Carbon Monoxide Production by Algae

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CO has been observed in the pneumatocysts of Nereocystis lutkeana (9) where it varied from 0.4 to 12.2 % of the total gases in the air bladders, depending on the specimen and the time of day. CO was not detected in the dead plant or macerated tissues which had been left to decompose or autolyse. These findings have been, in general, confirmed (11), although it has been found that the daily fluctuations in CO were due to pressure changes of the total gas, the relative concentrations of CO and nitrogen remaining constant.

The production of CO has been reported in other plants. It is produced by the growing leaves and stems of Medicago (16) and by Anacystis nidulans during illumination (4). Production during the enzymic degradation of rutin has been described (13, 15), and it has been detected during seed germination (12). The assimilatory fixation of CO by higher plants has been reported (1, 7).

Wittenberg (17) has reported the presence of CO in the floats of the Portuguese man-of-war and its production by a gas gland in the float when DL-serine was used as a substrate.

The present study describes experiments designed to gain information on the source of this unusual metabolite in algae, particularly in the brown alga, Egregia menziesii.

Materials & Methods

Plant Material. Algae used in this study were harvested at low tide from areas of the California coast between the 37th and the 38th parallels. Material was stored at −10° until used. Preliminary experiments revealed the presence of CO in the pneumatocysts of Egregia menziesii, and the fresh tissues of this alga were found to evolve measurable quantities of this gas. Because of the accessibility and abundance of this alga in the intertidal zone, it was used for the major part of this study. Algal tissues were macerated 5 minutes in a high-speed blender with equal amounts of 0.1 M potassium phosphate buffer, pH 7, or tested as whole tissue.

Carbon Monoxide Production. The sample to be tested was placed in a 50-ml tube with outlets at both ends. The volume in the tube was adjusted with potassium phosphate buffer solutions, leaving about 20 ml of free space above the sample. The outlets were fitted with tubing and clamped shut. At the end of the incubation period, the tube was connected through a trap containing 20 % potassium hydroxide to an evacuated 50-ml filter flask containing 5 ml of Ciuhandu's reagent (see below). As the CO-containing gases entered the evacuated filter flask, water was allowed to rise through the bottom inlet of the sample tube until it displaced all the gas into the trap and flask. The gas in the trap and tubing was then flushed into the reagent flask with air. Alternatively, the gas was flushed into a gas-tight syringe for transfer into cells for infrared analysis or through a Hopcalite tube (Available from the Mine Safety Appliance Co., Pittsburgh 8.) for semiquantitative analysis (8).

Assay of Carbon Monoxide. For more quantitative purposes, the method of Ciuhandu (2, 3) was employed. Standard curves were prepared with measured volumes of CO. The method is sensitive to quantities of less than 0.5 μ moles of CO per flask volume (approximately 45 ml) and useful for quantities up to 2 μ moles. Ether, petroleum ether, chloroform, and acetone produced some color with this reagent. These and solvents other than water were removed by vacuum distillation and samples were always tested in aqueous solution. With this method the average error for homogeneous solutions was less than 10 %, although data from fresh algal tissues had a much larger variation.

The Ciuhandu method appeared to be quite specific for CO. Nevertheless, when the concentration exceeded about one μ mole in 22.4 ml of gas, light absorption at 4600 m\(\mu\) was used to verify, qualitatively, the identity of the gas (10). Infrared determinations were made on a Perkins-Elmer Model 21 spectrometer using an absorption cell of 10 cm length.

Chromatography of Carbon Monoxide-Producing Compounds. Compounds involved in the production of carbon monoxide were separated and identified by ascending chromatography. When large quantities of material were to be isolated for testing purposes or for purification, a mixture was applied as a streak along the origin to Whatman No. 1 or No. 3 paper. After development, the active band was cut out and eluted from the strip with ethanol or subjected to further chemical testing directly on the paper. All chromatography and elution was done under nitrogen. Papers were dried in a stream of nitrogen which had been passed through a heated copper coil and into the drying chamber. Three solvents were used: 1. n-butanol, acetic acid, and water in the ratio 100:20.5:

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50. 2. pyridine, isopropyl alcohol, glacial acetic acid, and water in the ratio 8:8:1:4 and 3. water-saturated phenol.

Buffers used were 0.1 m potassium phosphate-citric acid, pH 2 to 6, 0.1 m potassium phosphate, pH 7, and 0.1 m sodium hydroxide-glycine, pH 10.

