Effects of Carbon Dioxide on Activity of Apple Mitochondria

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ABSTRACT

Effects of CO₂ on mitochondrial activity of apple (Malus pumila Mill. var. Richard Delicious) were studied in two ways. Immediate effects were determined by imposing 3 to 18% CO₂-bicarbonate mixtures on isolated apple mitochondria, and long-term effects were determined by extracting mitochondria from apples that had been stored for intervals in atmospheres containing 6 or 12% CO₂ plus 3% O₂. The CO₂-bicarbonate systems had immediate and broad effects on mitochondrial oxidations; 18% CO₂ stimulated malate oxidation about 10%; suppressed α-ketoglutarate, citrate, and NADH oxidations about 10%; and suppressed fumarate, pyruvate, and succinate oxidations about 32%. The effects of lower CO₂ concentrations varied with substrates. Mitochondria isolated from fruit stored in 6 or 12% CO₂ possessed a reduced capacity to oxidize added succinate or NADH, but retained a marked sensitivity to CO₂-bicarbonate mixtures. Respiratory control in these mitochondria was somewhat reduced, but CO₂ had not acted as a strong uncoupling agent.

Sauassure (23) demonstrated in 1804 that plants may be injured by exposure to high concentrations of CO₂. Although the precise nature of CO₂ injury has never been characterized, many responses of whole plants and excised plant parts have since been reported. One response often found is a suppression of respiration by high CO₂. This was first recorded by Kidd (12) and served as a basis for the development of controlled-atmosphere storage of apples and pears, since a moderate suppression of respiration by CO₂ is associated with a marked retardation of senescence of these fruits. However, excessive CO₂ causes injury to fruit, the effective concentration varying with kind of fruit, variety, temperature, and time of exposure. In apples, CO₂ injury initially appears as a brown, dry area in and around the core, and progressive deterioration of the fruit follows.

Despite numerous reports of a suppression of respiration in plants by CO₂, little is known of its role in this process. Hulme (9) reported that apples injured by CO₂ accumulated succinic acid. In pears, high CO₂ caused succinic and citric acids to accumulate (28); CO₂ also appeared to slow malate depletion, an effect later demonstrated in apples (13). Many other reports indicate changes in organic acid metabolism in various plant species due to excess CO₂.

These findings suggest that CO₂ can act as a controlling factor in the operation of the tricarboxylic acid cycle, and it has been suggested (8) that excess CO₂ inhibits mitochondrial oxidations, thereby diverting pyruvate to acetaldehyde and ethanol formation, substances known to accumulate in apples under excess CO₂ even though ample O₂ is present (26). These substances can be toxic in apples (24) and could be the proximal cause of CO₂ injury to them.

There is no direct evidence that CO₂ influences metabolism in apple mitochondria, but some evidence to this effect has been found in other plant systems. In Ricinus mitochondria, oxidation of succinic acid to fumarate was retarded by CO₂-bicarbonate mixtures containing more than 10% CO₂ (2, 4). There appeared to be competitive inhibition by succinic dehydrogenase by the CO₂ (3). The succinoxidase system in Iris rhizomes was sensitive to as low as 6% CO₂ (6). In addition, higher concentrations of CO₂, especially above 40%, inhibited the NAD-cytochrome c-reductase system and cytochrome c-oxidase (3). The sensitivity of cytochrome c-oxidase to CO₂ has been shown in several other plant species also (15).

Miller and Hsu (16) showed more far-reaching effects of CO₂-bicarbonate mixtures than had previously been reported. They found concentrations of 15% CO₂ to inhibit oxidations of citrate, isocitrate, malate, succinate, and NADH by cauliflower mitochondria. Phosphorylation was also inhibited by 15% CO₂.

This paper reports an investigation of the effects of increasing concentrations of CO₂ on the metabolism of apple mitochondria. Effects of CO₂-bicarbonate systems imposed directly on mitochondria and of CO₂-O₂ atmospheres imposed on whole fruit for extended periods have been determined.

