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## Carbon dioxide action on metabolism of organic and amino acids in crisphead lettuce \*

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### ABSTRACT

Tissue of crisphead lettuce (*Lactuca sativa* L.) was kept in air or air enriched with 5% to 20% CO<sub>2</sub> at 0°C for 2 to 9 days followed by transfer to air or the CO<sub>2</sub>-enriched atmosphere at 20°C for 1 day to study the mode of CO<sub>2</sub> action on metabolism of organic and amino acids. Exposure to 20% CO<sub>2</sub> reduced the extractable activity of succinate dehydrogenase. The 20% CO<sub>2</sub> treatment caused a decrease in pH, which partially inhibited the catalytic activity of succinate dehydrogenase and substantially activated glutamate decarboxylase. As a result, succinate and  $\gamma$ -aminobutyrate accumulated and concentrations of malate and glutamate decreased. Such effects on the organic and amino acids were more pronounced with higher temperature, higher CO<sub>2</sub> concentrations, and longer exposure to CO<sub>2</sub>. The accumulation of succinate and  $\gamma$ -aminobutyrate was induced by 15% or 20% CO<sub>2</sub> in all the three tested cultivars ('Climax', 'Salinas', and 'Winter Haven') and in both green and white tissues of lettuce, regardless of variation in their susceptibilities to CO<sub>2</sub> injury.

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Key words: Lettuce; *Lactuca sativa*; Organic acid; Amino acid; Controlled atmosphere; Physiological disorder

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### INTRODUCTION

Crisphead lettuce is very sensitive to CO<sub>2</sub> injury (Stewart and Uota, 1971; Brecht et al, 1973; Kader and Morris, 1977). Responses of lettuce to elevated CO<sub>2</sub> atmospheres included a decrease in titratable acidity, induction of phenylalanine ammonia-lyase (PAL), accumulation and oxidation of soluble phenolic compounds,

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and cell wall lignification (Singh et al., 1972; Siriphanich and Kader, 1985 and 1986; Ke and Saltveit, 1989). Under elevated CO<sub>2</sub> atmospheres, pH was decreased; but upon transfer to air a recovery in pH of the lettuce tissue was noted (Siriphanich and Kader, 1986).

The mode of CO<sub>2</sub> action on metabolism of organic and amino acids in plant tissue is unclear. Varied and sometimes contradictory effects have been reported (Weichmann, 1986), probably due to the differences in temperature, atmospheric composition, duration of exposure to CO<sub>2</sub>, and commodities used. Studies on apples and pears indicated that CO<sub>2</sub> reduced succinate dehydrogenase (SDH) activity and caused the accumulation of succinate (Hulme, 1956; Williams and Patterson, 1964; Frenkel and Patterson, 1973). On the other hand, McGlasson and Wills (1972) reported that CO<sub>2</sub> increased concentrations of malate, citrate, pyruvate, 2-oxoglutarate, glyoxylate, oxaloacetate, glutamate, and aspartate but not succinate in bananas. Wankier et al. (1970) found that increasing CO<sub>2</sub> concentration enhanced succinate and alanine contents, decreased malate and aspartate concentrations, but did not significantly influence citrate level in apricots and peaches. Saijo et al. (1989) reported that glutamate content decreased, while glutamine and  $\gamma$ -aminobutyrate concentrations increased in tomatoes kept in air enriched with 5% to 20% CO<sub>2</sub>.

In this research, we studied the interrelationships among changes in concentrations of organic and amino acids, intracellular pH, and regulation of related enzymes as influenced by CO<sub>2</sub> level, duration of exposure to CO<sub>2</sub>, temperature, cultivar, and tissue type of lettuce. Such information may help to elucidate the mechanism of CO<sub>2</sub> action on metabolism of organic and amino acids in plant tissue.

