RADIOACTIVE SULFUR TRACER STUDIES ON THE REPRODUCTION OF T4 BACTERIOPHAGE[1]

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Recent studies on the coli-phages T2 and T4 have added to our knowledge on the reproduction of viruses and suggest the following sequence of events: On adsorption the outer protein membrane of the infecting particle containing all its sulfur stays inert on the surface of the cell, and the phage nucleic acid, possibly combined with other sulfur-free compounds, penetrates into the cell (Hershey and Chase, 1952); from this material immature particles are formed, whose number increases by duplication (Luria, 1951); genetic recombination takes place between these particles which in time mature into complete phages (Visconti and Delbrück, 1953).

In this model an important step towards reproduction is the transformation of part of the parental phage into a noninfective, duplicating particle. This notion would be greatly strengthened if such particles could be isolated. Attempts have been made to look for P32-labeled, noninfective structures in T4 lysates obtained by adding cyanide at different times during the latent period. No evidence was found for the presence of any immature particles which sedimented with the mature phages, adsorbed on sensitive bacteria, or precipitated with anti-T4 serum (Maaløe and Stent, 1952).

This result suggests the following: Immature phage particles which contain phosphorus are either: (a) unstable outside the host cell; (b) so firmly attached to bacterial material that they are removed with the bacterial debris during purification; or (c) free and stable, but too small to be sedimented with the finished phages (in 60 minutes at 12,000 g) and without their antigenic and adsorptive specificities. It has been shown that these specificities belong to the protein membrane which does not enter the cell (Hershey and Chase, 1952). If the duplicating particles are small and do not yet possess protein membranes, they may belong, therefore, in the last category.

In the experiments with P32, we selected for structures with the properties of the protein membrane of the phage, and then looked for phosphorus associated with them. Different results might have been obtained if the protein itself had been labeled. Such experiments have now been carried out, again using the phage T4, but labeling with S35. The results indicate that noninfective, sedimentable particles which adsorb and precipitate with the specificity of the T4 phages are present in both premature and ordinary lysates. From the negative results obtained with P32 we conclude that these particles are phosphorus-free.

This finding presents a curious paradox. By inference we know that the first immature phage structure present inside the host cell contains nucleic acid but no sulfur; yet, when examining the products released from the cell when it lyses, we find that the only immature phage material our methods can detect contains sulfur but no nucleic acid. New techniques of preparation and identification are required before we may hope to isolate structures intermediate between these extremes.

MATERIALS AND METHODS

The cofactor requiring strain of T4r phage previously used was grown on *Escherichia coli*, strain B, at 37 C. The cultures were inoculated with about 10^7 washed bacteria from a 24 hour broth culture per ml, aerated for 2 to 2½ hours and infected with about 10 nonlabeled phage particles per bacterium when the density was approximately 10^8 cells per ml. Aeration was continued until lysis occurred or cyanide was added. Antibacterial serum was used to prevent adsorption of progeny phage on unlysed cells or debris as described by Maaløe and Stent (1952).

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The experiments of this paper are essentially similar to some of the P32 experiments previously reported. To make direct comparison possible the g-medium[4] then used was chosen as a basis. It was altered slightly for the present purpose: (1) chlorides were substituted for sulfates, (2) the concentration of phosphate buffer was raised to M/1,000, and (3) the concentration of methionine and cystine was lowered by a factor four (the usual casamino acid concentration of 0.1 per cent was reduced to 0.025 per cent and the medium supplemented with 0.075 per cent of a mixture of the sulfur-free amino acids in the proportions in which they occur in casein). In the modified medium the latent period and the burst size of the T4r were unchanged, being 22 to 23 minutes and 100 to 150, respectively, at 37 C. The casamino acid concentration was reduced because the original g-medium is unstable with respect to incorporation of inorganic S35 into phage. Media with a casamino acid concentration ranging from 0.1 per cent to zero and with a constant concentration of the sulfur-free amino acids were tried. Without casamino acid bacterial growth was slowed down considerably. In the concentration range 0.05 to 0.0125 per cent the media were relatively stable, growth was optimal, and incorporation of S35 was high and *independent* of the casamino acid concentration. When increasing this concentration to 0.1 per cent incorporation dropped sharply. It is clear, therefore, that in media containing methionine and cystine and inorganic S35, the effective specific activity cannot be calculated directly from the total concentration of sulfur. We have not attempted to obtain absolute values for the incorporation of S35 into phage.

