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Dependence of *in Vivo* Ethylene Production Rate on 1-Aminocyclopropane-1-Carboxylic Acid Content and Oxygen Concentrations[1]

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ABSTRACT

1-Aminocyclopropane-1-carboxylic acid (ACC) is aerobically oxidized in plant tissues to form ethylene by ethylene-forming enzyme (EFE). The effect of substrate (ACC and oxygen) concentrations on ethylene production rate by plant tissues was investigated. The K_m value for O_2 in ethylene production varied greatly depending on the internal ACC content. When ACC levels in the tissue were low (below its K_m value), the concentration of O_2 giving half-maximal ethylene production rate ($[S]_{0.5}$) ranged between 5 and 7%, and was similar among different tissues. As the concentration of ACC was increased (greater than its K_m value), $[S]_{0.5}$ for O_2 decreased markedly. In contrast, the K_m value for ACC was not much dependent on O_2 concentration, but varied greatly among different plant tissues, ranging from 8 micromolar in apple (*Malus sylvestris* Mill.) tissue to 120 micromolar in etiolated wheat (*Triticum aestivum*) leaf. Such a great variation was thought to be due to the different compartmentation of ACC within the cells in different tissues. These kinetic data are consistent with the view that EFE follows an ordered binding mechanism in which EFE binds first to O_2 and then to ACC.

The conversion of ACC[3] to ethylene in higher plants is carried out by an oxidative enzyme system which is generally referred to as EFE. Because EFE has not yet been isolated independent of intact cellular material (vacuoles, protoplasts, or tissue), all characterization of EFE has been carried out *in vivo* (15). The reaction catalyzed by EFE is shown by the following equation (9):

$$ACC + \tfrac{1}{2}\ O_2 \rightarrow C_2H_4 + CO_2 + HCN + H_2O$$

If the oxidation of ACC to ethylene in higher plants is catalyzed by the same enzyme system, it is expected that its K_m values for ACC and oxygen should be the same among different tissues under specified conditions. However, the reported apparent $[S]_{0.5}$ values of oxygen and ACC varied widely, ranging from 0.2 to 16% for oxygen (1–3, 8), and from 61 to 500 μM for ACC (5, 8, 11, 14), where the ACC concentration refers to the internal concentration of ACC within the tissues or external ACC concentration for the protoplast or vacuole preparations. Although many reports documented the relationship between ethylene production rates and external ACC concentrations in various plant tissues, these values were not considered, because external ACC concentrations do not reflect internal concentrations. Since EFE is a bi-substrate enzyme, the $[S]_{0.5}$ values for oxygen and ACC can vary as the concentration of the other substrate changes. Thus, the variation of $[S]_{0.5}$ values for EFE reported in the literature may have arisen in part from the different experimental conditions employed, namely $[S]_{0.5}$ values for ACC and for O_2 were determined under undefined O_2 and ACC concentrations, respectively.

In this study, we examined the $[S]_{0.5}$ values of oxygen and ACC for EFE in different tissues under defined ACC and O_2 concentrations.

MATERIALS AND METHODS

Plant Materials. Apples (*Malus sylvestris* Mill., var Golden Delicious) were harvested from a local orchard and were stored at 1°C. Apple plugs (1 cm in diameter and 2 cm in length) were prepared with a cork borer and a knife. After floating with shaking in 2% KCl solution for 10 min, plugs were blotted dry and were used in the experiments at room temperature (20°C); they exhibited steady ethylene production rates for at least 3 h.

Mungbean (*Vigna radiata* L.) hypocotyl segments (1 and 3 cm under hook) were prepared from 3-d-old etiolated seedlings grown in paper towel at 25°C in darkness, and used as experimental material.

Wheat (*Triticum aestivum*) seeds were germinated and seedlings grown in vermiculite at 25°C either in light (14 h light/10 h dark cycle) or in darkness for 9 d. Experimental materials consisted of 8-cm long segments cut from the tip of the leaves.