## MATERIALS AND METHODS

### *Materials and treatments*

Heads of lettuce grown under commercial conditions in Salinas and El-Centro, California, were harvested and vacuum cooled and then transported in an air-conditioned car to the Postharvest Laboratory of the Pomology Department at Davis. Four experiments were conducted to study the effects of CO<sub>2</sub> on organic and amino acids. In the first experiment, 'Salinas' lettuce tissues were held in air (control) and in air + 5%, 10%, 15%, or 20% CO<sub>2</sub> for 5 days at 0°C. In the second experiment, 'Salinas' lettuce tissues were exposed to air or air + 20% CO<sub>2</sub> at 0°C for 3, 6, or 9 days followed by transfer to air or air + 20% CO<sub>2</sub> at 20°C for 1 day. In the third experiment, lettuce tissues of the 'Climax', 'Salinas', and 'Winter Haven' cultivars were kept in air or air + 15% CO<sub>2</sub> for 6 days at 0°C. In the fourth experiment, green and white tissues of 'Salinas' lettuce were kept in air or air + 20% CO<sub>2</sub> at 0°C for 6 days followed by transfer to air at 20°C for 1 day. Three replicates were used per treatment in all the four experiments. For experi-

ments one, two, and three, 12- to 24-cm<sup>2</sup> midrib segments were cut from the leaves located one-third to two-third the distance between the outer and center leaves of lettuce heads. The segments were randomly distributed among treatments and kept in 4-l glass jars ventilated with humidified air or air plus the desired CO<sub>2</sub> concentrations. For experiment four, green and white tissue segments were cut separately from lettuce heads and kept under desired storage conditions. The required CO<sub>2</sub> concentrations were verified each day by analysis of a 10-ml gas sample using a Carle gas chromatograph (Model 111) with a thermal conductivity detector.

#### *Extraction, purification, and determination of organic acids*

The procedure was slightly modified from that of Brecht (1973) and Stumpf and Burris (1979). Fifty grams of lettuce tissues were chopped and preserved in boiling 95% ethanol (v/v). An internal standard (malonic acid) was added to the boiling ethanol for quantitative determinations of organic acids. The residue of the 95% ethanol extract was homogenized in 80 ml of 80% ethanol and then filtered. The residue left was resuspended in another 80 ml of 80% ethanol, allowing it to simmer for 10 minutes. The homogenate was filtered and combined with the previous filtrate. The filtrate was evaporated to about 100 ml, followed by adding petroleum ether to wash twice. After the ether washes were discarded, the sample was passed through a 10 × 200 mm Dowex 50-X 8 (H<sup>+</sup> form) column. The column was rinsed with 50 ml of water and the eluate which contained organic acids and sugars was collected. In order to remove sugars, the eluate was passed through a 10 × 200 mm Dowex 1-X 8 (formate) column followed by rinsing with 50 ml of water. Next, the organic acids were eluted with 60 ml of 6N formic acid and this eluate was evaporated to dryness. The residue was redissolved in 10 ml of 50% ethanol. A 2-ml aliquot was put into a serum vial and evaporated to dryness. One ml of trimethylsilyl (TMS) derivative (trimethylchlorosilane : hexamethyldisiloxane : pyridine, 1:4:5, v/v/v) was added into each sample vial. The vial was quickly capped, shaken for 1 hour at 30°C, and then stored at -20°C until used for analysis. The organic acids were determined by using a dual flame-ionization gas chromatograph which was equipped with a 12.5 × 1/8" stainless steel column packed with 2% QF-1 on 80-100 mesh (w/w). The temperature program was 145 to 225°C at 10°C · min<sup>-1</sup> and the detector and injector temperatures were 210 and 190°C, respectively. The flow rate of nitrogen, as a carrier gas, was 30 ml · min<sup>-1</sup>; the flow rates of air and hydrogen, gases for the flame ionization detector, were 250 and 25 ml · min<sup>-1</sup>, respectively.

#### *Determination of free amino acids*

The procedure for extraction and detection of amino acids was slightly modified from that of Butts (1972). In brief, 50 g of lettuce tissue was homogenized in 100 ml of water. Decanoic acid (capric acid) was added to the homogenate as internal

standard for quantification of amino acids. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at  $25\,000 \times g$  at  $4^{\circ}\text{C}$  for 25 min. The supernatant was frozen at  $-20^{\circ}\text{C}$  until used for analysis. Free amino acids were analyzed as trimethylsilyl derivatives using a two-column gas chromatograph with a dual flame ionization detector (Butts, 1972). Tentative identification of constituents was based on comparison of retention times with those of known standards. The identities of amino acids were further confirmed by a gas chromatograph/mass spectrometer.

