J. Amer. Soc. hort. Sci. 122(2):253-257. 1997.

# Elevated Carbon Dioxide Exposure Alters Intracellular pH and Energy Charge in Avocado Fruit Tissue

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ADDITIONAL INDEX WORDS. Nuclear magnetic resonance spectroscopy, ATP, ADP, $^{31}$P-NMR

**ABSTRACT. Changes in cytosolic and vacuolar pH, ATP, ADP, and the ATP:ADP ratio were measured in whole fruit or mesocarp disks of avocado *[Persea americana* (Mill.) cv. Hass] during brief exposures to elevated $CO_2$. Intact climacteric fruit exposed to air (21 % $O_2$), 20% $CO_2$ (17% $O_2$, balance $N_2$), or 40% $CO_2$ (13% $O_2$, balance $N_2$) had cytosolic pH values of 7.0, 6.6, and 6.4, respectively, while mesocarp disks had cytosolic pH values of 6.9, 6.7, and 6.4, respectively. The ß-ATP levels of intact climacteric fruit exposed to 20% $CO_2$ or 40% $CO_2$ for 2 h were reduced by 25% or 43%, respectively, relative to air-exposed fruit. HPLC analysis of nucleotide phosphates from preclimacteric avocados revealed that ATP levels and the ATP:ADP ratio increased in 40% compared to the air-stored fruit. However, 1 day after transfer to air, the effects of elevated $CO_2$ had dissipated. These modifications in cellular state could alter the activity of respiratory enzymes in fruit exposed to elevated $CO_2$ atmospheres.**

Short postharvest exposures of fruit to $CO_2$ levels >15% have many potential uses, such as alleviation of decay and reduction of insect infestation and certain physiological disorders in fruit (Ke and Kader, 1992; Truter and Eksteen, 1987). These brief $CO_2$ exposures may change the cytosolic pH, which is important in regulating the metabolism in plant tissue (Bown, 1985). To maintain the neutral pH of the cytoplasm, protons are transported across membranes or are consumed or produced by cellular metabolism. Proton pumps in the plasmalemma and tonoplast pump protons into the intercellular spaces and vacuole, respectively (Cerana et al., 1989; Kurkdjian and Guern, 1989; Torimitsu et al., 1984).

Various procedures are available to measure cellular pH (Kurkdjian and Guern, 1989); however, most of them are destructive, which eliminates the ability to measure the pH of the cytosol and vacuole separately. Nuclear magnetic resonance (NMR) spectroscopy

Received for publication 5 Mar. 1996. Accepted for publication 19 Sept. 1996. Research supported in part by U.S. Dept. of Agriculture research agreement no. 58-319R-3-004. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

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has been used as a nondestructive technique that can detect changes in plant metabolism. Roberts (1987) has detailed applying NMR spectroscopy elsewhere.

The most common use of NMR spectroscopy on plant organs has been conducted using $^{31}$P-NMR. Resonances from $^{31}$P nuclei of phosphorylated compounds are sensitive to the chemical environment around each P nucleus. NMR spectroscopy is not very sensitive, so only those compounds that are fairly abundant are measurable. $^{31}$P-NMR is useful in evaluating cytosolic and vacuolar $P_i$ contents, cytosolic pH (Roberts et al., 1980), and levels of selected phosphorylated compounds, such as glucose-6-P, ATP, and ADP. A drawback of this method is that relatively long periods (hours) of data acquisition are necessary to obtain an adequate signal:noise ratio.

Intracellular acidification of plant tissue has been measured during exposure to elevated $CO_2$ or low $O_2$ atmospheres by using $^{31}$P-NMR spectroscopy (Nanos and Kader, 1993; Siriphanich and Kader, 1986). Hess et al. (1993) reported that 20% $O_2$+ 80% $CO_2$ decreased the cytosolic pH of avocado fruit disks to 6.3 relative to 6.9 in air. Siriphanich and Kader (1986) found that 20% $CO_2$ decreased the cytosolic and vacuolar pH of cut lettuce *(Lactuca sativa* L.) pieces. Nanos and Kader (1993) reported that pear *(Pyrus communis* L.) fruit disks stored at 0.25% $O_2$ had cytosolic pH that was 0.4 pH units lower than the cytosolic pH of air (21% $O_2$)-stored fruit disks. The reason for the acidification is unclear, but it may be due to temporary lactic acid accumulation, $CO_2$ buildup, or disruption of proton pumps. Vacuolar pH cannot be accurately measured with this technique because the titration curves are insensitive at pH's <5.0 and the chemical shift of vacuolar $P_i$ depends on the ionic strength (Kurkdjian et al., 1985). Even so, $^{31}$P-NMR measurements indicated that vacuolar pH does not change in hypoxic maize *(Zea mays* L.) roots (Roberts et al., 1982).