MATERIALS AND METHODS

Preclimacteric apple fruits (Malus pumila Mill. var. Richard Delicious) stored for short periods at 0 C in air were used for studies involving the effects of CO₂-bicarbonate mixtures on mitochondrial activity. Other samples of these fruit were placed in controlled atmospheres containing either 6% CO₂ and 3% O₂, 12% CO₂ and 3% O₂, or air as a control. These fruit were stored for up to 16 weeks at 0 C and 90% relative humidity.

Apples were peeled and 350 to 400 g of tissue were gently grated into 750 ml of extraction medium using apparatus similar to that described by Romani et al. (21), except that a plastic grinding surface was substituted for a stainless steel screen. Extraction was at 0 to 4 C in prechilled apparatus. The medium, maintained at pH 7.8, consisted of 0.4 mM sucrose, 20 mM citrate, 10 mM KH₂PO₄, 0.75% (w/v) polyvinyl-pyrrolidone (PVP-40), 10 mM EDTA, and 10 mM cysteine hydrochloride (added immediately prior to extraction). The extract was strained through 50-μm mesh nylon fabric to remove the major portion of starch present (17, 22), and the filtrate was centrifuged for 10 min at 37,000 g. The pellet was resuspended in 30 ml of medium containing 0.4 mM sucrose, 10 mM EDTA, and 0.1 mM tris-HCl at pH 7.5. The homogenate was centrifuged for 10 min at 1000 g, and the supernatant was again centrifuged at

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Table I. Oxidation Rates and Respiratory Control Ratios of Mitochondria Isolated from Apple Fruit

Reaction mixtures contained: 0.25 m sucrose; 5 mM MgCl₂; 10 mM K₂HPO₄-KH₂PO₄; 10 mM tris-HCl pH 7.2; 3 mg/ml bovine serum albumin; 2 mg/ml yeast extract; and substrate as indicated. Respiratory control ratio was determined after addition of 0.3 μmole of ADP.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Conc</th>
<th>Rate of State 3 O₂ Uptake</th>
<th>Respiratory Control Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>μg atoms/min·mg protein</td>
<td>state 3/state 4</td>
</tr>
<tr>
<td>Citrate</td>
<td>16</td>
<td>25 ± 3</td>
<td>1.7 ± 0.0</td>
</tr>
<tr>
<td>Fumarate</td>
<td>16</td>
<td>35 ± 1</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>16</td>
<td>40 ± 6</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>16</td>
<td>48 ± 3</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>Malate</td>
<td>32</td>
<td>59 ± 4</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>Succinate</td>
<td>16</td>
<td>77 ± 0</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>NADH</td>
<td>1</td>
<td>162 ± 15</td>
<td>2.0 ± 0.0</td>
</tr>
</tbody>
</table>

37,000 g for 10 min. The pellet was suspended in 2 ml of 0.2 m sucrose at pH 7.2. This gave a 3 ml mitochondrial preparation generally containing 30 to 70 mg of protein, determined by the Lowry method (14). Total extraction time was 1.25 to 1.5 hr.

Oxidation rates and respiratory control ratios were measured polarographically at 28 °C with a recording oxygen cathode (Oxygraph Model KM, Gilson Medical Electronics) fitted with a Clarke-type electrode (Yellow Springs Instrument Co.) and Teflon (0.001 inch) membrane in a 1.5-ml cuvette. The reaction medium, following Bonner (5), consisted of 0.25 m sucrose, 5 mM MgCl₂, 10 mM KH₂PO₄-K₂HPO₄ buffer, 10 mM tris-HCl buffer (pH 7.2), and substrate at 16 mM (except NADH, 1 mM and malate, 32 mM). Also, bovine serum albumin (3 mg/ml) and yeast extract (2 mg/ml) (10) were added immediately prior to assay. Reaction times did not exceed 5 min. Respiratory control values were determined following addition of 3 μmole of ADP.

