing suspension:	
D43	146 ± 7.0
D43 + phage	138 ± 6.8
D/Pα	167 ± 7.5
P/Pα + phage	146 ± 7.0
Phage titer before adsorptions	7.0×10^9
Phage titer after adsorption with D43	6.8×10^8
Phage titer after adsorption with D/Pα	6.8×10^9

± values refer to standard deviation.

prepared as described above. To the other two tubes were added like amounts of D43 suspension. After allowing one-half hour for adsorption of phage and phosphates, the cells were thrown down by centrifugation and the sedimented cells washed six times with saline. Following the last washing, the packed cells were transferred to slides for the assay of radioactivity.

The results of this experiment are summarized in Table 1. The presence of bacteriophage did not influence the adsorption of phospate, and the two strains of bacteria did not differ in ability to adsorb the inorganic phosphates.

Experiment 2

To 15 ml. of infusion broth was added 1 ml. of radioactive KH_2PO_4 solution. The resulting mixture was then serially diluted 1/20, 1/80, 1/320, 1/1280, and 1/2560, each dilution being prepared in 10 ml. of broth. Each of the dilutions was divided into two equal parts of 5 ml. each. One portion of each dilution was added to a tube containing packed D43 cells which were free of phage (approximately 1×10^{11} cells per tube), and the other portion was added to a tube containing a like amount of packed D43 cells which had previously been exposed to Pα phage (approximately one particle of bacteriophage per cell). The cells in the tubes were suspended in the radioactive media by vigorous shaking and then allowed to stand one-half hour to allow adsorption of radioactivity. The suspensions were then centrifuged and the bacteria washed six times in saline. Following the last washing, the packed cells were transferred to slides for assay of radioactivity.

TABLE 2

Dilutions of radioactive medium	*Ct./min.*	*Packed cells Ct./min.*	*Packed cells + phage Ct./min.*
Undiluted	268,160	1,400	1,432
1/20	13,408	82	68
1/80	3,352	14	16
1/320	584	4	4
1/640	300	1	2
1/1280	154	2	2
1/2560	72	1	3
1/5120	31	3	1

Phage titer before adsorptions: 8×10^9
Phage titer after adsorptions: 7×10^8*

* Average values of all the tubes. Variation of titers was within the range of experimental error.

The results of this experiment are summarized in Table 2. As in Experiment 1, the presence of bacteriophage did not influence the adsorption of phosphate, nor did the two strains of bacteria differ in their ability to adsorb the inorganic phosphates.

Experiment 3

One-tenth ml. of a solution of radioactive KH_2PO_4 was added to 10 ml. of infusion broth. The medium was then inoculated with D43 and incubated at 37° C. for eighteen hours. Following incubation, the culture was centrifuged at 3000 r.p.m. for ten minutes, the supernatant decanted, and the sedimented cells washed six times with saline. The final sediment was suspended in 20 ml. of infusion broth with the addition of 0.1 ml. of stock P*a* phage, and the suspension was placed in the 37° incubator. After one and one-half hours of incubation, lysis was complete. The lysate was clarified by centrifugation and used in adsorption tests.

Dilutions of the solution of radioactive KH_2PO_4 were prepared in infusion broth. These dilutions were 1/10, 1/100, 1/200, 1/400, and 1/800, each in a volume of 20 ml. These solutions were used in the adsorption tests run in parallel with the test using the radioactive lysate of D43 cells prepared through the action of bacteriophage. The amount of cells used in each tube was one-sixth that used in the foregoing experiment, but otherwise the procedures of the experiments were identical.

The results of this experiment are summarized in Table 3 and will be discussed in later pages.

TABLE 3

Dilutions of P^{32} solution	*Ct./min.*	*Cells from corresponding solution after washing Ct./min.*
1/10	120,800	161
1/100	12,080	100
1/200	6,026	57
1/400	3,064	27
1/800	1,570	7
Lysate	77	91

Experiment 4

A radioactive lysate was prepared in a manner identical with that in Experiment 3. Three strains of bacteria were used in this experiment for

the adsorption tests: D43, D/Pα, and a strain of *Bacillus mesentericus* obtained from the stock culture of the Department of Bacteriology of the Yale University School of Medicine. These bacteria were grown in infusion broth and prepared for the adsorption tests as described under Materials and Methods. The number of cells of each strain used in each tube was approximately 1×10^{11}. The adsorption procedures used were as described in earlier experiments.

The results of this test are summarized in Table 4. This table shows that not all the phosphates released from bacterial cells on lysis by bacteriophage are associated with the latter, since cells which did not adsorb the virus took up as much radioactivity as did bacteria which adsorbed 90% of the bacteriophage.

TABLE 4

	Ct./min.		
Lysate	91	Titer of bacteriophage in lysate, before adsorption	1.1×10^{10}
Cells which had been suspended in lysate and then washed:		Titer of lysate after adsorptions with D43	9.8×10^{8}
D43	74 ± 5.4		
D/Pα	67 ± 5.2	Titer of lysate after adsorption with D/Pα	9.9×10^{9}
B. mesentericus	76 ± 5.5		
		Titer of lysate after adsorption with *B. mesentericus*	1.0×10^{10}

± values refer to standard deviation.

