THE EFFECT OF POLIOMYELITIS VIRUS TYPE I (MAHONEY STRAIN) ON THE PHOSPHORUS METABOLISM OF THE HELA CELL*

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There have been many investigations on the uptake of radioactive phosphorus by viral infected cells (1, 2), but the number of studies in which cells of animal origin are employed are few. To our knowledge, there are no studies on the effects of poliomyelitis virus on phosphorus metabolism in the HeLa cell. However, this cell has been successfully infected by the poliomyelitis virus (3, 4).

In this study we are reporting the results of uptake of radiophosphorus into cellular phosphorus fractions (inorganic, phospholipide, and nucleic acid) of the HeLa cell with and without exposure to poliomyelitis virus type I (Mahoney strain).

EXPERIMENTAL

Virus Experiment—15 days before the experiment, 1 X 10⁶ HeLa cells were inoculated into 200 ml. culture flasks, and 10 ml. of growth medium were added according to the procedure of Syverton et al. (5) and allowed to grow for 15 days at 36° in a stationary state. Twice weekly one-half of the medium in the culture flasks was replaced with fresh growth medium, which consisted of 60 parts of Hank’s balanced salt solution (5), 10 parts of 1 per cent Difco yeast extract, 30 parts of human serum and antibiotics (penicillin and streptomycin, 50 units per ml. and 50 γ per ml., respectively).

15 minutes before virus inoculation, the growth medium was discarded and the HeLa cells were rinsed three times with 10 ml. aliquots of Hank’s solution. The culture flasks to be inoculated with the virus or the antiserum¹ received 9 ml. of maintenance medium (5), those to be inoculated

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¹ 100 TCID₅₀ neutralized by 1:1600 dilution of the type I antiserum. Obtained from the National Foundation for Infantile Paralysis, Inc.
with virus plus antiserum mixture 8 ml., and those left uninfected 10 ml. The maintenance medium consisted of 90 parts of maintenance solution (5) and 10 parts of chicken serum. Cultures inoculated with virus received, in addition to the maintenance medium, 1.0 ml. of poliomyelitis virus $\left(10^{-5.24} \text{TCID}_{50}\right)$. Cultures inoculated with antiserum received 1.0 ml. of diluted antiserum (1:25) in addition to the maintenance medium, and those inoculated with a virus-antiserum mixture received 2 ml. of a solution containing 1.0 ml. of virus $\left(10^{-5.24} \text{TCID}_{50}\right)$ plus 1.0 ml. of antiserum (1:25) in addition to maintenance medium. The total volume in all cultures was 10 ml. The culture flasks were rotated slowly (20 cycles per minute) for 30, 60, 120, and 240 minutes before harvesting the cells. 30 minutes before the cells were harvested from the flasks of a given time interval, a 2.0 ml. aliquot was removed for the titration of the virus (6) and 1.0 ml. of NaH$_2$PO$_4$ was added. The final concentration of P$^{32}$ in each flask was 7.3 µc. per ml. The isotope remained in contact with the cells for 30 minutes regardless of the time of the addition of the virus.

At the time of harvesting, the cells were scraped from the walls of four culture flasks with a rubber policeman and pooled and uniformly mixed, and an aliquot was removed for cell counting and virus titration. The remainder of the cell suspension was transferred into two glass homogenizing tubes and centrifuged in the cold at 2000 $\times$ g for 1.5 minutes. The supernatant fluid was poured off and the cells were washed with physiological saline and recentrifuged in the cold at 2000 $\times$ g for 1.5 minutes. The wash fluid was added to the original supernatant solution and the cells were inactivated with 10.0 ml. of cold 10 per cent trichloroacetic acid. The cellular material was fractionated by the Schneider method (7) into acid-soluble (total and inorganic), phospholipide, and nucleic acid (hot trichloroacetic acid-soluble). In addition, inorganic phosphate of the supernatant medium was determined. The lipide was extracted with alcohol-ether and purified with chloroform (8). Chemical analyses were carried out on these fractions by the Fiske and Subbarow method (9) with the Beckman DU spectrophotometer. Inorganic phosphate of the extracellular fluid and the cellular acid-soluble phosphate were separated by the Delory method (10). Radioactivity measurements were made by a Geiger-Müller counter (supercaler, Tracerlab, Inc.) and the specific activities calculated$^3$ (8).

