RADIOACTIVE PHOSPHORUS TRACER STUDIES ON THE REPRODUCTION OF T4 BACTERIOPHAGE

II. KINETICS OF PHOSPHORUS ASSIMILATION

by

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INTRODUCTION

The characterisation of bacteriophages grown in the presence of the phosphorus isotope ^{32}P was the subject of the first paper of this series (MAALØE AND STENT[1]). The radioactivity contained in such phages was estimated after they had been isolated by means of differential centrifugation, or by precipitation by anti-phage serum or by adsorption onto receptive bacteria. The three methods of isolation gave nearly identical results, and it was concluded that irrespective of the time at which phage production is arrested, all phosphorus which can be recovered from a lysate by any of the three methods is part of infective phage particles.

The present study concerns the manner in which inorganic phosphorus from the growth medium is converted into bacteriophage phosphorus. It has been shown by COHEN[2] and by KOZLOFF AND PUTNAM[3] that at the time when a bacterium is infected with one of the phages, T2, T4, or T6, it has already assimilated about one third of all that phosphorus which eventually is incorporated into the progeny phage particles. We have extended these observations by examining the kinetics of the assimilation of phage phosphorus by bacteria before, as well as after, their in ection with the bacterophages T4r and T4r$^+$. These experiments indicate that all *phage* phosphorus assimilated before infection is first built into general bacterial constituents such as desoxyribonucleic acid (DNA) and ribonucleic acid (RNA) from which it is reclaimed after infection. We have also studied the course of the assimilation of phage phosphorus in experiments in which phage production was arrested early by means of addition of KCN and found that early and late assimilated phosphorus is mixed to a considerable extent before appearing in infective phage particles.

Kinetic studies on the phosphorus assimilation into bacteriophage T5 have just been published by LABAW[4] whose main conclusion, in contrast with our own, is that DNA and RNA *do not* contribute phosphorus to the T5 progeny. LABAW's experiments shall be discussed below in relation to the findings presented here.

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MATERIALS AND METHODS

The T4r bacteriophage, its bacterial host, *E-coli* strain B, and the synthetic growth medium "G" of low phosphate concentration are the same as described in the preceding paper. The methods for growing the phages under "one-step" conditions at 37° C and for arresting phage development by addition of KCN as well as the procedure for protecting free phages from inactivation by bacterial debris through addition of anti-bacterial serum are likewise described there. The T4r$^+$ phage was used in the parent strain from which our T4r strain was isolated.

The ^{32}P content of the bacteriophages in the radioactive lysates was determined after the phages had been isolated by differential centrifugation or by adsorption to heat-killed bacteria (HKB), as previously described. The expression *specific activity* refers to the number of counts per minute registered in a Geiger-Müller counter per mole of phosphorus in a sample. The specific activity of the growth medium is calculated from its total phosphorus concentration of $5 \cdot 10^{-4}$ moles/l, and that of the phages on the basis of the phosphorus content of $2.5 \cdot 10^{-17}$ g per particle, as given by HERSHEY, KAMEN, KENNEDY AND GEST[5].

EXPERIMENTAL

Assimilation of phosphorus

In the experiments to be presented in this study the chief variable is the time at which the specific activity of the growth medium of an uninfected or infected culture is changed. In most cases, radioactive phosphorus ^{32}P was added in order to raise suddenly the specific activity; in some experiments, excess non-radioactive inorganic phosphorus was added to media already containing ^{32}P in order to decrease the specific activity by a large factor.

If ^{32}P is already present when the medium is inoculated with bacteria, then the bacterial phosphorus will be of nearly the same specific activity as the medium when, 2½ hours later, the culture is infected with phage. In this case, also the phages grown on the labelled bacteria in the labelled medium obtain the specific activity of the medium. If the ^{32}P is added at some later time between inoculation of the culture and the time of lysis the specific activity of the phages will be less, and the ratio of their specific activity to that of the medium, the *fractional activity*, is a measure of the fraction of the phage phosphorus assimilated *after* the addition of the ^{32}P. If, conversely, the specific activity of the medium is high from the beginning and is then suddenly lowered, then the fractional activity measures the fraction of phage phosphorus assimilated *before* the time when the medium activity was lowered. The assimilation of phage phosphorus by the bacterial cells can thus be followed by changing the specific activity in a series of otherwise identical samples at various times between inoculation of the cultures and lysis of the infected bacteria.

In what follows below, we shall have frequent occasion to distinguish between that portion of the phage phosphorus which is assimilated by the bacteria before infection and that which is taken up by the cells after infection. For the sake of brevity, the former will be referred to as the *bacterial contribution* and the latter as the *medium contribution* of phosphorus. It should be emphasized that this distinction rests solely on the time of alteration of the specific activity of the medium and does not in any way presuppose any chemical or structural difference between these two phosphorus fractions.

Taking recourse to the principles outlined above we have determined the entire course of the assimilation of phage phosphorus in cultures infected with T4r. In order to study accurately the early stages of assimilation, when the interest is focussed on whether small amounts of phosphorus have *already* been taken up, the specific activity of the medium was reduced from an initially high value. At late stages, when it is im-

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portant to determine whether small amounts of phosphorus are still incorporated *after* a certain time, the specific activity of the medium was raised. Some intermediate points were determined by both methods. The *raising* of the specific activity was accomplished by adding small volumes of "G" medium containing an appropriate concentration of ^{32}P phosphate to non-radioactive medium. The *reduction* of the specific activity was effected by adding small volumes of concentrated non-radioactive phosphate buffer to the radioactive medium so as to raise the total phosphate concentration, and consequently lower the specific activity, by a factor of 100. Control experiments showed that the rate of uptake of ^{32}P actually decreased one-hundredfold immediately after this reduction of the specific activity of the medium.

