SYNTHESIS AND BIOSYNTHESIS OF ETHYLENE

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Ethylene, a "broad spectrum" physiological agent in plant metabolism, has excited new interest in recent years following accumulation of evidence which places it in the category of a plant hormone. Every plant tissue produces ethylene and is influenced by ethylene at some stage in its life cycle; yet the origin of ethylene in plants and the pathways of its biosynthesis are for the most part still unknown. There has been much activity in this field in the past 6 years, and a great advance has been made in the discovery of a precursor of ethylene in metabolism (10). A number of ethylene-forming model systems were developed and these have led to proposals of enzymes, intermediates and pathways for ethylene biosynthesis in vivo. In this discussion I am going to consider these model systems and suggested pathways and evaluate their pertinence to ethylene biosynthesis in tissues.

Lability of extracted ethylene-forming system

It was rather surprising to find that tissues such as apple and tomato fruit, which yield relatively large quantities of ethylene in vivo, gave virtually no ethylene upon homogenization. An example of results obtained in such experiments is shown in Table 1.

The large increase in ethylene production by tomato segments and slices compared to whole fruit is a unique injury effect in tomato tissue and will not be considered in this discussion. The point we wish to emphasize is that ethylene production drops precipitately from the intact tissue to homogenates, mitochondria, microsomes and supernatant fractions. Thus the ethylene-forming system does not survive destruction of the cell. Despite the use of various protective measures during homogenization (nitrogen atm, reducing agents, polyamide, etc.), it was not possible to preserve the ethylene-forming system in a cell-free homogenate. As far as I know no one has successfully extracted the ethylene-forming system from cells. This tells us that the ethylene-forming system in vivo is extremely delicate and cannot be extracted by usual biochemical techniques. We therefore adopted other approaches to this problem and developed some model systems for ethylene formation.

Table 1. Ethylene production by whole fruit, segments, slices, homogenates and subcellular particles of apples and tomatoes.

<table>
<thead>
<tr>
<th>Type of fruit</th>
<th>Whole segments</th>
<th>Slices</th>
<th>Homogenate</th>
<th>Mitochondria</th>
<th>Microsomes</th>
<th>Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple</td>
<td>29.6</td>
<td>35.4</td>
<td>20.3</td>
<td>6.8</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Tomato</td>
<td>4.4</td>
<td>58.6</td>
<td>102.0</td>
<td>2.2</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
</tr>
</tbody>
</table>

Model ethylene-forming systems

Peroxidized linolenic acid system. The first model system for ethylene production to be discovered used peroxidized linolenic acid as the source of ethylene. In this system the C-18 peroxidized polysaturated fatty acid was degraded in a reaction catalyzed by Cu++ and ascorbate to yield both saturated and unsaturated hydrocarbon gases (8). Actually the entire homologous series of hydrocarbons from methane to pentane and ethylene to pentane, and their isomers, were formed in addition to other hydrocarbons which we have not studied. However, ethylene and ethane were formed in greatest amount. The ratio of ethylene to ethane production was about 15:1. In a similar reaction system with Fe+++ replacing Cu++ , ethane was the predominant product of the reaction (ethane to ethylene about 4:1).

The degradation of linolenic acid occurs spontaneously at room temperatures (20-25°C) once the unsaturated fatty acid has been sufficiently peroxidized, but this degradation is considerably accelerated by Cu++ and ascorbic acid (Table 2). Fresh linolenic acid, which has not been peroxidized, yields virtually no ethylene or other hydrocarbon gases. Cuprous ions (Cu+) are the active catalysts and these are produced by interaction of Cu++ with ascorbic acid. Reduced copper or iron are the only metals tested which can catalyze this reaction. We also found that phosphate buffer pH 7.0 was just as effective as acetate buffer pH 4.5.

Table 2. Effect of peroxidation of linolenic acid on ethylene production in the copper-ascorbate system.

<table>
<thead>
<tr>
<th>System</th>
<th>µ ethane per 30 min</th>
<th>µ ethylene per 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh linolenic acid</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Peroxidized linolenic acid</td>
<td>1.10</td>
<td>16.72</td>
</tr>
</tbody>
</table>

Fig. 1. Proposed scheme for breakdown of peroxidized linolenic acid to ethylene.

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This type of breakdown of linolenic acid produces aldehydes and other products which can be further degraded by copper and ascorbic acid to ethylene and other hydrocarbon gases. Propanal was found to be the aldehyde product which is the immediate precursor of ethylene and ethane (11). The relative yield of ethylene from propanal and from peroxized linolenic acid is 860 to 1.