Isolation of Protoplast from Barley Leaves. Barley (*Hordeum vulgare*) seeds were germinated and grown in vermiculite at 25°C in light. After 4 to 5 d, top leaves (3–4 cm) were harvested. The abaxial epidermis of the leaves was peeled off with fine forceps, and the leaves were then floated in a Petri dish with the peeled sides down in 20 mL enzyme solution containing 2% cellulysin (Calbiochem), 1% pectolyase (Seishin Pharmaceutical, Japan), 1% macerase (Calbiochem), and 0.3% dextrin, all dissolved in 0.5 M mannitol (pH 5.5). After 40 to 60 min incubation at 25°C, the remaining debris was spun down with centrifugation at 500 *g* for 1 min. The supernatant was then centrifuged at 2000 *g* for 3 min, and the pellet was washed twice and resuspended with 0.5 M mannitol. The resulting suspension was counted for number of protoplasts on a hemacytometer under a light microscope.

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[3] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; EFE, ethylene forming enzyme which catalyzes the oxidation of ACC to ethylene; $[S]_{0.5}$, the substrate concentration which results in one-half maximal reaction rate; thus, when a reaction follows Michaelis-Menten kinetics, $[S]_{0.5} = K_m$. AIB, α-aminoisobutyric acid.

Estimation of $[S]_{0.5}$ for O_2. Apple plugs were put in 14-mL test tubes, which were sealed with a serum cap, and flushed through 13-gauge needles with a nitrogen or air (500 mL/min) stream for 5 min. After injecting the desired volumes of O_2 or N_2 into the tubes to modify the O_2 concentration, the sealed tubes were incubated for 10 to 15 min. Six or seven different oxygen concentrations were used successively on the same apple plugs, and the whole experiment was usually completed within 1.5 h. Three gas samples were taken at the beginning, the middle, and the end of the incubation period; the first and the last samples were used for ethylene determination, whereas the second sample was used for the O_2 determination. For mungbean hypocotyls, the same method was employed, except that, where indicated, hypocotyls were first preincubated with ACC at various concentrations to increase the endogenous ACC level, blotted dry, and their ethylene production rates under various O_2 concentrations were determined. Excised wheat leaves were floated in 1 mM ACC solution containing 0.01% Triton X-100 for 1 h, blotted dry and their ethylene production rate was determined following the same procedures as for apple plugs.

Estimation of K_m Values for ACC in Plant Tissues. All experiments were performed under air (21% O_2). Apple plugs were further cut into four pieces (0.5 cm in length, 1 cm in diameter), lots of two slices were randomly put into 14-mL test tube containing 0.5 mL of 0, 0.3, 1, 3, or 10 mM ACC in 2% KCl; each experiment was conducted in duplicate. In some experiments, apple plugs were vacuum infiltrated with ACC at various concentrations as described above. After equilibration for 1 h, ethylene production rates during the subsequent 10 min were measured.

Mungbean hypocotyls and wheat leaves were first incubated in different concentrations of ACC for 1 to 2 h, and their ethylene production rates in the subsequent 1 h incubation period were then determined. At the end of the incubation the tissues were washed with cold water for 5 min to remove ACC in the intercellular space and their internal ACC levels were determined as described previously (7). From the measured ethylene production rates and the corresponding internal ACC concentrations, K_m values for ACC were estimated. All experiments for estimation of K_m values for oxygen and ACC were repeated more than once. Although there were some variations between experiments, the same trends and conclusions were obtained. The data reported here were from a typical experiment.

Determination of Ethylene and Oxygen. Ethylene produced in the incubation flasks or tubes was determined by withdrawing a 1-mL gas sample and injecting into a gas chromatograph equipped with an alumina column and a FID detector. The concentration of oxygen was similarly determined with a gas chromatograph equipped with a molecular sieve column and a thermoconductivity detector.

RESULTS AND DISCUSSION

$[S]_{0.5}$ for O_2 in Apple and Other Tissues. The $[S]_{0.5}$ values for oxygen in apple tissue were determined to be 5 to 7% as shown in Figure 1. These values are considerably higher than those reported by Burg and his coworkers (2, 3). Burg and Thimann (3) in 1959 reported a value of 1 to 2% for O_2 where the incubation time was 1 h and the limit of GC sensitivity was 2.5 nL with a 2 mL gas sample. In a later report, Burg (2) gave a lower value of 0.2% for O_2, where the incubation period was 3 to 4 h. It should be pointed out that the ACC content of apple tissues increased under low oxygen concentrations (17) and stimulation of ethylene production by up to sixfold in various plant tissues under hypoxia has been reported (6). Under these conditions, when the internal ACC level is elevated the $[S]_{0.5}$ value can be erroneously underestimated. In order to minimize these possible errors we performed the ethylene production measurements using the same blotted-dry apple plugs which were incubated successively in different O_2 concentrations for a short period (10–15 min), and the O_2 levels were monitored by GC. Similar Michaelis-Menten kinetic curves were obtained when the ethylene production rates were measured either by successively increasing (Fig. 1) or decreasing (data not shown) O_2 concentration.

