Poliovirus Protein: Source of Amino Acids and Time Course of Synthesis*

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The techniques previously available for the study of purified animal viruses have not permitted the production under controlled conditions of sufficient virus for experiments involving degradative chemical analysis. The utilization of suspension cell cultures (1) and the development of a simple, convenient virus purification procedure (2) have now made possible an investigation of the questions of the origin and time of synthesis of virus protein. Type 1 poliovirus was propagated in HeLa S3 cells (3) differentially labeled with L-valine-Cl4, and the isotope content of the valine of the isolated virus protein was determined. The results indicate that the virus protein is assembled from the large intracellular amino acid pool of the cells, and that the synthesis of the virus protein begins almost at the same time as the appearance of mature virus, but proceeds thereafter at a more rapid rate than maturation.

EXPERIMENTAL

Materials and Methods

Propagation, Purification, and Assay of Virus—The cultivation of HeLa S3 cells in suspension, the infection of such cultures with Type 1 poliovirus, the isolation of purified virus from infected cultures, and the sedimentation of the purified virus in a centrifugally established CsCl gradient, have been described in the preceding paper (2). Viral assays by the plaque technique and determination of the course of viral maturation were likewise carried out as previously described (4).

Intracellular Amino Acid Pool—The intracellular pool was defined as the material soluble in cold trichloroacetic acid under the following conditions. Cell suspensions were centrifuged for 2 minutes at 1500 r.p.m., or until the pellet of cells was firmly packed. The pellet was resuspended in 5 to 10 ml of the supernatant fluid, transferred to a conical tube, and centrifuged again. The supernatant fluid was decanted, the pellet of cells suspended in 5 ml of cold 8% trichloroacetic acid, and the residue removed by centrifugation. The soluble fraction was extracted four times with 5 ml portions of ether to remove the tri-chloroacetic acid.

The aqueous fraction was evaporated to dryness under reduced pressure, and redissolved in water. Preparations from 1 ml of packed cells (2.5 × 106 cells) were of sufficient size for the isolation of amino acids by ion exchange chromatography. With samples of cells of this size, the contribution to the amino acid pool from contaminating medium was negligible. Protein hydrolysates were prepared from the trichloroacetic acid precipitate as described by Eagle et al. (5).

Chromatographic Methods—Cell pools, cell protein hydrolysates and media were analyzed for valine by ion exchange chromatography with a 50-cm column of Dowex 50 by an adaptation of the procedure of Pies et al. (6). Fractions of the column effluent were analyzed for amino acid with ninhydrin (7) and plated for determination of radioactivity (5).

The valine in hydrolysates of the viral protein was isolated on paper chromatograms developed in tert-amyl alcohol-2,4-lutidine-water (33:33:24 by volume) (6). The valine, which was located by treating a very narrow strip of the paper with ninhydrin, was eluted from the paper with water. The amount of valine in aliquots of eluate was determined by the colorimetric ninhydrin procedure (7) in a total volume of 1.2 ml. This procedure permitted the measurement of 0.01 µmoles of amino acid with reasonable accuracy, and the specific radioactivity of the valine derived from 250 µg of poliovirus protein could accordingly be determined.

Labelled Amino Acid—L-Valine uniformly labeled with C14 was obtained from the Schwarz Laboratories, Inc., and was used in the medium at a specific activity of around 20,000 c.p.m. per µmole.

Valine, an amino acid essential for growth of HeLa cells (8), which is neither synthesized nor degraded to any extent by these cells (9), was chosen for use because of the ease with which it can be isolated by paper and column chromatography.

RESULTS

Equilibration of Medium and Cell Pool—When cells previously grown in unlabeled medium are placed in a medium containing a labeled amino acid, there is a rapid equilibration between the medium and the intracellular pool, and the specific activity of a labeled amino acid in the medium and pool become virtually equal within 15 minutes. A similar result was obtained with cells infected with poliovirus. The infection was permitted to proceed for 8 hours, at which time viral production is largely completed, but release has just begun (4). Valine-C14 was added to the suspension of infected cells and aliquots were withdrawn for the preparation of pools at intervals thereafter. The specific activity of the valine of the pool was maximal within 5 minutes after the introduction of the labeled amino acid into

* A preliminary report of this work was presented before the American Society of Biological Chemists in April 1959.

the medium, indicating that, in infected cells as in growing cultures, changes in the composition of the medium are rapidly reflected in the composition of the pool.