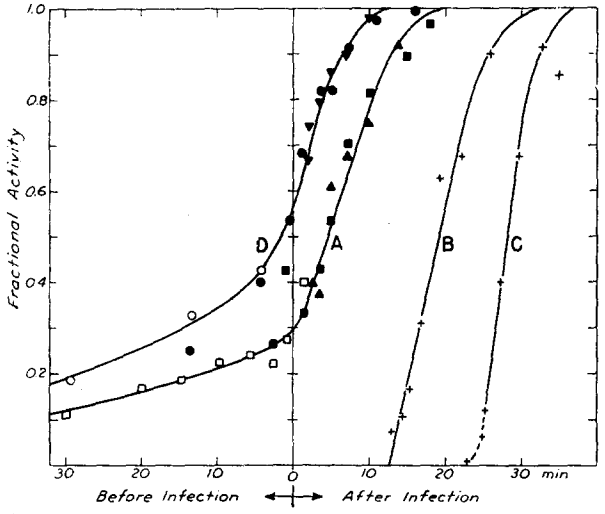

Fig. 1. Assimilation of phage phosphorus by bacteria before and after infection, 37° C.
Curve A: Fraction of bacteriophage phosphorus incorporated into phages of the final yield.
Curve B: Fraction of the final phage yield appearing intracellularly.
Curve C: Fraction of the final phage yield appearing extracellularly.
Curve D: Fraction of bacteriophage phosphorus incorporated into the first 15% of phages completed.

Symbols:

	Analysis by High-Speed Centrifugation Specific Activity		Adsorption to Heat-killed Bacteria Specific Activity
	raised	reduced	raised
Curve A	■	□	▲
Curve D	●	○	▼

The results of these experiments are presented as curve A in Fig. 1, in which the fraction of assimilated phage phosphorus is plotted against the time at which the specific activity of the medium was changed. Included in Fig. 1 are data from experiments in which the labelled phages were isolated by differential centrifugation as well as data from experiments in which isolation was achieved by adsorption to heat-killed bacteria; it is seen that the results obtained with these two methods agree well. The intracellular appearance of infective phage particles (DOERMANN[6]) and a one-step growth curve showing phage liberation in the course of spontaneous lysis are presented as curve B and C for comparison with the assimilation curve.

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The most interesting single point on curve A is perhaps the point corresponding to the time of infection ($t = 0$). The fraction of bacteriophage phosphorus assimilated by this time has been determined previously by COHEN[2] and by KOZLOFF AND PUTNAM[3] in experiments with the closely related phages T2r$^+$, T4r$^+$ and T6r$^+$. These workers found that about 70% of the phage phosphorus is derived from inorganic phosphate present in the medium at the time of infection and the remaining 30% from phosphorus assimilated by the bacteria before infection. Our results confirm this finding since curve A crosses the zero time axis at a level which indicates that about one-third of the phage phosphorus was assimilated by the bacteria before infection.

Bacterial contribution. The pre-infection part of curve A shows the assimilation of the phosphorus of the bacterial contribution during the 30 minutes preceding the infection. It is seen that half of this phosphorus is assimilated during a period of about 24 minutes, this being very nearly the generation time of our bacteria in "G" medium at 37° C. The uptake of phage phosphorus has also been followed during a period of altogether 100 minutes prior to infection. Throughout this period it is found that the total amount of bacterial phosphorus which can be used for phage synthesis increases at the rate of bacterial growth, *i.e.* when the number of bacteria doubles, the amount of phosphorus taken from the medium *which may later go into phages* also doubles. Hence it may be inferred that the phosphorus which is transmitted from the host to the virus progeny arises from endproducts of bacterial synthesis like DNA and RNA and not from a pool of low-molecular-weight substances which could be expected to turn over rapidly.

This conclusion agrees with KOZLOFF AND PUTNAM's finding that low-molecular-weight, so-called acid soluble phosphorus is not used preferentially for phage synthesis and is also in keeping with the observation made by WEED AND COHEN[7] that host nucleic acids appear in the phage progeny.

Kinetic experiments similar to ours have recently been carried out by LABAW[4] with T5 bacteriophage. LABAW believes that in his system the bacterial contribution is assimilated during the last few minutes before infection, and he consequently adopts a hypothesis like the one we have just discarded, *i.e.* that the bacterial contribution is derived mainly from intermediates of bacterial synthesis present in the cell at the time of infection. His conclusions are, however, based exclusively on experiments in which ^{32}P was *added* at different times before and after infection. As pointed out previously, this procedure is very insensitive if the question is whether, say, 80 or 90% of the total phage phosphorus is assimilated after the addition of ^{32}P. The relatively slow adsorption of T5, furthermore, makes it difficult to define precisely the time at which individual cells were actually infected. It would seem then that LABAW's experiments do not "preclude the bacterial desoxyribonucleic acid as the major source of the bacterial phosphorus contribution to T5 bacteriophage".

