

# Cyanide Metabolism in Relation to Ethylene Production in Plant Tissues<sup>1</sup>

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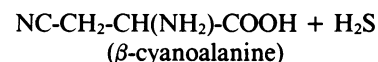
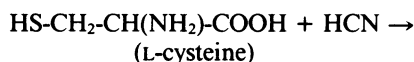
## ABSTRACT

HCN is the putative product of C-1 and amino moieties of 1-aminocyclopropane-1-carboxylic acid (ACC) during its conversion to ethylene. In apple (*Malus sylvestris* Mill.) slices or auxin-treated mungbean (*Vigna radiata* L.) hypocotyls, which produced ethylene at high rates, the steady state concentration of HCN was found to be no higher than 0.2 micromolar, which was too low to inhibit respiration (reported  $K_i$  for HCN to inhibit respiration was 10–20 micromolar). However, these tissues became cyanogenic when treated with ACC, the precursor of ethylene, and with 2-aminoxyacetic acid, which inhibits  $\beta$ -cyanoalanine synthase, the main enzyme to detoxify HCN; the HCN levels in these tissues went up to 1.7 and 8.1 micromolar, respectively. Although ethylene production by avocado (*Persea gratissima*) and apple fruits increased several hundred-fold during ripening,  $\beta$ -cyanoalanine synthase activity increased only one- to two-fold. These findings support the notion that HCN is a co-product of ethylene biosynthesis and that the plant tissues possess ample capacity to detoxify HCN formed during ethylene biosynthesis so that the concentration of HCN in plant tissues is kept at a low level.

Plant hormone ethylene is produced by all plants and in trace amounts elicits many physiological responses. Ethylene is biosynthesized in plants via the following sequence: methionine  $\rightarrow$  S-adenosylmethionine  $\rightarrow$  1-aminocyclopropane-1-carboxylic acid  $\rightarrow$  C<sub>2</sub>H<sub>4</sub> (19). In the biological oxidation of ACC<sup>2</sup> to ethylene, it has been shown that carboxyl carbon, C-1, and C-2,3 of ACC are metabolized into CO<sub>2</sub>,  $\beta$ -cyanoalanine derivatives, and ethylene, respectively (11, 12). Although no free HCN was identified, it was asserted that C-1 of ACC is initially liberated as HCN but is rapidly conjugated into  $\beta$ -cyanoalanine derivatives as soon as it is liberated. This notion was based on the observation that the metabolic fate of the C-1 of ACC during its conversion to ethylene was identical to that of administered HCN and that the amount of HCN-conjugates formed was equivalent to that of ethylene produced (11). Thus, the degradation of ACC into ethylene can be represented by the following equation: ACC +  $\frac{1}{2}$  O<sub>2</sub>  $\rightarrow$  C<sub>2</sub>H<sub>4</sub> + HCN + CO<sub>2</sub> + H<sub>2</sub>O. Since no free HCN was detected even in plant tissues which produced ethylene at very high rates, Peiser *et al.* (11) suggested that plants must have ample capacity to metabolize the HCN originating from ACC.

In higher plants, the key enzyme to detoxify HCN is  $\beta$ -

cyanoalanine synthase (EC 4.4.1.9), which catalyzes the following reaction:



$\beta$ -Cyanoalanine synthase is widely distributed in higher plants (9).  $\beta$ -Cyanoalanine thus formed is further metabolized to asparagine or to  $\gamma$ -glutamyl- $\beta$ -cyanoalanine (1–4, 6, 9, 11, 12).  $\beta$ -Cyanoalanine synthase has been purified about 4000-fold from blue lupin seedlings by Akopyan *et al.* (1); this enzyme is pyridoxal dependent and can be inhibited by AOA or 3-aminoxypropionic acid.

In this investigation, we have demonstrated that HCN does accumulate in a plant tissue which actively converts ACC to ethylene, when the conjugation of HCN to  $\beta$ -cyanoalanine is inhibited by AOA. By employing an isotope dilution method, we estimated that the steady state concentration of HCN in the tissues which actively produce ethylene was no higher than 0.2  $\mu$ M.

## MATERIALS AND METHODS

**Plant Materials.** Apple (*Malus sylvestris* Mill., var Golden Delicious) slices (1 cm in diameter, 0.5 cm in length) were prepared from fruits harvested from a local orchard and stored at 0°C. Avocado (*Persea gratissima*) were purchased from the local market. Mungbean (*Vigna radiata* L.) hypocotyl segments (2 cm long) were prepared from 3-d-old etiolated seedlings grown at 25°C.