In addition, $^{31}$P-NMR spectroscopy may be used to determine changes in cellular ATP and ADP levels, as reported by Bennett et al. (1987) in intact avocado fruit. An advantage of using NMR to observe *in vivo* changes in ATP is that artifacts arising from differences in tissue maturity and extractability are avoided (Young and Biale, 1967). A drawback of NMR is that it cannot differentiate between the 4 nucleotide di- or tri-phosphates (NDP/NTP). However, ATP and ADP are the most abundant. Nanos and Kader (1993) reported that 0.25% $O_2$-treated disks had a lower ATP:ADP ratio suggesting lower energy charge. Hess et al. (1993) reported that treatment of avocado disks with 20% $O_2$+ 80% $CO_2$ reduced ATP levels by 20% compared to the disks held in air. Environmental stresses, such as hypoxia (low $O_2$) or anoxia (no $O_2$), have caused a decrease in energy charge (Davies et al., 1985; Raymond and Pradet, 1980; Vanlerberghe et al., 1990). On transfer to air, energy charge returned to its initial value (Raymond and Pradet, 1980).

A more sensitive technique to measure changes in plant ATP and ADP levels is by high-performance liquid chromatography (HPLC) analysis (Brown and Davies, 1989; Meyer and Wagner, 1985). In addition, the specific NDP's and NTP's may be identified, which allows quantitation of ADP and ATP specifically.

Our objective was to evaluate the effects of 20% $CO_2$+ 17% $O_2$ and 40% $CO_2$+ 13% $O_2$ treatments on avocado fruit and fruit disk cytosolic pH, vacuolar pH, ATP and ADP levels, and the ATP:ADP ratio. These modifications in metabolism are discussed as

possible mechanisms by which elevated $CO_2$ may affect key respiratory enzymes (Lange and Kader, 1997).

## Materials and Methods

**PLANT MATERIALS.** 'Hass' avocado fruit were harvested in June at commercial maturity (≥24% dry mass) from an orchard in Santa Barbara County, Calif., and were transported to our laboratory in Davis, Calif., where they were stored at 10 °C for up to 1 week until initiation of the experiments. Fruit were selected for uniformity of size and freedom from defects. Individual fruit were selected for each experiment based on screening for uniform $C_2H_4$ production rates as described by Kerbel et al. (1988).

**$^{31}$P-NMR SPECTROSCOPY OF FRUIT DISKS.** Two 5 x 15-mm mesocarp disks were cut with a 15-mm internal diameter (ID) cork borer and were placed into a 2 x 18-cm NMR tube. The tube was capped and capillary tubing was used to circulate air (21% $O_2$), 20% $CO_2$ + 17% O, (balance $N_2$), or 40% $CO_2$ + 13% $O_2$ (balance $N_2$) around the disks' for 40 min at a flow rate of 30 mL-min$^1$. $^{31}$P-NMR scanning was conducted immediately at 20 °C using a spectrometer [Omega 300; General Electric (GE) NMR Instruments, Fremont, Calif.]. Two thousand transients were acquired using a 60° angle pulse, a 1.2-s pulse recycle time, 4096 complex time domain points, and a 10-KHz spectral width. Line broadening of 30 Hz was applied before Fourier transformation. Total acquisition time was 45 min. Chemical shifts in all spectra are reported relative to 85% $H_3PO_4$ as an external standard. A capillary tube containing 0.5 M methylene diphosphonic acid was inserted through the center of the disks to serve as an internal standard to help identify peaks. Peak assignments were as follows: I, glucose-6-phosphate (G-6-P); II, cytosolic phosphate ($P_i$); III, vacuolar $P_i$; IV, γ-P of ATP and ß-P of ADP; V, α-P of ATP and ADP; and VI, ß-P of ATP (Figs. 1 and 2). Cytosolic and vacuolar pH values were estimated by using standard curves of pH vs. chemical shift of 5 mM G-6-P and 5 mM potassium phosphate ($P_i$), respectively (Fig. 3). Three individual runs were conducted using separate sets of fruit disks kept under varying $O_2$-$CO_2$ regimes, and the average cytosolic and vacuolar pHs are reported in Table 1.