To determine the effects of CO₂ on mitochondria, aqueous solutions of NaHCO₃ were prepared in molar concentrations calculated by the Henderson-Hasselbach equation to produce pH 7.20 at 28 °C when in equilibrium with 3, 6, 12, and 18% CO₂ (27). CO₂ gas was slowly bubbled through the solutions for approximately 10 min until the pH was maintained at 7.20. These solutions were then used to prepare the oxidation media. Changes in pH due to loss of CO₂ from the medium during preparation and assay were minimal (less than 0.05 pH unit at 18% CO₂).

RESULTS

Mitochondrial Activity. The mitochondria obtained from the apple fruit were capable of oxidizing NADH and all the tricarboxylic acid cycle acids tested (Table I). Activity remained constant for at least 3 hr after extraction, during which time all assays were completed. Mitochondrial integrity appeared to be similar to that demonstrated for apples by Wiskich (29), except that our respiratory control ratios were higher (Table I). ATPase activity (25) of the mitochondria was relatively low (about 0.1 μmole Pi released/min·mg protein).

Effects of CO₂-bicarbonate Mixtures on Mitochondrial Activity. Oxidation rates in various CO₂-bicarbonate mixtures, adjusted for any "salt effect" (6) (which was about 10% inhibition at 18% CO₂ and 0% at 3% CO₂) are shown in Figure 1. These rates were established within a few seconds after addition of the mitochondria and were linear until ADP became limiting. Preliminary experiments with succinate indicated that CO₂ had no effect on the respiratory control ratio.

Increasing concentrations of CO₂ influenced oxidations of all substrates tested. With malate, a small stimulation was effected (Fig. 1A). This stimulation appeared to be saturated at low CO₂ concentrations, there being no significant difference among the effects of 6, 12, or 18% CO₂. In contrast, the oxidations of succinate, fumarate, and pyruvate were markedly suppressed by increasing levels of CO₂ (Fig. 1B). An almost linear response between 3% CO₂ and 18% CO₂ was observed, increasing to about a 30% inhibition at the 18% CO₂ level for all substrates. This inhibition was statistically significant at 6% CO₂ or greater for all three substrates and was significant at 3% CO₂ for succinate and fumarate.

Changes in Succinate and NADH Oxidation in Stored Fruit.

To assess the long term effects of CO₂ in the atmosphere surrounding intact fruit, mitochondrial oxidations of succinate and NADH, the most actively oxidized substrates (Table I) were determined after different storage intervals. Marked changes occurred with time in air-stored apples (Tables II and III). Succinate oxidation rose during the first 4 weeks of storage, leveled off for 2 weeks, and then declined steadily during the rest of the storage period. NADH oxidation was not determined during the time of the rise in activity, but it exhibited a decline with extended time in storage. The rise and subsequent decline in mitochondrial respiration closely resembled the respiratory climacteric, to which it probably corresponded (11). Mitochondria from fruit stored in 12% CO₂ and 3% O₂ also exhibited the rise and fall in oxidation of succinate, but the rise was suppressed and the decline was accelerated (Table II); furthermore, the decline in NADH oxidation was accelerated by this storage atmosphere (Table III). Storage in 6% CO₂ and 3% O₂ appeared to have little or no effect on succinate oxidation, but to increase the decline in NADH oxidation.

To see whether the effect of CO₂ in the atmosphere had a persistent effect on mitochondrial activity, after 16 weeks in...
the designated atmospheres all fruit were stored for an additional 4 weeks in air. This transfer did not change the trends of activity that were established during the preceding 16 weeks. If anything, the transfer to air intensified the decline in mitochondrial activity associated with the preceding exposures to CO₂.

Changes in Respiratory Control Ratios in Stored Fruit. Since CO₂ has been reported to affect oxidative phosphorylation (2), respiratory control of mitochondria from stored apples was assessed. The respiratory control ratio is the ADP-stimulated state 3 oxidation rate divided by the state 4 oxidation rate (7). By using four different substrates, in each case respiratory control declined during storage in air at 0 C (Table IV). Yet, the mitochondria clearly retained relatively strong respiratory control even at the end of the experiment.

