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Ethylene Biosynthesis

3. Evidence Concerning the Fate of C1–N1 of 1-Aminocyclopropane Carboxylic Acid[1]

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Evidence has been obtained that the product of ethylene biosynthesis from 1-aminocyclopropane carboxylic acid (ACC), aside from CO_2, is cyanide. This has been accomplished by synthesis of a carbon-13-labeled ACC, feeding to apple tissue, and isolation of the free amino acids. By enzymatic degradation to CO_2, followed by mass spectrometry, label is shown to be incorporated into the 4-carbon of asparagine. This occurs via well-known pathways for the metabolism of cyanide, and is confirmed by incorporation experiments using labeled cyanide. In conjunction with previous stereochemical studies, evidence for a sequential single-electron transfer pathway for ethylene biosynthesis has been considerably strengthened.

Ethylene is a plant hormone involved in regulating a number of important physiological processes including ripening, senescence, abscission, and germination. As such, its biosynthesis has received a great deal of attention. While methionine had been known as an ethylene precursor for some time, Yang has been a major contributor to filling in the gaps in its conversion to ethylene (*1*). He has shown it is first adenosylated and then is converted by a pyridoxal-dependent enzyme to 1-aminocyclopropane carboxylic acid (ACC). The latter conversion is the control point for the biosynthesis of the hormone, since the oxygen-requiring conversion of ACC to ethylene proceeds rapidly in all plant tissues. Yang's original work which proved ACC to be the ethylene precursor (*2*) established that C-2 and C-3 become ethylene, and it had been known that the carboxyl of methionine is released as CO_2. This is presumably also true of the carboxyl of ACC. Amazingly, the fate of C-2 of methionine (C-1 of ACC) had not been determined, though a lore developed that it was converted to formate and that the nitrogen was found as ammonia.

Yang was required to account for this in his work, and proposed a rather unlikely mechanism involving the nucleophilic opening of ACC by hydrogen peroxide at C-1 to produce a carbanion. He later proposed a nitrenium ion intermediate as well (*1*), which would undergo concerted fragmentation to ethylene and cyanoformate. The latter pathway has been probed in a purely chemical system

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(*3*) and seems reasonable, but the loss of stereochemistry in the biosynthetic reaction (*4, 5*) cannot be reconciled with a concerted reaction. However, hypochlorite oxidation of ACC yields ethylene with retention of stereochemistry (*4*). It is reasonable, though not proven, that the bleach oxidation involves the nitrene and that it does fragment concertedly. An electrochemical model reaction has also been developed which yields the same loss of stereochemistry as in the natural system and converts C-1 of ACC to cyanide (*5*).

This article reports the synthesis of C-1-labeled ACC and its evaluation in ethylene biosynthesis in apple, a generative tissue. Similar to results recently independently obtained by Walsh and Yang in mung bean and vetch (*6*), vegetative tissues, it is concluded that C1–N1 of ACC are indeed converted to cyanide during ripening ethylene biosynthesis, and that it is incorporated into the amino acid asparagine. The determination here relies on a coupled enzymatic degradation/GC/MS analysis which is complementary to the radiotracer/chemical degradation methodology used by Walsh and Yang.

MATERIALS AND METHODS

General. NMR spectra were recorded on Varian XL-100 and Nicolet NMC-300 spectrometers. Chemical shifts are given relative to internal $CHCl_3$ (7.25 ppm) or HOD (5.20 ppm). Ether was distilled from sodium/benzophenone prior to use. DMSO was vacuum-distilled from CaH_2. Washington red delicious apples were obtained locally. Sodium [^{13}C]cyanide and DL-[4-^{13}C]aspartic acid (99 and 90% enriched, respectively) were obtained from Merck Isotopes. Asparaginase, glutamate-oxaloacetate transaminase, 2-ketoglutaric acid, β-NAD, and formate dehydrogenase were obtained from Sigma. All solutions were degassed by four freeze–pump–thaw cycles prior to the addition of enzyme. Mass spectral analysis was conducted on a Hewlett–Packard Model 5995 GC/MS equipped with 6 ft × $\frac{1}{8}$ in. 80% Porapak N/20% Porapak Q steel column and using He as carrier gas.

