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LIPID HYDROPEROXIDES IN THE CONVERSION OF 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID TO ETHYLENE

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Key Word Index Lipoxygenase; linoleic acid hydroperoxide, 1-aminocyclopropane-1-carboxylic acid, ethylene, peroxy radical, pyridoxal phosphate

Abstract—Ethylene was formed from 1-aminocyclopropane-1-carboxylic acid (ACC) by a wholly defined system containing linoleic acid hydroperoxide (LOOH), Mn^{2+} and pyridoxal 5'-phosphate (PP) at pH 8.0. Under these conditions, neither oxygen nor lipoxygenase was required for ethylene production. Each component of the reaction mixture has been implicated as a cofactor required for aerobic *in vitro* conversion of ACC to ethylene by various plant preparations. Inhibition of ethylene formation by the α-tocopherol analogue, 2,2,5,7,8-pentamethyl-6-hydroxychroman, implies that free radicals are involved in the reaction. Peroxy radicals of LOOH were demonstrated to form in parallel with ethylene. Although PP was required for ethylene formation, peroxy radicals formed equally well with or without PP. This result implies that either a PP-ACC Schiff base reacts with peroxy radicals, or that peroxy radicals act through a PP-Mn^{2+} complex. Since the known stereochemistry argued against the former possibility [Pirrung, M. (1986) *Biochemistry* 25, 114], the latter is suggested. A possible mechanism is proposed involving oxidation of ACC by a PP Mn^{4+} LOO^- complex formed via sequential oxidation of Mn^{2+} by LOOH and peroxy radicals derived from LOOH

INTRODUCTION

1-Aminocyclopropane-1-carboxylic acid (ACC) is the immediate precursor of ethylene in plants [1, 2]. The physiological process by which higher plants convert ACC into ethylene has been the subject of intensive research activity in recent years, but characterization of the responsible enzyme or enzyme complex has been hampered by its sensitivity to cellular disruption. Most investigators agree that the lability of the ACC-ethylene enzyme is due to a requirement for membrane integrity [3]. Furthermore, Hoffman *et al.* [4] demonstrated that the *in vivo* process possesses a high degree of stereodiscrimination for one of four diastereomers of an ACC analogue, 1-amino-2-ethylcyclopropane-1-carboxylic acid (AEC); only (1*R*,2*S*)-AEC was effectively metabolized into 1-butene. Although many *in vitro* ACC to ethylene-converting preparations have been described, all of the cell-free preparations tested were not stereoselective in metabolizing AEC diastereomers into 1-butene [5-7], with the exception of a pea vacuole isolate [7].

In vitro preparations of the ethylene-forming reaction mixture often have many features in common, such as a requirement for the cofactors, manganous ion [5, 8-13] and pyridoxal 5'-phosphate (PP) [5, 10, 13]. Additionally, *in vitro* ethylene-forming activity (EFA) was stimulated by the presence of organic hydroperoxides [14, 15] or H_2O_2 [16]. Alternatively, a hydroperoxide was formed as an enzymic product during EFA [10, 13, 17]. Although the presence of hydroperoxide was not demonstrated directly, several *in vitro* systems included membranes, which may have been susceptible to sequential hydrolysis by acyl hydrolase and oxidation of released polyunsaturated fatty acids by lipoxygenase [5, 6, 8, 9, 11, 12, 18-21]. Whereas both the *in vivo* and *in vitro* EFA are inhibited by antioxidants, only the *in vitro* systems are sensitive to EDTA and thiols [3].

Two similar model systems have been reported recently that had virtually all the characteristics of *in vitro* EFA, and additionally afforded improved yields of ethylene from ACC. According to the method of Boller *et al.* [22], ACC was 70% converted to ethylene in the presence of Mn^{2+}, PP and H_2O_2 at pH 11.5. Bousquet and Thimann's [23] method of EFA utilized ACC, Mn^{2+}, PP, lipoxygenase and linoleic acid at pH 8.0, and was recently characterized further by Pirrung [24]. By use of this method a 9% yield of ethylene was obtained within 2 hr, which greatly exceeded the yield of *in vitro* EFA reported by others. Because there is increasing interest in the role of lipoxygenase in *in vitro* EFA [14, 15], particularly in response to wounding [17], we chose to study the method of Bousquet and Thimann [23] in order to understand the mechanism of lipoxygenase action in the conversion of ACC to ethylene.

