THE SYNTHESIS OF L-ASCORBIC ACID IN THE RAT FROM D-GLUCURONOLACTONE AND L-GULONOLACTONE*

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(Received for publication, June 15, 1956)

Previous studies have shown that the intact carbon chain of glucose is utilized for the synthesis of L-ascorbic acid in the rat (1-3). Evidence for this precursor relationship came from experiments in which the administration of carbon 1- and carbon 6-labeled glucose tracers resulted in the urinary excretion of L-ascorbic acid labeled predominantly in carbon 6 and carbon 1, respectively. Although these earlier studies were carried out in rats receiving Chloretone to increase their normal rate of L-ascorbic acid biosynthesis (4), recent results have shown that glucose is also a precursor of L-ascorbic acid in rats not treated with this drug (5).

Two possible pathways for the biosynthesis of L-ascorbic acid from glucose in rats have been investigated. One, involving L-sorbose as an intermediate, was ruled out in a previous isotopic study (6). The other pathway involving D-glucuronolactone and L-gulonolactone as intermediates is shown in the accompanying scheme.

![Chemical Reaction Scheme]

* This study was supported in part by the Josiah Macy, Jr., Foundation, New York, New York.
Some evidence for this latter scheme has been presented previously. Isherwood and coworkers (7) reported that the administration of D-glucuronolactone and L-gulonolactone to rats produced an increase in the urinary excretion of L-ascorbic acid. In addition, Horowitz and King (8) found that the administration of uniformly labeled D-glucuronolactone to Chloretone-treated rats resulted in the excretion of uniformly labeled L-ascorbic acid.

In the present investigation further evidence for this pathway of L-ascorbic acid biosynthesis was obtained by demonstrating that normal and Chloretone-treated rats are able to convert carboxyl-labeled D-glucuronolactone and L-gulonolactone to carboxyl-labeled L-ascorbic acid.1 Results were also obtained which indicate that neither compound is converted to L-ascorbic acid in guinea pigs. Incidental to this study, observations were made showing that L-gulonolactone is appreciably oxidized to CO₂ in guinea pigs and rats.

**EXPERIMENTAL**

*Radioactive Compounds*—D- and L-Gluconolactone-6-C¹⁴, sodium D- and L-Gluconolate-6-C¹⁴, and D-glucose-1-C¹⁴ were obtained from the National Bureau of Standards, Washington, D. C., and the compounds had specific activities of 0.68, 0.65, and 1.0 μC. per mg., respectively. L-Gulonolactone-1-C¹⁴ was synthesized by reducing sodium D-glucuronate-6-C¹⁴ with sodium borohydride (10).2 The L-gulonolactone-1-C¹⁴ had a specific activity of 0.17 μC. per mg. after recrystallization from glacial acetic acid. Material prepared in a non-radioactive trial synthesis had a melting point of 184–185° and an optical rotation of [α]²⁰ +53.6° (c 0.3, water) (10). Its elemental analysis was as follows:

\[ \text{C}_9\text{H}_{10}\text{O}_5 \]. Calculated, C 40.4, H 5.66; found, C 40.2, H 5.84

L-Gulonic acid-1-C¹⁴ was prepared by treating L-gulonolactone-1-C¹⁴ at 50° with a stoichiometric amount of NaOH in aqueous solution.

*Experimental Animals*—Male albino rats of the Wistar strain, weighing from 270 to 310 gm., were maintained on a basal diet of evaporated milk for at least 10 days before each experiment. In the experiments on the effect of Chloretone, 45 mg. of the drug were administered daily for a period of at least 4 days prior to the injection of the labeled compound. The Chloretone was given in a single dose by stomach tube as a homogenate in 1 ml. of evaporated milk. Male guinea pigs, weighing 250 to 300 gm., were maintained on a vitamin C-free diet supplemented daily with 5 mg.

1 A preliminary report of this work has been presented previously (9).

2 Some details of this synthesis were kindly supplied by Dr. H. S. Isbell of the National Bureau of Standards, Washington, D. C.
of L-ascorbic acid orally for 7 days prior to each experiment. The labeled compounds employed in the various experiments were dissolved in 1 ml. of water, and they were administered by intraperitoneal injection.