#### *Mitochondria isolation and assay of SDH*

Mitochondria were isolated from lettuce tissue using the method of Romani et al. (1969) with slight modification. Fifty g midrib tissue were macerated in 150 ml isolation medium which consisted of 50 mM potassium phosphate buffer (pH 7.2), 0.25 M sucrose, 5 mM EDTA, 4.5 mM  $\beta$ -mercaptoethanol, 0.2% soluble PVP (w/v) and 0.1% BSA (w/v). The pH of the slurry was continuously adjusted to 7.2 with small addition of 5N KOH. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at  $2000 g$  for 10 min. The supernatant was filtered through 4 layers of cheesecloth and centrifuged at  $10\,000 g$  for 5 min. This supernatant was discarded and the pellet was resuspended in 1 ml wash medium and homogenized with a glass microhomogenizer in the presence of 3 additional ml wash medium. The wash medium contained 50 mM potassium phosphate buffer (pH 7.2), 0.25 M sucrose and 0.1% BSA. The homogenate was centrifuged at  $2000 g$  for 5 min and the supernatant was recentrifuged at  $8000 g$  for 10 min. The pellet was resuspended in 0.5 ml wash medium. Aliquot of this suspension was diluted 1:5 with wash medium and used as the mitochondrial preparation.

SDH assay was performed using the method of Frenkel and Patterson (1973) with some modification. The reaction mixture contained 90 mM potassium phosphate buffer (pH 7.2), 3 mM KCN, 1.8 mM phenazine methosulphate, 0.03 mM dichlorophenol indophenol, 18 mM succinate, and 0.1 ml mitochondrial preparation. The decrease in  $A_{600}$  was followed at  $25^{\circ}\text{C}$  and was linear for at least 3 min. SDH activity was expressed as  $\mu\text{mol}$  of product formed per min per mg protein. The protein content of mitochondrial preparation was determined by the standard Bradford (1976) method.

In a separate experiment, a series of potassium phosphate solutions with various pH values were employed as assay buffers to study the influence of pH on SDH activity. To investigate the effect of *in vitro* addition of  $\text{CO}_2$  on the catalytic activity of SDH, the mitochondrial preparation and all other assay components were kept under a continuous flow ( $400 \text{ ml} \cdot \text{min}^{-1}$ ) of an air + 20%  $\text{CO}_2$  gas mixture in a thick plastic bag for 2 to 3 hrs. Then, a small hole was made to allow the insertion of a micropipetter to take the appropriate amount of each assay component. The hole was closed rapidly after the pipetting using a piece of tape. All the assay components were mixed quickly and the cuvette was flushed with air + 20%  $\text{CO}_2$ . The initial SDH activity was measured immediately. The pH of the assay mixture was determined following the enzyme measurement.

*Extraction and assay of glutamate decarboxylase (GDC)*

The procedure for extracting and assaying GDC from lettuce tissue was modified from Tsushida and Murai (1987). In brief, 4 g midrib tissue were homogenized in 16 ml of extraction buffer (pH 5.8) which contained 50 mM sodium phosphate and 0.02 mM pyridoxal-5'-phosphate. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 27 000 g for 20 min. The supernatant was retained as enzyme extract. To measure GDC activity, 0.5 ml of 60 mM sodium L-glutamate was added to 4.5 ml of enzyme extract in a test tube and the reaction mixture was incubated at 37°C. The enzyme reaction rate was constant for at least 2 h under these conditions. GDC activity was expressed as  $\mu$ mole of product formed per h per g fresh weight (FW). To study the influence of pH on GDC activity, the enzyme was extracted and assayed with a series of sodium phosphate buffers at various pH values.

## RESULTS AND DISCUSSION

*Effects of CO<sub>2</sub> on concentrations of organic and amino acids**Variation with CO<sub>2</sub> level*

The major organic acids in lettuce included citrate, fumarate, malate, and succinate. Exposure of 'Salinas' lettuce tissue to air + 20% CO<sub>2</sub> substantially increased succinate content, slightly decreased malate concentration, but did not significantly influence citrate or fumarate content over those of air control. Keeping lettuce tissue in air + 5%, 10%, or 15% CO<sub>2</sub> at 0°C for 5 days did not have significant effects on any of the organic acids (Table 1).