The phage material in the S35-containing lysates was purified in the Servall angle centrifuge or in the Spinco ultracentrifuge. The purified materials were characterized by relating the number of plaque forming phage particles per ml to the radioactivity, as measured directly, or after specific adsorption or precipitation of the phage as described by Maaløe and Stent (1952). The radioactivity measurements were made on samples dried on discs of lens paper; the efficiency of counting of S35 was about 5 per cent when self-absorption was negligible.

[4] This medium has the following composition (Maaløe and Watson, 1951): NaCl, 5 g; NH_4Cl, 1 g; $MgSO_4(7H_2O)$, 0.1 g; $CaCl_2$, 0.1 g; $FeSO_4(7H_2O)$, 0.01 g; DL-tryptophan, 0.01 g; glycerol, 2 g; gelatin, 0.01 g; tween 80, 0.2 g; casamino acids (Difco), 1 g; KH_2PO_4, 0.005 g; Na_2HPO_4, 0.01 g. To these substances were added 1,000 ml of distilled water and the pH adjusted to 7.1 with NaOH.

EXPERIMENTAL RESULTS

(1) *Evidence for the presence of noninfective particles in fresh lysates.* In the following experiments the radioactive sulfur was added to the medium at an activity of 10 to 20 μC of inorganic S35 per ml at the same time as the bacterial inoculum. After infection with phage, aeration was continued and at different times samples were withdrawn and mixed with KCN and antibacterial serum. The samples were held at 37 C until about 40 minutes after infection. Antibacterial serum was added to the remaining portion of the culture 20 minutes after infection. The labeled phage material was purified by two low and three high-speed centrifugations in a Servall angle centrifuge; of the infective phage particles, 90 to 95 per cent were regularly recovered after one hour at about 10,000 g.

The result of a typical experiment is shown in table 1. It is seen that the earlier phage growth is stopped, the greater the radioactivity per particle, e.g., at 15 minutes; when only about 10 per cent of the final phage yield has been formed, the purified lysate has approximately four times as many S35 counts per phage particle as are found after spontaneous lysis. This is true whether the radioactivity measurements are made directly on the purified lysates or on material specifically adsorbed on bacteria or precipitated with anti-T4 serum (columns 4 and 5).

These results suggest that the lysates contain, in addition to the finished phages, a number of S35-labeled, noninfective particles, which adsorb and precipitate with the specificity of the T4 phages. Under this assumption the decrease in radioactivity per plaque forming particle expresses the *change* in the ratio: (infective + noninfective particles)/(infective particles), with increasing phage yield. The data of table 1, however, do not permit estimation of the actual number of noninfective particles at any time. To obtain such estimates it is necessary to separate the two types of particles and evaluate the true radioactivity of the infective particles.

(2) *Estimation of the number of noninfective particles at different times during the latent period.* Since experiments with P32-labeled lysates failed

to indicate the presence of the noninfective particles, we assume that they are phosphorus-free and consequently contain no nucleic acid. It seemed likely, therefore, that they would be lighter than the complete phages and might be separated from them by some process of differential centrifugation.