In etiolated wheat leaves, the endogenous ACC content (40 nmol/g), and ethylene production rate (2 $nmol \cdot g^{-1}h^{-1}$) were so high (7) that $[S]_{0.5}$ could be estimated without providing ACC externally. Their O_2-dependent ethylene production curves appeared to obey the Michaelis-Menten kinetics as revealed by the double reciprocal plots which revealed a K_m value of 5.3% for O_2 (data not shown).

Some previous workers have suggested that a long incubation time is necessary for the tissues to reach equilibrium with atmospheric O_2 in the flask (2, 8). However, according to Cameron (4), the half-time required for apple plugs to reach equilibrium was less than 2 min. When transferred to an atmosphere of 2% oxygen apple plugs which produced ethylene at 0.98 $nL \cdot g^{-1}min^{-1}$ in 21% O_2, exhibited a decline in ethylene production rate to a steady rate of 0.15 $nL \cdot g^{-1}min^{-1}$ steady within 2 min. Conversely, when apple plugs placed in a flask were flushed with N_2 stream (500 mL/min) for 2 min, ethylene production ceased, and when O_2 was introduced to a final concentration of 3.5%, ethylene production was restored and reached 0.4 $nL \cdot g^{-1}min^{-1}$ within 3 min. These observations support the notion that a short period of time is required to reach equilibrium in apple plugs.

Since EFE is a bi-substrate enzyme, the apparent K_m value for O_2 can vary as the ACC level in the tissue varies (13). We have therefore examined the changes in apparent K_m values in the presence of different ACC levels, which were achieved by soaking apple plugs in various concentrations of ACC solution ranging from 0.3 to 10 mM. As the ACC concentrations increased, the K_m values for O_2 as determined from the corresponding slopes of the plots of $1/v$ *versus* 1/[oxygen] decreased (data not shown), confirming the notion that K_m values for oxygen vary with ACC levels. When the exogenous ACC concentration was raised to 10 mM, ethylene production rates were hardly dependent on oxygen concentration. Among the plugs which had been vacuum infiltrated with various concentrations of exogenous ACC, those infiltrated with 1 mM showed maximal ethylene production. A further increase in the exogenous ACC level resulted in a slight decrease in ethylene production. Table I shows the effect of varying ACC concentrations in infiltration solutions on the ethylene production rates under different O_2 atmospheres. Assuming that under 100% oxygen the dependence of ethylene production rate on internal ACC levels follows the Michaelis-Menten equation, the internal ACC concentration can be estimated as $[ACC] = K_m \times v/(V_m - v)$, where v is the ethylene production rate at a given ACC concentration and V_m is the ethylene production rate at a saturating ACC concentration, *i.e.* with 1 mM exogenous ACC concentration. The values for samples preloaded with 0, 0.1, and 0.3 mM exogenous ACC solutions were estimated to be 0.34, 1.6, and 2.2 K_m, respectively. These data indicate that the apparent K_m for oxygen is highly dependent upon ACC concentration.

Similar experiments were performed with mungbean hypocotyls. Since mungbean hypocotyls contained little ACC and produced very little ethylene, external ACC was administered by presoaking the tissues with various concentrations of ACC (Fig. 2). The K_m for oxygen in mungbean hypocotyls preloaded with 1 mM external ACC for 1 h (internal ACC = 30 nmol/g) was estimated to be 5.3% (Fig. 2A), but the values decreased as the concentrations of exogenous [ACC] increased (Fig. 2, B and C). When the internal ACC content was equal to its K_m value (about

