SHORT COMMUNICATIONS

Mode of Ethylene Formation by *Mucor hiemalis*

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INTRODUCTION

Ethylene is produced from methionine in combination with glucose by a common soil fungus, *Mucor hiemalis* (Lynch, 1972). The dynamics of ethylene formation by the fungus have been reported (Lynch & Harper, 1974). Many studies on the biosynthesis of ethylene by plants have led to the conclusion that methionine is the major if not the sole precursor (Yang & Baur, 1972) and that as in *M. hiemalis* the biosynthesis is favoured by high oxygen tensions (Mapson & Robinson, 1966; Burg, 1973).

Several *in vitro* ethylene-forming systems have been described. Lieberman, Kunishi, Mapson & Wardale (1965) put forward the first, which involved cuprous ions and ascorbate. Yang (1967) described two systems with peroxidase and specific phenols, whilst Yang, Ku & Pratt (1967) described another with FMN (flavin mononucleotide) and light. Generally methional (3-methyl-mercaptopropionaldehyde) and KMBA (α -keto- γ -methyl-mercaptopbutyric acid) are more effective precursors of ethylene than methionine in these systems. Ku & Leopold (1970) and Demorest & Stahmann (1971) showed that peptides with methionine end groups are also better precursors than methionine itself, a fact which is important *in vivo* because pools of free methionine are usually very low and a major pathway of D-amino acid assimilation is through the *N*-malonyl or *N*-acetyl acid (Pokorny, Marčenko & Keglevic, 1970; Rosa & Neish, 1968).

Mapson, March & Wardale (1969), from work with cauliflower florets, proposed that *in vivo* production of ethylene was governed by three enzymes, a transaminase which converts methionine to the keto acid, glucose oxidase which liberates H₂O₂ from glucose, and a peroxidase which uses the peroxide to liberate ethylene from the keto acid. However, the participation of KMBA *in vivo* has now been questioned (Baur & Yang 1969; Baur, Yang, Pratt & Biale, 1971; Lieberman & Kunishi, 1971) because in some tissues it is incorporated less efficiently than methionine, and furthermore there can be leakage of peroxidase into the surrounding medium, to give *in vitro* ethylene production from KMBA. This has led to statements that the *in vivo* biochemical pathway from methionine to ethylene remains unknown (Burg, 1973; Baur & Yang, 1969; Phan, 1971; Abeles, 1972).

METHODS

Mucor hiemalis (ATCC26035) was grown in shaken flasks or in a chemostat under conditions of air saturation as previously described (Lynch & Harper, 1974). The culture was filtered through a membrane filter (0.22 μ m; Millipore) to give the extracellular fraction. Portions (10 ml) of this preparation were incubated in the dark at 25 °C in universal bottles

Table 1. *Ethylene production by cultures and extracellular preparations of M. hiemalis grown in two different ways*

	Ethylene (nmol/ml/h)	
	Sealed flask	Chemostat
Growing culture	0.002	2.335
Extracellular preparation	0.124	0.022

(capacity 28 ml), which had a hole drilled in the metal cap to facilitate gas sampling. The caps and rubber liners were sterilized with 70 % alcohol to avoid ethylene release from the rubber which occurred on autoclaving. After 4 h a gas sample (1 ml) was taken and analysed by g.l.c. as described previously (Lynch & Harper, 1974).

RESULTS AND DISCUSSION

The extracellular preparation from Mucor hiemalis

During the death phase of *M. hiemalis* cultures a peak in ethylene production is usually observed (Lynch & Harper, 1974). Ethylene production might be due to the release of extracellular enzymes acting on methionine. When growing sealed flask cultures were filtered and the sterile extracellular preparation re-incubated, the rate of ethylene production was about 60 times greater than that from the growing sealed flask preparation; in chemostat cultures, the extracellular preparation produced about 100 times less than that of growing cultures (Table 1). In the former circumstances it seemed possible that ethylene formed in growing flask cultures was metabolized, but that there was less ethylene metabolism in the extracellular preparation. This possibility was discounted by showing that when uniformly labelled [^{14}C]ethylene was applied to growing flask cultures, it became distributed between the gas and liquid phases and not metabolized. Another explanation is that a volatile inhibitor of ethylene production was produced, which accumulated in the sealed flask but was removed by the continuous gas flow in the chemostat. When ethylene production from the extracellular preparation ceased, more ethylene was produced when the bottles were re-gassed with air, but at a slower rate. When the preparation was gassed with CO_2 , ethylene (6 p.p.m. in air, a concentration often found in flask cultures) or nitrogen, the rate of ethylene production was the same as that in air. Thus the inhibition could not have been caused by carbon dioxide, ethylene itself or lack of oxygen.