Results

Evolution of Carbon Monoxide by Fresh Algae.  
Table I lists a number of algae as well as two higher plants, Zostera marina and Medicago sativa, which were tested for CO production by their fresh tissues. There were considerable differences between samples collected at different times, but the various species are listed roughly in order of decreasing CO production. CO was evolved slowly, reaching a limit, under the conditions of our experiments, after 4 or 5 hours. If accumulated gases were flushed from the reaction vessel with fresh air or O₂, CO production resumed, reaching almost the same limit as before. The maximum attained in one experiment probably represented an equilibrium condition rather than the total capacity for CO production.

The amount of CO produced in Egregia menziesi did not decrease after storage for 2 to 5 months at −10°. There were no detectable seasonal variations in this gas production. Both stipes and fronds of Egregia were equally productive of CO, but in Nereocystis fronds evolved more gas per unit weight than stipes.

Preliminary experiments with Egregia demonstrated that killing the tissue by heating did not decrease the production of CO. The crude filtrate from macerated tissues strained through gauze also produced detectable CO. Boiling this extract for 5 minutes did not decrease its capacity to evolve CO. It appeared that the CO was a product of the decomposition of some compound in the alga, and not dependent upon a heat labile enzymic process.

Oxidative Nature of Carbon Monoxide Production. Since the amount of gas evolved by the alga was roughly proportional to the partial pressure of the O₂ present, testing was done in O₂ instead of air to increase the yield of CO. No CO was produced in CO₂, argon, or nitrogen atmospheres. Oxidants such as naphthaquinone and ferric chloride did not bring about CO production in a nitrogen atmosphere. It was apparent that the reaction which produced CO was oxidative in nature and required O₂ as the oxidant.

The gas was not bound in a heme type structure such as hemoglobin. When tissues were homogenized with potassium ferrocyanide according to the procedure of Wilks (16), additional evolution of CO with Medicago or Egregia was not detected.

Light Requirement for Carbon Monoxide Production. Wilks (16) found that the production of CO by Medicago required illumination with light in the region between 480 and 680 nm. CO production by fresh Egregia and by a sulfuric acid digest illuminated with approximately 2000 luxes from a tungsten lamp source was approximately double that produced in the dark, but light was not an obligatory requirement for the reaction.

Extraction of a Carbon Monoxide-Producing Compound From Egregia. When cold extraction of algal tissues with acetone, water, and 95% ethanol proved ineffective, an attempt was made to free the CO-yielding compound by refluxing macerated algae for 3 hours in 6 m sulfuric acid. Fresh 6 m sulfuric acid hydrolyzates evolved 50 to 75% of the CO produced by the original tissue under similar test conditions. Production of CO by both the plant and hydrolyzate doubled as the pH was increased from 2 to 10. In subsequent fractionation, solutions were tested at pH 10 to obtain a maximum amount of CO. The CO produced at pH 7 was measured on pooled fractions.

Isolation of a Carbon Monoxide-Producing Compound. A CO-producing compound was isolated from the 6 m sulfuric acid hydrolyzate by the following procedure: The solution was adjusted to pH 2 with barium hydroxide, barium sulfate removed by centrifugation, and the clear supernatant solution passed through an anion exchange column (Dowex-1-acetate, 100-200 mesh). Approximately 50% of the CO-generating capacity of the solution passed through the column. Similar treatment of a portion of the hydrolyzate with a cationic resin (Dowex-50-H, 200-400 mesh) removed none of the CO-generating capacity. Elution of the anionic exchange resin with 0.5 m acetic acid removed a CO-producing substance from the column which was precipitable with neutral lead acetate.

The lead precipitate did not decompose to CO in acid or alkaline solution. Removal of the lead by treating a suspension of the precipitate with hydrogen sulfide produced a yellow solution which turned darker in alkaline medium and evolved CO at pH 7 and 10. The regenerated compound reduced Tollen's
The regenerated solution was concentrated in vacuo with less than 10% loss of activity and further purified by ascending chromatography on Whatman No. 3 paper, using butanol-acetic acid-water solvent in a nitrogen atmosphere. A yellow band eluted with absolute ethanol was found to produce CO. The ultraviolet absorption spectrum of this material was determined in absolute ethanol, in an ethanol solution of sodium acetate plus boric acid, and in ethanolic sodium methoxide according to Jurd et al. (5,6) (fig 1). The observed shift in the absorption peak from 272 μ to 288 μ indicated the presence of o-dihydroxy groups.