Source of Amino Acid for Synthesis of Virus Protein—Previous studies from this laboratory had shown that poliovirus could be formed by full grown monolayer cultures in a medium lacking all amino acids except glutamine (10), and had suggested that the virus protein was synthesized from the free amino acids remaining in the intracellular pool under these conditions rather than from products of the degradation of cell protein (4, 11).

The use of the amino acid pool as the source of virus protein was confirmed by the experiments summarized in Table I. In Experiments A and B, the cell pool and protein were differentially labeled in the following manner. Cells previously grown in unlabeled medium were centrifuged and transferred, 1 hour before infection, to medium which contained valine-Cl4. There was no depletion of the valine from the medium in any experiments described, thus a constant reservoir of valine-C14 was afforded. At the time of infection, the cell pool was maximally labeled, while the specific activity of the valine in the cell protein was considerably lower.

The differential distribution of the label was maintained during the course of virus production. The valine of the purified virus isolated after 24 hours reflected the specific activity of the cell pool, which was less than 3% of that of the host cell protein. The small contribution of cell protein to the pool can be explained by the infected cell of the intracellular amino acid pool for virus protein synthesis and the rapid equilibration between amino acids in the medium and the pool, as indicated by the time course of the synthesis of viral protein. The addition of labeled amino acid into the medium of an infected culture after a part of the viral protein has been formed should result in a diminution of the specific activity of the corresponding amino acid in the virus relative to what it would have been if the label had been present from the beginning. If the reasonable assumption is made that there is negligible turnover of viral protein subsequent to maturation, the fraction of viral protein already formed when the time of addition of an amino acid label can be determined by measuring the specific activity of mature, purified virus in such a culture. In the experiments summarized in Table II and Fig. 1, infected cultures were divided into six equal parts, and valine-C14 was introduced at different times after infection. The virus was collected in 60 minutes, and the specific activity of the virus valine was determined.

The specific activity of the virus valine reflected that of the specific activity of the pool, indicating that it was derived in large part from this source.

A converse experiment was performed with cells grown for four generations in labeled medium and transferred to unlabeled medium 1 hour before infection (Experiment C, Table I). As before, the specific activity of the virus valine reflected that of the pool, which was less than 3% of that of the host cell protein. The small contribution of cell protein to the pool can be ascribed in large part to protein turnover (12) in the cells before and during the early stages of infection. The close agreement in specific activities of the pool and the virus protein in these two types of experiments indicates that the only portion of cell protein which is utilized for the synthesis of viral protein is that very small amount which enters the soluble pool by this process.

Time Course of Synthesis of Viral Protein—The exclusive use

![Figure 1](http://www.jbc.org/)

**Figure 1.** Time course of maturation of poliovirus, O, compared with the synthesis of virus protein, •, in two experiments. L-Valine-C14 was added at intervals to aliquots of an infected cell suspension. The percentage of virus matured was determined by dividing the average titer in each culture at each indicated time interval by the maximum titer, which in every culture occurred at 9 hours. The percentage of virus protein formed was obtained by dividing the specific activity (c.p.m. per optical density unit) in the purified virus from each aliquot by the maximum specific activity (1-hour culture) and subtracting from 100%.

**Table I**

Incorporation of L-valine-C14 into poliovirus from differentially labeled host cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time after infection</th>
<th>Total OD unit</th>
<th>Total PFU ( \times 10^5 )</th>
<th>OD unit</th>
<th>Total c.p.m.</th>
<th>c.p.m./OD unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Medium</td>
<td>0 hrs.</td>
<td>14,400</td>
<td>13,700</td>
<td>12,700</td>
<td>11,900</td>
<td>10,200</td>
</tr>
<tr>
<td>Cell pool</td>
<td>1 hr.</td>
<td>14,000</td>
<td>13,700</td>
<td>12,700</td>
<td>11,900</td>
<td>10,200</td>
</tr>
<tr>
<td>Cell protein</td>
<td>2 hrs.</td>
<td>13,900</td>
<td>13,700</td>
<td>12,700</td>
<td>11,900</td>
<td>10,200</td>
</tr>
<tr>
<td>Virus protein</td>
<td>3 hrs.</td>
<td>14,000</td>
<td>13,700</td>
<td>12,700</td>
<td>11,900</td>
<td>10,200</td>
</tr>
<tr>
<td>B. Cell pool</td>
<td>4 hrs.</td>
<td>13,700</td>
<td>13,400</td>
<td>12,400</td>
<td>11,600</td>
<td>9,900</td>
</tr>
<tr>
<td>Cell protein</td>
<td>5 hrs.</td>
<td>13,600</td>
<td>13,400</td>
<td>12,400</td>
<td>11,600</td>
<td>9,900</td>
</tr>
<tr>
<td>Virus protein</td>
<td>6 hrs.</td>
<td>13,600</td>
<td>13,400</td>
<td>12,400</td>
<td>11,600</td>
<td>9,900</td>
</tr>
<tr>
<td>C. Cell pool</td>
<td>7 hrs.</td>
<td>13,500</td>
<td>13,300</td>
<td>12,300</td>
<td>11,500</td>
<td>9,800</td>
</tr>
<tr>
<td>Cell protein</td>
<td>8 hrs.</td>
<td>13,400</td>
<td>13,200</td>
<td>12,200</td>
<td>11,400</td>
<td>9,700</td>
</tr>
<tr>
<td>Virus protein</td>
<td>9 hrs.</td>
<td>13,300</td>
<td>13,100</td>
<td>12,100</td>
<td>11,300</td>
<td>9,600</td>
</tr>
</tbody>
</table>