Malate was the most abundant organic acid whereas fumarate was present in much lower concentration in lettuce; citrate existed in moderate amount (Table 1). Malate is the major organic acid in many fruits and vegetables such as apples, pears, peaches, and tomatoes. In the tricarboxylic acid (TCA) cycle, fumarate → malate → oxaloacetate, the standard Gibbs free energy changes ( $\Delta G^{\circ}$ ) for these two steps are  $-0.9$  and  $+7.1$  kcal · mol<sup>-1</sup>, respectively (Stryer, 1981). This means

TABLE 1

Effect of elevated CO<sub>2</sub> levels on contents of organic acids in 'Salinas' lettuce tissue after 5 days at 0°C

Treatment	Content [mg · (100 g fresh weight) <sup>-1</sup> ]			
	Citrate	Fumarate	Malate	Succinate
Air	4.6a <sup>1</sup>	0.56a	43a	1.0b
5% CO <sub>2</sub>	4.9a	0.67a	39ab	1.0b
10% CO <sub>2</sub>	5.0a	0.62a	38ab	1.2b
15% CO <sub>2</sub>	4.9a	0.70a	39ab	1.3b
20% CO <sub>2</sub>	4.8a	0.69a	35b	2.6a

<sup>1</sup> Mean separation within columns by Duncan's test at  $P = 0.05$ .

TABLE 2

Effect of elevated CO<sub>2</sub> levels on contents of amino acids (alanine,  $\gamma$ -aminobutyrate, asparagine, glutamate, glycine, lysine, phenylalanine, serine, threonine and valine) in 'Salinas' lettuce tissue after 5 days at 0°C

Treatment	Content [mg·(100 g frsh weight) <sup>-1</sup> ]										
	Ala	$\gamma$ -AB	Asp	Glu	Gly	Leu	Lys	Phe	Ser	Thr	Val
Air	5.5a <sup>1</sup>	0.8c	13a	15a	3.7a	2.8a	8.1a	0.7a	9.0a	7.2a	2.6a
5% CO <sub>2</sub>	5.2a	1.0c	12a	14b	3.2a	3.6a	8.0a	0.9a	8.5a	7.3a	2.3a
10% CO <sub>2</sub>	5.1a	1.1c	13a	13b	3.7a	3.3a	7.6a	0.9a	8.0a	7.3a	2.4a
15% CO <sub>2</sub>	5.3a	1.4b	13a	14b	3.9a	3.3a	8.3a	0.8a	8.4a	7.3a	2.6a
20% CO <sub>2</sub>	5.5a	2.4a	13a	12c	3.7a	2.8a	7.8a	0.9a	7.8a	7.0a	2.6a

<sup>1</sup> Mean separation within columns by Duncan's test at *P* = 0.05.

that energetically, the conversion of fumarate to malate is a "downhill" reaction whereas the conversion of malate to oxaloacetate requires energy input. These energy changes favor the accumulation of malate.

Following storage at 0°C for 5 days, 15% or 20% CO<sub>2</sub> increased  $\gamma$ -aminobutyrate content in 'Salinas' lettuce tissue while 5% and 10% CO<sub>2</sub> had no significant effects (Table 2). All the elevated CO<sub>2</sub> atmospheres slightly decreased glutamate content. The elevated CO<sub>2</sub> atmospheres had only very slight or no effects on the other amino acids.

*Variation with duration of exposure to CO<sub>2</sub> and temperature*

The 20% CO<sub>2</sub> treatment increased succinate content in 'Salinas' lettuce tissue kept at 0°C (Fig. 1A). This effect became more pronounced as duration of exposure to CO<sub>2</sub> was extended. Succinate content further increased if the lettuce

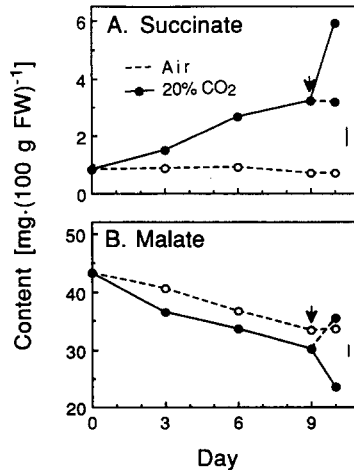


Fig. 1. Changes in contents of succinate and malate in 'Salinas' lettuce tissue exposed to air or air+20% CO<sub>2</sub> at 0°C for 3, 6, or 9 days followed by transfer (indicated by arrow) to air or air+20% CO<sub>2</sub> at 20°C for 1 day. The vertical bars represent pooled LSD values at *P* = 0.05.