**$^{31}$P-NMR SPECTROSCOPY OF INTACT FRUIT**. Avocado fruit were partially ripened by gassing with 10 µL-L$^{-1}$ ethylene for 2 d at 20 °C at a flow rate of 400 mL-min$^{-1}$. An individual fruit was heat-sealed into a 2.2-L freezer bag with inlet and outlet ports. Air (21 % $O_2$), 20% CO, + 17% $0_2$ (balance $N_2$), or 40% $CO_2$ + 13% $O_2$ (balance $N_2$) was circulated around the fruit at a flow rate of 100 mL-min$^{-1}$ for 2 h at 20 °C, while the packaged fruit was inside the sample chamber. Spectra were acquired at 20 °C using a 7 Tesla horizontal bore magnet ($^{3l}$P, frequency 121.62 MHz) manufactured by GE NMR Instruments. The transmitting and receiving probes consisted of a home-built, single-turn surface coil copper wire (4.3 cm in diameter) resting on a thin sheet of plexiglass. The avocado fruit rested on its side in a cylindrical holder under the plexiglass. Two capillary tubes filled with 0.5 M methylene diphosphonic acid were taped to the top of the surface coil and served as a chemical shift reference.

The data were collected using a 90° tip angle corresponding to an 80-µs pulse width. The total repetition rate was 2.2 s, and 1200 acquisitions were averaged for each run. The signal:noise ratio was improved by exponential linebroading with a 30-Hz time constant. Data were analyzed using the Omega 6.0.2 software (GE) resident on the

spectrometer. Three runs were conducted using separate fruit for each atmospheric treatment, and the average cytosolic/vacuolar pHs and reduction in ATP (percentage) are reported in Table 1.

**ADENINE NUCLEOTIDE EXTRACTION AND ANALYSIS BY HPLC**. Preclimacteric 'Hass' avocado fruit were treated for 4 d at 20 °C with humidified air, 20% $CO_2$ + 17% $O_2$, or 40% $CO_2$ + 13% $O_2$ at a flow rate of 100 mL-min$^{-1}$. The fruit then were transferred to humidified air for an additional day at 20 °C. Mesocarp tissue samples were frozen in liquid $N_2$ and placed at -80 °C until analyzed at a later date.

The Chromatographic analysis was performed using a Hewlett Packard (model 1050) HPLC system equipped with a diode array detector (model 1040M; Hewlett Packard, Wilmington, Del.) for monitoring simultaneously at 245 and 265 nm. Each sample was run through a 2-cm guard column (model LC-18-T; Supelco, Bellefonte, Pa.), 0.5-µm prefilter, and a microparticle reverse-phase (4.6 mm x 15 cm, 3-µm particle size) column (model C-l 8 Supelcosil LC-18-T; Supelco). Hewlett Packard ChemStation computer software (version A.02.00) was used to program the gradient. Reagent grade ATP, ADP, AMP, and IMP were obtained from Sigma Chemical Co. (St. Louis). Analytical reagent grade potassium dihydrogen phosphate ($KH_2PO_4$), dipotassium hydrogen phosphate ($K_2HPO_4$), tetrabutylammonium hydrogen sulfate, and perchloric acid, as well as HPLC grade methanol, were used for the mobile phase.