* Virus maturation began at 4 hours, and was complete at 9 hours; virus was harvested at 24 hours when release from cells was complete.

**Table II**

Effect of time of addition of L-valine-C14 to infected cultures on specific activity of purified poliovirus

<table>
<thead>
<tr>
<th>Hours after infection</th>
<th>Total OD unit</th>
<th>Total PFU ( \times 10^5 )</th>
<th>OD unit</th>
<th>Total c.p.m.</th>
<th>c.p.m./OD unit</th>
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<tr>
<td>1</td>
<td>1.699</td>
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<td>3648</td>
<td>2148</td>
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<tr>
<td>3</td>
<td>0.738</td>
<td>1.84</td>
<td>2.41</td>
<td>1569</td>
<td>2070</td>
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<tr>
<td>4</td>
<td>1.696</td>
<td>6.54</td>
<td>3.27</td>
<td>3315</td>
<td>1955</td>
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<tr>
<td>6</td>
<td>1.672</td>
<td>6.38</td>
<td>3.82</td>
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<td>641</td>
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<td>8</td>
<td>2.817</td>
<td>11.98</td>
<td>4.25</td>
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<td>90</td>
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<td>4.20</td>
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<td>30</td>
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</tbody>
</table>

* Fractions from CsCl gradients containing purified poliovirus were collected and the optical density at 260 mµ, infectivity and radioactivity determined. See text for experimental details.

† Optical density unit, 1 ml of material with optical density of 1 with a light path of 1 cm.

‡ PFU, plaque-forming units.
intervals into the aliquot. Since less than 0.1% of the cells in these experiments were uninfected 2 hours after addition of virus as judged by the inability to form clones, all cells could be expected to begin virus synthesis at approximately the same time. Purified virus was isolated from each culture after 21 hours as described (2). Each sample of purified virus was further characterized by sedimentation in a CsCl gradient, and infectivity, radioactivity, and optical density at 260 μm were measured in diluted fractions of the gradient. As previously described (2) the purified virus formed a single peak in the fractions of the gradient, and infectivity, radioactivity, and optical density were coincident.

The recovery of material from the different aliquots was reasonably uniform (Table II, Column 2), and the specific infectivity was fairly constant (Column 4). In contrast, the specific radioactivities expressed as counts per minute per unit of optical density at 260 μm (Column 6), declined sharply in the cultures to which label was added more than 4 hours after infection. The fraction of viral protein already formed and eventually incorporated into virus particles when label was added was determined by comparing the specific activity of the purified virus at each time period with the maximum value obtained when the label was added 1 hour after infection. The course of synthesis of virus protein determined by this method is compared with the specific activity of the purified virus at each time period with the maximum value obtained when the label was added 1 hour after infection. The course of synthesis of virus protein determined by this method is compared with the specific activity of the purified virus at each time period with the maximum value obtained when the label was added 1 hour after infection. The course of synthesis of virus protein determined by this method is compared with the specific activity of the purified virus at each time period with the maximum value obtained when the label was added 1 hour after infection. The course of synthesis of virus protein determined by this method is compared with the specific activity of the purified virus at each time period with the maximum value obtained when the label was added 1 hour after infection. The course of synthesis of virus protein determined by this method is compared with the specific activity of the purified virus at each time period with the maximum value obtained when the label was added 1 hour after infection. The course of synthesis of virus protein determined by this method is compared with the specific activity of the purified virus at each time period with the maximum value obtained when the label was added 1 hour after infection.

