Incorporation of 
Radioactivity from ¹⁴C-Sugars into Macromolecules in 
Poliovirus-infected or Guanidine-treated HeLa Cells

By I. L. GRAVES

Department of Epidemiology and Virus Laboratory, University of Michigan, 
Ann Arbor, Michigan, U.S.A.

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SUMMARY
¹⁴Carbon from glucose-U-¹⁴C was found to be incorporated into DNA, 
RNA and protein in HeLa cell cultures infected with poliovirus. This 
incorporation was partially inhibited by the 2nd hr of infection. The inhibition 
was related to the multiplicity of exposure to poliovirus and DNA was 
inhibited to a greater degree than RNA. In infected and normal HeLa 
cultures the rate of evolution of ¹⁴CO₂ from glucose-1-¹⁴C was 77 times 
greater than from glucose-6-¹⁴C. The incorporation of ¹⁴C from these sugars 
into macromolecules in normal and infected cultures did not reflect this 
difference, thus the ¹⁴C in the macromolecules comes from the glycolytic 
pathway rather than the hexosemonophosphate shunt. Concentrations of 
guanidine inhibiting poliovirus replication also inhibit the incorporation of 
¹⁴C from glucose-U-¹⁴C into DNA and RNA in normal and infected cultures. 
In cultures receiving both virus and guanidine, the inhibitory effects of each 
were not additive, suggesting that the viral inhibition of ¹⁴C incorporation 
requires some early step in virus replication.

INTRODUCTION
This study is concerned with the incorporation of ¹⁴C from uniformly labelled 
glucose, specifically labelled glucose and ribose into DNA, RNA, and protein in 
HeLa cells infected with type 1 poliovirus. The effects of guanidine, an inhibitor of 
poliovirus replication, on the incorporation of ¹⁴C from glucose into nucleic acids 
has also been examined.

The purpose of the investigation was to determine whether ¹⁴C from the distal 
precursor sugars would be incorporated into the macromolecules during infection or 
guanidine administration and, if so, which metabolic pathways are taken and whether 
virus infection causes a shift in the pathways utilized by the ¹⁴C precursors.

METHODS
Poliovirus and tissue culture. HeLa cells were grown in monolayers in Eagle's (1955) 
medium. MAHONEY strain of type 1 poliovirus was grown in HeLa cells and concen-
trated by centrifugation. Titrations were conducted in 2 oz prescription bottles and 
expressed as p.f.u./ml. The multiplicity of exposure in all experiments, except where 
otherwise indicated, was approximately 150 p.f.u./cell. African green monkey kidney 
cells (BS-C-1) were cultured by the method described by Hopps et al. (1963).
Extraction and separation of the polymers. Using the extraction method of Colter, Brown & Ellem (1962), the aqueous phase of a phenol extract of HeLa cells was found to contain DNA, RNA and polyglucose (Segovia et al. 1965). RNA and polyglucose precipitated from a 20% (v/v) ethanol, 1 M-NaCl solution (Segovia et al. 1965). Five per cent (v/v) trichloracetic acid solution was used to precipitate the RNA from the polyglucose (Graves et al. 1967; Graves, 1967a). The RNA precipitate was dissolved in 0.01 M-phosphate buffered saline, pH 7.2, reprecipitated and washed with cold trichloracetic acid and finally hydrolysed in trichloracetic acid (100° 10 min.). The hydrolysate (0.5 ml. containing similar quantities of radioactive material) was added to an appropriate amount of scintillation fluid (Kinard, 1957) and assayed for radioactivity in an Ansitron counter equipped with an external standard.