A benzoyl derivative of the CO-producing fraction, purified as described above, was prepared. Its melting point was 113° and its molecular weight, by the Rast Camphor method (14), 381±20. This derivative produced no CO until hydrolyzed, and then 1 mmole evolved 1.5 mmoles of the gas in 3 hours. It had a saponification number of 100 but instability of the saponified product made this observation difficult to interpret. Elemental analysis corresponded to an empirical formula of C_{22}H_{39}O_{6}:

Calculated: C, 70.00; H, 4.77; O, 25.40
Found: C, 70.7; H, 4.56; N, 0.22; O, 24.8

*Extraction of a CO-producing Compound by 0.01 m Hydrochloric Acid.* If the macerated algal tissue was refluxed for 2 hours in 0.01 m HCl an extract was obtained that would evolve at pH 10 about 10 to 20% as much CO as the original tissue. This extract produced no gas at pH 7. The CO-producing substance could be recovered from the extract by precipitation with neutral lead acetate. When it was regenerated with hydrogen sulfide and refluxed for 2 hours in 3 m HCl under nitrogen, a product was formed that evolved CO at pH 7.

Chromatographic comparisons were made (table II) between the hydrolyzed material from the HCl extract and the anionic material from the sulfuric acid hydrolysate. \( R_f \) values for the two fractions as well as those of known polyphenolic compounds are given in table II. The material isolated from the sulfuric acid hydrolysate compared very closely with the hydrolyzed material from the 0.01 m HCl extract.

**Table II**

\( R_f \) Values of Carbon Monoxide-Producing Fractions & Polyphenols

<table>
<thead>
<tr>
<th></th>
<th>Butanol-acetic acid-water</th>
<th>Water-saturated phenol</th>
<th>Pyridine-isopropanol-acetic acid-water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anionic material from</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sulfuric acid hydrolyzate</td>
<td>0.62</td>
<td>0.63</td>
<td>0.69</td>
</tr>
<tr>
<td>Hydrolyzed material from</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01 m HCl extract</td>
<td>0.65</td>
<td>0.60</td>
<td>0.51-0.69**</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>0.63</td>
<td>0.53</td>
<td>...</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0.66</td>
<td>0.94</td>
<td>...</td>
</tr>
</tbody>
</table>

* All \( R_f \)'s were determined by chromatography on Whatman No. 1 paper in a \( N_2 \) atmosphere.

** Streaking occurred.
Discussion

Several algae as well as higher plants have been shown to produce CO. The gas not only reached easily detectable concentrations in the air bladders of these algae but was evolved by fresh fronds, stipes, and macerated tissues. Considerable variation was observed between specimens and different collections of *Egregia menziesii* as seen in two representative values given in table I. This variation was not related to seasonal change.

Production of CO by heat-treated tissues and the requirement for oxygen indicate that the reaction is probably a non-enzymic oxidation.

The properties of a CO-producing compound isolated from a 6 M sulfuric acid hydrolyzate suggest a polyphenolic compound. The instability of this compound in sodium methoxide and other alkaline solutions is characteristic of phenols with o-dihydroxy groups, such as gallic acid and pyrogallol.

Sufficient evidence was not found to relate the isolated CO-producing compound to the in vivo process. Chromatographic evidence was obtained to show that the compound was similar to a substance that could be extracted by milder treatment with dilute HCl and then hydrolyzed. It is possible that the plant contains this compound and it is enzymically hydrolyzed before it decomposes further to CO. Accumulation of the hydrolyzed compound would account for the heat stable production of CO.

Summary

I. Carbon monoxide production has been demonstrated in *Egregia menziesii*. several other algae and in the higher plants, *Zostera marina* and *Medicago sativa*.  
II. The ability to produce carbon monoxide is not destroyed by heating the tissues.  
III. Oxygen is required for carbon monoxide production by *Egregia menziesii*.  
IV. A carbon monoxide-producing compound can be extracted by refluxing the macerated algal tissues in 0.01 M hydrochloric acid or by hydrolysis with 6 M sulfuric acid.  
V. An anionic, carbon monoxide-producing compound with the properties of a polyphenol has been isolated from a sulfuric acid hydrolyzate. The elemental analysis and the molecular weight, 381 ± 20, for a benzyol derivative of the compound are reported.

Literature Cited