ABSTRACT

In the dark, leaf tissue of crisphead lettuce (Lactuca sativa L.) metabolized 14CO to 14CO2 and acid-stable products. Tissue incubated at 2.5°C for 3.5 hours and 48 hours converted about 1% and 17%, respectively, of the applied 14CO to 14CO2, and incorporated about 0.04% and 0.6% of the 14C in acid-stable products. Examination of soluble acid-stable products from 14CO and 14CO2-treated leaf tissue revealed that the labeling patterns of both treatments were identical during the 3.5-hour and the 48-hour incubation periods. Malate, citrate, and aspartate together comprised 70% or more of the soluble radioactivity from both treatments. Incorporation of radioactivity from CO into soluble acid-stable products during a 3-hour incubation period at 2.5°C was inhibited 90% by adding 3% nonradioactive CO2. These results indicate that in head lettuce in the dark 14CO is metabolized primarily to 14CO2 which is the precursor of acid-stable products. In leaf discs at 2.5°C, the apparent Kₘ for CO oxidation to CO2 was 5.3 micromolars per liter and the Vₘₐₓ was 9.7 nanomolars per gram per hour. The mitochondrial fraction of the leaf homogenate was the most active fraction to oxidize CO to CO2, and this activity was heat-labile and cyanide-sensitive.

MATERIALS AND METHODS

Plant Material. Crisphead lettuce (Lactuca sativa L.) was obtained from a local market. Leaf discs (1.1 cm in diameter) or strips (1 x 4 cm) were prepared using a cork borer or a razor blade.

14CO Generation. 14CO was generated from sodium [14C]formate (New England Nuclear, 52 mCi/mmol). A desired aliquot of the radioactive sodium formate solution was pipetted into a 5.3 ml test tube and taken to dryness under N2 stream. The tube was sealed with a rubber stopper and slightly evacuated. After injecting 0.25 ml concentrated H2SO4, the tube was heated for 15 min at 90°C to generate CO. After cooling, the gas in the tube was transferred via a small tubing (2 mm diameter) with attached hypodermic needles on both ends to a sealed, evacuated 100 ml volumetric flask containing a few ml of 6 N KOH to absorb any contaminating 14CO3.

14CO Measurement. 14CO was measured by oxidizing it to 14CO2 (14). Oxidation was performed in an enclosed 50 ml Erlenmeyer flask containing a 14 x 45 mm shell vial and a few granules (approx. 0.2 g) of I2O5. The flask was sealed with a serum stopper and slightly evacuated. A gas sample of 14CO and 0.5 ml of unlabeled CO as carrier were injected into the flask which was then heated on a sand bath at 100°C for 2.5 h. After cooling, 0.2 ml ethanolamine:2-ethoxyethanol (1:1, v/v) was injected into the shell vial to absorb the 14CO2 which was measured by liquid scintillation counting. This method gave quantitative conversion of CO to CO2 as verified by GC.

Comparison of Labeled Products from 14CO and 14CO2. Labeled products from 14CO- and 14CO2-treated lettuce were examined for two incubation periods, 3.5 and 48 h, which were conducted as separate experiments. Five g of leaf strips were placed in 50-ml test tubes for the 3.5 h incubation experiment and 2 g of leaf discs were placed in 50-ml Erlenmeyer flasks for the 48 h experiment. Test tubes and flasks were then sealed with serum stoppers and covered with aluminum foil. Either 14CO (approx 2.6 x 10⁷ cpm, final concentration 109 μl/l in the 3.5 h experiment and approx. 2 x 10⁸ cpm, final concentration 84 μl/l in the 48 h experiment) or 14CO2 (approx. 2.5 x 10⁹ cpm, final concentration 22 μl/l in the 3.5 h experiment and approx. 6 x 10⁹ cpm, final concentration 53 μl/l in the 48 h experiment) was injected into the test tubes or flasks and then incubated for the specified time at 2.5°C. At the end of the incubations gas samples were taken for 14CO and 14CO2 determination, and the leaf discs were homogenized and extracted in 80% (v/v) ethanol containing 0.4 M acetic acid. After concentration the extracts were fractionated into neutral, cationic, and anionic fractions by passing through columns of ion exchange resins Dowex 50 (H+) and Dowex 1 (hydroxyl). Paper chromatography and electrophoresis were used to separate the cationic (amino acid) and anionic (organic acid) fractions. Aaspatic and glutamic acids (acidic amino acids) were separated from the other amino acids using paper electrophoresis at pH 4. The neutral amino acids (including asparagine and glutamine) were subjected to acid hydrolysis with asparagine and glutamine being converted to aspartic and glutamic acids, which were then separated using