Experiment 5

Two radioactive bacterial lysates were used in this experiment. One was prepared in a manner identical with that used in Experiments 3 and 4. The other was prepared by sonic disintegration of the cells, as follows: One ml. of a sterile solution of radioactive KH_2PO_4 was pipetted into 20 ml. of infusion broth. This medium was inoculated with D43 and incubated at 37° C. for twenty-four hours. At the end of this time, the cells were thrown down by centrifugation and the sedimented cells washed six times in saline. After the last washing, the cells were re-suspended in 10 ml. of infusion broth and transferred to a lusteroid cup cut down to fit the cup of a Raytheon Magnetostrictor. This apparatus was that described by Shropshire.[27] The lusteroid cup was placed in the Magnetostrictor, surrounded by water, and the current was turned on. The apparatus was run

at its maximum frequency for a period of twenty minutes, after which the contents of the lusteroid cup were transferred to a high speed centrifuge and spun at 15,000 r.p.m. for one-half hour. The supernatant, which was slightly opalescent, was diluted with an additional 10 ml. of infusion broth and then used in the adsorption tests.

The bacterial cells used in the tests were prepared as described in Materials and Methods. Two strains were used, D43 and D/Pα. Three tubes containing packed D43 cells and three containing packed D/Pα cells where placed in a rack. To one tube of each set were added 10 ml. of Pα bacteriophage titering 9×10^9 phage particles per ml. After allowing one-half hour for adsorption of the virus, the suspensions were centrifuged and the supernatant decanted.

The radioactive medium prepared by disintegrating bacteria in the Magnetostrictor was divided into four equal portions of 5 ml. each. The contents of one tube of D43 treated with bacteriophage and the contents of one tube of D/Pα treated with bacteriophage were each suspended in one of the 5 ml. portions of this radioactive medium. The contents of one tube of untreated cells of each strain were likewise mixed with 5 ml. portions of the same medium. Each of the remaining tubes of D43 cells and D/Pα cells was mixed with 5 ml. of lysate prepared by the use of bacteriophage. All the mixtures were allowed to stand one-half hour and then centrifuged at 3,000 r.p.m. for ten minutes. The supernatants were decanted and the sedimented cells washed six times with saline. The final sediments were transferred to slides for assay of radioactivity.

The results of this experiment are presented in Table 5 and are discussed below.

TABLE 5

CT./MIN.
MAGNETOSTRICTOR LYSATE (M. L.) 105
PHAGE LYSATE (P. L.) 92

Preliminary treatment of cells	*Cells suspended in M.L. and then washed*	*Ct./min.*	*Cells suspended in P.L. and then washed*	*Ct./min.*
Treated with phage	D43	10 ± 2.8		
None	D43	7 ± 2.6	D43	76 ± 5.5
Treated with phage	D/Pα	10 ± 2.8		
None	D/Pα	13 ± 2.9	D/Pα	73 ± 5.3

± values refer to standard deviation.

Summary and discussion of experiments of Section I

Examination of Tables 1, 2, and 3 shows that when given amounts of non-metabolizing bacteria were suspended in radioactive, inorganic-phosphate solution assaying from 6,000 to 18,000 ct./min. per 0.1 ml., phosphates were adsorbed by the bacteria to give counts ranging from 82 to 176 ct./min. However, according to the results shown in Table 5, when the suspending medium contained radioactive phosphates assaying at 105 ct./min. per 0.1 ml. resulting from the mechanical disruption of radioactive bacteria, phosphates were adsorbed to give counts ranging from 7 to 13 ct./min. Comparable counts were adsorbed from solutions of inorganic phosphates assaying at 317 to 1,500 ct./min. On the other hand, lysates prepared through the action of bacteriophage on bacteria which had previously been allowed to grow and metabolize in the presence of radioactive phosphates seem to have contained relatively much larger amounts of adsorbable radiophosphorus compounds (Tables 3, 4, and 5). These lysates assayed at 77 to 92 ct./min. per 0.1 ml., and the bacteria allowed to adsorb phosphates from this media assayed at 67 to 91 ct./min. This amount adsorbed corresponds to the amounts adsorbed by similar quantities of bacteria from inorganic solutions of radioactive phosphates assaying at approximately 6,000 to 13,000 ct./min. per 0.1 ml. That these differences were not due to the influence of bacteriophage on the ability of non-metabolizing bacteria to adsorb radiophosphorus compounds was indicated by the experiments whose results are presented in Tables 1, 2, and 5. In these experiments, the presence of bacteriophage did not influence the amount of radiophosphorus taken up by bacteria, either of a phage-sensitive strain or of a phage-resistant strain, whether these bacteria were heat-killed or merely prevented from growing by being kept cold.

The data presented in Tables 4 and 5 further indicate that the radiophosphorus compounds present in lysates of radioactive bacteria prepared through the action of bacteriophage are not all associated with the latter, since bacteria of strains which did not adsorb phage acquired just as much radioactivity as did bacteria which adsorbed 90% of the phage present in suspension. Furthermore, most of the radiophosphorus compounds present in the lysates in these experiments were not adsorbed, either by bacteria which did or by those which did not adsorb bacteriophage.