**Effect of Virus Infection on Protein Synthesis**—The effect of virus infection on the relative rates of uptake of L-valine-C14 into the protein of control and uninfected cultures are described in Table III. In accord with the experiments of Salzman and Lockhart (13), there was no evidence of a stimulation of net synthesis or protein turnover in the infected cell during the early periods of infection (14, 15). Moreover, the rate of protein turnover, which must be considered to be responsible for the labeling of protein in this experiment since no increase in protein occurred, declines sharply 2 hours after infection.

**DISCUSSION**

The studies of Siddiqi et al. (16) with bacteriophages and Commeron et al. (17) with tobacco mosaic virus clearly indicated that in those systems, virus protein was a product of synthesis de novo by the infected host cell, and that isotopically labeled small molecules were used in preference to the protein of the cell. A similar conclusion was drawn with respect to the poliovirus-HeLa cell system on the basis of virus yields in cells depleted of their amino acid pool (4, 11). The present experiments with differentially labeled cells provide direct evidence that poliovirus protein is a newly formed material in the infected HeLa cell, and that little or none of the cell protein is utilized for virus synthesis.

The classic studies of Herehey et al. (18, 19) in phage infected bacteria indicate that the first newly formed viral precursor molecules are DNA, followed by the production of protein. Elegant immunologic studies with bacteriophage have shown that virus protein is formed almost entirely during the course of maturation, not before. The data of DeMars (20) and Rountree (21) suggest that protein formation proceeds at a slightly more rapid rate than maturation, while Lanni (22) reports a perfectly parallel rate.

Our experiments similarly demonstrate that the poliovirus protein which is incorporated into viral particles is not synthesized until maturation begins. This suggests the possibility that maturation awaits virus-protein synthesis. This would be implied more strongly if the time course of polio-RNA synthesis were found to precede that of the protein. With two other animal viruses (23, 24), infectious RNA has been shown to accumulate in infected cells before the onset of maturation. It must be remembered that the only protein assayed in our experiments for its time of synthesis is that which is incorporated into whole virus particles. Thus it is possible that poliovirus antigens which are not incorporated into virus may appear before maturation begins. The data of Roizman et al. (25) suggest this as a possibility but are not sufficient to permit a firm conclusion on this point.

**SUMMARY**

1. Evidence is presented that the amino acids utilized in the synthesis of poliovirus in infected HeLa cells come from the intracellular free amino acid pool.

2. The synthesis of poliovirus protein which is incorporated into virus particles begins at the same time as intracellular maturation, but proceeds at a more rapid rate.

3. Protein synthesis and protein turnover are inhibited in infected cells after 2 hours of infection as judged by a depression in the ability to incorporate labeled amino acids.

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**Table III**

<table>
<thead>
<tr>
<th>Hours after suspension</th>
<th>Infected</th>
<th>Uninfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>label added</td>
<td>mg protein/10</td>
<td>c.p.m./mg</td>
</tr>
<tr>
<td>1</td>
<td>10.43</td>
<td>789</td>
</tr>
<tr>
<td>2</td>
<td>10.58</td>
<td>402</td>
</tr>
<tr>
<td>3</td>
<td>11.09</td>
<td>170</td>
</tr>
<tr>
<td>4</td>
<td>10.30</td>
<td>259</td>
</tr>
<tr>
<td>5</td>
<td>11.02</td>
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</tr>
<tr>
<td>6</td>
<td>10.36</td>
<td>126</td>
</tr>
<tr>
<td>11</td>
<td>10.48</td>
<td>17</td>
</tr>
</tbody>
</table>

* The 6 × 10⁶ cells were harvested and suspended in serum-free medium at 4 × 10⁶ cells per ml (See "Methods"). The cells were divided into two portions, one of which was infected with 1.2 × 10⁶ plaque-forming units, while the other served as an uninfected control; 10 ml (4 × 10⁶ cells) were withdrawn from both cultures at intervals and allowed to incorporate L-valine-C14 for 1 hour. Incorporation was stopped by addition of trichloroacetic acid to 5%. The total protein and the radioactivity incorporated into protein is described in the two cultures. Maturation of virus was 60% complete by 6 hours and complete by 11 hours.
REFERENCES

Poliovirus Protein: Source of Amino Acids and Time Course of Synthesis
James E. Darnell, Jr. and Leon Levintow