After preliminary experiments, the following solutions and conditions were used as the standard procedure for the analysis of ATP and its breakdown products as previously reported by Watanabe et al. (1989) with some modifications. The first eluant was 0.1 M potassium phosphate buffer containing 4 mM tetrabutylammonium hydrogen sulfate (pH 6.0) as the ion pair reagent (buffer-1); the second was 0.07 M potassium phosphate buffer (pH 7.2) containing 30% (v/v) methanol (buffer-2). For the first 2.5 min, 100% of buffer-1 was run, followed for the next 2.5 min by a linear gradient from 0% to 30% of buffer-2, then 5 min of a linear gradient from 30% to 60% of buffer-2. The next gradient step was to increase the buffer-2 from 60% to 100% of buffer-2 for 3 min, followed by an additional 4 min at 100% buffer-2. The final steps included a linear gradient from 100% to 0% buffer-2 for 3 min, followed by a 2 min wash of 100% buffer-1 between samples to equilibrate the system. The total acquisition time for each run was 20 min. The flow rate was 1.0 mL-min$^{-1}$, and the column temperature was 23 to 25 °C.

Ten grams of frozen (-80 °C) avocado tissue were ground into pieces with a mortar and pestle and were extracted with 15 mL of l N cold perchloric acid and 200 µL of AMP at 1 mg-mL$^{-1}$ (internal standard) in a homogenizer (Polytron; Brinkmann Instruments, New York). AMP was used as an internal standard because in preliminary tests no AMP was detected. The homogenate was centrifuged at 10,000x g for 15 min, and the supernatant was filtered through four layers of cheesecloth. The pellet was re-extracted in 15 mL l N perchloric acid and then centrifuged. The supernatants were combined and neutralized with ≈1.2 g solid potassium carbonate and then centrifuged at 10,000 x *g* for 10 min. To remove the neutral lipid from the avocado supernatant, two rounds of hexane extraction in a separatory funnel were necessary. The resulting supernatant was filtered through a 0.22-µm filter, and 20 µL were injected into the HPLC. Assignment of the peaks was achieved by retention time analysis and co-injection with standards. ATP and ADP were detected at 265 nm without interference from IMP.

## Results and Discussion

$^{3l}$P-NMR measurements on avocado fruit disks showed a shift in the resonance of G-6-P, cytosolic $P_i$, and vacuolar $P_i$ from 5 ppm to 3 ppm due to the elevated $CO_2$ treatments (Fig. 1). Using a standard curve in which the chemical shifts of G-6-P and P. were determined at several pH levels (Fig. 3A), cytosolic and vacuolar pH levels were estimated (Table 1). The chemical shifts in G-6-P were used to estimate cytosolic pH, whereas the shifts of inorganic potassium phosphate were used to estimate vacuolar pH (Fig. 3 A and B).