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In Vivo $K_m$ for Oxidation of CO to CO$_2$. One g of leaf disks with 0.5 ml water was placed in 25-ml Fernbach flasks which were wrapped with aluminum foil, stoppered, and placed at 2.5°C. After temperature equilibration, various amounts of $^{14}$CO$_2$ were injected into the flasks. Thirty min later, a 3-ml gas sample was taken for $^{14}$CO$_2$ determination.

Oxidation of CO by Various Fractions of Leaf Homogenate. A modified procedure of Stokes et al. (15) was used to obtain a leaf homogenate. Leaves were homogenized in a juice extractor (Acme Supreme) with a solution containing phosphate buffer (pH 7.5; 10 mM), mannitol (0.35 M), EDTA (1 mM), and NaHSO$_3$ (3 mM) and filtered through eight layers of cheesecloth. Three fractions were obtained by centrifugation: A pellet fraction (1,200g spin for 4 min), a mitochondridial fraction (pellet from 10,000g spin for 10 min) and a supernatant fraction (supernatant after 10,000g spin). Pellet and mitochondrial fractions were washed twice in PO$_4$- mannitol buffer and resuspended in PO$_4$-mannitol plus 0.75 mM MgCl$_2$. Portions of each fraction were incubated in 25 ml flasks for 6 h at 20°C with $^{14}$CO$_2$ (10$^6$ cpm, final concentration 0.84 µl/l). The $^{14}$CO$_2$ produced was absorbed in 6 × KOH held in center wells. The KOH was then acidified with lactic acid and the $^{14}$CO$_2$ released was absorbed in 0.5 ml of ethanolamine:2-ethoxyethanol (1:1, v/v) mixture, the radioactivity of which was determined by liquid scintillation counting. Protein was determined by the method of Bradford (3).

RESULTS AND DISCUSSION

As shown in Table I, when lettuce tissue was exposed to $^{14}$CO in the dark at 2.5°C for 3.5 or 48 h, the primary metabolite was CO$_2$, though a small amount of radioactivity was found in acid-stable products. Inasmuch as both $^{14}$CO and $^{14}$CO$_2$ were present, the question arises whether the radioactive acid-stable products were derived from $^{14}$CO, $^{14}$CO$_3$, or both. One approach to answer this question is to compare the pattern of labeled products between $^{14}$CO- and $^{14}$CO$_2$-treated leaf tissue. The labeled soluble products were the same between these two treatments for both the 3.5 and the 48 h incubation. This suggests that it was $^{14}$CO$_2$ derived from $^{14}$CO and not $^{14}$CO per se which was fixed. The soluble fraction most heavily labeled in the $^{14}$CO and $^{14}$CO$_2$ treatments was the anionic fraction.

For most experiments, the leaf tissue was incubated at 2.5°C because this is a good commercial storage temperature for lettuce. However, leaf disks treated with $^{14}$CO or $^{14}$CO$_2$ incubated at 2.5 or 20°C for 48 h were compared with respect to the distribution of label in the neutral, anionic, and cationic fractions. No major differences between the two temperatures within each treatment or between the $^{14}$CO and $^{14}$CO$_2$ treatments were observed (data not shown). As expected, more $^{14}$CO was oxidized to $^{14}$CO$_2$ at the higher temperature (about 50% at 20°C compared with about 17% at 2.5°C in the 48 h incubation), but less $^{14}$CO was incorporated in both treatments at 20°C, presumably due to the greater dilution of $^{14}$CO$_2$ by respiratory CO$_2$.