**Virus for Experiment**—Poliomyelitis virus type I, Mahoney strain, was maintained by HeLa passage and stored at $-70^\circ$. Freshly passed and

$^2$ TCID$_{50}$ = the dilution of the virus just sufficient to kill 50 per cent of the 5 day-old cell cultures in the test.

$^3$ Specific activity = per cent of dose of P$^{32}$ inoculated $\times 10^{-9}$ per cell/mg. of P $\times 10^{-9}$ per cell.
pooled virus was used and titrated by serial dilution, the stationary HeLa tube method being used, with eight tubes per dilution. The TCID_{50} was determined to be 10^{-6.24} according to the Reed-Muench formula (6). For the experiment, a sufficient quantity of virus was thawed, diluted 1:10 with maintenance solution, and 1.0 ml. was added to each culture flask containing the HeLa cells.

**Antiserum for Experiment**—Poliomyelitis antiserum type I was obtained from the National Foundation for Infantile Paralysis, Inc. The serum was diluted 1:25 with Hank's solution and 1.0 ml. was added to the appropriate flasks.

**Results**

Through the use of multiple labeling studies with the aid of C^{14}-choline and inorganic P^{32}, it has been demonstrated that the incorporation of either isotope into phospholipide is a measure of the synthesis of phospholipide (11). Similar experimental procedures with C^{14}-adenine and P^{32} have demonstrated that various components of the nucleic acid molecule do not turn over independently (12). Hahn and Hevesey (13) have also shown that nucleic acid will not exchange with P^{32} when mixed with the latter in vitro. It is thus reasonable to assume that measurement of the radioactivity or the specific activity of the phospholipide and the nucleic acids of the HeLa cell during the ascending portion of the incorporation curve would be a measurement of synthesis of these components (14).

At any rate, under the conditions of our experiments, an increase in the formation of phospholipides and nucleic acids may be reasonably assumed when higher values for the total radioactivity are found, and the specific activity values are also higher, or at least not lower, than in the control cells (15).

It may be observed from the data of the uptake of P^{32} into nucleic acid and phospholipides in the HeLa cells maintained in growth medium that 30 minutes represent a period of incorporation which falls on the ascending portion of the incorporation curve (Table I). It may thus be assumed, in view of the results reported above, that we are measuring synthesis of these compounds in these cells.

HeLa cells bathed in maintenance medium show only slight incorporation of P^{32} (Table II). This is consistent with the findings that maintenance fluid is not conductive for cell growth and proliferation.4

In Table III are tabulated the results of P^{32} uptake into the various phosphorus fractions in the HeLa cells in which the bathing fluid is maintenance medium and the cells are exposed to poliomyelitis virus, a mixture

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4 Ross, J., and Syverton, J. T., private communication; thesis, Department of Bacteriology, University of Minnesota (1957).
of poliomyelitis virus and antiserum, and antiserum for varying lengths of time. The incorporation of P³² into the nucleic acid and the phospholipide fraction is accelerated many-fold as the time of virus contact is extended. A rapid incorporation of P³² into these organic compounds after 2 hours of virus exposure was evident from the increased specific activity values. A small but perceptible increase of specific activity during the early time periods of virus exposure was also evident. Cellular inorganic and the total acid-soluble phosphate specific activities for the infected cells were also higher than those of the controls.