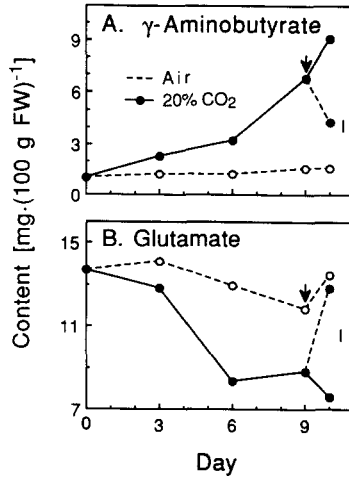


Fig. 2. Changes in contents of  $\gamma$ -aminobutyrate and glutamate in 'Salinas' lettuce tissue exposed to air or air + 20% CO<sub>2</sub> at 0°C for 3, 6, or 9 days followed by transfer (indicated by arrow) to air or air + 20% CO<sub>2</sub> at 20°C for 1 day. The vertical bars represent pooled LSD values at  $P = 0.05$ .

tissue was transferred from 20% CO<sub>2</sub> at 0°C to 20% CO<sub>2</sub> at 20°C for a day. If lettuce tissue was transferred from 20% CO<sub>2</sub> at 0°C to air at 20°C, however, succinate concentration did not change.

Malate content gradually decreased in tissues stored at 0°C (Fig. 1B). The tissue exposed to 20% CO<sub>2</sub> had lower malate content than that of air control tissue. Malate content further decreased if the tissue was transferred from 20% CO<sub>2</sub> at 0°C to 20% CO<sub>2</sub> at 20°C. In the 20% CO<sub>2</sub>-treated lettuce tissues, an increase in succinate content was usually accompanied by a decrease in malate concentration ( $r = -0.96$ ,  $P < 0.01$ ). If the lettuce tissue was transferred from 20% CO<sub>2</sub> at 0°C to air at 20°C, malate content increased.

When kept in air + 20% CO<sub>2</sub> at 0°C,  $\gamma$ -aminobutyrate content increased in lettuce tissue as duration of exposure was extended (Fig. 2A). The  $\gamma$ -aminobutyrate content further increased when the tissue was transferred from 20% CO<sub>2</sub> at 0°C to 20% CO<sub>2</sub> at 20°C; but the content decreased when the 20% CO<sub>2</sub>-treated tissue was transferred to air at 20°C. The 20% CO<sub>2</sub> treatment caused a decrease in glutamate concentration (Fig. 2B). The glutamate content returned to near its original level following removal of the lettuce tissue from 20% CO<sub>2</sub> at 0°C to air at 20°C. The negative correlation between  $\gamma$ -aminobutyrate content and glutamate concentration of 20% CO<sub>2</sub>-treated tissue was significant ( $r = -0.82$ ,  $P < 0.05$ ).

#### *Differences among cultivars and between tissue types*

After storage at 0°C for 6 days, increased concentrations of succinate and  $\gamma$ -aminobutyrate were noted in 15% CO<sub>2</sub>-treated tissues from all the three lettuce cultivars (Table 3). It was suggested that the accumulation of succinate might be the reason for CO<sub>2</sub> injury in apples and pears (Hulme, 1956; Williams and Patterson, 1963). On the other hand, Menegus et al. (1989) reported that rice (with

TABLE 3

Variation in contents of succinate and  $\gamma$ -aminobutyrate among 'Climax', 'Salinas', and 'Winter Haven' lettuce cultivars (A) and between green and white tissues of 'Salinas' lettuce (B). Measurements were done after storage in air or air + 15% or 20% CO<sub>2</sub> at 0°C for 6 days

Cultivar	Tissue	Content [mg · (100 g fresh weight) <sup>-1</sup> ]			
		Succinate		$\gamma$ -aminobutyrate	
<b>Experiment A</b>		Air	15% CO <sub>2</sub>	Air	15% CO <sub>2</sub>
Climax		0.3c <sup>1</sup>	1.8a	0.0c	2.9a
Salinas		0.3c	1.3ab	0.0c	2.2ab
Winter Haven		0.3c	2.0a	0.6c	1.7b
<b>Experiment B</b>		Air	20% CO <sub>2</sub>	Air	20% CO <sub>2</sub>
Salinas	Green	0.4c	5.3a	1.6b	9.1a
Salinas	White	0.4c	2.8b	0.2c	9.3a

