Plant Physiol. (1983) 73, 784–790
0032-0889/83/73/0784/07/$00.50/0

Bicarbonate/CO_2-Facilitated Conversion of 1-Aminocyclopropane-1-carboxylic Acid to Ethylene in Model Systems and Intact Tissues[1]

Received for publication March 14, 1983 and in revised form July 6, 1983

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ABSTRACT

Bicarbonate markedly enhances ethylene production from 1-aminocyclopropane-1-carboxylic acid (ACC) in model chemical systems where the conversion is free radical-mediated, in thylakoid membrane suspensions of *Phaseolus vulgaris* L. cv Kinghorn where the reaction is light-dependent, and in microsomal membrane suspensions and intact tissues where the reaction is enzymically mediated. In two model systems generating free radicals—the Fenton reaction and a reaction mixture containing xanthine/xanthine oxidase, $NaHCO_3$ (200 millimolar) increased the formation of ethylene from ACC by 84-fold and 54-fold, respectively. Isolated thylakoid membranes also proved capable of ACC-dependent ethylene production, but only upon illumination, and this too was enhanced by added $NaHCO_3$. As well, light-induced inhibition of ACC-dependent ethylene production by leaf discs was relieved by adding 200 millimolar $NaHCO_3$. Finally, $NaHCO_3$ (200 millimolar) augmented ACC-dependent ethylene production from young carnation flowers by about 4-fold, and the conversions of ACC to ethylene by microsomes isolated from carnation flowers and etiolated pea epicotyls were higher by 1900 and 62%, respectively, in the presence of 200 millimolar $NaHCO_3$.

This increased production of ethylene appears not to be due to bicarbonate or CO_2-induced release of the gas from putative receptor sites, since the addition of $NaHCO_3$ to sealed reaction mixtures after the ACC to ethylene conversion had been terminated had no effect. Spin-trapping studies have confirmed that bicarbonate does not facilitate the formation of free radicals thought to be involved in the conversion of ACC to ethylene. Nor did bicarbonate alter the physical properties of the membrane bilayer, which might indirectly modulate the activity of the membrane-associated enzyme capable of converting ACC to ethylene. Rather, bicarbonate appears to directly facilitate the conversion of ACC to ethylene, and the data are consistent with the view that CO_2 derived from bicarbonate is the active molecular species.

Increased production of ethylene in response to enhanced levels of CO_2 has been demonstrated for detached leaves and leaf discs of cocklebur (15), intact sunflower plants (6, 12), tobacco leaf discs (1, 2, 18), and detached senescing oat leaves (14). The effect is obtained when CO_2 is supplied either directly as a gas or indirectly as bicarbonate, and no increase in ethylene production is obtained when CO_2 is removed with a KOH trap (15, 18). White light also affects ethylene production by leaf tissue, presumably through modulation of endogenous CO_2 levels. For example, exposure of leaf tissue to white light has been found to inhibit ethylene production relative to controls maintained in darkness (11, 14, 15, 18). Moreover, light inhibition of ethylene production can be overcome by the addition of $NaHCO_3$ or CO_2 (15, 18).

Kao and Yang (18) have proposed that CO_2 affects ethylene production in leaf tissue by facilitating its enzymic formation from ACC.[3] However, an alternative explanation has been proposed by Grodzinski *et al.* (15), who contend that CO_2 could be displacing bound ethylene, thereby promoting its release from the tissue. During photosynthesis, for example, the internal concentration of CO_2 within the leaf cells would be reduced, resulting in increased binding and metabolism of ethylene and less detectable production. In an effort to distinguish between these possibilities, we have examined the ability of $NaHCO_3$ to facilitate the conversion of ACC to ethylene in two chemical systems and in several biological systems capable of producing the gas from exogenous ACC. We also report data indicating that the bicarbonate effect is not a consequence of ethylene displacement from microsomal membranes, which possess ethylene binding sites (28, 29) and are capable of producing ethylene from ACC (24, 25).

MATERIALS AND METHODS

Chemicals. ACC, DPH, xanthine, xanthine oxidase, and catalase were obtained from Sigma Chemical Co. Ferrous sulfate septahydrate, sodium bicarbonate, sodium chloride, sodium formate, and sodium bisulfite were purchased from J. T. Baker Chemicals Ltd. *Cis*-PNA, *trans*-PNA, and TMA-DPH were obtained from Molecular Probes Ltd. H_2O_2 (30%,v/v) was from BDH Chemicals Ltd. and DMPO from Aldrich Chemical Co. DMPO was purified prior to use with activated charcoal according to Buettner and Oberley (10).

Plant Material. Thylakoid membranes were isolated from primary leaves of *Phaseolus vulgaris* L. (cv Kinghorn) grown under greenhouse conditions in a mixture of sand, peat moss, and soil (1:1:2) at a mean temperature of 26°C with a 16-h photoperiod. Primary leaves were harvested after 2 weeks of growth. To prepare chloroplasts, 20 g fresh weight of leaf tissue

[1] This research was supported by the Natural Sciences and Engineering Research Council of Canada. D. G. M. and R. L. L. are recipients of NSERC postgraduate scholarships.

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[3] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; *cis*-PNA, *cis*-parinaric acid; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; DMSO, dimethyl sulfoxide; DPH, 1,6-diphenyl-1,3,5-hexatriene ; ESR, electron spin resonance; THF, tetrahydrofuran; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene; Tiron, 1,2-dihydroxybenzene-3,5-disulfonic acid; *trans*-PNA, *trans*-parinaric acid.

were cut with scissors into 70 ml of extraction buffer containing 0.3 M sorbitol, 1 mM $MgCl_2$, 1% BSA, and 50 mM Tricine, pH 8.0, and homogenized with a Sorvall Omnimixer for 5 s. The homogenate was filtered through eight layers of cheesecloth and centrifuged for 2 min at 2000*g*. The chloroplast pellet was resuspended in wash buffer (0.3 M sorbitol and 50 mM Tricine, pH 8.0) and centrifuged at 2000*g* for 2 min. Thylakoid membranes were obtained by resuspending the chloroplast pellet in 50 mM Tricine, pH 8.0, for 10 min to osmotically disrupt the envelope membranes (16). The released thylakoids were then pelleted by centrifugation at 4000*g* for 5 min and washed twice by resuspension in 50 mM Tricine, pH 8.0, and centrifugation at 4000*g* for 5 min. Thylakoid membranes were then resuspended in wash buffer at a concentration of 2 mg of Chl/ml. Chl was determined as described by Arnon (3).