These experiments indicate, then, that in suspensions of bacteria lysed through the action of bacteriophage there are present phosphorus-containing compounds other than those directly associated with the bacteriophage, which are not present in the inorganic phosphate solutions used in the

experiments and which are not present in normal living bacteria, or at least are not liberated from them by mechanised disruption. No attempt was made to elucidate the nature of such compounds. It was clear, therefore, that any radioactivity acquired by bacteriophage in such systems would be measured together with that of components foreign to the phage, whose radioactivity would then in effect be masked.

In subsequent experiments, differential centrifugation as well as dialysis were used to eliminate, in so far as possible, contaminating radioactive components, in order to allow the activity of the radiophosphorus acquired by bacteriophage to be determined.

Section II

Studies on the Source of Phosphorus in Bacteriophage

In this group of experiments an attempt was first made to determine whether compounds of phosphorus, existing in the bacterial cells prior to infection with bacteriophage, could serve as a source of phosphorus for bacteriophage. Further experiments were then carried out to compare the relative amount of phosphorus in bacteriophage derived from the forementioned source with the amount derived from the medium in which the bacteria and the bacteriophage were suspended.

Experiments 1, 2, and 3

Nine ml. of sterile infusion broth were pipetted into a sterile tube, and one ml. of radioactive Na_2HPO_4 solution was added. The resulting medium was inoculated with D43 and incubated at 37° C. After eighteen hours the cells of the resulting culture were sedimented by centrifuging and washed six times in infusion broth. After the final washing, the cells were resuspended in 20 ml. of infusion broth, and 0.1 ml. of stock Pα was added. The resulting mixture was incubated at 37° C. for one and one-half hours, when lysis was complete. Bacterial debris was removed by centrifugation, and the clarified lysate was transferred to a high speed centrifuge. This centrifuge was an International Centrifuge, No. 1, type SB, with a multispeed attachment and a built-in refrigerating unit, and was operated at 16,000 r.p.m. It was found that 95 to 99% of the virus could be sedimented at this speed and recovered. The supernatant fluids were decanted and the remaining pellets taken up in sterile 0.85% saline solution. The resulting suspension was again centrifuged at 3,000 r.p.m. in an angle head centrifuge and the supernatant again transferred to the high speed centrifuge. This process was repeated through four cycles of differential centrifugation. The final suspension, which was colorless and only slightly opalescent, was transferred to a sac made of dialyzing cellophane and dialyzed against running water at 4° C. for seventy-two hours. By the end of this time, a light precipitate appeared,

which was removed by centrifuging at 3,000 r.p.m. The sediment was tested for its phage content, which was found to be less than 1% of the phage present in the supernatant and was therefore discarded.

The supernatant, the volume of which was 20 ml., was divided into two equal portions of 10 ml. each. To one portion were added packed D43 cells, approximately 1×10^{11} in number, which had been washed in physiological saline. To the other portion were added a like number of packed, washed D/Pα cells. Adsorption was allowed to proceed for approximately thirty minutes, after which the cells were sedimented by centrifuging. The sediment was washed six times in saline and finally spread on slides for assay of radioactivity. On the following day, when the results of the phage titrations became known, it was noted that no phage had been removed from the suspensions either by D43 or by D/Pα cells. The above adsorption procedure was repeated, with the same results.

To each of the supernatants resulting from the second adsorption was added 1.0 ml. of infusion broth, and adsorption was allowed to take place for a third time. The supernatant resulting from the sedimenting of the third mixture of purified lysate and D/Pα cells was mixed with D43 cells and allowed to stand for thirty minutes. All mixtures were tested as above, that is, the suspensions were centrifuged and the sedimented cells washed six times and then spread on a slide for assay of radioactivity. The supernatants from all adsorption mixtures were tested for phage content and radioactivity.

The results of this experiment are summarized in Table 6. It is of interest to note that some adsorption factor seems to be necessary in order that phage Pα be adsorbed by bacteria of a sensitive strain. Previous to this work, only certain members of the "T" phages have been reported to require an adsorption factor, which was found by T. F. Anderson to be tryptophane.[1] No attempt was made in the present study to determine the nature of the adsorption factor described above.

Four cycles of differential centrifugation followed by dialysis removed approximately 97% of the radioactivity of the lysate prepared through phage action on radioactive bacteria.

When radioactive lysates, purified as described above, were mixed with washed bacteria, no adsorption of bacteriophage took place, whether the bacteria used were of phage-sensitive or of phage-resistant strains. At the same time, very little radioactivity was taken up by these bacteria. When a small amount of infusion broth was mixed with the purified lysate, the phage-sensitive bacteria adsorbed approximately 90% of the bacteriophage as well as some radioactivity, whereas the phage-resistant strain did not adsorb a significant amount of bacteriophage and acquired much less radioactivity.