Medium contribution. The assimilation rate of phage phosphorus increases very sharply within a minute or so after infection. Whereas immediately before infection only about 0.6% of the total bacteriophage phosphorus was assimilated per minute, we find that already two minutes after infection this rate has risen to about 5% per minute, or to a value eight times greater.

It is very unlikely that this sudden increase in assimilation rate reflects an equally great increase in the total uptake of phosphorus of the cells, which, until they were infected, grew under optimal conditions. LABAW, MOSLEY AND WYCKOFF[8] report, furthermore, that the phosphorus uptake of T2r$^+$ infected bacteria is no greater than that

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of uninfected cells. It is likely that the same is true for cells infected with T4r, and it must therefore be concluded that the *fraction* of the assimilated phosphorus used for phage synthesis increases greatly within one to two minutes after infection. Such a change in the phosphorus metabolism as a consequence of infection has, in fact, already been described and called "phosphorus shunt" by COHEN[9], who found that the rate of DNA synthesis is increased while RNA synthesis is suppressed in bacteria infected with T2. Our findings indicate that *the phosphorus shunt is established almost as soon as the bacterium is infected* and not some eight to ten minutes later when DNA synthesis can first be demonstrated.

A simple calculation may be made to estimate roughly what fraction of the *total* phosphorus uptake of the infected cell is used for phage synthesis. It may be assumed that after infection, at least during the first few minutes, the cell takes up phosphorus at the same rate as before infection. This rate is about $5 \cdot 10^{-16}$ g per cell per minute. An average production of 150 T4r particles per cell corresponds to $3.8 \cdot 10^{-15}$ g of phosphorus, so that our estimated rate of assimilation of *phage* phosphorus after infection of 5% per minute represents about $1.8 \cdot 10^{-16}$ g per cell per minute. These figures indicate that about one third of the phosphorus taken up during the first minutes after infection is incorporated into *finished* phages. The true fraction of phosphorus assimilated by the cells and then diverted into channels directed towards phage synthesis is probably greater. Thus an appreciable, if not a dominant, part of the phosphorus economy of the cells is affected by the "phosphorus shunt".

Between 12 and 13 minutes after infection, *i.e.*, at the time when the first finished phage particles appear intracellularly, we find that 85% of the total phage phosphorus has been taken up. The assimilation curve, which up to this time is almost linear, then bends gradually to reach 95–100% assimilation at 18 minutes. It has recently been shown that the time when individual bacteria in a culture infected with T4r phages contain their first finished phages varies from about 13 to 19 minutes after infection at 37° C (BENTZON, MAALØE AND RASCH[10]). Hence it may be assumed that individual infected bacteria have assimilated *all* the phosphorus which is to appear in subsequently completed phages at the time when they contain their *first* infective phage particles.

The assimilation curve A of Fig. 1 may now be compared with curve B, the intracellular appearance of finished phages. The distance between these curves shows that phosphorus added to the medium 18 minutes or more after infection fails to be incorporated into the particles of the phage yield, despite the fact that phages are still being finished as much as 10–12 minutes later. The simplest interpretation of this observation is that during this late phase of phage production *it takes at least 10–12 minutes before a newly assimilated phosphorus atom can become part of a complete phage particle*. The question of whether phosphorus continues to be assimilated and started on its way towards incorporation into phage particles during these last 10 minutes, or whether phosphorus assimilation and synthesis of phage material stop when there is not time enough left to achieve incorporation into complete phage particles is considered next.

It should be recalled that profound changes in the physiological state of an infected cell coincide with the intracellular appearance of the first finished phages: KCN will induce lysis only if added after that time (DOERMANN[11]), and a process with a characteristic temperature coefficient is initiated at this critical time, which controls the time of spontaneous lysis (MAALØE[12]). These physiological changes might well be due to or coupled

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with loss of the ability to assimilate phosphorus from the medium. We have attempted to answer this question by studying the uptake of phage phosphorus of cells infected with the bacteriophage strain T4r+ under conditions of controlled lysis inhibition.

Phosphorus assimilation of lysis inhibited bacteria

Bacteria infected with T4r or T4r+ phages at low multiplicities (*i.e.*, with an average of one phage or less per cell) give rise to identical one-step growth curves. If cells infected with T4r+ phage are reinfected at some later stage with T4 phage, the one-step curve is altered in two ways: the latent period is increased, and the burst size is raised by a factor of two or more (DOERMANN[11]). The lysis is inhibited by the secondary infection. Cultures primarily infected with the r-mutant used in all the preceding experiments

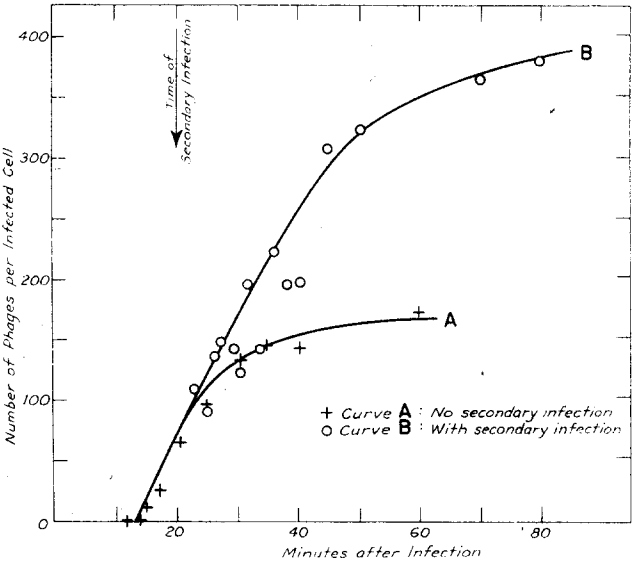

Fig. 2. The effect of lysis inhibition on the intracellular appearance of T4r+ bacteriophage, 37° C.
Primary infection: one T4r+ phage per bacterium.
Secondary infection: ten ultraviolet light inactivated T4r phages per bacterium