The scintillation fluid contained the following ingredients: 80 g. naphthalene, 5 g. 2,5-diphenyloxazole (PPO), 0.05 g. P-bis[2-(5 phenyl-oxazolyl)]-benzene (POPOP), p-dioxane q.s. to 1 l. Using this system, the specific activities (counts/μg./min.) of macromolecules from poliovirus and/or guanidine-treated cultures were compared with those from control cultures. The RNA content was quantitatively determined by the orcinol method (Dische, 1955). The DNA precipitated in 50% (v/v) ethanol (Segovia et al. 1965). Following precipitation, the DNA was purified and its radioactivity determined in the same manner as the RNA. The DNA was quantitatively determined with the Burton (1956) method. Protein was retrieved from the phenol-saline interphase and purified by two chloroform-methyl alcohol (2:1) extractions, one cold trichloracetic acid, and one hot (100°; 15 min.) trichloracetic acid, extraction. It was washed again in cold trichloracetic acid, dissolved in 0.1 M-NaOH and quantitatively measured with the Lowry method (Lowry et al. 1951).

Cultures which contained 2 × 10⁷ cells yielded approximately 1000, 1400 and 2800 μg. of DNA, RNA, and polyglucose respectively. Eighty per cent of the total ¹⁴C count in the aqueous phase of the phenol extract was recovered in the macromolecules.

Evolution of ¹⁴CO₂ from specifically labelled glucose. Cheldelin (1961) has described the methods for delineation of metabolic pathways using specifically labelled ¹³C compounds. The following experiments use glucose-1-¹⁴C and glucose-6-¹⁴C. The rate of evolution of ¹⁴CO₂ from glucose-1-¹⁴C is a measure of the activity of the hexose-monophosphate shunt whereas the evolution from glucose-6-¹⁴C is a measure of the activity of the glycolytic and Krebs cycle pathways. Wood, Katz & Landou (1963) have, however, described some possible limitations in these interpretations.

The HeLa cells (4.2 × 10⁶/culture) used in ¹⁴CO₂ evolution experiments were cultured in Eagle's medium in 250 ml. French square milk dilution bottles. Ten min. before the experiment was begun, the sodium bicarbonate buffered medium was replaced with tris (0.007 M final concentration) buffered medium containing 0.01 of the normal glucose concentration. It was found that this method would maintain a pH of 6.9 in the presence of a constant stream of water-saturated air flowing over HeLa cell cultures for at least 20 hr at 37°. Two μc of either glucose-1-¹⁴C or glucose-6-¹⁴C in 0.4 ml. of PBS was added to 11.6 ml. of the tris-buffered medium and a stream of water-saturated air was passed over the cultures. The ¹⁴CO₂ evolving from the HeLa cell cultures was trapped during 30 min. intervals in 3 ml. of 2 M-NaOH and no less than 0.3 ml. of the NaOH was added to 10 ml. of scintillation fluid and assayed for radioactive count.

Radioactive ¹⁴C compounds. Uniformly (-U-) labelled glucose (2.18 mc/mm), glucose-
Radioactivity from $^{14}$C sugars into macromolecules

$^1$-$^{14}$C (1.5 mc/mm²) and glucose-6-$^{14}$C (1.6 mc/mm²), were obtained from Volk Radiochemical Co. Ribose-1-$^{14}$C (0.05 mc/3.2 mg) was obtained from New England Nuclear Corporation. Each cell culture received, as a pulse label for 1 hr, 3 µc of a radioactive compound. The time of labelling varied as indicated in the specific experiments. During the period of exposure to the isotope, the Eagle’s medium was replaced with fresh medium containing 0.01 of the normal amounts of glucose in each experiment unless otherwise indicated.

Guanidine hydrochloride. This compound (lot 43A) was obtained from Eastman Kodak, Rochester, N.Y.

RESULTS

The inhibitory effect of poliovirus infection upon the incorporation of $^{14}$C from $^{14}$C-sugars into DNA, RNA and protein of HeLa cells

To learn whether radioactivity from glucose-U-$^{14}$C, glucose-1-$^{14}$C, glucose-6-$^{14}$C and ribose-1-$^{14}$C would be incorporated into macromolecules, 3 µc of each were used to pulse-label infected and control cultures during the 2nd to the 3rd hr of infection.