**Incubation Conditions.** Apple slices were presoaked in 2% KCl solution containing specified chemicals in a 50-mL flask for 2 h, blotted dry, and then incubated at 25°C in a 10-mL flask sealed with a serum cap. Each flask contained a plastic center well with 200  $\mu$ L of 0.1 N NaOH to trap the released HCN. Mungbean hypocotyls were similarly treated as apple slices except that they were preincubated in 5 mL 50 mM Mes buffer (pH 6.1) and blotted dry before incubation.

**Determination of Ethylene.** One ml gas sample was withdrawn from the flask and assayed with a GC (11).

**Colorimetric Determination of HCN.** HCN was analyzed mainly by a modified method developed by Lambert *et al.* (7). To 200  $\mu$ L of trap solution (0.1 N NaOH) were added 100  $\mu$ L 1 M acetic acid, 1 mL 0.25% succinimide/0.025% N-chlorosuccinimide reagent, and 200  $\mu$ L 3% barbituric acid in 30% pyridine, and the reaction mixtures were then shaken vigorously. After 10 min, the absorbance at 580 nm was determined. This method can measure HCN down to 0.1 nmol. In a second method developed by Guilbault and Kramer (5), cyanide was measured based on its catalytic activity to form a purple compound by reacting with *p*-nitrobenzaldehyde and *o*-dinitrobenzene. To 50

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<sup>2</sup> Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AOA, 2-aminoxyacetic acid; EFE, ethylene forming enzyme; [I]<sub>0.5</sub>, the inhibitor concentration that inhibits 50% of the enzyme activity.

$\mu\text{L}$  trap solution (0.5 N NaOH), 500  $\mu\text{L}$  0.1 M *p*-nitrobenzaldehyde (dissolved in Cellosolve) and 500  $\mu\text{L}$  0.1 M *o*-dinitrobenzene (dissolved in Cellosolve) were added. Cyanide content was estimated by following the rate of increase in absorbance at 560 nm in a spectrophotometer.

**Estimation of Tissue [HCN] by Isotopic Dilution.** Radioactive NaCN (0.8 nmol, 38 nCi) was added to the tissues, which were then homogenized with water. After centrifugation at 5000g for 2 min, the supernatant was transferred into a 25 ml flask, which was then sealed with a rubber serum cap fitted with a plastic center well containing 200  $\mu\text{L}$  of 0.1 N NaOH solution. The supernatant was acidified to pH 1 by the injection of  $\text{H}_2\text{SO}_4$  solution. The flasks were kept at room temperature for 24 h to allow HCN to be distilled into the NaOH trap solution. Ten  $\mu\text{L}$  of the trap solution was assayed for its radioactivity by scintillation counting, and the remaining solution was employed for the HCN assay by the modified Lambert method as described above. The amount of HCN initially present in the tissue homogenate was calculated from the following equation: the amount of HCN in the tissue = (the amount of labeled NaCN added)  $\times$  ( $C_o - C$ )/ $C$ , where  $C_o$  is the specific radioactivity of NaCN added to the tissue, and  $C$  is the specific radioactivity of HCN recovered in the trap. In order to improve the recovery of HCN during the homogenization, 10 mM of AOA solution was used as the homogenization medium to inhibit further metabolism of HCN, and the homogenate was kept at low temperature to minimize the loss of HCN into the gas phase. Normally about 10 to 20% of HCN was recovered with this procedure.

**Assay of  $\beta$ -Cyanoalanine Synthase Activity in Apple.** Apple tissue was homogenized with 2.5 ml 0.1 M Tris buffer (pH 8.5) per g tissue. After centrifugation at 10,000g for 10 min, the supernatant was employed for the enzyme assay. The enzyme assay was modified from Miller and Conn's method (9). The reaction was performed in a sealed 14-mL test tube containing 0.2 mL enzyme solution, 0.8 mL Tris buffer (0.1 M, pH 8.5), 25 mM L-cysteine, and 25 mM NaCN in a total volume of 1 mL. After incubation at 35°C for 30 min, the reaction was stopped by injecting 100  $\mu\text{L}$  20 mM *N,N*-dimethyl-*p*-phenylenediamine in 7.2 N HCl and 100  $\mu\text{L}$  30 mM  $\text{FeCl}_3$  in 1.2 N HCl through the serum cap. The color developed due to the presence of  $\text{H}_2\text{S}$  was read at 650 nm, using  $\text{Na}_2\text{S}$  as the standard.