Respiratory control was affected by storage in 12% CO₂ and 3% O₂ (Table V). During the 6- to 12-week period of storage, the mitochondria from the 12% CO₂ fruit were less tightly coupled than those from air-stored fruit. However, after 16 weeks there was no longer any significant difference between fruit from air or 12% CO₂ and 3% O₂, and the respiratory control values were still relatively high for apple mitochondria. Thus, it appears that CO₂ imposed an earlier decline in respiratory control but did not markedly disrupt oxidative phosphorylation.

Having established that mitochondria from freshly harvested apples are sensitive to CO₂ (Fig. 1), it was of interest to determine the effects of storage time and atmosphere on this sensitivity. Since both activity and CO₂ sensitivity were high with succinate as substrate, mitochondria extracted from different atmospheres were assayed on succinate in 12% and 18% CO₂ bicarbonate systems. Mitochondria from air-stored fruit lost at least 50% of their sensitivity to CO₂ by the end of the experiment (Table VI). Mitochondria from fruit stored in 12% CO₂ and 3% O₂ also lost at least 50% of their sensitivity, but the loss occurred at a slower rate. During the first 12 weeks of storage, mitochondria from the 12% CO₂-stored fruit were al-
ways more sensitive to CO₂ than those from air-stored apples. It is evident that 12% CO₂ in the atmosphere surrounding intact apples did not reduce the sensitivity of extracted mitochondria to CO₂ inhibition, but that this sensitivity declined with fruit senescence.

Occurrence of CO₂ Injury in Fruit. After 12 and 16 weeks in storage, about 10% of the apples held in 12% CO₂ and 3% O₂ exhibited CO₂ injury in the core area, but all fruit from 6% CO₂ and 3% O₂ or air were free of injury. After 16 weeks in the test atmospheres plus 4 weeks in air, 90% of the fruit from 12% CO₂ and 3% O₂ and 85% of those from 6% CO₂ and 3% O₂ possessed injury symptoms, whereas all of those stored in air remained free of core browning.

DISCUSSION

These experiments demonstrate that CO₂ can have a strong controlling effect on apple mitochondrial activity. CO₂-bicarbonate systems modified without any lag period the oxidation of all seven substrates tested. The suppressed oxidations of succinate and citrate could explain the reported accumulations of these acids under high CO₂ (9, 28). However, the mild stimulation of malate oxidation suggests that the effect of high CO₂ in delaying malate consumption in apples (13) is an indirect response, probably due to an over-all reduction in mitochondrial activity.

The effect of CO₂ on mitochondria clearly was not due to an effect on a single enzyme, such as succinic dehydrogenase, although the succinoxidase system in apple mitochondria is very sensitive to CO₂. Many enzyme systems must be sensitive to CO₂ to different degrees. Such a widespread effect of CO₂ on mitochondrial metabolism could be attributed to either structural or configurational changes of the mitochondrion, or to pH changes within it. Although the pH of the assay medium was maintained at a constant level for all CO₂ concentrations, CO₂ diffuses freely through membranes (18) and could change the internal pH, thus affecting enzyme activities and oxidation rates. This change should occur very quickly, and the responses shown in Figure 1 occurred without any lag period.

Long term exposure of intact fruit to CO₂ evoked changes in activity of extracted mitochondria. The accelerated decline in capacity to oxidize succinate or NADH with time suggests that mitochondrial damage was occurring. Furthermore, the intensification of this decline rather than a lessening or reversal of it when fruit were transferred to air indicates that the damage was irreparable. Noteworthy, however, was the sparing of the CO₂-sensitivity during this decline in activity.

Prolonged exposure to CO₂ reduced oxidative phosphorylation during much of the storage period. While CO₂ clearly did not act as a strong uncoupler of phosphorylation, the observed effect could have been consequential, reducing the energy level within the cell. Atkinson (1) has discussed the possible regulatory mechanisms whereby a drop in the cellular energy level would stimulate sugar utilization. In our study, we did find evidence of accelerated sugar utilization in the apple maintained in 12% CO₂ and 3% O₂, to be presented in a later paper. If despite accelerated sugar utilization the cellular energy remained at a reduced level, structural changes within the cell or mitochondria or both might occur and lead to progressive loss of mitochondrial activity.

LITERATURE CITED