TABLE 6

Assays of radioactivity		*Phage titers*	
	Ct./min.	Original lycate	9.0×10^9
Medium	157,000	After 4th centrifuging	8.8×10^9
Original lysate	2,010	After dialysis	8.5×10^9

Lysate purified by centrifugation

Times centrifuged	*One*		*Two*		*Three*		*Four*	
Portion of centrifuged lysate assayed	*Supernatant*	*Pellet suspension*	*Supernatant*	*Pellet suspension*	*Supernatant*	*Pellet suspension*	*Supernatant*	*Pellet suspension*
Counts/minute .	1820	125	35	100	19	83	4	82

Purified lysate mixed with cells

Times mixed	*One*		*Two*		*Three*		*Four*
Types of cells used in mixture	D43	D/Pα	D43	D/Pα	D43	D/Pα	D43
Counts/minute	10	11	12	8	153	14	169
Phage titer of supernatant	7.1×10^9	7.5×10^9	8.1×10^9	7.6×10^9	7.8×10^8	6.9×10^9	6×10^8

The above experiment was repeated twice (Experiments 2 and 3) under identical conditions, using the same solution of radioactive phosphate, and the results were almost identical in each case after correction was made for radioactive decay and for a uranium glass standard count of 360 ct./min. Since both phage-sensitive and phage-resistant strains of bacteria took up little radioactivity under conditions in which neither strain adsorbed bacteriophage, whereas phage-sensitive bacteria took up much more radioactivity under conditions in which bacteriophage was adsorbed, it may be concluded that the radioactivity adsorbed under the latter conditions was directly associated with the bacteriophage particles. This indicates that compounds of phosphorus, present in bacteria and existing prior to infection with bacteriophage, can serve as a source of phosphorus for the latter. However, these experiments do not show whether bacteriophage can also utilize phosphorus compounds synthesized by the bacteria after infection or whether bacteriophage in the presence of sensitive bacteria can utilize phosphates existing in the medium in which the bacteria and the virus are suspended. The following group of experiments was designed to test the above possibilities.

Experiments 4, 5, and 6

Except as otherwise stated, the procedures described below were followed in each of Experiments 4, 5, and 6.

To each of three tubes, labeled *1, 2,* and *3,* were added 9 ml. of sterile infusion broth. One ml. of radioactive H_3PO_4 solution was pipetted into tubes *1* and *3,* and 1 ml. of saline was pipetted into tube *2.* Each tube was inoculated with a drop of a twenty-four-hour infusion broth culture of D43 and placed in the 37° C. incubator. Eighteen hours later, the cells in tube *1* were sedimented by centrifuging and washed six times in sterile infusion broth. The washed cells were finally suspended in 19 ml. of infusion broth with the addition of 1 ml. of saline and 0.1 ml. of stock bacteriophage Pα. Into tube *2* were pipetted 9 ml. of infusion broth, 1 ml. of radioactive phosphate solution, and 0.1 ml. of stock bacteriophage Pα. Into tube *3* were pipetted 9 ml. of infusion broth, 1 ml. of saline, and 0.1 ml. of stock bacteriophage Pα. These mixtures were placed in the 37° C. incubator for one and one-half hours, at the end of which time lysis was complete. The lysates were clarified by centrifugation and the supernatants decanted. The bacteriophage present in the suspensions was then sedimented in the high speed centrifuge and the supernatants discarded. The pellets were suspended in phosphate buffer and again centrifuged at 3,000 r.p.m. This procedure of low speed centrifugation followed by high speed centrifugation was repeated twice more, the pellets being resuspended each time in fresh sterile phosphate buffer. The final pellet suspensions were removed to dialyzing cellophane sacs and dialyzed against running water at 4° C. for eighty-four hours. At the end of that time, the dialyzed lysates were centrifuged at 3,000 r.p.m. for fifteen minutes to remove precipitated material and the supernatants decanted. The volume of each lysate was made up to 50 ml. with sterile infusion broth.

Each of the purified Lysates *1, 2,* and *3* was divided into two equal portions of 25 ml. each. One portion of Lysate *1* was added to a tube containing packed D43 cells approximately 1×10^{11} in number, and the other portion was added to a tube containing a like amount of packed D/Pα cells. Lysates *2* and *3* were treated in an identical manner. The cells were suspended by thorough mixing with the aid of pipettes and the suspensions allowed to stand one-half hour. After this time the cells were sedimented by centrifuging and the supernatants decanted into tubes containing fresh-packed cells, but in reverse order, *i.e.,* the supernatant resulting from the centrifugation of a mixture of lysate and packed D43 cells was decanted into a tube containing packed D/Pα cells, and the supernatant resulting from the centrifugation of a mixture of lysate and packed D/Pα cells was decanted into a tube containing packed D43 cells. These mixtures were treated in the same manner as the former ones. The cells which were thrown down by centrifugation of the mixtures were washed six times in saline and finally transferred to slides for assay of radioactivity.

In Experiment 6, reversal of the order of adsorption was carried out a third time. That is, lysates which had been exposed to D43 and D/Pα cells in that order were mixed again with D43 cells, and lysates which had been exposed to D/Pα and D43 cells in that order were mixed again with D/Pα cells.

The results of Experiments 4, 5, and 6 are summarized in Table 7.