**Table I**
Specific Activities of Phosphorus Fractions of HeLa Cell in Growth Medium*

<table>
<thead>
<tr>
<th>Time of incubation of P³²</th>
<th>Extracellular inorganic</th>
<th>Cold acid-soluble cellular inorganic</th>
<th>Total cold acid-soluble</th>
<th>Phospholipide</th>
<th>Nucleic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mg. of P per cell × 10⁻⁹</td>
<td>Specific activity</td>
<td>Mg. of P per cell × 10⁻⁹</td>
<td>Specific activity</td>
<td>Mg. of P per cell × 10⁻⁹</td>
</tr>
<tr>
<td>min.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>84.99</td>
<td>151</td>
<td>0.37</td>
<td>90.0</td>
<td>6.24</td>
</tr>
<tr>
<td>45</td>
<td>142.43</td>
<td>156</td>
<td>0.61</td>
<td>102.0</td>
<td>7.66</td>
</tr>
</tbody>
</table>

* Average values of a pool of two cultures in triplicate.

Specific activity = per cent of dose of P³² inoculated × 10⁻⁹ / mg. of P × 10⁻⁹ per cell. Radioactivity per cell = per cent of dose of P³² inoculated × 10⁻⁹. The dose of P³² inoculated 65.58 μc. per culture.

**Table II**
Specific Activities of Phosphorus Fractions of HeLa Cell in Maintenance Medium*

<table>
<thead>
<tr>
<th>Time of incubation of P³²</th>
<th>Extracellular inorganic</th>
<th>Cold acid-soluble cellular inorganic</th>
<th>Total cold acid-soluble</th>
<th>Phospholipide</th>
<th>Nucleic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mg. of P per cell × 10⁻⁹</td>
<td>Specific activity</td>
<td>Mg. of P per cell × 10⁻⁹</td>
<td>Specific activity</td>
<td>Mg. of P per cell × 10⁻⁹</td>
</tr>
<tr>
<td>min.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>230</td>
<td>44.13</td>
<td>2.25</td>
<td>27.95</td>
<td>9.20</td>
</tr>
<tr>
<td>30</td>
<td>246</td>
<td>39.13</td>
<td>2.32</td>
<td>28.38</td>
<td>9.35</td>
</tr>
<tr>
<td>40</td>
<td>412</td>
<td>40.28</td>
<td>3.39</td>
<td>30.22</td>
<td>14.68</td>
</tr>
</tbody>
</table>

* Average values of a pool of three cultures in triplicate.

Specific activity = per cent of dose of P³² inoculated × 10⁻⁹ per cell / mg. of P × 10⁻⁹ per cell. Radioactivity per cell = per cent of dose of P³² inoculated × 10⁻⁹. The dose of P³² inoculated 65.58 μc. per culture.
### Table III

**Specific Activities of Phosphorus Fractions of HeLa Cell with and without Exposure to Poliomyelitis Virus in Maintenance Medium**

<table>
<thead>
<tr>
<th>Time of virus exposure</th>
<th>Extracellular cold acid-soluble inorganic cellular inorganic % of P</th>
<th>Cold acid-soluble cellular inorganic % of P</th>
<th>Total cold acid-soluble Specific activity</th>
<th>Phospholipide Specific activity</th>
<th>Nucleic acids Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>Control: 150 101.36 0.72 46.60 14.57 6.83 4.49 0.10 9.17 0.14</td>
<td>Virus: 139 94.81 0.77 43.51 13.28 7.32 4.51 0.08 9.58 0.14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>Control: 127 104.38 0.78 45.08 11.12 7.36 4.56 0.13 10.12 0.13</td>
<td>Virus: 152 97.66 0.89 37.70 15.45 5.81 4.42 0.08 7.93 0.22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>Control: 85 111.52 0.65 43.09 14.78 5.16 3.34 0.20 4.54 0.33</td>
<td>Virus: 155 91.38 1.08 38.06 14.01 7.22 5.07 0.11 8.90 0.47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>240</td>
<td>Control: 117 95.75 0.99 33.57 18.76 5.12 4.09 0.18 4.29 0.28</td>
<td>Virus: 132 99.02 1.15 53.64 16.09 13.87 3.57 0.90 4.40 2.20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ Antiserum: 121 103.97 1.12 55.71 19.31 7.89 3.93 0.67 4.34 1.13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Average values of a pool of four cultures in triplicate. P<sup>32</sup> added to the medium 30 minutes before the cells were harvested.

