ETHYLENE FORMATION FROM METHIONAL MEDIATED BY LIPID HYDROPEROXIDES

KEITH JOHN ALLSOP


CENTRE FOR NFLD. STUDIES
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OF NEWFOUNDLAND

ETHYLENE FORMATION FROM METHIONAL MEDIATED BY LIPID HYDROPEROXIDES

by

Keith John Allsop, B.Sc.

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Department of Biochemistry
Memorial University of Newfoundland

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ABSTRACT

Ethylene could be formed by a model system containing lipoxygenase, linolenate, sulphite and methional. The system had an optimal pH of 7.8. Free radicals formed during the lipoxygenase-catalyzed oxidation of linolenate were thought to initiate sulphite oxidation. Hydroxyl radicals formed during sulphite oxidation reacted with methional. Ethylene was one of the products of the reaction.

Ethylene could also be formed from a model system containing linoleic acid hydroperoxide, sulphite and methional. The pH optimum was 5.0. It is thought that two species were responsible for converting the methional to ethylene, the hydroxyl radical, formed during sulphite oxidation and singlet oxygen which probably arose from the collision of two sec-peroxy radicals.

Studies of oxygen uptake by a LAHPO/sulphite system showed that the hydroxyl radical was formed during sulphite oxidation and may be a chain carrying species. Because both hydroxyl radicals and methional inhibited sulphite oxidation initiated by LAHPO it was thought that methional was reacting with hydroxyl radicals in our system. Because inhibitors of sulphite oxidation only partially inhibited ethylene formation it was thought that another species, not formed during sulphite oxidation could also be responsible for the conversion of methional to ethylene.

The evidence for singlet oxygen production by linoleic acid hydroperoxide and sulphite came from three techniques - thin layer analysis of the products of diphenylfuran oxidation, inhibitor studies on diphenylfuran oxidation using fluorescence spectrophotometry and chemiluminescence. Diphenylfuran was converted by linoleic acid hydroperoxide

and sulphite to cis-dibenzoylethylene, the product formed when diphenylfuran reacts with singlet oxygen. Both diphenylfuran oxidation and chemiluminescence were inhibited by singlet oxygen quenchers and antioxidants but not inhibitors of sulphite oxidation.

Several simple experiments were performed to show that singlet oxygen could react with methional to produce ethylene. Light, methylene blue, and methional produce ethylene by a singlet oxygen reaction. Other singlet oxygen producing systems, H_2O_2 and OCl^-, peroxidase, H_2O_2 and Cl^- also converted methional to ethylene.

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ABBREVIATIONS

BHA	Butylatedhydroxyanisole
BHT	Butylatedhydroxytoluene
DABCO	1,4-diazabicyclo (2.2.2) octane
oDBB	o-dibenzoylbenzene
cis-DBE	cis-dibenzoyethylene
trans-DBE	trans-1,2-dibenzoylethylene
DMF	Dimethylfuran
DMSO	Dimethylsulphoxide
DPF	2,5-diphenylfuran
DPIBF	1,3-diphenylisobenzofuran
EDTA	Disodium (ethylenedinitrilo) tetracetate
αKMBA	α-keto- -methylthiobutyric acid
LAHPO	Linoleic acid hydroperoxide
NDGA	Nordihydroguiaretic acid
SOD	Superoxide dismutase

INTRODUCTION

Ethylene is a gas produced by many plant tissues. It is involved in a wide variety of physiological effects including breaking of dormancy in seeds, inhibition or stimulation of growth of stems, induction of flowering, gravitational responses, leaf senescence and fruit ripening (1). Perhaps because of its economic importance the effect most studied is fruit ripening.

The changes usually associated with fruit ripening are softening of the fruit flesh, hydrolytic conversion of storage materials and changes in pigments and flavours. In some fruits these changes occur over a few days but in citrus fruits these changes may take months. The fruits that ripen over a short period of time usually experience a burst of ethylene production and a burst of respiration just before ripening occurs. These fruits are known as climacteric fruits. The slow ripening fruits do not experience the burst of ethylene production or the burst of respiration and are known as non-climacteric fruits.

Burg and Burg (2) concluded that ethylene was the ripening hormone on the basis of experiments in which the ripening of banana fruits was inhibited by placing them in a partial vaccuum. The reintroduction of oxygen did not restore ripening but the addition of ethylene did. Ethylene causes increased synthesis of many enzymes which are responsible for the changes that occur during ripening (1).

The problem this thesis is concerned with is how is ethylene synthesized? The precursor of ethylene was unknown for many years.

The possible precursors proposed included acetate, linolenic acid, propanal, ethanol, glucose, acrylic acid, β alanine, organic acids and methionine (3). Now the only two seriously considered are linolenic acid and methionine and there is evidence for both hypotheses.

In 1964, Lieberman and Mapson (4) showed that ethylene could be formed from peroxidated linolenic acid and cuprous ion. Galliard et al (5) later showed that ethylene could be formed from apple extracts incubated with linolenic acid and ascorbate. What was probably occuring was that lipoxygenase was oxidising the linolenic acid to linolenic acid hydroperoxide which was then broken down to ethylene by the cuprous ion. The ability of extracts from apples at different stages of their development to produce ethylene from linolenic acid correlated with the ethylene production by the whole fruit (6). This probably reflects the fact that lipoxygenase activity rises and falls at about the same time as the burst of ethylene production which occurs at the climacteric stage (7).

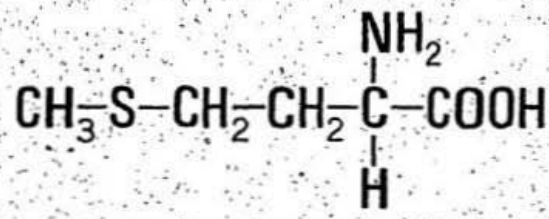
Enzymatic reactions discriminate between compounds containing different mass isotopes, especially when carbon dioxide or bicarbonate is a reactant (8). Because of this lipids in higher plants have a higher percentage of ^{12}C than do carbohydrates or proteins (9). Laties has shown that the ethylene produced by avocados at the late climacteric and postclimacteric stages has a $^{13}C/^{12}C$ ratio typical of plant lipids and not plant carbohydrates or protein (10), indicating a fatty acid origin for the ethylene.

It is generally believed however that methionine is the precursor of ethylene in higher plants. At the same time as they showed linolenic acid could act as an ethylene precursor, Lieberman and Mapson showed that ethylene could also be formed from a model system containing cupric ion, ascorbate and methionine (4). Methionine was deaminated and decarboxylated to methional by a Strecker degradation (11) catalysed by cuprous ion. Methional was then broken down to ethylene, possibly by hydroxyl radicals.

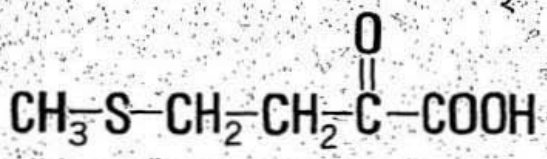
Plant tissue fed with methionine produced more ethylene than controls (3). Later Lieberman et al showed that ^{14}C-methionine was converted to ^{14}C-ethylene with 60% efficiency by apple slices (12). Working with cauliflower florets Mapson and Wardale (13) showed that ethylene formation was stimulated by the addition of methionine. Ethylene could also be formed from cell-free extracts although in this case methional was a better precursor. Investigation of the cell-free extract showed that the components of the ethylene producing system were a peroxide generating system (glucose and glucose oxidase), a peroxidase, a phenol, a sulphinic acid and either methional or α-ketomethylthiobutyric acid (13, 14, 15, 16, 17). The structures of the methionine derivatives are given in Figure 1. Mapson and Wardale also showed that αKMBA could be formed from methionine in the presence of a mitochondrial transaminase and an acceptor keto acid (17).

Yang investigated a model system containing peroxidase, hydrogen peroxide or manganous ions, sulphite, a phenol and methional (18). The peroxidase was thought to oxidise the phenol to the phenoxy radical which then initiated the aerobic oxidation of

Methionine



αKMBA



Methional

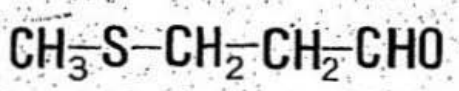


Figure 1 Structures of methionine and related compounds.

sulphite. Free radicals such as the hydroxyl radical (.OH), the superoxide radical ($O_2^{\cdot-}$) or the hydroperoxy radical ($HO_2^{\cdot}$) were thought to react with methional to produce ethylene. It was thought that these reactions could be occurring inside the cauliflower florets. However Liebeman and Kunushi (19) claimed that ethylene production by the cauliflower florets was caused by different components leaking into the buffer surrounding the tissue.

Several other pieces of evidence suggest that the model system proposed by Yang does not operate in plants. Methional, although it is a good precursor of ethylene in cell free extracts of cauliflower florets and in Yang's model system is a poor precursor in intact tissue (20). αKMBA is utilized 100 x more effectively than methionine by the peroxidase system, but αKMBA is utilized by apple and cauliflower tissue with about the same efficiency as methionine (17,20). If αKMBA is an intermediate in the conversion of methionine to ethylene this could be interpreted as meaning that the conversion of methionine to αKMBA occurs very quickly in plants and that the conversion of αKMBA to ethylene is rate-limiting.

In the model system monophenols or m-diphenols promote ethylene production but in intact tissue these have no effect on ethylene production (21). This is hardly surprising since if the peroxidase system were operating in plants the phenols would probably have limited access to the ethylene producing system. Added phenols would probably be unable to get to the site of ethylene biosynthesis.

Finally there is no correlation between levels of peroxidase in pea stem and the amount of ethylene produced by different parts of the stem (22). Mapson and Wardale (7) also reported that peroxidase

levels fell during ripening of tomato fruits while ethylene production increased. It must be remembered that peroxidase has several isoenzymes. Only one of these could be responsible for ethylene production and overall levels of peroxidase may vary differently to the levels of one isoenzyme. However the weight of evidence suggests that a peroxidase is not involved in ethylene biosynthesis.