In barley (12) and bean leaves (1, 2), serine in addition to sucrose was heavily labeled after exposure to $^{14}$CO in the light. Bidwell and Beebe (1) concluded that in bean leaves sucrose was labeled by $^{14}$CO either via serine, which does not involve the intermediacy of CO$_2$, or via the oxidation of CO to CO$_2$, which in turn was incorporated into sucrose via 3-phosphoglyceric acid. However, in corn leaves $^{14}$CO was incorporated exclusively via CO$_3$ and 3-phosphoglyceric acid into sucrose (1). This was attributed to differences between C$_3$ and C$_4$ plants. In barley (12) and bean leaves (1), it was suggested that $^{14}$CO incorporation into serine occurred via the tetrahydrofolate system.

Bidwell and Fraser (2) found that in the dark $^{14}$CO$_2$ was the main product of $^{14}$CO metabolism in bean leaves as we observed in lettuce. However, contrary to our results, they found marked differences in labeled products when $^{14}$CO and $^{14}$CO$_2$ were administered. They reported that with $^{14}$CO 50% of the soluble radioactivity was recovered as sucrose and 22% as serine; with $^{14}$CO$_2$ no label was recovered in either of these compounds, but label was recovered in aspartate, malate, and citrate, which accounted for 77% of the radioactivity. Their results suggest that most of the $^{14}$CO was incorporated into serine and other products via a CO$_2$-independent pathway, as had been observed in the light; however, the authors suggested that some CO was incorporated via oxidation to CO$_2$, since aspartate was labeled. The results of our experiments indicate that most, if not all, of the CO was fixed after being oxidized to CO$_2$ since the labeling pattern between the CO and CO$_2$ treatments was essentially the same. We observed that aspartate, malate, and citrate together accounted 70% or more

Table I. Distribution of $^{14}$C into Products from Leaf Tissue Exposed to $^{14}$CO or $^{14}$CO$_2$

<table>
<thead>
<tr>
<th>Labeled Products</th>
<th>3.5 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}$CO</td>
<td>1.19</td>
<td>16.7</td>
</tr>
<tr>
<td>$^{14}$CO$_2$</td>
<td>0.04</td>
<td>0.61</td>
</tr>
<tr>
<td>Insoluble</td>
<td>0.004</td>
<td>1.12</td>
</tr>
</tbody>
</table>

Table II. Effect of Exogenous Nonradioactive CO$_2$ upon the Conversion of $^{14}$CO to $^{14}$CO$_2$ and upon the $^{14}$C Incorporation into Soluble Acid-Stable Products

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Exposure Time</th>
<th>$^{14}$CO$_2$ Produced</th>
<th>$^{14}$C Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}$CO</td>
<td>3</td>
<td>249</td>
<td>15.5</td>
</tr>
<tr>
<td>$^{14}$CO + 3 $^{14}$CO$_2$</td>
<td>3</td>
<td>292</td>
<td>1.5</td>
</tr>
<tr>
<td>$^{14}$CO</td>
<td>48</td>
<td>2,733</td>
<td>57.2</td>
</tr>
<tr>
<td>$^{14}$CO + 3 $^{14}$CO$_2$</td>
<td>48</td>
<td>3,166</td>
<td>32.0</td>
</tr>
</tbody>
</table>

* The radioactivity in the neutral amino acid fraction including serine and alanine contained 8% and 5% of the soluble radioactivity from the $^{14}$CO and $^{14}$CO$_2$ treatments, respectively; radioactivity in the individual amino acids was not determined.
of the radioactivity in the \(^{14}\text{C}\)O and \(^{14}\text{C}\)O\(_2\) treatments for both incubation periods as Bidwell and Fraser (2) found for the CO\(_2\) treatment. In the 48 h experiment, we observed only a small amount (2%) of radioactivity in serine for both CO and CO\(_2\) treatments. Due to low radioactivity recovered in the 3.5 h experiment, we were unable to separate and identify labeled serine.

