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Improving the Keeping Quality of Agricultural Commodities Under Controlled Atmosphere

A. Apelbaum, A. Kader, S.F. Yang

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A. ABSTRACT

The research deals with three aspects of controlled atmosphere storage of fresh produce: a. The effect of various gas compositions, high CO₂ treatment and storage procedures, on storability of selected commodities. b. The physiological, biochemical and pathological consequences of introducing carbon monoxide to controlled atmosphere storage. c. Factors affecting biosynthesis of the ripening and senescence hormone, ethylene. During the course of this study the following results were obtained: a simple method for the determination of resistance to gas diffusion in plant organs was developed, in tomato fruits, for example, the resistance of the intact fruit was shown to be 7,800 seconds per centimeter. A gas composition was determined to improve storability of apricot and peaches; in the case of immature and over-mature Patterson and Tilton apricots, storage at 2% O₂ and 5% CO₂ at 1°C improved storability, compared with air storage. Rapid CA or high CO₂ pre-storage treatment improved storability of Golden Delicious apples and slowed down the accumulation of 1-aminocyclopropane-1-carboxylic acid (ACC) in the fruit compared with slow CA.

Addition of 11% carbon monoxide to peaches held in CA inhibited completely growth of Monilinia fructicola and reduced internal breakdown incidence and severity while no significant increase in CO₂ and C₂H₄ or enhanced softening was recorded. In grapes, 10% CO in air reduced respiration and C₂H₄ production retarded berry browning and softening. In lettuce leaves, CO is metabolized primarily to CO₂ which is a precursor of acid-stable products. Malate, citrate and aspartate comprised 70% of the labeled CO found in the acid-stable product. The mitochondria was the most active fraction in oxidizing CO to CO₂.

Leafy tissue subjected to water deficit accumulate ACC and within 4 h produce ethylene at a rate 30-fold higher than the control. This increase in ethylene production was due to increased activity of both ACC synthase and the ethylene-forming enzyme. Polyamines inhibit ethylene in leafy tissue and apple fruits. This inhibition was found to be due to the inhibition of macromolecular synthesis caused by polyamines. The antifungal antibiotics, sinefungin and A9145C, which are analogs of S-adenosylmethionine, inhibited the activity of ACC-synthase in tomato fruits. In green tomatoes, sinefungin was as effective as aminoethoxyvinylglycine. Mitochondrial fraction from leafy tissue was found to efficiently produce ethylene from ACC. The reaction was enhanced by Mn²⁺ and inhibited by KCN, EDTA, NaN₃, CoCl₂, n-propylgallate, 2,4-DNP structural analogs of ACC, and anaerobic conditions.

B. OBJECTIVES OF THE RESEARCH

1. To determine the physiological and biochemical changes associated with a short prestorage treatment with relatively high CO₂.
2. To investigate the effects of holding selected commodities under elevated CO₂ with special emphasis on the physiological disorders alleviated or aggravated by CA, and genotypic differences in tolerance to elevated CO₂.
3. To determine the effect of accumulated ethylene and other volatiles under CA conditions, with special emphasis on the role of water loss- and wound-induced ethylene production. To explore the mechanism of the induction and possible control of these processes.
4. To determine the relationship between ascorbic acid, phenolic compounds, and the discoloration induced by increased CO₂ or reduced O₂.
5. To clarify the interaction between ethylene and CO₂ in fruit ripening and physiological disorders, especially chilling injury.
6. To study the effect of temporary exposure to anaerobic conditions before storage in CA on physiological and biochemical changes in selected commodities.
7. To determine the effects of introducing carbon monoxide to CA storage on metabolic changes associated with ripening and physiological disorders and to study the fate of this compound in the tissue.

This research report covers 12 studies aiming at the improvement of the keeping quality of agricultural commodities under controlled atmosphere. The report on each study includes a brief introduction, detailed description of the methodology and materials used, results, discussion and relevant bibliography.

1. The effect of controlled atmosphere and high CO₂ prestorage treatment on biochemical changes and produce quality in selected commodities

1.1 Determination of Resistance to Gas Diffusion in Fruits

Our studies deal with the effects of various applied gases on plant tissue, and with emanation of gases from the tissue. Therefore, we found that it was necessary to study the resistance of plant tissue to gas diffusion and to develop a simple method to determine this resistance.

Production of ethylene by plant tissues is usually expressed as the amount emanating over a given time period per unit weight (for example, $\mu\text{l kg}^{-1} \text{ h}^{-1}$). However, the rate of ethylene emanation would only equal the production rate if the system is at the steady-state. Furthermore, it is the internal concentration of ethylene and not the ethylene production rate which governs its biological activity in the tissue. Consequently, if the ratio of internal ethylene concentration to ethylene production rate is not constant from tissue to tissue, then production rates cannot be used to compare the ethylene status of different tissues. Because the skin of an organ presents a much greater barrier to diffusion than the pulp (4), it can be assumed that the primary factor which regulates the internal concentration of a gas for any given production rate is the resistance of the skin to diffusion. Estimation of internal concentrations from production rates therefore requires knowledge of skin resistances.

Burg and Burg (4) defined a 'resistance factor' (R') which could be estimated for bulky plant organs as the ratio of internal concentration to the rate of production when the organ was at the steady-state;

$$R' = \frac{[\text{ethylene}]_{\text{internal}}}{ds/dt/M} \quad (1)$$

where $[\text{ethylene}]_{\text{internal}}$ is the concentration of ethylene inside the tissue and $ds/dt/M$ the rate of ethylene production per unit mass. This equation is

valid only if the external concentration of ethylene is zero. It should be noted that this resistance factor is expressed per unit weight of tissue and not per unit surface area, yet it is the latter which limits the escape of gases from a tissue. Once R' is known, Burg and Burg (4) reasoned that internal ethylene could be estimated from steady-state production rates as follows:

$$[\text{ethylene}]_{\text{internal}} = R'(ds/dt/M) \quad (2)$$

This approach is valid provided that the tissue can be idealized as a hollow sphere where internal concentrations are uniform, resistance to diffusion within the sphere is insignificant in comparison to the resistance across the skin, and the skin has an insignificant thickness relative to the radius of the organ (5). Although the steady-state method has been used to estimate the resistance factors of a number of plant organs (see Ref 1), there are some drawbacks to the method. Probably the most serious drawback to the steady-state method is that it requires an accurate estimate of the internal concentration of ethylene. Although this might be simple for an organ with internal cavities (e.g. cantaloupe, tomato), it can be extremely difficult for more solid organs (e.g. avocado, potato, banana). By definition, the tissue must be at steady-state and this could prevent an accurate measurement of resistance in fruits undergoing a climacteric rise or fall in ethylene production. For instance, Burg and Burg (3) used the steady-state method to estimate that greater than 60% of the gas exchange in tomato fruit occurred through the stem scar. However, they were unable to quantify this relationship more precisely because they could not determine to what extent the treatment (stem scar blocked) affected production rates. Also, for many plant tissues, the rate of ethylene production may be too low to measure

(e.g. preclimacteric fruits, vegetative tissue). Finally, it should be re-emphasized that R' is only an empirical value without conventional dimensions, and is not constant with changes in the surface to volume ratio.

We report here a method to estimate quantitatively the resistance coefficients based on kinetic analysis of the efflux of preloaded gases from plant tissues. This method is analogous to the measurement of membrane permeability by kinetic analysis of the efflux of specific ions. We chose ethane for preloading because it is neither produced nor metabolized to a significant degree by the tissue, it should have properties similar to ethylene, and it can be quickly and accurately determined at low concentrations by gas chromatography.

Experimental

Tomato (Lycopersicum esculentum Mill. cv. 'Ace') fruits were obtained locally and stored at 15°C. Cucumber (Cucumis sativus L. var. anglicus Bailey) fruits wrapped in a plastic film were obtained in a local market.

For the efflux method, plant organs such as tomato fruits were first placed in a jar purged with a constant flow of air containing 500-1,000 $\mu\text{l/l}$ ethane. The length of time required to reach equilibrium depended on the organ employed; 3 to 4 h were sufficient for untreated tomatoes. To initiate an experiment, a single fruit was transferred to a second, ethane-free jar (9 L) which was then quickly enclosed at time zero. At the time of sealing, a diaphragm pump attached to the jar was turned on to ensure rapid mixing. The pump circulated approximately 500 L/h, and the gas in the jar was mixed within seconds. For determination of ethane, 1-ml gas samples were withdrawn at regular intervals through a septum attached to the jar. After a sufficient number of samples were taken (usually 20-30), the pump was turned

off and adequate time was allowed to let the whole system come to equilibrium. The concentration of ethane in the jar was then remeasured.

Theoretical Background. As pointed out by Burg and Burg (4), diffusion of gas from plant tissue should behave according to Fick's Law, which can be written as follows:

$$ds/dt = (C_{in}^t - C_{out}^t)A/R \quad (3)$$

where ds/dt is the rate of efflux ($nl\ s^{-1}$), R is the resistance coefficient ($s\ cm^{-1}$), A is the surface area of the tissue (cm^2), and C_{out}^t and C_{in}^t are the concentrations (nl/ml) outside and inside the tissue, respectively, at time t .

$$s_{out}^t = V_{out} \times C_{out}^t \quad (4)$$

where s_{out}^t is the total amount (nl) of ethane which escaped from the fruit to the jar at time t , and V_{out} (cm^3) represents the volume of the jar minus the volume taken up by the plant organ. The derivative of equation 4 with respect to time reveals that:

$$ds/dt = V_{out}(dC_{out}/dt) \quad (5)$$

Also, since the total mass of ethane is constant in the closed system at all times, and since under our experimental conditions $V_{out} \gg V_{in}$:

$$V_{in}C_{in}^t + V_{out}C_{out}^t = C_{out}^{\infty}(V_{in} + V_{out}) = C_{out}^{\infty}V_{out} \quad (6)$$

where V_{in} is the volume inside the tissue accessible to ethane and C_{out}^{∞} is the concentration of ethane in the jar after equilibrium is established (i.e. at time infinity). Substituting equations 5 and 6 into equation 3, and integrating from time zero to time t for C_{out}^t , the following equation

can be obtained:

$$\ln(1 - \frac{C_{out}^t}{C^\infty}) = - \frac{A}{R \times V_{in}} \times t \quad (7)$$

For convenience let:

$$k = \frac{A}{R \times V_{in}} \quad (8)$$

then:

$$\ln(1 - \frac{C_{out}^t}{C^\infty}) = -k \times t \quad (9)$$

The first-order efflux rate constant, k , can be directly determined as the negative slope of a plot of $\ln(1 - C_{out}^t/C^\infty)$ versus t .

A rearrangement of equation 8 shows how R can be calculated from k :

$$R = \frac{A}{k \times V_{in}} \quad (10)$$

From equations 1, 3 and 10, it can be shown that:

$$R' = \frac{M}{k \times V_{in}} \quad (11)$$

or that:

$$R' = \frac{R \cdot M}{A} \quad (12)$$

If mass, surface area, and V_{in} are known, R and R' can be calculated from k . The surface area of tomato was estimated from the radius on the assumption that they were spheres. Cucumbers were assumed to be cylinders, and the surface area was estimated from the radius and length.

V_{in} in equation 6 represents the volume inside the tissue accessible to

ethane gas, including the entire volume of air space (intercellular plus cavity) in the fruit plus approximately 10% of the volume of the liquid portion of the tissue, since the partition (water/gas) coefficient of ethylene is 0.10 at room temperature. The air and liquid space of a tomato fruit can be estimated by determining the entire volume of the fruit before and after homogenization. Alternately, equation 6 reveals that V_{in} can also be calculated directly from the data collected for ethane efflux and the initial concentration of ethane equilibrated with the tissue. At time zero, since $C_{out}^0 = 0$, equation 6 simplifies to:

$$V_{in} = \frac{V_{out}}{C_{in}^0} \times C_{out}^{\infty} \quad (13)$$

Thus, V_{in} can be estimated from the C_{out}^{∞} (the concentration of ethane at equilibrium for each fruit after an efflux run), and the ratio of V_{out} (the volume of the jar) to C_{in}^0 (the internal concentration of ethane in the fruit at time zero, which can be assumed to be equal to the concentration of the loading gas).

Results and Discussion

Comparison of the steady-state and ethane efflux methods. Table I shows a comparison of resistance factors of green and red tomato fruits obtained by the steady-state method of Burg and Burg (4) (equation 1) and the ethane efflux method as described above and using equation 12. The close agreement between the two methods support the assumptions made during the derivation of the ethane efflux method. Using equation 13, the V_{in}/A ratio, which was used to calculate R from k, was determined as 0.135.

Mode of ethylene diffusion from tomato fruits. The ethane efflux method allows a direct determination of resistance to ethylene diffusion of plant organs and was employed to examine the characteristics of gas diffusion in tomato fruits. Typical efflux curves for tomato fruits either untreated or with stem scars blocked with vaseline are shown in Figure 1, in which the fraction of ethane released is plotted as a function of time. A plot of $\ln(1 - C_{out}^t / C^\infty)$ versus time (Fig. 1) was used to obtain values for k using equation 9 (Table II). The straight lines with intercepts near zero are indicative of monophasic diffusion. This suggests that in tomato fruits, the movement of gases within the tissue is not limiting relative to movement out of the tissue, which supports assumptions made during the derivation of the equations. The total resistance of untreated fruit can be considered to consist of two parts, one representing the stem scar and the other the skin. With the stem scar covered with vaseline, the resistance measured should represent that of the skin. Because the diffusion of gases through the stem scar and skin are in parallel, the first-order rate constants for the entire fruit should be the summation of the values of the stem scar and the skin. No significant differences were noted between green and red tomato fruits; for both stages of ripening, the stem scar accounted for approximately 97% of the gas exchange (Table II). It has long been recognized that fruit with the stem scar blocked ripen considerably slower than those not treated (2), presumably because of the increase in CO_2 level and concurrent decrease in O_2 level within the tissue which result from the marked increase in resistance to diffusion. It is seen that the resistance of the skin is approximately 1,000 times that of the stem scar (Table II). The ethane efflux method can also be used to quantitate the effect of waxes on gas diffusion. It was found that one coat of a wax used commercially on tomatoes

Table I. A Comparison of Resistance Factors for Green and Red Tomato Fruits Estimated from the Steady-State Method and the Ethane Efflux Method

Stage of Tomato	R' Value	
	Steady-state	Ethane efflux
	g h/ml	
Green	2.9 ± 0.7	2.1 ± 0.6
Reg	2.9 ± 0.5	2.3 ± 0.6

FIG. 1. Plots of ethane efflux versus time for treated (stem scar blocked) and untreated tomato fruits.

Table II. Diffusion of Ethane through Whole Fruit, Fruit Skin, or Stem Scar of Green and Red Tomato

First order rate constants for whole fruit and for fruit skin were determined by measuring ethane efflux of untreated fruits and fruits whose stem scars were covered with vaseline, respectively. First-order rate constant for stem scar was calculated by subtraction. Percent diffusion contribution and R were calculated from k .

Tomato Part	k	Diffusion Contribution	R
	$s^{-1} \times 10^6$	%	$s\text{ cm}^{-1} \times 10^{-3}$
Green			
Skin	3.4	3	270
Stem scar	123	97	0.22
Whole fruit	126	100	7.4
Red			
Skin	3.4	3	280
Stem scar	91	97	0.30
Whole fruit	94	100	7.8

Table III. First-order Rate Constants and Resistance Coefficients for Shrink-Wrapped and Unwrapped English Cucumber Fruits Obtained by Ethane Efflux Method

R value for plastic film was calculated by subtraction. V_m/A ratio was estimated as 0.155 cm.

	k	R	Resistance Contribution
	$s^{-1} \times 10^{-4}$	$s\text{ cm}^{-1} \times 10^{-3}$	%
Shrink-wrapped (skin + film)	2.8	23.0	100
Unwrapped (skin)	56	1.1	5
Plastic film		21.9	95

had no significant effect on gas exchange and even two coats increased resistance only slightly (data not shown). The results would not be surprising if the applied wax did not adequately block the stem scar.

Resistance of unwrapped and shrink-wrapped cucumbers. The ethane efflux method was utilized to investigate the effect of a plastic shrink wrap on ethane diffusion from English cucumbers. As shown in Table III, the film increased the resistance by a factor of 20 and provided about 95% of the resistance. Using equation 13, the value for V_{in}/A was determined to be 0.155 cm. It should be noted that the diffusion of gas through the cucumber skin and the plastic film is in series. Thus, the overall resistance coefficient should be the summation of the resistance of each of these components.

These examples demonstrate that the ethane efflux method provides a direct and simple method for estimation of resistance to diffusion of tissues. The method does not necessarily require that the loading gas be at equilibrium with the organ prior to an efflux run if an independent method is used to estimate V_{in}/A ratios. However, initial equilibrium might be advisable, particularly if an organ is found to exhibit multiphasic diffusion patterns. It should be noted that V_{in} could be underestimated by methods using equation 13 if a significant portion of ethane leaked out of the organs during the transfer from the loading jar to the efflux jar. The good agreement between the ethane efflux method and the steady-state method suggests that resistance to ethylene and ethane are similar. Preliminary work shows that the ethane efflux method can be readily adapted for use with stem, root, and bulky tissues. The information obtained from the method can be utilized for understanding various phenomena which involve ethylene diffusion. The method allows investigations concerning the paths of diffusion from plant tissues as shown for the stem scar of tomato. The effects of waxes and films

can also be quantitated. Furthermore, first-order rate constants can be used to determine the length of time it would take for the gas phase of a tissue to come to equilibrium with an externally introduced gas. Conversely, it would be possible to estimate the length of time for a treated commodity to lose the gas. Such information is of value to the postharvest physiologist. Using R or R' , which are interconvertible according to equation 12, it is possible to estimate internal concentrations of ethylene from production rates when the system is at steady-state. Due to its ease and adaptiveness the efflux method provides a quantitative approach to the study of diffusion of ethylene and other gases in plant tissues.

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1.2 The Effect of Various O_2 and CO_2 Compositions and Ethylene on the Quality of Canning Apricots and Clingstone Peaches

California apricots and clingstone peaches are harvested for canning on the basis of their skin and flesh color, respectively, and are normally processed within one day of harvest. Currently, when canning capacity is exceeded by daily harvested tonnage, processors may store the fruit a few days (apricots) or up to 2 wks (cling peaches) at $0-2^{\circ}C$ in air. As more restrictions on water use and waste disposal are imposed, longer storage may become necessary to regulate processed quantity per day. In addition, increased use of mechanical harvesting may result in a wide range of maturities at harvest, including minimally mature fruit which require ripening before canning.

Previous work (Salunkhe et al., 1966; Wankier et al., 1970; Claypool and Pangborn, 1972) has shown that canning quality of apricots can be affected by cultivar, maturity, and length and type of storage. Claypool and Pangborn (1972) tested Blenheim and Tilton apricots and judged Tilton to have limited storage potential compared to Blenheim. In this study we compare Tilton to Patterson, a recently introduced cultivar in California.

Anderson et al. (1969) recommended a controlled atmosphere (CA) of 1% O_2 plus 5% CO_2 for storage of fresh market peaches for longer than three weeks duration. Claypool and Davis (1959) found no beneficial effect of 1 or 2.5% O_2 and observed that flavor ratings of canned peaches decreased as the CO_2 concentration increased above 5%. Most previous studies on the effect of maturity or ripeness on canning quality (Salunkhe et al., 1966; Boggess et al., 1974) have been limited to air storage without added ethylene.

The objectives of this study were (1) to explore the possibility of maintaining good quality in storage through use of controlled atmospheres (CA), and (2) to evaluate the possible use of ethylene (C_2H_4) treatments

to ripen green fruits before canning in order to achieve faster and more uniform ripening.

Experimental

Source of fruit and sorting by maturity. Apricots (Patterson and Tilton) and clingstone peaches (Loadel, Andross, Klamt, Carolyn and Halford) were obtained from Tri-Valley Growers (Modesto, CA). The fruits were hand harvested and sorted in the orchard into three maturities, designated M1, M2 and M3. These were defined as follows: (M1) hard fruit with overall green color; (M2) firm fruit with yellow to yellow-orange ground color on the cheeks and little or no green on the suture or ends; and (M3) moderately soft fruit with an overall yellow-orange ground color. The initial color and firmness measurements corresponding to these maturity stages are shown in Table 1 (apricots) and Table 3 (cling peaches).

Handling. Fruits were transported to Davis within 24 hr of harvest and stored at 0°C in air if overnight holding was required. Peaches were dipped in 20°C fungicidal suspension of benomyl and 2,6-dichloro-4-nitroaniline, a treatment which Wells (1972) has shown to be very effective in controlling decay caused by Monilinia and Rhizopus fungi. All fruits were sorted for freedom from bruising and decay, and for uniformity of size range and maturity stage among replicates before being placed in 9.5-liter jars fitted with rubber stoppers with inlet and outlet tubes. Three replicates of each treatment were prepared for both the storage and ripening experiments.