In an attempt to resolve the conflict between linolenic acid and methionine as precursors of ethylene Mapson et al (23) carried out a study in which radioactively-labelled linolenic acid and methionine were fed to apple discs, tomato discs and cauliflower florets at different stages of their development. Labelled methionine was converted to labelled ethylene by all fruits at all stages of their development. When labelled linolenic acid was given to the fruits no labelled ethylene was produced. However in apple discs unlabelled linolenate increased the formation of ^{14}C-ethylene from ^{14}C-methionine. It was suggested that linolenate may play a secondary role in ethylene production, and that lipoxygenase was also involved.

Mapson and Wardale then showed the presence of an enzyme with lipoxygenase activity in tomatoes (7). They also showed that ethylene could be formed from a model system containing peroxidase, p-hydroxybenzoate, benzene sulphinic acid, lipoxygenase, linolenate and αKMBA or methional. Lipoxygenase was essential for this system. Sulphite can replace the sulphinic acid. Appreciable amounts of ethylene could be formed in the absence of peroxidase and the phenol.

They then went on to measure peroxidase and lipoxygenase levels in tomato fruits at the preclimacteric, the onset of the climacteric, the climacteric and postclimacteric stages. Lipoxygenase activity increased at the onset of the climacteric (just prior to the burst of ethylene synthesis) but peroxidase activity declined steadily throughout all stages of development. However this result may be atypical. Other workers have reported that peroxidase levels increase during ripening (1).

In view of the evidence suggesting that lipoxygenase activity is more closely related to ethylene production than is peroxidase activity we decided to investigate a model system containing lipoxygenase, linolenate, sulphite and methional. We thought that this system would produce ethylene because it has been known since 1961 that lipoxygenase, when catalyzing the oxidation of its substrate can initiate sulphite oxidation (24). The system should be similar to the model system of Yang.

MATERIALS AND METHODS

Where possible chemicals were purchased from the Sigma Chemical Company. The lipoxygenase obtained from Sigma was soyabean lipoxygenase (Type I). 2,5-diphenylfuran (DPF) was supplied by Eastman Organic Chemicals. Trans-1,2-dibenzoylethylene (trans-DBE), 1,3-diphenylisobenzofuran (DPIBF) and o-dibenzoylbenzene (oDBB) were supplied by the Aldrich Chemical Company. Linoleic acid hydroperoxide (LAHPO) was prepared by the method of O'Brien (25) and was a mixture of the 9- and 13- DL hydroperoxides.

Ethylene Production

Incubations were carried out in 25 ml Erlenmeyer flasks sealed with rubber septa. The incubation mixture for the synthesis of ethylene contained 1,340 units/ml lipoxygenase, 1 mM linolenate, 4 mM sulphite, 1 mM methional and 1 mM EDTA in 1 ml 50 mM phosphate buffer pH 7.8. The flasks were incubated for 30 mins at 30°C.

A non-enzymic system was also found to produce ethylene. The non-enzymic system contained 1 mM LAHPO, 1 mM sulphite and 1 mM methional in 1 ml 50 mM acetate buffer pH 5.0. The non-enzymic reaction was much faster but less extensive than the enzymic system. The incubation time was reduced to one minute, so great care had to be taken to ensure that the buffer was at a constant temperature (20°C) and that the contents of the flask were mixed thoroughly. The reaction was started by the injection of 10 µl of 100 mM LAHPO through the septum into the reaction mixture. The incubation was carried out in a rapidly shaking, constant temperature water bath.

At the end of the incubation period 0.5 mls of gas was withdrawn using a Hamilton 1 ml gas tight syringe. The sample was analyzed by gas chromatography using the flame ionization detector on a Pye Unicam Gas Chromatograph. The glass column, 1.5 m x 0.6 mm was packed with Chromosorb 102 obtained from the Johns-Manville Company. The operating temperature was 98°C and the carrier gas used was nitrogen at a pressure of 20 p.s.i. and a flow rate of 40 ml/min. Ethylene was estimated quantitatively by injecting into the gas chromatograph a known amount of a standard ethylene mixture purchased from Applied Science Labs.

Oxygen Uptake

Oxygen uptake by a reaction mixture containing 0.1 mM LAHPO, 2 mM sodium sulphite, 1 mM EDTA and 50 mM phosphate buffer pH 6.0 was measured on a Clark-type oxygen electrode at 20°C.

Diphenylisobenzofuran (DPIBF) Oxidation

3.0 ml of 50 mM phosphate buffer pH 6.0 containing 1 mM EDTA and 20 µl of 5% Triton x -100 were placed in a cuvette. 2 µl of a 100 mM solution of DPIBF dissolved in acetone was added to the cuvette. The basic reaction mixture also contained 0.1 mM sulphite and 0.1 mM LAHPO. The disappearance of DPIBF was followed by the decrease in absorbance at 420 nm.

Purification of DPIBF

The DPIBF was found to contain impurities of oDBB, the product of DPIBF oxidation by singlet oxygen. It was purified by thin layer chromatography on silica gel G plates activated by heating to 110°C for 1 hour. All steps were done in the dark to prevent the spontaneous

conversion of DPIBF to oDBB. Benzene was used as the mobile phase. Under these conditions DPIBF moved very close to the solvent front while oDBB had an Rf of 0.35. The DPIBF band was scraped off and re-dissolved in benzene. The benzene solution was decanted and evaporated to dryness. The DPIBF was stored in the dark.

Identification of Products of DPIBF Oxidation

The products of DPIBF oxidation were extracted with chloroform and spotted on silica gel plates under the same conditions used for the purification of DPIBF. The products of DPIBF oxidation could be seen under UV light.

Chemiluminescence

5 ml of 50 mM phosphate buffer, pH 6.0 was placed in a glass scintillation vial. The chemiluminescence produced during the reaction between 0.4 mM LAHPO and 1 mM sulphite was measured using a scintillation counter (Beckman Model LS-233) with a digital readout, with the machine in the out-of-coincidence mode. The counts were recorded every ten seconds for the first minute and then every half minute for four minutes.

Oxidation of Diphenylfuran - Identification of Products

The product of the reaction between DPF and singlet oxygen, cis-DBE (26), could not be obtained commercially. DPF was incubated in the presence of two known singlet oxygen-producing systems : 5 mM H_2O_2 and 5 mM NaOCl (27), and 0.5 mM potassium bromide, 0.5 mM hydrogen peroxide and 0.031 units/ml lactoperoxidase (27). Presumably these reactions would convert DPF to cis-DBE.

2 mM LAHPO, 2 mM sodium sulphite and 1 mM EDTA were also incubated

with 1 mM DPF in 1 ml of 50 mM acetate buffer pH 4.5.. The DPF stock solution was 100 mM and dissolved in either acetone to DMSO. 10 µl of stock solution was added to the reaction mixture. The mixture was incubated for 5 minutes at 20°C. Products were extracted with 1 ml chloroform. The chloroform extract was dried and the residue was transferred to silica gel plates with small volumes of chloroform. The plates were developed for one hour in a solvent system consisting of heptane-dioxane, 3:1. The products were then examined under UV light.

Spectrophotometric Determination of Rate of DPF Oxidation

The oxidation of DPF was followed spectrophotometrically by measuring the decrease in emission at 368 nm (slit 6 mµ) with excitation at 333 nm (slit 6 mµ) using a Perkin Elmer fluorescence spectrophotometer MPF 2A. A typical reaction mixture contained 3 mls 50 mM acetate buffer pH 4.5, 33 µM LAHPO, 33 µM sulphite 200 µM EDTA and 6.6 µM DPF. The DPF stock solution (10 mM) is dissolved in acetone or DMSO. 2 µl of this is added to the reaction mixture.

RESULTS

Lipoxygenase-Catalyzed Ethylene Formation

Ethylene was formed from a reaction mixture containing lipoxygenase, linolenate, sulphite and methional. The reaction was completed within one hour under the conditions described in "Materials and Methods". The pH-dependence of the reaction is shown in Figure 2 and the time course of the reaction is shown in Figure 3. Some ethylene was formed even in the absence of lipoxygenase. This was thought to be due to impurities of linolenic acid hydroperoxide already present in the linolenic acid solution. Bubbling the stock solution of fatty acid with nitrogen prior to use inhibited the non-enzymic reaction probably by preventing lipid peroxidation. The non-enzymic reaction was greater at an acidic pH than at an alkaline pH.

Table 1 shows the effects of various reagents on the rate of conversion of methional to ethylene by the system containing lipoxygenase. Over the first 30 minutes the rate of ethylene formation was 0.8 nmoles/min. The figures in the table represent the percentage of this rate in the presence of the reagent. Only 5% of the methional was converted to ethylene.

Since hydroxyl radicals can react with methional to produce ethylene (28) the hydroxyl radical scavengers mannitol (29) and ethanol (30) were tested for their ability to inhibit the reaction. Mannitol completely inhibited ethylene production but ethanol which is an efficient hydroxyl radical scavenger only inhibited the reaction 40%.