**Linolenic acid and ethylene production in tissues.** We speculated that ethylene may be formed in tissues by the following sequence of reactions:

bound linolenic acid $\rightarrow$ lipase $\rightarrow$ free linolenic acid $\rightarrow$ lipoxidase $\rightarrow$ peroxized linolenic acid $\rightarrow$ propenal $\rightarrow$ copper enzyme $\rightarrow$ ethylene

This scheme was based on the assumption that linolenate was bound as a lipid in membranes and could be released by lipase in a controlled reaction. The free unsaturated acid would then be subject to peroxidation and degradation to propenal and finally to ethylene, by lipoxidase and a hypothetical copper-mediated enzyme. Our experiments on the effect of various reducing agents on apple tissue slices suggest that ethylene production is enhanced by oxygen and suppressed by nitrogen, antioxidants, reducing agents, and copper chelating agents. These experiments also show that cytoplasmic particles from apples produce ethylene, in the presence of fresh unoxidized linolenic acid, copper, and ascorbic acid. However, this system may be considered a model ethylene-forming system in which lipoxidase, contained in the cytoplasmic particles, causes peroxidation of linolenic acid which then reacts with Cu$^{2+}$ and ascorbic acid to give ethylene.

Additional suggestive evidence which tended to link linolenic acid to ethylene production in vivo was provided by Meigh et al. (18) who observed an increase in lipoxidase activity in apple peel tissue just prior to the rise in ethylene production. It was further observed by others (4) that ethylene production was stimulated in apple peel disks upon addition of linolenic acid and lipoxidase. However, as with cytoplasmic particles, this system is in reality only a model system in which peroxidation of linolenate is accentuated.

Although these experiments and others, which show an increase in lipids prior to the climacteric rise in apple peel disks (4), tend to link peroxidation of linolenic acid with ethylene production, crucial experiments with labeled substrates show no such linkage. C-14 labeled linolenic acid (16) and C-14 propenal (1) do not yield C-14 labeled ethylene. The labeled fatty acid was absorbed by the tissue and converted to C-14 carbon dioxide, as was labeled propenal, but no labeled ethylene was detected. We must therefore conclude that linolenic acid does not convert to ethylene in living cells, and the model system for ethylene production from linolenic acid does not have a counterpart in living tissues.

**Metionine and methionine-related ethylene-forming systems.** The second ethylene-forming model system discovered in our laboratory at Beltsville utilized methionine as substrate (9). Once again cupric ions and ascorbate acted as catalysts to degrade methionine to ethylene. Unlike the linolenate model system, ethylene was the only hydrogen peroxide formed in the reaction.

The essential features of the methionine model system are shown in Table 3. Either an aerobic atmosphere or H$_2$O$_2$ is needed for this reaction. In fact more ethylene is formed in the presence of added H$_2$O$_2$ than in the aerobic H$_2$O$_2$-free system. The total inhibition of ethylene formation by catalase implicates H$_2$O$_2$ as an intermediate in this reaction. Hydrogen peroxide must be formed by reaction of ascorbate and Cu$^{2+}$ ions in aerobic aqueous solutions as shown below.

1) ascorbate $+ 2$ Cu$^{2+}$ $\rightarrow$ dehydroascorbate $+ 2$ Cu$^+$ $+ 2$ H$^+$
2) $2$ Cu$^+$ $+ H^+$ $+ O_2$ $\rightarrow$ $2$ Cu$^{2+}$ $+ H_2$O$_2$

By the use of C-14 methionine we established that ethylene comes from carbon 3 and 4 of methionine. The reaction mechanism may be considered as a splitting of both ends of the molecule leaving the middle ethylenic group behind, as shown below:

$$\text{CH}_3\text{S-CH}_2\text{CH}_2\text{CH-COO} + \text{Cu}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{CH}_3\text{S-CH}_2\text{CH}_2\text{CHO} + \text{CO}_2 + \text{NH}_3$$

The breaking out of the carboxyl group and the amino group can occur by the Strecker degradation, in which amino acids are degraded to the aldehyde of one less carbon atom, in the presence of H$_2$O$_2$ and metal ions. The breaking of the S to carbon 4 bond could occur after an electron withdrawing complex of copper and sulfur is formed in the molecule. A proposed sequence of reactions from methionine to ethylene is outlined below:

$$\text{CH}_3\text{SCH}_2\text{CH}_2\text{CH-COOH} + \text{Cu}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{CH}_3\text{SCH}_2\text{CH}_2\text{CHO} + \text{CO}_2 + \text{NH}_3$$

$$\text{Cu}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{CH}_3\text{S} + \text{CH}_2\text{CH}_2 + \text{CO}_2$$

This scheme was partially deduced from the ability or inability of various compounds structurally similar to methionine to form ethylene with Cu$^{2+}$ and ascorbate (Fig. 2).