Conversion to L-Ascorbic Acid—The conversion of the various precursors to L-ascorbic acid in normal rats and guinea pigs was determined by estimating the amount of $^{14}C$-L-ascorbic acid present in the animal 24 hours after administration of the labeled compounds. This was done as follows: The animals were sacrificed, and their livers, adrenals, spleens, testes, and kidneys were removed. The pooled sample of the various tissues of each animal was homogenized in the cold with about four times its weight of 5 per cent trichloroacetic acid, and the protein residue was removed by centrifugation. L-Ascorbic acid present in the supernatant fluid was determined by titration of an aliquot with indophenol dye (11). The L-ascorbic acid in the remaining supernatant fluid was isolated by an ion exchange method (4, 12) after the addition of a weighed quantity of carrier L-ascorbic acid (100 to 200 mg.). The specific activity of the L-ascorbic acid present originally in the tissue sample was calculated from the specific activity of the isolated L-ascorbic acid and the amount of L-ascorbic acid present before addition of carrier. The per cent incorporation of the various labeled precursors into L-ascorbic acid was obtained by multiplying the calculated specific activity (expressed as per cent of dose per mg.) by the body pool of L-ascorbic acid, 10 mg. per 100 gm. body weight for rats (4) and 5.4 mg. per 100 gm. body weight for guinea pigs. The results of these experiments give minimal values for the incorporation of the various labeled compounds into L-ascorbic acid, since no correction is made for the amount of labeled L-ascorbic acid metabolized and excreted in the urine during the 24 hour period after their administration. This correction would be relatively small, however, since L-ascorbic acid is slowly metabolized and excreted by normal rats and guinea pigs with a half life of about 3 days in both species (4, 13).

The conversion of the various labeled compounds to L-ascorbic acid in Chloretone-treated rats was determined by measuring the incorporation of $^{14}C$ into urinary L-ascorbic acid collected during a 24 hour period after administration of the labeled compounds (1, 6).

Degradation Procedure—The $^{14}C$ in carbon 1 of L-ascorbic acid was obtained as CO$_2$ by decarboxylation with hot mineral acid (1).

Measurement of Radioactivity—The method for collection and preparation of samples and their assay for $^{14}C$ were described previously (6). The radioactive purity of the L-ascorbic acid isolated from urine and tissues was established by finding constant specific activity of the L-ascorbic acid

* Dayton, P. G., and Burns, J. J., to be published.
and its 2,4-dinitrophenylsazone derivative after successive crystallizations (1, 6).

**Results**

The incorporation of C\(^{14}\) into body L-ascorbic acid of normal rats was measured 24 hours after the administration of D-glucose-1-C\(^{14}\), D-glucuronolactone-6-C\(^{14}\), L-gulonolactone-1-C\(^{14}\), sodium D-glucuronate-6-C\(^{14}\), and sodium L-gulonate-1-C\(^{14}\) (Table I). It will be noted that the per cent incorporation of D-glucuronolactone-6-C\(^{14}\) and L-gulonolactone-1-C\(^{14}\) averaged 2.2 and 8.1 per cent, respectively, compared to 0.045 per cent for D-glucose-1-C\(^{14}\). No C\(^{14}\) was detected in L-ascorbic acid after administration of D-glucuronate-6-C\(^{14}\) and L-gulonate-1-C\(^{14}\), indicating that the lactone structures are apparently required for L-ascorbic acid biosynthesis.