Storage experiment. The effects of CA on storage life at 1.1°C, ripening of M1 fruit at 20°C after storage, and canning quality were compared to air storage using three maturities of each cultivar. Individual lots of fruits

were exposed to one of four different atmospheres: air control, 2% O_2 in N_2 , 5% CO_2 in air, and 2% O_2 plus 5% CO_2 in N_2 . The gases were mixed in a flow-through system using needle valve type flowmeters and the total flow rates delivered to each jar adjusted so that CO_2 accumulation from respiration remained below 0.2%. Gas mixtures were monitored by gas chromatography. The relative humidity in the jars was maintained at 95-100% by bubbling the gases through water before entering the sample chamber. Storage times were 1, 2, and 3 wk for apricots and 2 and 4 wk for cling peaches at $1.1^{\circ}C$.

Ripening experiment. Ripening responses at $20^{\circ}C$ in air, and in air containing 100 ppm ethylene for the initial 48 hr of ripening, were compared using M1 and M2 fruits. Samples to be ripened were held at $20^{\circ}C$ until color and hand-held firmness were judged indicative of optimum stage for immediate canning, i.e., M3. Gas mixing, flow rate adjustment, monitoring of mixes and attainment of desired relative humidity were as mentioned above.

Evaluation and canning of samples. Samples of ten fresh fruits per replicate were evaluated initially and after the various treatments. Initial and post-treatment samples were also sent to Tri-Valley Growers for canning. A syrup consisting of one-third sucrose, one-third high fructose corn syrup, and one-third corn syrup was used. Fruits were cooked in continuous rotary cookers at $100^{\circ}C$ for 14 min. Evaluations of canned fruits from the ripening experiment and from the CA storage treatments were performed after about 10 months storage at $20^{\circ}C$.

Color. The skin color of fresh and canned fruits was measured with a Gardner XL-23 Color Difference Meter. The instrument was calibrated with a standard white reference plate ($X = 81.7$, $Y = 84.1$, $Z = 97.9$). One reading was taken of each cheek on a fruit.

Firmness. With flesh exposed by peeling a small area of skin on each cheek, a UC Firmness Tester with a 0.79-cm tip was used to measure the fruit flesh firmness.

Chemical analysis. Fruit samples were homogenized in a Waring Blendor, centrifuged for 20 min at 13,200 x g and the supernatant juice decanted. Soluble solids content (SSC) of juice and syrup was measured with an Abbe refractometer. The pH of the juice was measured directly with a Corning, pH Meter 130. Total titratable acidity (TTA) was determined by diluting a 6.00 g aliquot of juice with 50 ml deionized water and titrating to pH 8.2 with 0.1 N NaOH. Results were expressed as percent malic acid. All samples of canned fruit were rinsed for one minute in deionized water and drained for two minutes to remove the canning syrup before chemical analyses were performed.

Discarded fruits. Percent discard is the average of three replicates and includes fruit which were unsuitable for canning due to either physiological or pathological deterioration.

Sensory evaluation by a taste panel. A taste panel of 11 individuals scored canned fruit samples for firmness, sweetness, sourness, and flavor intensity by marking an unscaled 10-cm line labeled at opposite ends with 'none' or 'extreme'. The judges were trained prior to testing by being given fruit samples representative of the ranges of the various attributes as well as definitions of the terms. These were as follows: "firmness" the force required to penetrate the fruit with the back molars; "sweetness", the taste associated with sucrose; "sourness", the taste associated with acids; "flavor intensity", the relative intensity of the flavor associated with a fresh apricot or cling peach.

Judges were given three pieces of each sample placed under red light

to minimize color bias and labeled with random three-digit numbers. A maximum of ten samples were tasted per day. Judges were instructed to expectorate samples and to rinse with water between samples.

Statistical analysis. Data were treated by analysis of variance (ANOVAR) with least significant difference (LSD) between treatment means determined at the 5% level.

Results and Discussion

Apricots. The 'a' color value, which indicates the relative greenness or redness of a sample, gave the best correlation with firmness readings and changed to a greater degree during ripening than either 'Rd' or 'b' values, which measure relative whiteness/blackness and yellowness/blueness, respectively. Thus, 'a' values seem a better measure of ripeness and are included in the data presented here while 'Rd' and 'b' values are not.

The 2% O₂ plus 5% CO₂ CA storage treatment was the most consistently effective in retaining the quality attributes of fresh apricots and was therefore chosen for further evaluation of canned samples. For this reason data are presented for this CA treatment only.

Effect of controlled atmosphere. CA storage was more effective than air storage in retarding the loss of greenness by fresh M1 and M2 fruits (Table 1). CA storage had no effect on the SSC of fresh apricots, but did result in greater firmness, lower pH, and higher TTA, as well as lower decay than air storage.

When objective evaluations of the canned fruits were performed these differences between air- and CA-stored fruits remained generally similar. CA-stored fruits were lower in 'a' values at M1 and M2 stages with no significant differences among M3 fruits (data not shown). The pH of CA-stored

Table 1—Compositional and quality attributes of fresh apricots as related to storage treatment at 1.1°C

Variable	Treatment	Days	Patterson			Tilton		
			M1 ^a	M2	M3	M1 ^a	M2	M3
Color ('a' value)		0	7.3	17.6	26.9	-4.9	17.3	26.2
	Air	7	6.3	24.4	27.1	-2.1	18.0	24.8
		14	21.7	25.5	27.3	16.4	19.4	25.3
		21	14.0	25.6	28.1	8.6	21.5	24.7
	CA	7	7.3	21.5	28.8	-4.2	18.1	26.0
		14	16.5	23.8	27.2	11.1	19.9	26.6
		21	11.4	24.0	28.6	10.6	18.3	25.6
	LSD @ 5%		2.1	1.5	1.2	2.2	1.5	NS
	Firmness (kg)							
Firmness (kg)		0	5.6	3.6	0.9	7.9	4.0	1.3
	Air	7	4.1	1.5	0.4	5.7	2.3	0.7
		14	0.6	1.5	0.7	1.0	2.0	0.7
		21	1.5	1.5	0.7	1.4	2.1	0.8
	CA	7	4.7	2.7	1.0	6.4	3.3	0.8
		14	1.8	2.0	1.0	2.5	2.5	0.9
		21	3.2	2.3	0.9	2.7	2.7	1.1
	LSD @ 5%		0.6	0.4	0.2	0.6	0.6	0.2
	SSC (%)							
SSC (%)		0	10.4	12.6	16.7	8.8	12.0	14.6
	Air	7	10.7	14.0	16.3	4.2	12.4	9.8
		14	10.6	14.2	15.7	8.8	12.5	15.3
		21	10.4	13.8	15.4	8.9	12.3	14.8
	CA	7	10.2	13.6	15.8	7.0	12.5	14.9
		14	10.6	14.3	15.9	8.7	12.5	15.3
		21	10.5	13.5	15.3	9.0	12.4	15.3
	LSD @ 5%		NS	0.6	0.8	0.9	NS	0.9
	pH							
pH		0	3.74	3.88	4.28	3.89	4.17	4.37
	Air	7	3.69	3.98	4.26	4.01	4.20	4.28
		14	3.96	4.09	4.36	4.04	4.21	4.35
		21	3.79	4.22	4.43	4.02	4.34	4.50
	CA	7	3.79	3.99	4.14	3.93	4.11	4.29
		14	3.85	4.02	4.18	4.04	4.17	4.31
		21	3.73	4.06	4.24	3.85	4.21	4.38
	LSD @ 5%		0.04	0.07	0.11	0.06	0.07	0.10
	Titratable Acidity (%)							
Titratable Acidity (%)		0	1.34	1.09	0.60	1.45	0.84	0.54
	Air	7	1.46	0.89	0.62	0.81	0.49	0.45
		14	1.20	0.78	0.56	1.05	0.65	0.46
		21	1.37	0.69	0.55	1.05	0.67	0.45
	CA	7	1.50	1.00	0.72	1.07	0.62	0.50
		14	1.39	0.90	0.69	1.10	0.66	0.48
		21	1.43	0.87	0.65	1.11	0.78	0.55
	LSD @ 5%		0.08	0.08	0.06	0.20	0.06	0.09
	Discard (%)							
Discard (%)		0	—	—	—	—	—	—
	Air	7	0	0	0.8	0	0	0
		14	0	0	5.3	20.0	0	6.7
		21	3.3	0	16.9	4.2	0	14.2
	CA	7	0	0	0.4	0	0	0
		14	0	0	0.9	0	0	1.0
		21	0.8	0	3.4	4.2	0	6.7
	LSD @ 5%		NS	NS	6.0	NS	NS	3.8

^a Ripened for 0, 4, and 2 or 0, 5, and 4 days at 20°C after removal from air storage or CA storage, respectively.

Table 2—Compositional and quality attributes of fresh apricots ripened in air or air plus 100 ppm ethylene for 48 hr at 20°C

Variable	Treatment	Patterson		Tilton	
		M1	M2	M1	M2
Ripening time (days)	Air	8	6	8	6
	C ₂ H ₄	7	5	7	5
LSD @ 5%		NS	NS	NS	NS
Color ('a' value)	Initial	5.0	22.0	-0.2	8.8
	Air	16.0	28.6	14.5	18.4
	C ₂ H ₄	20.3	28.0	16.5	20.4
LSD @ 5%		2.5	1.2	2.9	1.1
Firmness (kg)	Initial	4.9	3.0	5.4	3.6
	Air	2.9	1.0	1.0	1.3
	C ₂ H ₄	1.0	0.5	0.5	0.5
LSD @ 5%		0.2	0.3	0.2	0.05
SSC (%)	Initial	8.8	11.6	10.6	11.5
	Air	9.6	12.8	9.9	11.1
	C ₂ H ₄	9.3	12.6	9.8	11.3
LSD @ 5%		NS	0.5	0.6	NS
pH	Initial	3.72	3.95	4.03	4.14
	Air	3.78	4.21	4.20	4.28
	C ₂ H ₄	3.94	4.12	4.31	4.24
LSD @ 5%		0.14	0.06	0.05	0.05
Titratable acidity (%)	Initial	0.82	0.58	0.66	0.57
	Air	0.66	0.47	0.58	0.52
	C ₂ H ₄	0.73	0.45	0.55	0.53
LSD @ 5%		0.10	0.03	0.05	0.02

fruits was slightly lower and the acidity higher than air-stored fruits. The taste panel judges found CA-stored M1 fruits firmer than air-stored fruits, but the firmness differences between air- and CA-stored M2 and M3 fresh fruits were essentially lost after canning. The taste panel detected little or no differences in sweetness, sourness, or flavor intensity between the two treatments, but off-flavors were more frequently noted in air than in CA fruits. These data are in agreement with the results of Claypool and Pangborn (1972) indicating that CA retards development of off-flavors.

The potential applicability of CA to storage of canning apricots appears to depend on the condition of the fruit to be stored. For fruit of the M2 maturity stage, CA has little beneficial effect over air storage, but fruits picked at the M1 and M3 stages may benefit from CA storage. CA storage results in a firmer M1 canned product with no perceptible detrimental effect on flavor. In addition, whereas decay may be a problem in air-stored M1 and M3 fruits, it is effectively retarded by CA storage.

A potentially important effect of CA storage concerns the retention of low pH values compared to air storage. This is especially apparent after longer storage of the higher maturity fruits. The high pH values recorded in canned M3 apricots from the 3-wk storage treatment (4.3) are approaching the upper limit for safe canning (4.5). CA storage may allow the use of lower cooking times and/or temperatures than would otherwise be necessary to safeguard against possible contamination, thereby resulting in better textural and nutritive quality.

Effect of ethylene treatments. Fresh apricots were significantly softer and less green after treatment with ethylene even though ripened for one day less than air-ripened fruits (Table 2). There were no differences in SSC, TTA, or percent discard between the two ripening treatments; dif-

Table 3—Compositional and quality attributes of fresh peaches as related to storage treatment at 1.1°C

Variable	Treatment	Days	Carolyn			Halford		
			M1 ^a	M2	M3	M1 ^a	M2	M3
Color ('a' value)		0	4.8	18.7	24.8	1.5	10.5	21.4
	Air	14	8.9	12.3	20.4	8.0	10.0	18.3
		28	7.6	13.4	18.6	9.4	10.8	18.1
	CA	14	10.4	13.8	19.1	8.4	8.6	16.8
		28	8.9	11.4	17.9	7.9	8.8	16.2
	LSD @ 5%		2.0	0.9	1.9	2.2	1.8	1.6
Firmness (kg)		0	4.8	4.2	3.4	4.0	3.2	3.5
	Air	14	2.7	4.3	3.4	2.2	3.3	3.2
		28	3.8	4.1	2.8	3.2	3.1	2.7
	CA	14	2.5	4.1	3.6	2.4	3.2	3.4
		28	3.3	4.1	2.8	3.0	3.3	3.0
	LSD @ 5%		0.4	NS	NS	0.3	NS	0.5
SSC (%)		0	9.6	11.1	10.7	9.3	9.9	14.1
	Air	14	8.9	9.9	10.3	9.1	9.8	12.5
		28	8.6	10.4	9.8	9.6	9.3	12.1
	CA	14	8.5	9.9	10.3	9.3	9.7	12.4
		28	8.9	9.9	10.2	8.9	9.6	12.2
	LSD @ 5%		NS	0.3	0.8	NS	NS	0.7
pH		0	3.76	3.88	3.99	3.91	4.01	4.03
	Air	14	3.99	3.93	4.24	4.21	4.16	4.28
		28	4.03	4.14	4.44	4.25	4.29	4.58
	CA	14	3.98	3.99	4.27	4.19	4.20	4.31
		28	3.97	4.19	4.65	4.32	4.41	4.53
	LSD @ 5%		0.02	0.08	0.15	0.07	0.06	0.07
Titratable acidity (%)		0	0.82	0.58	0.50	0.66	0.57	0.50
	Air	14	0.67	0.57	0.42	0.60	0.51	0.42
		28	0.63	0.44	0.29	0.51	0.45	0.30
	CA	14	0.62	0.51	0.41	0.59	0.52	0.42
		28	0.65	0.44	0.26	0.52	0.45	0.29
	LSD @ 5%		0.05	0.07	0.05	0.04	0.05	0.02
Discard (%)		0	—	—	—	—	—	—
	Air	14	0	0	0	0	0	0
		28	6.7	1.4	18.1	22.7	1.4	0
	CA	14	0	0	0	0	0	0
		28	6.7	0	4.2	8.0	2.8	0
	LSD @ 5%		NS	NS	NS	10.1	NS	NS

^a Ripened for 3 or 4 and 3 days at 20°C after removal from air storage or CA storage (14 and 28 days), respectively.

Table 4—Compositional and quality attributes of fresh peaches ripened in air or air plus 100 ppm ethylene for 48 hr at 20°C

Variable	Treatment	Carolyn		Halford	
		M1	M2	M1	M2
Ripening time (days)	Air	8	6	8	5
	C ₂ H ₄	8	5	8	5
LSD @ 5%		NS	NS	NS	NS
Color ('a' value)	Initial	4.8	18.7	1.5	10.5
	Air	12.2	19.3	10.1	15.0
	C ₂ H ₄	11.1	22.2	10.6	13.5
LSD @ 5%		3.8	1.3	2.3	NS
Firmness (kg)	Initial	4.8	4.2	3.9	3.2
	Air	2.9	2.8	2.9	2.6
	C ₂ H ₄	2.7	3.0	2.9	2.7
LSD @ 5%		0.4	0.4	0.1	0.2
SSC (%)	Initial	9.6	11.1	9.3	9.9
	Air	8.7	9.8	9.0	10.1
	C ₂ H ₄	8.2	9.4	8.4	9.9
LSD @ 5%		1.0	0.4	0.3	NS
pH	Initial	3.76	3.88	3.91	4.01
	Air	3.93	4.08	4.13	4.16
	C ₂ H ₄	3.92	4.10	4.09	4.08
LSD @ 5%		0.05	0.06	0.05	0.08
Titratable acidity (%)	Initial	1.17	0.75	1.07	0.97
	Air	1.25	0.68	0.94	0.77
	C ₂ H ₄	1.07	0.62	0.85	0.70
LSD @ 5%		0.06	0.06	0.12	0.05

ferences in pH were small.

After canning, the ethylene-treated fruits were still higher in 'a' value but no significant differences were observed in SSC or TTA. While C_2H_4 -treated fruits had a higher (by about 0.2 units) pH than air control M1 fruits, no differences in pH were noted in the M2 fruits. The taste panel found ethylene-treated M1 fruits softer than those from air while the firmness differences seen in fresh M2 fruits were no longer detectable. In addition, canned M1 fruits were judged less sour when ripened with ethylene. Consequently, the ethylene-treated M1 fruits were also judged to have less flavor intensity than air-ripened fruit. Panel members found no differences among M2 fruits canned after the two ripening treatments (data not shown).

The results of the ripening experiment indicate that ethylene treatment (100 ppm for 48 h at 20°C) accelerates the ripening process compared to air storage. Even though the ethylene-treated fruit were held for one day less at 20°C than the air-ripened fruit, they were softer and showed less green color. It seems possible that manipulation of holding time, temperature, and/or ethylene concentration can be used to develop ripening schedules which could be applied to achieve faster and more uniform ripening of canning apricots.

Clingstone peaches. The 'a' color value, which indicates the relative greenness or redness of a sample, gave the best correlation with firmness readings and has been shown to correlate well with flavor and visual quality by Leonard et al. (1961). It thus seems to be a better measure of ripeness than either 'Rd' or 'b' values, and therefore only 'a' values are presented here.

The 2% O_2 plus 5% CO_2 storage treatment was the most effective in retarding color change and development of decay in fresh peaches and was therefore

chosen for further evaluation of canned samples. Data are presented for this CA treatment and for Carolyn and Halford peaches only. The relative responses of the other three cultivars will be discussed.

Effect of controlled atmosphere. There were small or no significant differences between air- and CA-stored fruit in terms of color, firmness, SSC, pH and TTA for each storage duration (Table 3). However, CA storage was more effective than air storage, in some cases, in lowering the incidence of decay observed after 4 wk at 1.1°C.

Canned M3 Halford peaches from CA storage showed higher 'a' values than air-stored fruits. Canned M1 fruits which were held in CA had a slightly higher pH than air-stored fruits. Otherwise, the objective evaluation revealed no statistically significant differences between the two storage treatments. The taste panel judges also detected no significant differences between fruits from the two storage treatments except that CA-stored M3 peaches were scored firmer than air-stored fruits.

An expert panel from Tri-Valley Growers subjectively rated canned Carolyn peaches good to very good in flavor and appearance with M1 fruits from CA storage judged to be of significantly better overall appearance than air-stored fruits (data not shown). Canned Loadel peaches were rated good while Halford and Klamt peaches scored low and Andross peaches were intermediate in appearance and flavor quality attributes.

An important result of this study is the observed variation in responses to storage among the cultivars tested. It seems that the most important factor in the successful storage of cling peaches may be the cultivar that is chosen. Large differences were found with respect to off-flavors, flesh browning, and bruising. Both Wankier et al. (1970) and Watada et al. (1979) cited development of off-flavors as a major factor limiting CA storage of

fresh market peaches. We observed no significant increase in off-flavors in CA-stored canning cling peaches compared to air. Among the five cultivars, Halford and Klamt were badly discolored and exhibited off-flavors described as "fishy" and "caramelized" by the judges. Andross, Loadel, and Carolyn fruits ranged from good to excellent in both appearance and flavor after four weeks storage followed by canning, regardless of the storage atmosphere.

There was also a wide range of susceptibility to decay among the cultivars which was most serious in M1 fruits ripened after storage. Alternaria was most common on these fruits, while Monilinia was more common on fruits affected during storage or the ripening experiment. While Andross, Carolyn, and Halford peaches were relatively unaffected by decay, Klamt peaches were heavily infected, and Loadel peaches were subject to such heavy decay that none of the M1 fruits transferred to 20°C after 4 wk storage were salvageable after a few days at room temperature.

When M1 fruits were ripened immediately after harvest, decay was not a significant problem. Therefore, the much greater amounts of decay observed in M1 fruits ripened after storage compared to immediate ripening indicate that when long-term storage of cling peaches is necessary, green fruits should probably be ripened before storage rather than after. Although CA storage proved to offer only small benefit over air storage in terms of canning quality, it would tend to reduce decay compared to air storage if initial sorting and ripening of green fruits is not feasible.

It is known that susceptibility of fruits to chilling injury is lessened with increasing maturity (Fidler, 1968; Lyons, 1973) and accelerated decay incidence is a common symptom of chilling injury in other commodities upon transfer to room temperature (Lyons, 1973; Ryall and Pentzer, 1974). Therefore, the decay observed in this study may have been a symptom of chilling injury to the M1 peaches.

