ETHYLENE PRODUCTION FROM METHIONINE AS MEDIATED BY FLAVIN MONONUCLEOTIDE AND LIGHT*

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Although numerous reports on ethylene evolution from subcellular preparations have appeared, the immediate precursor of ethylene in plant tissue and the nature of the ethylene producing system remain obscure and uncharacterized (Abeles, 1966; Hansen, 1966; Lieberman et al., 1966; Shimokawa and Kasai, 1966; Thompson and Spencer, 1966; Wooltorton et al., 1965). Abeles and Rubinstein (1964) reported the production of ethylene when they mixed an enzyme and a non-protein fraction isolated from pea seedlings. In addition to this enzyme-catalyzed reaction, they also observed that Fe^{++} and FMN alone could cause non-enzymatic evolution of ethylene from their non-protein fraction at a rate greatly exceeding that of the enzyme-catalyzed reaction. Although we were unable to confirm their enzymic reaction, FMN-mediated ethylene production was confirmed, the active substrate in pea seedling extracts which produced ethylene in the presence of FMN and light was identified as methionine, and the two-carbon skeleton of ethylene was shown to be derived from carbons 3 and 4 of methionine.

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EXPERIMENTAL

Purification and Identification of an Ethylene-Producing Substrate from Pea Seedlings

Homogenates of etiolated pea seedlings (epicotyls and roots) were prepared as described by Abeles and Rubinstein (1964) and fractionated as described in Table I. The reaction mixtures consisted of the equivalent of 1.0 g of original seedling extract, 50 µmoles of sodium acetate, 0.5 µmole of FMN, 100 µmoles of glucose, and 0.15 unit of glucose oxidase, made to 1.3 ml in 25-ml Erlenmeyer flasks fitted with rubber serum caps. The flasks were usually incubated at room temperature under the ordinary room lights (approx. 150 foot candles at 25°). Ethylene produced in the reaction was sampled after one hour with a hypodermic syrings and determined by gas chromatography, using an activated alumina column and a flame ionization detector (Lyons *et al.*, 1962). Activity of the fractions is defined as millimicroliters of ethylene produced in one hour.

No ethylene was produced in the dark or in the absence of FMN, and the efficiency of production dropped about 85% when glucose oxidase was omitted from the reaction mixture. It was later found that flushing the flask with nitrogen gas could substitute for the addition of glucose and glucose oxidase, but addition of H_2O_2 could not. Over 100% of the original cation-fraction activity was recovered in the zone of Rf 0.39-0.52, where methionine and valine are found. No activity was apparent in zones with higher Rf values, but some was detected in zones of lower Rf. The recovery of over 100% of the original activity may indicate the elimination of inhibitors.

Authentic methionine was found to be an excellent substrate for production of ethylene under the described conditions; valine was inactive. An aliquot of the cation fraction was therefore cochromatographed on paper with 0.1 mµmole of uniformly labeled ^{14}C-methionine (25 mµc). The paper was first developed with phenol:water (100:40 w/w) and then with n-butanol:acetic acid:water (4:1:5, v/v). The methionine spot was located by autoradiography, eluted, and analyzed for ethylene production. Over 85% of the activity of the cation fraction was recovered in the methionine spot. No detectable ethylene was produced from a control spot containing only the same amount of ^{14}C-methionine.

TABLE I

Purification of the Ethylene-Producing Substrate from Pea Seedlings

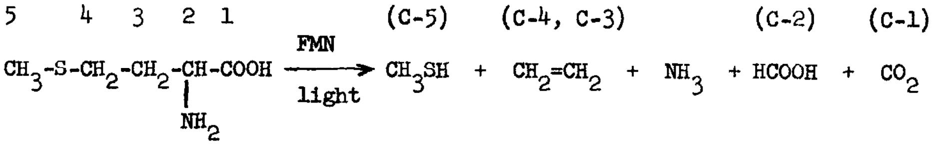
Purification stage	Ethylene production $m\mu l$	Purification stage	Ethylene production $m\mu l$
1. Supernatant	10.0	4. Paper chromatography of cation fraction	
Pellet	1.0		
2. Protein fraction	0.1	Rf 0 - 0.13	0.7
Non-protein fraction	9.8	0.13 - 0.26	0.8
		0.26 - 0.39	0.7
3. Neutral fraction	0.4	0.39 - 0.52	13.3
Anion fraction	0.5	0.52 - 0.65	0.0
Cation fraction	13.1	0.65 - 0.80	0.0
		0.80 - 1.00	0.0

1. Homogenate of pea seedlings fractionated into supernatant and pellet by centrifugation at 15,000 x g for 20 min.
2. Supernatant separated into protein and non-protein fractions by passage through a column of Sephadex G-25.
3. Non-protein fraction separated into neutral, anion, and cation fractions on ion exchange resin columns (Dowex, 50 H^+ form, and IR-4B, OH^- form).
4. Cation fraction separated by paper chromatography with n-butanol:acetic acid:water (4:1:5, v/v) as the developing solvent. Zones eluted with 50% ethanol.