cannot be inhibited by reinfection. In his original studies of the phenomenon of lysis inhibition DOERMANN[11] concluded that primary as well as secondary infection had to be by an r+-phage for inhibition to take place; with our T4 strains we find that lysis can be inhibited equally well by secondary infection with T4r and T4r+ phages. Furthermore, it is now known that the particles effecting the secondary infection need not be active in the sense of being able to form plaques; the capacity to cause lysis inhibition is retained after inactivation by ultraviolet light or X-rays (WATSON[13]).

In Fig. 2 we present curves showing the intracellular development of phages in two aliquots of a culture infected with an average of one T4r+ particle per bacterium. The aliquot represented by curve A received no secondary infection, and it is seen that the phage yield increased up to about 30 minutes after infection; this curve is essentially similar to curve B of Fig. 1, showing the intracellular phage development in T4r infected

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bacteria. The aliquot represented by curve B in Fig. 2 was infected secondarily with an average of ten ultraviolet light inactivated T4r particles per cell 20 minutes after the primary infection*. It is seen that the secondary infection caused phage production to continue at a constant rate until about 50 minutes after the primary infection, resulting in a final yield more than twice that of the non-inhibited aliquot.

We now return to the question of whether the assimilation of phosphorus ceases when the first finished phages appear in the cells, or whether the cells continue to assimilate phosphorus and produce more phage precursor material after that time.

If assimilation and production of phage material continue, then lysis inhibition can be viewed simply as an inhibition of the processes which normally causes the cells to lyse between 23 and 30 minutes after infection. If on the other hand, assimilation and production stop half-way through the normal latent period then lysis inhibition must also function as a stimulus to the cells to resume the synthesis of phage material. Curve B of Fig. 2 already points in the direction that synthesis continues, for it appears to be a straight continuation of curve A. If the synthesis of new phage material in the bacterial culture had stopped gradually between 12 and 18 minutes after infection and not been resumed until after re-infection at 20 minutes, then this pause should have expressed itself at a later stage as a lag in the intracellular appearance of finished phage.

As a more critical test of this point we have determined the amount of phage phosphorus assimilated during one-minute periods throughout the latent period of bacteria infected primarily with T4r$^+$, and re-infected *20 minutes* later to produce lysis inhibition.

A bacterial culture grown to 10^8 cells/ml in non-radioactive "G" medium was concentrated five times and infected with an average of one T4r$^+$ particle per cell. Two minutes were allowed for adsorption before the culture was diluted to the original density. ^{32}P was added to aliquots of this culture at different times and one minute after this addition each sample received enough concentrated, non-radioactive phosphate buffer to reduce the specific activity by a factor of forty. 20 minutes after the primary infection, each sample was re-infected with an average of 10 ultraviolet inactivated T4r particles per cell. 12 minutes later anti-bacterial serum was added to the cultures to prevent re-adsorption to bacteria and bacterial debris of newly liberated phage.

Table I shows the results of this experiment. In the first column are listed the one-minute intervals during which the specific activity in the different samples was held at the high level; the second column shows the fractional activities of the phages isolated from the individual samples. If it is assumed that the uptake of phage phosphorus is, in fact, constant at all times, then the specific activities of the phages can be corrected for the *residual* ^{32}P-assimilation occurring after the specific activity of the medium has been reduced. These corrected fractional activities, presented in the third column, may be considered to represent the fractions of the total amount of phage phosphorus assimilated during the different time intervals. The fact that these figures are constant seems to justify the correction procedure. We conclude, therefore, that phage phosphorus was assimilated at a constant rate throughout, even though lysis inhibition was established as late as 20 minutes after infection.

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* It should be noted in this connection that LESLEY, FRENCH AND GRAHAM[14] have shown that phages adsorbed more than a few minutes after a primary infection are broken down to a certain extent on the surface of the bacterium, so that about 50% of their phosphorus appears as material of low molecular weight in the medium. It thus seems either that the part of the phage particle subject to this breakdown is not required for lysis inhibition, or that the breakdown process itself causes it.