Microsomal membranes capable of converting ACC to ethylene were isolated from epicotyl sections of dark-grown pea seedlings and from cut carnation flowers (*Dianthus caryophyllus* L. cv White-Sim; Yonder Atkin, Leamington, Ontario, Canada) as described previously (24, 25), except that the isolated membranes were dialyzed at 2°C overnight against 2 mM Epps buffer, pH 8.5, in order to eliminate an endogenous cytosolic inhibitor of ethylene production from ACC (24). The flowers were cut at the commercial stage and maintained for 4 d in deionized H_2O under continuous illumination at 22°C before being used for membrane isolation. By this time, the petals were fully expanded and showing no symptoms of petal-inrolling.

Assays for Ethylene Production and ACC Levels. Ethylene production from ACC in the presence of the Fenton reagent or xanthine/xanthine oxidase was carried out essentially as described by Legge *et al.* (22). For the Fenton reaction, the assay mixture contained 25 μM $FeSO_4$, 0.03% H_2O_2, 1 mM ACC, and specified concentrations of $NaHCO_3$ in 1 ml of 0.3 M sorbitol-50 mM Tricine, pH 8.0. For the xanthine/xanthine oxidase reaction, the assay contained 0.2 mM xanthine, 1 mM ACC, 0.05 units xanthine oxidase, and specified concentrations of $NaHCO_3$ in 1 ml of 0.3 M sorbitol-50 mM Tricine, pH 8.0. In some experiments, catalase at a concentration of 5 units/ml was also added. The reaction mixtures were sealed in 12 × 75-mm test tubes and incubated for 1 h at room temperature. At the end of this period, a 1.0-ml gas sample was removed from the head space and analyzed isothermally (65°C) in a Perkin-Elmer Series 900 gas chromatograph fitted with an AlO_3 column and a flame ionization detector.

For measurements of ethylene production from ACC in the presence of thylakoid membranes, the reaction mixture contained 1 mM ACC, thylakoid membranes (0.2 mg Chl), and specified concentrations of $NaHCO_3$ in 1 ml of 0.3 M sorbitol-50 mM Tricine, pH 8.0. The reaction mixtures were again sealed in 12 × 75-ml test tubes and placed 10 cm in front of a 150-w light bulb (10 w/m^2) for 1 h at room temperature. A 3-cm water filter was placed between the test tubes and the light bulb to protect the reaction mixtures from heat generated by the light bulb. At the end of this period, a 1-ml gas sample was removed from the head space for ethylene measurements. For measurements of ethylene production from ACC in the presence of pea and carnation microsomes, the assay contained 200 μg of membrane protein, 1 or 2 mM ACC, and specified concentrations of $NaHCO_3$ in 1 ml of 2 mM Epps buffer, pH 8.5. The reaction mixtures were again sealed in 12 × 75-ml test tubes and incubated for 1 h at 31°C, and a 1-ml gas sample from the head space was analyzed for ethylene. Membrane protein was measured as described by Bradford (9).

Levels of ethylene produced by discs from 2-week-old primary bean leaves were also determined. Eight discs (0.7-mm diameter) were placed adaxial side up in a 25-ml Erlenmeyer flask containing 2 ml of 0.3 M sorbitol-50 mM Tricine, pH 8.0, and specified concentrations of $NaHCO_3$. The flasks were sealed with rubber septa and incubated in the light (10 w/m^2 from fluorescent bulbs) for 1 h. For measurements of ethylene production from segments of pea epicotyl grown under etiolating conditions as described previously (25), 10 segments 3 cm in length were placed upright in test tubes (12 × 75 ml) containing 1 ml of 2 mM Epps buffer, pH 8.5, and specified concentrations of $NaHO_3$, and incubated for 1 h at room temperature in the light (10 w/m^2 from fluorescent bulbs). Ethylene production from cut carnation flowers was measured by placing single flowers in 100-ml glass jars fitted with serum stoppers for 1 h at room temperature in the light (10 w/m^2 from fluorescent bulbs). The glass jars contained 10 ml of 2 mM Epps buffer, pH 8.5, and specified concentrations of $NaHCO_3$. In each case, 1-ml gas samples from the head space were analyzed for ethylene. ACC levels in carnation petal tissue were measured as described by Lizada and Yang (21).

ESR Spin Trapping. Free radicals formed during the conversion of ACC to ethylene in the presence of the Fenton reagent and specified concentrations of $NaHCO_3$ were detected as spin adducts of DMPO added to the reaction mixture at a concentration of 100 mM. ESR spectra were recorded on a Varian E-12 ESR spectrometer at room temperature as described (22).

Fluorescence Depolarization. Fluorescence depolarization of isolated membranes labeled with fluorescent probes was carried out essentially as described previously (30). Stock solutions of *cis*-PNA and *trans*-PNA (4 mM in absolute ethanol) as well as DPH (2 mM in THF) and TMA-DPH (2 mM in DMSO) were flushed with N_2 and stored at −20°C. For membrane labeling, aliquots of the stock solutions were diluted 1000-fold in 0.3 M sorbitol-50 mM Tricine, pH 8.0, or 2 mM Epps buffer, pH 8.5, by stirring vigorously for 15 min, and equal volumes of fluorescent probe solution and membrane suspension were mixed to give a final probe concentration of 2 μM for *cis*-PNA and *trans*-PNA and 1 μM for DPH and TMA-DPH. Upon addition of probe to the membrane suspension, the mixture was quickly vortexed for 10 to 15 s. The final concentration of membrane was 20 μg Chl/ml for thylakoid membranes and 25 μg protein/ml for microsomal membranes.