An additional experiment was conducted to examine whether \(^{14}\text{C}\)O or \(^{14}\text{C}\)O\(_2\) is the species being incorporated into lettuce. If \(^{14}\text{C}\)O\(_2\) derived from \(^{14}\text{C}\)O is being incorporated, it is expected that the addition of large amounts of nonradioactive CO\(_2\) should result in reduced \(^{14}\text{C}\) incorporation. The results of Table II support such a view. When 3% CO\(_2\) was introduced with \(^{14}\text{C}\)O (final concentration 53 \(\mu\)l/l), the \(^{14}\text{C}\) incorporation was reduced by about 90% during a 3-h exposure period, but only about 45% during a 48 h incubation (Table II). To evaluate the effect of exogenous CO\(_2\) and \(^{14}\text{C}\)O\(_2\) incorporation, it is important to consider the endogenous CO\(_2\) production rate from respiration. The respiratory rate of cut lettuce stored at 2.5°C was estimated to be approximately 11 \(\mu\)l g\(^{-1}\) h\(^{-1}\), which would give about 0.13% and 2.1% CO\(_2\) after incubation for 3 and 48 h, respectively. Thus, exogenous addition of 3% CO\(_2\) would greatly reduce the specific radioactivity of CO\(_2\) during the 3-h incubation, but such an effect was much less significant during the 48 h incubation because of the accumulation of large amounts of respiratory CO\(_2\). This experiment and the experiment comparing the labeling pattern of the \(^{14}\text{C}\)O and \(^{14}\text{C}\)O\(_2\) treatments (Table I) indicate that the incorporation of CO proceeds through the intermediary of CO\(_2\).

During the course of this investigation, we observed that both the conversion of \(^{14}\text{CO}\) to \(^{14}\text{C}\)O\(_2\) and the incorporation of \(^{14}\text{C}\)O into acid-stable products were inhibited to the same extent by a given concentration of cyanide (data not shown). Although these data are in parallel with the view that CO is incorporated via oxidation to CO\(_2\), they cannot be regarded as evidence, because serine hydroxymethyl transferase, which may be involved in the fixation of CO into serine, is also inhibited by cyanide (10). Hence, \(^{14}\text{CO}\) is the major product of \(^{14}\text{C}\)O metabolism (Table I) and the oxidation of \(^{14}\text{CO}\) to \(^{14}\text{C}\)O\(_2\) is an important step leading to labeled acid-stable products, we have examined the dependence of CO oxidation on CO concentrations by leaf discs at 2.5°C. From the data of Figure 1 it is estimated that the system had an apparent \(K_m\) of approximately 5.3 \(\mu\)l/l and a \(V_{\text{max}}\) of 9.7 nl g\(^{-1}\) h\(^{-1}\). It should be noted that the concentration of CO employed in commercial modified atmosphere for lettuce is at least 2% (17), which is much higher than the apparent \(K_m\) of 5.3 \(\mu\)l/l. Under such conditions, we can estimate that CO is oxidized at a rate of about 10 nl g\(^{-1}\) h\(^{-1}\) at 2.5°C.

Fractionation of a lettuce leaf homogenate indicated that the mitochondrial fraction was the most effective fraction to oxidize CO to CO\(_2\) (Table III). These activities were heat-labile and the mitochondrial fraction was sensitive to CN\(^{-}\) inhibition. Under our assay conditions (0.8 \(\mu\)l/l final CO concentration and at 20°C), the homogenate catalyzed the oxidation of CO to CO\(_2\) only at 1/200 the rate of that of the leaf disc.

The oxidation of CO to CO\(_2\) appears to be a widespread phenomenon among living organisms including bacteria (8, 13), animals (7), algae (5), and higher plants (1, 2). In algae, Chappelle (5) observed that this reaction required molecular O\(_2\), which was effectively inhibited by cyanide, azide, and hydroxylamine, and low light increased the oxidation rate several fold over that of the dark rate. Earlier, Breckenridge (4) had shown that CO was oxidized in muscle tissue by Cyt oxidase. Our results, showing that the highest oxidation activity was localized in the mitochondrial fraction, are consistent with such a view.

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