TABLE 7

Materials assayed	*Count/Minute* a	b	c
Medium	275,000	285,000	284,600
Crude lysate 1	5,225 ± 41.7	5,560 ± 43.0	4,760 ± 39.9
Purified lysate 1	58 ± 4.4	135 ± 6.7	46 ± 3.4
Purified lysate 2	36 ± 3.5	54 ± 4.2	35 ± 3.4
Purified lysate 3	46 ± 3.9	171 ± 7.6	51 ± 4.1

	Bacteriophage Titers x 10^8					
	Crude Lysates			*Purified Lysates*		
	a	b	c	a	b	c
Lysate 1	40	90	150	41	100	200
Lysate 2	45	96	170	44	89	240
Lysate 3	34	110	140	40	90	200

Strain of Cells	*Lysate No.*	*First Adsorption* *Washed Cells Ct./Min.* a	b	c	*Supernatant Ct./Min.* a	b	c	*Bacteriophage Titer after Adsorption* x 10^8 a	b	c
D43	1	1790	2285	1530	50	132	41	3.5	19.0	19.0
D/Pa	1	210	580	208	47	151	38	40.0	72.0	200.0
D43	2	495	430	278	33	50	30	3.4	18.0	21.0
D/Pa	2	147	200	246	37	50	35	43.0	65.0	220.0
D43	3	1810	2362	1670	38	172	47	3.6	9.3	18.0
D/Pa	3	114	520	191	43	168	49	35.0	69.0	190.0
		Second Adsorption								
D43	1	3200	2010	1310	48	128	32	3.7	18.0	18.0
D/Pa	1	60	530	154	50	133	35	4.2	20.0	17.0
D43	2	589	412	266	31	52	36	3.0	18.0	16.0
D/Pa	2	48	236	222	32	47	32	3.3	15.0	19.0
D43	3	3090	2082	1340	39	164	39	3.1	20.0	16.0
D/Pa	3	54	510	154	40	168	46	3.5	13.0	18.0
		Third Adsorption								
D43	1			104						15.0
D/Pa	1			92						16.0
D43	2			81						
D/Pa	2			93						
D43	3			72						14.0
D/Pa	3			88						16.0

Counts given in this table are corrected for a uranium glass standard count of 360 ct./min. and for radioactive decay.

Bacteriophage titers given are corrected to correspond to the original volume of lysate.

a = results of Experiment 4; b = results of 5; c = results of 6.

The lysates used in these experiments were of three types: Lysates No. 1 were prepared through the action of bacteriophage on cells which had previously been grown in radioactive media; Lysates No. 2 were prepared through the action of bacteriophage on bacteria which were not radioactive but which were suspended in radioactive media at the time of infection; Lysates No. 3 were prepared from bacteria which were grown in radioactive media and remained suspended in those media at the time of infection with bacteriophage.

The lysates were purified by differential centrifugation and dialysis, after which they were mixed with both phage-sensitive and phage-resistant cells. The phage-sensitive bacteria removed 90% of the bacteriophage activity from these lysates and acquired three to ten times as much radioactivity as did the phage-resistant cells, which did not remove a significant amount of bacteriophage from suspension. Approximately four to six times as much radioactivity was taken up by phage-sensitive cells from the lysates prepared from radioactive bacteria as from the lysates prepared from non-radioactive bacteria suspended in radioactive media.

Following the above series of adsorptions, both series were carried out with the same samples of the lysates, in which the order of adsorption was reversed. Again the phage-sensitive cells removed 90% of the bacteriophage present in the lysates and at the same time adsorbed much more radioactivity than did the phage-resistant cells, which did not take a significant amount of phage out of suspension. Here, too, approximately four to six times as much radioactivity was taken up by phage-sensitive cells from lysates derived from radioactive cells as from lysates derived from non-radioactive cells suspended in radioactive media.

When the order of adsorption was reversed for a third time, *i.e.,* after 90% of the bacteriophage had been removed from all samples of the lysates, the amount of radioactivity taken up by the phage-sensitive bacteria was considerably reduced and now equalled the amount taken up by the phage-resistant cells. In other words, whenever conditions were such that no bacteriophage was adsorbed, a relatively small amount of radioactivity was acquired by the adsorbing cells; and when bacteriophage was adsorbed, a much larger amount of radioactivity was acquired. This seems to confirm earlier conclusions, namely, that the difference between the amount of radioactivity taken up by phage-sensitive cells and phage-resistant cells represents radioactive phosphorus directly associated with the bacteriophage in suspension. However, the major part of the radioactive phosphorus present

in the purified lysates was not removed when 90% of the bacteriophage was removed from suspension. This indicates that the majority of the radiophosphorus in such lysates was not associated directly with bacteriophage and that the methods used were not adequate to purify the bacteriophage completely. That considerable purification of the lysates took place through centrifugation and dialysis is shown by the fact that almost 99% of the radioactivity present in the crude lysates prepared from radioactive bacteria suspended in non-radioactive media is lost by these methods without appreciable loss of bacteriophage activity.

Experiment 7

In general, the procedures followed in this experiment were similar to those in the three preceding ones. However, prior to testing the radioactivity acquired by the bacteriophage by means of adsorption to phage-sensitive cells, a series of six preliminary adsorptions, using phage-resistant cells, was performed on each sample of the lysates. This was done in an attempt to remove the contaminating, non-specifically adsorbing radioactive components which were not eliminated by differential centrifugation and dialysis of the bacteriophage lysate. The results of this experiment are summarized in Table 8.

From the data presented in this table, it can be seen that there was considerable loss of bacteriophage during the purification procedures prior to the preliminary adsorptions with D/Pα cells. Preliminary adsorption by bacteria of strain D/Pα did not remove a significant amount of radioactivity or bacteriophage. Following these adsorptions, bacteria of strain D43 were mixed with the lysates and were found to remove 90% of the remaining bacteriophage and to acquire a significantly larger amount of radioactivity than did the cells of a phage-resistant strain. However, the major portion of radioactivity still remained in the purified lysates after all the adsorption procedures.