Steady state fluorescence depolarization (*P*) was measured at room temperature using an SLM 8000 spectrofluorometer. For DPH and TMA-DPH, an excitation wavelength of 360 nm and an emission cutoff filter of 418 nm were used. For *cis*-PNA and *trans*-PNA, excitation wavelengths of 325 and 320 nm, respectively, were used, and emitted fluorescence was passed through a 370-nm cutoff filter. When measurements for thylakoid membranes were being recorded, the emitted fluorescence was passed through an additional band filter (transmitting from 400–450 nm) to eliminate Chl fluorescence.

RESULTS

Ethylene can be generated chemically from ACC in the presence of $OH^\cdot$ formed either by the Fenton reaction (Fe^{2+} and H_2O_2) or in the presence of xanthine and xanthine oxidase (22). In the xanthine/xanthine oxidase reaction, $OH^\cdot$ is apparently formed through the Haber-Weiss reaction (22, 26). When $NaHCO_3$ (200 mM) was added to these chemical systems, ethylene production from ACC increased by 84-fold in the Fenton reaction and by more than 54-fold in the presence of xanthine and xanthine oxidase (Table I). ACC plus $NaHCO_3$ alone produced no ethylene, and no ethylene was produced by the Fenton reaction with bicarbonate unless ACC was also present (Table I). As well, both Fe^{2+} and H_2O_2 had to be present to obtain the bicarbonate enhancement of ethylene production by the Fenton reaction, indicating that bicarbonate facilitates the $OH^\cdot$-mediated conversion of ACC to ethylene (Table I). Catalase is known to scavenge H_2O_2 generated through spontaneous dismutation of $O_2^{\cdot-}$, thereby minimizing the conversion of $O_2^{\cdot-}$ to $OH^\cdot$ through

Table I. *Effects of $NaHCO_3$ and Various Other Sodium Salts on ACC-Dependent Production of Ethylene in Model Chemical Systems*

Reaction Mixtures	Ethylene[a]
	nl/h·reaction tube
Fenton reaction[b]	
Fenton mixture plus ACC	0.53 ± 0.02
Fenton mixture plus ACC and 200 mM $NaHCO_3$	42.0 ± 1.4
Fenton mixture minus Fe^{2+} plus ACC and 200 mM $NaHCO_3$	2.18 ± 0.11
Fenton mixture minus H_2O_2 plus ACC and 200 mM $NaHCO_3$	0.04 ± 0.02
Fenton mixture plus 200 mM $NaHCO_3$	ND[c]
ACC plus 200 mM $NaHCO_3$	ND
Fenton mixture plus ACC and 200 mM NaCl	0.73 ± 0.03
Fenton mixture plus ACC and 200 mM Na acetate	0.80 ± 0.03
Fenton mixture plus ACC and 200 mM Na formate	0.72 ± 0.02
Fenton mixture plus ACC and 200 mM $NaHSO_3$	0.02
X/XO reaction[b,d]	
X/XO mixture plus ACC	0.35 ± 0.03
X/XO mixture plus ACC and 200 mM $NaHCO_3$	19.3 ± 1.0
X/XO plus ACC and catalase	0.02 ± 0.01
X/XO mixture plus ACC, 200 mM $NaHCO_3$, and catalase	0.14 ± 0.01

[a] Standard errors of the means for three separate experiments are indicated.

[b] Conditions outlined in "Materials and Methods."

[c] Not detectable.

[d] X/XO, xanthine/xanthine oxidase.

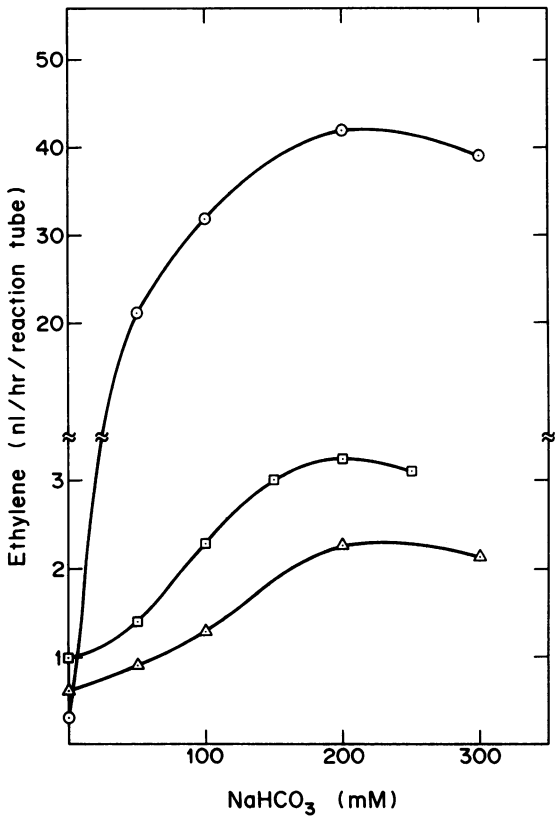

FIG. 1. Concentration dependence of the $NaHCO_3$ enhancement of ACC-dependent ethylene production by the Fenton reaction, illuminated thylakoid membranes, and microsomal membranes from pea epicotyls. (O), Fenton reaction containing 25 μM $FeSO_4$, 0.03% H_2O_2, and 1 mM ACC in wash buffer, pH 8.0; (□), mirosomal membranes from pea epicotyls (200 μg protein) and 1 mM ACC in 1 ml of 2 mM Epps buffer, pH 8.5; (Δ), illuminated thylakoid membranes (0.2 mg Chl) and 1 mM ACC in 1 ml of wash buffer, pH 8.0.

the Haber-Weiss reaction (22). Accordingly, the sensititivity to catalase of the bicarbonate effect in the xanthine/xanthine oxidase system again suggests that bicarbonate is facilitating the OH^--mediated conversion of ACC to ethylene (Table I).