The incorporation of C\(^{14}\) into urinary L-ascorbic acid during 24 hours following administration of D-glucose-1-C\(^{14}\), D-glucuronolactone-6-C\(^{14}\), and L-gulonolactone-1-C\(^{14}\) to Chloretone-treated rats was measured (Table II). It will be noted that the incorporation of D-glucuronolactone-6-C\(^{14}\) and L-gulonolactone-1-C\(^{14}\) into urinary L-ascorbic acid averaged 1.2 and 3.6 per cent, respectively, compared to 0.45 per cent for glucose-1-C\(^{14}\). However, these are minimal values for the incorporation of these precursors into L-ascorbic acid, since about one-half the total L-ascorbic acid synthesized daily by Chloretone-treated rats is not excreted in the urine but is further metabolized (4). The actual values for the incorporation of the various

### Table I

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Compound</th>
<th>Dose of labeled compound</th>
<th>Per cent of dose in L-asorbic acid*</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-84</td>
<td>D-Glucose-1-C(^{14})</td>
<td>35.0</td>
<td>0.055</td>
</tr>
<tr>
<td>R-85</td>
<td>&quot;</td>
<td>35.0</td>
<td>0.035</td>
</tr>
<tr>
<td>R-20</td>
<td>D-Glucuronolactone-6-C(^{14})</td>
<td>8.2</td>
<td>2.3</td>
</tr>
<tr>
<td>R-22</td>
<td>&quot;</td>
<td>8.2</td>
<td>2.0</td>
</tr>
<tr>
<td>R-31</td>
<td>L-Gulonolactone-1-C(^{14})</td>
<td>11.9</td>
<td>9.1</td>
</tr>
<tr>
<td>R-32</td>
<td>&quot;</td>
<td>11.9</td>
<td>7.2</td>
</tr>
<tr>
<td>R-53</td>
<td>D-Glucuronate-6-C(^{14})†</td>
<td>5.1</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>R-78</td>
<td>&quot;</td>
<td>5.1</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>R-49</td>
<td>L-Gulonate-1-C(^{14})†</td>
<td>12.2</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>R-52</td>
<td>&quot;</td>
<td>12.2</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

* For method of calculation see the experimental section.
† Sodium salt.
compounds into L-ascorbic acid are, therefore, about twice those reported in Table II; that is 0.9, 2.4, and 7.2 per cent for D-glucose, D-glucuronolactone, and L-gulonolactone, respectively. These values for the incorpora-

**Table II**

*Incorporation of C\(^{14}\) into Urinary L-Ascorbic Acid during 24 Hours after Administration of Various Labeled Compounds to Chloretone-Treated Rats*

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Compound</th>
<th>Dose of labeled compound</th>
<th>Per cent of dose in L-ascorbic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-1*</td>
<td>D-Glucose-1-C(^{14})</td>
<td>10.0</td>
<td>0.50</td>
</tr>
<tr>
<td>R-2*</td>
<td>&quot;</td>
<td>10.0</td>
<td>0.40</td>
</tr>
<tr>
<td>R-5A</td>
<td>D-Glucuronolactone-6-C(^{14})</td>
<td>16.1</td>
<td>1.1</td>
</tr>
<tr>
<td>R-5B</td>
<td>&quot;</td>
<td>16.1</td>
<td>1.3</td>
</tr>
<tr>
<td>R-42</td>
<td>L-Gulonolactone-1-C(^{14})</td>
<td>6.4</td>
<td>3.9</td>
</tr>
<tr>
<td>R-43</td>
<td>&quot;</td>
<td>6.4</td>
<td>3.8</td>
</tr>
<tr>
<td>R-96</td>
<td>&quot;</td>
<td>6.4</td>
<td>3.1</td>
</tr>
</tbody>
</table>

*Previously published experiments (5).*

**Table III**

*Distribution of C\(^{14}\) in L-Ascorbic Acid after Administration of Various Labeled Compounds to Normal and Chloretone-Treated Rats*