Superoxide dismutase, an enzyme which catalyzes the dismutation of superoxide radicals had no effect on the rate of ethylene formation from

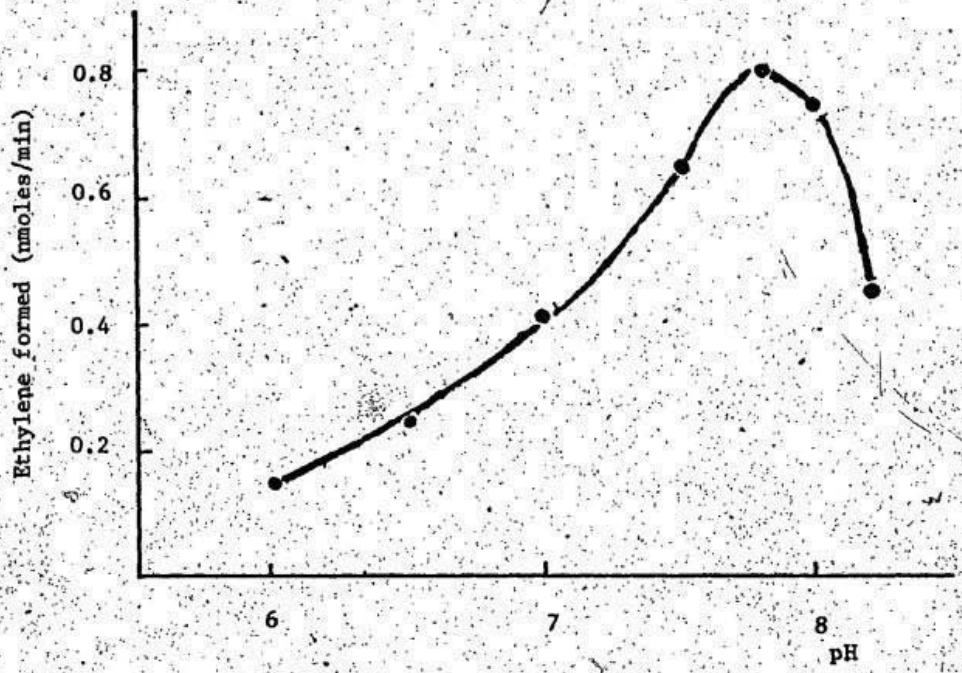


Figure 2 Dependence of lipoxygenase-catalyzed ethylene formation on pH.

Reaction mixtures contained, 1340 units/ml lipoxygenase, 1mM linolenate, 4 mM sulphite, 1 mM methional and 1 mM EDTA in 1 ml 50 mM phosphate buffer incubated for 30 minutes at 30°C.

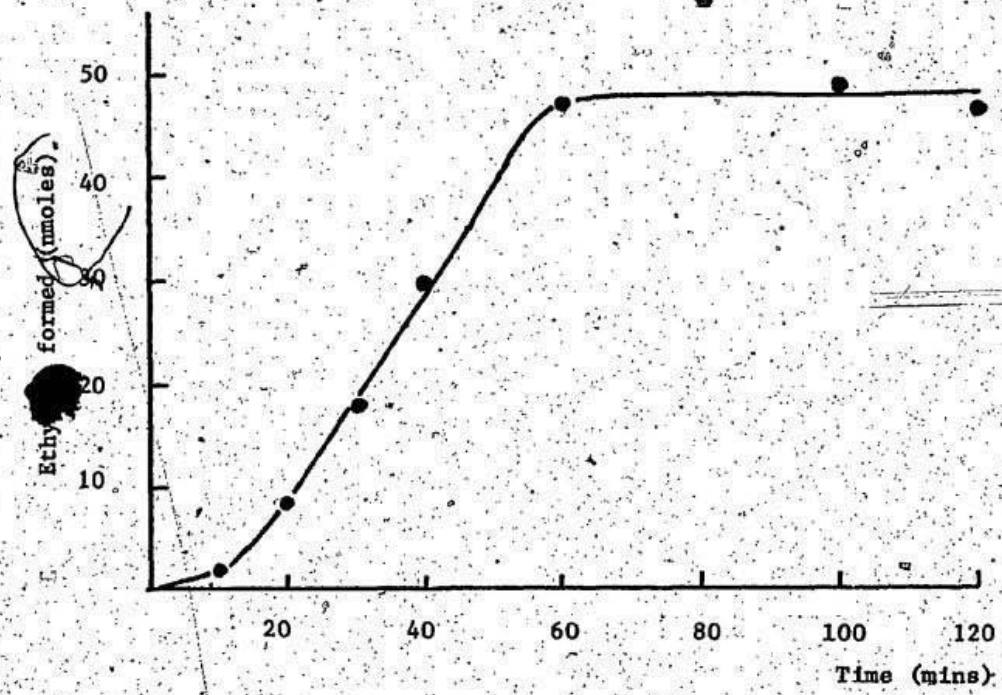


Figure 3 Time-course of lipoxygenase-catalyzed ethylene formation at pH 7.8.

The conditions used are the same as for figure 2.

Table I

Ethylene Formation Catalyzed by Lipoxygenase

Addition	Rate of Ethylene Formation
Complete System	100
Mannitol 0.1 M	0
Ethanol 0.1 M	58
SOD 10 µg/ml	100
Bilirubin 0.1 mM	13
DPF 1 mM	100
Azide 10 mM	100
Catalase 1000 units/ml	100
Catechol 20 µM	67
Catechol 1 mM	8
Resorcinol 20 µM	100
Resorcinol 1 mM	40

The complete system contained 1,340 units/ml lipoxygenase, 1 mM linolenate, 4 mM sodium sulphite, 1 mM methional and 1 mM EDTA in 1 ml 50 mM phosphate buffer pH 7.8 contained in 25 ml Erlenmeyer flasks sealed with a rubber septum. Flasks were incubated for 30 minutes at 30°C. Under these conditions ethylene was formed at a rate of 0.8 nmoles/min.

methional. Superoxide radicals may be produced during the autooxidation of sulphite but, would not appear to be necessary for the synthesis of ethylene. Catalase had only a small effect on ethylene production so hydrogen peroxide would not appear to play a major role in ethylene synthesis.

Singlet oxygen scavengers such as 2,5-diphenylfuran (26) and azide (31) were also ineffective. Bilirubin is a singlet oxygen quencher (32) but is also probably a free radical scavenger and its inhibitory action was probably due to its free radical scavenging ability. Catechol is another singlet oxygen and free radical scavenger (33) which was a good inhibitor of ethylene production. However it is also an inhibitor of lipoxygenase and this may be how it is acting here.

Resorcinol which stimulates the peroxidase-containing ethylene-generating system of Yang did not have a similar effect on our system.

Non-Enzymic Ethylene Formation

Replacement of linolenic acid and lipoxygenase by 1 mM linoleic acid hydroperoxide also resulted in ethylene formation. The reaction had different characteristics than the enzymic reaction. The optimal pH was 5.0 instead of 7.8. The pH dependence of the reaction is shown in Figure 4.

With a LAHPO concentration of 1 mM maximum ethylene production occurred when the sulphite concentration was 1 mM. The amount of ethylene synthesized increased linearly up to this point but after a sulphite concentration of 1 mM was reached further increases in sulphite concentration did not increase the amount of ethylene formed.

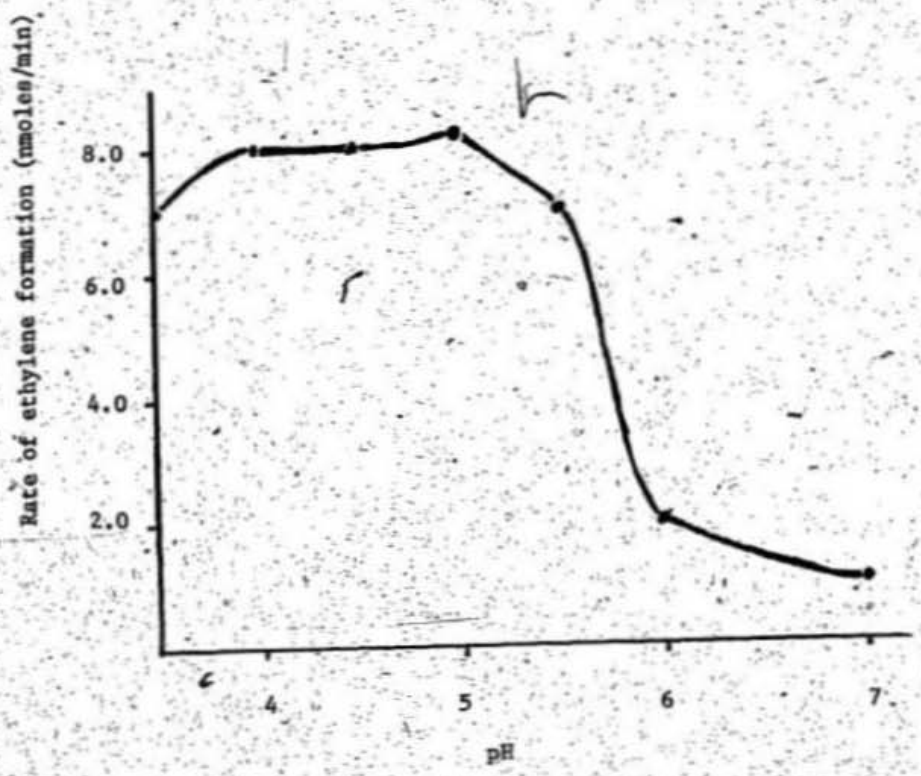


Figure 4 pH curve of non-enzymic ethylene formation.

Similarily when the sulphite concentration was 1 mM maximum ethylene production occurred with a LAHPO concentration of 1 mM. The reaction was much faster than the enzymic reaction, being completed within two minutes instead of sixty. The time course of the reaction is shown in Figure 5. The incubation time was reduced to one minute. Although the ethylene was formed at a much faster rate in the non-enzymic reaction the total amount of ethylene produced was only about one-third the amount produced by the enzymic system. The rate of formation was 8.1 nmoles/min and 14.5 nmoles of ethylene were produced when the reaction had gone to completion - an efficiency of conversion of methional to ethylene of only 1.5%.

It was thought that the non-enzymic system would be easier to study since the rate of ethylene formation would not be affected by changes in the activity of the enzyme which could occur by the addition of some solvents or reagents. Table 2 shows the effects of various reagents on the synthesis of ethylene by the non-enzymic system.