[*2-^{13}C*]*Ethyl glycinate hydrochloride.* This was obtained from [2-^{13}C]glycine (90% enriched, KOR isotopes) in 67% yield on a 13-mmol scale by following the procedure of Ellsworth (*7*). ^{1}H NMR (D_2O): δ 4.65 (2H, q, J = 6.8), 4.27 (2H total, 10%s, 90%d, J = 142.8), 1.67 (3H, t, J = 6.8).

N-[*2-^{13}C*]*Formyl ethyl glycinate.* This was obtained from the ester above by the procedure of Weinstock (*8*) on a 9-mmol scale in quantitative yield after Kugelrohr distillation (1 Torr, 180°C). ^{1}H NMR ($CDCl_3$): δ 8.26 and 8.22 (1H, brs), 4.23 (2H, q, J = 7.1), 4.11 (2H total, 10%d, J = 5.3, 90% dd, J = 5.2, 141.2), 1.28 (3H, t, J = 7.1).

[*2-^{13}C*]*Ethyl isocyanoacetate.* This was prepared from the formyl compound above by the procedure of Weinstock. A 62% yield was obtained on a 9-mmol scale after Kugelrohr distillation (18 Torr, 150°C). ^{1}H-NMR ($CDCl_3$): δ 4.28 (2H, q, J = 7.1), 4.20 (2H, 10%s, 90%d, J = 146.0), 1.32 (3H, t, J = 7.1).

[*1-^{13}C*]*Ethyl isocyanocyclopropane-1-carboxylate.* This was prepared from the above ester using the procedure of Schöllkopf (*9*). A 23% yield was obtained on a 5.5-mmol scale after Kugelrohr distillation (150°C, 18 Torr). This yield is unusu-

ally low for this procedure, and we routinely obtained 50–60% in runs on unlabeled material. The compound showed NMR data identical to those reported by Schöllkopf, except for broadening of the cyclopropane hydrogens due to coupling to carbon.

[1-^{13}C]Aminocyclopropane-1-carboxylic acid. To 175.1 mg of the above prepared isocyanoester was added 2 ml of 4.5 M KOH, and the solution was refluxed under nitrogen for 5 h. The reaction mixture was cooled, neutralized with concentrated HCl, and loaded onto a 15-g column of Dowex 50 (H^+). The column was eluted with 100 ml of H_2O, and then the amino acid was eluted with 200 ml 1 M NH_4OH and 100 ml H_2O. Concentration provided 102.3 mg (80%) of the title compound. This compound showed proton NMR data identical with ACC except for broadening of the cyclopropane protons by ^{13}C coupling (δ 1.71, 1.61). The compound showed a single ^{13}C NMR resonance at 36.3 ppm relative to internal DMSO (39.56 ppm).

Feeding experiments. A plug of tissue was cut from an apple with a 15-mm cork borer. This plug was sliced into 5-mm-thick discs, and 10–15 g of these were placed flat on the bottom of a 250-ml Ehrlenmeyer flask. [1-^{13}C]ACC (30–60 μmol) in 4–8 ml 2% sucrose was added and the flask was sealed with a serum cap. From the same sample of apple a similar array was prepared in a Petri dish, and this tissue was treated with Na^{13}CN (1.5–40 μmol) in 2% sucrose. A third sample was prepared in a 250-ml Ehrlenmeyer flask which contained only apple tissue and sucrose solution. These were incubated in the dark at room temperature, and the production of ethylene was followed by gas chromatography (*5*). For samples which included ACC, 0.5–1.7 μmol of ethylene was produced. The control was usually ca. 5% of this value. After an incubation period of 12–30 h, the tissue of each sample was individually frozen in dry ice, homogenized with a Waring blender, centrifuged to remove solids, and submitted to Dowex 50 (H^+) ion-exchange chromatography. The basic fraction was eluted from the column with 1 M NH_3 and concentrated, usually providing between 15 and 30 mg of amino acids. The NMR spectrum of this mixture showed ACC as a major component, as well as signals in the 3-ppm region corresponding to asparagine.