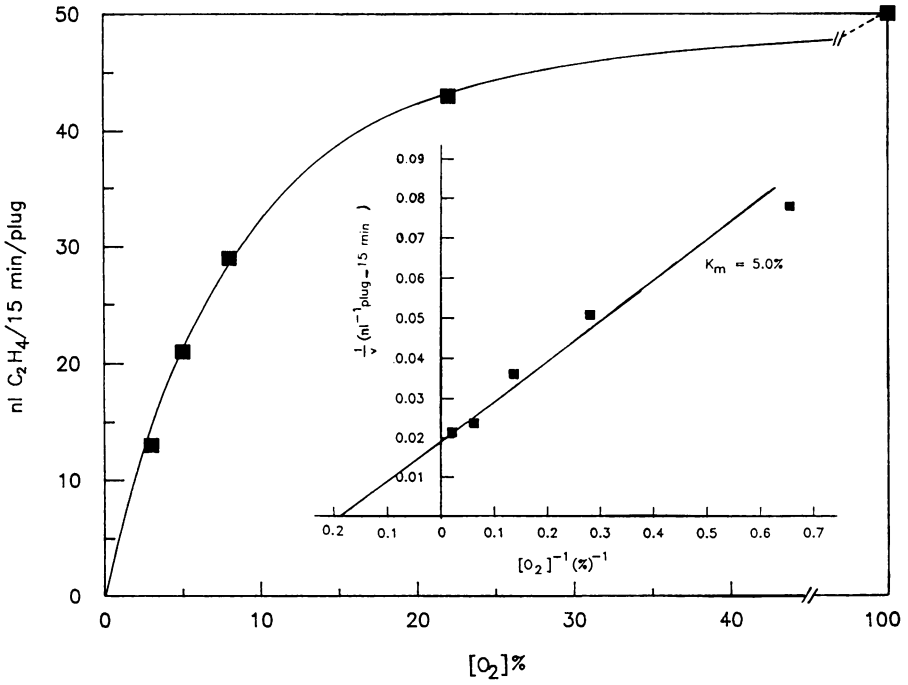

FIG. 1. Dependence of ethylene production on oxygen concentration in apple plug. Apple plugs (1 cm in diameter, 2 cm in length) were enclosed in a 15-mL test tube, and their ethylene production rates were successively determined under increasing oxygen concentrations. Inset is the double reciprocal plot of the same data. Similar results were obtained when ethylene production rates were successively determined under decreasing oxygen concentrations.

Table I. *Effect of Exogenous ACC Concentration on O_2-Dependent Ethylene Production in Apple Plugs*

Apple plugs prepared from a single fruit were vacuum infiltrated with various concentrations of ACC as indicated. One h after infiltration, the ethylene production during the subsequent 10 min under 100, 21, and 3% O_2 was successively determined. The endogenous ACC content in plugs without exogenous ACC infiltration was determined to be 3.3 nmol/g.

Exogenous ACC	C_2H_4 Production (O_2 concentration, %)			Estimated K_m for O_2[a]
	100	21	3	
mM	$nL \cdot g^{-1} h^{-1}$			%
0	86	79	28	6.2
0.1	209	186	109	2.8
0.3	235	186	153	1.6
1	342	346	279	0.7
10	332	337	300	0.3

[a] K_ms for O_2 with different ACC concentrations were estimated from the Michaelis-Menten equation, $K_m = (V_m \times [S]/v) - [S]$, where V_m was assumed to be the rate at 100% O_2, and v, the rate at 3% O_2, and [S] = 3%. Since this estimate is based only on one point, the value should be regarded as a crude measure of K_m.

30 nmol/g, as will be described later), the apparent K_m for oxygen was about 5%.

For the bi-substrate enzymes, it is recognized that an increase in the concentration of one substrate may result in a decrease in apparent K_m of the other substrate (13). In the present study, when the ACC concentration was increased to a higher level, the apparent K_m for O_2 approached zero. These data indicate that the affinity of EFE for oxygen increased markedly as the internal ACC content increased.

It is probable that the lower K_m values for O_2 reported previously by Burg (2, 3) could have resulted from higher ACC content in the apples they employed. An increase in ACC content under hypoxia (6, 17) or under storage (10) has been documented. Thus, a longer incubation period may bring about hypoxia and consequently result in higher ACC content. It should be emphasized that during the storage of apple fruits the ACC content may increase from less than 0.1 to higher than 30 nmol/g (10). Apples are commercially stored under 1 to 4% O_2. Based on our present finding, we may predict that the effectiveness of low O_2 to inhibit ethylene production is greater when the ACC levels in apples are low, and this effectiveness decreases as their ACC levels increase.