Table I. The specific activity* of polymers isolated from poliovirus-infected and control cultures

<table>
<thead>
<tr>
<th>Sugar</th>
<th>DNA</th>
<th>RNA</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected control</td>
<td>Infected control</td>
<td>Infected control</td>
</tr>
<tr>
<td>Glucose-1-$^{14}$C</td>
<td>0.19</td>
<td>1.5</td>
<td>0.26</td>
</tr>
<tr>
<td>Glucose-6-$^{14}$C</td>
<td>0.10</td>
<td>1.2</td>
<td>0.25</td>
</tr>
<tr>
<td>Glucose-U-$^{14}$C</td>
<td>0.11</td>
<td>1.5</td>
<td>0.29</td>
</tr>
<tr>
<td>Ribose-1-$^{14}$C</td>
<td>0.16</td>
<td>2.4</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>0.17</td>
<td>2.2</td>
</tr>
</tbody>
</table>

* Radioactive counts/µg./min.
† The time of pulse labelling was the 2nd to the 3rd hr of infection.
‡ a and b are duplicate experiments.

The specific activities (radioactive count/µg./min.) of the extracted macromolecules are listed in Table I. The macromolecules from infected cultures incorporated $^{14}$C from each of the sugars. This result indicates that the pathways for $^{14}$C utilization are open during the 2nd to the 3rd hr of infection. Glucose-1 and glucose-6 contributed $^{14}$C to a similar degree to most macromolecules. However, virus infection partially inhibited the incorporation of $^{14}$C irrespective of the source of the $^{14}$C or the type of macromolecule. These facts initiated the following lines of enquiry: (1) at what time in the infectious sequence does the inhibition of incorporation of $^{14}$C occur? (2) Does the inhibition occur only in HeLa cells? (3) What is the effect of the multiplicity of exposure of poliovirus upon the incorporation of $^{14}$C from glucose-U-$^{14}$C? (4) Since $^{14}$C from glucose-1-$^{14}$C and glucose-6-$^{14}$C is incorporated to a similar extent, does the hexosemonophosphate shunt or do the glycolytic and Krebs cycle pathways contribute most of the $^{14}$C that is incorporated into the macromolecules of infected and normal HeLa cells? (5) What effect does guanidine, a known inhibitor of poliovirus, have on the incorporation of $^{14}$C from glucose-U-$^{14}$C into nucleic acids from uninfected HeLa cells as well as cells receiving both poliovirus and guanidine?
The time of inhibition and cell type specificity

Cultures of HeLa cells were infected with poliovirus and pulse labelled at hourly intervals with glucose-U-14C. The nucleic acids were extracted and their specific activities determined. The results are listed in Table 2 and are expressed as percentage of control values. The average specific activity of DNA and RNA extracted from control cultures was 1.7 and 3.7 respectively. In most experiments the inhibition due to virus infection occurred during the 2nd hr of infection but it was never complete. BS-C-1 cells also showed inhibition of 14C incorporation, indicating that the inhibitory effect is not limited to HeLa cells.

Table 2. Incorporation of 14C from glucose-U-14C into nucleic acids extracted from cultures infected with poliovirus for various times

<table>
<thead>
<tr>
<th>Interval*</th>
<th>DNA</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-2 hr</td>
<td>2-3 hr</td>
</tr>
<tr>
<td>Expt 1</td>
<td>78</td>
<td>43.2</td>
</tr>
<tr>
<td>,, 2</td>
<td>61.6</td>
<td>44</td>
</tr>
<tr>
<td>,, 3</td>
<td>108</td>
<td>35.5</td>
</tr>
<tr>
<td>,, 4*</td>
<td>89.4</td>
<td>42.3</td>
</tr>
</tbody>
</table>