Enzymatic degradation of amino acids to CO_2. A modification of the procedure of Cooney (*10*) was used. A "decarboxylation reagent" solution was prepared in 5 ml of degassed 0.2 M citrate buffer, pH 5.5, by adding 250 mg of α-ketoglutaric acid, 25 μl of $ZnSO_4$ solution (prepared by diluting 7 g of $ZnSO_4 \cdot 7H_2O$ to 10 ml), and 100 μl (200 units) of glutamate-oxaloacetate transaminase. The degradation cocktail was composed of 0.5 ml of the decarboxylation reagent, 0.5 ml of the amino acid sample in degassed water, and 100 μl of asparaginase solution (100 U/ml in citrate buffer). Incubation was conducted in a 5-ml round-bottomed flask sealed with a rubber septum under a nitrogen atmosphere for 2–2.5 h at 50°C. The sample was cooled under nitrogen and acidified with 100 μl H_2SO_4.

Mass spectral analysis. This was conducted using a SIM procedure. The spectrometer was tuned at $m/e = 50$, and several GC/MS runs were conducted on CO_2 in air prior to each run to determine the natural abundance control. Samples (1 ml) were injected via a gas-tight syringe, with CO_2 showing a retention time of ~1 min at 60°C, flow = 25 ml/min. Integration of the data was performed without mass

axis dithering or a SIM window. This required determining the fractional mass at which the M^+ and M^{+1} peaks were maximum prior to each run.

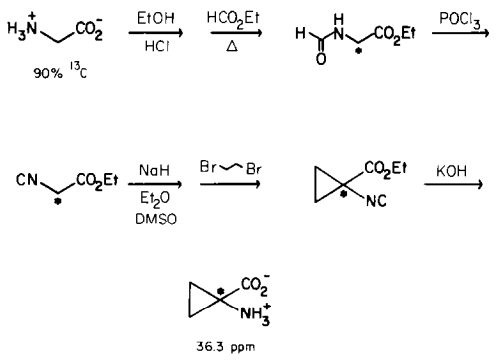
Data. Gas samples were analyzed for M^{+1}/M^+ ratios at least in triplicate. While the theoretical natural abundance ratio is 1.16%, we commonly measured values of ca 1.20% on CO_2 in air. The M^{+1}/M^+ ratio in each unknown was measured, which ranged from 1.43 to 1.70% depending on the conversion of ACC to ethylene. The absolute standard deviations of these values were 0.03%. The average of the replicates was divided by the measured M^{+1}/M^+ ratio in air to arrive at the enrichment factors. These ranged from 1.06 to 1.35 (five runs). The average enrichment factor was 1.216 per μmol ethylene. This compares to a standard value of 1.24 per μmol of labeled asparagine. This value was obtained by degrading under the above protocol blanks to which had been added [4-^{13}C]lithium aspartate (0.5–5 μmol). The incorporation of label from ACC to asparagine is thus 1.216/1.24 = 90%. The data from a representative experiment will be used to illustrate the procedure. Ethylene (1.12 μmol) was produced from [1-^{13}C]ACC in a 24-h feeding. The isolated amino acids were degraded to CO_2, which showed an isotope ratio of 1.71% (±0.02%). A sample of CO_2 in air showed an isotope ratio of 1.27% (±0.003%). The enrichment factor was thus 1.34. When a micromole of [4-^{13}C]Asp was degraded the enrichment factor was 1.24. The unknown therefore contained 1.34/1.24 = 1.08 μmol $^{13}CO_2$. Feeding of ^{13}C-labeled cyanide (1–40 μmol) in seven separate experiments led through asparagine to CO_2, in which the enrichment factor was 1.07–1.25.