(a) The Involvement of Hydroxyl Radicals and their Source

Hydroxyl radical scavengers partially inhibited ethylene synthesis. Hydroxyl radicals are thought to be generated during sulphite oxidation by the following series of reactions (18,45).

$$ROOH + HSO_3^- \longrightarrow RO\cdot + OH^- + HSO_3\cdot \qquad (i)$$

$$HSO_3\cdot + O_2 \longrightarrow SO_3 + H^+ + O_2^-\cdot \qquad (ii)$$

$$O_2^-\cdot + H^+ + SO_3^{2-} \longrightarrow SO_3^-\cdot + HO_2^- \qquad (iii)$$

$$HO_2^- + H^+ \longrightarrow H_2O_2 \qquad (iv)$$

$$H_2O_2 + O_2^-\cdot \longrightarrow OH\cdot + OH^- + O_2 \qquad (v)$$

Superoxide radicals are produced when oxygen reacts with the bisulphite radical reaction (ii). The superoxide radical can then react with sulphite to produce $SO_3^-\cdot$. Thus a chain reaction occurs. In this scheme the superoxide radical is the chain carrying species but it is

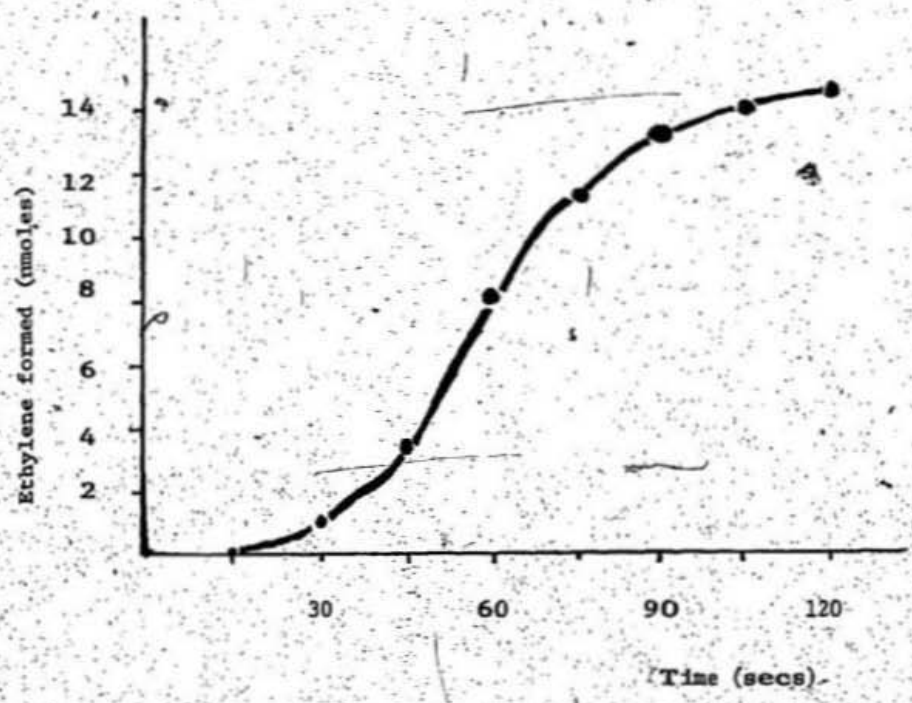


Figure 5 Time-course of non-enzymic reaction.

Table 2

Ethylene Formation by the Non Enzymic System

Addition	Ethylene formed after one minute
None	100
n-butanol 10 mM	50
n-butanol 50 mM	50
Ethanol 10 mM	62
Ethanol 50 mM	60
Mannitol 50 mM	35
Catalase 1000 units/ml	70
Hydrogen Peroxide 0.8 mM	180
Superoxide Dismutase 10 µg/ml	100
Anaerobic conditions	50
Acetone 70 mM	66

The complete system contained 1 mM LAHPO, 4 mM sulphite, 1 mM methional and 1 mM EDTA in 1 ml of acetate buffer pH 5.0 contained in 25 ml Erlenmeyer flasks sealed with a rubber septum. Flasks were incubated at 20°C for one minute. Under these conditions ethylene was formed at a rate of 8.1 nmoles/min.

thought that other species such as the hydroxyl radical can also carry on the chain reaction by reacting with bisulphite to produce the bisulphite radical ($HSO_3\cdot$). In support of this it is found that both superoxide dismutase and hydroxyl radical scavengers inhibit oxygen uptake by the LAHPO/sulphite system (see Table 3). Superoxide dismutase catalyzes the dismutation of superoxide radicals according to the following equation

$$O_2\cdot^- + O_2\cdot^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

Catalase also inhibits oxygen uptake by the LAHPO/sulphite system, consistent with the hypothesis that reaction (v) is the source of the hydroxyl radicals.

Singlet oxygen quenchers such as azide and DABCO do not inhibit oxygen uptake. In fact they enhance it though the reason for this is unknown. Histidine, methionine and tryptophan are also singlet oxygen scavengers (36) but they can also react with hydroxyl radicals (30) and hydrogen peroxide. These amino acids probably inhibit oxygen uptake because of their ability to scavenge hydroxyl radicals and hydrogen peroxide.

The antioxidants BHA and NDGA also inhibit oxygen uptake but only at relatively high concentrations. Methional also inhibits oxygen uptake possibly because of its hydroxyl radical scavenging ability (34).

The data are consistent with the hypothesis that the Haber-Weiss reaction (reaction (v)) is the source of the hydroxyl radicals.

In 1970 Beauchamp and Fridovich (28) used a superoxide radical generating system (xanthine and xanthine oxidase) to show that hydroxyl radicals could react with methional to produce ethylene. They proposed the Haber-Weiss reaction as the source of the hydroxyl radicals. Bors et al (34) confirmed that hydroxyl radicals reacted with methional very quickly and also showed that superoxide radicals only reacted slowly

Table 3

Oxygen Uptake During the Interaction of Sulphite and LAHPO

Addition	Rate of Oxygen Uptake
None	100
n-propanol 67 mM	25
Mannitol 0.1 M	20
Catalase 1000 units/ml	60
SOD 10 µg/ml	50
Acetone 50 mM	10
Azide 50 mM	130
DABCO 10 mM	140
Histidine 20 mM	50
Methionine 20 mM	40
Tryptophan 1 mM	10
BHA 1 mM	50
NDGA 1 mM	40
Methional 2 mM	30

Oxygen uptake was measured using a Clark-type oxygen electrode. The complete system contained 0.1 mM LAHPO, 2 mM sulphite, 1 mM EDTA in 1.9 mls 50 mM phosphate buffer pH 6.0. At 20°C with no additions the initial rate of oxygen uptake was 770 µmoles/min.

with methional. Lieberman et al (12) showed that hydrogen peroxide and methional would produce ethylene but the concentration of hydrogen peroxide used was very high (17 mM).

As mentioned before hydroxyl radical scavengers partially inhibited ethylene synthesis. With both n-butanol and ethanol a maximum inhibition was obtained. Catalase also inhibited ethylene synthesis and hydrogen peroxide stimulated it. However superoxide dismutase had no effect. Anaerobic conditions inhibited ethylene synthesis 50%.

These results suggested to us that some species formed during sulphite oxidation was at least partially responsible for the conversion of methional to ethylene. Superoxide radicals and low concentrations of hydrogen peroxide cannot convert appreciable amounts of methional to ethylene. The hydroxyl radical can convert methional to ethylene (28,34) and is probably the species responsible here.

Because hydroxyl radical scavengers only partially inhibited ethylene biosynthesis and because anaerobic conditions, which would be expected to completely inhibit sulphite oxidation, only partially inhibited ethylene biosynthesis it was thought that another species, not formed during sulphite oxidation may be responsible for converting methional to ethylene.

(b) Involvement of Singlet Oxygen in the Conversion of Methional to Ethylene

Azide is a singlet oxygen quencher which inhibits the formation of ethylene from methional (see Table 4). This cannot be due to its action on sulphite oxidation since it stimulates oxygen uptake by an unknown mechanism. DABCO which forms a charge-transfer complex with singlet oxygen behaves in a similar manner.

Two of the best inhibitors of ethylene synthesis were methionine

Table 4

Effect of Singlet Oxygen Traps and Antioxidants on Ethylene Formation by the Non Enzymic System

Addition	Rate of Ethylene Formation
None	100
Azide 10 mM	48
Histidine 10 mM	45
DABCO 10 mM	90
Methionine 2 mM	40
Methionine 20 mM	6
Tryptophan 50 µM	20
Tryptophan 1 mM	0
Ethoxyquin 10 µM	60
Ethoxyquin 50 µM	3
Butylatedhydroxyanisole (BHA) 100 µM	65
Propylgallate 100 µM	70
Nordihydro guiaretic acid (NDGA) 100 µM	60

Conditions are the same as those employed for Table 2.

and tryptophan. This is probably because they can react with both singlet oxygen (35) and hydroxyl radicals (30). Histidine is another amino acid which can react with both singlet oxygen and hydroxyl radicals. However it is not such a potent inhibitor of ethylene biosynthesis.

The antioxidants, BHA, propylgallate and NDGA also inhibited ethylene synthesis at concentrations which were ineffective in inhibiting uptake. The antioxidants can react with radicals formed from the fatty acid hydroperoxides, such as RO· and ROO·. It is possible that the singlet oxygen arises from the interaction of some of these radicals.

Ethoxyquin was the most effective antioxidant in inhibiting ethylene biosynthesis.

(c) The Source of the Singlet Oxygen

That the singlet oxygen was derived from the hydroperoxides was suggested by the fact that antioxidants such as BHA, propylgallate and NDGA inhibited ethylene formation at concentrations which were not effective in inhibiting sulphite oxidation.

Several methods were used to attempt to demonstrate that singlet oxygen was being formed during the reaction between LAHPO and sulphite.