Specific activity = per cent of dose of P<sup>32</sup> inoculated × 10<sup>-9</sup> per cell / mg. of P × 10<sup>-9</sup> per cell. Radioactivity per cell = per cent of dose of P<sup>32</sup> inoculated × 10<sup>-9</sup>. The dose of P<sup>32</sup> inoculated 59.5 μc. per culture.

### Table IV

**Comparison of Virus Titer with That of Specific Activities of Nucleic Acid and Phospholipide**

<table>
<thead>
<tr>
<th>Time of virus exposure</th>
<th>Virus titer: × 10&lt;sup&gt;14&lt;/sup&gt;</th>
<th>Per cent change</th>
<th>Specific activity nucleic acid</th>
<th>Per cent change</th>
<th>Specific activity phospholipide</th>
<th>Per cent change</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.15</td>
<td>0.0</td>
<td>0.14</td>
<td>0.0</td>
<td>0.08</td>
<td>0.0</td>
</tr>
<tr>
<td>60</td>
<td>0.51</td>
<td>236.0</td>
<td>0.22</td>
<td>57.2</td>
<td>0.08</td>
<td>0.0</td>
</tr>
<tr>
<td>90</td>
<td>120</td>
<td>1460.0</td>
<td>0.47</td>
<td>236.0</td>
<td>0.11</td>
<td>32.0</td>
</tr>
<tr>
<td>210</td>
<td>2.34</td>
<td>1460.0</td>
<td>2.18</td>
<td>1460.0</td>
<td>0.90</td>
<td>1000.0</td>
</tr>
</tbody>
</table>

Virus titer = 1/TCID<sub>50</sub> (the dilution of the virus just sufficient to kill 50 per cent of the 5 day-old cell cultures in the test). The 30 minute titer represents the minimal viral titer in the medium in these experiments. The drop in titer from the initial values indicates the penetration or adsorption of the virus into or on the cell. An increase in virus titer in medium subsequent to the 30 minute interval represents newly made or desorbed virus. Per cent change is calculated according to the average difference between the value of the 30 minute interval with the values of each subsequent time interval.
The virus titer and the specific activity in the medium as a function of time of virus contact with the cell are compared in Table IV. The virus titer and the increase in labeling of the nucleic acids trace out similar curves after the initial control period. The initial drop of viral titer in the medium is the result of a rapid penetration or adsorption of the virus on the cells (16-18). The increase of virus titer and the nucleic acid labeling with P$_{32}$ is almost identical for each time period. Those cells bathed in antiserum alone incorporated P$_{32}$ into the various fractions at about the same rate as the control cultures. On the other hand, the rate of incorporation of P$_{32}$ into the HeLa cells exposed to a mixture of virus and antiserum (with antiserum sufficient to neutralize only a portion of the virus) was above that for the control cultures but below those cell cultures exposed to the virus alone (Table III).

**DISCUSSION**

The HeLa cell does not grow and divide when bathed in maintenance medium. This can be determined by counting the actual number of cells in cultures with the passage of time. The low incorporation rate of P$_{32}$ into the several cellular fractions of these cells in maintenance fluid may be related to this failure to grow and divide (Table II). The effect of the growth medium may be observed by a comparison of the uptake of P$_{32}$ into the nucleic acid and phospholipide (Tables I and II). In the presence of virus and a medium which cannot supply the materials for normal cell growth, a large incorporation of P$_{32}$ occurs. What does the rapid incorporation of P$_{32}$ into the several cellular fractions in viral infected cells in maintenance medium represent? It is undoubtedly not a result of growth and proliferation of the cells, since viral infection of these cells causes a rapid decline of cell number, lysis being complete in 12 to 36 hours with the viral dosage used in these experiments (5, 19). It may represent an unsuccessful attempt by the cell to replace metabolites and maintain itself in the presence of a destructive agent. Finally, the rapid incorporation of P$_{32}$ may represent the synthesis of intracellular viral components. The experiments reported here cannot distinguish unequivocally between these two alternatives. However, it has been shown (20) that the bacterial purines, C$_{14}$-adenine and C$_{14}$-guanine, are used intact for the synthesis of viral nucleic acid. This lends credence to the idea of transfer of bacterial nucleic acid to viral nucleic acid. It has been further shown that bacterial nucleic acid P (deoxyribonucleic acid) (DNA) is most likely a source of viral (phage) nucleic acids (2).

