BIOCHEMICAL STUDIES OF VIRUS REPRODUCTION
IV. THE FATE OF THE INFECTING VIRUS PARTICLE*

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Despite frequent speculation concerning the mode of action of viruses, few biochemical studies of the virus-host cell relationship have been undertaken, and the sequence of steps in virus multiplication remains largely unknown. Any tenable theory of virus reproduction must consider both the rôle and the fate of the original infecting virus particle, and, at present, little evidence is available on this subject. Though the isotopic tracer technique lends itself to a study of this problem through the use of labeled virus, only one early and inconclusive experiment of this nature has been reported (2). In the course of study of virus precursors in the Escherichia coli T₅ bacteriophage system (3-6), procedures were developed for the preparation of P³²-labeled bacteriophage which made tracer experiments feasible under conditions of controlled infection. The research described below reveals that, while in the course of bacteriophage multiplication a significant amount of the phosphorus of the infecting virus particle appears in the progeny, much of the P is liberated into the medium in a soluble form. The isotopic data, together with observations in bacteriophage genetics (7-10), indicate that the original infecting virus particle does not survive reproduction.

EXPERIMENTAL

Procedure

Preparation of P³²-Labeled Bacteriophage—T₅r⁺ bacteriophage was labeled by growth on E. coli in broth containing 2 to 4 μc. of P³² per ml. After purification (4, 5) the radioactive phage was used for infection of bacteria actively growing in nutrient broth. The progeny of the labeled infecting phage was isolated as related below, and the radioactivity was measured in all the solutions obtained in the course of purification. The conditions for growth of the phage and the methods of assay and of measuring radioactivity have been previously described (4-6). About 95 per cent of the P found in the purified phage is present as desoxyribonucleic acid (DNA) (5). Preparations of bacteriophage obtained by these meth-

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ods have been shown to be essentially homogeneous in the ultracentrifuge and in the electrophoresis apparatus (4, 5). The labeled virus used for inoculation possessed somewhat lower infectivity (gm. of N per plaque-forming particle) than that customarily obtained. This was apparently due to the radioactivity incorporated into the phage. However, prior to infection 98 per cent of the radioactivity of the purified virus concentrate was sedimentable with the virus at 20,000 \( \times g \), and, upon infection, the adsorption of radioactivity by the bacteria paralleled the adsorption of the phage. Previous experiments had demonstrated that not more than 0.1 per cent of the DNA phosphorus of the phage exchanges with the inorganic phosphate of the medium (6).

**Injection with Labeled Virus**—Bacteria, actively growing in isotope-free broth at a concentration of about \( 2 \times 10^8 \) cells per ml., were infected with labeled virus at a phage to bacterium ratio of about 3:1. Under these conditions bacterial multiplication ceases (4, 11, 12), lysis inhibition ensues (11), and the phage produced represents for the most part a single generation of virus. In several experiments the bacteria were first singly infected with labeled virus (one phage per bacterium) and additional unlabeled virus was added at a later time.

**Isolation of Phage Progeny**—The isolation of the newly formed virus was accomplished by one of two methods: (1) In the first experiments described, the infected bacteria were separated from unadsorbed phage 15 minutes after infection by 10 minutes centrifugation at 4000 \( \times g \). The bacteria were resuspended in sterile broth and incubated with aeration at 37° overnight. The virus liberated was purified by repeated differential centrifugation in the high speed angle centrifuge, one step at 2000 \( \times g \) for 15 minutes to remove bacterial detritus and unlyzed bacteria, another at 20,000 \( \times g \) for 2 hours to sediment the phage. In this method the radioactivity was measured in the crude lysate, in the supernatants obtained at low and high speed, and in the phage concentrate. (2) In other experiments the bacteria were first singly infected with labeled virus (one phage per bacterium) and additional unlabeled virus was added at a later time. In other experiments the bacteria were infected with labeled virus (one phage per bacterium) and additional unlabeled virus was added at a later time. In larger volumes of culture were employed the unadsorbed phage was not removed and the newly formed phage was purified as previously described (4); i.e., by preliminary filtration through a Mandler candle, followed by concentration in the Sharples supercentrifuge and then differential centrifugation as above. The two procedures yielded similar products. The low speed supernatant solution in the first method is comparable to the filtered lysate in the second, and the high speed supernatant solution is analogous to the Sharples effluent.