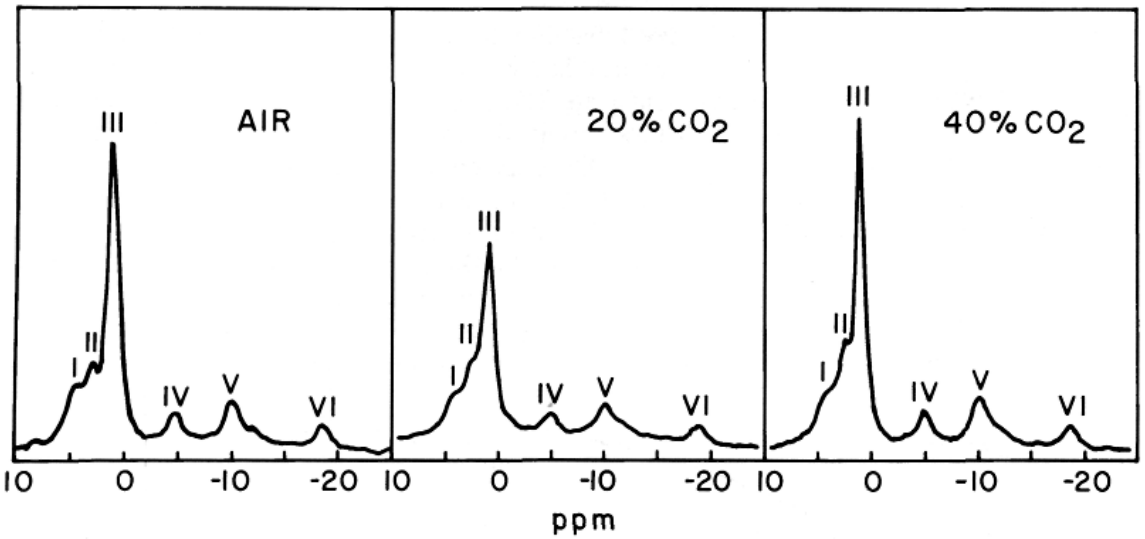


Fig. 1. Effects of air (21% $O_2$), 20% $CO_2$ + 17% $O_2$, and 40% $CO_2$ + 13% $O_2$ for 45 min at 20 °C on 'Hass' avocado fruit disks. Chemical shifts of glucose-6-phosphate (G-6-P), cytosolic inorganic phosphate ($CytP_i$), and vacuolar inorganic phosphate ($VacP_i$) were measured by $^{31}$P-NMR spectroscopy. Peak assignments are as follows: I, G-6-P; II, $CytP_i$; III, $VacP_i$; IV, γ-P of ATP and ß-ADP; V, α-P of ATP and ADP; and VI, ß-P of ATP.

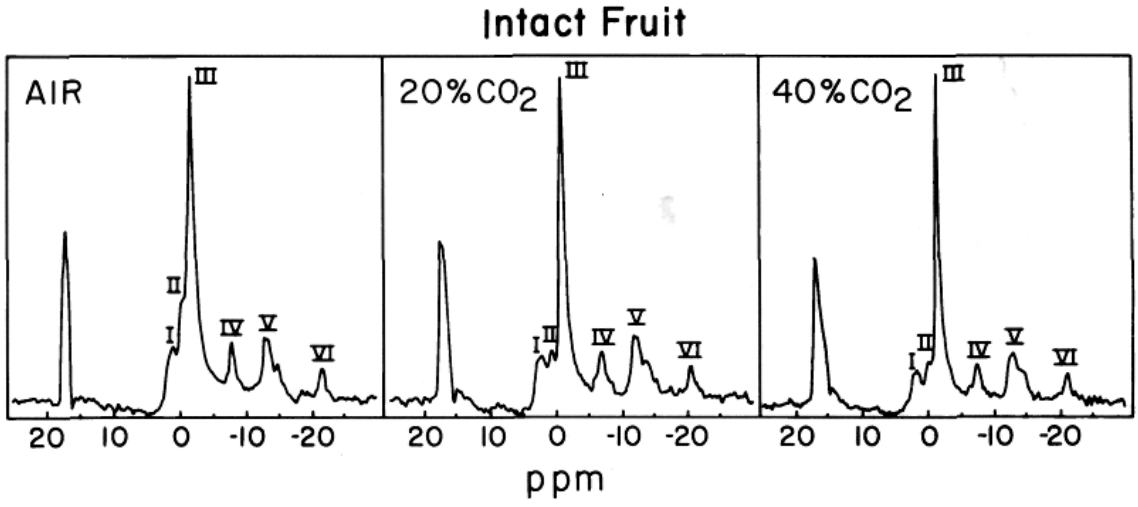


Fig. 2. Effects of air (21% $O_2$), 20% $CO_2$ + 17% $O_2$, and 40% $CO_2$ + 13% $O_2$ for 2 h at 20 °C on 'Hass' avocado fruit disks. Chemical shifts of glucose-6-phosphate (G-6-P), cytosolic inorganic phosphate ($CytP_i$), and vacuolar inorganic phosphate ($VacP_i$) were measured by $^{31}$P-NMR spectroscopy. Peak assignments are as follows: I, G-6-P; II, $CytP_i$; III, $VacP_i$; IV, γ-P of ATP, and ß-ADPt; V, α-P of ATP and ADP; and VI, ß-P of ATP. The first peak represents the 0.5 M methylene diphosphonic acid internal standard.