More radioactivity was taken up by D43 bacteria from lysates derived from radioactive bacteria than from a lysate derived from non-radioactive bacteria suspended in radioactive medium. Furthermore, the amount of radioactivity acquired by D43 from lysates derived from radioactive bacteria suspended in a non-radioactive medium approximately equalled the amount taken up from the lysates derived from radioactive bacteria suspended in a radioactive medium.

TABLE 8

Assay of radioactivity		*Phage titers*		
Material assayed	*Ct./min.*		*Before purification*	*After purification*
Medium	275,000			
Purified lysate 1	65 ± 4.7	Lysate 1	5.8×10^{9}	1.7×10^{9}
Purified lysate 2	25 ± 2.9	Lysate 2	7.2×10^{9}	1.8×10^{9}
Purified lysate 3	81 ± 5.2	Lysate 3	6.0×10^{9}	1.5×10^{9}

Adsorption No.	*Strain of cells*	*Lysate No.*	*Radioactivity of washed cells ct./min.*	*Radioactivity of supernatant ct./min.*	*Phage titer after adsorption*
	D/Pa	1	61		
1	D/Pa	2	59		
	D/Pa	3	54		
	D/Pa	1	53		
2	D/Pa	2	55		
	D/Pa	3	61		
	D/Pa	1	38		
5	D/Pa	2	33		
	D/Pa	3	43		
	D/Pa	1	29	67	1.0×10^{9}
6	D/Pa	2	23	22	1.1×10^{9}
	D/Pa	3	22	72	8.0×10^{8}
	D43	1	201	63	1.1×10^{8}
7	D43	2	34	23	1.0×10^{8}
	D43	3	204	65	1.2×10^{8}

Counts given were corrected for a U. G. count of 360 and for radioactive decay of from 0.85 mc/ml. stock solution to 0.25 mc/ml.

Discussion

Various investigators have, in the past, used differential centrifugation and dialysis to obtain purified bacteriophage[6, 18, 26] and have then performed chemical analyses on the material so obtained. Although chemical and physical determinations of homogeneity were carried out and the materials obtained were shown to be more or less pure according to these standards, it was felt that substances other than bacteriophage may have been present which were indistinguishable from the latter through ordinary chemical and physical tests. Therefore, biological methods in combination with the usual physical methods for detection of radioactive tracer elements were used in this work rather than the less sensitive chemical methods. The results of

such experiments confirmed the suspicion that differential centrifugation and dialysis did not eliminate all contaminating substances. These procedures were found to eliminate approximately 95% of the phosphorus compounds present in the crude lysate, but, when 90% of the bacteriophage was removed from the resulting "purified" bacteriophage suspension by means of specific adsorption with a sensitive strain of cells, it was found that most of the phosphorus compounds still present after centrifugation and dialysis still remained behind. This seems to indicate that most of the material remaining in suspension in the lysates purified by the above methods was not bacteriophage.

A certain degree of error was introduced by the fact that not all of the P^{32} taken up by cells suspended in radioactive lysates was part of the bacteriophage adsorbed by the cells. This was shown by the fact that cells which were not capable of adsorbing bacteriophage did acquire some radioactivity and that the phage-sensitive strain of bacteria, in the absence of the co-factor necessary for phage adsorption, likewise gained some radioactivity in an amount approximately equal to that shown by the phage-resistant cells. However, when a medium containing the co-factor was added to the above system, phage-sensitive cells picked up much more radioactivity than in the absence of the co-factor, indicating that the difference was due to the radioactive phosphorus contained in the adsorbed bacteriophage particles. That this difference was not due to differences in the ability of phage-sensitive and phage-resistant strains of bacteria to adsorb phosphate or to increased adsorbing ability of the bacteria due to the presence of bacteriophage was also shown in Section I of the experimental work.

It was also found that adsorption of bacteriophage by phage-sensitive bacteria could not be performed directly from the unpurified lysates for the purpose of measurement of the P^{32} content of the bacteriophage, since comparatively large amounts of other phosphorus-containing materials were present in the lysate which were adsorbed to the bacteria at the same time as the phage particles themselves were adsorbed. These contaminating materials were adsorbed by phage-resistant strains of organisms as well as by the phage-sensitive strain, and therefore no conclusion could be drawn as to the amount of P^{32} contained by the bacteriophage particles themselves. When the lysates were partially purified by the methods mentioned above, most of the non-specifically adsorbing radiophosphorus compounds were eliminated.

It is of interest to note that lysates of bacteria where lysis was induced by bacteriophage contain compounds, other than bacteriophage, qualita-

tively different from those present in normal bacteria which were lysed by mechanical means. This is indicated by the fact that, when phage-resistant strains of bacteria were added to lysates entirely comparable in their content of P^{32} but produced by bacteriophage on one hand and by mechanical disruption on the other, the added bacteria adsorbed much more radioactivity from the former lysate than from the latter. No attempt was made to determine the cause of this difference.