**Results**

**Multiple Infection with Labeled Phage**—The distribution of radioactivity in the solutions obtained after multiple infection of \( E. coli \) with \(^{32}\)P-labeled
bacteriophage $T_4$ and purification of the progeny is shown in Table I. In this experiment 800 ml. of broth containing actively growing bacteria at a concentration of $2.5 \times 10^8$ cells per ml. were inoculated with 2 ml. of radioactive phage having a titer of $3.1 \times 10^{11}$ particles per ml. and a radioactivity of 50,000 counts per minute per ml. 15 minutes after infection the unadsorbed phage was removed by centrifugation. At this time 60 per cent of the phage and 67 per cent of the radioactivity had been adsorbed by the bacteria. The titer of the final lysate was $5.6 \times 10^{10}$ phage per ml. About 25 ml. of phage having a titer of $8.5 \times 10^{11}$ particles per ml. and an infectivity of $10^{-15.97}$ gm. of N per plaque were obtained on purification by differential centrifugation in the angle centrifuge. The radioactivity of the phage was calculated by multiplying the radioactivity per purified virus particle by the titer of the lysate. Practically all of the radioactivity of the recovered phage were in the nucleic acid fraction.

**Table I**

*Distribution of Radioactivity after Multiple Infection of E. coli with $P^{32}$-Labeled Bacteriophage $T_4$*

| Material                        | Titer (phage per ml.) | Radioactivity (c.p.m. per ml.) | per cent | c.p.m. per phage $\times 10^{-6}$ 
|--------------------------------|------------------------|--------------------------------|----------|----------------------------------
| Lysate                         | 5.8                    | 84.6                           | 100      | 1.4                              
| Low speed supernatant          | 6.2                    | 76                             | 89.8     | 1.2                              
| High speed supernatant         | 0.027                  | 41.5                           | 49.2     | 150                             
| Phage concentrate              | 85                     | 33.1* (485.2)                  | 39.1     | 0.57                            
| Purified phage concentrate     | 120                    | 34.5* (718)                    | 40.8     | 0.59                            

* Corrected to original volume; actual values given in parentheses.

per cent of the added radioactivity appeared in the phage progeny, and this value was unchanged after a second cycle of centrifugation. Half of the total radioactivity was found in the high speed supernatant solution. This solution was essentially free of phage and the radioactivity per phage particle in this supernatant solution was about 300 times as great as in the purified phage concentrate.

**Mixed Infection with Labeled and Unlabeled Phage**—In a second experiment (Table II), the bacteria were first infected with labeled phage at a phage to bacterium ratio of 1. 3 minutes after infection, 68 per cent of the phage and 67 per cent of the added radioactivity had been adsorbed. At this time unlabeled phage was added to the infected bacterial suspension, so that there were two additional phage particles per bacterium. The purpose of the experiment was to ascertain whether the first phage particle adsorbed contributed more radioactivity to the progeny than did
virus adsorbed at a later time. However, the same proportion of the radioactivity was found in the purified virus progeny as previously.

**Growth of Phage without Removal of Unadsorbed Labeled Phage**—Another series of experiments performed without removal of the unadsorbed phage, and by using the supercentrifuge for preliminary concentration of the phage, gave essentially similar results. The data are summarized in Table III. Despite the lack of removal of unadsorbed phage, the percentage of the added radioactivity that appeared in the newly formed virus was similar but slightly lower than in the previous experiments. As before, no difference in the result was found whether each bacterium was infected simultaneously with several labeled virus particles (multiple infection, Experiments I to III, Table III) or whether it was infected first with one labeled particle, and 3 minutes later unlabeled phage was added to the culture (Experiment IV).

**Nature of Unsedimentable Phosphorus**—In Experiment III described above it was found that 45 per cent of the P\textsuperscript{32} in the Sharples effluent could not be precipitated by cold 5 per cent trichloroacetic acid and, hence,

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**Table II**

<table>
<thead>
<tr>
<th>Material</th>
<th>Titer</th>
<th>Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( \text{c.p.m. per ml.} \times 10^{10} )</td>
</tr>
<tr>
<td>Lysate</td>
<td>6.6</td>
<td>38.2</td>
</tr>
<tr>
<td>Low speed supernatant</td>
<td>5.8</td>
<td>32.4</td>
</tr>
<tr>
<td>High “”</td>
<td>0.041</td>
<td>13.2</td>
</tr>
<tr>
<td>Phage concentrate</td>
<td>105</td>
<td>16.0* (257.6)</td>
</tr>
</tbody>
</table>

* Corrected to original volume; actual values given in parentheses.