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TABLE I

RATE OF PHOSPHORUS INCORPORATION INTO VIRUS PARTICLES IN T4r+ INFECTED CULTURES BEFORE AND AFTER LYSIS INHIBITION

Period after infection during which culture was exposed to high specific activity *minutes*	*Specific activity ratio of bacteriophages*	*Specific activity ratio corrected for residual ^{32}P assimilation, or fraction of final bacteriophage P assimilated per min.*
Before Lysis Inhibition		
3–4	0.020	0.012
4–5	0.022	0.014
5–6	0.023	0.014
6–7	0.022	0.014
9–10	0.017	0.011
12–13	0.021	0.015
14–15	0.020	0.014
16–17	0.017	0.012
18–19	0.017	0.013
After Lysis Inhibition		
22–23	0.015	0.012
24–25	0.012	0.010
26–27	0.014	0.013
28–29	0.015	0.014
30–31	0.011	0.011

Since T4r and T4r+ phages behave exactly alike except with respect to the phenomenon of lysis inhibition, we may assume that until lysis inhibition was established 20 minutes after infection, the conditions in the T4r+-infected cells were exactly the same as in the cells infected with the r-mutant. If this assumption is correct, then *T4r-infected cells continue to produce phage material at an undiminished rate at least until 20 minutes after infection.*

Phosphorus assimilation and synthesis of phage nucleic acids thus continue undisturbed during some time subsequent to the uptake of *all* that phosphorus which manages to get incorporated into finished phages. Some of the phage precursor material produced is thus wasted in non-lysis inhibited infections. In lysis-inhibited systems, however, in which the host cell-phage complex is preserved beyond the normal latent period these precursors find incorporation into infective phage particles because of the longer time afforded for their completion. The conclusion that the phenomenon of lysis inhibition concerns only the retardation of lytic reactions and does not intervene directly in phage production supports the view that once initiated, the lytic processes in an infected cell proceed independently of phage production. Previously this idea was based chiefly on the wide distribution of the burst sizes of individual cells (DELBRÜCK[15]), the finding that infected bacteria lyse in the presence of proflavine without production of active phages (FOSTER[16]), and on the difference between the temperature coefficients of the lytic reaction and of phage production (MAALØE[12]; BENTZON, MAALØE AND RASCH[10]).

Assimilation of phosphorus incorporated into early phages

It was shown in the first paper of this series that all phosphorus isolated from

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lysates by differential centrifugation, by adsorption to HKB and by precipitation by anti-phage serum belonged to infective particles. The results were the same whether phage growth was stopped early by addition of KCN or whether lysis was allowed to proceed spontaneously. This fact makes it possible to study with these techniques not only the kinetics of the assimilation of the phosphorus contained in the final phage yield but also the *distribution* of the assimilated phosphorus to phages completed within the host cells at different times. We may therefore now consider such questions as: 1. is the phosphorus of the bacterial contribution concentrated mainly in the phages completed early, and 2. do the phosphorus atoms of the medium contribution appear in the successively completed phage particles in the order of their assimilation?

1. Recent work by WEED AND COHEN[7] indicates that the early finished phages (henceforth referred to as "early phages") receive a larger fraction of their pyrimidines from the bacterial DNA than does an average phage particle of the final yield. It was to be expected, therefore, that the phosphorus of the bacterial contribution would be incorporated predominantly into the early phages. We have confirmed this expectation by an experiment in which ^{32}P was added at the time of infection and the culture then divided into samples in which phage growth was arrested at different times by addition of KCN. The specific activity of the phages produced in these samples was determined after isolation by differential centrifugation, as previously described.

The results of this experiment are presented in Table II showing the bacterial contribution to various early fractions of the final phage yield. It is seen that *the earlier the phages are completed, the greater the fraction of their phosphorus which has been assimilated before infection.* The phosphorus of the early phages, however, is not supplied *exclusively* by the bacterial contribution. A considerable fraction, about 40%, of the phosphorus of the first 16% of the phage yield is derived from the medium contribution.

TABLE II

BACTERIAL PHOSPHORUS CONTRIBUTION TO BACTERIOPHAGES ISOLATED AT DIFFERENT TIMES DURING THE LATENT PERIOD

Fraction of final phage output in infected cells	*Fraction of bacteriophage phosphorus assimilated before infection*
0.16	0.59
0.26	0.52
0.52	0.49
0.74	0.51
0.98	0.41
1.00	0.33
2.10 ($T4r^+$)	0.24

Table II also shows the bacterial contribution to phages isolated after spontaneous lysis of a culture infected with $T4r^+$ under conditions of lysis inhibition. The phage yield in this experiment was equivalent to 210% of the normal yield of a T4r lysate. The average bacterial contribution of 24% to these r^+ phages is somewhat lower than the average contribution of 30–40% to r phages; it is, nevertheless, high enough to show that *some* phosphorus from the bacterial contribution is used in the synthesis of r^+ phages completed later than any of the phages of a normal T4r lysate.

2. It was seen in Table II that a considerable fraction of the phosphorus of even

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the earliest phages is assimilated from the medium after infection. We have studied the kinetics of assimilation of this phosphorus of the early phages in order to see whether the atoms first assimilated all appear in the phages first finished, or whether some mixing occurs of phosphorus assimilated from the medium at different times.

As in the assimilation measurements on the phages of the final yield, reported as curve A of Fig. 1, the specific activity of the growth medium was raised or lowered at different times before and after infection of a bacterial culture with T4r phages. In the present experiments, however, KCN was added 15 minutes after infection in order to arrest phage growth when, according to curve B of Fig. 1, about 15% of the final phage yield is present within the cells. The specific radioactivity of the phages was again determined after isolation by means of differential centrifugation or adsorption to HKB. The fractions of the total phage phosphorus assimilated after the time of alteration of the specific activity were, as before, estimated from the fractional activities.