The bicarbonate-induced enhancement of ethylene production in the Fenton reaction proved to be dependent upon the concentration of $NaHCO_3$. Maximum enhancement was obtained at 200 mM $NaHCO_3$ (Fig. 1), the same concentration of bicarbonate that caused maximum stimulation of ethylene production *in situ* in detached leaves and leaf disks of cocklebur (15). The enhancement effect of $NaHCO_3$ in the Fenton reaction also appeared to be highly specific in that the sodium salts of chloride, acetate, and formate at a concentration of 200 mM caused only a slight elevation of ethylene levels, and 200 mM $NaHSO_3$ actually inhibited ethylene formation (Table I). Indeed, further experiments demonstrated that $NaHSO_3$ when present with $NaHCO_3$ (200 mM) in a molar ratio of 1:20 inhibited ethylene formation in the Fenton reaction by as much as 80%.

To further examine the mechanism by which bicarbonate might be enhancing the conversion of ACC to ethylene in the Fenton system, the ESR spectra of DMPO spin adducts were examined in the presence and absence of 200 mM $NaHCO_3$ as described by Legge *et al.* (22). When DMPO was added to the Fenton reaction in the absence of ACC, a spectrum representing the $OH^·$ spin adduct of DMPO was formed (Fig. 2A). $NaHCO_3$ (200 mM) reduced the amplitude of the spectrum by about 80% (Fig. 2B). Hydroxyl radicals are known to react readily with $NaHCO_3$ to produce the $CO_3^{-·}$ radical (13, 19). However, no spin adduct of DMPO other than that attributable to $OH^·$ could be detected. The $CO_3^{-·}$ and $CO_2^{-·}$ radical species have strong transient absorption peaks at 600 (with a molar extinction coefficient of 2900 M^{-1} cm^{-1}) and 260 nm (with a molar extinction coefficient of 2250 M^{-1} cm^{-1}), respectively (19), and hence the Fenton reaction system containing 200 mM $NaHCO_3$ was scanned from 200 to 700 nm. However, no evidence was obtained for the presence of either of these radical species. When ACC was added to the Fenton reaction mixture, an additional spectrum tentatively identified as the DMPO adduct of a carbon-centered radical derived from ACC (22) was superimposed on the spectrum for the $OH^·$ adduct of DMPO (Fig. 2C); bicarbonate (200 mM) reduced the amplitude of the hydroxyl radical spin adduct, but had no effect on the adduct for the ACC-derived radical (Fig. 2D).

The effect of bicarbonate on ethylene production by discs from primary bean leaves was also tested. Low levels of ethylene were obtained from the discs under both light and dark regimes (Fig. 3A). ACC enhanced ethylene production in both the light and dark regimes but, in accordance with earlier obervations (11, 14, 15, 18), light inhibited the formation of ethylene from ACC relative to levels obtained in darkness (Fig. 3A). Moreover, this inhibition was largely overcome by the addition of 200 mM $NaHCO_3$ (Fig. 3A). Treatment of leaf discs with 200 mM $NaHCO_3$ alone did not result in any significant increase in ethylene production (Fig. 3A).

In view of the light sensitivity of ACC-dependent ethylene production from leaf discs, the ability of isolated thylakoid membranes to convert ACC to ethylene in the presence and absence of $NaHO_3$ was examined. Thylakoid membranes maintained in darkness produced only trace levels of ethylene even in the prescence of ACC and 200 mM $NaHCO_3$ (Fig. 3B). In the light, addition of ACC to the thylakoid membranes resulted in significant levels of ethylene being produced, and 200 mM bicarbonate caused a further 5-fold enhancement in ethylene production (Fig. 3B). Again, the enhancement effect appeared to be a

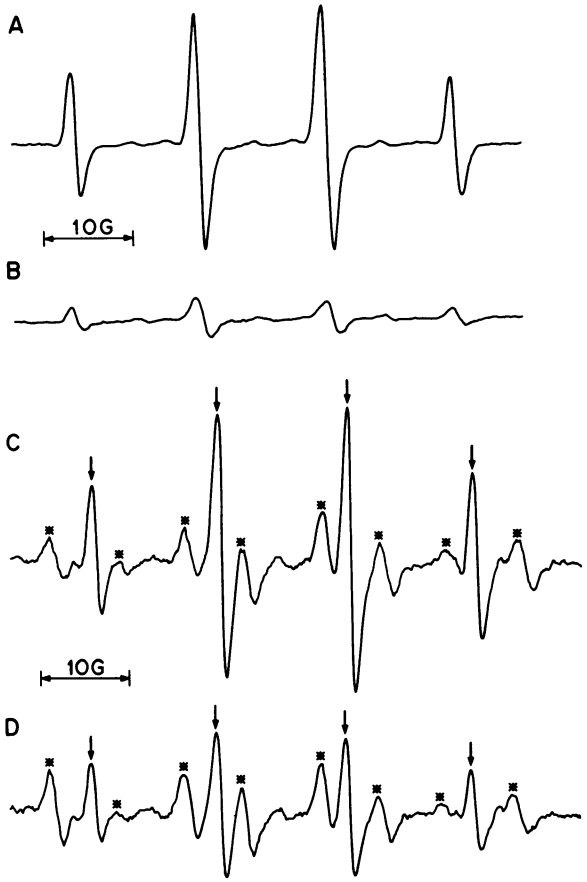

FIG. 2. Electron spin resonance spectra of the DMPO spin adducts formed in the Fenton reaction system. A, $OH^{\cdot}$ spin adduct formed in the absence of ACC; B, $OH^{\cdot}$ spin adduct formed in the absence of ACC but in the presence of 200 mM $NaHCO_3$; C, spin adducts formed when 35 mM ACC was added to the Fenton reaction system. Components of the spectrum derived from the hydroxyl adduct are designated by arrows and those attributable to the putative carbon-centered radical of ACC (22) are designated by asterisks; D, spin adducts formed when 35 mM ACC and 200 mM $NaHCO_3$ were added to the Fenton reaction system. Components of the spectrum derived from the hydroxyl adduct are designated by arrows and those attributable to the putative carbon-centered radical of ACC (22) are designated by asterisks.