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\( ^1 \) When large numbers of phage are used to infect large numbers of bacteria at a given multiplicity (ratio of phage to bacteria), the virus content is distributed among the bacteria in different multiplicities. The actual spread of the multiplicities is a Poisson distribution. Thus it can be calculated that, in the experiment in which bacteria were infected first with one labeled phage and then with two unlabeled phages, 64 per cent of the labeled phages were the first virus particles to be adsorbed by bacteria (the other labeled particles being the second or third phages adsorbed by multiply infected bacteria). However, in the experiment in which the ratio of labeled phage to bacteria was 3, the fraction of the total radioactivity contained in all the first particles adsorbed by each bacterium was 32 per cent. Accordingly, if only the first phage adsorbed contributed its P to the progeny, the radioactivity of the liberated virus should have been twice as great in the first experiment above as in the second.
was apparently not nucleic acid or attached to protein. Half of the radioactivity of the effluent which was soluble in cold trichloroacetic acid was precipitable as inorganic phosphate by alkaline magnesia mixture. The results indicate that much of the nucleic acid P of the original infecting virus particle was now present in the medium in a low molecular weight form. The same conclusion is obtained by inspection of the figures for total radioactivity per phage particle found in the purified concentrate compared to that in the Sharples effluent (see Table I, the last column).

**Table III**

*Distribution of Radioactivity after Injection of E. coli with P^32-Labeled Bacteriophage T₆*

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Crude lysate</th>
<th>Filter candle</th>
<th>Sharples effluent</th>
<th>Phage progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>% radioactivity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I*</td>
<td>100</td>
<td>15</td>
<td>63</td>
<td>22</td>
</tr>
<tr>
<td>II*</td>
<td>100</td>
<td>9</td>
<td>60</td>
<td>31</td>
</tr>
<tr>
<td>III*</td>
<td>100</td>
<td>11</td>
<td>57</td>
<td>32</td>
</tr>
<tr>
<td>IV†</td>
<td>100</td>
<td>0</td>
<td>70</td>
<td>31</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C.p.m. per phage ( \times 10^9 )</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>I*</td>
<td>1.9</td>
<td>140</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>II*</td>
<td>5.7</td>
<td>760</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>III*</td>
<td>0.59</td>
<td>18</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>IV†</td>
<td>1.4</td>
<td>230</td>
<td>0.44</td>
<td></td>
</tr>
</tbody>
</table>

* Multiple infection.
† Single infection with labeled phage, secondary infection with unlabeled phage.

**DISCUSSION**

Although all hypotheses of virus reproduction assume some sort of autocatalytic reaction (13), it cannot yet be said with certainty whether viruses multiply by logarithmic replication, by successive linear duplication, or otherwise. It seems probable, however, that, for all viruses so far studied, a single particle is sufficient to initiate an infection under ideal conditions of susceptibility of the host (14, 15). Indeed, for the coliphages (16) each active phage particle will give one plaque (phage colony). The original particle disappears within the cell and after reproduction cannot be distinguished from its progeny. In the first isotopic experiments on the fate of the infecting virus particle, Stanley (2), using P^32-labeled tobacco mosaic virus, found that most of the radioactivity finally appeared in normal components of the plant cell and only a small percentage appeared in the newly isolated virus. He assumed that but a small fraction of the
inoculating virus entered the cell as intact infectious virus and concluded that the incorporation of P₃₂ into new virus from active virus could not be distinguished from incorporation from virus disintegration products. This quandary is resolved in our experiments by the removal of unadsorbed phage. The E. coli bacteriophage system, moreover, possesses the advantage that multiple infection of the bacteria with phage of the wild type (r⁺) strain produces prolonged inhibition of lysis and gives large yields of virus comprising, for the most part, a single generation (4, 11, 12).

In all the experiments consisting of multiple infection of bacteria with labeled phage, the bulk of the radioactivity (49 to 63 per cent) was found in the medium as unsedimentable low molecular weight P. These results indicate that disintegration of the infecting virus particles occurs. The latter conclusion is corroborated by the finding that the radioactivity per phage particle in the supernatant solutions was at least 100-fold greater than that in the purified virus concentrate. About 10 to 15 per cent of the isotope was associated with filterable bacterial débris, and a significant portion (22 to 42 per cent) appeared in the isolated virus.