* The interval of exposure to glucose-U-14C was measured from the time of addition of poliovirus. The incorporation is expressed as percent of control values.
† BS-C-1 cells were used in Expt 4.
Radioactivity from $^{14}$C sugars into macromolecules

The effect of graded multiplicities of poliovirus on the incorporation of $^{14}$C from glucose-U-$^{14}$C into DNA, RNA and protein

Figure 1 illustrates the effect of increasing multiplicities of poliovirus on the inhibition of incorporation of $^{14}$C from glucose-U-$^{14}$C into the macromolecules. The pulse-labelling was during the 2nd to the 3rd hr of infection. With DNA, the inhibition was more dependent on the multiplicity of exposure than RNA and protein. At the highest multiplicity (186 p.f.u./cell) incorporation of $^{14}$C into DNA was inhibited 50% more than into RNA.

The average specific activity of the proteins from control cultures was 0.6 counts/µg./min. and an average of 700 µg. of protein was used to determine the radioactive count.

The rate of $^{14}$CO₂ evolution from glucose-1-$^{14}$C and glucose-6-$^{14}$C in uninfected and poliovirus-infected cultures

The data in Table 1 show that $^{14}$C from either glucose-1 or glucose-6 is incorporated into most macromolecules to a similar extent. If the relative activities of the hexose-monophosphate shunt and the glycolytic and Krebs cycle pathways were known, it would be possible to infer which pathway contributes most of the $^{14}$C to the macromolecules. To measure these activities, 2 µC of glucose labelled in either the 1st or 6th position were added to infected (multiplicity of exposure = 290 p.f.u./cell) and control cultures. Figure 2 illustrates that the rate of $^{14}$CO₂ liberation from glucose-1 was greater than that from glucose-6. The total amount of $^{14}$CO₂ liberated during successive 30 min. intervals from one infected and two control cultures is presented. In addition, the rate of $^{14}$CO₂ liberation from a pulse label of glucose-6-$^{14}$C during the 2nd to the
3rd and 3rd to the 4th hr of infection was not influenced by poliovirus infection (multiplicity of exposure = 252 p.f.u./cell). The incorporation of $^{14}$C from specifically labelled sugars (Table I) was measured during the 2nd to the 3rd hr of infection and at this time the hexosemonophosphate shunt (Fig. 2) liberated 77 times more $^{14}$CO$_2$ than the glycolytic and Krebs cycle pathways. Poliovirus infection did not influence the rate of $^{14}$CO$_2$ evolution during this period. These experiments (Table I, Fig 2) show that even though the $^{14}$C from the 1st position was liberated 77 times more rapidly, the amount of $^{14}$C which was incorporated into the macromolecules was similar.

**The inhibition of incorporation of $^{14}$C from glucose-U-$^{14}$C into nucleic acids extracted from cultures treated with guanidine for various times and concentrations**

Since guanidine is a known inhibitor of poliovirus replication, the effect of this compound on normal cultures was examined. In this HeLa cell system, guanidine at 75 µg./ml. completely inhibited poliovirus multiplication.

Table 3. *The inhibition of incorporation* of $^{14}$C from glucose-U-$^{14}$C into nucleic acids extracted from cultures treated with guanidine for various times

<table>
<thead>
<tr>
<th>Nucleic acid extracted</th>
<th>Interval†</th>
<th>0–1 hr</th>
<th>1–2 hr</th>
<th>2–3 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Guanidine (µg./ml.)</td>
<td>75</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>DNA</td>
<td>Expt 1</td>
<td>81.6</td>
<td>72.2</td>
<td>81.6</td>
</tr>
<tr>
<td></td>
<td>Expt 2</td>
<td>N.D.</td>
<td>N.D.</td>
<td>87.6</td>
</tr>
<tr>
<td></td>
<td>Expt 3</td>
<td>100</td>
<td>51.7</td>
<td>58.8</td>
</tr>
<tr>
<td>RNA</td>
<td>Expt 1</td>
<td>78.4</td>
<td>75.8</td>
<td>72.8</td>
</tr>
<tr>
<td></td>
<td>Expt 2</td>
<td>N.D.</td>
<td>N.D.</td>
<td>87.3</td>
</tr>
<tr>
<td></td>
<td>Expt 3</td>
<td>81.5</td>
<td>75.5</td>
<td>56.8</td>
</tr>
</tbody>
</table>