After it was established that bacteriophage could obtain phosphorus from compounds contained in the bacterial cells, the question arose as to whether this was the main source or whether the medium could satisfy part or all of the phosphorus requirements of the bacteriophage. In order to resolve this question, three types of bacteria-bacteriophage systems were used:

1. Bacteria were allowed to metabolize in the presence of radio-phosphates. The cells were subsequently washed and suspended in a medium free from radioactivity and then infected with virus. After lysis occurred, the bacteriophage which had been grown under these conditions was tested for the presence of P^{32}. In this system, the only source of P^{32} for the bacteriophage consisted of P^{32} contained in the bacterial cells prior to infection with virus.

2. Non-radioactive bacteria were suspended in a medium containing P^{32} and were then infected with virus. The chief source of P^{32} for the bacteriophage here, of course, consisted of compounds present in the medium. During the period prior to lysis, the bacteria did have an opportunity to incorporate P^{32} which the virus may have subsequently been able to utilize, but control experiments not given in the report showed that the amount acquired by the bacteria in the time prior to lysis could not have been over one-fourth the amount obtained by the cells used in system 1.

3. Radioactive bacteria prepared as in system 1 were allowed to remain in medium containing P^{32}. These cells were infected with virus, and therefore the bacteriophage had the opportunity to obtain P^{32} from both sources, the cells and the medium.

After the virus had been grown in the above three sets of conditions, it was found that virus grown under the conditions in systems 1 and 3 contained much more radioactivity than did virus grown as in system 2. Furthermore, the radioactivity of virus grown as in system 1 equalled that of virus grown as in system 3. These results indicated that the bacteria alone furnished the phosphorus compounds for the virus particles. If the medium had furnished any phosphorus, virus grown as in system 3 would have contained more P^{32} than that grown as in systems 1 or 2. If the medium furnished the major portion or all of the phosphorus contained in the virus,

the bacteriophage grown under the conditions of system 2 would have contained more radioactivity than the virus resulting from system 1 but would have been approximately equal in P^{32} content to the virus resulting from system 3. The fact that bacteriophage grown in non-radioactive cells suspended in radioactive medium contained some radioactivity might be explained by the fact that, prior to lysis, the bacteria in this system did have some opportunity to grow and metabolize P^{32} compounds which were subsequently utilized by the bacteriophage.

These results seem to conflict with those obtained by Cohen[3, 4, 5] and by Putnam and Kozloff.[25] These investigators, working with r+ viruses of the "T" series, found that the main source of phosphorus for bacteriophage was to be found in the medium rather than in preformed compounds of phosphorus existing in bacteria prior to infection. There seem to exist several possible explanations for the discrepancies between the results of the above authors and those of the present investigations: (a) The investigations of Cohen and of Putnam and Kozloff were carried out on so-called "lysis inhibiting" strains of bacteriophage described by Hershey[16, 17] and by Doermann.[10] No turnover in ribonucleic acid was found to accompany the synthesis of desoxyribonucleic acid during the growth of bacteriophage. However, working with other viruses, many workers have found this not to be the case. Gratia, Brachet, and Jeener,[15] for example, found that cells of silkworms infected with the virus of silkworm jaundice showed a rapid turnover of ribonucleic acid accompanying the synthesis of desoxyribonucleic acid. Thus, the host cells may have furnished the materials necessary for formation of the desoxyribonucleic acid of the virus. This may have been the case in the present investigations, where a strain of bacteriophage was used which did not belong to the lysis inhibiting group. It therefore seems possible that bacterial viruses of other than lysis inhibiting strains have the ability to organize bacterial enzymes in such a manner as to bring about synthesis of viruses, using preformed bacterial compounds as a substrate. (b) Differential centrifugation and dialysis were used by the above-mentioned authors to purify their bacteriophage preparations. These procedures seem to have been adequate to give preparations showing chemical and physical homogeneity, but, in view of the present findings, the preparations may not have been biologically homogeneous. (c) In the investigations presented here, the concentration of cells infected by bacteriophage was fairly high. The resulting lysate showed an average yield of virus of three to five bacteriophage particles per host cell. In the work of Cohen and of Putnam and Kozloff, the average yield per cell was twenty to thirty

virus particles. When low concentrations of bacterial cells are used and infection of these cells takes place in optimal media, it is not unusual to obtain burst sizes ranging from one hundred to three hundred per cell. Thus it may well be that with changes of environment, corresponding changes in the metabolic processes occur which can determine the source of materials used in the growth of bacteriophage.

Conclusions

1. The presence of bacteriophage does not influence the adsorption of phosphates by non-metabolizing bacteria.

2. During the course of growth of bacteriophage and lysis of the host cells, compounds of phosphorus are formed which are not directly associated with the bacteriophage particles and which differ in nature from those found in normal cells.

3. The usual methods of purifying bacteriophage by differential centrifugation and dialysis are not always adequate for the purification of bacteriophage.

4. Under conditions where the burst size is small, the source of phosphorus of the bacteriophage described in this work is to be found in phosphorus compounds existing in bacterial cells prior to their infection with bacteriophage.

References

1 Anderson, T. F.: The role of tryptophane in the adsorption of two bacterial viruses on their host, *E. coli.* J. Cellul. Physiol., 1945, *25,* 17-26.

2 Cohen, S. S.: Cytoplasmic particles of chorio-allantoic membrane and their relations to purified preparations of influenza virus. Proc. Soc. Exp. Biol., N. Y., 1944, *57,* 358-360.