Although it could not be determined by direct experiment whether the radioactivity in the liberated phage was contained in unaltered original virus particles or was distributed among the progeny, the weight of the evidence favors the latter hypothesis. Hershey (8) has demonstrated that more than one particle of the same phage type can participate in reproduction inside the same bacterial cell. Delbrück and Bailey (17) have shown that, when a single bacterium is infected with two coliphages of closely related strains, both phages are adsorbed, and each multiplies successfully. Our own experiments indicate that, when bacteria are infected, first with one labeled particle and 3 minutes later with two unlabeled phages, the same fraction of the radioactivity appears in the isolated virus as when bacteria are simultaneously infected with several labeled phage particles. These data demonstrate (1) that several virus particles of the same or closely related strains may reproduce in a single cell without interference but not without interaction (see below), and (2) that the first particle adsorbed does not exert an inhibiting effect on other particles when the primary infection precedes secondary infection by only 3 minutes. Since each adsorption is equally fruitful, it seems probable that each multiplying virus meets the same fate; namely, disintegration during the

Not all the bacteria lyse simultaneously. It has been suggested that the first phages liberated are secondarily adsorbed on as yet unlysed cells, causing delayed lysis (11). On rupture of the latter bacteria, the newly formed but secondarily adsorbed phage may remain attached to the bacterial débris, accounting for the fraction of the radioactivity usually found in that material (10 to 15 per cent).

Dulbecco (10) presents evidence that the maximum number of particles of bacteriophage that can participate in intracellular growth in a single cell is 8 to 10.
process of reproduction. Further support for the view that the original virus particle is disrupted is found in the one-step growth curve studies of Doermann (18). By inducing premature lysis of infected bacteria through the use of sonic vibration or chemical agents, Doermann found that the average intracellular virus content is far less than 1 in the first half of the latent period (12).

The most plausible interpretation of our data is that all infecting virus particles are destroyed during reproduction, each contributing essential phosphorylated material to the progeny. Using the idea of recombination (17), Luria and Dulbecco (7, 9) have suggested from studies of coliphage inactivated by ultraviolet light that a transfer of “self-reproducing units” may take place, resulting in reactivation of the inactive particles. The ultraviolet light-absorbing loci involved in this interaction between two individually non-infective particles number 30 to 50 and have been compared to genes, and the virus particle itself to a “gene complex.” Moreover, Hershey and Rotman (8, 19) have suggested that T2, T4, and T6 coliphages are capable of mutual transfer of hereditary characteristics such as plaque type, host range, etc., when adsorbed in the same host cell. For example, if a single cell is infected with two virus particles bearing different genetic markers, each phage reproduces, but all possible “recombinants” are likewise obtained. Both workers, from two different lines of evidence, have arrived at the idea of independently multiplying subunits. Indeed, Luria (7) has suggested that reproduction takes place by independent reproduction of a number of units and incorporation of these into the final phage particles. Further evidence for the existence of “immature” phage particles is summarized by Cohen (12).

The P32 found in the isolated virus may be combined in one of the genetic units postulated by Hershey and Rotman (8, 19) and Luria and Dulbecco (7, 9). If this assumption is correct, these units must contain about one-third of the phosphorus of the phage; for this is the fraction appearing in the progeny. Finally, it should be pointed out that quantitatively the infecting virus contributes only a minute portion of the P of the progeny.

**SUMMARY**

The fate of the infecting virus particle has been investigated with P32-labeled *Escherichia coli* bacteriophage T6. The labeled virus used for infection was prepared by growth on *E. coli* in broth containing radioactive phosphorus and was purified by differential centrifugation. Bacteria in isotope-free broth were infected with the purified radioactive phage under conditions producing largely a single generation of virus. The greater part of the isotope was found in the medium as unsedimentable low molecular P, but a significant portion appeared in the liberated virus. The
fraction of the radioactivity appearing in the liberated virus was the same, whether bacteria were multiply infected simultaneously with several labeled phage particles or mixedly infected successively with labeled and unlabeled bacteriophage. The results indicate that the original infecting particle is disrupted within the cell and that about one-third of its phosphorus is transferred to the progeny. The significance of this conclusion is discussed in relation to the hypothesis that bacteriophage reproduction takes place by independent reproduction of a number of subunits and incorporation of these into the final phage particles.

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