RESULTS

Bousquet and Thimann's [23] method of conversion of ACC to ethylene was modified to replace lipoxygenase and linoleic acid with the product of their reaction, linoleic acid hydroperoxide (LOOH). With the modified method, a 9% yield of ethylene was obtained within 2 hr at saturating levels of LOOH (Fig. 1). At this saturating concentration of LOOH (2 mM), 22 mol of LOOH were required to produce 1 mol of ethylene from ACC; however, at 0.25 mM concentration of LOOH the molar ratio

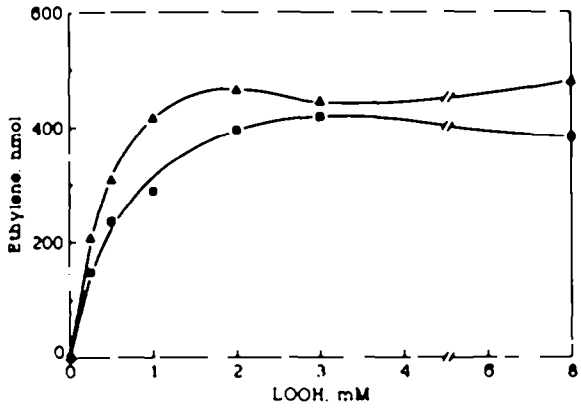


Fig 1 Ethylene evolution from ACC (2 hr incubation) as a function of LOOH concentration under either an Ar (▲——▲) or air (■ — ■) atmosphere. In addition to LOOH, each reaction contained 1 mM ACC, 0 1 mM Mn^{2+}, 0 1 mM PP and 10 mM NaHEPES (pH 8) Reaction volume 5 ml Values are means (n = 2–4).

of LOOH to ethylene produced was decreased to 6 Compared to incubation under an Ar atmosphere, air was slightly inhibitory to EFA Without LOOH no ethylene was evolved (Table 1)

The modified system showed properties similar to the lipoxygenase-linoleic acid system defined by Bousquet and Thimann [23] EFA was greatly diminished by omission of either Mn^{2+} or PP (Table 1) In our hands, the omission of PP resulted in a greater decrease of EFA than observed by Bousquet and Thimann [23], but our result was in agreement with recent findings [24]. EFA was strongly inhibited by EDTA and dithiothreitol (Table 1). However, a quantity of dithiothreitol (6 mM) in excess of LOOH (4 mM) was required to terminate EFA, whereas, dithiothreitol at 1 mM concentration was ineffective in inhibiting EFA Bousquet and Thimann [23] noted inhibition of EFA with the antioxidant, n-propylgallate and in the present study, the antioxidant, 2,2,5,7,8,-pentamethyl-6-hydroxychroman (PMHC), also strongly inhibited EFA (Table 1).

When ethylene was measured during the first few minutes, a significant lag time was noted with 0.67 mM LOOH, but the lag was nearly abolished by increasing LOOH to 4 mM (Fig. 2). As measured by a decrease in conjugated diene absorption at 234 nm, there also was a lag in the decomposition of LOOH that paralleled the lag in EFA (data not shown).

Since there appeared to be a temporal relationship in LOOH decomposition and EFA, the fate of LOOH was investigated further. Extracts ($CHCl_3$-CH_3OH) of reac-