3 Cohen, S. S.: The synthesis of bacterial viruses in infected cells. *Cold Spring Harbor Symposia on Quantitative Biology,* 1947, *12,* 35-49.

4 Cohen, S. S.: The synthesis of bacterial viruses. I. The synthesis of nucleic acid and protein in *Escherichia coli B* infected with T_2r+ bacteriophage. J. Biol. Chem., 1948, *174,* 281-293.

5 Cohen, S. S.: The synthesis of bacterial viruses. II. The origin of the phosphorus found in desoxyribonucleic acid of T_2 and T_4 bacteriophage. J. Biol. Chem., 1948, *174,* 295-303.

6 Cohen, S. S. and Anderson, T. F.: Chemical studies on host-virus interactions. I. The effect of bacteriophage adsorption on the multiplication of its host, *Escherichia coli B.* With an appendix giving some data on the composition of bacteriophage T_2. J. Exp. M., 1946, *84,* 511-523.

7 Cohen, S. S. and Fowler, C. B.: Chemical studies on host-virus interactions. III. Tryptophane requirements in the stages of virus multiplication in the *Escherichia coli*—T_2 bacteriophage system. J. Exp. M., 1947, *85,* 771-784.

8 Cohen, S. S. and Fowler, C. B.: Chemical studies in host-virus interactions. V. Some additional methods of determining nutritional requirements for virus multiplication. J. Exp. M., 1948, *87,* 275-282.

9 Curnen, E. C. and Horsfall, F. L.: Studies on pneumonia virus of mice (PVM). III. Hemagglutination by the virus; the occurrence of combination between the virus and a tissue substance. J. Exp. M., 1946, *83,* 105-131.

10 Doermann, A. H.: Lysis and lysis inhibition with *Escherichia coli* bacteriophage. J. Bact., Balt., 1948, *55,* 257-276.

11 Fitzgerald, R. J. and Babbit, D.: Studies on bacterial viruses. I. The effect of certain compounds on the lysis of *Escherichia coli* by bacteriophage. J. Immun., Balt., 1946, *52,* 121-125.

12 Fitzgerald, R. J. and Lee, M. E.: Studies on bacterial viruses. II. Observations on the mode of action of acridine in inhibiting lysis of virus infected bacteria. J. Immun., Balt., 1946, *52,* 127-135.

13 Fowler, C. B. and Cohen, S. S.: Chemical studies in host-virus interactions. IV. A method of determining nutritional requirements for bacterial virus multiplication. J. Exp. M., 1948, *87,* 259-274.

14 Furth, J. and Kabat, E. A.: Immunological specificity of material sedimentable at high speed present in normal and tumor tissues. J. Exp. M., 1941, *74,* 247-261.

15 Gratia, A., Brachet, J., and Jeener, R.: Etude histochimique des acides nucléiques au cours de la grasserie du ver à soie. C. rend. Soc. biol., 1945, *139,* 72-75.

16 Hershey, A. D.: Mutation of bacteriophage with respect to types of plaques. Genetics, 1946, *31,* 620-640.

17 Hershey, A. D.: Spontaneous mutation in bacterial viruses. *Cold Spring Harbor Symposia on Quantitative Biology,* 1946, *11,* 67-77.

18 Hook, A. E., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W.: Isolation and characterization of the T_2 bacteriophage of *Escherichia coli.* J. Biol. Chem., 1946, *165,* 241-257.

19 Kabat, E. A. and Furth, J.: Chemical and immunological studies on the agent producing leukosis and sarcoma of fowls. J. Exp. M., 1940, *71,* 55-70.

20 Knight, C. A.: A sedimentable component of allantoic fluid and its relationship to influenza viruses. J. Exp. M., 1944, *80,* 83-99.

21 Price, W. H.: Bacteriophage formation without bacterial growth. I. Formation of staphylococcus phage in the presence of bacteria inhibited by penicillin. J. Gen. Physiol., 1947, *31,* 119-126.

22 Price, W. H.: Bacteriophage formation without bacterial growth. II. The effect of niacin and yeast extract on phage formation and bacterial growth in the presence of penicillin. J. Gen. Physiol., 1947, *31,* 127-133.

23 Price, W. H.: Bacteriophage formation without bacterial growth. III. The effect of iodoacetate, fluoride, gramicidin and azide on the formation of bacteriophage. J. Gen. Physiol., 1947, *31,* 135-139.

24 Price, W. H.: Phage formation in *Staphylococcus muscae* cultures. I. A factor necessary for phage formation. J. Gen. Physiol., 1948, *31,* 233-238.

25 Putnam, F. W. and Kozloff, L. M.: On the origin of virus phosphorus. Science, 1948, *108,* 386-387.

26 Sharp, D. G., Hook, A. E., Taylor, A. R., Beard, D., and Beard, J. W.: Sedimentation characters and pH stability of the T_2 bacteriophage of *Escherichia coli.* J. Biol. Chem., 1946, *165,* 259-270.

27 Shropshire, R. F.: Turbidometric evaluation of bacterial description by sonic energy. J. Bact., Balt., 1947, *53,* 685-693.

28 Stanley, W. M., Knight, C. A., and DeMerre, T. J.: Les virus: Études biochimiques et biophysiques récentes. Act. med.-chirur., 1945, *6,* 3-81.