* Incorporation is expressed as percentage of control values.
† The times of addition of glucose-U-$^{14}$C were measured from the start of the guanidine treatment.
N.D. = Not determined.

Table 4. *The specific activity* of RNA and DNA isolated from HeLa cells

<table>
<thead>
<tr>
<th>Nucleic acid</th>
<th>No treatment</th>
<th>Virus</th>
<th>Virus + guanidine†</th>
<th>Guanidine†</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>8.3</td>
<td>5.5</td>
<td>5.6</td>
<td>6.0</td>
</tr>
<tr>
<td>DNA</td>
<td>4.4</td>
<td>2.9</td>
<td>2.9</td>
<td>2.5</td>
</tr>
</tbody>
</table>

* Incorporation of $^{14}$C (counts/µg./min.) from glucose-U-$^{14}$C which was present in the cultures during the 2nd to the 3rd hr of infection.
† Guanidine (75 µg./ml.) was added at the same time as the virus. The glucose in the medium was 0.01% of normal in all cultures at the time guanidine was added. Note that the inhibitory effects of guanidine and virus infection are not additive.

The data in Table 3 are expressed as percentage of control, non-treated values and show the inhibitory effect of guanidine on the incorporation of $^{14}$C into DNA and RNA extracted from normal HeLa cells. In each of the three experiments the DNA was isolated from the same cultures as the RNA. The cultures were pulse labelled at hourly intervals following treatment. Note that in all of these experiments the guanidine dose and inhibition response was discontinuous. For example, in Expt 1, the inhibition of incorporation into DNA and RNA was apparent after 1 hr of guanidine treatment.
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(75 $\mu$g./ml.) and the inhibition increased during the following 2 hr of incubation. However, treatment with graded amounts of guanidine for 3 hr did not result in an orderly dose response. Treatment with 37.5 $\mu$g./ml. guanidine resulted in an inhibition of incorporation into DNA of 18.4 %; 75 $\mu$g. resulted in an inhibition of 43.3 %; 300 $\mu$g./ml. did not increase the degree of inhibition of incorporation of $^{14}$C into DNA. The inhibition of incorporation into RNA imposed by guanidine was also discontinuous which, in both instances, indicates that it acts at multiple sites.

The inhibition of incorporation of $^{14}$C from glucose-$U$-$^{14}$C in infected, guanidine-treated cultures

Table 4 lists the specific activities of RNA and DNA extracted from four different cultures: control (no treatment), infected, infected and guanidine-treated, and guanidine-treated. Guanidine (75 $\mu$g./ml.) was added at the same time as the virus. At 0 hr the glucose concentration in all cultures was reduced to 0.01 of normal. The cultures were pulse labelled during the 2nd to the 3rd hr of infection. The inhibition of $^{14}$C incorporation into RNA and DNA in the guanidine-treated culture was of the same order of magnitude as observed in the culture receiving virus and guanidine. The inhibitory effects of virus infection and guanidine treatment were not additive.

DISCUSSION

Gifford & Syverton (1957) found that oxygen uptake and production of CO$_2$ under anaerobic conditions were not altered early in the infection of HeLa cells with type I poliovirus. Their results are in keeping with the present findings that the rates of $^{14}$CO$_2$ evolution from specifically labelled ($^{14}$C-1, $^{14}$C-6) glucose were not affected by poliovirus infection. It seems apparent that the glucose metabolic pathways examined here do not shift during poliovirus infection.