Estimation of K_m Values for ACC in Various Plant Tissues. Unless otherwise specified, all the K_m values for ACC were estimated under an atmosphere of 21% O_2. Incubation of most plant tissues in 1 mM exogenous ACC for 1 to 2 h did not result in maximal ethylene production rates, indicating that the ACC levels within their tissues have not attained a saturating level. This is because the incubation period was too short to allow equilibrium between exogenous ACC and the cells. However, in excised green wheat leaves, administration of 0.2 mM exogenous ACC from the cut ends for 2 h resulted in steady and maximal ethylene production rates. In this case ACC was fed through the transpiration stream, and depending on the feeding period the internal ACC concentration could become even higher than that of the external feeding solution.

When barley leaf protoplasts were incubated with various concentrations of external ACC for 2 h, ethylene production rates became maximal at 0.2 mM ACC, and the K_m for ACC was estimated to be 20 μM (data not shown).

The $[S]_{0.5}$ value for ACC in plant tissues has been routinely determined based on the dependence of the ethylene production rate on the internal ACC content following external administration of various ACC concentrations. The $[S]_{0.5}$ for internal ACC was found to be 20 μM (20 nmol/g fresh weight) in green wheat leaves (data not shown), 34 μM in mungbean hypocotyl (Fig. 3), and 16 μM in green oat leaves (14). There are, however, some difficulties in this method: first, it is impossible to ensure all intercellular ACC has been removed before the assay for internal ACC content; second, we can only obtain the concentrations as nmol/g fresh weight, but not as concentration; third, the observed $[S]_{0.5}$ represents the overall value but does not indicate the true value, if ACC (either endogenously produced or exogenously administered) is compartmentalized and thus not evenly distributed within the cells. In some tissues, such as mungbean hypocotyls, the endogenous ACC level can be increased by auxin