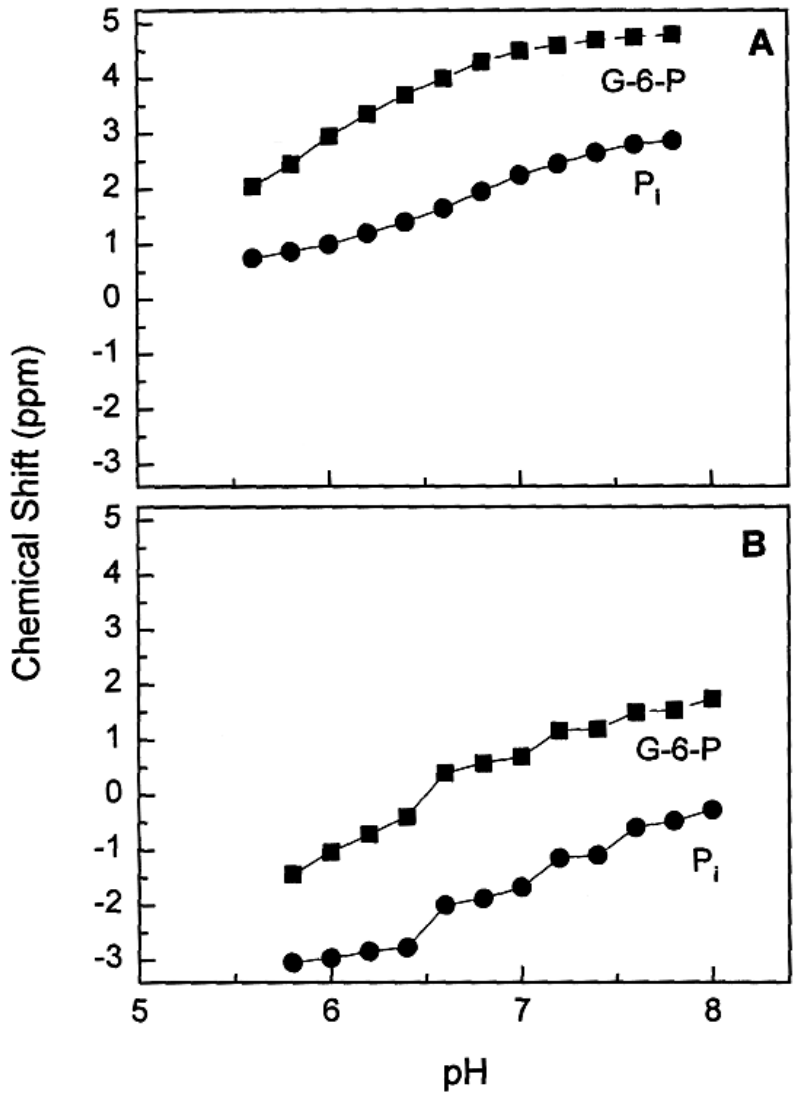


Fig. 3. Calculated effects of pH on chemical shifts of glucose-6-phosphate (G-6-P) and inorganic phosphate ($P_i$) measured by NMR spectroscopy using 'Hass' avocado (**A**) fruit disks or (**B**) intact fruit. These data were based on standard solutions of 5 mM G-6-P and potassium phosphate.

Fruit disks held in 20% $CO_2$ + 17% $O_2$ had a 0.2-unit decrease in cytosolic pH relative to disks held in air, whereas disks held in 40% $CO_2$ + 13% $O_2$ had a 0.5-unit decrease in cytosolic pH. In a similar study using intact avocados treated for 2 h, there were decreases in cytosolic pH of 0.4 (20% $CO_2$) and 0.6 (40% $CO_2$) pH units (Table 1 and Fig. 2). Vacuolar pH either decreased slightly (fruit disks) or not at all (intact fruit) under elevated $CO_2$ atmospheres (Table 1). $^{31}$P-NMR spectroscopy was not a sensitive technique to estimate vacuolar pH changes (Nanos and Kader, 1993).

The decrease in cytosolic pH in this study is in agreement with reports of Lakshminarayana and Subramanyan (1970), who reported the acidification of mango fruit by elevated $CO_2$ but contrasts reports by Siriphanich and Kader (1986) and Lebermann et al. (1968) in which lettuce and broccoli *(Brassica olerácea* var. *italica*