Ethylene Production from Methionine and Allied Compounds

Methionine and its allied compounds were tested for their efficiency in ethylene production under conditions similar to those described for pea seedling extracts, except that 350 foot candles of daylight fluorescent light were supplied from below. In addition to methionine, active substrates included ethionine, homocysteine, homocystine, α-hydroxy-γ-methylmercaptobutyric acid, γ-methylmercaptopropionaldehyde, and S-methylmercaptopropanol. Methionine sulfoxide, methionine sulfone, cysteine, S-methylcysteine, serine, homoserine, acrolein, and ethanethiol were inactive. The optimum pH for the production of ethylene from methionine was between 8 and 9. No cofactors other than FMN and light were required; hydrogen peroxide, oxygen, Cu^{++}, Ni^{++}, Co^{++}, and Fe^{++} were inhibitory. Riboflavin could replace FMN, but FAD showed only 10% of the activity of FMN. When methionine was used as the substrate, methanethiol and dimethyldisulfide were identified as products by gas chromatography, and ammonia was found with Nessler's reagent. The only ninhydrin-positive compound produced was methionine sulfoxide, as revealed by paper chromatography of the reaction products. (Production of this compound from methionine, mediated by riboflavin and light, has been reported by Nickerson and Strauss, 1961.)

Since both homocysteine and homocystine were efficient substrates for the production of ethylene in this system, the methyl group of methionine appears not to convert to ethylene. To determine which carbons of methionine give rise to ethylene, C-1- and C-2-labeled ^{14}C-methionine and uniformly labeled ^{14}C-methionine were compared. As shown in Table II, C-1- and C-2-labeled methionines did not yield significant radioactivity in the ethylene produced, while uniformly labeled methionine did; therefore, the ethylene must come from the C-3 and/or C-4 carbons. Because the specific activity of the ethylene obtained in our system accounts for about 2/5 of that of the uniformly labeled methionine used, we conclude that the C-3 and C-4 carbons are converted to ethylene as a unit. With the additional evidence (Table II) that C-1 gave rise mainly to carbon dioxide and C-2 to formic acid, and that ammonia, methanethiol, and dimethyldisulfide were additional products, we suggest the following reaction for the production of ethylene from methionine:



$$\overset{5}{CH_3}\text{-S-}\overset{4}{CH_2}\text{-}\overset{3}{CH_2}\text{-}\underset{NH_2}{\overset{2}{CH}}\text{-}\overset{1}{COOH} \xrightarrow[\text{light}]{\text{FMN}} \overset{(C\text{-}5)}{CH_3SH} + \overset{(C\text{-}4,\ C\text{-}3)}{CH_2{=}CH_2} + NH_3 + \overset{(C\text{-}2)}{HCOOH} + \overset{(C\text{-}1)}{CO_2}$$

The methanethiol may then be oxidized to dimethyldisulfide. Conversion of methionine ranged from 50 to 80%.

Ethylene production from methionine in the presence of Cu^{++}, H_2O_2, and ascorbate has been reported by Lieberman et al. (1965); they also showed that ethylene was derived from carbons 3 and 4, but their maximum conversion was only 8 to 11%.

TABLE II

Production of Ethylene from ^{14}C-Methionine

Position of label	Methionine cpm/mμmole	Ethylene recovered μmole	Ethylene recovered cpm/mμmole	Radioactivity (10^{-3} x cpm) CO_2	HCOOH	HCHO	Residue
C-1	1026	0.49	9.1	817	5.3	2.0	214
C-2	1141	0.53	3.1	51.1	472	78.8	577
Uniform	971	0.30	446	95.8	-	-	-

Reaction mixtures contained 1 μmole of methionine, 1 μmole of FMN, and 50 μmoles of phosphate buffer (pH 8.0) made to 1.3 ml in 50-ml Erlenmeyer flasks fitted with a center well and a rubber serum cap (Katz et al., 1954). The flasks were incubated for an hour at room temperature while illuminated from below with 350 foot candles of light (daylight fluorescent).

For the measurement of radioactivity, ethylene was absorbed in mercuric acetate (Gibson and Crane, 1963), and CO_2 was absorbed in ethanolamine (Katz et al., 1954). Formic acid was converted to CO_2 by $HgCl_2$ oxidation, and formaldehyde by NaIO and $HgCl_2$ oxidation (Sakami, 1955). The residue was converted to CO_2 by persulfate oxidation (Katz et al., 1954). Radioactivity of each fraction was counted in a Packard liquid scintillation counter.

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