(i) DPIBF Oxidation

1,3-diphenylisobenzofuran (DPIBF) is converted by singlet oxygen to o-dibenzoylbenzene. Thin layer chromatography was used to identify the products of DPIBF oxidation which occurred during the reaction between LAHPO and sulphite. The results are illustrated in Figure 6. DPIBF was oxidised to the same product formed when it reacts with singlet oxygen.

The oxidation of DPIBF was then followed spectrophotometrically at

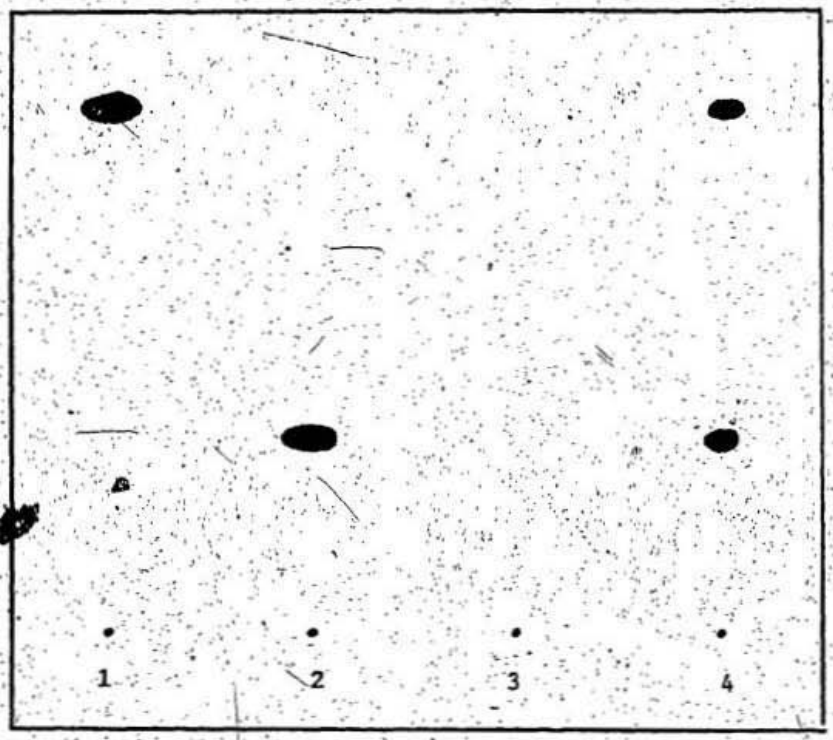


Figure 6 Identification of products of DPIBF oxidation by LAHPO and sulphite.

1. DPIBF
2. oDBB
3. LAHPO and sulphite
4. LAHPO, sulphite and DPIBF

Reaction conditions are described in "Materials and Methods".

420 nm. The results of the experiments are shown in Table 5. DPIBF oxidation was not inhibited by the hydroxyl radical scavengers, mannitol, ethanol, n-propanol or n-butanol. The singlet oxygen quencher, azide and the singlet oxygen traps, dimethylfuran, tryptophan and methionine also had no effect on DPIBF oxidation. As mentioned previously tryptophan and methionine also react with hydrogen peroxide, therefore this species is also probably not involved in DPIBF oxidation.

The only class of compounds which effectively inhibited DPIBF oxidation were the antioxidants. These were very effective at low concentrations. It has been shown by Hawco *et al* (37) that *sec*-peroxy radicals can initiate DPIBF oxidation. This is probably the reaction occurring here. The *sec*-peroxy radicals may arise from reaction (viii).

$$RO\cdot + ROOH \longrightarrow ROH + ROO\cdot \qquad \text{(viii)}$$

Antioxidants inhibit DPIBF oxidation by scavenging ROO· or RO· (38).

DPIBF oxidation was used to attempt to demonstrate the involvement of singlet oxygen. Instead it provided evidence for the formation of *sec*-peroxy radicals.

(ii) Chemiluminescence

The next method used to detect the presence of singlet oxygen during the reaction between LAHPO and sulphite was chemiluminescence. Light is emitted when two molecules of singlet oxygen collide (39) and this can be detected under the conditions described in "Materials and Methods". The reaction between LAHPO and sulphite was found to be accompanied by a strong chemiluminescence. The effect of various reagents on the chemiluminescence is shown in Table 6. The dimol collision of singlet oxygen presumably occurs in the gas phase as the concentration of singlet

Table 5

DPIBF Oxidation by LAHPO and Sulphite
Followed Spectrophotometrically

Addition	Relative Rate of DPIBF Oxidation
None	100
Mannitol 0.1 M	100
Ethanol 0.1 M	95
n-propanol 50 mM	100
n-butanol 50 mM	100
Azide 20 mM	100
Dimethylfuran (DMF) 2 mM	100
Tryptophan 1 mM	100
Methionine 1 mM	100
BHA 5 µM	33
BHA 100 µM	0
NDGA 5 µM	30
NDGA 100 µM	0
Propylgallate 10 µM	35
Ethoxyquin 1 µM	10
Methional 50 µM	35

The complete reaction mixture contained 67 µM DPIBF, 1 µM EDTA, 20 µl of 5% Triton X-100, 0.1 mM LAHPO and 0.1 mM sulphite in 3.0 mls of 50 mM phosphate buffer pH 6.0. Under these conditions the initial rate of DPIBF oxidation was 11 µmoles/min.

Table 6

Chemiluminescence after 10 seconds Produced by LAHPO and Sulphite

	Counts
Buffer alone	140,000
Complete system	790,000
Addition	
Ethanol 50 mM	385,000
Methanol 50 mM	651,000
Methionine 0.4 mM	453,000
Azide 20 mM	201,000
DMF 2 mM	442,000
Histidine 20 mM	320,000
DABCO 20 mM	678,000
BHA 1 mM	266,000
BHT 1 mM	246,000
Methional 0.4 mM	136,000
Boiling chip	2,153,200

The complete system contained 0.4 mM LAHPO, 1 mM sulphite and 1 mM EDTA in 5 mls of 50 mM phosphate buffer pH 6.0.

oxygen needed in the aqueous phase would be too high (40). It is interesting to note that placing a boiling chip in the scintillation vial enhanced the chemiluminescence 3-fold, possibly by encouraging the formation of small oxygen bubbles on its surface. The singlet oxygen quencher, azide, and the singlet oxygen traps, dimethylfuran (DMF) and histidine all inhibited the chemiluminescence. DABCO had little effect. It would be expected to enhance the chemiluminescence by stabilizing the singlet oxygen in a charge-transfer complex. The antioxidants BHA and BHT also inhibited the chemiluminescence. The best inhibitor of the chemiluminescence was methional which inhibited completely at 0.4 mM.

(iii) The Conversion of DPF to cis-DBE

Diphenylfuran (DPF) reacts with singlet oxygen to give cis-dibenzoylethylene (cis-DBE) (26). Figure 7 shows the results obtained in experiments designed to determine the product of DPF oxidation by LAHPO and sulphite. Known singlet oxygen producing reactions converted DPF to a product which is presumably cis-DBE. It has the same Rf value as authentic cis-DBE using the same solvent system (26). It is also present as an impurity of trans-DBE. Enough cis-DBE was formed to perform an approximate quantitative analysis in the presence of various reagents. Table 7 gives estimates of the amount of cis-DBE formed.

Azide, DABCO and methionine all inibited the conversion of DPF to cis-DBE indicating that it is singlet oxygen and not free radicals which converts DPF to cis-DBE. Antioxidants also inhibited cis-DBE production. Mannitol catalase and SOD had no effect. Acetone, an efficient inhibitor of sulphite oxidation had no effect on singlet oxygen production.

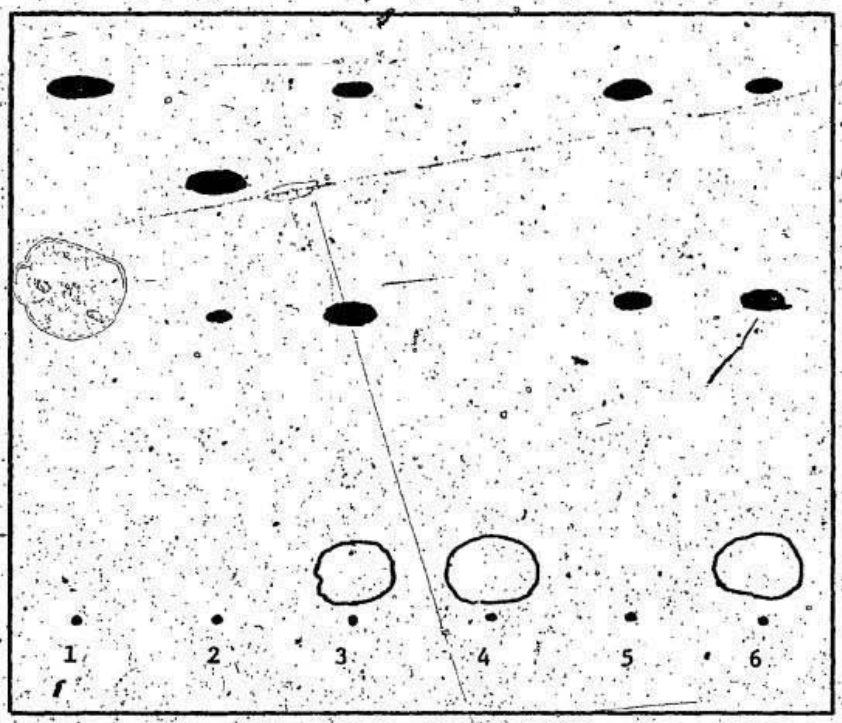


Figure 7 Identification of the products of DPF oxidation by LAHPO and sulphite.

1. DPF
2. trans-DBE
3. LAHPO, sulphite and DPF
4. LAHPO and sulphite
5. Hydrogen peroxide, sodium hypochlorite and DPF.
6. Lactoperoxidase, hydrogen peroxide, potassium bromide and DPF.