Although the rate of $^{14}$CO$_2$ liberated via the hexosemonophosphate shunt was 77 times that of the glycolytic and Krebs cycle pathways, the amount of $^{14}$C incorporated into the macromolecules from infected and control cultures did not reflect this difference. If, in HeLa cells, the hexosemonophosphate shunt were a major pathway of the macromolecule precursors, glucose-1-$^{14}$C would contribute less $^{14}$C than glucose-6-$^{14}$C. A reason for this is that the $^{14}$CO$_2$ from the glucose-1-$^{14}$C would escape from the system more rapidly, leaving less $^{14}$C for incorporation into the macromolecules. If, however, the glycolytic pathway contributes most of the $^{14}$C, the specific activity of the macromolecules would be similar whether glucose was labelled in the 1st or the 6th position. The reason for this is that after cleavage of the glucose molecule, each three-carbon moiety would have one $^{14}$C label irrespective of whether glucose was labelled in the 1st or the 6th position. This one $^{14}$C in each three-carbon moiety would have an equal chance of being incorporated into the macromolecules. Within the limits of this system (Wood et al. 1963) the data indicate that in HeLa cells the glycolytic scheme is the major pathway taken by precursors of host macromolecules in poliovirus-infected and normal cells.

Most experiments (Table 2) indicate that poliovirus infection inhibits the incorporation of $^{14}$C from glucose-$U$-$^{14}$C into macromolecules as early as the 1st to the 2nd hr of infection. Since $^{14}$CO$_2$ evolution (Fig. 2) was not affected by virus infection, the specific reactions that are inhibited by virus infection probably involve the products
of the glycolytic pathway at some point(s) following decarboxylation of the precursor moieties.

The time during infection when the incorporation of the more proximal DNA precursor, thymidylate, is inhibited also helps delineate the point(s) where poliovirus infection inhibits host DNA synthesis. Using the same line of HeLa cells and strain of poliovirus, the incorporation of [3H]thymidylate was not inhibited by 3 hr after infection (Ackermann & Wahl, 1966). This is 2 hr later than the inhibition of incorporation of 14C from glucose-U-14C into DNA. These facts suggest that poliovirus infection inhibits the host’s distal precursor reactions before the proximal DNA precursor reactions are inhibited.

At the higher multiplicities of exposure to poliovirus (Fig. 1), DNA was inhibited to a greater degree than RNA or protein. During the 2nd to the 3rd hr of infection experiments using specifically labelled sugars (Table 1) as well as glucose-U-14C (Table 2) showed more inhibition of 14C into DNA than RNA. This may indicate that the distal precursor reactions leading to host DNA synthesis are more sensitive to inhibition by virus infection than those leading to RNA synthesis.

Guanidinium is thought to inhibit the synthesis of poliovirus RNA polymerase (Lwoff, 1965). Guanidinium’s inhibition of 14C incorporation into nucleic acids opens the question of whether this effect also contributes to inhibiting poliovirus replication. This may be tenable since normal HeLa cells are affected by concentrations of guanidinium which completely inhibit virus replication.

In poliovirus-infected, guanidinium-treated cultures, the incorporation of thymidylate, a more proximal DNA precursor, was similar to that of control, non-treated cultures (Ackermann, Davies & Wahl, 1967). These observations support the view that the distal rather than the proximal DNA precursor reactions are more sensitive to guanidinium’s action. However, in glycogen synthesis guanidinium did inhibit the incorporation of the proximal precursor glucose (Graves & Ackermann, 1966; Graves 1967b).

The inhibitory effects of guanidinium and of virus infection on the incorporation of 14C into DNA and RNA were not additive (Table 4). This suggests that the inhibition of 14C incorporation into RNA and DNA detected soon after infection requires active virions. It appears that the partial inhibition of 14C from sugars into macromolecules is related to and dependent on some early event in the sequence of virus replication.

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REFERENCES


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