Effect of ethylene treatment. Only small differences were found in rate or uniformity of ripening between the ethylene- and air-ripening treatments. However, ethylene-ripened fruits were somewhat lower in SSC, pH and significantly lower in acidity than air-ripened fruit (Table 4). Decay was not a problem in this experiment in contrast to the M1 fruit ripened after storage (Table 3).

Objective evaluation of the canned Halford peaches showed no significant differences between air- and ethylene-ripened fruits. The taste panel, however, judged M1 fruits both firmer and more sour when treated with ethylene, while ethylene-treated M2 fruits were scored as softer and less sour than air-ripened fruit.

The Tri-Valley Growers panel found no significant differences between fruits from the two ripening treatments but rated M1 and M2 Carolyn fruits good and very good, respectively, in flavor and rated both maturities excellent in overall appearance.

When ripening of cling peaches is desired before canning, there is apparently little to be gained from ethylene treatment. Fruits ripened in air, and in air after a 2-day ethylene treatment at 20°C, were essentially equal in appearance (including decay) and flavor quality. Although the ethylene treatment used in this study did not accelerate the ripening process, use of a longer duration and/or a higher ethylene concentration may potentially exhibit this effect. Otherwise, it would seem that simply holding the fruits in air at 20°C will suffice to allow ripening which produces peaches of good flavor and appearance when canned.

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1.3 Influence of Various Storage Atmospheres and Storage Procedures on
1-Aminocyclopropane-1-Carboxylic Acid Concentration in Relation to Flesh
Firmness in 'Golden Delicious' Apple

'Golden Delicious' apples soften at a relatively high rate in air storage or in conventional CA storage ("slow CA") in which the required atmosphere is established slowly by fruit respiration. Excellent firmness retention in this cultivar was achieved, however, when a "prestorage high CO₂ treatment" (5) or a "rapid CA" storage procedure (8) was used; both were equally effective (9).

The role of ethylene in initiating fruit ripening and softening is well-known (1). In postclimacteric apples, C₂H₄ is synthesized by the sequence: methionine → S-adenosylmethionine (SAM) → 1-aminocyclopropane-1-carboxylic acid (ACC) → C₂H₄ (3). During ripening of various climacteric fruits, increased C₂H₄ synthesis is accompanied by increased ACC concentration in the tissues (6). However, both the conversion of SAM to ACC and the conversion of ACC to C₂H₄ are restricted in preclimacteric fruit tissues (2,6). Ethylene production and flesh softening of apples during ripening in air at 15° to 20°C also are suppressed by treatments of preclimacteric apples with 1-3% O₂ or 15-20% O₂ + 20-30% CO₂ (10). ACC synthesis in these tissues is delayed initially, but not prevented, and eventually ACC is accumulated in large quantities (10).

This paper describes the changes of ACC levels in the fruit tissue in relation to the rates of flesh softening in 'Golden Delicious' apples stored in air and CA, preceded by a "prestorage high CO₂", "rapid CA", or "slow CA" storage procedure.

Experimental

Samples of 'Golden Delicious' apples (5 one-fruit replicates per sample for each storage treatment and examination date) were obtained from 5 trees (one apple per tree) in a commercial orchard at Summerland, B.C. on 23 Sept. 1982. They were cooled in 0°C air for 48 hr and then sealed in 3, gas-tight cabinets (0.6 x 1.2 x 2.4 m) placed at 0° on 25 Sept. The storage atmosphere of 2.5% O₂ + 1.5% CO₂ in the "rapid CA" cabinet was established within 24 hr of sealing by use of N₂ and CO₂ gases; it was established in 25 days through fruit respiration in the "slow CA" cabinet. The "17% CO₂ + rapid CA" samples were exposed to an atmosphere of 5% O₂ + 17% CO₂ during the first 10 days followed by storage in 2.5% O₂ + 1.5% CO₂. All CA samples were transferred to 1 cabinet on 12 Oct. and the storage atmosphere was maintained at 2.5% O₂ + 1.5% CO₂ throughout the remainder of the storage period. The air storage samples were kept continuously in a 0° cold storage room (12 x 8.5 x 3.7 m). The concentrations of C₂H₄ inside the storage cabinets were neither controlled nor monitored.

Two samples of fruit for each storage treatment and removal date were removed after 0, 28, 59, 88, and 118 days of storage; and internal C₂H₄ concentration, severity of CO₂ injury, flesh firmness, and ACC concentration were determined on each fruit of the 1st and 2nd sample (5 one-fruit replicates) immediately upon removal from storage and after 1 day in 20°C air, respectively.

A 1-ml gas sample was withdrawn with a syringe from the seed cavities of each apple submerged under water, and C₂H₄ concentration was determined by gas chromatography. Internal C₂H₄ concentrations are given only for those fruit which were kept for 1 day in open air at 20°C after removal from storage, because they would be correlated directly to the C₂H₄ production

rates (4). Internal C_2H_4 measurements were not made on fruit immediately after removal from the CA cabinets, as they would represent the sum of the rate of C_2H_4 production and concentration inside the storage cabinet.

The skin surface areas afflicted with CO_2 injury were determined for each fruit. Flesh firmness was determined on each fruit with a Magness-Taylor penetrometer (2 measurements made on opposite pared sides, 11.1-mm tip). For ACC determination, 2 opposite sectors of cortical tissue from each fruit (about a quarter of an apple weighing 50-60 g) were diced and blended with a Waring blender, in 3 ml of 2% HCl (w/v) per g of fruit tissue. The homogenate then was filtered through Whatman No. 4 filter paper under suction from a water aspirator. The ACC concentration in the extract was assayed according to the method of Lizada and Yang (11) after neutralizing with NaOH.

Results and Discussion

The low levels of ACC (0.3 nmol/g) and the absence of measurable C_2H_4 in 'Golden Delicious' apples picked at the preclimacteric stage on 23 Sept. 1982 (Fig. 1) suggest that synthesis/availability of ACC is a limiting factor regulating C_2H_4 production. It has been shown that preclimacteric fruit tissue lacks capability not only for ACC synthesis but also for the conversion of ACC to C_2H_4 (2,6). In this study, the ACC level in fruit stored in $0^\circ C$ air increased markedly (to 39 nmol/g after 27 days) during the 1st month of air storage, then declined sharply (to 7.5 nmol/g after 59 days) during the 2nd month of storage and remained at a low and constant level (3.7 and 4.9 nmol/g after 88 and 118 days, respectively) during the 3rd and 4th month of storage (Fig. 1). The results suggest that the enzyme responsible for ACC synthesis was synthesized at maximum rate during the 1st month of storage in 0° air. The rapid and steady conversion of ACC to C_2H_4 also could be a

Fig. 1 Changes in flesh firmness and l-aminocyclopropane-1-carboxylic acid (ACC) and ethylene (C₂H₄) concentrations in stored 'Golden Delicious' apples in relation to the storage atmospheres (air vs. CA), storage procedures ("rapid CA" vs. "slow CA" vs. "prestorage high CO₂"), and storage duration.

cause of reduced ACC levels in later periods of storage. Whether the induction of ACC synthase, which catalyzes the formation of ACC from SAM (12), during the early part of air storage at 0° is triggered by the removal of fruit from the tree, by chilling of fruit in the cold storage, and/or by other factors is yet to be determined.

The internal C_2H_4 concentration in fruit stored in 0°C air for 2 and 3 months was much higher than that in fruit stored for only 1 month. A decline in ACC level during the 2nd and 3rd month suggested that the ethylene-forming enzyme, which converts ACC to C_2H_4 , was not fully developed until the 3rd month of air storage.

The firmness data (Fig. 1) confirm previous reports (8,9) that the softening rate in 'Golden Delicious' apples is related to the storage atmospheres and the rapidity of establishing the CA conditions. The effectiveness of "rapid CA", "high CO_2 ", "slow CA", or air storage in maintaining flesh firmness appeared to be related closely to their inhibitory effects on the synthesis/accumulation of ACC and on the subsequent conversion of ACC to C_2H_4 (Fig. 1).

Both "rapid CA" and "high CO_2 prestorage treatment" were more effective than "slow CA" in suppressing the rise of ACC level which occurred during the 1st month of CA storage (Fig. 1). This is expected because it took 25 days to establish the required low- O_2 CA conditions under the "slow CA" storage procedure, during which time an active synthesis of ACC must have occurred. Only small and fairly comparable amounts of ACC and C_2H_4 were detected in fruit subjected to a "rapid CA" or a "prestorage high CO_2 " storage procedure (Fig. 1). This could explain, at least in part, why the rates of flesh softening in these fruit samples were lowest and comparable to one another (Fig. 1) (9). Fruit samples from "slow CA", which were fir-

mer than those from air storage but less firm than those from the "rapid CA" or the "prestorage high CO_2 " storage procedure, accumulated ACC at an intermediate rate (Fig. 1).

The low levels of ACC and C_2H_4 in the fruit stored with a "rapid CA" or "prestorage high CO_2 " storage procedure indicate that the onset of ripening was delayed and the ripening processes were suppressed throughout the storage period, thereby restricting the synthesis of ACC, the formation of C_2H_4 , and the softening process. Continuous exposure of preclimacteric apples to elevated CO_2 or low O_2 atmospheres delays ACC synthesis initially, but eventually results in an accumulation of ACC (10), due possibly to an inhibitory effect of high CO_2 on the formation of ACC (10) and the inhibitory effects of low O_2 (3,10) and high CO_2 (10) on the conversion of ACC to C_2H_4 .

In conclusion, the rapid accumulation of ACC and the subsequent, high rates of C_2H_4 production are related closely to the rapid flesh softening observed in fruit stored in air; these processes can be suppressed greatly by storage of fruit in CA, provided the fruit were exposed to low O_2 and/or high CO_2 regimes immediately following harvest. CO_2 injury has been a problem associated with the "prestorage high CO_2 " storage procedure (7), and in this study about 70% of the CO_2 -treated fruit (9% of the total skin surfaces) exhibited CO_2 injury. Consequently, 'Golden Delicious' apples designated for late marketing should be stored in CA using the simple and safe "rapid CA" storage procedure (8,9), in order to suppress the synthesis of ACC and C_2H_4 to reduce the loss of flesh firmness, and to maximize the storage life of the fruit.

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2. The physiological, biochemical and pathological consequences of introducing carbon monoxide to controlled atmosphere storage and the fate of CO absorbed by the tissue

2.1 The Effect of Carbon Monoxide on Postharvest Decay, Respiration, Ethylene Production, and Quality of Peaches Held in Controlled Atmospheres

The effectiveness of carbon monoxide (CO) as a fungistatic gas is pathogen dependent and is greatly enhanced when combined with reduced- O_2 atmospheres (7). The minimum concentrations required to achieve decay control appear to be 5% CO in less than 5% O_2 (6,7,15). Reduced O_2 alone did not influence growth of Monilinia fructicola (13,15), but its growth and spore germination were suppressed by 10 to 20% CO_2 atmospheres (3,16). Although CO_2 levels above 5% can harm peach fruit quality (2,11), Anderson et al. (1) reported that flesh breakdown and discoloration were delayed by storing peaches at $0^\circ C$ in a controlled atmosphere (CA) of 1% O_2 + 5% CO_2 rather than in air. Smith and Anderson (14) found that while CA slightly reduced decay on peaches during storage at 0° , it had no effect on their decay following transfer to 18.3° .

CO mimics C_2H_4 effects and can enhance fruit ripening when added to air (4,8,10). Kader et al. (9) reported that 5 or 10% CO added to air increased CO_2 and C_2H_4 production rates and hastened ripening of mature-green tomatoes. However, when CO was added to 4% O_2 atmosphere, its effect on ripening was very small. They also reported that tomato fruits subjected to 5 or 10% CO + 4% O_2 maintained their sugar and acid content better than control fruits. El-Goorani and Sommer (6) found no off-flavors attributable to 9% CO in apples or oranges, but strawberries developed moderate off-flavor under CA or CA + 9% CO, which was attributed to reduced O_2 + elevated CO_2 and not added CO.

We report here the effects of CO added to reduced O_2 and/or elevated CO_2 on decay due to Monilinia fructicola, respiration, ethylene production, and internal breakdown, and on flesh color, firmness, and composition of peaches.

Experimental

'Fay Elberta' peaches were picked at shipping maturity (on the basis of ground color) from the Wolfskill Experimental Orchard, Winters, Calif. Fruits were sorted to eliminate defects and were matched by color, size and touch-firmness into lots of 10 to 20 fruits each. Three replicates (lots) were used per treatment. All experiments were carried out at 5°C to increase the deterioration rate and the incidence and severity of internal breakdown over that of fruits held at 0° (1).

Monilinia fructicola was originally isolated from rotting peach fruits and was maintained at about 4°C on potato-dextrose agar (PDA) slants. Its pathogenicity was verified by fruit inoculation. In vitro tests used PDA (20 ml per plate) in Petri dishes which were inoculated at the center with 6-mm mycelial plugs from the edge of a 7-day-old culture. Lids were raised with a bent sterile wire to facilitate maintenance of the cultures at the atmospheres of the CA chambers (4.5-liter glass jars). Growth was measured as radial expansion of colonies in 6 replicates per treatment after 14 days at 5°.

For in vivo studies, fruits were washed, surface-sterilized by immersion in sodium hypochlorite solution (500 µg/ml) for 5 min, and air-dried. Plugs (6-mm in diameter) of a 6-day-old culture were inserted with a dissecting needle into 10-mm-deep wounds in fruit tissue. Inoculated fruits (20 per treatment) were placed in 4.5-liter glass jars which were ventilated with a continuous flow (100 ml/min) of the desired gas mixture. After 14 days at 5°C, fruits were examined for rot development by measuring the diameter of the lesions. These fruits were then placed at 20° in air and re-examined after 3 days.

The desired O₂, CO₂, and CO mixtures were provided by metering appro-

priate amounts of air, N_2 , CO_2 , and/or CO in a flow-through system using capillary tubes or needle valves as flow meters. Gas mixtures were verified by thermal conductivity gas chromatography. The CA treatments used were: 1) air control; 2) air + 11% CO; 3) air + 5% CO_2 ; 4) air + 5% CO_2 + 11% CO; 5) 4% O_2 ; 6) 4% O_2 + 11% CO; 7) 4% O_2 + 5% CO_2 ; and 8) 4% O_2 + 5% CO_2 + 11% CO. These gas mixtures were monitored and adjusted every 2 days to ensure that the actual compositions were within 10% of the intended mixtures.

Three replicates of 10 fruits each per treatment were placed in 4.5-liter jars which were ventilated with a humidified stream of the desired gas mixture at flow rates selected to ensure that CO_2 did not accumulate above 0.4%. CO_2 production was determined every 2 days at 5°C and daily at 20°C by the Claypool-Keefer colorimetric method (5), and C_2H_4 production was measured using a flame-ionization gas chromatograph. It was possible to determine CO_2 production only for those samples that did not have elevated CO_2 as part of their atmosphere.

Three replicates of 20 fruits each per treatment were used for quality evaluation. Fruits were evaluated initially, after 14 days at 5°C, and after an additional 3 days at 20°C in air. Flesh color and firmness were determined on individual fruits by a Gardner Color Difference Meter (CDM) and a UC Fruit Firmness Tester with a 7.9-mm plunger, respectively. Fruits were cut across the equatorial diameter and subjectively scored for internal breakdown symptoms on a scale of 1 = none to 5 = extreme. A combined sample of flesh wedges taken from all fruits per replicate was used for compositional analyses. Soluble solids content (SSC) was determined with a Bausch & Lomb ABBE-3L refractometer, and titratable acidity was determined by titrating 6 g of juice to pH 8.1 with 0.1 N NaOH.

Results and Discussion

The addition of 11% CO to air reduced in vitro growth rate of Monilinia fructicola by 41% (Table 1). CO₂ (5%) added to air stimulated growth of the fungus by about 25%; even in CO, 5% CO₂ increased growth about 25% over CO alone. In 4% O₂, fungal growth was not inhibited, but addition of 5% CO₂ to it reduced growth, and addition of 11% CO prevented growth for 14 days at 5°C.

Brown rot development on peaches in air was delayed by 5% CO₂ and by 11% CO. While 4% O₂ alone had no effect, the addition of 11% CO to 4% O₂ + 5% CO₂ completely inhibited brown rot development during 14 days at 5°C. Once these fruits were transferred to air at 20° rot development resumed, but the relative differences among treatments remained about the same after 3 days.

We have noted the effectiveness of added CO (5-11%) + reduced O₂ (2-5%) in decay control on uninoculated peaches in several experiments during the 1978 to 1981 seasons (data not included). Other researchers (6,7,9,12,15) have reported on the fungistatic effects of CO on various fungi, especially when combined with reduced O₂ levels.

CO added to air stimulated CO₂ production by peaches during storage at 5°C for 14 days (Fig. 1). Fruits held under 4% O₂ had a lower respiration rate than those held in air, but it was increased by adding 11% CO. These results are similar to those reported on mature-green tomatoes (9). Upon transfer to air at 20°, large increases in CO₂ production rates occurred (Table 2). On the first day at 20°, fruits which were subjected to 5% CO₂ + air with or without 11% CO had a higher CO₂ production rate than most of the other treatments, which may indicate some degree of CO₂ injury. By the third day at 20°, no significant differences in respiratory rates were observed among treatments, except that fruits that had been in air + 5% CO₂ continued to exhibit a higher rate.

Table 1. Growth of *Monilinia fructicola* cultured on potato dextrose agar medium and the rate of brown rot development on inoculated peach fruits after 14 days at 5°C under various controlled atmospheres with or without added CO.

Treatment			Mean ¹ <i>in vitro</i> growth rate (mm/day)	Mean ² rate of rot development <i>in vivo</i> (mm/day)
O ₂ %	CO ₂ %	CO%		
Air	—	—	0.63 b ^a	1.98 ab
Air	—	11	0.37 d	1.28 d
Air	5	—	0.78 a	1.69 c
Air	5	11	0.47 c	1.21 d
4	—	—	0.61 b	2.05 a
4	—	11	0 e	0 e
4	5	—	0.48 c	1.79 bc
4	5	11	0 e	0 e

¹Means of 6 replicates.

²Means of 20 fruits.

^aMean separation in columns by Duncan's multiple range test, 1% level.

Table 2. Effects of various CA ± CO treatments for 14 days at 5°C on CO₂ and C₂H₄ production rates (means of 3 replicates) by 'Fay Elberta' peaches during subsequent holding for 3 days in air at 20°.

Treatment			ml CO ₂ /kg-hr		μl C ₂ H ₄ /kg-hr	
O ₂ %	CO ₂ %	CO%	Day 1	Day 3	Day 1	Day 3
Air	—	—	32.6b ^a	33.0b	37.1b	33.7b
Air	—	11	33.8ab	32.2b	47.5a	46.1a
Air	5	—	35.5a	40.6a	22.9cd	17.0d
Air	5	11	35.9a	36.2b	34.1b	38.5b
4	—	—	34.2ab	34.1b	25.7c	28.5c
4	—	11	29.5c	34.2b	27.6c	27.7c
4	5	—	27.5c	32.5b	18.1d	12.8d
4	5	11	34.0ab	32.6b	22.3cd	11.7d

^aMean separation in columns by Duncan's multiple range test, 5% level.

Table 3. Effects of controlled atmospheres with or without CO on color, firmness, composition, and internal breakdown of 'Fay Elberta' peach fruits held for 14 days at 5°C under indicated atmospheres followed by 3 days in air at 20°.

Evaluation time	Treatment			Color (CDM "a" value)	Firmness (kg)	SSC (%)	pH	Titratable acidity (%)	Internal breakdown score ¹
	O ₂ %	CO ₂ %	CO%						
Initial	---	---	---	5.4	4.2	11.0	4.03	0.46	1.0
After 2	Air	---	---	8.4a ^b	2.1b	10.4a	4.15a	0.37a	2.8ab
wks at	Air	---	11	8.1a	1.8a	10.6a	4.17a	0.37a	2.1a
5° + 3	Air	5	---	6.9c	2.2b	10.4a	4.17a	0.36a	3.8c
days at	Air	5	11	6.4c	2.7c	10.7a	4.03b	0.45b	2.7ab
20°C	4	---	---	7.5b	3.1cd	11.1b	4.15a	0.40a	3.3b
	4	---	11	7.5b	3.0cd	11.2b	4.15a	0.40a	2.6ab
	4	5	---	6.1d	3.2d	10.9ab	4.13a	0.39a	3.7c
	4	5	11	6.9c	2.8c	10.8ab	4.17a	0.37a	3.9c

¹Scoring system: 1 = none; 2 = slight; 3 = moderate; 4 = severe; 5 = extreme.

^aMean separation in columns by Duncan's multiple range test, 5% level.