Plenck), respectively, treated with elevated $CO_2$ had increased homogenate pH. Siriphanich and Kader (1986) found a decrease in cytosolic pH of lettuce leaf disks when measured by $^{3l}$P-NMR, as in our study. These contradicting results between tissue types may be due to the major difference between the physiology of a fruit vs. that of a vegetative tissue, such as lettuce leaves or broccoli stems and florets. Another explanation may be that homogenate pH from broccoli or lettuce is not a good estimate of fruit cystolic pH. Siriphanich and Kader (1986) suggested that these differences may be due to greater buffering capacities in some plant tissues relative to others that allow maintenance of a higher pH and survival under moderately elevated $CO_2$ atmospheres. The rationale for intracellular acidification of plant tissue by exposure to elevated $CO_2$ is that carbonic acid dissociates to bicarbonate and hydrogen ion, which could cause the pH of cells to drop. The pH of vacuolar space may have been higher in whole fruit compared to fruit disks due to wounding of disk tissue and, hence, acidification of the surrounding tissue.

Lower ß-ATP signals were detected in avocado fruit tissue treated with elevated $CO_2$ compared to tissue kept in air (Table 1 and Fig. 3) as previously reported by Siriphanich and Kader (1986) in lettuce leaf tissue. Treatment with 40% $CO_2$ resulted in a roughly 2-fold reduction in ATP relative to treatment with 20% $CO_2$ (Table 1). This decrease in ATP content was estimated using just the chemical shift peak from ß-ATP (Fig. l, Peak VI) since that was the only peak without interfering phosphorus signals from other compounds. Reduction in ATP (percentage) within avocado fruit disks was not reported since the results were variable and lacked precision due to a low signal: noise ratio (Fig. 1). One possible explanation for the reduced ATP content in fruit treated with elevated $CO_2$ is that the reduction may be due to an uncoupling effect on oxidative phosphorylation (Fanestil et al., 1963). Thus, $CO_2$ could be limiting the energy supply needed for survival of plant tissue.

HPLC determination of ATP and ADP concentrations in avocado fruit treated with air, 20% $CO_2$, or 40% $CO_2$ at 20 °C for up to 4 d revealed that ATP and ADP levels changed in a parallel manner (Fig. 4A). On day 2, fruit treated with 20% $CO_2$ had higher ATP and ADP contents relative to fruit held in air, whereas on day 4, fruit treated with 40% $CO_2$ had the highest ATP and ADP levels. After transfer to air for 1 d, the differences in ATP and ADP levels dissipated. The ATP:ADP ratio of fruit treated with 40% $CO_2$ was higher than that for fruit held in air on day 4, but after transfer to air for 1 d (day 5), air and 40% $CO_2$-stored fruit had similar ATP:ADP ratios that were higher than fruit held in air. These findings are in contrast to the reduction in ATP that was observed in the $^{31}$P-NMR studies (Table l, Fig. 4A). However, these experiments differed greatly in the physiological age of the fruit and the treatment duration. The HPLC study on ATP content occurred over 5 d and used preclimacteric fruit, whereas the NMR study occurred over *2* h and was conducted on climacteric fruit. The NMR study addressed the rapid, direct effects of elevated $CO_2$ on avocados, while the longer term HPLC study took into account indirect, residual responses to elevated $CO_2$ exposure.