## RESULTS AND DISCUSSION

Apple slices were prepared from apple fruits which produced ethylene at a high rate (2–8 nmol/g·h). Thus, if apple tissue is not capable of detoxifying HCN and if this HCN does not diffuse out of the tissue, HCN concentration within the tissue would increase at a rate of 2 to 8  $\mu\text{M}/\text{h}$ . It should be noted that the  $K_i$  value of HCN for the HCN-sensitive enzymes, such as Cyt *c* oxidase, are in the 10  $\mu\text{M}$  range (16). Since the  $\text{pK}_a$  of HCN is 9.3, the cyanide in plant tissues exists predominantly in the form of HCN, which is also volatile. As shown in Table I, when apple slices were presoaked in the absence or presence of AOA or ACC, no HCN was trapped from the gas space of the incubation flasks. However, a significant amount of HCN was recovered when the tissues were treated with both AOA and ACC. These results are in accord with the current notion about ACC metabolism in apple. Postclimacteric apple fruit tissue is characterized by its high ACC turnover rate because apple fruit produces ethylene at high rate (2–8 nmol/g·h), while maintaining a relatively low ACC level (2–10 nmol/g). Thus, a sustained high rate of ethylene production requires continuous ACC synthesis (19). Although AOA inhibits the conversion of HCN into  $\beta$ -cyanoalanine by inhibiting  $\beta$ -cyanoalanine synthase (1), HCN was not detected in the gas phase. This can be explained on the basis that AOA also strongly inhibits the conversion of SAM to ACC (20), which serves as the donor of both ethylene and HCN. In contrast,

Table I. Effect of AOA and ACC Treatments on Ethylene Production and HCN Accumulation in Apple Slices and Mungbean Hypocotyls

For each treatment, eight apple slices weighing 2 g were presoaked for 2 h in 2% KCl (control) or 2% KCl containing AOA (5 mM), ACC (1 mM), or AOA (5 mM) + ACC (1 mM), as indicated. The slices were blotted dry and transferred to enclosed flasks fitted with center wells containing NaOH. After incubation for 2 h, the ethylene accumulated in the gas phase and the HCN trapped in the NaOH solution were assayed. HCN concentration in the tissue was estimated by the isotope dilution method. For the mungbean hypocotyls, conditions were the same as for apple slices except that 10 segments (0.9 g) each were presoaked in 5 ml 50 mM Mes buffer (pH 6.1) containing AOA (5 mM), ACC (10 mM), or AOA (5 mM) + ACC (10 mM) and were incubated for 4 h.

| Treatment          | $\text{C}_2\text{H}_4$ | HCN             |           |
|--------------------|------------------------|-----------------|-----------|
|                    |                        | In gas phase    | In tissue |
|                    |                        | <i>nmol/g</i>   |           |
| Apple              |                        |                 |           |
| Control            | 7                      | ND <sup>a</sup> | 0.2       |
| AOA                | 0.2                    | ND              | 0.1       |
| ACC                | 18                     | ND              | 0.2       |
| AOA + ACC          | 14                     | 1.6             | 1.7       |
| Mungbean hypocotyl |                        |                 |           |
| Control            | 0.1                    | ND              | ND        |
| AOA                | 0.02                   | ND              | ND        |
| ACC                | 24                     | 0.3             | 1.9       |
| AOA + ACC          | 17                     | 2.4             | 8.1       |

<sup>a</sup> Not detectable or less than 0.1 nmol of HCN in the trap.

when exogenous ACC was administered along with AOA, high ethylene production was maintained, and the tissue became cyanogenic as indicated by the recovery of HCN from the gas phase. In the presence of exogenous ACC, ethylene production was enhanced, but the apple tissue remained noncyanogenic (Table I), presumably because the tissue had ample capability to detoxify the HCN produced during ethylene production.