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Compound</th>
<th>Per cent of total C(^{14}) in L-ascorbic acid in carbon 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-20</td>
<td>D-Glucuronolactone-6-C(^{14})</td>
<td>85</td>
</tr>
<tr>
<td>R-22</td>
<td>&quot;</td>
<td>83</td>
</tr>
<tr>
<td>R-31</td>
<td>L-Gulonolactone-1-C(^{14})</td>
<td>87</td>
</tr>
<tr>
<td>R-32</td>
<td>&quot;</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloretone-treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-5A</td>
<td>D-Glucuronolactone-6-C(^{14})</td>
<td>88</td>
</tr>
<tr>
<td>R-5B</td>
<td>&quot;</td>
<td>92</td>
</tr>
<tr>
<td>R-42</td>
<td>L-Gulonolactone-1-C(^{14})</td>
<td>84</td>
</tr>
<tr>
<td>R-96</td>
<td>&quot;</td>
<td>85</td>
</tr>
</tbody>
</table>

tion of labeled D-glucuronolactone and L-gulonolactone to L-ascorbic acid in the Chloretone-treated rat are similar to those obtained for the incorporation of these lactones into L-ascorbic acid in the non-drug-treated animal (Table I). However, it should be noted that there is a marked difference in the incorporation of labeled glucose into L-ascorbic acid in
normal and Chloretone-treated rats which is a reflection of the increased amounts of L-ascorbic acid synthesized in the drug-treated animal (4). The distribution of C\textsuperscript{14} in the L-ascorbic acid synthesized after administration of D-glucuronolactone-6-C\textsuperscript{14} and L-gulonolactone-1-C\textsuperscript{14} to normal and Chloretone-treated rats was measured (Table III). The L-ascorbic acid isolated in the various experiments was labeled predominantly in carbon 1, indicating that D-glucuronolactone and L-gulonolactone are

\begin{table}
\centering
\caption{\textit{C\textsuperscript{14} in Body L-Ascorbic Acid 24 Hours after Administration of Labeled Compounds to Guinea Pigs}}
\begin{tabular}{l|l|c|c}
\hline
Experiment No. & Compound & Dose of labeled compound & Per cent of dose in L-ascorbic acid* \\
\hline
G-25A & D-Glucuronolactone-6-C\textsuperscript{14} & 4.9 & <0.1 \\
G-25B & " & 4.9 & <0.1 \\
G-36 & L-Gulonolactone-1-C\textsuperscript{14} & 11.7 & <0.2 \\
G-37 & " & 11.7 & <0.2 \\
\hline
\end{tabular}
\end{table}

* For method of calculation see the experimental section.

\begin{table}
\centering
\caption{\textit{C\textsuperscript{14} in Expired CO\textsubscript{2} and Urine during 24 Hour Period after Intraperitoneal Administration of L-Gulonolactone-1-C\textsuperscript{14} to Guinea Pigs and Rats}}
\begin{tabular}{l|l|c|c}
\hline
Experiment No. & Species & Per cent of dose in \\
& & CO\textsubscript{2} & Urine \\
\hline
R-31 & Rat & 69 & 29 \\
R-32 & " & 68 & 22 \\
G-36 & Guinea pig & 55 & 30 \\
G-37 & " & 67 & 25 \\
\hline
\end{tabular}
\end{table}

converted to L-ascorbic acid without appreciable fragmentation of their carbon chains.

The incorporation of C\textsuperscript{14} into body L-ascorbic acid of guinea pigs was measured 24 hours after the administration of D-glucuronolactone-6-C\textsuperscript{14} and L-gulonolactone-1-C\textsuperscript{14} (Table IV). It will be noted that no detectable incorporation of either compound was observed in guinea pig, values being less than one-twentieth those in rats.

The excretion of C\textsuperscript{14} in respiratory CO\textsubscript{2} and urine was compared following administration of L-gulonolactone-1-C\textsuperscript{14} to guinea pigs and rats (Table V). L-Gulonolactone is appreciably oxidized to CO\textsubscript{2} in guinea pigs and rats; most of the C\textsuperscript{14} in CO\textsubscript{2} was recovered within 5 hours after the dose. In
contrast, sodium gulonate-1-C\textsuperscript{14} is oxidized to CO\textsubscript{2} to a considerably lesser extent than L-gulonolactone-1-C\textsuperscript{14} as shown by the results of the following experiment: Two rats each received a 12 mg. intraperitoneal dose of sodium L-gulonate-1-C\textsuperscript{14} and the amounts of C\textsuperscript{14} in respiratory CO\textsubscript{2} and urine were determined over a 24 hour period. Essentially all the injected C\textsuperscript{14} was found in the urine, less than 10 per cent of the dose being present in the respiratory CO\textsubscript{2}.