TABLE 1

Radioactivity per phage particle after centrifugation of lysates at 10,000 g

KCN ADDED AT TIME, t=	FRACTION OF FINAL PHAGE YIELD AT TIME, t	S35 COUNTS/MIN/PLAQUE FORMING PARTICLE × 10^7		
		In lysate purified by centrifugation	In material adsorbed on HKB*	In material precipitated by anti-T4 serum
15	0.13	13.0	10.8	10.4
17	0.25	8.9	7.0	6.8
20	0.58	5.7	3.4	4.8
No	1.00 (1)	3.0	2.0	2.4
	1.00 (1)	2.9		2.1
KCN	1.00 (2)	3.1	1.8	
	1.00 (2)	2.7	1.7	

* HKB, means bacteria of strain B grown overnight in broth, washed, resuspended in buffer containing NaCl and $MgSO_4$, and heated to 70 C for 30 minutes. These bacteria were used in a concentration of about 10^9 cells per ml, and tryptophan was added to sensitize the phage. With heat-killed B/4 bacteria, adsorption of radioactivity was less than 10 per cent of that obtained with the sensitive B strain.

(1) Lysates purified after addition of 10 ultraviolet light inactivated T4r+ particles per T4r particle; (2) lysates purified without added carrier phage. The identical values obtained with and without carrier phage show that no appreciable amount of S35 compounds could adsorb on the phage and so cause an artificial increase in the radioactivity per particle.

The experiment designed to test this possibility was similar to the one described above, except that the lysates were purified in a Spinco centrifuge at 40,000 g in order to collect possible particles smaller than phage. Each high-speed centrifugation lasted 40 minutes, and to minimize the loss of sedimented material, 0.3 to 0.5 ml out of the 10 ml supernatant was always left to cover the pellet. Four such centrifugations were necessary to remove inorganic S35 sufficiently from the pellet material. Table 2, column 3, shows that the result at this stage is very similar to that presented in table 1. The lysates, therefore, cannot contain significant amounts of labeled material which can be isolated in the Spinco centrifuge but which fails to sediment at 10,000 g during one hour in the Servall centrifuge.

TABLE 2

Radioactivity per phage particle after differential centrifugation of purified lysates

KCN ADDED AT TIME, t=	FRACTION OF FINAL PHAGE YIELD AT TIME, t	RADIOACTIVITY PER PLAQUE FORMING PARTICLE (IN ARBITRARY UNITS)			(S35 OF NONINFECTIVE PARTICLES)/(S35 OF FINAL YIELD OF INFECTIVE PHAGE)*
		In material sedimented in 40 min at 40,000 g	In supernatant after subsequent 30 min at 10,000 g	In material recovered after 4 × 30 min at 10,000 g	
15	0.08	4.6	7.8	1.1	0.3
17	0.18	3.2	4.8	1.2	0.4
20	0.40	1.7	2.6	1.2	0.3
No KCN	1.00	1.3 (1) (1.2–1.35)	2.2 (1) (2.0–2.3)	1.0(1)	0.3

* Calculated from corresponding values of columns 2 and 3; e.g., 0.08(4.6 − 1) = 0.29.

(1) These estimates are derived from four independent experiments, including that which gave the rest of the data of this table. The figures in parens show the variation between these experiments.

All samples were tested by precipitation with anti-T4 serum: in samples containing sedimented material (columns 3 and 5), about 75 per cent of the radioactivity was precipitated, while in the supernatants (column 4) 50 to 60 per cent were recovered in the precipitates. The general trend of the experiment thus is the same whether the radioactivity per particle is based on measurements on the purified lysates or on specifically precipitated material.

The purified lysates then were spun four times successively for 30 minutes at about 10,000 g in the Servall centrifuge. About 75 per cent of the infective particles were recovered in each centrifugation to give a final recovery of 30 to 40 per cent. If the assumption that the noninfective particles sediment more slowly is correct, these particles should be eliminated gradually from the pellet material with the result that the radioactivity per phage particle should decrease. Conversely, in the supernatant the radioactivity per infective particle should increase. Columns 3 to

5 of table 2 show that both changes occur, and that selection for the relatively heavy phages reduced the radioactivity per infective particle to approximately the same low value in all cases. This value, which may be taken as an estimate of the true radioactivity per complete phage particle, has been chosen as unit. The amount of sulfur present in each sample in excess of that of the finished phages has been calculated from the figures of columns 2 and 3. Column 6 shows that in terms of sulfur the amount of noninfective material is constant at all times. If we assume that each noninfective particle contains as much sulfur as a finished phage—as is the case with the protein membranes—the figures of table 2 are direct estimates of the ratio: (infective + noninfective particles)/(infective particles). In this case the figures of column 6 represent the numbers of noninfective particles as fractions of the final phage yield.