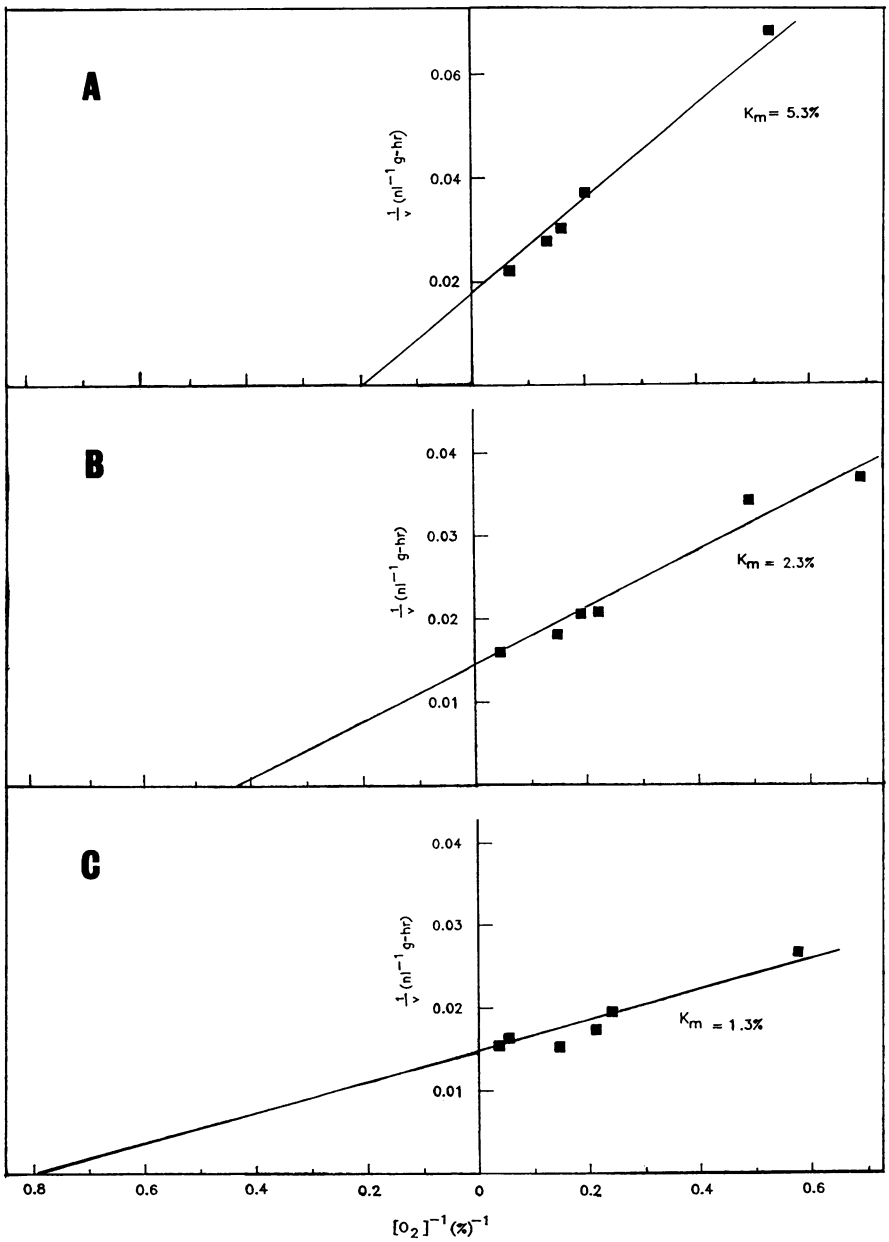

FIG. 2. Double reciprocal plots of ethylene production rate vs oxygen concentration in mungbean hypocotyls. Mungbean hypocotyl segments (2 cm in length) which had been preincubated with ACC were blotted dry, placed in 15-mL test tubes and their ethylene production was determined successively at different oxygen concentrations. A, Hypocotyls were preloaded with 1 mM ACC for 1 h, resulting in an internal ACC level of 30 nmol/g; B, hypocotyls were preloaded with 10 mM ACC for 2 h, resulting in an internal ACC content of 335 nmol/g; C, hypocotyls were preincubated with 30 mM ACC for 1.5 h, resulting in an internal ACC content of 1768 nmol/g.

treatment (16), which increases ethylene production by increasing the synthesis of ACC without influencing EFE. In one experiment, we incubated mungbean hypocotyls with 0.5 mM IAA + 10 μM benzyladenine for 3 h, and their ethylene production rate (v) and internal ACC content ([S]) were determined. In order to determine the ethylene production rate under saturating ACC concentration (V_m), the segments were incubated with various external ACC concentrations up to 30 mM to obtain the V_m value. Assuming that their ethylene production rates (v) in relation to their corresponding internal ACC concentration ([S]) follows the Michaelis-Menten equation, $v = V_m[S]/(K_m + [S])$, the K_m value for ACC can be calculated when [S] and V_m values are known. We obtained a K_m value of 25 μM. Yoshii and Imaseki (16) treated mungbean hypocotyls with various IAA concentrations and determined the relationship between ethylene production rates and internal ACC levels. Based on their data, a K_m value of 35 μM for ACC was calculated.

In apples, we estimated the K_m value for ACC by taking advantage of their high ethylene production rate and the various endogenous ACC contents among different apples. It should be noted that different fruits may differ not only in ACC content but also in EFE activity, which is directly related to V_m. Thus, for each apple, we assayed its ethylene production rate (v), its endogenous ACC level ([S]) and V_m; V_m was estimated by providing each apple plug with saturating levels of ACC either by soaking with 5 mM of external ACC for 1 h or by vacuum infiltration with 0.5 mM ACC. When the Michaelis-Menten equation is rearranged in the form of $V_m/v = K_m/[S] + 1$, a plot of V_m/v against 1/[S] for different apples should result in a straight line, the slope of which is the K_m. In this experiment we employed 13 different apples, which were from three different sources, with internal ACC levels ([S]) ranging from 1.1 to 14.1 nmol/g, ethylene production rates (v) from 34 to 196 nL$\cdot$g^{-1}h^{-1} and V_m from 72 to 637 nmol$\cdot$g^{-1}h^{-1}. Such a plot for these 13 different apples is shown in Figure 4, which reveals a K_m of 8.1 μM. These results indicate that though the quantity of EFE or V_m varied widely among different fruits, their K_m for ACC remains constant.