Curve D of Fig. 1 presents the results of these experiments. It is seen that in the post-infection part of this graph curves A and D are almost parallel, with D preceding A by about 6 minutes. The assimilation of the phosphorus of the early phages seems to be complete about 10 minutes after infection, or some 5 minutes before phage production was stopped by KCN. Curve D intersects the zero time axis at a level which indicates that 60% of the phosphorus of the early phages has been assimilated before infection, compared to a bacterial contribution of about 35% to the phages of the final yield represented by curve A. These results are in fair agreement with those reported in Table II.

Finally, curve D shows the course of the assimilation of the bacterial contribution. As in the case of the final phage yield we find that about half the bacterial contribution was assimilated within one bacterial division period prior to infection. Since a large fraction of the phosphorus of the phages considered here was taken up before infection, the rate of its assimilation could be determined with good accuracy. It is, therefore, possible to assert that even in the case of the early phages, the phosphorus of the bacterial contribution is derived mainly from general bacterial constituents and not from short-lived intermediate compounds.

DISCUSSION

In the following we shall summarize and discuss our findings in terms of the diagram presented as Fig. 3, which incorporates all data relevant to the assimilation of the phosphorus of the early as well as the total phage yield. The ordinate in the diagram represents the *rate* of intracellular appearance of finished phage particles or of assimilation of phage phosphorus. The basic unit in this presentation is the total amount of phage or of phage phosphorus in the final yield and the rates are accordingly expressed as fractions of this unit produced or assimilated per minute. As abscissa is chosen the time in minutes after infection, and hence *areas* on the diagram represent amounts of phage or phage phosphorus expressed as fractions of the basic unit.

The intracellular appearance of the finished phages is presented as the field ABCD, which is derived by differentiation of curve B of Fig. 1. Field ABCD, like curve B, shows that phages begin to appear 12–13 minutes after infection and are then finished at a constant rate of 7.5% per minute for about 10 minutes; after that period the rate

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decreases to reach zero about 30 minutes after infection. The area of field ABCD is by definition 1 since it represents the total amount of phage or of phage phosphorus in the final yield.

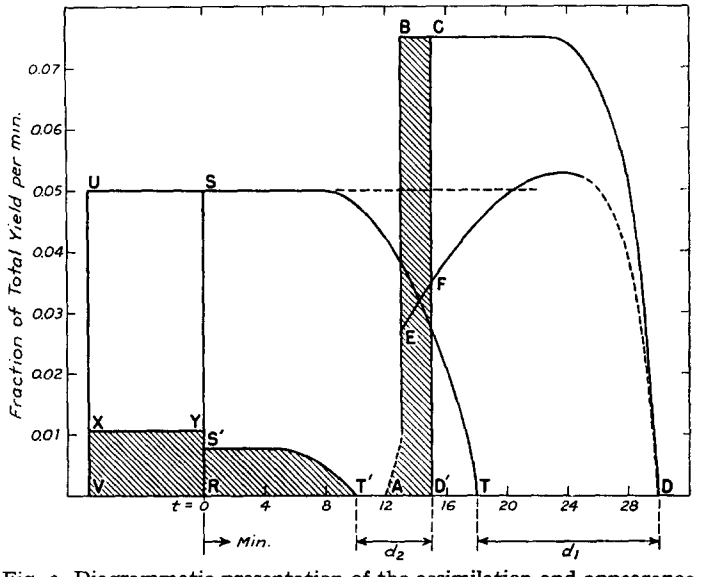

Fig. 3. Diagrammatic presentation of the assimilation and appearance in finished particles of phage phosphorus.

Field ABCD: total amount of phage or phage phosphorus
Field ABCD′: "early" phages, constituting 15% of total
Field AEFD: medium contribution to total phage yield
Field AEFD′: medium contribution to early phages
Field EBCD: bacterial contribution to total phage yield
Field EBCF: bacterial contribution to early phages
Field RST: Assimilation of medium contribution to total yield
Field RS′T′: Assimilation of medium contribution to early phages
Field RSUV: Amount of bacterial contribution to total yield
Field RYXV: Amount of bacterial contribution to early phages
d_1: minimal development time of phosphorus of late phages
d_2: minimal development time of phosphorus at early phages

ABCD has been subdivided in two ways:

First, it is divided vertically into areas corresponding to early and late phages. The field ABCD′, on the left edge of ABCD, depicts those 15% of the final phage yield which were completed during a period of 2–3 minutes preceding the addition of KCN. The area of field ABCD′ accordingly is 0.15 units.

Secondly, ABCD is divided horizontally to show how the phage phosphorus of the final yield is made up of bacterial and medium contributions. This subdivision is made on the basis of the figures of Table II, showing the bacterial contribution to various early fractions of the total phage yield. The lower field, AEFD, represents the medium contribution and the upper field, EBCD, the bacterial contribution to the total phage phosphorus. Similarly, the fields AEFD′ and EBCF represent the medium and bacterial contributions to the phosphorus of the early phages.

The assimilation of the phosphorus of the medium contributions may now be pictured in a similar way:

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By plotting the slope of the post-infection part of the assimilation curve, A, of Fig. 1 we obtain the curve ST, and the field RST then represents the assimilated phosphorus which subsequently constitutes the medium contribution of the finished phages (field AEFD). The area of RST is, of course, equal to that of AEFD, and since both represent the medium contribution to the total phage yield, each area equals 0.65 units or 65% of the area of ABCD.