![Active molecules](image)

![Inactive molecules](image)

**Fig. 2.** Active and inactive substrates in ethylene-forming model system.

Whenever the S atom in the molecule is encumbered, as in methionine sulfone or methionine sulfoxide, there is no ethylene production. In homocysteine the combination of cupric ions and the SH group is a far more stable adduct and does not cause splitting of the S-C bond. Of the active molecules, methionol and the $\alpha$-keto-$\gamma$-methyl thiolobutyrate (K MBA) are far more active than methionine, in keeping with their roles as possible intermediates between methionine and ethylene. Ethionine and the hydroxy analog of methionine show approximately the same activity as methionine, in the model system.

A similar model ethylene-forming system in which methionine is degraded by a flavin mononucleotide (FMN)-light system was described by Yang et al. (23). Except for some minor differences the light-driven free radical degradation of methionine is essentially the same as the copper-ascorbate catalyzed system.

**Conversion of methionine to ethylene in tissues**

When C-14 methionine labeled in carbons 3 and 4 was fed to apple tissue slices, the C-14 label was readily observed in ethylene produced by the tissue. Methionine stimulated ethylene production mainly in senescent or post-climacteric apple tissue slices (10). Ethylene production was also stimulated by methionine in tissues of tomato and banana fruit and in pea seedlings (3). Significant stimulation of ethylene production by apple tissue was shown also in the presence of the $\alpha$-OH analog of methionine. However, ethionine, methionol and K MBA had virtually no influence on ethylene production by tissue slices. Ethionine does not stimulate ethylene production in tissues because the enzyme involved may be highly specific for methionine and cannot react with ethionine. Also since methionol and K MBA are inactive in stimulating ethylene production in tissues, we believe they are not intermediates between methionine and ethylene.

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**Table 3. Hydrogen peroxide and oxygen as factors in methionine model system.**

<table>
<thead>
<tr>
<th>Flask content</th>
<th>Atm</th>
<th>C$_2$H$_4$ (µl/30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Buffer $^2$ + Methionine (5 µmoles) + Cu$^{2+}$ (5 µmoles) + Ascorbate (50 µmoles)</td>
<td>N$_2$</td>
<td>0.0</td>
</tr>
<tr>
<td>2) Buffer $^2$ + Methionine (5 µmoles) + Cu$^{2+}$ (5 µmoles)</td>
<td>N$_2$</td>
<td>0.0</td>
</tr>
<tr>
<td>3) Buffer $^2$ + Methionine (5 µmoles) + Cu$^{2+}$ (5 µmoles) + Ascorbate (50 µmoles)</td>
<td>N$_2$</td>
<td>6.7</td>
</tr>
<tr>
<td>4) Buffer $^2$ + Methionine (5 µmoles) + Cu$^{2+}$ (5 µmoles) + Ascorbate (50 µmoles)</td>
<td>Air</td>
<td>3.2</td>
</tr>
<tr>
<td>5) Buffer $^2$ + Methionine (5 µmoles) + Cu$^{2+}$ (5 µmoles) + Ascorbate (50 µmoles) + H$_2$O$_2$</td>
<td>Air</td>
<td>5.4</td>
</tr>
<tr>
<td>6) Buffer $^2$ + Methionine (5 µmoles) + Cu$^{2+}$ (5 µmoles) + Ascorbate (50 µmoles) + Catalase (30,000 units)</td>
<td>Air</td>
<td>0.0</td>
</tr>
</tbody>
</table>

$^2$0.1M phosphate buffer pH 7.0 in total vol of 5 ml.
demonstrated conclusively that methionine is a precursor of ethylene in higher plant tissues. What remains to be established are the intermediates and enzymes involved in the pathway from methionine to ethylene.