<sup>1</sup> Mean separation within pairs of columns of each experiment by Duncan's test at  $P = 0.05$ .

higher resistance to anoxia) accumulated more succinate than wheat (with lower resistance to anoxia) in response to anaerobic stress. Although 'Climax' is much more sensitive to CO<sub>2</sub> injury than 'Salinas' and 'Winter Haven' (Siriphanich, 1984), these differences cannot be explained by differences in concentrations of succinate and  $\gamma$ -aminobutyrate. The accumulation of succinate and  $\gamma$ -aminobutyrate appears to be a common response to elevated CO<sub>2</sub> stress.

Exposure to 20% CO<sub>2</sub> at 0°C for 6 days increased succinate and  $\gamma$ -aminobutyrate contents in both green and white tissues of 'Salinas' lettuce, and the green tissue had higher succinate concentration than that of white tissue (Table 3). White tissue is more susceptible to CO<sub>2</sub> injury than green tissue (Brecht, 1973; Siriphanich, 1984), but this difference can not be explained on the basis of differences in succinate and  $\gamma$ -aminobutyrate contents.

#### Regulation of related enzymes by CO<sub>2</sub>

Succinate dehydrogenase (SDH) catalyzes the conversion of succinate into fumarate. This enzyme was sensitive to pH change with its optimum activity at pH 8.3 (Fig. 3). Previous work (Siriphanich and Kader, 1986) with <sup>31</sup>P-NMR technique

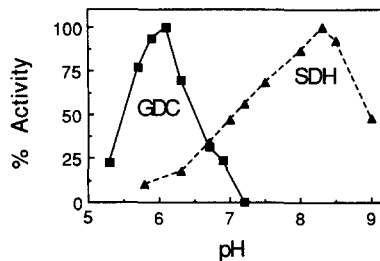


Fig. 3. Effects of assay pH on relative activities of succinate dehydrogenase (SDH) and glutamate decarboxylase (GDC) from lettuce tissue.



indicated that exposure to 20% CO<sub>2</sub> caused a decrease in cytoplasmic pH from 6.7 to 6.3. Although we did not know the exact pH in mitochondria where SDH is located, we could expect that in this metabolically active organelle the normal physiological pH would be higher than the cytoplasmic pH of 6.7. This is based on the fact that an H<sup>+</sup>-ATP pump is located in the inner membrane of mitochondria, which pumps H<sup>+</sup> out of the organelle and maintains a pH gradient across the membrane. When CO<sub>2</sub> is dissolved in water, the major components from the dissolved CO<sub>2</sub> are CO<sub>2</sub>, HCO<sub>3</sub><sup>-</sup>, and H<sup>+</sup> (Umbreit, 1949). The production of H<sup>+</sup> by dissolved CO<sub>2</sub> would cause a reduction in pH of the solution. The magnitude of this reduction depends on the composition (such as weak acids) of the solution and the "buffering" capacity of the plant cells (related to H<sup>+</sup>-ATP pump). Therefore, exposure of lettuce tissue to 20% CO<sub>2</sub> would reduce mitochondrial pH to a certain degree. In the range of pH 6 to 8, a reduction in pH would inhibit SDH activity (Fig. 3).

In vitro addition of 20% CO<sub>2</sub> to the assay components inhibited the catalytic activity of SDH by 28%, which was associated with a reduction of assay pH from 7.2 to 7.0 (Table 4). According to Umbreit (1949), the HCO<sub>3</sub><sup>-</sup> concentration in the water solution at pH near 7 from a 20% CO<sub>2</sub> atmosphere was 0.1 to 0.2 mM at a temperature range of 0 to 20°C. The effect of NaHCO<sub>3</sub> at 0.1 to 5.0 mM on the catalytic activity of SDH was very slight and not significant (Table 4). Since 5.0 mM NaCl also had such a slight effect, HCO<sub>3</sub><sup>-</sup> did not appear to be the major component from dissolved CO<sub>2</sub> to inhibit the catalytic activity of SDH. All the NaHCO<sub>3</sub> and NaCl solutions used did not significantly affect pH of the assay mixtures. The in vitro analyses from Fig. 3 and Table 4 implied that the production of H<sup>+</sup> from dissolved CO<sub>2</sub> and the reduction in pH was most likely the major reason for inhibiting the catalytic activity of SDH by exposure to 20% CO<sub>2</sub>.