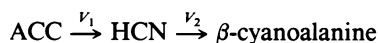
By employing the isotope dilution method, we determined the HCN concentration in apple slices and in mungbean hypocotyls after the treatments with AOA and/or ACC. Table I also shows the relationship between ethylene production, HCN released into the gas phase, and HCN remaining in apple tissue following various treatments for 2 h. Control apple slices released no HCN, and their internal [HCN] was estimated to be no higher than 0.2  $\mu\text{M}$ , whereas ACC-plus-AOA treated slices released a significant amount of HCN, and the tissue [HCN] increased to 1.7  $\mu\text{M}$ . Similar results were obtained in mungbean hypocotyls. Following the treatment with 10 mM ACC, ethylene production increased 200-fold and the amount of HCN became detectable in the gas phase as well as in the tissue. This trend became more pronounced when mungbean hypocotyls were treated simultaneously with both AOA and ACC (Table I), where the tissue [HCN] went up to 8.1  $\mu\text{M}$ . In order to verify the validity of the Lambert's method we employed, we compared the amount of HCN recovered from mungbean hypocotyls as determined by the Lambert's method and that determined by the method developed by Guilbault and Kramer (5). Since the HCN values obtained from these two independent methods agreed well, we assumed that our HCN assays were valid.

We have also examined the effect of AOA and ACC on the  $\beta$ -cyanoalanine synthase activity extracted from the apple tissue. As expected, AOA significantly inhibited the enzyme activity with an  $[\text{I}]_{0.5}$  of about 0.1 mM. Moreover, ACC at high concentration also exhibited an inhibitory effect on the enzyme activity; at 10 mM the inhibition was about 50% (data not shown).

In order to examine the changes in the capability to detoxify

HCN during the ripening process, we followed the changes in extractable  $\beta$ -cyanoalanine synthase in avocado fruit at different ripening stages, and the results are shown in Table II. In spite of the fact that ethylene increased 1000-fold at the climacteric stage,  $\beta$ -cyanoalanine synthase activity only doubled; no HCN was detected in the gas phase or in the tissue. Similar results were reported in senescing carnation flower by Manning (8) who observed that both EFE and  $\beta$ -cyanoalanine synthase activities increased during the flower senescence; however, the increase in ethylene production was several hundred-fold, whereas the increase in  $\beta$ -cyanoalanine synthase activity was only two-fold. These observations suggest that these plant tissues possess a high basal level of  $\beta$ -cyanoalanine synthase, which is capable of detoxifying HCN that is generated during ethylene production from ACC.

Since plant hormone ethylene is produced by all plants, and cyanide is a co-product of ethylene biosynthesis, it is not surprising that  $\beta$ -cyanoalanine synthase is not confined to cyanogenic plants but occurs widely throughout the plant kingdom. A major source of cyanide is from the hydrolysis of cyanogenic glycosides in those plant species which accumulate them. For those plant tissues that do not accumulate cyanogenic glycosides, but produce ethylene at a high rate, ethylene biosynthesis can be the major source of HCN (17). Hence, when the ethylene production rate and the capability to detoxify HCN in a tissue are known, it is possible to estimate the steady state [HCN] level within the tissue. The overall reaction is shown below:



where  $v_1$  = HCN production rate = C<sub>2</sub>H<sub>4</sub> production rate, and  $v_2$  = the rate of HCN metabolism to  $\beta$ -cyanoalanine. Assuming that the rate of HCN metabolism follows the Michaelis-Menten kinetics, then

$$v_2 = \frac{V \times [\text{HCN}]}{K_m + [\text{HCN}]},$$

where  $V$  is the maximum rate of HCN metabolism at saturating concentration of HCN and is therefore related to the amount of  $\beta$ -cyanoalanine synthase. The  $K_m$  for  $\beta$ -cyanoalanine synthase *in vitro* has been estimated to be about 0.5 mM (1, 6). Since this  $K_m$

Table II. Changes in Ethylene Production Rate, Tissue Cyanide Content, and Extractable  $\beta$ -Cyanoalanine Synthase Activity in Avocado Fruits at Different Stages of Ripening

| Stage of Ripening | Ethylene      | HCN             | $\beta$ -Cyanoalanine Synthase Activity |
|-------------------|---------------|-----------------|---|
|                   | nmol/g·h      | nmol/g          | nmol/mg protein·h                       |
| Prelimacteric     | 0.004 ± 0.004 | ND <sup>a</sup> | 159 ± 14                                |
| Climacteric peak  | 4.25 ± 1.08   | ND              | 282 ± 27                                |
| Postclimacteric   | 1.75 ± 0.21   | ND              | 139 ± 5                                 |

<sup>a</sup> Not detectable or less than 0.1 nmol HCN/g.