DISCUSSION

The results of this investigation are in agreement with a pathway of L-ascorbic acid biosynthesis from D-glucose via D-glucuronolactone and L-gulonolactone in both normal and Chloretone-treated rats. For instance, finding that carboxyl-labeled D-glucuronolactone and L-gulonolactone are converted to L-ascorbic acid labeled chiefly in carbon 1 indicates that the carbon chain of these lactones is converted without fragmentation to L-ascorbic acid. Since no information is available on the turnover and pool sizes of D-glucuronolactone and L-gulonolactone, it is not possible at the present time to estimate how much of the total L-ascorbic acid synthesized each day in normal and Chloretone-treated rats originates via these compounds.

The most likely intermediate involved in the conversion of L-gulonolactone to L-ascorbic acid is either 2-keto- or 3-keto-L-gulonolactone. Since these compounds undergo spontaneous enolization to L-ascorbic acid (14), their role in the biosynthesis of L-ascorbic acid in rats was not evaluated in this study. Experiments in rats with C\textsuperscript{14}-labeled 2-keto-L-gulonic acid indicate, however, that this acid is not an intermediate in the conversion of L-gulonolactone to L-ascorbic acid.\textsuperscript{3}

The results of this investigation, indicating that D-glucuronolactone and L-gulonolactone are not converted to L-ascorbic acid in the guinea pig, point out the possible missing biochemical reaction in this species and perhaps in man needed for the synthesis of L-ascorbic acid. A more definitive conclusion concerning the specific biochemical reaction which is missing will be possible when information is available on whether or not D-glucuronolactone can be converted to L-gulonolactone in the guinea pig.

It has been known for considerable time that certain drugs such as Chloretone and various barbiturates when administered to rats markedly increase the urinary excretion of L-ascorbic acid (15). Results of a previous turnover rate study showed that this increase in excretion of L-ascorbic acid results from an actual acceleration in its rate of synthesis (4). The results of the present study suggest that the primary effect of Chloretone is to increase the synthesis of D-glucuronolactone which is then utilized for the synthesis of L-ascorbic acid. Support for this interpretation comes
from the observation that Chloretone administration to rats produces a marked increase in the excretion of both L-ascorbic acid and D-glucuronic acid (16). In the guinea pig, however, Chloretone administration results only in an increase in the excretion of D-glucuronic acid, since this species is unable to synthesize L-ascorbic acid. At this time no conclusion can be drawn on the actual mechanism by which a drug, such as Chloretone, accelerates the synthesis of D-glucuronolactone. However, the ability of a drug to be conjugated as a glucuronide is apparently not required, since barbital, which is excreted completely unchanged in the urine, markedly increases the urinary excretion of both D-glucuronic acid and L-ascorbic acid by rats and of D-glucuronic acid by guinea pigs.4

SUMMARY

Carboxyl-labeled D-glucuronolactone and L-gulonolactone are converted in normal and Chloretone-treated rats to carboxyl-labeled L-ascorbic acid, indicating that the intact carbon chain of these lactones is utilized for the biosynthesis of L-ascorbic acid. Carboxyl-labeled D-glucuronic acid and L-gulonic acid, however, are not converted to L-ascorbic acid in the rat, indicating the importance of the lactone structure in the biosynthesis of L-ascorbic acid.

No conversion of L-gulonolactone and D-glucuronolactone to L-ascorbic acid was detected in the guinea pig, an indication of the possible missing biochemical reaction in this species needed for the synthesis of L-ascorbic acid.

Carboxyl-labeled L-gulonolactone was found to be extensively oxidized to CO₂ in both rats and guinea pigs. However, no appreciable oxidation of carboxyl-labeled sodium L-gulonate was observed.

The authors of the paper gratefully acknowledge the assistance of Dr. Erwin H. Mosbach in carrying out the synthesis of the labeled L-gulonolactone.

BIBLIOGRAPHY


4 Unpublished observations from our laboratory.