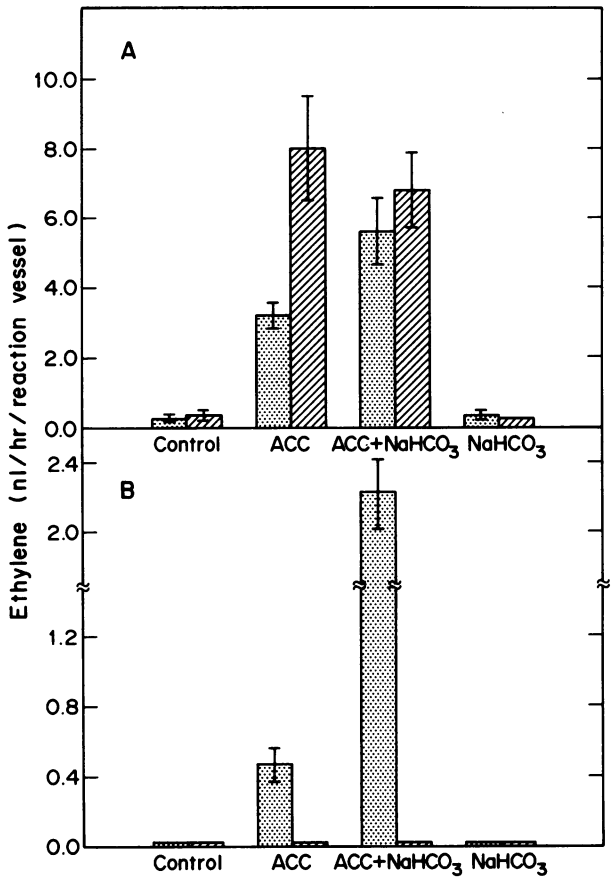

FIG. 3. Effects of illumination and $NaHCO_3$ on ethylene production by leaf discs and isolated thylakoid membranes from 2-week-old primary bean leaves. A, Bean leaf discs incubated in light and dark in 25-ml Erlenmeyer flasks containing 2 ml of wash buffer. B, Thylakoid membranes (0.2 mg Chl/ml) in 1 ml of wash buffer sealed in 6-ml test tubes and incubated in light and dark. Final concentrations of ACC and $NaHCO_3$ were 1 and 200 mM, respectively. Light (stippled bars); dark (hatched bars). Standard errors of the means for three separate experiments are shown; $n = 3$.

specific response to bicarbonate in that various other sodium salts (chloride, acetate, and formate) had little effect on ethylene levels, and $NaHSO_3$ almost completely inhibited its formation (Table II). The effect of bicarbonate on the thylakoid-mediated conversion of ACC to ethylene was also concentration-dependent, with optimum levels of ethylene being obtained at 200 mM $NaHCO_3$ (Fig. 1).

Ethylene production by epicotyl segments cut from etiolated pea seedlings was not differentially sensitive to light and dark regimes. Moreover, whereas ACC caused a 6- to 7-fold increase in ethylene production under both light and dark conditions, there was very little further enhancement upon addition of 200 mM $NaHCO_3$ (Fig. 4A). However, microsomal membranes isolated from the pea epicotyl segments, which have been previously characterized as a model system capable of converting ACC to ethylene (25), were responsive to bicarbonate. $NaHCO_3$ (200 mM) raised the level of ethylene production from ACC in the presence of the microsomal membranes by about 62% (Fig. 4B). The enhancement effect appeared to be a specific response to bicarbonate in that sodium chloride had no effect and $NaHSO_3$ inhibited ethylene formation (Fig. 4B). Heat-denatured microsomes in the absence of $NaHCO_3$ were incapable of converting ACC to ethylene.

Table II. *Effects of $NaHCO_3$ and Various Other Sodium Salts on Ethylene Production from ACC by Illuminated Thylakoid Membranes*

Reaction Mixtures	Ethylene[a]
	nl/h·reaction tube
Control[b]	0.55 ± 0.01
Control plus 200 mM $NaHCO_3$	2.22 ± 0.28
Control plus 200 mM NaCl	0.61 ± 0.05
Control plus 200 mM Na acetate	0.35 ± 0.01
Control plus 200 mM Na formate	0.52 ± 0.01
Control plus 200 mM $NaHSO_3$	0.03

[a] Standard errors of the means for three separate experiments are indicated.

[b] Contained thylakoid membranes (0.2 mg Chl) and 1 mM ACC in 1 ml of wash buffer.