The estimates of table 2 indicate that lysates obtained by adding cyanide at 15 minutes contain 3 to 4 noninfective particles per finished phage. After spontaneous lysis approximately the inverse ratio is found. These two types of lysate have been tested for their killing effect on bacteria. No difference in killing efficiency per plaque forming particle was detected from which we may deduce that the maximum killing efficiency of the noninfective particles is about 0.2.

(3) *Assimilation of phage sulfur from the medium.* By adding a labeled compound to cultures at different times before and after infection with phage, the assimilation of the compound and its incorporation into phage can be studied. Experiments with P32 and T4r phages have shown that on an average it takes about 14 minutes for a newly assimilated phosphorus atom to become part of a finished phage particle (Stent and Maaløe, 1953). The 14 minutes may be evaluated graphically as the average distance between the phosphorus assimilation curve and the curve showing the intracellular appearance of finished phage particles. Since these curves are roughly parallel, a good estimate is obtained by reading the distance between the 50 per cent points on the curves (6 to 7 and 20 to 21 minutes, respectively). Similar experiments have been made with S35. The sulfur assimilation curve also is found to be nearly parallel to the phage appearance curve. The distance between these curves is, however, only 6 to 7 minutes as measured between the 50 per cent points. The average time spent by phosphorus and sulfur atoms between assimilation and incorporation into finished phage particles is thus very different, being about 14 and 6 to 7 minutes, respectively.

Spontaneously lysed cultures to which S35 was added 12 or 16 minutes after infection have been purified and then centrifuged 4 times for 30 minutes at 10,000 g. As in the experiments of table 2 it was found that the radioactivity per phage particle decreased by 20 to 25 per cent as a result of the last four centrifugations. The infective and the noninfective particles, therefore, must be labeled to approximately the same extent even when the isotope is added late. This finding excludes the possibility that the noninfective particles found at all times constitute a background of early-made, incomplete phages which never achieve maturation; if this were the case, the noninfective particles would have remained unlabeled when S35 was added as late as 12 or 16 minutes after infection.

DISCUSSION

Problems concerning the interpretation and implications of the experimental results will now be discussed:

(a) For our conclusions it is essential that the material isolated from the lysates is labeled uniformly. The following considerations show that this is obtained: (1) The concentration of organic sulfur can be raised or lowered by a factor of two without affecting the incorporation of S35 into phage. This means that the amount of methionine and cystine used up during phage growth has no measurable effect on the specific activity in the medium. (2) The results obtained after final isolation of the phage show that early and late produced phages have the same specific activity (table 2).

(b) The evidence for the existence of labeled particles other than finished phages rests on the decrease in radioactivity per plaque forming particle with increasing phage yield (table 1). This change could be explained alternatively by assuming that in some way labeled material became adsorbed to the finished phages, more being found with the early than with the late ones. This possibility is unlikely as we know the lysates do not contain labeled material which will adsorb on unlabeled phages introduced after lysis (table 1), and it is ruled out completely by the experi-

ments which show that the radioactivity of the early phages can be reduced to that of the later ones by further purification (table 2).

(c) The noninfective particles have certain properties in common with finished phages. It is interesting, therefore, to compare them with other noninfective particles which have been reported. These are the protein membranes, or "ghosts", obtained by osmotic shock (Anderson, 1949), and the so-called "doughnuts". The latter name arises from the appearance of these particles in electron micrographs; they have been found in the material obtained on breaking open cells by decrease in pressure just before the appearance of the first finished phages (Levinthal and Fisher, 1952), and also by causing lysis to occur in the presence of proflavine (DeMars *et al.*, 1953). Present evidence is insufficient to say that these two kinds of doughnuts are the same, but we shall treat them together here. The known properties of these noninfective particles, which all lack nucleic acid, are listed below:

	SEDIMENT READILY, BUT MORE SLOWLY THAN PHAGE	ADSORB ON SENSITIVE BACTERIA	REACT WITH WHOLE ANTIPHAGE SERUM	KILLING EFFICIENCY ON BACTERIA
"Ghosts"	+	+	+	0.3–0.9
"Doughnuts"	+	−	+	<0.1
S35 particles	+	+	+	<0.2

The close resemblance between ghosts and S35 particles suggests that the latter also might be degeneration products of finished phages. The S35 particles could be imagined to arise from phages which after lysis adsorbed on debris, lost their nucleic acid, and desorbed again. The experiments of this paper show that desorption can account for the formation of at most a small fraction of the S35 particles: when cyanide is added at 15 minutes, 70 to 80 per cent of the isolated particles are found to be noninfective; if all particles were initially infective, dilute lysates, in which adsorption is negligible, should yield at least 5 times as many active phage per bacterium as undiluted lysates. Actually, when antibacterial serum is used, these yields are identical.

The fact that doughnuts adsorb poorly, if at all, on sensitive bacteria indicates that our preparations contain relatively few of them. If a large proportion of the S35 particles were doughnuts, the radioactivity per phage particle should be lower and more uniform in adsorption experiments than in precipitation experiments. Columns 4 and 5 of table 1 show no such difference. There are two reasons why doughnuts probably contribute little S35 to our purified preparations: their sulfur content, and their mass as estimated from light scattering measurements, are lower than those of the complete phage particles. Thus, a large fraction of the doughnuts may be eliminated from the pellet material during the initial 3 or 4 centrifugations, and the rest would contribute little to the total radioactivity of our preparation. Thus we can conclude that the S35 particles are larger than doughnuts and probably represent a later stage in phage development. Consequently, they may be difficult to distinguish from finished phages in the electron microscope.

(d) The possibility must be considered that the immature particles in the lysates are side products of phage synthesis. A certain fraction of the maturing particles might be defective and their protein parts constitute the immature particles. The rate of assimilation of phage sulfur shows that membrane material is made continuously, and defective particles would tend to accumulate during phage synthesis. Since the number of immature particles is the same at all times, this possibility can be ruled out. Alternatively, a number of particles might be made early which never achieve maturation but form a constant background of immature particles. That this is not so is shown by the fact that labeled immature particles are found even when S35 is added so late that the early phages remain unlabeled.

During the time interval studied finished phages are produced at a contant rate. Since the number of immature particles also is constant during this period, it seems reasonable that they represent a stage in normal phage synthesis.

(e) The model of phage reproduction presented in the introduction conceives of immature, genetically complete particles, probably rich in nucleic acid. These particles duplicate and develop into mature phages by acquiring the antigenic and adsorptive properties prescribed by their genetic constitution. The paradox brought out by our findings is that lysates do not seem to contain such particles at any stage of development prior to full maturation, but instead con-

tain a relatively large number of immature particles with antigenic and adsorptive specificity and without nucleic acid.

In terms of the isotopes used, the problem is thus: why we fail to isolate at intermediate stages of maturation particles which contain both phosphorus and sulfur. Two possible solutions to this problem are:

(1) The intermediate stage is of very short duration. Few particles in this stage would coexist at any given time and, therefore, they would escape detection. This notion implies that the nucleic acid containing particles and the protein membranes are made separately and that the immature particles in the lysates are nonattached membranes.

(2) Immature particles remain in the intermediate stage for an appreciable period, but they are unstable. Upon lysis they break down into soluble nucleic acid and empty membranes as mature phages do under osmotic shock. Under this model we are not forced to imagine that the protein membranes are made separately; they may be formed directly on the surface of the immature nucleic acid containing particles.

SUMMARY

The experiments indicate the presence in both premature and ordinary T4 lysates of noninfective particles containing sulfur. These particles adsorb on bacteria and precipitate with anti-T4 serum in a way similar to finished phage. However, they are somewhat lighter and have no appreciable killing effect on bacteria. Previous experiments with P32 show that they do not contain nucleic acid.

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