The concentrations of reagents are given in "Materials and Methods".

Table 7

Conversion of DPF to cis-DBE in the Presence of Various Inhibitors

	Approximate Amount of cis DBE Formed
None	+ + + +
Azide 20 mM	+
DABCO 20 mM	+
Methionine 10 mM	+ +
BHT 1 mM	+
NDGA 200 µM	+
BHA 100 µM	+ +
Ethoxyquin 100 µM	+ +
Mannitol 10 mM	+ + + +
Catalase 1000 units/ml	+ + + +
SOD 10 µg/ml	+ + + +
Methional 1 mM	+ +
None (DPF stock solution dissolved in DMSO)	+ + + +
10 µl Acetone (DPF dissolved in DMSO)	+ + + +

The basic reaction mixture contained 2 mM LAHPO, 2 mM sulphite, 1 mM EDTA and 1 mM DPF in 1 ml 50 mM acetate buffer pH 4.5. DPF could be seen under a UV lamp.

(iv) DPF Destruction

As well as cis-DBE production, the destruction of DPF was followed using fluorescence spectrophotometry as described in 'Materials and Methods'. Table 8 gives details of the effects of various reagents. The singlet oxygen quenchers, azide and DABCO both inhibited DPF destruction. Some antioxidants, NDGA and BHA, were good inhibitors but BHT is poor. Hydroxyl radical scavengers (ethanol and mannitol) had little effect.

I have presented several lines of evidence which show that singlet oxygen is being formed during the reaction between LAHPO and sulphite. Because sulphite oxidation can be inhibited without inhibiting singlet oxygen production, and because of the inhibition of singlet oxygen production by low concentrations of antioxidants it is thought that the singlet oxygen is derived from the fatty acid hydroperoxides. Singlet oxygen traps and quenchers also inhibit the conversion of methional to ethylene. However we do not know whether the singlet oxygen is reacting with methional to produce ethylene or whether the singlet oxygen is necessary for the generation of some other species which can convert methional to ethylene. To demonstrate that singlet oxygen could in fact react with methional several simple experiments were performed.

Methylene blue is a photosensitizer. When excited by visible light it can transfer its energy to oxygen exciting it to the singlet state (36). A Clark-type oxygen electrode containing 5 μM methylene blue and 1 mM methional in 1.9 mls 50 mM phosphate buffer, pH 7.0 was irradiated with orange light. A rapid oxygen uptake was observed. The rate of oxygen uptake was enhanced 7-fold when the phosphate was dissolved in

Table 8.

Destruction of DPF Measured by Fluorescence Spectrophotometry

	Rate of DPF Oxidation (nmoles/min)
None	22.1
5 mM Azide	3.0
20 mM DABCO	15.2
10 µl Acetone	22.0
67 µM NDGA	2.5
67 µl BHT	18.7
67 µM BHA	5.3
20 µl Ethanol	20.2

The basic reaction mixture contained 33 µM LAHPO, 33 µM sulphite, 200 µM EDTA and 6.6 µM DPF in 3 mls 50 mM acetate buffer pH 4.5.

D_2O (pD 7.3).

In a second experiment a 25 ml Erlenmeyer flask which contained 1 ml 50 mM phosphate buffer, pH 7.0, 5 μM methylene blue and 1 mM methional was sealed with a rubber septum was irradiated with orange light. Ethylene formation was measured as previously described. Ethylene was formed at a rate of 2.7 nmoles/min. In D_2O buffer the rate of ethylene formation was 13.6 nmoles/min, approximately a five-fold increase. Singlet oxygen has a longer half-life in D_2O than in ordinary water. Because the rates of oxygen uptake and ethylene formation are enhanced in D_2O it is probably singlet oxygen which is reacting with methional to produce ethylene. If the triplet state of the photosensitizer or some other species were reacting with methional no enhancement of the rate of oxygen uptake or ethylene formation would be seen.

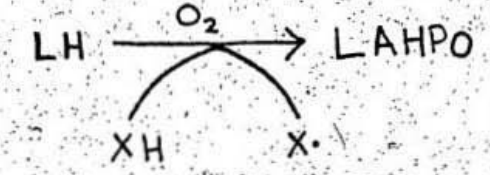
Several other reaction mixtures also converted methional to ethylene : lactoperoxidase, hydrogen peroxide and potassium bromide; hydrogen peroxide and sodium hypochlorite; and LAHPO and Ce^{4+}. The first two reactions are known to produce singlet oxygen (27). The third reaction probably produces singlet oxygen by the two following reactions:

$$ROOH + Ce^{4+} \longrightarrow ROO\cdot + Ce^{3+} + H^{+} \qquad (ix)$$

$$2ROO\cdot \longrightarrow R'O + {}^1O_2 + R''OH \qquad (x)$$

DISCUSSION

Yang's model system for the biosynthesis of ethylene contained horseradish peroxidase, hydrogen peroxide or Mn^{2+}, sulphite, a phenol and methional. Sulphite oxidation was initiated by the phenoxy radical formed by the enzyme. In our model system sulphite oxidation is thought to be initiated by free radical intermediates formed during the oxidation of linolenate by lipoxygenase. Lipoxygenase can also catalyze co-oxidation reactions which can form free radicals according to the following mechanism.



Both our model system and Yang's model system have an optimal pH of 7.8. The pH of a tomato homogenate is usually about 4.5 although this is due mainly to organic acids accumulating inside the vacuolar space (41). The cytoplasm and organelles could have a pH well above 4.5 and it is possible that our model system could operate. It must be remembered that in our experiments soyabean lipoxygenase was used which has an optimal pH of 9.0. Tomato lipoxygenase has an optimal pH of 6.3 - 6.5.

However there are several reasons why the lipoxygenase system could not be operating in plants. Inorganic sulphite is not usually found in plants. The organic sulphinic acids such as cysteine sulphinic acid or methane sulphinic acid can be found (16) but these are in very small quantities - not sufficient to account for the large increase in ethylene production which occurs during the climacteric stage of fruit ripening.

Another objection to the model system is that it will not form ethylene from methionine, only from αKMBA or methional. There is evidence to suggest that methional or αKMBA are not intermediates in the conversion of methionine to ethylene in plants (see Introduction). Methional is not found in fruits (42). In fact it is toxic to fruit tissues (42). However there is also not enough free methionine in plants to sustain the increase in ethylene at the climacteric stage of fruit ripening (43). Presumably this is synthesized as required. Yang (44), showed that the conversion of methionine to ethylene required both ATP and pyridoxal phosphate. He suggested that methionine was first adenylated and then broken down to ethylene and other products by a pyridoxal phosphate dependent γ-elimination.

If methional were the precursor of ethylene in plants and its reaction with the hydroxyl radical, the lipid peroxy radical or singlet oxygen were the ethylene producing reaction, nature has designed a very inefficient way of making ethylene. As can be seen from Table 3 only 50 nmoles of ethylene were formed from 1 μmole of methional. The efficiency of the conversion of methional to ethylene is only 5%. The other products of methional oxidation were not identified but the main product was presumably the sulphoxide, the major product in Yang's peroxidase-catalyzed system (45).

No enzyme or enzyme pathway has yet been found which can directly convert methionine to ethylene. The enzymes which so far have been proposed as the 'methionine cleaving enzymes' - lipoxygenase and peroxidase only serve to produce the appropriate free radicals. It is possible, however, that in the plant ethylene production occurs via a free radical mediated reaction.

(a) The Production of Hydroxyl Radicals

According to Davies (46), LAHPO and sulphite can react together in three different ways according to the pH (see Figure 8). At alkaline pH's LAHPO reacts with sulphite by a nucleophilic displacement (Sn2 reaction). At acidic pH's LAHPO reacts with bisulphite to produce the corresponding alcohol and sulphate but the reaction proceeds via an intramolecular rearrangement of a peroxysulphurous ester to a sulphuric ester. Only at a pH of about 1.0 is the free radical reaction supposed to occur. However oxygen was taken up by a reaction mixture containing LAHPO and sulphite at a pH as high as 7.8. At pH 5.0 oxygen uptake was almost instantaneous. Apparently the free radical reaction occurs at higher pH's than thought by Davies.

One possible pathway of free radical-mediated sulphite oxidation is given on page 19. Hydroperoxides, superoxide radicals and probably hydroxyl radicals are capable of reacting with bisulphite or sulphite to produce the species $HSO_3^{\cdot}$ and $SO_3^{\cdot-}$ which can then react with oxygen. Since hydroxyl radical scavengers inhibited oxygen uptake and methional is a known hydroxyl radical scavenger it was concluded that methional was reacting with hydroxyl radicals in our system.

The oxygen uptake data is consistent with the scheme on page 19. However it was found that superoxide dismutase did not inhibit ethylene formation. If this result were to be believed it would mean that the hydroxyl radicals could not possibly arise from the Haber-Weiss reaction (v).

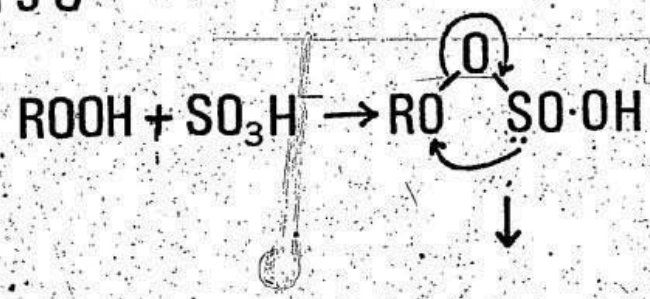
$$H_2O_2 + O_2^{\cdot-} \longrightarrow OH^{\cdot} + OH^- + O_2 \qquad (v)$$

Superoxide radicals are used in the reaction and are also necessary for the production of hydrogen peroxide. Hydrogen peroxide is necessary

pH 9·0

$$ROOH + SO_3^{2-} \rightarrow ROH + SO_4^{2-}$$

pH 5·0



$$ROOH + SO_3H^- \rightarrow RO\,SO{\cdot}OH$$

$$H_2SO_4 + ROH \xleftarrow{H_2O} RO{\cdot}SO_2OH$$

pH 1·0

$$ROOH + HSO_3^- \rightarrow RO\cdot + OH^- + HSO_3$$

Figure 8 Reactions of LAHPO with sulphite which occur at different pH's.

for at least some of the ethylene synthesis since ethylene synthesis is inhibited by catalase and stimulated by hydrogen peroxide.