Peroxidase ethylene-forming systems that utilize methional or KMBA model system. Yang (24) described an enzymatic model system for ethylene formation in which pure horse radish peroxidase (HRP) catalyzed the conversion of methional or KMBA to ethylene. This system required a phenol, H2O2 or Mn++ and sulphite as cofactors. Hydrogen peroxide can be substituted for Mn++ in this reaction and the system is 100% dependent on sulphite ions. Whereas some phenols are important components of this system, ortho-diphenols are inhibitory. Very significantly this system could not utilize methionine.

A proposed mechanism for degradation of methional in the HRP-catalyzed system involves a free radical chain reaction starting from oxidation of the phenol by the peroxidase-H2O2 system. This is followed by oxidation of sulphite which propagates a free radical chain reaction. Ultimately the radicals react with methional to produce a methional radical which causes degradation of the molecule to dimethyl disulphide, formic acid and ethylene (24).

Peroxidase ethylene-forming system in tissues. The experiments of Mapson and colleagues suggested the apparent similarity between ethylene production in tissues and the HRP-ethylene-forming model system (12, 13). Mapson’s group, working with cauliflower tissue, isolated and purified a peroxidase enzyme from cauliflower florets which produced ethylene in a system containing an ester of p-coumaric acid, H2O2 and sulphite. They demonstrated the presence of glucose oxidase in cauliflower tissue which in reaction with glucose in the tissue could supply H2O2 for the reaction. Furthermore they also isolated and identified the methyl ester of p-coumaric acid and methanal sulphonic acid in cauliflower florets (14). All the cofactors of the HRP-ethylene-forming system were present in cauliflower florets, in addition to peroxidase. However, cauliflower floret tissue could not be stimulated in its ethylene production, by additions of methional.

Peroxidase enzymes which could form ethylene with methional, in a system supplemented with a phenol, sulphite and H2O2 or Mn++, were also obtained from apple (22) and tomato tissue (7). These systems could use either methional or KMBA as substrate for ethylene production, but could not convert methionine to ethylene.

More recently Mapson et al (15) reported that cauliflower florets were stimulated in their ethylene production when incubated in a solution containing KMBA. Furthermore, such systems when supplied with C14 KMBA or C14 methionine showed much greater incorporation (about 4 times more) of C14 into ethylene from KMBA than from methionine. These workers concluded that KMBA was the natural intermediate between methionine and ethylene.

The scheme proposed by Mapson et al for the in vivo synthesis of ethylene is shown below in Fig. 3.

Table 4. Comparison of ethylene production by cauliflower florets in presence of KMBA and methionine in “wet” and “dry” systems and the effect of catalase in the “wet” system.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Wet system'x</th>
<th>Dry system'y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no KMBA)</td>
<td>5.3</td>
<td>6.9</td>
</tr>
<tr>
<td>Methionine (1 mM)</td>
<td>6.4</td>
<td>5.8</td>
</tr>
<tr>
<td>KMBA (1 mM)</td>
<td>37.0</td>
<td>7.1</td>
</tr>
<tr>
<td>Control + Catalase (15,000 units)</td>
<td>6.3</td>
<td>---</td>
</tr>
<tr>
<td>Methionine + Catalase</td>
<td>8.4</td>
<td>---</td>
</tr>
<tr>
<td>KMBA + Catalase</td>
<td>11.5</td>
<td>---</td>
</tr>
</tbody>
</table>

'x'Wet systems were incubated in 3 ml 0.1M phosphate buffer pH 6.8.
'y'Dry systems were incubated 1 hr in buffer containing 1 mM KMBA prior to removal, blotting dry and incubated in dry state.

We also found that cauliflower floret tissue lacks peroxidase into the buffer solution, and in the presence of KMBA and the cofactors, p-coumaric acid, sulphite and H2O2, forms a potent exogenous ethylene-producing system. Finally, we showed that stimulation of ethylene by KMBA in 3 tissues was dependent, basically, on the presence of peroxidase in the surrounding buffer (Table 5).

Table 5. Stimulation of ethylene production by KMBA and peroxidase leakage in filtrates of cauliflower, tomato, and apple tissue, in 0.1M phosphate buffer pH 6.5.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control KMBA</th>
<th>Control KMBA due to KMBA</th>
<th>Peroxidase Activity of buffer A460nm/5ml/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cauliflower</td>
<td>0.51</td>
<td>4.47</td>
<td>+776</td>
</tr>
<tr>
<td>Tomato</td>
<td>47.65</td>
<td>65.13</td>
<td>+37</td>
</tr>
<tr>
<td>Apple</td>
<td>5.66</td>
<td>5.16</td>
<td>-9</td>
</tr>
</tbody>
</table>