The conversion of ACC to ethylene by young carnation flowers was not affected by light and dark regimes, but was enhanced by $NaHCO_3$. Exogenous ACC raised ethylene production from less than 0.1 nl/h·flower to 2.5 nl/h·flower in the light and to 1.6 nl/h·flower in darkness, and 200 mM $NaHCO_3$ induced a further 7- to 8-fold increase (Fig. 5A). $NaHO_3$ had no effect in the

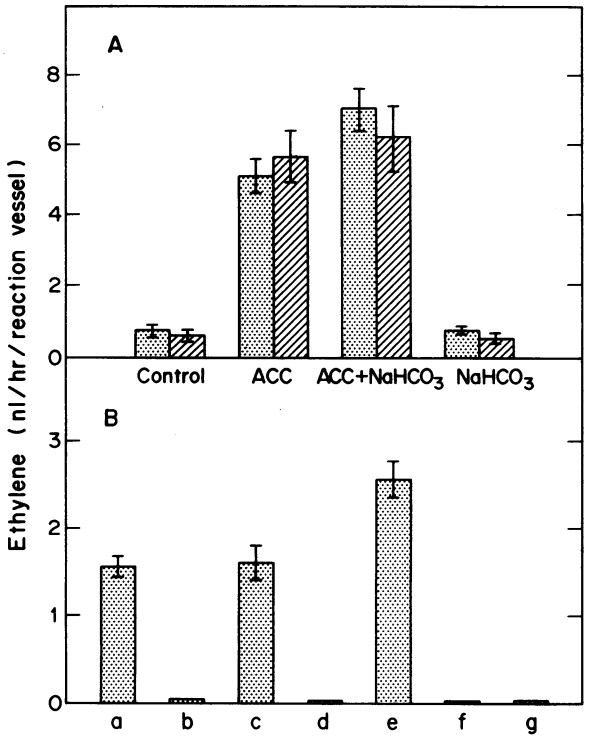

FIG. 4. Ethylene production by tissue segments and microsomal membranes isolated from etiolated pea epicotyls. A, Pea epicotyl segments incubated in sealed test tubes containing 1 ml of 2 mM Epps buffer, pH 8.5, in light (stippled bars) and dark (hatched bars); concentrations of ACC and $NaHCO_3$ were 1 and 200 mM, respectively. B, Microsomal membranes membranes (200 μg protein/ml) in 1 ml of 2 mM Epps buffer, pH 8.5: (a) plus 1 mM ACC; (b) heat-denatured membranes plus 1 mM ACC; (c) plus 1 mM ACC and 200 mM NaCl; (d) plus 1 mM ACC and 200 mM $NaHSO_3$; (e) plus 1 mM ACC and 200 mM $NaHCO_3$; (f) plus 200 mM $NaHCO_3$; (g) heat-denatured membranes plus 200 mM $NaHCO_3$.

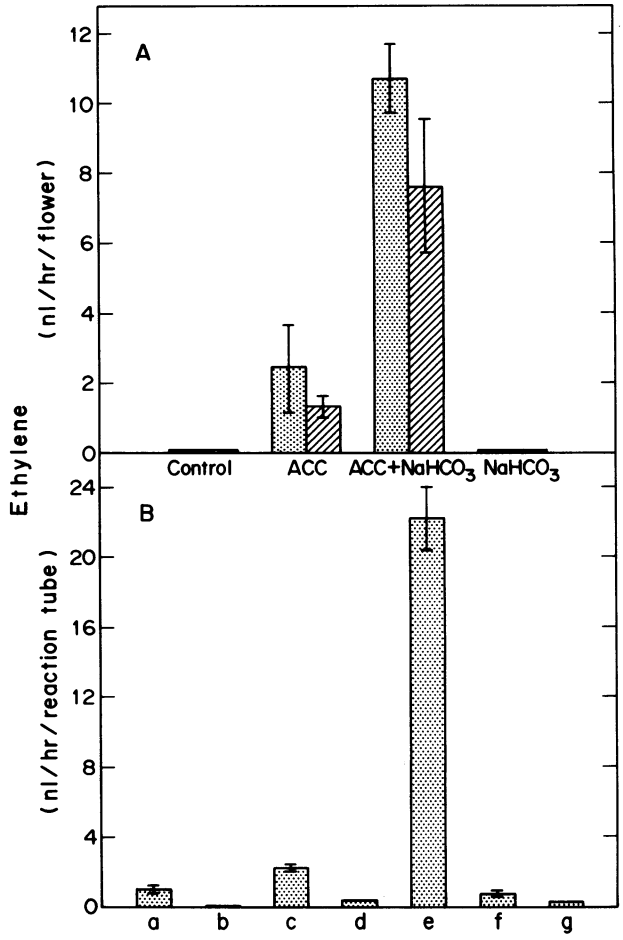

FIG. 5. Ethylene production by cut carnation flowers and microsomal membranes isolated from carnation flowers. A, Carnation flowers incubated in glass jars containing 10 ml of 2 mM Epps buffer, pH 8.5, in light (stippled bars) and dark (hatched bars). Concentrations of ACC and $NaHCO_3$ were 2 and 200 mM, respectively. B, Microsomal membranes (200 μg protein/ml) in 1 ml of 2 mM Epps buffer, pH 8.5: (a) plus 2 mM ACC; (b) heat-denatured membranes plus 2 mM ACC; (c) plus 2 mM ACC and 200 mM NaCl; (d) plus 2 mM ACC and 200 mM $NaHSO_3$, (e) plus 2 mM ACC and 200 mM $NaHCO_3$; (f) plus 200 mM $NaHCO_3$; (g) heat-denatured membranes plus 200 mM $NaHCO_3$.

absence of ACC (Fig. 5A). Analysis of the carnation petal tissue for ACC content after the 1-h incubation in the presence of either ACC alone or ACC plus $NaHCO_3$ demonstrated that the bicarbonate-induced enhancement of ethylene production was not attributable to an increased uptake of ACC into the petals in the presence of $NaHCO_3$. Microsomal membranes from carnation flowers are also capable of converting ACC to ethylene (24), and also responded to bicarbonate. Indeed, 200 mM $NaHCO_3$ increased microsome-mediated ethylene production from ACC by ≃20-fold, and again the enhancement appeared to be a specific response to ACC and was heat-denaturable (Fig. 5B).