The fresh growth medium produced no ethylene so the organism must release an enzyme or chemical intermediate in the medium which leads to ethylene formation. By treating the preparation with various agents which inhibit enzyme activity (Table 2) it was shown that the latter was the more likely. A stimulation in ethylene production by Fe^{3+} in the extracellular preparation was observed, but there was an even greater stimulation by Fe^{2+} ; there was an inhibition with Cu^{2+} and Mn^{2+} (Table 2). Acid stimulated the process, whereas it was inhibited by alkali. After adjusting the preparation to alkaline pH before making it acid, an even greater stimulation was observed than when it was adjusted to acid pH directly. This might be explained by the drop in pH causing more Fe^{3+} to become dissociated from the citrate complex. On raising the pH there might also have been dissociation to give precipitation as the hydroxide. When the alkaline solution was acidified, more Fe^{2+} was probably available. Hence the breakdown of the intermediate in the extracellular preparation might be Fe-dependent and not pH-dependent. Possible intermediates in ethylene

Table 2. *Characteristics of ethylene production by the extracellular preparation from Mucor hiemalis*

Treatment	Inhibition (–) or stimulation (+) of ethylene production (%)
Enzyme inhibitors	
Boiling (10 min)	+ 708
Autoclaving (10 min)	0
Trichloroacetic acid (15 % v/v)	0
pH change	
6 to 12	– 50
6 to 1	+ 951
6 to 1 to 12	+ 284
6 to 12 to 1	+ 5550
Metal ions (at 10 times basal medium concn)	
Cu ²⁺ (0.02 mg/ml)	– 18
Mn ²⁺ (0.2 mg/ml)	– 21
Fe ³⁺ (0.2 mg/ml)	+ 2820
Fe ²⁺ (0.2 mg/ml)	+ 24300
Chemical treatments (50 mg/ml)	
2,4-Dinitrophenylhydrazine	– 94
Ninhydrin	– 57
Semicarbazide HCl	– 48

formation are methional and KMBA. When the extracellular preparation was treated with reagents which would inactivate methionine (ninhydrin), methional (semicarbazide) and KMBA (2,4-dinitro-phenylhydrazine), ethylene formation was inhibited (Table 2).

The amino-acid oxidase system

In the pathway proposed by Mapson *et al.* (1969), which involves the conversion of methionine to the keto acid by a transaminase, the possibility that the step could be achieved *in vivo* by amino acid oxidase was rejected because there was no increased oxygen uptake when methionine was added to an ethylene-producing mitochondrial system. However in living *M. hiemalis* it appears that amino acids could be catabolized by a transaminase or an amino acid oxidase; in the yeast *Trigonopsis variabilis*, it has been shown that amino acid oxidase functions aerobically on D-methionine whilst transaminase functions anaerobically on L-methionine (SentheShanmuganathan & Nickerson, 1962). When D-amino acid oxidase from hog kidney (1 mg/ml) was added to the sterile medium used in the growth of *M. hiemalis*, ethylene was formed from D-methionine at 0.5 nmol/ml/h. The enzyme requires oxygen, and shaking the bottles to increase oxygen transfer doubled the rate of ethylene production; however, when the atmosphere in the bottle was replaced with nitrogen, ethylene production was only halved.

If the enzyme action is not significant, the flavin coenzyme (flavin adenine dinucleotide, FAD) which produces ethylene in the presence of light might be (Yang *et al.* 1967). When light was completely excluded from the amino-acid oxidase system, there was a 94 % inhibition of ethylene formation. Illumination from above the bottle caps at 15000 lux resulted in a 33-fold stimulation.

The flavin/light system

When amino acid oxidase was replaced by FMN at concentrations between 0.1 and 10 mg/ml, ethylene was formed only in the light and was dependent on the concentration of FMN. It was also found that methional was a better substrate than methionine itself ($\times 6$) but *N*-acetylmethionine was less efficient ($\times 0.4$). However, these effects are pH-dependent (Ku & Leopold, 1970). It should be noted that *N*-acetylmethionine and methional in the medium form ethylene in the absence of flavin, albeit at a slower rate, in the presence (approx. 5 %) or absence (approx. 2 %) of light. Amino acid oxidase has 2 mol FAD/mol enzyme; thus the effective FAD concentration in the amino acid oxidase system was 0.2 mg/10 ml. At this concentration it gives ethylene from methionine at 0.5 nmol/ml/h, whereas the FMN/light system at 0.2 mg FMN/10 ml gives ethylene at 5.0 nmol/ml/h. However, it has been shown that FAD gives rise to ethylene at about 5 % of the rate of FMN (Yang *et al.* 1967). Thus it appears that the efficiency of ethylene production per mole of flavin by the two systems is of the same order of magnitude. The results obtained would be consistent with the FAD of an amino acid oxidase in *M. hiemalis* reacting with light to form ethylene from methionine. We have observed that light promotes ethylene production in growing flask cultures (J. M. Lynch and S. H. T. Harper, unpublished observations), but the reported experiments on ethylene production by *M. hiemalis* (Lynch & Harper, 1974) are for dark-grown cultures. Thus even if the FAD/light system operates in *M. hiemalis*, an additional system must operate.

A consequence of the process of extracellular ethylene formation by *M. hiemalis* in the soil is that the intermediate could be formed by the organism at one site, possibly near the soil surface where there is more substrate and light, and then be transported by rainfall to a site remote from the organism, where it breaks down to ethylene.

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