CO in air stimulated C_2H_4 production by fruits during the second week of storage at $5^{\circ}C$ (Fig. 2). All CA treatments reduced C_2H_4 production rates, with the combination of 4% O_2 + 5% CO_2 having the greatest effect. In the presence of 5% CO_2 , C_2H_4 production remained near 0 during the first week of storage. In all the CA treatments, adding 11% CO slightly increased C_2H_4 production relative to each CA treatment alone during the latter part of the storage period. When fruits were transferred to air at 20° , C_2H_4 production rates increased, and fruits which were subjected to CO added to air $\pm$ 5% CO_2 continued to exhibit higher C_2H_4 production rates than those which were held in air $\pm$ 5% CO_2 (Table 2). CO added to 4% O_2 $\pm$ 5% CO_2 had no significant effect on C_2H_4 production rates during the 3 days at 20° relative to comparable treatments without CO.

Although CO mimicked C_2H_4 effects in stimulating CO_2 and C_2H_4 production by the fruits held in air, its effects were minimal when added to 4% O_2 , which is the optimum fungistatic treatment.

After 14 days at $5^{\circ}C$ + 3 days at 20° , fruits from all treatments had a lower green flesh color (higher CDM "a" values) and were softer than fruits at harvest, but changes in SSC, pH, and titratable acidity were small (Table 3). Slight to severe symptoms of internal breakdown were observed after all treatments. Nearly all CA treatments retarded changes in flesh color and firmness. Fruits from the reduced O_2 treatments retained their firmness better than those held in air. CO added to air or 4% O_2 + 5% CO_2 slightly hastened fruit softening in comparison with fruits held in comparable treatments without CO, but CO added to 4% O_2 had no significant effect on flesh color or firmness relative to those held in 4% O_2 (Table 3). We also found no significant differences among fruits of all treatments in total sugars, total phenolics, or total ascorbic acid contents (data not included).

Fig. 1. Respiration rates of 'Fay Elberta' peaches (means of 3 replicates) as influenced by reduced O₂ and/or 11% CO during holding at 5°C for 14 days; LSD at 5% = 0.9 ml/kg-hr.

Fig. 2. Effects of controlled atmospheres with or without added CO on ethylene production rates by 'Fay Elberta' peaches (means of 3 replicates) during holding at 5°C for 14 days; LSD at 5% = 0.4 μl/kg-hr.

Exposure of 'Fay Elberta' peaches to 5% CO₂ resulted in higher scores of internal breakdown (flesh browning, mealy texture). CO slightly reduced the symptoms, especially flesh browning, in every case except when combined with 4% O₂ + 5% CO₂. The effect of CO appears to be a secondary effect, possibly on reducing symptoms of flesh browning, and not an inhibitory effect on the physiological injury of low temperatures. Our data indicate no beneficial effect of CA in reducing or alleviating internal breakdown of peaches as has been reported by Anderson et al. (1). This may be due to cultivar differences in response to CA and in susceptibility to internal breakdown.

We conclude that the use of CO combined with reduced O₂ atmospheres as a fungistatic treatment on peach fruits will not detrimentally influence their quality attributes. Commercial use of such treatment for decay control will depend on availability of alternative methods such as postharvest fungicides and on development of adequate technological procedures that would minimize the hazards of CO to humans during postharvest handling of horticultural commodities.

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2.2 The Effect of Carbon Monoxide on Postharvest Quality and Storage Life of Grapes Held in Air or Controlled Atmospheres

SO₂, although effective in retarding the activity of decay-causing organisms in grapes, including Botrytis cinerea Pers., is very corrosive to metals, injurious to most other fresh fruits, and causes injury to grapes if used excessively (4,5,10,11). The optimum SO₂ concentration used in grapes is usually a compromise between that which will provide acceptable control of decay and that which will not cause excessive injury (10).

Controlled-atmosphere (CA) storage has been tested for decay control and extension of the storage life of grapes (13). Uota (16) showed that CA alone did not provide adequate decay control under conditions of relatively high humidity; however, combining CA (5% O₂ and 10% CO₂) with the application of 1000 ppm SO₂ at 7-day intervals gave almost complete decay control. Nassar (8) found that elevated CO₂ concentrations (up to 15%) increased browning of berries and resulted in the accumulation of α-amino butyric acid and succinic acid and reduction of aspartic and glutamic acids in grapes, but SO₂ fumigation did not change the organic and amino acids examined. Nelson (9) concluded that CA was not very promising for commercial grape storage, since grapes kept at less than 15% CO₂ had higher decay than those subjected to SO₂ fumigation; while 15% CO₂ controlled the decay, browning of the berries made it unsatisfactory. Harvey and Uota (5) also found that CA alone did not control decay of grapes unless the CO₂ levels were increased or the O₂ levels were reduced to the point where physiological disorders may occur.

CO is slightly fungistatic in air and often highly fungistatic in reduced O₂ (<5%) atmospheres (1,2,3,6,7,14,15). Kader et al. (6) found that CO (5% and 10%) + 4% O₂ atmospheres retarded growth of B. cinerea on inoculated

tomatoes harvested at the mature-green or pink stage and held at 12.5°C for up to 14 or 10 days, respectively. CO + CA inhibits decay caused by B. cinerea on cherries (14), strawberries (1,3), apples (15), and kiwifruits (14). Peiser et al. (12) found that CO was not directly metabolized by lettuce leaf discs but was converted, in part, into CO₂ which was metabolized into organic acids and other constituents.

The goals of this study were to evaluate the possible use of CO alone or in combination with CA (2% O₂ with or without 5% CO₂) in suppressing decay and prolonging the storage life of 'Thompson Seedless' grapes and to compare these treatments with the use of SO₂ either by direct fumigation or through generators.

Experimental

'Thompson Seedless' grapes were harvested from the Univ. vineyard at Davis, Calif. on August 20, 1981, and August 27, 1982 from vines that were girdled, thinned, and sprayed with 20 ppm gibberellic acid at full bloom and fruit set. Fruit clusters were sorted to eliminate defects and were matched into lots by color, size, and appearance. Three replicates (3 kg each) were used per treatment and for initial evaluation. Quality evaluations were made initially and then monthly during the storage period. They included overall appearance, decay incidence, browning of berries, and drying of stems using subjective scoring systems. Berry firmness was measured on 20 fruit per replicate using subjective scoring in 1981 and a UC Fruit Firmness Tester with a 4.8-mm plunger and berry skin removed in 1982. Soluble solids content (SSC) and pH were measured on juice samples. Titratable acidity (TA) was measured by titrating 10 ml juice to pH 8.1 with 0.133 N NaOH.

Grapes were kept in 2 controlled-temperature rooms, one for the CA treatments and the other for the SO_2 treatments to avoid SO_2 contamination in the first room. Fruit kept in air or CA at 0°C were placed in 10-liter, metal chambers that were ventilated with a continuous flow of the desired gas mixture. Control and SO_2 -treated fruit were packed in shipping containers and placed on shelves at 1° .

The desired O_2 , CO_2 , and CO mixtures were provided by metering appropriate amounts of air, N_2 , CO_2 and/or CO in a flow-through system using needle valves and capillary tubes as flow meters. Gas mixtures were verified and monitored every few days by analyzing them with a thermal conductivity gas chromatograph to ensure that the actual compositions were within 10% of the intended mixtures throughout the storage duration. Air and all gas mixtures were humidified by bubbling them through water before entering the CA chambers. The flow rates used were selected to ensure that CO_2 produced by the fruit did not accumulate above 0.4%. The CA treatments used in 1981 were: air control; air + 10% CO; 2% O_2 + 5% CO_2 ; and 2% O_2 + 5% CO_2 + 10% CO. CA treatments in 1982 were: air control; 2% O_2 ; and 2% O_2 + 10% CO.

SO_2 treatments in 1981 were: air control; conventional SO_2 fumigation treatment (SO_2/F); and in-package SO_2 -generating pads (SO_2/G). Two 2-stage pads (4,10) were used for each container (3 replicates of 3 kg each for a total of 9 kg of grapes) in the SO_2/G treatment. One pad was placed below and the 2nd on top of the packed fruit, and containers were wrapped in polyethylene film to restrict ventilation and to provide high humidity for the release of SO_2 . For the SO_2/F grapes were fumigated with 0.2% (v/v) SO_2 for 20 min in a fumigation chamber immediately after packing and subsequently with 0.1% SO_2 for 20 min at weekly intervals. In 1982, only the SO_2/G treatment was used and storage was at 0°C .

Respiration rate (CO_2 production) of grapes kept in air, 2% O_2 and 2% O_2 + 10% CO , and C_2H_4 production by fruits kept under all the CA treatments were measured every 2 or 3 days for 2 months, using gas chromatography.

After one month of storage in 1982, a taste panel, consisting of 6 trained judges, evaluated the sensory attributes of grapes from the 3 replicates of the 4 treatments using a simple triangle test. All possible combinations were used for each panelist in each replicate. The panelists subsequently were asked to score each sample for browning, bleaching, firmness, sulfury flavor, and off-flavor by marking 10-cm lines labeled near their ends by "none" and "extreme" on the score sheet. Judges were trained for 2 days before each monthly evaluation period. Consistent references of grapes exhibiting extreme browning, bleaching, or sulfury flavor were presented along with the samples and all evaluations (3 replicates) were made by the same judges to minimize variability. Judges received 4 samples of 20 fruit each per day (one session), in random order. Samples were presented to judges in individual booths under white lights. Judges were instructed to rinse their mouths with distilled water between samples and to expectorate samples.

Results and Discussion

CO added to air reduced CO_2 production by grapes; the effect was more pronounced during the 2nd month than during the first month of storage at 0°C (Fig. 1A). Both the 2% O_2 and the 2% O_2 + 10% CO atmospheres also reduced respiration rate during the 2nd month, but had no significant effect during the first month of storage (Fig. 1B). The 2- to 3-fold increase in CO_2 production rate by grapes kept in air may have been due to incidence and spread of decay.

Fig. 1. Effects of CA treatments on respiration rates of 'Thompson Seedless' grapes during holding at 0°C for 60 days; A = 1981 study and B = 1982 study. LSD (1) and (2) are for the first and 2nd month of storage, respectively.

Fig. 2. Effects of CA treatments on ethylene production rates by 'Thompson Seedless' grapes kept at 0°C for 60 days; A = 1981 study and B = 1982 study. LSD (1) and (2) are for the first and 2nd month of storage, respectively.

Fig. 3. Effects of storage conditions on the overall appearance quality (9 = excellent; 7 = good; 5 = fair; 3 = poor; 1 = very poor) of 'Thompson Seedless' grapes. LSD (1) is for the first 2 months and LSD (2) is for the last 2 months of storage (1981 study).

Fig. 4. Effects of storage conditions on decay incidence in 'Thompson Seedless' grapes. LSD (1) is for the first 2 months and LSD (2) is for the last 2 months of storage (1981 study).

CO effect on CO_2 production by fruits appears to be dependent upon their respiratory pattern. CO reduced CO_2 production by nonclimacteric fruits, as shown for grapes (Fig. 1A, B) and strawberries (3). On the other hand, CO stimulates CO_2 production by climacteric fruits, such as tomatoes (6) and peaches (7), perhaps due to its C_2H_4 -mimicking characteristics and its stimulation of C_2H_4 production by these fruits.

Ethylene production rates by grapes were very low in all treatments (Fig. 2). Differences among treatments during the first month of storage were small and mostly not significant. During the 2nd month at 0°C , there was a 4- to 6-fold increase in C_2H_4 production by fruit kept in air (Fig. 2), which may have been due to spread of decay. All CA treatments, with or without CO, reduced C_2H_4 production rates, but CO-containing atmospheres enhanced the effect. The effectiveness of CO in reducing C_2H_4 production rates may have been largely due to its decay control effects, since it stimulates C_2H_4 production by other fruits (6,7,14).

The deterioration rate was fastest in grapes kept in air at either 0° or 1°C (Fig. 3). There were no significant differences among treatments other than the air control during the first month of storage. After 2 months, grapes kept under 2% O_2 + 5% CO_2 deteriorated to an overall appearance score below 5 (fair); thus, this treatment was discontinued along with the 2 air controls. Decay was the main cause of deterioration in these and other treatments (Fig. 4). The effectiveness of CO added to air in controlling decay diminished following 2 months at 0° (Fig. 4). Although SO_2/F was very effective in preventing decay (Fig. 4), it did not differ from the air + CO treatment in overall appearance of grapes (Fig. 3) due to the pronounced browning caused by SO_2/F , especially during the 3rd and 4th months of storage (Table 1). The overall appearance of grapes held under CA + CO

Table 1. Effects of storage conditions on quality attributes of 'Thompson Seedless' grapes (1981 study).

Storage conditions			Browning ^a (score)	Firmness ^b (score)	SSC (%)	pH	TA (% tartaric acid)
Duration (months)	Temp (°C)	Treatment					
---	---	Initial	1.0	5.0	21.8	3.34	0.74
2	0	Air control	2.0 a ^a	3.5 b ^a	21.1 ab	3.42 a	0.74 a
		Air + 10% CO	0.5 c	3.7 ab	20.2 bc	3.46 a	0.72 a
		CA(2% O ₂ + 5% CO ₂)	1.5 b	3.3 bc	19.6 c	3.43 a	0.73 a
		CA + 10% CO	1.3 b	4.0 a	21.6 a	3.50 a	0.70 a
	1	Air control	2.1 a	3.1 c	21.7 a	3.45 a	0.72 a
		SO ₂ /F	2.3 a	3.2 c	21.9 a	3.52 a	0.68 a
		SO ₂ /G	1.1 bc	3.5 b	21.7 a	3.54 a	0.67 a
3	0	Air + 10% CO	0.9 d	3.6 c	20.1 f	3.48 b	0.64 b
		CA + 10% CO	2.3 e	3.5 c	20.8 c	3.61 c	0.64 b
	1	SO ₂ /F	3.5 f	2.7 f	21.8 d	3.51 b	0.69 c
		SO ₂ /G	1.8 e	2.8 f	21.9 d	3.47 b	0.63 b
4	0	Air + 10% CO	1.1 g	2.8 g	20.4 i	3.51 d	0.57 d
		CA + 10% CO	3.1 h	3.2 g	20.1 i	3.68 f	0.56 d
	1	SO ₂ /F	4.5 i	1.7 i	22.0 g	3.63 e	0.63 e
		SO ₂ /G	2.3 h	2.3 h	21.6 h	3.53 d	0.57 d

^aBrowning scores: 1 = none; 2 = slight; 3 = moderate; 4 = severe; 5 = extreme.^bFirmness scores: 1 = very soft; 2 = soft; 3 = intermediate; 4 = firm; 5 = very firm.^cMean separation in columns for each storage duration by LSD test, 5% level.

Table 2. Effects of storage conditions on decay incidence and quality attributes of 'Thompson Seedless' grapes (1982 study).

Storage conditions at 0°C			Flesh firmness (N)	Sensory evaluation scores ^a				
Duration (months)	Treatment	Decay (%)		Firmness	Browning	Bleaching	Off- flavor	Sulfury flavor
2	Air control	21.1 c ^y	2.6 c	6.1 a	3.7 a	1.6 a	1.3 a	3.0 a
	2% O ₂	9.2 d	3.0 d	6.3 a	1.3 b	1.3 ab	1.7 a	2.9 a
	2% O ₂ + 10% CO	0.2 e	3.0 d	6.1 a	1.3 b	0.9 b	1.4 a	4.1 a
	SO ₂ /G	0.1 e	2.6 c	5.2 b	4.5 a	3.7 c	1.6 a	3.1 a
3	2% O ₂ + 10% CO	0.2 f	2.9 e	5.9 c	1.5 c	1.1 d	1.3 b	3.4 b
	SO ₂ /G	0.1 f	2.8 e	5.8 c	3.6 d	2.3 e	1.4 b	4.4 c
4	2% O ₂ + 10% CO	0.4 g	2.5 f	6.1 d	2.6 e	1.4 f	2.4 c	4.6 d
	SO ₂ /G	0.2 g	2.6 f	5.4 e	5.6 f	2.9 g	1.7 d	3.7 e

^aScoring system: 0 = none to 10 = extreme.^yMean separation in columns for each storage duration by LSD test, 5% level.

seemed slightly better than that of the SO_2/G -treated fruit after 3 or 4 months in storage, but the difference was not significant statistically (Fig. 3). By the end of 4 months, overall appearance scores of grapes stored under CA + CO were significantly better than those of fruits subjected to the SO_2/F or air + CO treatments. CO added to CA was markedly more effective than CO in air and was as effective as the 2 SO_2 treatments for decay control (Fig. 4). In the 1982 study, the 2% O_2 + 10% CO treatment was as effective as the SO_2/G for decay control for up to 4 months at 0° (Table 2). As has been noted with other fruits (1,3,6,7,14,15), CO is more fungistatic when combined with reduced O_2 levels.

No significant differences in browning, pH, and TA and only small differences in firmness and SSC were noted among all treatments after one month in storage in the 1981 study (data not shown). Grapes subsequently kept in air + 10% CO exhibited the least brown discoloration, followed by those kept under CA + 10% CO and SO_2/G treatments (Table 1). SO_2/F -treated berries showed the most severe browning, probably due to accelerated senescence and the extra handling involved in repeated fumigation treatments. After 3 or 4 months in storage, browning of the berries kept in CA + CO was greater than that of berries in the SO_2/G and air + CO treatments (Table 1), which may have been due to elevated CO_2 as has been reported by others (8,9).

Grapes kept under CO added to air or CA softened at a slower rate than the SO_2 -treated grapes (Table 1). No significant differences in pH or TA were noted among treatments after 2 months in storage and SSC was comparable among treatments except for slightly lower SSC values in fruit subjected to air + CO or CA. After 3 and 4 months, SO_2 -treated fruit contained more SSC than CO-treated fruit (Table 1), probably due to greater water loss in the SO_2 -treatments because of lower relative humidity, especially in the SO_2/F treatment. Differences in pH and TA were small after 3 and 4 months.

In 1982, sensory evaluation using triangle tests showed that after one month of storage, judges were able to differentiate only between the SO₂/G and the 2% O₂ + 10% CO-treated fruit (data not shown). SO₂-treated berries were scores lower in firmness and higher in browning and bleaching after 2 months than those from the other treatments (Table 2). After 3 months of storage, SO₂-treated fruit were scored higher in browning, bleaching, and sulfury flavor than 2% O₂ + 10% CO₂-treated fruits. CO-treated fruits were scored higher in firmness and lower in browning and bleaching, at the end of 4 months in storage, but were rated slightly higher in off-flavor and sulfury flavor than SO₂-treated berries (Table 2).

In conclusion, 10% CO in a 2% O₂-atmosphere appeared to be the best CA treatment, controlling decay as well as the currently used SO₂ treatments, while providing better quality maintenance of grapes than SO₂ during storage for up to 4 months. Further evaluations of the advantages and disadvantages of CO vs. SO₂ in relation to fruit quality and environmental safety considerations must be made before CO can be recommended for long-term storage of grapes.

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2.3 The Fate of Carbon Monoxide in Lettuce Leaf Discs

Recently much interest has been directed towards the use of carbon monoxide for prolonging the storage life of fruits and vegetables as well as meats (9, 16, 17). During transit or temporary storage, CO has been used as a supplement in modified atmosphere to inhibit discoloration of cut surfaces in lettuce and to reduce decay development in several fruits and vegetables. Under appropriate conditions CO has little or no adverse effects upon fruits and vegetables based on visual and sensory evaluations.

Absorption and metabolism of CO has been examined in algae (5) and higher plants (1, 2, 6, 11, 12). CO has been reported to be incorporated into acid stable products by two different pathways. In some plants CO *per se* is fixed (1, 2, 12) while in other cases it is first metabolized to CO₂ and this CO₂ is fixed (1, 2). In addition to the differences in CO metabolism attributed to different plants, light or darkness may also influence the metabolism of CO (2).

Since CO is presently being used commercially as a component of modified atmosphere during transit and storage of shredded lettuce and since its pathway of metabolism can vary with species and environmental conditions, the present study was conducted to examine the metabolism of CO in lettuce tissue in the dark.

Experimental

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.

^{14}CO generation. ^{14}CO was generated from sodium [^{14}C]formate (New England Nuclear, specific activity 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 N_2 stream. The tube was sealed with a rubber stopper and slightly evacuated. After injecting 0.25 ml concentrated H_2SO_4 , 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 $^{14}\text{CO}_2$.

^{14}CO measurement. ^{14}CO was measured by oxidizing it to $^{14}\text{CO}_2$ (14). Oxidation was performed in an enclosed 50 ml Erlenmeyer flask containing a 14 x 45 mm shell vial and a few granules (ca. 0.2 g) of I_2O_5 . The flask was sealed with a serum stopper and slightly evacuated. A gas sample of ^{14}CO 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 ethanol-amine:2-ethoxyethanol (1:1, v/v) was injected into the shell vial to absorb the $^{14}\text{CO}_2$ which was measured by liquid scintillation counting. This method gave quantitative conversion of CO to CO_2 as verified by gas chromatography.