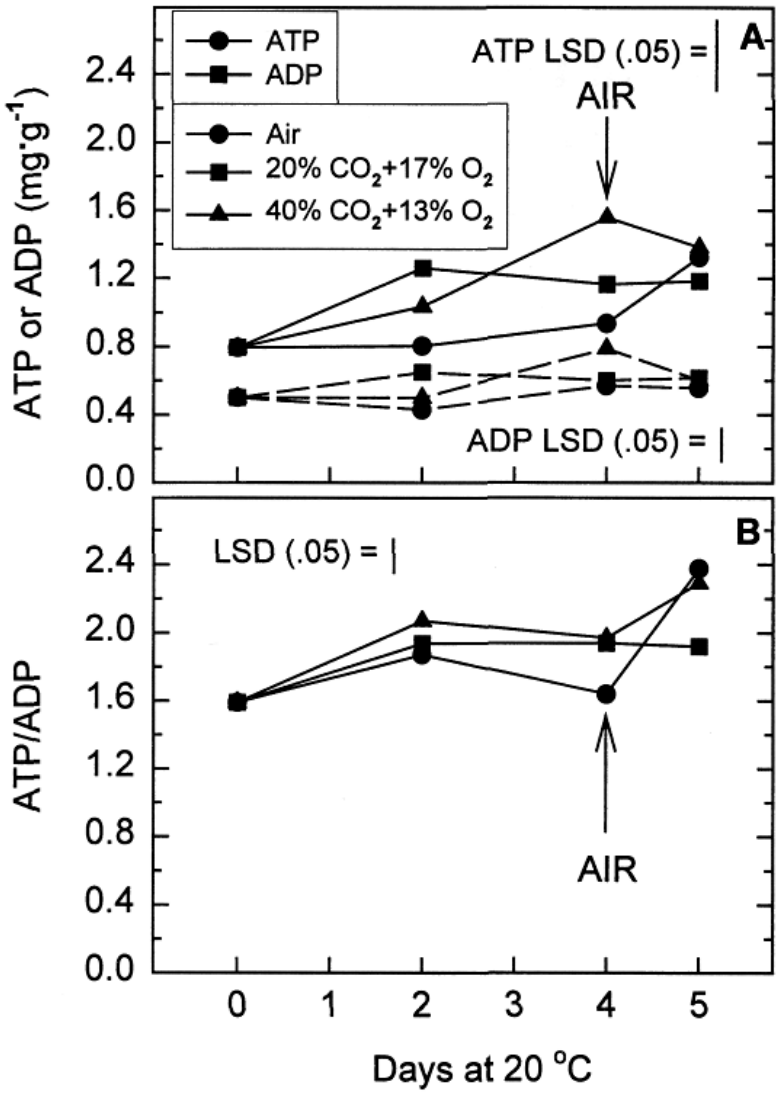


Fig. 4. Changes in (**A**) ATP and ADP concentrations (based on fresh mass) and (**B**) ATP : ADP ratios in preclimacteric 'Hass' avocados kept at 20 °C during 4 d of treatment with air (21% $O_2$), 20% $CO_2$ + 17% $O_2$, and 40% $CO_2$ + 13% $O_2$, and after transfer to air for 1 d.

Table 1. Changes in pH and ATP levels as determined by $^{31}$P-NMR spectroscopy in 'Hass' avocado fruit disks and intact fruit kept at 20 °C in air (21% $O_2$), 20% $CO_2$ + 17% $CO_2$, or 40% $CO_2$ + 13% $O_2$ for 45 min (disks) or 2 h (fruit).

| | Preclimacteric fruit disks | | Intact climacteric fruit | | |
|---|---|---|---|---|---|
| Treatment | Cytosolic pH | Vacuolar pH | Cytosolic pH | Vacuolar pH | Reduction in ATP (%) |
| Air | 6.90 | 6.20 | 7.00 | 7.00 | 0 |
| 20% $CO_2$ + 17% $O_2$ | 6.70 | 6.00 | 6.60 | 7.00 | 25 |
| 40% $CO_2$ + 13% $O_2$ | 6.40 | 6.00 | 6.40 | 7.00 | 43 |
| $LSD_{0.05}$ | 0.10 | 0.05 | 0.17 | 0.08 | 6 |

Since $CO_2$ uncoupled oxidative phosphorylation (Fanestil et al, 1963; Palet et al, 1991), one would expect less ATP to accumulate in $CO_2$-stressed tissues as shown in Table 1. An accumulation of ATP may be a plant defense-related response to the physiological lesion that may occur to membranes and enzymes under enviromental stresses such as elevated $CO_2$ exposure. This defensive response may be necessary to produce more energy-related compounds (such as ATP) to accommodate the additional energy needs of cellular repair.

ATP and ADP have regulated an array of respiratory enzymes, and the changes of these metabolites resulting from hypoxic treatment can play an important role in regulating respiration (Raymond et al., 1987). The decrease in cytosolic pH in $CO_2$-treated avocado tissue also can affect the activity of a number of respiratory enzymes (Turner and Turner, 1980; Wiskich, 1980). We found that a decrease in pH of 0.2 to 0.6 units could decrease the *in vitro* activity of key respiratory enzymes by 30% to 85% (Lange and Kader, 1997). Elevated $CO_2$ acidifies avocado fruit tissue and changes ATP and ADP levels depending on the duration of $CO_2$ exposure and maturity of the fruit.

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