Exposure of lettuce to 20% CO<sub>2</sub> for 2 to 9 days reduced the extractable activity of SDH (Fig. 4). This reduction in SDH extractable activity might be due to a suppression of SDH biosynthesis or a modification of the enzyme structure or conformation by the 20% CO<sub>2</sub> treatment since the extraction and assay pH was kept constant at 7.2 for the samples from both air and 20% CO<sub>2</sub> treatments (Fig. 4).

TABLE 4

Effects of in vitro addition of CO<sub>2</sub>, NaHCO<sub>3</sub>, or NaCl to assay mixture on catalytic activity of succinate dehydrogenase (SDH) from lettuce tissue

Addition	pH	% SDH activity
Air control	7.2a <sup>1</sup>	100a
20% CO <sub>2</sub>	7.0b	72b
0.1 mM NaHCO <sub>3</sub>	7.2a	94a
0.5 mM NaHCO <sub>3</sub>	7.2a	96a
5.0 mM NaHCO <sub>3</sub>	7.2a	94a
5.0 mM NaCl	7.2a	94a

<sup>1</sup> Mean separation within columns by Duncan's test at *P* = 0.05.

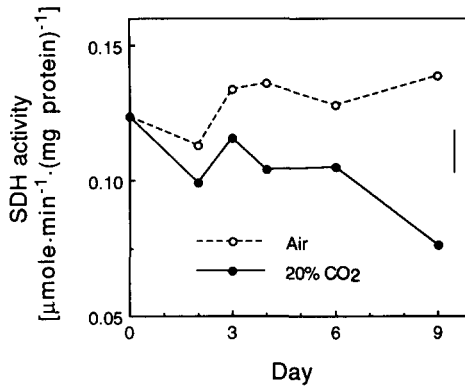


Fig. 4. Effects of exposure to air or air + 20% CO<sub>2</sub> at 0°C for 2, 3, 4, 6, or 9 days on extractable activity (determined at pH 7.2) of succinate dehydrogenase (SDH) from lettuce tissue. The vertical bar represents pooled LSD value at  $P = 0.05$ .

Since SDH is responsible for converting succinate into fumarate, an inhibition of SDH activity by exposure to 20% CO<sub>2</sub> would result in the accumulation of succinate. On the other hand, the inhibition of SDH could partly contribute to the reduction in malate concentration since the recycling of succinate to malate was reduced. After 9 days at 0°C, succinate concentration in lettuce tissue exposed to 20% CO<sub>2</sub> increased about 0.2 mM while malate content decreased about 0.9 mM; whereas in air control tissue, malate concentration decreased about 0.7 mM without an increase in succinate level (Fig. 1). This suggested that 0.7 mM of malate lost was not due to succinate accumulation; instead, it might have been used as a substrate for respiration or as a carbon source for biosynthesis of amino acids and proteins.

Glutamate decarboxylase (GDC) catalyzes the decarboxylation of glutamate into  $\gamma$ -aminobutyrate. Wallace et al. (1984) reported that GDC was not associated with any organelle or membrane component; instead, this soluble enzyme appeared to be located in cytoplasm. GDC was very sensitive to pH change (Fig. 3) and exposure to 20% CO<sub>2</sub> caused a reduction in cytoplasmic pH from 6.7 to 6.3 in lettuce tissue (Siriphanich and Kader, 1986). GDC was present in lettuce tissues exposed to air or 20% CO<sub>2</sub> (Fig. 5A). If this enzyme was extracted and assayed at its optimum pH 5.8, there was no significant difference in GDC activity between air and 20% CO<sub>2</sub> treatment (Fig. 5A). However, if we mimicked the in vivo cytoplasmic pH change and extracted and assayed GDC at pH 6.7 and 6.3 for the lettuce tissues exposed to air and 20% CO<sub>2</sub>, respectively, significant difference occurred (Fig. 5B). It appeared that GDC was much less active in air control tissue under normal cytoplasmic pH. Exposure to 20% CO<sub>2</sub> caused a reduction in cytoplasmic pH, which in turn activated GDC and subsequently resulted in the accumulation of  $\gamma$ -aminobutyrate and a decrease in glutamate concentration. The activation of GDC and the decarboxylation of glutamate into  $\gamma$ -aminobutyrate may