Comparison of labeled products from ^{14}CO and $^{14}\text{CO}_2$. Labeled products from ^{14}CO - and $^{14}\text{CO}_2$ -treated lettuce were examined for two incubation periods, 3.5 h 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 ^{14}CO (ca. 2.6×10^7 cpm, final concentration 109 $\mu\text{l/l}$) in the 3.5 h experiment and ca. 2×10^7 cpm, final concentration 84 $\mu\text{l/l}$ in

the 48 h experiment) or $^{14}\text{CO}_2$ (ca. 2.5×10^6 cpm, final concentration 22 $\mu\text{l/l}$ in the 3.5 h experiment and ca. 6×10^6 cpm, final concentration 53 $\mu\text{l/l}$ 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 ^{14}CO and $^{14}\text{CO}_2$ 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. Aspartic 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 paper electrophoresis at pH 4. Radioactivity was located using a radioscanner and the products were identified by CO-migration with standards.

In vivo Km for oxidation of CO to CO_2 . One g of leaf discs 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 were injected into the flasks. Thirty minutes later, a 3 ml gas sample was taken for $^{14}\text{CO}_2$ 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 8 layers of cheesecloth. Three fractions were obtained by centrifugation: A "pellet" fraction (1200g spin for 4 min), a mitochondrial 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^{3-} -mannitol buffer and resuspended in PO_4^{3-} -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 (10^5 cpm, final concentration 0.84 $\mu\text{l/l}$). The $^{14}\text{CO}_2$ produced was absorbed in 6N KOH held in center wells. The KOH was then acidified with lactic acid and the $^{14}\text{CO}_2$ 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 h or 48 h, the primary metabolite was CO_2 , though a small amount of radioactivity was found in acid-stable products. Since both ^{14}CO and $^{14}\text{CO}_2$ were present, the question arises whether the radioactive acid-stable products were derived from ^{14}CO , $^{14}\text{CO}_2$ or both. One approach to answer this question is to compare the pattern of labeled products between ^{14}CO -treated and $^{14}\text{CO}_2$ -treated leaf tissue. The labeled soluble products were the same between these two treatments for both the 3.5 h and the 48 h incubation. This suggests that it was $^{14}\text{CO}_2$ derived from ^{14}CO and not ^{14}CO *per se* which was fixed. The soluble fraction most heavily labeled in the ^{14}CO and $^{14}\text{CO}_2$ treatments was the anionic fraction.

For most experiments the leaf tissue was incubated at 2.5 C since this is a good commercial storage temperature for lettuce. However, leaf discs treated

Table I. Distribution of ^{14}C Into Products From Leaf Tissue Exposed to ^{14}CO or $^{14}\text{CO}_2$.

For the 3.5 h incubation, 5 g of leaf tissue were incubated in 50 ml test tubes containing ^{14}CO (2.6×10^7 cpm, final concn. $109 \mu\text{l/l}$) or $^{14}\text{CO}_2$ (2.5×10^6 cpm, final concn. $22 \mu\text{l/l}$) at 2.5 C. In the 48 h incubation, 2 g of leaf discs were incubated in 50 ml Erlenmeyer flasks containing ^{14}CO (2×10^7 cpm, final concn. $84 \mu\text{l/l}$) or $^{14}\text{CO}_2$ (6×10^6 cpm, final concn. $53 \mu\text{l/l}$) also at 2.5 C.

Labeled Products	3.5 h		48 h	
	^{14}CO	$^{14}\text{CO}_2$	^{14}CO	$^{14}\text{CO}_2$
	% of ^{14}C supplied			
CO_2	1.19	-	16.7	-
Soluble fraction	0.04	2.98	0.61	6.38
Insoluble	0.004	0.13	0.07	1.12
	% of soluble radioactivity			
Neutral fraction	2	3	3	5
Cation fraction				
Aspartate	19	27	17	10
Glutamate	7	6	7	7
Asparagine	3	2	6	14
Glutamine	4	1	5	10
Serine	- ^a	- ^a	2	2
Alanine	- ^a	- ^a	1	2
Unknown	0	0	2	1
Anion fraction				
Malate	20	16	40	28
Citrate	32	39	17	21
Unknown	5	1	0	0

^aThe radioactivity in the neutral amino acid fraction including serine and alanine contained 8% and 5% of the soluble radioactivity from the ^{14}CO and $^{14}\text{CO}_2$ treatments, respectively; radioactivity in the individual amino acids were not determined.

with ^{14}CO or $^{14}\text{CO}_2$ incubated at 2.5 C 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 $^{14}\text{CO}_2$ treatments were observed (data not shown). As expected, more ^{14}CO was oxidized to $^{14}\text{CO}_2$ 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}C was incorporated in both treatments at 20 C, presumably due to the greater dilution of $^{14}\text{CO}_2$ 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 Bebee (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_2 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 $^{14}\text{CO}_2$ 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 $^{14}\text{CO}_2$ were administered. They reported that with ^{14}CO 50% of the soluble radioactivity was recovered as sucrose and 22% as serine; with $^{14}\text{CO}_2$ 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

Table II. Effect of Exogenous Non-radioactive CO₂ Upon the Conversion of ¹⁴CO to ¹⁴CO₂ and Upon the ¹⁴C Incorporation Into Soluble Acid-stable Products.

Lettuce leaf discs (2 g in 50 ml flask) were exposed to ¹⁴CO (1.3 x 10⁷ cpm, final concn. 53 µl/l) with or without the addition of 3% non-radioactive CO₂ for 3 h and 48 h at 2.5 C in the dark. Values are the average of duplicate samples.

<u>Treatment</u>	<u>Exposure time</u> h	¹⁴ CO ₂ <u>produced</u> cpm x 10 ⁻³	¹⁴ C <u>incorporation</u> cpm x 10 ⁻³
¹⁴ CO	3	249	15.5
¹⁴ CO + 3% CO ₂	3	292	1.5
¹⁴ CO	48	2,733	57.2
¹⁴ CO + 3% CO ₂	48	3,166	32.0

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 contained 70% or more of the radioactivity in the ^{14}CO and $^{14}\text{CO}_2$ 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}CO or $^{14}\text{CO}_2$ is the specie being incorporated into lettuce. If $^{14}\text{CO}_2$ derived from ^{14}CO is being incorporated, it is expected that the addition of large amounts of non-radioactive CO_2 should result in reduced ^{14}C incorporation. The results of Table II support such a view. When 3 % CO_2 was introduced with ^{14}CO (final concentration 53 $\mu\text{l/l}$), the ^{14}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}CO 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 $\mu\text{l}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$, 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

Figure 1. Lineweaver-Burke plot of the dependence of CO oxidation to CO_2 upon CO concentrations in lettuce leaf discs. The incubation temperature was 2.5 C.

Table III. CO Oxidation Activity of Various Fractions of a Lettuce Leaf Homogenate.

Fractions were isolated according to procedure given in "Materials and Methods" and portions of each fraction were incubated in 25 ml flasks for 6 h at 20 C with ^{14}CO (10^5 cpm, final concn. $0.84 \mu\text{l/l}$). $^{14}\text{CO}_2$ produced was absorbed in 6N KOH in center wells and then radioactivity was determined.

Fraction	$^{14}\text{CO}_2$ Produced	
	Total cpm	cpm/mg protein
Pellet	1112	1059
Pellet, boiled	12	11
Mitochondria	2572	3429
Mitochondria + 1 mM CN^-	70	93
Mitochondria, boiled	1	1
Supernatant	93	264
Supernatant, boiled	5	14

experiment comparing the labeling pattern of the ^{14}CO and $^{14}\text{CO}_2$ 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}CO to $^{14}\text{CO}_2$ and the incorporation of ^{14}CO 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).

Since $^{14}\text{CO}_2$ is the major product of ^{14}CO metabolism (Table I) and the oxidation of ^{14}CO to $^{14}\text{CO}_2$ 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 Fig. 1 it is estimated that the system had an apparent K_m of approximately 5.3 $\mu\text{l/l}$ and a $V_{\max}$ of 9.7 $\text{nl}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$. 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 $\mu\text{l/l}$. Under such conditions, we can estimate that CO is oxidized at a rate of about 10 $\text{nl}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ 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 $\mu\text{l/l}$ 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 discs.

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 oxygen, 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 cytochrome oxidase. Our results that the highest oxidation activity was localized in the mitochondrial fraction are consistent with such a view.

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2.4 The Effect of Carbon Monoxide on Respiration, Amino Acids, Sugars and

Ascorbic Acid Level in Shredded Lettuce Held in Controlled Atmospheres

Much interest has been recently directed towards the use of carbon monoxide for prolonging the storage life of fruits and vegetables, as well as meats (Kader et al., 1978; Woodruff, 1977; Wolfe, 1980). During transite or temporary storage of vegetables and fruits, CO has been used as a supplement in modified atmosphere to inhibit discoloration of cut surfaces in lettuce and to reduce decay development in several fruits and vegetables. Under appropriate conditions CO has little or no adverse effects upon fruits and vegetables based on visual and sensory evaluations. Absorption and metabolism of CO has been examined in algae (Chappelle, 1962) and higher plants (Krall and Tolbert, 1957; Chappelle and Krall, 1961; Bidwell and Fraser, 1972; Kortschak and Nickell, 1973; Bidwell and Bebee, 1974). Krall and Tolbert (1957) observed that ^{14}CO was mainly incorporated into serine in barley leaves in the light, but dark fixation was too small to determine labeled products. In algae, $^{14}\text{CO}_2$ was the primary product of ^{14}CO metabolism in both light and dark although nonvolatile products from dark fixation were not examined (Chappelle, 1962). Exposing bean leaves to ^{14}CO , Bidwell and Fraser (1972) concluded that in the light most of the ^{14}CO was reduced and incorporated into serine and this was subsequently converted to sucrose; in the dark, over 90% of the ^{14}CO was converted to $^{14}\text{CO}_2$ although the labeling pattern of the acid-stable products suggested that primarily ^{14}CO and not $^{14}\text{CO}_2$ was being incorporated.

Since CO is presently being used commercially as a component of modified atmosphere during transit and storage of shredded lettuce, the present study was conducted to examine the effect of CO on compositional changes in shredded lettuce and to determine to what extent CO is incorporated in lettuce leaf discs.

Experimental

Two different experimental conditions were used to study the effects of CO on crisphead lettuce: experiments conducted in flasks with controlled CO, designated "controlled CO", and experiments conducted in polyethylene bags designated "modified CO". Lettuce for the controlled CO experiments was obtained from a local market, cut into approximately 1 x 3 cm pieces and 50 g placed in 250 ml Erlenmeyer flasks. Control flasks contained air and treated flasks contained approximately 3.4% CO. Samples were held at 2.5°C and assays were run at 0, 5, 10, 15 and 20 days. All flasks were flushed with air when the CO₂ concentration reached 2.5% (in no flasks did the [CO₂] rise higher than 3.0% during the experiment) and the same level of CO was reintroduced into the treated flasks. Flushing was necessary every 2 to 3 days. Shredded lettuce packaged in polyethylene bags obtained from Red Coach was used in the modified CO experiments. Control bags contained air only and treated bags contained elevated O₂ (ca. 30%) and CO (ca. 7%). The lettuce was held at 2.5°C and samples taken at 2, 9, 16 and 23 days after the lettuce was processed and bagged.

Assay for ascorbic acid, amino acids, reducing sugars and total sugars: The procedure of Terada et al. (1978) was followed for determination of ascorbic acid. Amino acid content was determined by the method of Yemm and Cocking (1954). Reducing sugars and total sugars were determined by the procedures of Somogyi (1952) and Dubois et al. (1956), respectively.

Respiration rate: Respiration rate was determined periodically by measuring the CO₂ concentration accumulated in the flasks or bags with a gas chromatograph unit.

CO determination: Gas samples from flasks or bags were periodically taken

and their CO concentration was determined with a gas chromatograph unit.

^{14}CO generation: ^{14}CO was prepared from sodium [^{14}C]formate with concentrated H_2SO_4 .

^{14}CO measurement: ^{14}CO was measured by oxidizing it to $^{14}\text{CO}_2$ quantitatively with I_2O_5 (National Academy of Science, 1977). $^{14}\text{CO}_2$ was assayed by a liquid scintillation counter after absorption in ethanolamine.

Comparison of labeled products from ^{14}CO and $^{14}\text{CO}_2$: Two g of lettuce leaf discs (11 mm diameter), which were prepared with a cork borer, were placed into a 50 ml Erlenmeyer flask which was sealed with a serum stopper and wrapped with aluminum foil. Either ^{14}CO (ca. 2×10^7 cpm, final concentration 84 $\mu\text{l/l}$) or $^{14}\text{CO}_2$ (ca. 6×10^6 cpm, final concentration 53 $\mu\text{l/l}$) was injected into the flasks which were incubated for 48 h at 2.5°C with continuous shaking. At the end of incubations, gas samples were taken for ^{14}CO and $^{14}\text{CO}_2$ determination. Then leaf discs were ground in 80% (v/v) ethanol in 0.4 M acetic acid, and the extracts were allowed to set at room temperature for 2 days. After concentration, the extracts were fractionated into neutral, cationic and anionic fractions by ion exchange resins. The amino acids in the cationic fraction and the organic acids in the anionic fraction were further identified by paper chromatography and electrophoresis. Radioactivity was located using a radioscanner and the products were identified by co-migration with standards.

Dependence of the rate of CO oxidation to CO_2 on CO concentration: One g leaf discs with 0.5 ml water were placed in 25 ml Fernbach flasks and the flasks were wrapped with aluminum foil, stoppered and placed at 2.5°C . After temperature equilibration, various amounts of ^{14}CO were injected into the flasks. Thirty min later, a 3 ml gas sample was taken for $^{14}\text{CO}_2$ determination.

Results

Respiration rates for the controlled CO experiment are given in Figure 1. No apparent differences between the air and CO treatments were observed. The fluctuations in respiration rate were presumably linked in some way with flushing the flasks with air. Usually the respiration rate was lowest just prior to flushing and highest just after flushing. This may have resulted from suppression of the respiration rate by the build-up of CO₂ or the manipulation of the flasks during flushing may have stimulated the respiration rate.

Changes in gas composition in the bags of shredded lettuce during the MA experiment are given in Figures 2 and 3. For both the CO-treated and air samples, the oxygen level decreased to 1 to 2% after 7 to 8 days and the CO₂ level was near 10% after 6 days of storage and only slowly increased subsequently. In the CO-treated samples, the CO level dropped slowly from the initial 7% to near 2% after 22 days. Due to the difficulty of taking gas samples from these bags, there might be considerable errors in the values presented in these figures.

Ascorbic acid levels were slightly lower in CO samples compared to those in air in both controlled and modified CO experiments (Figs. 4 and 5). It should be noted that initial ascorbic acid levels were low in both experiments, which may have resulted from cutting the lettuce. Ascorbic acid levels in uncut lettuce were usually 1 to 3 times higher than what was found here (Wu, 1978). There was also a general trend of decreasing levels during storage in both treatments over the course of the experiments.

No significant differences between air and CO samples were observed for amino acids, reducing or total sugars in both the controlled and modified CO experiments (Figs. 4 and 5). The amount of reducing sugars was

Figure 1. Respiration rates of air samples (○) and CO samples (○) of shredded lettuce during the course of the controlled CO experiment.

essentially the same as the total sugars in the bagged lettuce (Fig. 5) indicating the absence of sucrose. This was verified by results from paper chromatography where only glucose and fructose was found.

As shown in Table I, when lettuce leaf discs were exposed to ^{14}CO , its primary metabolite was CO_2 (16% of total radioactivity), though a small amount of radioactivity (0.6%) was found in acid-stable products. However, since both ^{14}CO and $^{14}\text{CO}_2$ were present, the question arises whether the radioactive acid-stable products were derived from ^{14}CO , $^{14}\text{CO}_2$, or both. One approach to answer this question is to compare the pattern of labeled products between ^{14}CO -treated and $^{14}\text{CO}_2$ -treated leaf discs. Since the labeling pattern between these two is similar (Table I), this would indicate that it was $^{14}\text{CO}_2$ derived from ^{14}CO and not ^{14}CO per se which was fixed. The ratio of ^{14}CO in the soluble vs. insoluble fractions in the ^{14}CO -treated discs was comparable to that in $^{14}\text{CO}_2$ -treated leaf discs with values of 8.7 and 5.7, respectively. In the soluble fraction, the products labeled were qualitatively the same and quantitatively similar in both treatments. In both treatments, the anionic fraction contained the most radioactivity, with malate being the predominate labeled product. In another experiment (data not shown), the cationic fraction was slightly higher in radioactivity than the anionic fraction, but again the distribution of label in the neutral, anionic and cationic fractions was comparable in the ^{14}CO and $^{14}\text{CO}_2$ -treated discs and the labeled products in the soluble fraction were identical in the two treatments.

For most experiments the leaf discs were incubated at 2.5°C since this is a good commercial storage temperature for lettuce. However, a comparison was made between 2.5° and 20°C with respect to the distribution of label in the neutral, anionic and cationic fractions. No major differences between

Figure 2. Changes in concentration of CO_2 (●), O_2 (○) and CO (Δ) in the CO -treated samples of polyethylene-bagged lettuce during the course of the modified CO experiment.

Figure 3. Changes in concentration of CO_2 (●) and O_2 (○) in the air samples of polyethylene-bagged lettuce during the course of experiment.

Figure 4. (right) Changes in concentrations of total sugars, reducing sugars, amino acids and ascorbic acid in lettuce during the controlled CO₂ experiment.

Figure 5. (left) Changes in concentrations of total sugars, reducing sugars, amino acids and ascorbic acid in lettuce during the modified CO₂ experiment.

the two temperatures within each treatment or between the ^{14}CO and $^{14}\text{CO}_2$ treatments were observed (data not shown). As expected, more ^{14}CO was oxidized to $^{14}\text{CO}_2$ at the higher temperature (about 55% at 20°C compared with about 17% at 2.5°C), but less ^{14}C was incorporated in both treatments at 20°C , presumably due to the greater dilution of $^{14}\text{CO}_2$ by respiratory CO_2 .

In barley (Krall and Tolbert, 1957) and bean leaves (Bidwell and Fraser, 1972; Bidwell and Bebee, 1974), serine in addition to sucrose was heavily labeled after exposure to ^{14}CO in the light. Bidwell and Bebee (1974) 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_2 and 3-phosphoglyceric acid into sucrose (Bidwell and Bebee, 1974). This was attributed to differences between C_3 and C_4 plants. In barley (Krall and Tolbert, 1957) and bean leaves (Bidwell and Bebee, 1974), it was suggested that ^{14}CO incorporation into serine occurred via the tetrahydrofolate system.

In the dark, Bidwell and Fraser (1972) found that $^{14}\text{CO}_2$ 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 $^{14}\text{CO}_2$ were administered. They reported that with ^{14}CO , 50% of the soluble radioactivity was recovered in sucrose, but no label was recovered in sucrose with $^{14}\text{CO}_2$; only ^{14}CO labeled serine and only $^{14}\text{CO}_2$ labeled malate and phosphoglycerate. These results suggest that ^{14}CO was incorporated into serine as had been observed in the light. One main difference between our work and that of Bidwell and Fraser was the duration of exposure, and temperature; 48 h at 2.5°C in our experiments, and 20 min at room temperature in theirs. However, the labeling pattern we observed from both ^{14}CO

Table I. Distribution of ^{14}C Into Products From Leaf Discs Exposed to ^{14}CO or $^{14}\text{CO}_2$.

Two g of leaf discs were incubated with either ^{14}CO (20×10^6 cpm, final conc. $84 \mu\text{l/l}$) or $^{14}\text{CO}_2$ (6×10^6 cpm, final conc. $53 \mu\text{l/l}$) for 48 h at 2.5°C .

Labeled Products	^{14}CO -treatment	$^{14}\text{CO}_2$ -treatment
	<u>% of ^{14}C supplied</u>	
CO_2	16.7	-
Soluble fraction	0.61	6.38
Insoluble fraction	0.07	1.12
	<u>% of soluble radioactivity</u>	
Neutral fraction	3	5
Cation fraction	40	46
Aspartate	17	10
Glutamate	7	7
Asparagine	6	14
Glutamine	5	10
Serine	2	2
Alanine	1	2
Unidentified Cation	2	1
Anion fraction	57	49
Malate	40	28
Citrate	17	21

Figure 6. Dependence of the rate of CO oxidation to CO₂ on the CO concentration.

and $^{14}\text{CO}_2$ -treated leaf discs was very similar to that found by Benson and Calvin (1950) in barley leaves exposed to $^{14}\text{CO}_2$ for 50 min in the dark. They observed that malate was the primary labeled product and the other labeled amino acids found were the same as we found in lettuce leaf discs. The direct incorporation of CO without the involvement of CO_2 did not appear to be a significant pathway since the radioactivity in serine from CO was low and essentially the same as that recovered from $^{14}\text{CO}_2$ -treated leaf discs.