To assess the prospect that $NaHCO_3$ might be enhancing ethylene production from ACC by perturbing membrane lipids and thus altering the activity of the membrane-associated enzyme that converts ACC to ethylene, various fluorescent probes were used to monitor the lipid environments in different regions of thylakoid and carnation microsomal membranes following treatment with bicarbonate. Measurements of *P* (the degree of polarization) for membranes labeled with DPH, which probes the interior hydrocarbon region of the membrane, and with TMA-DPH, a probe which is anchored at the lipid-water interface, indicated that bicarbonate had no apparent effect on the physical organization of the lipid bilayer. Similarly, there were no significant changes in *P* for membranes labeled with *cis*-PNA, which partitions equally between liquid crystalline and gel phase lipid, or with *trans*-PNA, which partitions preferentially into gel phase lipid, following treatment of either thylakoid membranes or carnation microsomal membranes with $NaHO_3$.

The prospect that ethylene formed from ACC remains bound, either specifically or nonspecifically, to microsomal and thylakoid membranes and is released by $NaHCO_3$ was also examined. Carnation microsomes, pea epicotyl microsomes, and thylakoid membranes were incubated for 2 h in standard reaction mixtures, and 200 mM $NaHO_3$ was added either at the beginning of the reaction or after 1 h. As expected, bicarbonate enhanced ethylene production in all three systems over the 2-h incubation period when added at time 0, and to a lesser extent when added after 1 h (Fig. 6, A–C). Ethylene production from ACC was almost totally inhibited when thylakoid membranes were placed in darkness, and when 20 mM Tiron (a scavenger of O_2^-; 25) was added to either microsomal system (Fig. 6D). To determine whether bicarbonate was releasing bound ethylene, production by each membrane system was terminated after 1 h, and the terminated reaction mixture was incubated in the presence or absence of 200 mM $NaHCO_3$ for an additional hour (Fig. 6, E and F). Ethylene levels measured after the 2-h period in these inhibited systems were not significantly different in the presence

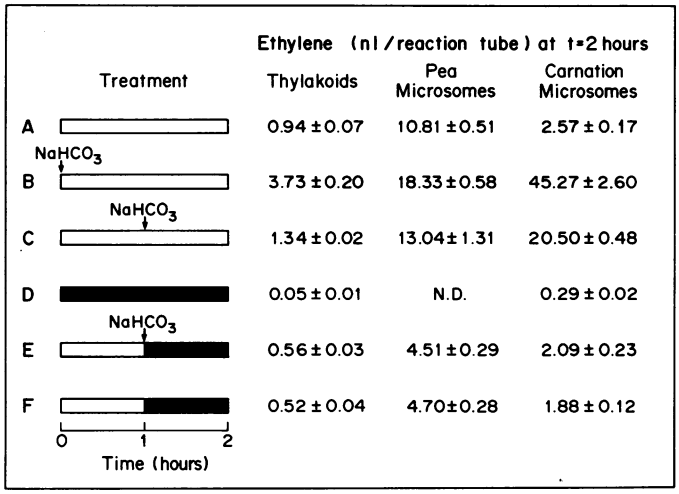

FIG. 6. Effect of $NaHCO_3$ on the release of ethylene from putative membrane receptors. Thylakoid membranes (0.2 mg Chl/ml) in wash buffer and carnation microsomal membranes (200 μg protein/ml) in 2 mM Epps buffer, pH 8.5, were incubated at room temperature for 2 h in the standard 1-ml reaction mixtures containing 1 mM ACC in the case of thylakoids and 2 mM ACC in the case of microsomes. Microsomal membranes (200 μg protein/ml) from pea epicotyls were incubated at 31°C in 1 ml of 52 mM Epps buffer, pH 8.5, containing 10 mM ACC for 2 h. $NaHCO_3$ was added at final concentrations of 200 mM for the thylakoid and carnation microsomal systems and 300 mM for the pea microsomal system. The reaction for the thylakoid system was terminated by placing the reaction mixtures in darkness. The reaction for both microsomal membrane systems was terminated by adding Tiron (20 mM final concentration). A, Membranes plus ACC incubated for 2 h; B, membranes plus ACC plus $NaHCO_3$ incubated for 2 h; C, membranes plus ACC incubated for 1 h at which point $NaHCO_3$ was added and incubation continued for another hour; D, membranes plus ACC; reactions terminated at zero time and incubated for 2 h; E, membranes plus ACC incubated for 1 h at which point the reactions were terminated, $NaHCO_3$ was added and incubation was continued for another hour; F, membranes plus ACC incubated for 1 h at which point the reactions were terminated and incubated for another hour. Standard errors of the means for three separate experiments are indicated; $n = 3$.

or absence of 200 mM $NaHCO_3$ (Fig. 6, E and F), indicating that $NaHCO_3$ is not releasing ethylene bound to the membranes.

DISCUSSION

Bicarbonate markedly enhances ethylene production from ACC in chemical systems where the conversion is driven by free radicals (Fenton reaction, xanthine/xanthine oxidase); in thylakoid membrane suspension where the reaction is dependent upon light; and in microsomal membrane suspensions and intact tissues where the reaction is enzymically mediated. It has been proposed that bicarbonate could achieve this effect by promoting release of bound ethylene (15) or facilitating the actual conversion of ACC to ethylene (18). The former prospect is consistent with previous observations that CO_2 can displace bound [^{14}C] ethylene from putative receptor sites of both intact tissue and cell-free preparations (7, 28). However, Sisler (28) has calculated that the level of ethylene released from these specific receptor sites is too low to permit ordinary chemical detection. Thus, even if CO_2 does release bound ethylene, the contribution of such ethylene to the increased production measured in the presence of exogenous bicarbonate would be insignificant. Sisler (29) has also demonstrated that 69% of the ethylene binding sites are located in a 12,000 to 100,000g fraction, which is essentially a microsomal fraction. Accordingly, the experiments reported in the present study, in which the ability of bicarbonate to release ethylene from microsomal membranes was tested, also indicate that the increased levels of ethylene detected in the presence of $NaHCO_3$ cannot be attributed to CO_2-mediated release of bound ethylene.