The present study and others indicate that the observed K_m values for ACC in different tissues varied somewhat among

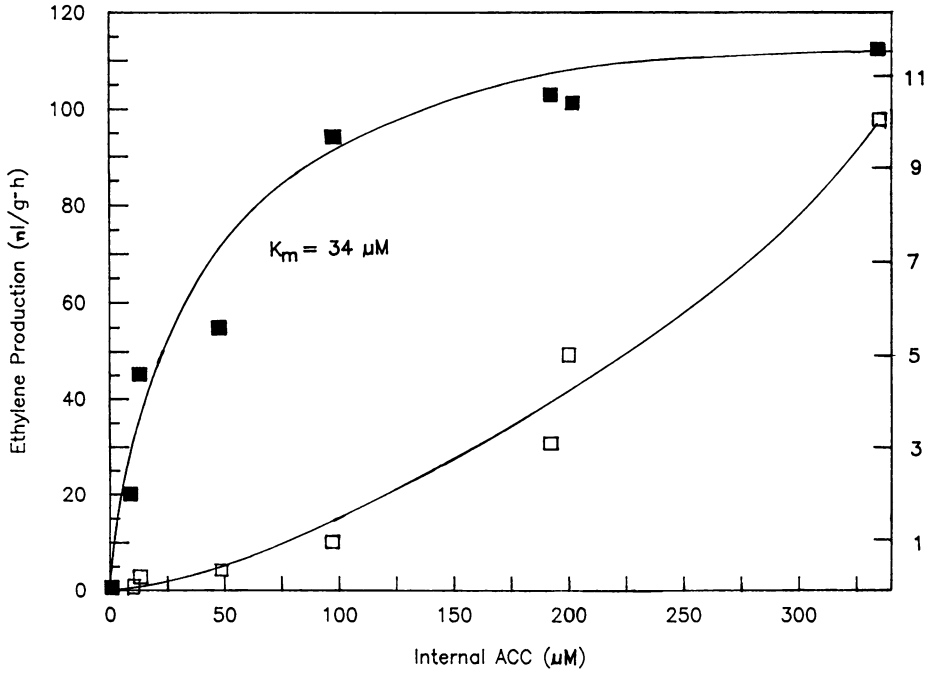

FIG. 3. Dependence of ethylene production rates on ACC contents in mungbean hypocotyls. Mungbean hypocotyls which had been incubated with 0, 0.1, 0.3, 1, 3, and 10 mM ACC for 2 h were assayed for their ethylene production rates and internal ACC contents. The dependence of ethylene production rates (■) on internal ACC concentration and the relationship between internal and external ACC (□) are shown.

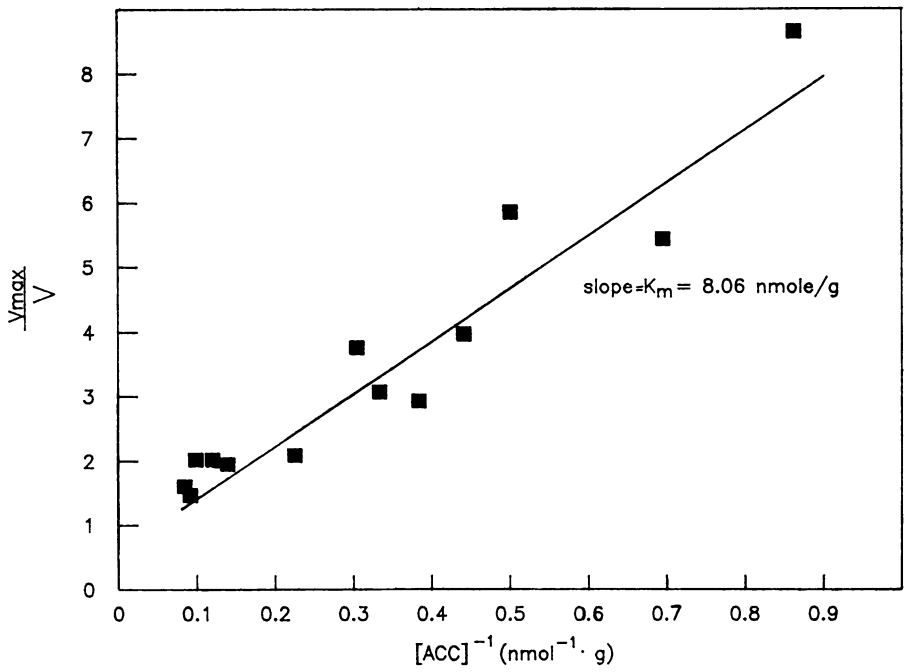

FIG. 4. V_{max}/v *versus* 1/[ACC] plot for 13 different apples.