Since $^{14}\text{CO}_2$ was observed to be the major product of ^{14}CO metabolism (Table I) and the production of $^{14}\text{CO}_2$ from ^{14}CO appears to be an important step leading to labeled acid-stable products, we examined the dependence of CO oxidation on CO concentrations by leaf discs at 2.5°C . It was estimated that the system had an apparent K_m of approximately $5 \mu\text{l/l}$ and a V_{max} of $9.2 \text{ nl}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ (Fig. 6). In the experiment of Table I, in which the leaf discs were incubated for 48 h at 2.5°C with $84 \mu\text{l/l}$ of CO, the oxidation rate of CO was $7.3 \text{ nl}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$. This value is in agreement with the V_{max} determined in Figure 6.

The oxidation of CO to CO_2 appears to be a widespread phenomenon of living organisms including bacteria (Hegeman, 1980; Meyer and Schlegel, 1979), animals (Fenn, 1970), algae (Chappelle, 1962) and higher plants (Bidwell and Bebee, 1974; Bidwell and Fraser, 1972). In algae, Chappelle (1962) observed that this reaction required molecular oxygen, was effectively inhibited by cyanide, azide and hydroxylamine, and low light intensity increased the oxidation rate several fold over that of the dark rate.

Conclusions

1. No differences in the respiration rates were observed between air controls and CO-treated lettuce.

2. No differences in the amino acid, reducing sugar or total sugar levels between air controls and CO-treated lettuce were observed. The ascorbic acid level of CO-treated lettuce appears to be slightly lower than that of air control.
3. The major metabolism of CO in lettuce tissue in the dark was oxidation to CO₂ which was, in turn, incorporated into other acid-stable products, among which malate was the predominate product. This conclusion was supported by the observations that the incorporated products of CO and CO₂ were identical.
4. The oxidation of CO to CO₂ in lettuce tissue was dependent on the concentration of CO. The concentration of CO which gave half maximal activity was estimated to be 5 μ l/l. At higher CO concentrations, it was estimated that lettuce oxidized CO to CO₂ at a rate of 216 μ l/kg/day at 2.5°C and 700 μ l/kg/day at 20°C.

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3. Studies on endogenous and exogenous factors affecting the biosynthesis of the ripening hormone, ethylene, with special emphasis on delaying tissue senescence.

3.1 Effect of Water Loss on Ethylene Production in Leafy Tissue

Subjecting plants or detached leaves to water deficit stress results in a surge in ethylene production (4,8,16,18). In avocado fruits it has been shown (3) that an accelerated loss of fresh weight due to water deficit hastened the peak of ethylene production and thus resulted in earlier fruit ripening. Increased ethylene production by tissues which normally produce very little ethylene has been reported to occur as a response to the stresses represented by drought, mechanical wounding, and radiation (4,10,11,13,16,19, 24). As ethylene is a plant hormone known to be involved in many processes, including growth, development, ripening, and senescence (1); this stress-induced ethylene may assist the plant to cope with stress conditions. It has been suggested that under drought conditions ethylene production would cause leaf abscission, and thereby reduce water loss (16).

Adams and Yang (2) have studied ethylene biosynthesis in apple tissue and established the following biosynthetic pathway: methionine $\rightarrow$ SAM³ $\rightarrow$ ACC $\rightarrow$ ethylene. The validity of this pathway has since been confirmed in other systems (20), including auxin-induced ethylene production (23) and stress-induced ethylene production (6,24). The present study was conducted to determine which step in the ethylene biosynthetic pathway is responsible for the surge of ethylene production caused by water deficit stress.

Experimental

Plant material. Wheat (Triticum aestivum L. cv. Anza) seeds were soaked for 3 h in distilled H₂O and sown in vermiculite. Seedlings were grown in a growth chamber under a 14-h photoperiod with a light intensity of 80 $\mu\text{E m}^{-2} \text{s}^{-1}$ provided by a mixture of cool-white and Gro-lux tubes at 22°C and a RH of 70%. Four h after the beginning of the ninth light period, primary leaves

(10-12 cm long) were excised above the coleoptile and trimmed to 8 cm.

Water Stress. The technique used was described by Wright (18). Batches of excised leaves (1 g) were allowed to wilt while spread in Petri dishes at room temperature (20-22°C) and 55% RH. When the leaves reached the desired degree of water stress, as indicated by loss of fresh weight (it took about 30 min to reach 9% weight loss and 10-15 min to reach 2% weight loss), they were inserted into 12-ml glass test tubes (15 x 90 mm) which were then closed with serum caps for the desired period. Nonstressed leaves used as controls were inserted in test tubes containing several drops of water to maintain high RH. The sealed tubes were kept in the growth chamber at 22°C under light. Gas samples (1 ml) were taken periodically from each tube for ethylene analysis. The tubes were flushed with ethylene-free air every 2 h. After the final sample was taken, the leaves were reweighed to determine loss of fresh weight during the sampling period. The total water loss, expressed as percent of initial fresh weight, was used as an expression of the degree of water stress.

Feeding Technique. Leaves were placed in small beakers with the cut surfaces dipped in aqueous solutions of the precursor or inhibitor at the concentrations indicated. The beakers were placed for 3 to 4 h in a growth chamber under light as described above and the leaves were then subjected to water stress as described.

Chemicals. SAM was obtained from Boehringer, and AVG was a gift from J.P. Scannel (Hoffman-La Roche). ACC was purchased from Calbiochem, CHI from Sigma, and SP-Sephadex from Pharmacia Fine Chemicals.

Determination of Ethylene. Gas samples (1 ml) withdrawn from the tubes to be tested were injected into a gas chromatograph equipped with an alumina column and flame ionization detector.

Determination of ACC. The tissue was homogenized in 70% ethanol. After centrifugation, the ethanol was evaporated under vacuum at 40°C. The residue was dissolved in 1 ml water, and an aliquot was used for assay of ACC according to the method of Lizada and Yang (12). The sample to be assayed was placed in a 10-ml test tube in an ice bath, 1 μ mol HgCl_2 was added, and the volume was brought to 0.4 ml with water. The tube was then sealed with a rubber serum cap. Approximately 100 μ l cold mixture of 5% NaOCl and saturated NaOH (2:1, v/v) was injected into the tube. The reaction mixture was kept in ice for 5 to 10 min, the tubes were shaken vigorously, and the ethylene liberated was assayed by GC. The efficiency of the conversion of ACC to ethylene by this method in the presence of the ethanol extract of the tissue was determined by adding a known amount of authentic ACC as an internal standard. The yield was usually between 66 and 70%. The amount of ACC was calculated from the quantity of ethylene liberated and the conversion efficiency.

Determination of SAM. SAM was isolated according to the method of Glazer and Peale (9). Batches of wheat leaves (3 g) were homogenized and extracted with 15 ml cold 10% TCA (w/v). After centrifugation at 10,000g for 10 min at 4°C, a known amount of [^{14}C]SAM was added as an internal standard used to monitor recovery during purification. The supernatant was extracted three times with water-saturated ether to remove the TCA. One ml 0.15 M HCl was added to the sample and it was passed through a column (1 x 5 cm) of SP-Sephadex C-25 equilibrated with 10 mM HCl . The column was washed with 0.15 M HCl until the effluent showed no absorption at 257 nm. SAM was then eluted with 15 ml 0.5 M HCl and the eluate was collected in 2-ml fractions which were used for determination of total SAM. Profiles of radioactivity in the various fractions were found to correspond to adsorptivity at 257 nm.

To verify the presence of SAM, the adsorption spectrum of the eluate was compared with that of authentic SAM. The concentration of SAM was determined spectrophotometrically, assuming a molar adsorptivity of 15,000 at 257 nm. Recovery, based on the radioactivity of SAM, ranged from 65 to 72%. All experiments were repeated three times with two or three replicates for each treatment. The data presented are results obtained from a representative experiment.

Results and Discussion

Wheat leaves which were excised 4 h after the beginning of the ninth light period and kept in a humid atmosphere produced ethylene at a low rate ranging from 0.3 to 0.6 $\text{nl}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ for 24 h after excision (Fig. 1). If these leaves were subjected to a water deficit stress, increased ethylene production was observed. Stress ethylene production was small but apparent when water loss reached 2% of the initial fresh weight. This production increased progressively to a maximum rate when the water loss reached 9%, but then declined as the water loss continued (Fig. 2). The maximum rate of ethylene production for leaves which had lost 9% of their initial fresh weight was about 37 $\text{nl}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ 4 h after the end of the stress period (Fig. 1). Thereafter, ethylene production declined, falling to 8 $\text{nl}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ after 12 h and to the control level after 24 h (Fig. 1). Similar results have been reported by Wright (18) with wheat leaves and by others with various tissues (4,8,11,16).

The ACC content of nonstressed wheat leaves was very low ranging between 0.4 and 0.6 $\text{nmol}\cdot\text{g}^{-1}$ tissue during 24 h after the leaves were harvested (Fig. 1). In leaves stressed by water deficit, the ACC content first increased and subsequently declined as the stress was increased. These stress-

FIG. 1. Time course for the effect of water stress on ethylene production and ACC content in wheat leaves. Leaves were subjected to water stress conditions until they lost 9% their initial fresh weight (about 30 min at 22°C and 55% RH) and enclosed in sealed, 12-ml test tubes (1 g each). Nonstressed control leaves were enclosed in test tubes containing several drops of water to maintain high RH. Ethylene production and ACC content were determined periodically. The tubes were flushed with ethylene-free air every 2 h to prevent O₂ depletion and CO₂ accumulation. Vertical bars indicate $\pm$ SE limits. The values of ACC and ethylene were expressed per g of initial fresh weight.

FIG. 2. Effect of the severity of water stress on ethylene production and ACC content in wheat leaves. Leaves were subjected to water stress conditions at 22°C and 55% RH for various lengths of time until they reached the indicated weight loss. Ethylene production and ACC content were determined 4 h after the end of the water stress treatment.

-induced changes in ACC content paralleled the changes in the rate of ethylene production (Fig. 1). The sharp rise in both ethylene production and ACC content became apparent when weight loss reached about 2% initial fresh weight, and continued to a peak value at a loss of about 9% initial weight (Fig. 2). The sharp reversal in the changes in ACC content and rate of ethylene production as the loss of weight exceeded 9 or 10% initial weight may reflect damage to the tissue.

When ACC was administered to nonstressed leaves, ethylene production was increased 70-fold (Table I). In this, as in other tissues (7,20), ACC is probably the rate-limiting factor for ethylene production. The induction of ethylene production by water deficit stress, as by auxin treatment (22, 23) or by stress conditions such as waterlogging (6) and wounding (24), seems to cause an increase in ACC synthesis which ultimately results in increased ethylene production. This view was further supported by the analysis of the content of SAM and ACC. While water stress stimulated ethylene production and caused accumulation of ACC, it did not affect the level of SAM (Table II). These data are consistent with the view that the step converting SAM to ACC is rate-limiting and is accelerated by stress, whereas the step converting methionine to SAM is not rate-limiting, thereby the level of SAM can be maintained even when the conversion of SAM to ACC is accelerated. When AVG, a known inhibitor of ACC synthase, which catalyzes the conversion of SAM to ACC (2,5), was applied to wheat leaves prior to water stress treatment, it inhibited both ACC accumulation and the surge of ethylene production by 90% (Table I). Since the accumulation of ACC in the tissue seems to be the direct cause for increased ethylene production, it is reasonable to assume that the decline in ethylene production after 4 h (Fig. 1) was due to the depletion of ACC below a critical level. However,

Table I. Effect of CHI, AVG and ACC on Ethylene Production and ACC content in Water-Stressed Wheat Leaves

Leaves were preincubated for 4 h with the cut ends dipped in H₂O or in solutions containing the precursor or inhibitor at the indicated concentration. At the end of the preincubation period, leaves were subjected to water stress conditions until they lost 9% their initial fresh weight (about 30 min at 22°C and 55% RH). Ethylene production and ACC content were determined 4 h after the end of the water stress treatment. Results presented are the mean $\pm$ SE of three replications.

Treatment	Ethylene $\text{nl}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$	ACC $\text{nmol}\cdot\text{g}^{-1}$
Nonstressed leaves		
H ₂ O	0.5 $\pm$ 0.08	0.4 $\pm$ 0.03
ACC, 0.5 mM	22.2 $\pm$ 1.7	126 $\pm$ 6
AVG, 0.05 mM	0.3 $\pm$ 0.04	0.5 $\pm$ 0.02
ACC + AVG	21.7 $\pm$ 1.9	106.0 $\pm$ 10
Water-stressed leaves		
H ₂ O	35.2 $\pm$ 2.3	12.8 $\pm$ 2.2
ACC, 0.5 mM	61.1 $\pm$ 5.0	126 $\pm$ 9
AVG, 0.05 mM	3.5 $\pm$ 0.2	1.2 $\pm$ 0.2
ACC + AVG	58.4 $\pm$ 4.7	115 $\pm$ 10
CHI, 0.5 mM	0.8	0.4

Table II. Effect of Water Loss on Ethylene Production, ACC Content, and SAM Content in Wheat Leaf

Leaves were subjected to stress conditions until 9% initial fresh weight was lost. Ethylene production, ACC content, and SAM content were determined 4 h after the water stress treatment. Results presented are the mean $\pm$ SE of three replications.

Treatment	Ethylene $\text{nl}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$	ACC $\text{nmol}\cdot\text{g}^{-1}$	SAM
Non-water-stressed	0.6 $\pm$ 0.08	0.5 $\pm$ 0.03	38
Water-stressed	32.0 $\pm$ 2.5	17.0 $\pm$ 2.2	39

the depletion of ACC could not be attributed entirely to its conversion to ethylene. ACC content of the tissue increased to $14 \text{ nmol} \cdot \text{g}^{-1}$ 4 h after the end of the stress treatment and rapidly decreased thereafter, declining to $1 \text{ nmol} \cdot \text{g}^{-1}$ at the end of a 24-h incubation period. During the course of the accumulation and decline of ACC, however, less than 9 nmol ethylene were produced. These data suggest that ACC might be metabolized by some pathway other than ethylene production. The stimulatory influence of water deficit stress was not restricted to ACC synthesis. In addition to increased ACC synthesis, the capacity of the system to convert exogenous ACC to ethylene was approximately 3 times greater in water deficit-stressed leaves than in controls (Table I).

The formation of ACC from SAM is known to be catalyzed by ACC synthase (5,21), and any increase in ACC must involve either activation or de novo synthesis of this enzyme. Our attempts to isolate and study the activity of ACC synthase from wheat leaves were not successful. If de novo synthesis of proteins is involved in water stress-induced ethylene production, it is expected that application of CHI, a known inhibitor of protein synthesis, would inhibit the stress-induced ACC accumulation and ethylene production. In tissue incubated with 0.5 mM CHI for 3 h prior to stressing treatment, both ACC synthesis and ethylene production were effectively blocked (Table I). The present results suggest that during the course of ethylene synthesis induced by water stress de novo synthesis of an enzyme, probably ACC synthase, is induced and is in turn responsible for the accumulation of ACC and the increase in ethylene production. The evidence indicates that the pathway of the biosynthesis of water stress-induced ethylene and its induction, are similar, if not identical, to those of stress ethylene induced by auxin (23) or mechanical wounding (24). The biochemical mechanism by which

these different stimuli result in stimulation of the same biochemical step remains to be elucidated.

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3.2 Polyamines Inhibit Biosynthesis of Ethylene in Leafy Tissue and Apple Fruit Protoplasts

Attention has been focused on the role of polyamines in plant metabolism following studies implicating them as essential growth factors (16). Many reports have since appeared showing that polyamines can delay senescence of plant tissue. For example, exogenous application of polyamines retards the aging of leaf protoplasts (5,6) and preserves Chl in thylakoid membranes (15) during dark-induced senescence of detached leaves. Also, polyamines inhibit development of RNase and protease activity in barley leaf discs (15). The mechanism by which polyamines exert these effects is unknown. These effects, however, are the opposite of those caused by the plant growth regulator, ethylene, which promotes senescence of many plant tissues (8).

We investigated the effect of polyamines on ethylene biosynthesis, partly because polyamines and ethylene derive from the same precursor, S-adenosyl-methionine (1,2,16), and because of the possibility that polyamines regulate plant metabolism by influencing the biosynthesis of ethylene.

Experimental

Chemicals. ACC was purchased from Calbiochem; putrescine-HCl, spermidine-HCl came from Sigma; Biofluor liquid scintillation fluid came from New England Nuclear; and (^{14}C -3,4)L-methionine came from Research Products International Corp.

Plant Material. Apple fruits (Malus sp. cvs. Golden Delicious and Anna) were harvested at the preclimacteric stage and stored at 0°C until used or ripened at 20°C. Discs (1.0 cm in diameter and 2 mm thick) were cut from fruits at various stages of ripening. Six discs (1 g) were incubated in a

25-ml Erlenmeyer flask containing 3 ml incubation medium consisting of 600 mM sorbitol in 10 mM phosphate (pH 7). In some cases, Tris-HCl (pH 7) was used.

Protoplast Isolation. Protoplasts were prepared from Golden Delicious fruit tissue. A 3-g sample of apple discs was incubated overnight in 9 ml 800 mM sorbitol containing 100 mM CaCl_2 , 10 mM Mes (pH 6.0), 10 $\mu\text{g/ml}$ chloramphenicol, 5 $\mu\text{g/ml}$ Fungizone, and 5% (w/v) Cellulysin (3). The macerate was filtered through glass wool; protoplasts were allowed to settle for 20 min and then transferred with a Pasteur pipette to fresh medium without Cellulysin. Aliquots (1-ml) of protoplasts were placed in 25-ml flasks and brought to a final volume of 2.1 ml with the appropriate solutions, according to the treatments described in Table I.

Pinto beans (Phaseolus vulgaris L.) and tobacco (Nicotiana tabacum L.) plants were grown in a greenhouse under natural lighting at temperatures ranging between 25 and 30°C. Fully expanded leaves were harvested and washed in running tap water. Leaf discs (1 cm diameter) were cut with a corkborer and floated abaxially down under light in Petri dishes containing 0.1 mM IAA or 1 mM ACC in water. This procedure is referred to as preincubation. At the end of the preincubation, the discs were transferred to 25-ml Erlenmeyer flasks containing IAA or ACC and various polyamines to be tested as described for each experiment.

Ethylene Determination. All of the Erlenmeyer flasks were flushed with air between successive determinations, and gas samples were withdrawn with airtight hypodermic syringes. Ethylene was determined by GC.

(^{14}C)Methionine Feeding Experiment. Apple discs (1 g) were incubated for 5 h in 125-ml flasks with 5 ml of a solution containing 5 μCi (^{14}C -3,4)methionine (40 mCi/mmol), 600 mM sorbitol, and 10 mM phosphate buffer (pH 7.0). A

gas sample was withdrawn from each flask for ethylene determination. A second 3-ml gas sample was then withdrawn and injected into a scintillation vial (sealed with a rubber serum cap) containing 3 ml 0.1 M mercuric acetate at 0°C in methanol. Next, 10 ml Biofluor scintillation fluid was added to the vial, and radioactivity was assayed by scintillation spectrometry.

All experiments were repeated at least three times, with three replicates for each treatment. The data presented are the means of three replicates of a representative experiment.

Results

Effect of Applied Diamine and Polyamines on Ethylene Production. The diamine and polyamines incorporated into the incubation medium markedly inhibited ethylene production in apple discs (Fig. 1) but did not affect respiration (data not shown). At least 4 h of incubation were needed for the polyamines to significantly inhibit ethylene production in the discs. The diamine, putrescine, was the least potent inhibitor (Figs. 1 and 2); at 0.1 mM, it had no effect, and, at 1 mM, it increased ethylene production slightly during the first 6 h before inhibiting production sharply after 24 h. In contrast, 1 or 10 mM of the polyamines spermidine and spermine inhibited ethylene production by 40 to 68% after 6 h and by 90 to 100% after 24 h.

The extent to which the polyamines inhibited ethylene production depended largely on the ripeness of the fruit (Fig. 3). After 6 h of incubation, 10 mM spermine inhibited ethylene production in slices from early climacteric fruit by 85%, in those from midclimacteric fruit by 65%, and in those from climacteric-peak fruit by only 50% (Fig. 3). Addition of 10 mM Ca^{2+} to the medium did not significantly change the rate of ethylene production in the

Table 1. Effect of Spermine and Spermidine on Ethylene Production by Apple Protoplasts

Protoplasts were isolated from midclimacteric fruit and handled and treated as described in "Materials and Methods."