The lateral displacement of the fields RST and AEFD expresses the average length of the "dark" period following assimilation, during which a phosphorus atom passes through the various intermediate stages on its way to become part of a finished phage particle. We will refer to this period as *the development time of phosphorus atoms*. Since the two fields are of similar width, it may be assumed that the *average* development time of phosphorus atoms assimilated at different times after infection does not vary much. This common average development time measured by the horizontal distance between the centers of gravity of the fields RST and AFGD is found to be about 14 minutes. The distance, d_1, between the points T and D on the abscissa indicates that towards the end of phage production the *minimum* development time is about 12 minutes.

The experiments with lysis-inhibited cultures had shown that T4r-infected cells continue to synthesize phage material at a constant rate until at least 20 minutes after infection. This finding is indicated in Fig. 3 by the dotted line extending the upper, horizontal boundary of the field RST to the right. The area of fields under the dotted line and to the right of the curve ST thus represent phosphorus atoms assimilated so late that phage synthesis had stopped before their development time was over.

Next we shall consider the assimilation of the medium contribution to the *early* phages: The field AEFD′ was shown to represent the appearance of this phosphorus in finished phages. In order to show diagrammatically how this phosphorus was assimilated, the slope of curve D of Fig. 1, multiplied by 0.15 (the fraction of the final yield represented by the early phages) has been plotted as curve S′T′ on Fig. 3. We thus obtain the field RS′T′, representing the assimilated phosphorus which eventually constitutes the medium contribution to the early phages (field AEFD′). The areas of fields RS′T′ and AEFD′ are, of course, equal.

The *order* preserved among phosphorus atoms during the dark period may now be discussed in terms of the distribution of the development time. If the conversion of phosphorus from inorganic phosphate of the medium to nucleic acid phosphorus of infective phage particles proceeded along a rigid assembly line, then all phosphorus atoms in a given cell should spend the same length of time in completing this process. If, on the other hand, the progress of these atoms during synthesis is disordered, then some atoms may have shorter and other atoms longer development times.

It must be realized that in experiments conducted with mass cultures variation in the development time may arise from differences in the speed with which different individual bacteria carry out their assimilatory processes, quite aside from variation due to mixing of phosphorus within individual cells. Such differences between bacteria probably exist, since the first 15% of the final phage output to appear intracellularly is found within only a minor fraction of all the infected bacteria (BENTZON, MAALØE AND RASCH[10]). Hence the early phages can be said to originate predominantly from "fast" cells, in which they represent not the first 15% but perhaps the first 30% of the final phage output of these bacteria.

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The fast cells may, therefore, also possess shorter development times for the processing of phosphorus. Since *no* cells contain any finished phage prior to 12 minutes after infection, however, it is clear that in even the fastest cell there must be some phosphorus atoms with development times of at least 12 minutes. The width of field RS'T' indicates that the medium contribution to the early phages is assimilated over a period of ten minutes, in contrast to the two or three minutes (indicated by the distance AD') during which these phages appear as infective particles within the cells. Hence there must be some phosphorus atoms within one and the same infected bacterium which have development times as different as 5 minutes (distance d_2) and 12 minutes (distance RA). It may be concluded, therefore, that the early phage particles of individual cells contain phosphorus atoms assimilated at very different times. The synthesis of the phosphorus containing phage structures thus does not proceed along a rigid assembly line.

The average development time of the phosphorus atoms appearing in the early phages appears to be only 9–10 minutes, as compared to the overall average development time of 14 minutes of phosphorus atoms of the final yield. The minimum development time of the phosphorus of early phages is only 5 minutes (d_2), as compared to 12 minutes (d_1) for the latest phages finished. Hence it appears that phosphorus assimilated by fast bacteria has lower average as well as shorter minimum development times.

We shall briefly consider how the mixing of phosphorus assimilated at different times may be imagined to occur. At all stages of phage synthesis precursor material may accumulate to form pools from which material is withdrawn at random for further processing. It is obvious that the degree of mixing due to such pools depends both on the total amount of pool material and the distribution of this material over a number of pools. The more pool material present and the fewer the consecutive pools over which the material is spread, the greater is the mixing to be expected. The distribution of development times observed in these experiments then points to the existence of a substantial amount of pool material during phage production. One might speculate as to whether one such pool might be constituted of immature phage particles undergoing genetic recombination. Such considerations, however, presuppose that appreciable quantities of phosphorus are transferred when genetic material is exchanged in the course of bacteriophage reproduction, an assumption concerning which no information is as yet available.

It now remains to consider the bacterial contribution. It has been suggested that there is a fundamental difference between the phosphorus-containing structures which the phages receive from the host cell and the structures synthesized from phosphorus assimilated after infection. Under this view about 30% of the phosphorus-containing structures of the phage particle are thought to constitute "essential" material which only the bacterial cell can supply (EVANS[17]). The observation that early phages receive much more phosphorus from the host cell than do late phages suggests, however, that every part of a phage particle can be constituted of phosphorus assimilated before as well as after infection. We rather envisage, therefore, that bacterial and medium contributions subsequent to a certain stage in their development towards phage phosphorus, follow identical pathways and are mixed together by the same mechanism which is responsible for the mixing of early and late assimilated phosphorus of the medium contribution.