The Haber-Weiss reaction was first proposed in 1934 by Haber and Weiss but since then several people have shown that the reaction is very slow based on both theoretical (47) and experimental evidence (48). If a pathway for hydrogen peroxide production not involving superoxide radicals could be demonstrated one possible reaction for the generation of hydroxyl radicals could be

$$H_2O_2 + HSO_3^- \longrightarrow OH\cdot + SO_4^{2-} + 2H^+$$

An analagous reaction would be

$$ROOH + HSO_3^- \longrightarrow OH\cdot + SO_3 + ROH$$

but this is only speculation.

(b) The Production of Singlet Oxygen

Singlet Oxygen is also supposed to arise from the Haber-Weiss reaction (49) and it has been shown that this is theoretically possible (50). However neither catalase nor SOD inhibited singlet oxygen production as followed by cis-DBE production, DPF disappearance or chemiluminescence.

Catalase, SOD and hydroxyl radical scavengers all inhibit sulphite oxidation. However none of these inhibit singlet oxygen formation. Therefore singlet oxygen cannot arise during sulphite oxidation.

Howard and Ingold (51) showed that singlet oxygen could arise from the collision of two sec-peroxy radicals (see Figure 9). The mechanism was proposed by Russell in 1957 (52). The sec-peroxy radicals could arise from the following reactions

$$ROOH + HSO_3^- \longrightarrow RO\cdot + HSO_3\cdot + OH^- \qquad (i)$$

$$RO\cdot + ROOH \longrightarrow ROH + ROO\cdot \qquad (viii)$$

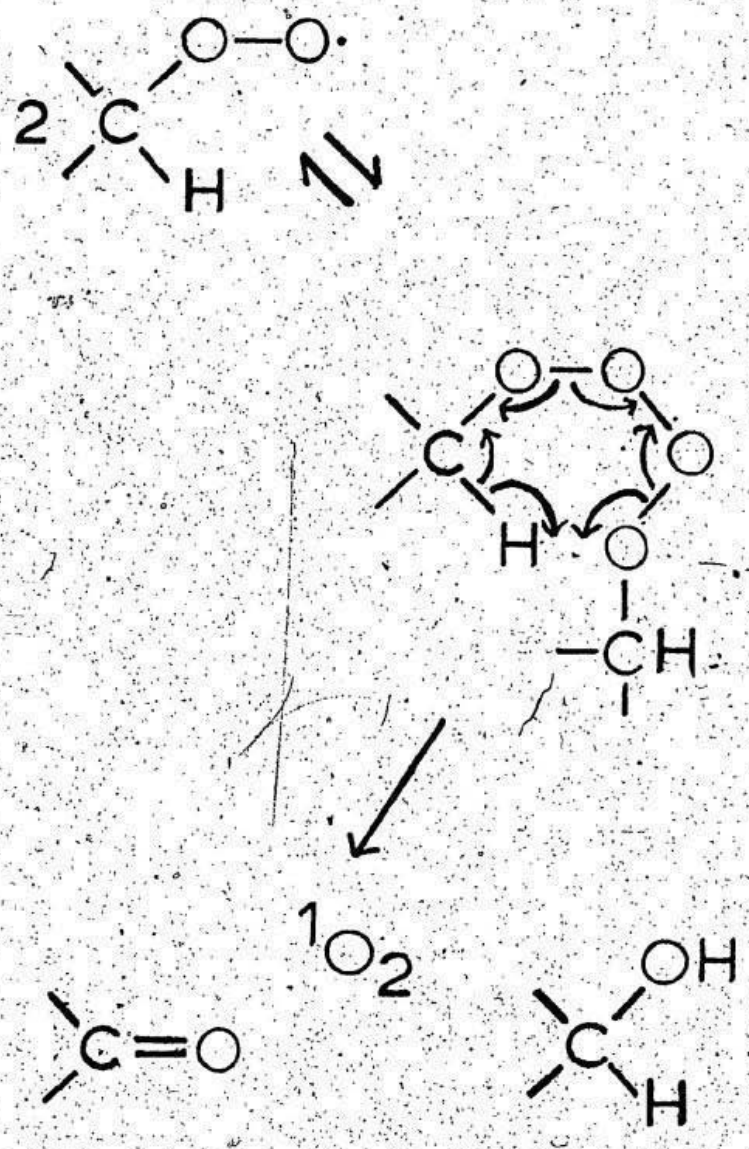


Figure 9 The self reaction of two sec-peroxy radicals.

It has been proposed (37) that ROO· can react with DPIBF. From the experiments where DPIBF oxidation was followed spectrophotometrically it was concluded that ROO· was being formed by our system.

Peroxy radicals can arise by another pathway (reactions (vi) and (vii).

$$RO\cdot \xrightarrow{\beta \text{ scission}} R'CHO + R''\cdot \qquad \text{(vi)}$$

$$R''\cdot + O_2 \longrightarrow R''OO\cdot \qquad \text{(vii)}$$

However this is a primary peroxy radical. The reaction between two primary peroxy radicals does not produce singlet oxygen (51).

Cumene hydroperoxide is a tertiary hydroperoxide. It is a more efficient initiator of sulphite oxidation than is LAHPO. However at pH 7.4 chemiluminescence is observed during the reaction between LAHPO and sulphite but not between cumene hydroperoxide and sulphite. The lack of a hydrogen atom makes it impossible for tertiary peroxides to interact by the Russell mechanism. This is further evidence that the singlet oxygen is derived from the collision of two sec-peroxy radicals and does not arise during the oxidation of sulphite.

(c) The Conversion of Methional to Ethylene by the Model System

One of the problems associated with inhibitor studies is that it is difficult to find inhibitors which are specific for one species. As far as is known azide and DABCO only quench singlet oxygen and do not react with hydroxyl radicals, alkoxy radicals or hydroperoxy radicals. The lower alcohols methanol, ethanol, propanol and butanol are thought to be specific hydroxyl radical scavengers. The antioxidants, BHT, BHA, NDGA are thought to react with lipid peroxy radicals.

Azide partially inhibits the conversion of methional to ethylene. It does not inhibit sulphite oxidation, and presumably it does not affect

the production of singlet oxygen from the hydroperoxide. DABCO behaves in a similar manner. Therefore singlet oxygen reacts with methional to produce ethylene but is not totally responsible for ethylene synthesis. BHA, NDGA and propyl gallate also partially inhibited ethylene formation confirming the hypothesis that singlet oxygen does derive from the hydroperoxide. Ethoxyquin is a far better inhibitor of ethylene formation than any of the three others tried. This is presumably because it also reacts with other free radical species, possibly the hydroxyl radical or other species induced in sulphite oxidation.

The hydroxyl radical scavengers, ethanol and n-butanol also only partially inhibit ethylene formation although they are very good hydroxyl radical scavengers (30) and also good inhibitors of sulphite oxidation. However mannitol which is also a hydroxyl radical scavenger is a much better inhibitor of ethylene formation. It may also inhibit singlet oxygen production.

The best inhibitors of ethylene production were the amino acids, methionine and tryptophan. These two and histidine are known to react with singlet oxygen (36). Methionine can also react with hydrogen peroxide (12). However all three are also very efficient hydroxyl radical scavengers (30). All the data is consistent with the theory that in the model system methional is converted to ethylene by two species. The hydroxyl radicals formed during sulphite oxidation are thought to be responsible for about half of the ethylene production. Singlet oxygen derived from the hydroperoxides is thought to be responsible for the other half.

(d) The Conversion of Methionine to Ethylene in the Plant

None of the model systems described so far can use methionine as substrate. Neither hydroxyl radicals nor singlet oxygen can react with methionine to give ethylene. The LAHPO, sulphite reaction cannot convert methionine to ethylene so presumably ROO· and RO· cannot react with methionine to produce ethylene.

Several compounds have been suggested as intermediates in the pathway from methionine to ethylene. αKMBA was one proposed intermediate and this possibility was discussed in the introduction. Another suggested intermediate was S-adenosylmethionine. This was suggested by Yang in 1974 (44). Yang proposed that S-adenosylmethionine was broken down by a pyridoxal phosphate dependent γ-elimination reaction which also required oxygen.

αKMBA and methional can both be utilized by Yang's model system (53) and our model system to produce ethylene. Methionine can be converted to αKMBA enzymically (7) and it is possible that methionine could also be converted to methional enzymically. There are several other ways in which methionine could be converted to methional.

It has recently been shown that methionine can be converted to methional when safflower oil is heated briefly to 200°C (54). This treatment prevents further autoxidation of the fatty acids. Methional was shown to be the agent acting as an antioxidant. Heating methionine with soyabean protein also results in the formation of methional (55). This was thought to be due to catalytic amounts of glucose, which when heated in the presence of air can form the active group which can catalyze the reaction known as the Strecker degradation (see Figure 10).