	Ethylene Production					
	0 to 2 h		2 to 4 h		4 to 20 h	
	nl/g	%	nl/g	%	nl/g	%
Control	30	100	23	100	81	100
Spermidine, 1 mM	26	86	22	98	68	83
Spermine, 1 mM	25	75	18	78	39	48

FIG. 1. Effect of diamine and polyamines on ethylene production by apple tissue. Discs from Golden Delicious apple fruits in midclimacteric were incubated in 25-ml flasks (6 discs weighing 1 g/flask) containing 600 mM sorbitol in 10 mM phosphate buffer (pH 7.0), 10 μ g/ml D-chloramphenicol, and the amines (putrescine, spermidine, and spermine) at 1 mM. Rate of ethylene produced in control samples at 0 to 2, 2 to 4, 4 to 6, and 6 to 24 h were 45, 52, 55, and 37 nl/g·h, respectively.

FIG. 2. Relation between concentration of putrescine, spermidine, and spermine and percentage inhibition of ethylene production in midclimacteric apple tissue. Methods and procedure were the same as described in legend to Figure 1. Rates of ethylene production were recorded after 6 h of incubation, when rate in control sample was 65 nl/g·h.

FIG. 3. Effect of spermine on ethylene production by apple fruits of different ripeness. Discs from preclimacteric, midclimacteric, or climacteric apple fruits (cv. Anna) were incubated in 25-ml flasks containing 600 mM sorbitol in 10 mM Tris-HCl (pH 7.0) in the absence and presence of 10 mM spermine. For those respective stages, ethylene production rates were 22, 155, and 242 μ l/kg·h in whole apples and 15, 76, and 92 nl/g·h in control discs, as measured at 6 h.

control during 6 h of incubation (data not shown). However, the presence of Ca^{2+} , greatly relieved the inhibitory effects of 0.1 and 10 mM spermine (Fig. 4) and spermidine (data not shown). In the presence of Ca^{2+} , putrescine at 10 mM was totally ineffective as an inhibitor (data not shown).

Spermine and spermidine also inhibited ethylene production in apple cortex protoplasts (Table I). Between 4 and 20 h of incubation, spermine, the larger polyamine, was more effective in inhibiting ethylene production than was spermidine (52% versus 17%). The moderate inhibitory effect of polyamines on ethylene production by the protoplasts as compared to tissue slices was probably due to high Ca^{2+} (100 mM) content in the incubation medium; such high concentrations of Ca^{2+} are required for survival of apple protoplasts (3).

The conversion of (^{14}C -3,4)L-methionine to $^{14}\text{C}_2\text{H}_4$ in apple tissue was inhibited by 1 mM spermine or spermidine (Fig. 5). Inhibition was noticed within 2 h of incubation, and it increased gradually to about 60% during the next 4 h.

The diamine and polyamines also inhibited IAA-induced ethylene production in pinto bean leaf discs (Fig. 6) much the same as they did in apple tissue, without affecting CO_2 evolution. At 5 mM, both spermidine and spermine reduced ethylene production noticeably (about 20%) within 2 h of incubation and maximally after 6 h (about 80%). Putrescine was much less inhibitory, as previously noted. In most cases, prolonging the incubation to 24 h only slightly increased the inhibitory effect of the polyamines and partially reversed that of putrescine.

The conversion of ACC to ethylene in tobacco leaf discs was stimulated between 10 and 30% by putrescine, spermidine, and spermine, when applied at 0.1 mM (data not shown). However, increasing the concentration of spermine

FIG. 4. Effect of Ca^{2+} on the inhibition of ethylene production in apple tissue by spermine. Spermine concentrations were 0.1 mM, (○); 1 mM, (○); or 10 mM, (Δ); with (---) or without (—) 10 mM CaCl_2 . Rate of ethylene produced in control samples between 0 to 2, 2 to 4, 4 to 6, and 6 to 24 h was 50, 55, 64, and 42 nl/g·h, respectively.

FIG. 5. Effect of spermidine and spermine on the conversion of [^{14}C -3,4]methionine to labeled ethylene in apple tissue. Golden Delicious apple discs were incubated in 25-ml flasks (6 discs weighing 1 g/flask) containing 600 mM sorbitol in 10 mM phosphate buffer (pH 7), 5 μCi [^{14}C -3,4]methionine (49 mCi/mmol), and 1 mM spermidine or spermine.

FIG. 6. Effect of a diamine and polyamines on auxin-induced ethylene production. Pinto bean leaf discs were preincubated for 1 h in light with 0.1 mM IAA, and then the indicated amines were added at concentrations of 1 mM (---) and 5 mM (—). Further incubation was carried out in the dark. Gas samples were withdrawn periodically, and ethylene content was determined by GC. Control values minus IAA were negligible (1 nl/g·h); with IAA, control values were 40, 34, and 20 nl/g·h at 2 to 4, 4 to 6, and 6 to 24 h, respectively.

FIG. 7. Effect of a diamine and polyamines on ACC-induced ethylene production in tobacco leaves. Tobacco leaf discs (6/flask) were preincubated in light in 25-ml flasks for 1 h with 1 mM ACC, and then the indicated amines were introduced to the media at concentrations of 1 (---) or 10 mM (—). The flasks were sealed with serum caps and incubated in the dark, and gas samples were withdrawn periodically. Rate of ethylene produced in control samples with 1 mM ACC was 57, 51, 59, and 177 nl/g·h at 0 to 2, 2 to 4, 4 to 6, and 6 to 24 h, respectively.

or spermidine to 1 mM resulted in a 20 to 30% inhibition in 4 h and 50 to 60% inhibition in 24 h (Fig. 7). At 10 mM, both of these polyamines severely inhibited ethylene production by about 90% in 24 h (Fig. 7). Putrescine was inhibitory only at 10 mM.

Discussion

This study demonstrates that polyamines inhibit ethylene biosynthesis in apple fruit slices and protoplasts, and IAA-induced ethylene production in pinto bean leaf discs. One of the reasons for this inhibition could be charge neutralization of the plant membrane, since high concentrations of Ca^{2+} prevented or partly reversed the polyamine effect. However, calcium by itself had no effect on ethylene production in apple tissue during a 6-h incubation period, by which time polyamine inhibition was already evident.

The polyamine effect may be directed at the step for converting ACC to ethylene (1). This step appears to be associated with a particulate fraction of the cell (7,11). The fact that ethylene production in situ (particularly the ACC-to-ethylene step) is susceptible to osmotic shock (4,12,14), cold shock (4,14), lipophilic membrane perturbants (4, 9-11,14), and ionic and nonionic detergents (1,4,12,14) indicates involvement of a membrane function in ethylene biosynthesis. Therefore, polyamines may interact with the plant membranes in a way that inhibits ethylene evolution by a physical mechanism. Polyamines may bind ionically to membrane targets, causing conformational changes that impair the functionality of the ethylene-synthesizing system. This hypothesis appears to be incompatible with a report (6) that polyamines stabilize the integrity of membranes and, thus, may enable them to retain their functions, including ethylene production. However, this contradiction may not be real if a specific membrane microenvironment is required for

ethylene biosynthesis, as suggested earlier (4,9-12,14), and if polyamines alter that microenvironment.

On the other hand, although ethylene biosynthesis seems to require membrane stability and integrity, ripening (climacteric) fruits, whose membranes are presumably undergoing dynamic changes (13,17), sharply increase their ethylene production. Moreover, the inhibiting effect of polyamines on ethylene production is more pronounced at the early stages of ripening (Fig. 3), i.e. before ethylene production has entered its presumed autocatalytic stage. Therefore, changes in, or degradation of, certain membrane components or compartments specific for polyamines may lead to less effectiveness of polyamines during progressive senescence as inhibitors of ethylene production.

The reduced ethylene production by polyamine-treated tissues seen in our study may have been (a) due to a general nonspecific effect of polyamines on cellular membranes; (b) directly responsible for retarding senescence; or (c) a consequence of retarded senescence.

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3.3 Inhibition by Polyamines of Macromolecular Synthesis in Apple Fruits and Its Implication for Ethylene Production and Senescence Processes

Naturally occurring diamines and polyamines have been shown to play an important role in the regulation of cell division and growth in procaryotic organisms and higher animals (7,8).

Smith and his colleagues established the important role polyamines play in higher plants in maintaining the ion balance in cases of potassium deficiency (2). A more recent report pointed out the correlation among increased putrescine content, and the high rate of cell division in plants (11).

In a series of papers, Galston, Altman, and co-workers (2,3,10,12,13) have shown that exogenously applied polyamines and some of their precursors stabilize protoplasts against lysis, inhibit the dark-induced rise in RNase and protease activity, and reduce the rate of Chl loss in leaves and protoplasts. Supportive evidence for the protective role of polyamines at the membrane level was presented by Popovic et al. (19), who showed that spermine prevented the loss of Chl from thylakoid membranes during dark-induced senescence of detached leaves.

Altman and Bachrach (2) showed that applied polyamines also inhibited wound-induced RNase activity in potato tubers and focused attention on the decrease in level of endogenous polyamines with the aging of dwarf bean and radish leaves.

All these effects provoked by polyamines oppose those produced by ethylene, a hormone known to be associated with plant senescence. Furthermore, Apelbaum et al. (4) reported that applied polyamines at concentrations similar to those that retard senescence (2,13), inhibited ethylene production in several plant tissues.

The pathway for ethylene biosynthesis has recently been described in

more detail. Lieberman et al. (15) showed that methionine is the precursor for ethylene and additional information on the intermediates was presented by Adams and Yang (1). These discoveries established the following pathway for ethylene production:

Yu et al. (23) and Apelbaum et al. (5) showed that the ACC level in the tissue is the rate-limiting factor in the pathway of ethylene production in mung bean stems and apple fruit. Apelbaum et al. (5,6) also showed that the integrity of membrane structure is required for the continuous conversion of ACC to ethylene. Thus, it appears that the two crucial reactions in this pathway were the formation of ACC from SAM and the degradation of ACC to ethylene.

The present work was undertaken in an attempt to understand the nature of the inhibition of ethylene biosynthesis by polyamines and to determine at which step in ethylene biosynthesis it occurs.

Experimental

Chemicals. 1,3-Diaminopropane dihydrochloride (mol wt, 147.2), putrescine dihydrochloride (mol wt, 161.1), cadaverine dihydrochloride (mol wt, 175.1), spermidine trihydrochloride (mol wt, 254.6), spermine tetrahydrochloride (mol wt, 348.2), and ACC were purchased from Sigma.

Uniformly labeled L-(¹⁴C)leucine was purchased from Schwartz/Mann, Orangeburg; (5-³H)uridine from the Radiochemical Centre, Amersham, U.K.; and uniformly labeled (¹⁴C)uridine from New England Nuclear. Instagel II liquid scintillation fluid was purchased from Packard Instrument Inc.

Plant Material. Apple (*Malus domestica* Borkh., cv. Golden Delicious) fruits were harvested at the preclimacteric stage and stored at 0°C until used.

Fifteen to 18 h before the beginning of the experiment, apples were transferred to 22°C. The fruit was enclosed in glass jars for 1 h, and ethylene production by the whole fruit was determined as described below. Discs, 9 mm in diameter and 2 mm thick, were cut and incubated for various periods of time at 25°C.

Incubation Conditions. Six discs (approximately 1.7 g) were incubated in a 25-ml Erlenmeyer flask containing 2 ml of 0.6 M sorbitol in 10 mM Na-phosphate buffer (pH 7.0) with or without the indicated concentration of the diamines or polyamines. Chloramphenicol was added to give a final concentration of 10 µg/ml. L-(¹⁴C)leucine (0.5 µCi/ml), (¹⁴C)uridine (0.5 µCi/ml), or (5-³H)uridine (4 µCi/ml) was added 2 h before the end of the incubation.

Ethylene Determination. Flasks were flushed with air before being closed with a rubber cap. A 1-ml sample of the gas contained in the flasks was analyzed by gas chromatography after a 2-h accumulation period.

Rate of Macromolecular Synthesis. A 2-h pulse of radioactive precursor was used throughout, at the end of which the flasks were rapidly cooled to 0°C, and the radioactive incubation medium removed. The apple discs were washed three times with cold (0°C) incubation medium, transferred to a glass homogenizer, and homogenized at 0°C in 2.5 ml of distilled H₂O. The homogenate was centrifuged for 5 min at 1500 g, the pellet was discarded, and the supernatant was used for further analysis.

Aliquots (0.1 ml) of the supernatant were spotted on Whatman No. 3 paper strips, dried, and counted with Instagel II fluid in a liquid scintillation spectrometer to determine the total uptake of precursor. In parallel, aliquots, 0.5 ml, were brought to 10% v/v with TCA and allowed to precipitate for 20 min at 2°C. The precipitates were collected on Whatman GF/C filters

by vacuum filtration, washed twice with TCA 10%, twice with TCA 5%, and once with absolute ethanol. Filters were dried under an IR lamp and counted with Instagel II liquid scintillation fluid.

ACC Level Determination. ACC was determined in the same supernatant used for the incorporation studies by the chemical degradation method of Lizada and Yang (16). The efficiency of ACC conversion to ethylene was 70 to 80% under our conditions.

Results and Discussion

Diamines and polyamines inhibited ethylene production and incorporation of radioactively labeled leucine or uridine into TCA-insoluble material in apple fruit discs, while having little or no effect on the ACC content of the tissue (Table I).

The inhibitory effect was in general more pronounced with higher mol wt amines. After 6 h of incubation in the presence of 5 mM 1,3-diaminopropane, ethylene production was inhibited by 40% and the incorporation of labeled leucine by 56%. However, the polyamine spermine, under the same conditions, inhibited ethylene production by 70% and incorporation of leucine and uridine by 83% and 56%, respectively. Spermidine and spermine displayed similar inhibitory properties with respect to the parameters studied (Table I).

The diamines had no effect on the ACC content of the tissue, while the polyamines spermidine and spermine slightly reduced the ACC level in the tissue.

The inhibitory effect of spermine (Fig. 1) on ethylene production and similarly on labeled uridine incorporation progressed rapidly until 6 h and at much lower rate until 24 h. However, the inhibition of ethylene production and leucine incorporation was already noted after 2 h of incubation -

Table I Effect of Diamines and Polyamines on Ethylene Production, ACC Content, and Macromolecular Synthesis

Treatment	C_2H_4	ACC	Incorporation into TCA-Insoluble Material	
			(^{14}C)Leucine	(5- 3H)Uridine
			%C	
Control	100 (a) ^a	100 (a)	100 (a)	100 (a)
1-3-Diaminopropane	60 (b)	90 (a)	44 (b)	97 (a)
Putrescine	54 (b)	109 (a)	63 (c)	56 (b)
Cadaverine	56 (b)		34 (b)	72 (c)
Spermidine	28 (c)	84 (b)	19 (d)	45 (d)
Spermine	31 (c)	87 (b)	17 (d)	42 (d)

^aValues followed by different letters are significantly different (5% level) as determined by Duncan's multiple range test.

Apple discs were incubated for 6 h in the presence of 5 mM concentration of the indicated amines as described in "Materials and Methods." The rate of ethylene production in controls was 5.09 nmol/g·h and the level of ACC was 4.10 nmol/g L-(^{14}C)leucine and (5- 3H)uridine incorporation in controls were 4,010 and 1,794 cpm, respectively, which represent 22.4 and 7.1% of the total uptake, respectively.

40% and 85% inhibition, respectively - whereas the effect on uridine incorporation was apparent only after 4 h, when 30% inhibition was observed. The effect of spermine on leucine incorporation was more pronounced than that on uridine incorporation, after 6 h of incubation; leucine incorporation was inhibited by 90% and uridine by 64%.

When apple discs were incubated with different concentrations of spermine (Fig. 2), ethylene production and leucine incorporation were progressively inhibited with the increase in concentrations of the polyamine. These inhibitory effects were observed within a relatively narrow range of concentrations. Spermine at 0.1 mM inhibited ethylene production by 14% and leucine incorporation by 55%, while lower concentrations of the polyamines were ineffective. An almost maximal effect was obtained with 1 mM spermine, where

FIG. 1. Time course of inhibition of ethylene production and macromolecular synthesis by 5 mM spermine. The effect of spermine (5 mM) treatment on ethylene production ($\circ$ — $\circ$), L- $[^{14}C]$ leucine ($\circ$ — $\circ$) and $[^{14}C]$ uridine ($\circ$ — $\circ$) incorporation into TCA-insoluble materials in apple discs were determined as described in "Materials and Methods." Vertical bars indicate $\pm$ SE limits.

FIG. 2. Effects of different concentrations of spermine on C_2H_4 production and L- $[^{14}C]$ leucine incorporation. Apple discs were incubated for 6 h in the presence or absence of the indicated concentrations of spermine. Ethylene production ($\circ$ — $\circ$) and L- $[^{14}C]$ leucine incorporation ($\circ$ — $\circ$) were determined as described in "Materials and Methods." Vertical bars indicate $\pm$ SE limits.

ethylene production was inhibited by 60% and leucine incorporation by 90%.

In all cases, spermidine displayed effects similar to those of spermine (data not shown).

It has been well established that the biosynthesis of ethylene is inhibited by protein synthesis inhibitors (14). More recently, both the formation of ACC and the conversion of ACC to ethylene have been shown to require continuous de novo protein synthesis (23,24).

Our results show that polyamines inhibit macromolecular synthesis when applied exogenously at concentrations between 0.1 and 10 mM, and it is conceivable that this inhibition of macromolecule synthesis may be responsible, at least in part, for the observed inhibition of ethylene production reported by Apelbaum et al. (4) and Suttle (21). It has been shown that Ca^{2+} ions are capable of relieving in part the inhibition produced by polyamines on ethylene production (4). These results, taken together with those in the present communication, suggest that the polyamines affect ethylene biosynthesis by interfering with at least two processes, protein synthesis and membrane integrity.

The conversion of ACC to ethylene has been shown to be inhibited rapidly by 10 mM spermidine or spermine in tobacco leaves (4) and in soybean hypocotyls (21). The fact that the ACC level was not observed to increase in apple discs, even though ethylene production was inhibited by 80%, would indicate that the polyamines must also inhibit the formation of ACC, probably by interference with the continuous de novo synthesis of ACC synthase, which was shown to be required in order to maintain the level of ACC in the tissue (23).

The inhibition of macromolecular synthesis produced by polyamines, as reported here, might have, in addition to the effects on ethylene biosynthesis, effects of a more general nature. It has been shown that applied 0.1 to 10 mM

polyamines retarded the dark-induced senescence phenomena, like the decrease in Chl degradation, or the reduction in the increase of RNase and protease (3,9,13), as well as wound-induced increase in RNase (2). However, similar effects were reported for cycloheximide, a well-known protein synthesis inhibitor. Cycloheximide at concentrations that inhibit 80% incorporation of ^{14}C into protein proved to be a very powerful inhibitor of the dark-induced senescence (22). It inhibited protease synthesis in senescing oat leaves (18) and the loss of Chl from leaflets at Anacharis canadensis (Elodea) in the dark (17). It is conceivable then that the above reported effects produced by 0.1 to 1 mM polyamines are partially due to a pharmacological effect resulting from a lack of continuous de novo protein synthesis in the tissue.

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3.4 Inhibition of ACC Synthase Activity by Antifungal Antibiotics and Siba

Ethylene has long been known to have a profound effect on plants (1). The pathway of ethylene biosynthesis has been gradually elucidated and can be summarized in the sequence: Methionine $\rightarrow$ S-adenosylmethionine (SAM) $\rightarrow$ 1-amino-cyclopropane-1-carboxylic acid (ACC) $\rightarrow$ ethylene (2). The rate-limiting enzyme of this process was shown to be ACC synthase (2,3). This enzyme, which converts SAM to ACC, was described by Boller et al. (4) and by Yu et al. (5) in extracts of tomato fruit.

ACC synthase showed a K_m between 13 μ M (4) and 20 μ M (5) with respect to its substrate, SAM, and it showed a strict requirement for pyridoxal phosphate. Thus, inhibitors of enzymes requiring pyridoxal phosphate as a co-factor, such as aminoxycetic acid and aminoethoxyvinylglycine (AVG), were shown to inhibit efficiently ACC synthase (2). Some analogs of SAM, like S-adenosylethionine and S-adenosylhomocysteine (SAH), were also shown to act as inhibitors in the ACC synthase reaction, although 50 μ M SAH was needed to bring about 50% inhibition.(5).

The naturally occurring antifungal antibiotics Sinefungin and A9145C, and the synthetic nucleoside Siba (5'-deoxy-5'-5-S-isobutylthioadenosine), form part of a family of compounds which are analogs to SAM and SAH (Fig. 1). These compounds have been shown to inhibit in vivo the proliferation of a tumor virus (6) and parasites (7), and to inhibit in vitro a number of tRNA methylases, N-methyl transferases and O-methyl transferases (6,8). More recently, another type of transmethylation reaction involving the creation of a cyclopropane ring at a site of unsaturation of a membrane phospholipid fatty acid has been shown to be strongly inhibited by Sinefungin and A9145C (9).