various tissues: 14 to 20 μM in leaf tissues (14); 25 to 35 μM in mungbean hypocotyls (Fig. 3 and Ref. 16); 8 μM for apple (Fig. 4), 66 μM for pea epicotyls (11), 20 μM for barley protoplast (data not shown), and 61 μM for pea leaf vacuoles (5). If we assume that the real K_m values for EFE with respect to ACC in different tissues are the same, then these different K_m values for ACC observed among different tissues could be due to different subcellular compartmentation of ACC with respect to EFE. In mungbean hypocotyls, the K_m (25–35 μM) values estimated from the tissues where ACC synthesis was induced endogenously by auxin treatment are similar to the K_m (34 μM) values observed in those hypocotyls where ACC was supplied exogenously. In this case the exogenously administered ACC and endogenously synthesized ACC are probably similarly compartmented with respect to EFE. However, this was not the case in etiolated wheat leaves, where the K_m for ACC was estimated to be 120 μM (data not shown), which was 10 times higher than that in apples. One explanation is that most of the ACC accumulated in etiolated wheat leaves (7) is localized inside the vacuole (5), and thus becomes unavailable to interact with EFE. In contrast, O_2 diffuses readily into the cells, and there is no compartmentation problem. Although etiolated wheat leaves exhibited a very high K_m value for ACC, its K_m value for oxygen is similar to those of other tissues.

General Discussion. In a bi-substrate enzyme, an increase in the concentration of one substrate could result in a decrease in the apparent K_m of the other substrate (13). In the present study, we have shown that when the O_2 concentration was increased from 21 to 100%, there was little influence on the apparent K_m for ACC. However, when the ACC concentration was increased

Table II. *Effect of EFE Inhibitors on O_2-Dependent Ethylene Production*

Apple plugs were infiltrated with 50 μmol AIB or 5 μmol $CoCl_2$ where indicated. After 2 h, tissues were incubated successively under 3, 21, and 100% of O_2 for 5 min and ethylene produced was determined. The data represent mean ± SE of four replicates. K_m values were estimated as described in Table I.

	C_2H_4 Production (O_2 concentration, %)			Estimated K_m for O_2
	100	21	3	
	$nL \cdot g^{-1} h^{-1}$			%
Experiment 1				
Control	88 ± 5	73 ± 4	28 ± 2	6.4
AIB	33 ± 5	31 ± 4	22 ± 2	1.5
Experiment 2				
Control	109 ± 3	87 ± 3	34 ± 3	6.6
Co^{2+}	24 ± 5	19 ± 5	7 ± 2	7.3

to a high level, the apparent K_m for O_2 decreased greatly and approached zero. These data indicate that the affinity of EFE for oxygen increased markedly, when the internal ACC content increased. These data conform to an ordered bi-substrate mechanism in which oxygen binds to EFE before ACC binds. Such a reaction scheme is further supported by the observations that administration of AIB, a competitive inhibitor of EFE with respect to ACC (12), resulted in a lowered ethylene production rate and a lower apparent K_m for oxygen (Table II). These results are fully expected if oxygen first binds to EFE forming EFE-O_2 complex, which then reacts with AIB or ACC. Thus, the presence of AIB or ACC facilitates the formation of the EFE-O_2 complex and consequently lowers the K_m for oxygen, but AIB inhibits ethylene production because AIB competitively inhibits the binding of ACC to the EFE-O_2 complex. In contrast, application of Co^{2+}, an inhibitor of EFE but structurally unrelated to ACC (15), did not result in lowering the K_m value for O_2. Confirmation of such a reaction mechanism awaits the isolation of cell-free EFE. Although our kinetic data are in agreement with an ordered bi-substrate mechanism, other more complicated mechanisms cannot be ruled out.

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