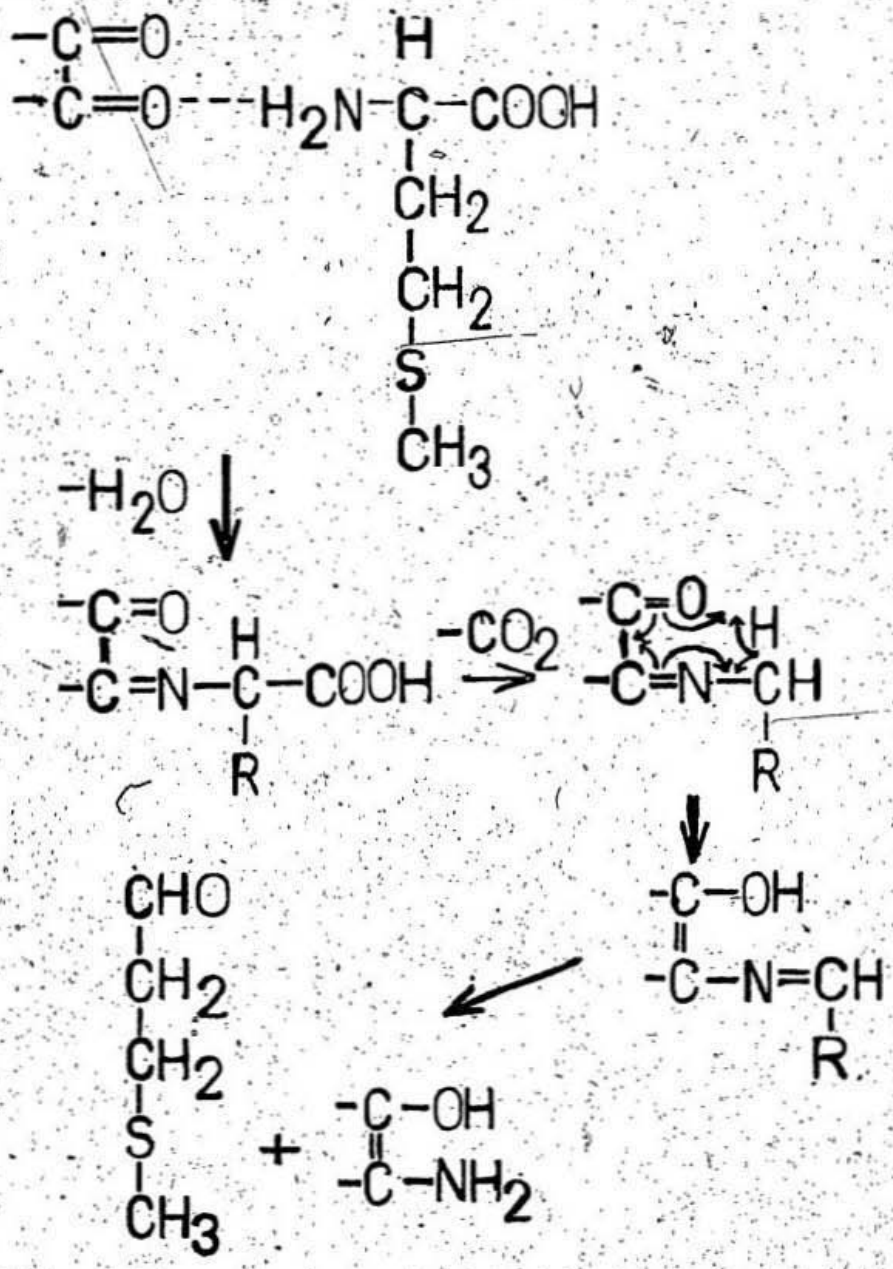


Figure 10 The Strecker degradation of methionine.

There are two possible explanations why LAHPO and sulphite did not convert methionine to ethylene. Either the breakdown of LAHPO did not lead to the formation of products with the active group or the methionine was converted mainly to the sulphoxide.

If similar reactions occur when ethylene is being formed the lipid peroxides (presumably located in the membranes) will first have to break down to the compounds containing the group capable of catalyzing a Strecker degradation. These presumably would then diffuse into the cytoplasm of the cell where they could catalyze the conversion of methionine to methional. At the same time singlet oxygen could be produced by the collision of two sec-peroxy radicals. Singlet oxygen is fairly stable in non-aqueous environments. It could diffuse from the lipid bilayer to the cytoplasm where it could convert methional to ethylene. Methional would be destroyed as quickly as it was formed and would likely be undetectable.

(e) Peroxides in Plants

Frenkel has shown that levels of hydrogen peroxide and fatty acid hydroperoxides increase just prior to ripening and fall when ripening begins (56).

Solomos and Laties (57) showed that if preclimacteric bananas or avocados are treated with cyanide, cyanide-insensitive respiration is invoked, ethylene synthesis increases and the fruits eventually ripen. Ethylene also stimulates respiration. Since ethylene synthesis is autocatalytic it is possible that a product of cyanide insensitive respiration may be involved in ethylene biosynthesis. Recently it has been shown that in mung bean hypocotyls the cyanide insensitive respiration reduces oxygen only partially to hydrogen peroxide (58). The hydrogen peroxide may arise from the dismutation of superoxide radicals. If

superoxide radicals and hydrogen peroxide are both present then singlet oxygen may also be formed. This could cause lipid peroxidation.

There is some evidence that the breakdown of lipid peroxides occurs during ripening and that the breakdown of lipid peroxides is necessary for ripening to occur. Maguire and Haard (59) showed that lipofuscin pigments accumulate in fruits as they ripen. These pigments have characteristic absorption and fluorescence properties and are thought to be due to cross-linked polymers of proteins and fatty acid moieties formed during the free radical-mediated breakdown of fatty acid hydroperoxides. Baker et al (60) showed that propylgallate, an antioxidant could inhibit the ripening of tomatoes.

Free radicals seem to be intimately involved in both ripening and senescence. The conversion of methionine to ethylene via methional would play a central role in both processes. Methional can react with hydroxyl radicals (34), singlet oxygen and probably ROO·. Ethylene can react with hydroxyl radicals (61), singlet oxygen (40), and alkoxy radicals (46). Ethylene probably has a dual function - the initiation of ripening and the delaying of senescence.

(f) Stress Ethylene

Ethylene is produced by many plant tissues in response to wounding or stress (1). According to Abeles (1), "the primary function of stress ethylene is to accelerate abscission of organs damaged by disease, insect, drought and temperature". However it also plays a role in protecting the rest of the tissue from damage to one part of it. If a fruit is gashed there will be damage to cells surrounding the gash. This may lead to lipid peroxidation and ethylene production by the mechanisms outlined. If the sycamore fruit is pierced ethylene production is increased and

ripening is enhanced (62). Scar tissue forms around the wound but does not spread to the remainder of the fruit. Ethylene and methional may be the protective agents.

(g) Hydrogen Peroxide and Ethylene Production

The hydrogen peroxide produced by the cyanide-insensitive pathway could be used to convert methionine to methional in a reaction which proceeds via a chloramine which hydrolyses in aqueous solutions (63). This reaction is catalyzed by a peroxidase which can use chloride ion as a substrate.

$$H_2O_2 + Cl^- + H^+ \xrightarrow{\text{myeloperoxidase}} HOCl + H_2O \qquad (x)$$

$$HOCl + R\,CH\,(COO^-)\,\overset{+}{N}H_3 \longrightarrow H^+ + H_2O + RCH(COO^-)NHCl \qquad (xi)$$

$$RCH(COOH)NHCl + H_2O \longrightarrow RCHO + CO_2 + {}^+NH_4 + Cl^- \qquad (xii)$$

The product of cyanide-insensitive respiration, hydrogen peroxide, had been difficult to detect because catalase and peroxidases were present which immediately destroyed it. It is possible that one of the peroxidases present in the mitochondria of plants is a chloroperoxidase capable of carrying out the above reaction. It is interesting to note that this reaction also produces singlet oxygen.

$$HOCl + H_2O_2 \longrightarrow H_2O + {}^1O_2 + Cl^- \qquad (xiii)$$

So the system should be capable of converting methionine to ethylene via the chloramine and methional.

In summary I would like to outline the sequence of events which I think occurs in fruits when they ripen. In my opinion the 'switch' for ripening is an increase in lipoxygenase activity. This increase probably results from a combination of several factors. It may be mediated by phytochrome which can control lipoxygenase levels (64), or hormonal changes from the tree which in turn could be due to changes in the

environment. The increase in lipoxygenase activity increases the levels of peroxides in cell membranes which cause them to become disorganized. Interfering with membrane organization increases cyanide-insensitive respiration (57). Levels of hydrogen peroxide rise. The hydrogen peroxide is either used to convert methionine to ethylene by a peroxidase-halide system or indirectly by synthesis of more lipid peroxides. The ethylene produced perturbs membrane organization further and the level of cyanide-insensitive respiration increases. Thus ethylene formation is autocatalytic.

SUMMARY AND CONCLUSIONS

Two possible precursors of ethylene in fruits are linolenic acid hydroperoxide and methionine. Lipoxygenase may also be involved. A model system has been described in which linoleic acid hydroperoxide and sulphite can be used to form ethylene from methional. The mechanism of the reaction has been investigated.

Both hydroxyl radicals and singlet oxygen can react with methional to produce ethylene. The hydroxyl radical arises during sulphite oxidation but the singlet oxygen is formed when two sec-peroxy radicals collide. Using DPBF oxidation it was shown that methional also reacts with either RO· or ROO··.

Several methods were used to confirm singlet oxygen production by LAHPO and sulphite: the conversion of DPF to cis-DBE, DPF oxidation (followed on the fluorescence spectrophotometer) and chemiluminescence. Inhibitor studies on all three methods confirmed that the singlet oxygen did not arise during sulphite autoxidation but arose from the collision of two sec-peroxy radicals.

A pathway has been outlined by which methionine can be converted to ethylene. Sulphite is not necessary for the pathway. In the model system sulphite merely serves to produce the alkoxy radical from LAHPO. In the plant the alkoxy radical could be produced during the breakdown of lipid peroxides, although the natural initiator of peroxide breakdown is unknown.

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