Controls. Control degradations and GC/MS analyses were conducted on blanks of amino acids obtained from incubation in the absence of ACC, on pure asparagine, on water, on [1-^{13}C]ACC, and on [1-^{13}C]ACC plus the amino acids isolated from a blank. In no case was any statistically significant enrichment observed for CO_2 produced after enzymatic degradation.

RESULTS

In order to assess the fate of C1–N1 of ACC in ethylene biosynthesis, an isotopically labeled compound was required. It was obtained by the outstanding technology of Schöllkopf. The synthetic route (Scheme I) from commercially available [2-^{13}C]glycine proceeded in this instance in less than 10% overall yield, but we have obtained as high as 50% yield in unlabeled runs.

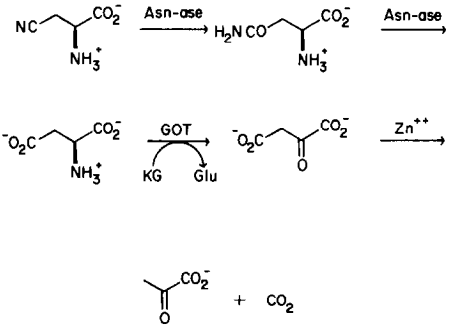
Presuming that C1–N1 of ACC are, in fact, converted to cyanide ion during ethylene biosynthesis, a reasonable question is whether this will damage the plant. In fact, cyanide metabolism in higher plants has been fairly well studied (*11*), with possible metabolites including thiocyanate and asparagine. The former would arise through the action of the enzyme rhodanese (*12*), while the latter is known to be produced through the intermediacy of β-cyanoalanine. This is considered to arise in the enzyme β-cyanoalanine synthase from Michael addition of cyanide to an aminoacrylate–pyridoxal Schiff's base derived from cysteine. This leads to incorporation of the cyanide carbon into the 4-position of β-cyanoalanine and asparagine. Since there is evidence that the carbon of thiocyanate may also be



SCHEME I.

incorporated into carbon-4 of asparagine (*11e*), analysis for label of the amino acid fraction from an incubation experiment with [1-^{13}C]ACC was chosen as a first goal.

Feeding of [1-^{13}C]ACC to apple tissue followed by isolation of the free amino acids by homogenization and ion-exchange chromatography provides recovered ACC as a major component of a mixture which includes asparagine. The amount of label in its 4-position is a measure of the production of cyanide from ACC. While the separation of this complex amino acid mixture and specific degradation of Asn would be extremely difficult and tedious to accomplish chemically, the selection of a single substrate among a host of others and execution of selective transformations on it is the forte of enzymes. Thus, the degradation and analysis of the amino acid mixture by a modification of a protocol advanced by Cooney (*10*) was performed. This is summarized in Scheme II, and involves the hydrolysis of β-cyanoalanine and/or asparagine to aspartate by asparaginase, followed by conversion of aspartate to oxaloacetate by glutamate-oxaloacetate transaminase. The substrate specificity for this enzyme is quite high (it shows no pyruvate transaminase activity, for example) and it does not affect [1-^{13}C]ACC (*vide infra*). Once oxaloacetate is obtained, it is readily decarboxylated by zinc ion. While this procedure sounds laborious, in fact all four processes may be conducted simulta-



SCHEME II.

neously in a single vessel, with the net result that the 4-carbon of asparagine or β-cyanoalanine is evolved as CO_2.[2]

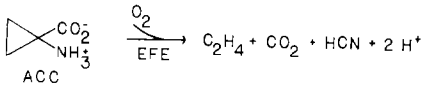
Analysis by mass spectrometry of the CO_2 so produced should signal incorporation of label from [1-^{13}C]ACC into asparagine by an increase in the M^{+1} ion. Naturally, because unlabeled asparagine from other sources exists in the tissue, this enrichment will not be complete. In fact, a much more serious problem is the pervasity of CO_2 in the atmosphere and many of the reputedly pure gases which are routinely used in the laboratory. With scrupulous care to minimize the dilutive effect of atmospheric and dissolved CO_2, it proved possible to observe significant enhancement of the M^{+1} signal of CO_2 and to determine the amount of labeled amino acid in each sample. The sensitivity of the method as demonstrated herein is at least 0.5 μmol. While obviously not as sensitive as radiotracer methods, this technique is more than capable of answering the questions posed here concerning the products of ethylene biosynthesis.