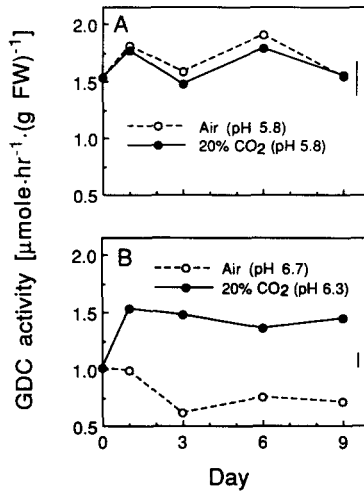


Fig. 5. Effects of exposure to air or air + 20% CO<sub>2</sub> at 0°C for 1, 3, 6, or 9 days and extraction and assay pH on activity of glutamate decarboxylase (GDC) from lettuce tissue. The vertical bars represent pooled LSD values at  $P = 0.05$ .

have two physiological functions. First, it may partly counteract a decrease in pH by the stress CO<sub>2</sub> level through removing one carboxylic group from glutamate and therefore plays a regulating or “buffering” role in controlling intracellular pH. Second, the formation of  $\gamma$ -aminobutyrate may serve as a way to temporally store nitrogen and to avoid its loss under elevated CO<sub>2</sub> stress, since after transferring lettuce tissue from 20% CO<sub>2</sub> back to air,  $\gamma$ -aminobutyrate concentration decreased and glutamate content recovered to near its original level (Fig. 2). In plants,  $\gamma$ -aminobutyrate transaminase could transfer the amino group from  $\gamma$ -aminobutyrate to  $\alpha$ -ketoglutarate to form glutamate (Saijo et al., 1989; Tokunaga et al., 1976). Tsushida and Murai (1987) reported that transaminases were inhibited at reduced pH and were active at normal physiological pH.

## CONCLUSIONS

Based on the results presented in this paper, a proposed mode of CO<sub>2</sub> action on metabolism of organic and amino acids in lettuce is shown in Fig. 6. In this model, the most significant effects of CO<sub>2</sub> are the suppression and/or inhibition of SDH and the activation of GDC. Elevated CO<sub>2</sub> concentration reduces the extractable activity of SDH; it also causes a decrease in pH, which partially inhibits SDH activity. This reduction and inhibition of SDH activity results in succinate accumulation and partly contributes to a decrease in malate concentration. The activation of GDC by a decrease in pH through dissolved CO<sub>2</sub> causes a reduction in glutamate content and the accumulation of  $\gamma$ -aminobutyrate, which may play a regulatory role in response to elevated CO<sub>2</sub> stress.

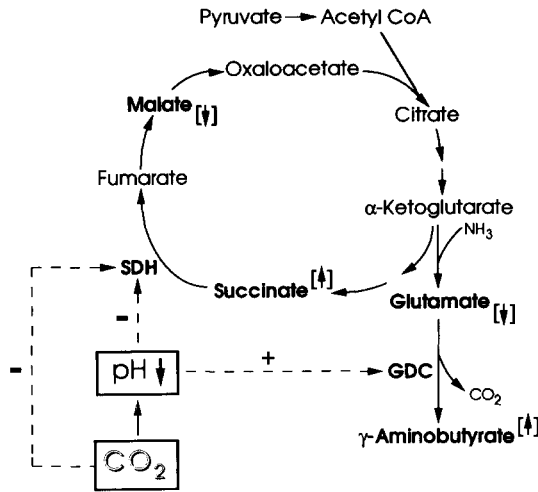


Fig. 6. Proposed mode of CO<sub>2</sub> action on metabolism of organic and amino acids in lettuce. Abbreviations and symbols: GDC, glutamate decarboxylase; SDH, succinate dehydrogenase; +→, activation; -→, suppression and/or inhibition; [↑], content increased by CO<sub>2</sub>; [↓], content decreased by CO<sub>2</sub>.

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