It is of interest that the inorganic P in the experiments described here was incorporated into the nucleic acids of the cell at the same rate as the increase of virus titer in the medium (Table IV). However, the cells
themselves are being lysed. This suggests further that the incorporation of the P\textsuperscript{32} into organic components was used for purposes other than maintaining the cell integrity. In Table IV a comparison of the virus titer with the specific activity of the cell nucleic acid and phospholipide was made. The percentage increase of labeling of the nucleic acids with that of the increase in virus titer in the medium suggests that the increase in nucleic acid labeling is a measure of intracellular viral nucleic acid. In this regard it is of interest that Cohen's data (21) suggest that the absolute bacterial DNA is related to virus titer in the early time periods of the infection.

A progressive increase of lipide phosphorylation with extended viral infection was demonstrated (Table III). The reason for, or the fate of, the newly formed phospholipide molecules is not known. 4 hours after viral infection, the incorporation of P\textsuperscript{32} into phospholipide is 5.0 times that of the phospholipide of the control cells. Moldave (22) had also found that infection of mouse brain cells with Theiler's GD VII virus resulted in an increase of the specific activity in the lipide P by a factor of 1.3 times that of the controls. It is of interest that Cohen (1) found no P\textsuperscript{32} incorporation into phospholipide of bacteria infected with phage. The possibility of inorganic P\textsuperscript{32} contaminating the phospholipide, and thus giving the latter a high specific activity, can be discounted on the grounds that contamination would be equal in all experimental groups. This is not the case. In addition, \textit{in vitro} experiments in this laboratory show that the inorganic P\textsuperscript{32} is not carried over into chloroform from the alcohol-ether.

The control cultures as well as the infected cultures show decreasing amounts of nucleic acid phosphorus per cell as the incubation progresses. This may be the result of the presence of the foreign protein (chicken serum) in the medium. It has been shown that foreign protein in the medium, bathing liver cells \textit{in vitro}, does affect the metabolism of the cell (23). In the present experiments chicken serum was used in place of human serum to exclude the possibility of homotypic antibodies to the virus employed. It is highly likely that the foreign protein has caused a progressive loss of organic phosphorus from the cell.

The effectiveness of the antiserum in neutralizing the virus and thus reducing the uptake of P\textsuperscript{32} was tabulated in Table III. The amount of antiserum added to the virus was chosen so as to give only partial neutralization of the virus. In this way, it was possible to assess the effect of virus-antiserum interaction. It is evident that the antiserum which was present in amounts sufficient to give only partial neutralization was adequate to prevent these cells from behaving as a typical infected cell culture, and their response was therefore intermediate between control
and infected cells. The results obtained from these experiments suggest that the uptake of P32 into the nucleic acid fraction of the HeLa cell might be utilized for titrating virus or antiserum. This aspect of the problem is being further investigated.

SUMMARY

The uptake of radiophosphorus (P32) into cellular phosphorus fractions (inorganic, phospholipide, and nucleic acid) by the HeLa cell, the HeLa cell exposed to poliomyelitis virus plus antiserum, and antiserum alone has been investigated.

The uptake of radiophosphorus by HeLa cells exposed to the poliomyelitis virus was accelerated into the nucleic acid and lipide fractions.

The increased uptake into nucleic acids has been related to the increase of virus titer in the medium.

BIBLIOGRAPHY

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