When [1-^{13}C]ACC is processed by apple tissue, incorporation of 90% of the label (based on ethylene produced) into asparagine is observed. That this occurrence proceeds through the intermediacy of cyanide needed next to be established. When labeled cyanide is fed to apple tissue, incorporation of label into asparagine occurs as evidenced by enhancement of the M^{+1} peak of CO_2 after enzymatic degradation. While the amount of label incorporated is not as great as when cyanide is produced biosynthetically, this can be explained through poorer uptake of cyanide compared to ACC.

Another experiment which strongly connects ethylene production with incorporation of label into asparagine was suggested by Yang.[3] Incubation of apple tissue with [1-^{13}C]ACC at 50°C, conditions which suppress ethylene biosynthesis by ~85%, leads to no significant incorporation of label into CO_2 after asparagine degradation. Furthermore, cyanide metabolism is not impeded by these conditions.[3] Controls also show that [1-^{13}C]ACC, which was present in all of the amino acid samples, is not converted to $^{13}CO_2$ by the degradation protocol.

DISCUSSION

The results presented herein have established the fate of C1–N1 of ACC in ethylene biosynthesis. The stoichiometry of ethylene biosynthesis during ripening is established as in Eq. [1].



[1]

This is a novel view compared to the dogma that has been promulgated based on a negative experiment and an abstract (*13*). This view held that formate and ammo-

[2] While this procedure does not differentiate between labeled β-cyanoalanine and asparagine, label in either provides evidence for the intermediacy of cyanide.

[3] The Davis, Cambridge, and Stanford groups became aware of each others' efforts only after each was in the penultimate stage of the work. Manuscripts were exchanged.

nia are the products of C1–N1 of ACC during ethylene biosynthesis in apple tissue.[4] The raison d'etre of β-cyanoalanine synthase, about which its discoverers said "the physiological significance of this enzyme in organisms not known to have cyanogenic compounds is obscure," is now clear. All plants may produce ethylene, and the byproducts of its biosynthesis must be metabolized.

An intriguing aspect of these results concerns how they fit into the picture of ethylene's physiological actions. For example, the onset of the climacteric phase in fruit in which respiration increases and ripening begins is believed to be caused by ethylene but can be promoted by exogenous cyanide (*14*). The climacteric phase is marked by a shift from cytochrome oxidase as the terminal electron acceptor in glycolysis to a cyanide-insensitive respiratory system (*15*). From the current perspective, an imperative for the shift may be suggested, or perhaps cytochrome blockage causes the shift. In another example, cyanide has been implicated in seed germination (*16*), a process which has also been strongly linked with ethylene (*17*).[5] While likely to face opposition much as did ethylene (*18*), perhaps someday cyanide may be considered a hormone in its own right.

While evidence has recently been offered that nitrenium ions and radical cations (*5*) are chemically competent intermediates in ethylene biosynthesis, two independent studies show the natural reaction proceeds by a nonconcerted mechanism (*4, 5*). It was proposed (*5*) that this result can only be accommodated by the radical cation intermediate, and it was established that a nonconcerted mechanism operates in this model. Since it is also established herein and elsewhere (*6*) that the biosynthetic reaction, like the electrochemical model reaction, produces cyanide, the evidence for a sequential single-electron transfer pathway for ethylene biosynthesis seems extremely strong.

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[4] In fact, an attempt to discover labeled formate in anionic fractions using formate dehydrogenase/β NAD to produce CO_2 was unsuccessful; the endogenous formate levels were too great.

[5] For a review of all of the affectors of germination, see R. B. Taylorson and S. B. Hendricks (1977) *Annu. Rev. Plant. Physiol.* **28,** 331.

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