Table III. Steady State HCN Levels in Unripe and Ripe Apples

The values of [HCN] calculated = 500  $\mu$ M  $\times$   $v_1/V$ ; see the text for detail. The values of [HCN] observed represent the determined HCN concentrations in the tissue; the methods employed were as those described in Table I.

| Stage  | C <sub>2</sub> H <sub>4</sub> ( $v_1$ ) | Cyanoalanine Synthase ( $V$ ) | $v_1/V$ | [HCN] Calculated | [HCN] Observed |
|--------|---|-------------------------------|---------|------------------|----------------|
|        | nmol/g·h                                |                               |         | $\mu$ M          |                |
| Unripe | 0.01                                    | 630                           | 1:63000 | 0.01             | <0.1           |
| Ripe   | 3.3                                     | 1650                          | 1:500   | 1.0              | 0.2            |

value is very large in relation to [HCN] observed in plant tissues (Table I), the relation becomes  $v_2 = V \times [\text{HCN}]/K_m$ . At steady state,  $v_1 = v_2 = V \times [\text{HCN}]/K_m$ . By rearrangement, we obtain

$$[\text{HCN}] = (v_1/V) \times K_m.$$

The above equation predicts that as the ethylene production rate increases, [HCN] will increase linearly, if  $V$  (or the amount of  $\beta$ -cyanoalanine synthase) and  $K_m$  remain unchanged.

Various enzymes are known to be sensitive to HCN inhibition (14), including cytochrome oxidase. The concentration of HCN which gives 50% inhibition of cyanide-sensitive respiration in plant tissues has been estimated to be 10 to 20  $\mu$ M (16). Thus, for the plant tissue to maintain [HCN] below the safe level of 1  $\mu$ M,  $V$  ( $\beta$ -cyanoalanine synthase activity) should be at least 500 times larger than  $v_1$  (ethylene production rate), if the *in vivo*  $K_m$  value for  $\beta$ -cyanoalanine synthase is similar to the reported *in vitro* value of 500  $\mu$ M. In the experiment of Table III with a ripe apple fruit, the *in vivo*  $v_1$  (ethylene production rate) was 3.3 nmol/g·h, whereas *in vitro*  $V$  (extractable  $\beta$ -cyanoalanine synthase activity) was 1650 nmol/g·h. By applying the above equation, [HCN] can be estimated to be 1.0  $\mu$ M, which is 5 times higher than the [HCN] value of 0.2  $\mu$ M experimentally determined (Table III). This discrepancy indicates that either the *in vivo*  $K_m$  value for  $\beta$ -cyanoalanine synthase was much lower than the *in vitro* value of 500  $\mu$ M or the *in vitro*  $\beta$ -cyanoalanine synthase activity was underestimated due to the inactivation or incomplete recovery of the enzyme activity during the extraction procedures.

It should be pointed out that the estimated [HCN] represents the average concentration in the tissue. It is generally thought that ethylene is synthesized in the tonoplast or plasma membrane (19), whereas  $\beta$ -cyanoalanine synthase is localized mainly in mitochondria (1, 17). Thus, the different compartmentation of these two enzymes would result in uneven distribution of HCN within the cell.

Based on an early hypothesis of Solomos and Laties (15) that ethylene induces the cyanide-resistant respiration and this cyanide-resistant respiration operates in ripening fruits, several authors (10, 13) have recently proposed that the increased ethylene biosynthesis during fruit ripening may result in increased HCN level, which in turn inhibits cytochrome oxidase and triggers cyanide-resistant respiration. However, the present study shows that the HCN level in ripening fruits does not increase to a level sufficient to inhibit cytochrome oxidase. Recent work from Laties and his associates (16, 18) has revealed that the respiration in preclimacteric as well as climacteric avocado fruit is mediated by the cytochrome respiratory path and that there is no engagement of the cyanide-resistant path in either tissue. Thus, the hypothesis that ethylene biosynthesis participates in the induction of the cyanide-resistant respiration as advanced by Pirrung and Brauman (13) is not supported by experimental evidence. Recently, Mizutani *et al.* (10) also determined the cyanide content in apple fruit by oxidizing the cyanide in the extract to BrCN and measuring the BrCN by GC. Although they noted that the method was not specific, they reported that the cyanide content in apple fruit during development ranged from 4  $\mu$ g/g (0.15 mM) to 8  $\mu$ g/g (0.3 mM). Since these levels are 10 times higher than the  $K_i$  value (10  $\mu$ M) for HCN to inhibit cytochrome-mediated respiration, the respiration in preclimacteric and climacteric apple tissues would have proceeded via the cyanide-resistant path. However, the respiration in fruit tissues has been shown to be cyanide sensitive (16, 18). This observation invalidates the results reported by Mizutani *et al.* (10).

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