WTomato and apple tissues were incubated at 30°C in 5 ml buffer in 5 ml buffer in 25 ml sealed flasks, containing vials with 1 ml 10% KOH. Cauliflower tissue was incubated in 3 ml buffer.

x3 g florets.

x4 g 0.5 cm cubes of pericarp tissue (half-ripe).

x4 apple plugs about 1 g.
Tomato and cauliflower tissue, both of which allowed leakage of peroxidase to the surrounding buffer, exhibited increases in ethylene production in the presence of KMBA. The increase with cauliflower tissue was very great due undoubtedly to its relative high hcon of sulphate. However, the lack of increase in ethylene production by apple tissue (which is a potent ethylene producer) was due to the absence of leakage of peroxidase to the surrounding buffer KMBA solution.

We therefore conclude that the stimulation of ethylene production in cauliflower florets caused by KMBA is due to an artificial external system, analogous to the HRP ethylene-forming model system, which forms in the buffer solution surrounding the tissue. The components of this system, peroxidase, cocomeric acid, and glucose-glucose oxidase, are presumed to leak from the cauliflower tissue during incubation in the buffer solution containing KMBA. Consequently it does not appear that KMBA is an intermediate between methionine and ethylene.

Additional related experiments with catalase suggest that peroxidase is not involved in the biosynthesis of ethylene from methionine, since catalase inhibits the external peroxidase system but has little or no influence on the natural endogenous ethylene-forming system.

Rhizobitoxine as an inhibitor of ethylene production

In cooperation with Lowell Owens, we recently found that rhizobitoxine (19), an inhibitor of methionine biosynthesis in the bacterium Salmonella typhimurium, inhibited ethylene production 75% in sorghum seedlings and apple tissue slices. This inhibition was only partially relieved by addition of methionine (Table 6).

<table>
<thead>
<tr>
<th>Additions</th>
<th>No rhizobitoxine (umoles)</th>
<th>Rhizobitoxine (0.012M) Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>25.7</td>
<td>6.2</td>
</tr>
<tr>
<td>Methionine (1mM)</td>
<td>30.7</td>
<td>15.0</td>
</tr>
<tr>
<td>KMBA (1mM)</td>
<td>32.7</td>
<td>7.7</td>
</tr>
</tbody>
</table>

The fact that the inhibition by rhizobitoxine could not be relieved by methionine strongly suggests that the inhibition occurs between methionine and ethylene. The proposed structure of rhizobitoxine suggests an analog of methionine which may account for this inhibition.

Since 75% of ethylene production is inhibited by rhizobitoxine, then practically all the ethylene produced by the tissue arises through the pathway inhibited by the toxon. We showed by use of C-14 methionine that rhizobitoxine acts by inhibiting the conversion of methionine to ethylene (20). In Penicillus digitatum where methionine is not a precursor of ethylene in metabolism (6), rhizobitoxine does not inhibit ethylene production. We conclude, therefore, that methionine is the major if not the sole precursor of ethylene in higher plants.

Other proposed pathways

Although methionine is established as a precursor of ethylene in higher plants, it does not appear to be a precursor of ethylene in the mold P. digitatum (6). The middle carbons of the 4 carbon Krebs cycle acids, succinic, fumaric, and malic have been suggested as precursors of ethylene in P. digitatum. However, the incorporation of carbons from these acids is very low and we question whether these acids represent direct precursors of ethylene in metabolism. What is clear, however, is that the pathway for ethylene production in the mold is different from that in higher plants.

There has also been a proposal that γ-alanine is a precursor of ethylene in higher plant tissue (21). The conversion of γ-alanine to ethylene was only 0.002% and its conversion to ethylene could not be confirmed in other laboratories (3). The pathway from γ-alanine to ethylene is presumed to go via acrylic acid, which we found to be a poor precursor of ethylene, when added to tissue slices.

To date methionine is the only established precursor of ethylene in higher plant tissues. Moreover, as we indicated from experiments with rhizobitoxine, methionine appears to be the major if not sole natural precursor of ethylene in higher plants. Despite what appeared to be promising data linking peroxidase and KMBA, as enzyme and intermediate, respectively, between methionine and ethylene, our recent experiments cast considerable doubt on this linkage. The inhibition of ethylene production with rhizobitoxine may suggest the involvement of a pyridoxal phosphate-mediated enzyme, but at present the biochemical pathway between methionine and ethylene remains unknown.

Literature Cited