Thus, the bicarbonate enhancement of ACC-dependent ethylene evolution appears likely to be effected through a more efficient conversion of ACC to ethylene. The prospect that bicarbonate facilitates this conversion indirectly by altering the physical properties of the membrane lipid bilayer so as to modulate the activity of the enzyme converting ACC to ethylene appears to be ruled out by the finding that treatment of carnation microsomal membranes with 200 mM $NaHCO_3$ has no significant effect on DPH, TMA-DPH, *cis*-PNA, or *trans*-PNA polarization values. Several lines of evidence from both intact tissues and model systems suggest that ethylene formation from ACC may be mediated by free radicals. For example, the reaction can be inhibited *in situ* by radical scavengers (4, 5), is driven by $OH^{\cdot}$ in a strictly chemical system consisting of ACC and the components of the Fenton reaction (22), and appears to be facilitated by $O_2^{\cdot-}$ when catalyzed by isolated microsomal membranes (25). Thus, it is conceivable that bicarbonate might enhance ACC-dependent ethylene production by facilitating the formation of these reactive species of oxygen. However, spin-trapping experiments with the chemical system, in which the conversion of ACC to ethylene is driven by $OH^{\cdot}$ formed through the Fenton reaction, indicated that bicarbonate actually reduces the pool size of $OH^{\cdot}$. Moreover, bicarbonate had no effect on levels of the ACC-derived radical thought to be an intermediate in the chemical conversion of ACC to ethylene (Fig. 2, C and D; 22). The reduced pool size of $OH^{\cdot}$ presumably reflects quenching by $NaHCO_3$, although increased levels of either $CO_3^{\cdot-}$ or $CO_2^{\cdot-}$, which are known to be products of reactions involving $OH^{\cdot}$ and $NaHCO_3$ (13, 19), were not detected. Moreover, if $CO_2^{\cdot-}$ were responsible for the enhanced conversion of ACC to ethylene in the Fenton reaction system, the addition of sodium formate, which reacts with $OH^{\cdot}$ to produce $CO_2^{\cdot-}$ (8, 27) should have increased ethylene production rather than having no effect.

The effect of bicarbonate on ethylene production from ACC is concentration-dependent, and maximum stimulation of ethylene production was achieved with 200 mM bicarbonate for leaf discs (15), with the chemical system using the Fenton reaction, and with microsomal and thylakoid membranes (Fig. 1). Maximum stimulation of ACC-dependent ethylene production in leaf discs can also be achieved by direct application of 1.5 to 3% CO_2 (18). If this CO_2 were converted entirely to bicarbonate in the system used by Kao and Yang (18), the maximum HCO_3^- concentration would be 33 mM. Since 200 mM HCO_3^- is required for maximal enhancement, it appears that CO_2 is a much more potent stimulator of ACC-dependent ethylene formation than HCO_3^-. At physiological pH and over the pH range employed in the *in vitro* reactions examined in the present study, the equilibrium among CO_2, HCO_3^-, and CO_3^{2-} favors HCO_3^-. Under these conditions, the equilibrium concentration of CO_2 in the head space over 200 mM HCO_3^- would be about 3%. It therefore seems likely that the effect observed with HCO_3^- is attributable to CO_2. This contention is supported by the fact that introduction of only 0.1% CO_2 into the head space above the Fenton reaction enhanced the conversion of ACC to ethylene by about 2-fold.

In view of the apparent role of CO_2 in facilitating the conversion of ACC to ethylene, it is conceivable that the inhibitory effect of light on this conversion in leaf discs reflects partial depletion of endogenous CO_2 pools by photosynthetic fixation. The fact that light-induced inhibition of ACC-dependent ethylene formation can be overcome by addition of bicarbonate supports the contention that the inhibitory effect does reflect

partial depletion of CO_2 pools. The fact that photosynthetic fixation can influence ethylene formation suggests that chloroplasts may also be capable of forming ethylene from ACC. Konze and Elstner (20) have reported that isolated thylakoid membranes produce ethylene from methionine upon illumination by a mechanism contingent upon the formation of ·OH through the Haber-Weiss reaction. In the present study, we demonstrate that illuminated thylakoid membranes also convert ACC to ethylene, and that this conversion can be stimulated by added bicarbonate. However, since this reaction only occurs in the light, it is presumably free radical-mediated and nonenzymic. Bean leaf discs and carnation flowers both responded to exogenous bicarbonate by producing more ACC-dependent ethylene, suggesting that in both instances availability of CO_2 was a rate-limiting factor. By contrast, ACC-dependent ethylene production by etiolated pea epicotyl segments proved to be only marginally responsive to bicarbonate. This may simply reflect a high respiratory activity in this tissue, which would lead to correspondingly high endogenous levels of bicarbonate. Indeed, ACC-dependent ethylene production by microsomal membranes isolated from pea epicotyls, which are in effect removed from endogenous sources of bicarbonate, did prove responsive to exogenous $NaHCO_3$.

In conclusion, $NaHCO_3$ clearly promotes the conversion of ACC to ethylene, presumably by serving as a source of CO_2. This proved to be true for intact tissues; for microsomal membranes, which serve as an *in vitro* model system capable of enzymically converting ACC to ethylene (24, 25); and for chemical model systems able to convert ACC to ethylene through a free radical mechanism (22). Notwithstanding this common response to bicarbonate, the extent to which these model systems are a true simulation of the *in situ* conversion remains unclear (17). Since the conversion of ACC to ethylene is enhanced by HCO_3^- in chemical systems where no enzyme is present, it is conceivable that CO_2 achieves its effect by interacting directly with ACC, perhaps by forming an ACC-carbamate complex (23).

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