Since the conversion of SAM to ACC and methylthioadenosine involves an

elimination of an α -H along with a γ -substituent (1-3 elimination) giving rise to a cyclopropane ring (5), it was of interest to study the effect of analogs of SAM and SAH on the synthesis of ACC.

Experimental

Sinefungin and A9145C were a gift from Dr. R. Nagarajan, E. Lilley Research Laboratories (Indianapolis, IN 46285, USA). Siba was a gift from Prof. E. Lederer, Institut de Chimie des Substances Naturelles (CNRS), 92290 Gif sur Yvette, France.

Enzyme Extractions. Red or green tomatoes (Faculty 16 variety) were sliced to 0.5 cm width and were incubated overnight in 10 mM MES buffer, pH 6.1, 0.6 M sorbitol and 10 μ g/ml chloramphenicol. The slices were then washed twice with cold water and homogenized in a Sorvall Omnimixer Blender at 2°C in the presence of 2 mM DTT, 1 μ M pyridoxal phosphate, 0.1 M Na phosphate buffer, pH 7.9. The homogenate was pressed through four layers of cheese-cloth, brought to 0.1 M $MgCl_2$ to precipitate pectins, and centrifuged at 27,000 x g for 20 min. The supernatant fluid was concentrated under pressure by ultrafiltration through an Amicon YM-10 membrane. The concentrated enzyme was then passed through a Sephadex G-25 column equilibrated with 20 mM Na phosphate buffer, pH 7.9, 0.4 mM DTT, 2.5 μ M pyridoxal phosphate. The protein containing fraction was used as the source of enzyme. Protein was determined according to Bradford (11).

Enzyme Assay. The standard reaction mixture consisted of 50 mM Epps buffer, pH 8.5, 150 μ M SAM, 200 μ l enzyme (70-100 μ g protein) and equilibration buffer in a total volume of 600 μ l. Reactions were incubated at 30°C for 1 h and were terminated by the addition of 0.1 ml of cold 10 mM $HgCl_2$. Tubes

were then sealed with serum caps and ACC formed was determined by conversion to ethylene according to Lizada and Yang (12). The efficiency of conversion of ACC to ethylene was 70 to 80% in all our experiments. A sample of gas was taken to determine the concentration of ethylene by gas chromatography.

Results and Discussion

Sinefungin, Siba or A9145C, which are structural analogs of SAM (Fig. 1), inhibited the activity of ACC synthase from tomato fruit when assayed in the presence of SAM as substrate. These compounds, however, were not converted to ACC by either extract from red or green tomatoes. The inhibitory effect of these SAM analogs on the activity of ACC synthase isolated from red tomatoes is shown in Figure 2, as a function of their molar concentration. For comparison we have included in this study treatments with AVG, which is a known potent inhibitor of ACC synthase (4). Among the analogues tested, Sinefungin was found to be the most potent inhibitor of ACC synthase. It showed an I_{50} of 25 μ M. Siba was less potent with an I_{50} of 95 μ M, and A9145C was the least effective showing an I_{50} of 0.85 mM. In extracts from red tomatoes, AVG was a more potent inhibitor of ACC synthase than the SAM structural analogs tested showing an I_{50} of 1.7 μ M. However, ACC synthase extracted from green tomato fruits was far more sensitive to Sinefungin than that extracted from red tomatoes. In the case of enzyme from green tomatoes, Sinefungin was as effective as AVG, showing an I_{50} of 1.0 μ M, which is identical to that for AVG (Table I). Siba had the same I_{50} for ACC synthase obtained from green or red tomatoes.

The inhibition produced by Sinefungin or Siba, as well as AVG, can be reversed almost completely by passing the inhibited enzyme through a G-25 column (Table II). Thus indicating that the inhibitors tested are not

Table I Sensitivity to AVG and Sinefungin of ACC synthase from red and green tomatoes

	I_{50} (μ M)	
	<u>Green</u>	<u>Red</u>
AVG	1.0	1.7
Sinefungin	1.0	25.0
Siba	100.0	95.0

ACC synthase preparation from green tomatoes with a specific activity of 1.4 nmoles/mg protein/h, or ACC synthase preparation from red tomatoes (15 nmoles/mg protein/h) were incubated with increasing concentrations of inhibitors as described under Experimental.

Table II Reversibility of the inhibition of ACC synthase caused by various inhibitors

Inhibitor	Enzyme activity (% control)	
	Directly	After G-25 Sephadex chromatography
None	100.0	100.0
AVG, 2.5 M	50.7	91.8
Sinefungin, 100 μ M	29.5	86.3
Siba, 208 μ M	68.3	119.0

ACC synthase (21.6 nmoles/mg protein/h) obtained from red tomatoes was preincubated with the indicated inhibitors and assayed directly or after G-25 Sephadex chromatography.

covalently bound to ACC synthase.

The fact that the structural analogs of SAM did not serve as a substrate for ACC synthase is in accordance with the results obtained by Boller et al. (4) and Yu et al. (5) indicating that both the sulfonium function and the adenosine moiety of the SAM molecule are essential components of an active substrate for ACC synthase.

When tested for their ability to inhibit ACC synthase activity, Sinefungin was found to be the most potent and A9145C the least potent. In contrast, the unsaturated molecule, A9145C, was reported to be the most potent inhibitor of transmethylation reactions (9). Yu et al. (5) have shown that several sulfur-containing SAM analogs are capable of inhibiting ACC synthase. The data presented herein indicated that the presence of a sulfur atom in the molecule is not a prerequisite for the inhibition, since Sinefungin which lacks a sulfur atom is a more potent inhibitor than Siba which has one.

Differences in sensitivity to AVG between red and green tomatoes have been reported by Baker et al. (10), where the production of ethylene by green tomato tissue was 1.6 to 2.2-fold more sensitive to AVG than that of red tomato tissue. Our results showed that Sinefungin inhibition of ACC synthase activity followed a similar pattern, where a 25-fold higher molar concentration was required to achieve 50% inhibition in red tomatoes as compared with green tomatoes.

In conclusion, we report here that the naturally occurring antibiotic Sinefungin is a new and potent inhibitor of ACC synthase that is at least twice as effective as SAH (5). Siba and especially Sinefungin may prove of importance in further understanding the mechanism of ethylene biosynthesis.

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3.5 Factors Affecting Ethylene Production in Plant Mitochondria

In a recent study we demonstrated the formation of ethylene from its precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), in the mitochondrial fraction isolated from etiolated pea seedlings (1). A 2- to 3-fold increase in the rate of this reaction was obtained with sonicated submitochondrial particles (SMP) over the rate measured with intact mitochondria. Since SMP are inside-out vesicles of the inner mitochondrial membrane (2,3), it was concluded that biosynthesis of ethylene from ACC in plant mitochondria is associated with the inner mitochondrial membrane.

Ethylene formation in plant organs or in plant tissues has been largely characterized by the use of specific inhibitors of ethylene formation, structural analogs of the substrates of the reaction, and various treatments of the ethylene-forming tissue which affected the reaction. The identification of intermediates in the biosynthetic pathway leading to ethylene formation (4-6) made it possible to gain more information about the site of inhibition of some of the inhibitors, the nature of the reaction it inhibited and the factors involved in it.

Aminoethoxy-vinylglycine (AVG) and aminoxyacetic acid (AOA), which are known to be inhibitors of pyridoxal enzymes, inhibit the conversion of S-adenosylmethionine (SAM) to ACC (7,8). This step precedes the final step of ethylene biosynthesis, namely, ACC conversion to ethylene. Cobalt ion strongly inhibits ethylene production, both in apple tissues and in mung bean hypocotyls (9). Co^{2+} was found to promote many growth processes which are inhibited by ethylene and which were shown to inhibit ethylene formation in mung bean hypocotyls while, at the same time, ACC was accumulated (10). It has therefore been suggested that Co^{2+} inhibits the final step of ACC conversion to ethylene. Ethylene synthesis was also inhibited under anaerobic

conditions. The conversion of ACC to ethylene (11) was considered the O_2 -dependent step of this reaction. This step was also considered as the site of inhibition of the uncouplers 2,4-dinitrophenol and CCCP (12,13).

Structural analogs of ACC (13) and of other intermediates in the reaction (14,15) were added in the ethylene-forming system in apple slices, cauliflower and tomato tissue. Of these analogs, both methional and α -keto- γ -methylthiobutyric acid (KMB), which are structural analogs of methionine, may be degraded to ethylene under certain conditions (16,17). The early suggestions that they are intermediates in the pathway of ethylene biosynthesis were contradicted when differences in the nature of labeled by-products were found between the system which uses labeled methionine and the one utilizing labeled KMB (18).

Here we used SMP in an attempt to characterize and compare the ethylene-forming system in the cell-free preparation, to the one in the intact tissue. The use of SMP was preferred over the use of intact mitochondria, since the former seems to have the ethylene-forming system in an orientation which is exposed to the medium and hence it was not necessary to consider transport of ACC across the membrane as a kinetic limitation.

ACC conversion to ethylene by submitochondrial particles were found to be specific to ACC as a substrate and was abolished when inhibitors of the reaction in vivo were added to the assay medium.

Experimental

Plumular hooks from 7-day-old etiolated pea seedlings (Pisum sativum var. Kelvedon Wonder) were excised and homogenized, the mitochondrial fraction was isolated, and submitochondrial particles were prepared as in (1).

The conversion of ACC to ethylene was assayed in 1.0 ml of reaction mix-

ture which, unless otherwise mentioned, contained: 100 mM Na-Hepes buffer at pH 8.0, 0.1 mM MnCl_2 , 10 mM ACC and submitochondrial particles (at a final concentration of 0.2–0.4 mg protein/ml). The reaction mixture was placed in a test tube, closed with a rubber septum immediately after the addition of the substrate, and placed in a shaking water bath at 28°C. Ethylene production was measured as in (1), at 20-min intervals. Ethylene formation was linear with time for at least 1 h. Negligible amounts of ethylene were obtained with heat-denatured control samples.

Results and Discussion

Effect of pH and of Divalent Cations on ACC Conversion to Ethylene by Submitochondrial particles. Figure 1 depicts the dependence of the rate of ethylene formation by SMP on the pH of the medium. A maximal value was obtained at pH 8.0. This activity was inhibited by more than 90% when either Tris or Tricine buffer was used instead of Hepes buffer at the same pH. Since Tricine is known to bind divalent cations (19), its inhibitory effect could be due to the removal of divalent cations, which are necessary in the reaction. Table 1 describes the effect of various divalent cations on ethylene formation by SMP, MnCl_2 caused a more than 20-fold increase in the rate of ACC conversion to ethylene over the control, whereas CaCl_2 , MgCl_2 or CuCl_2 had a relatively small effect on this reaction. It has been reported that ethylene formation from ACC by a soluble fraction isolated from etiolated pea seedlings, is greatly enhanced by the addition of MnCl_2 to the reaction mixture (20). The mitochondrial ethylene-formation activity reported here is similar to the previously reported soluble system not only in its dependence on MnCl_2 and in the concentration of MnCl_2 which yields maximal activation (Fig. 2), but also in the pH value obtained for optimal ethylene-formation activity (21).

Table 1

The effect of divalent cations on ethylene formation from 1-aminocyclopropane-1-carboxylic acid in submitochondrial particles

Addition	Cation concentration (mM)	Ethylene formation (nmol · mg protein ⁻¹ · h ⁻¹)
—	—	1.15
MgCl ₂	1.0	1.60
	5.0	1.62
CaCl ₂	1.0	2.14
CuCl ₂	1.0	2.55
MnCl ₂	0.1	31.66
	1.0	20.00

Ethylene formation was determined in a 1.0-ml reaction mixture containing 100 mM Na-Hepes at pH 8.0, 10 mM KCl, 10 mM ACC, 25 μ l submitochondrial particles (15.5 mg protein/ml) and various divalent cations, as indicated, at 28°C

Table 2

Effect of uncouplers and of various inhibitors on ACC conversion to ethylene in submitochondrial particles

Addition	Concentration	Ethylene formation (nmol · mg protein ⁻¹ · h ⁻¹)	% of control
—	—	8.22	100
Anaerobiosis	—	1.86	22
FCCP	10 μ M	5.76	70
CCCP	10 μ M	6.92	84
DNP	5 mM	6.15	75
EDTA	0.5 mM	u.d.	—
KCN	0.1 mM	2.76	33
	1.0 mM	0.88	11
CoCl ₂	5 μ M	4.27	52
	50 μ M	1.07	13
NaN ₃	1 mM	1.36	16
<i>n</i> -Propyl gallate	5 μ M	2.05	25
	20 μ M	0.33	4

Effect of Uncouplers and of Different Inhibitors on the Conversion of ACC to Ethylene by Submitochondrial Particles. Uncouplers of oxidative phosphorylation were shown to inhibit ethylene formation in ethylene-producing tissues at concentrations which cause complete uncoupling in isolated mitochondria. Addition of 0.1 mM 2,4-dinitrophenol to mung bean hypocotyls caused about 85% inhibition of both ACC- and IAA-dependent ethylene formation (12). The uncoupler, CCCP, inhibited ethylene formation in the same system at a much lower concentration ($I_{50} = 5 \mu\text{M}$). Similar results were obtained in apple slices and in etiolated pea seedlings (13), where a significant reduction in ATP concentration was demonstrated concomitantly. As seen in Table 2, CCCP, FCCP and 2,4 DNP caused only a slight inhibition of ethylene production by SMP under similar conditions. Similar results were obtained with intact mitochondria (not shown). The reason for the different responses of the isolated mitochondrial system and the ethylene-forming tissue to the addition of uncouplers is still obscure. One possible explanation may be that ACC conversion to ethylene in vivo involves an ATP-dependent step which precedes the conversion reaction itself, either directly or indirectly (e.g., the transport of ACC in vivo to the intracellular site of conversion).

As mentioned above, metal chelators inhibit ethylene formation in SMP. The inhibitory effect of EDTA, as well as other metal chelators, has already been demonstrated in apple tissues (4). In SMP, ethylene formation is inhibited by EDTA (Table 2), and this inhibition can be reversed when the concentration of Mn^{2+} in the assay medium is elevated (not shown). More information on the nature of the ethylene-forming enzyme is needed in order to understand the role of Mn^{2+} in the mechanism of the reaction.

KCN, n-propyl gallate and CoCl_2 , which are known to inhibit ethylene production in ethylene-forming tissue (4,9,10,13), were effective even at

Fig. 2. Effect of MnCl_2 concentration on the conversion of ACC to ethylene. Reaction mixture was as described in section 2. MnCl_2 was added at variable concentrations as indicated in the figure. SMP was added at a final protein concentration of 0.4 mg/ml.

Fig. 1. Effect of pH on ACC conversion to ethylene. The reaction mixture (1.0 ml) contained 100 mM buffer at the indicated pH, 10 mM KCl, 0.1 mM MnCl_2 , 10 mM ACC and 0.24 mg SMP. Pipes buffer was used between pH 6.0 and 7.0, Hepes buffer was used between pH 7.0 and 8.0 and CAPS buffer was used between pH 8.0 and 9.0.

Table 3

Ethylene formation by submitochondrial particles in the presence of various substrates

Addition	Ethylene formation (nmol · mg protein ⁻¹ · h ⁻¹)	% of control
10 mM ACC	8.22	100
10 mM cyclopropane carboxylic acid	u.d.	—
10 mM ACC + 10 mM cyclopropane carboxylic acid	2.04	25
10 mM cyclopropyl- methylecarbinol	u.d.	—
10 mM ACC + 10 mM cyclopropylmethyl- carbinol	5.92	72
10 mM KMB	0.19	2
10 mM ACC + 10 mM KMB	11.04	134

lower concentrations in isolated submitochondrial particles (Table 2). The mode of action of these inhibitors in the ethylene-formation reaction is not known; however, n-propyl gallate is a free radical scavenger and it has been thought for a long time that its inhibitory effect on ethylene formation in tissues indicates that free radicals are involved in the conversion of ACC to ethylene (22). It should be noted that n-propyl gallate has recently been shown (23) to inhibit the mitochondrial alternate electron transport pathway at concentrations similar to those which inhibit ethylene formation in SMP (Table 2). KCN is a very well known inhibitor of cytochrome oxidase, the enzyme which carries out the final step in the chain of oxidation reactions in the mitochondria. The sensitivity of the reactions to anaerobic conditions, together with the inhibitory effect of KCN on the reaction, are in line with the suggestion made about a decade ago that cytochrome oxidase may be involved in the reaction (11).

The Effect of Structural Analogs of ACC and of KMB on Ethylene Formation in Submitochondrial Particles. Table 3 shows the effect of two analogs of ACC, cyclopropane carboxylic acid and cyclopropylmethylcarbinol, on ethylene formation in SMP. Both analogs inhibited ethylene formation from ACC, but their mode of inhibition is still under investigation. These compounds did not inhibit ethylene formation in either apple cortex or bean leaf (13). This may be due to the slow penetration of these substrates into the cytoplasm of the cell.

SMP converts KMB to ethylene at a rate of about 2% that of the conversion of equimolar concentrations of ACC to ethylene (Table 3). In contrast, conversion of KMB to ethylene by homogenates of etiolated pea seedlings (21) (Fig. 2b) was reported to occur at a rate similar to that of ACC conversion to ethylene under the same conditions. Whereas very slow degradation of KMB to ethy-

lene was observed when the former was added to SMP (Table 3), it caused a synergistic enhancement of ACC conversion to ethylene in this system. The reason for the activation effect is still obscure. Degradation of KMB to ethylene was previously attributed to oxidases present in many of the ethylene-forming tissues (23). It has also been demonstrated in a model system developed in (24), in which horseradish peroxidase, sulfite, specific phenols and manganese or catalytic amounts of H_2O_2 converted methional or KMB to ethylene.

We may thus conclude that SMP converts ACC to ethylene in a mode which is rather specific to ACC and is similar both in its characteristics and its sensitivity to inhibitors, to the system described in vivo.

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D. DESCRIPTION OF THE COOPERATION

A close cooperation among the investigators has already been established while the principal investigator spent a sabbatical leave in the Davis campus. During this period, mutual research interest in the field of CA storage of agricultural perishables resulted in fruitful and successful collaboration which initiated studies aimed at improving the keeping quality of agricultural perishables under CA atmosphere. This reported cooperative research is a direct outcome of the collaboration. The three investigators have been working for many years in the field of post harvest senescence and deterioration of agricultural perishables. However, each one of the three investigators has developed a different approach to the study emphasizing the technological, physiological and biochemical aspects. The fruitful collaboration in the form of a BARD research project contributed to the mutual accomplishment of the research objectives.

During the first year of the project, the principal investigator spent a month in Davis for exchange of information and development of techniques to be employed in the project. On the third year of the project, Dr. S.F. Yang visited the principal investigator's laboratory, where joint evaluation of the results and intensive discussions were held between the investigators.

E. BENEFIT TO AGRICULTURE

Postharvest losses of fresh fruits and vegetables - which occur after considerable effort, energy and expense have been invested in producing and harvesting a crop - represent one of the great wastes in agriculture. These losses are inordinately great because of the characteristic perishable nature of most harvested fruits and vegetables, due to normal physiological aging and consequent susceptibility to pathological attack by microorganisms. Losses occur at harvest, during handling, transportation, storage and in retail shops, as well as in the consumer's home. The social and economic significance of these losses is expressed in unavailability of good quality fruits and vegetables, especially out of season, and in the high price which limits widespread use of these nutritive foods. A report to the United States Congress determined that 20 percent of all food produced in the United States is lost or wasted. Losses of the more perishable fruits and vegetables may be much greater, approaching 30 percent of the harvested crop. This is a serious problem in both the United States and Israel and in many countries throughout the world.

Current technology to counteract postharvest losses involves massive use of refrigeration, special packaging, controlled atmospheres and chemical treatments. Refrigeration is very costly and cannot be used for many fruits and vegetables which suffer physiological injury at low, but non-freezing, temperatures. All current postharvest technology for preserving crops after harvest involves alteration of the external environment of the crop. These include either controlled atmospheres, such as low pressure or increased CO₂ and reduced O₂, lowered temperatures, or special packaging to provide physical protection. Also use of various chemical agents as dips, washes or sprays improve protection against pathological invasion and eliminate or reduce the microbial load already present on the tissues. Bet-

ter understanding of the role of various factors affecting fruit and vegetable ripening, physiological disorders and deterioration under CA conditions will enable us to optimize CA storage conditions and apply the CA technology to commodities that presently do not benefit from CA.

The results of this study expanded and deepened our knowledge of the role of the various factors affecting ripening, physiological disorders and deterioration of the perishable commodities during CA storage. Such knowledge will allow the optimization of the use of the increasingly expensive energy spent on handling and storage of high quality agricultural produce and make possible the use of CA for commodities that presently do not benefit from CA storage.

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