The bacterial contribution to the early phages may be considered further to test this hypothesis. Since 60% of the phosphorus of the early phages and only 35% of that

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of all phages were assimilated before infection (see Fig. 1), the early phages contained

$$\frac{0.60}{0.35} \times 0.15 = 0.25$$

of the total bacterial contribution. On the other hand, the fraction of the phosphorus assimilated from the medium immediately after infection which reaches the early phages may be estimated from the ratio of the ordinates RS′ and RS of Fig. 3. This ratio is

$$\frac{0.008}{0.050} = 0.16,$$

showing that similar shares of the total bacterial contribution and of phage phosphorus assimilated shortly after infection appear in the early phages. It is reasonable, therefore, to regard the bacterial contribution as a block of phosphorus started at infection time along the phage production line along the same channel as phosphorus recently assimilated from the medium.

We have presented the bacterial contribution in Fig. 3 to the left of the zero time axis as the field RSUV, having an arbitrary height equal to that of the field RST representing the medium contribution. The width of RSUV is so that its area amounts to 0.35 units or 35% of that of the field ABCD, the total phage phosphorus. The bacterial contribution to the early phages is indicated by the field RVXY, the height of which is chosen so as to make the area of the double field VXYS′T′, representing all the phosphorus of the early phages, equal to the area of field ABCD′, representing all the early phages at the time of their appearance. It is seen that RY, the height of field VXYR, is not very different from RS′, expressing graphically the conclusion that similar shares of the bacterial contribution and of the first assimilated portion of the medium contribution are allocated to the early phages.

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SUMMARY

1. Prior to infection the phosphorus which eventually is incorporated into T4r bacteriophages is assimilated from the medium at the rate of bacterial growth. Before being used for phage synthesis this phosphorus must therefore have been part of general bacterial constituents like the nucleic acids.

2. Phosphorus assimilated before infection appears in all the phages of the progeny but predominantly in the early finished particles.

3. Within one to two minutes after infection the rate of assimilation of phage phosphorus increases by a factor of about eight. Synthesis of phage material thus seems to start very shortly after infection.

4. The average time spent by phosphorus atoms between assimilation and incorporation into infective phage particles is about fourteen minutes. Early finished phages contain a mixture of phosphorus assimilated just after infection and relatively late assimilated phosphorus. The time elapsing between assimilation and incorporation of phosphorus atoms thus varies considerably.

5. Bacteria infected with T4r phages probably continue to produce phage material at an undiminished rate until at least twenty minutes after infection. Some of this material will not have time enough to be incorporated into finished phage particles before lysis.

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RÉSUMÉ

1. Avant l'infection, le phosphore qui sera, plus tard, incorporé dans le bactériophage T4r est assimilé à partir du milieu avec la vitesse de la croissance bactérienne. Avant d'être utilisé pour la synthèse des phages, ce phosphore doit donc avoir fait partie des constituants généraux des bactéries, telles que les acides nucléiques.

2. On trouve le phosphore assimilé avant l'infection dans tous les phages de la progéniture mais surtout dans les particules dont le développement était terminé le premier.

3. Une ou deux minutes après l'infection la vitesse d'assimilation de phosphore de bactériophage devient huit fois plus grande. Il semble donc que la synthèse de la substance de phage commence peu de temps après l'infection.

4. Le temps moyen qui s'écoule entre l'assimilation des atomes de phosphore et leur incorporation dans les particules infectieuses de bactériophage est de quatorze minutes. Les phages terminés les premiers contiennent un mélange de phosphore assimilé juste avant l'infection et de phosphore assimilé relativement tard. Le temps qui s'écoule entre l'assimilation des atomes de phosphore et leur incorporation varie donc considérablement.

5. Des bactéries infectées par le bactériophage T4r continuent probablement de produire de la substance de phage à la même vitesse pendant au moins vingt minutes après l'infection. Une partie de cette substance n'aura pas le temps d'être incorporée dans des particules de phage terminées avant que la lyse ne commence.

ZUSAMMENFASSUNG

1. Vor der Infektion wird der Phosphor, welcher später vom T4r-Bakteriophagen aufgenommen werden soll, aus dem Medium mit der Geschwindigkeit des Bakterienwachstums assimiliert. Bevor er für die Phagensynthese gebraucht wurde, muss dieser Phosphor also ein Teil allgemeiner Bakterienbestandteile, wie Nukleinsäuren, gewesen sein.

2. Vor der Infektion assimilierten Phosphor findet man in allen Phagen, vor allem aber in den zuerst fertig entwickelten Teilchen.

3. Innerhalb von ein oder zwei Minuten nach der Infektion wird die Geschwindigkeit der Phagenphosphor-Assimilation achtmal grösser. Es scheint also, dass die Synthese von Phagen-Substanz sehr rasch nach der Infektion beginnt.

4. Die Durchschnittszeit zwischen Assimilation und Einverleibung in die infektiösen Phagenteilchen beträgt für Phosphoratome ungefähr vierzehn Minuten. Bakteriophagen, welche früh fertig entwickelt sind, enthalten ein Gemisch von gerade vor der Infektion assimiliertem und verhältnismässig spät assimiliertem Phosphor. Die Zeitspanne zwischen der Assimilation und der Einverleibung der Phosphoratome variiert also bedeutend.

5. Mit T4r infizierte Bakterien setzen wahrscheinlich die Bildung von Phagensubstanz mit unveränderter Geschwindigkeit bis mindestens 20 Minuten nach der Infektion fort. Ein Teil dieser Substanz wird nicht in fertige Phagenteilchen einverleibt werden können bevor die Lyse eintritt.

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