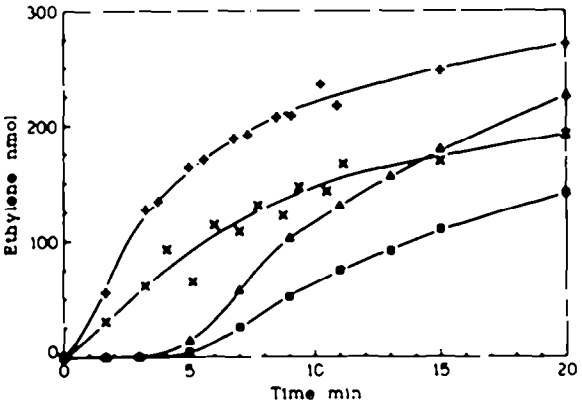


Fig 2 Lag in ethylene formation from ACC in the presence of 0 67 mM LOOH compared to the lack of a significant lag in the presence of 4 mM LOOH (▲ – ▲) 0 67 mM LOOH under Ar, (■ ■) 0 67 mM LOOH under air; (+ — — +) 4 mM LOOH under Ar, (× - - ×) 4 mM LOOH under air Included in the reaction mixtures were 0 1 mM $MnSO_4$, 1 mM ACC, 0.1 mM PP and 10 mM NaHEPES (pH 8), reaction volumes were 5 ml Values with 0 67 mM LOOH were means (n = 2).

Table 1 Effect of various reagents on ethylene-forming activity

	Ethylene formation					
	30 min		60 min		120 min	
Reaction mixture	nmole	% of control	nmole	% of control	nmole	% of control
Control*	364	100	449	100	494	100
Control plus						
EDTA (1 mM)	0	0	0	0	0	0
DTT (6 mM)	0	0	0	0	0	0
(1 mM)	393	108	477	106	529	107
PMHC (0 91 mM)	5	1	10	2	12	2
DABCO (10 mM)	378	104	499	111		
Histidine (10 mM)	569	156	571	127		
Control minus						
LOOH	0	0	0	0	0	0
PP	19	5	23	5	30	6
$MnSO_4$	10	3	30	7	30	6
$MnSO_4$ and PP	0	0	0	0	0	0

*Control was composed of 0.1 mM $MnSO_4$, 0 1 mM PP, 1 mM ACC, 4 mM LOOH in 10 mM NaHEPES buffer (pH 8) under an Ar atmosphere Volume of reaction 5 ml

tion aliquots were examined by TLC. TLC showed that LOOH decomposed gradually with time of reaction, but no major decomposition product was discerned. The streaking of some products on TLC plates suggested that polymerization was possible. When the extracted material was examined by GC after making the appropriate derivative, it was discovered that the undecomposed LOOH had been isomerized. That is, the sample of LOOH, originally containing 97–98% (9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid, had isomerized into a mixture of the 13-hydroperoxide and 9-hydroperoxy-10,12-octadecadienoic acid, presumably via peroxy radicals according to the mechanism of Chan *et al.* [25]. The isomerization of hydroperoxide (under air) occurred in parallel with EFA, including a probable lag at the 0.67 mM concentration of LOOH (Fig. 3). As with EFA, Mn^{2+} was an absolute requirement for isomerization (Fig. 3). Under Ar the isomerization, which mechanistically requires O_2 (see Discussion), was markedly inhibited (data not shown).

Although PP is required for EFA, it is not necessarily required for isomerization of LOOH; that is, isomerization occurred as readily with or without PP (data not shown).

Inhibitors of singlet oxygen, 1,4-diazabicyclo[2.2.2]-octane (DABCO) [26] and histidine [27], each at 10 mM concentration under an Ar atmosphere, did not inhibit EFA. DABCO was without effect, and histidine stimulated EFA (Table 1).

A system known to generate alkoxy radicals from LOOH using a $FeCl_3$–cysteine redox cycle [28] afforded very low yields of ethylene. Replacement of $MnSO_4$ with hematin, haemoglobin and cytochrome *c* also did not lead to significant amounts of ethylene.

DISCUSSION

From a model system composed of 20 mM ACC, lipoxygenase and linoleic acid at pH 9.0, Kacperska and Kubacka-Zebalska [17] obtained only a 0.08% yield of ethylene after 5 hr incubation. Our initial experiments with a similar system also proved to be inefficient in producing ethylene from ACC (results not given). These data contrasted markedly with those of Bousquet and Thimann [23], who obtained a 9% yield of ethylene from 1 mM ACC within 2 hr by inclusion of two additional cofactors, Mn^{2+} and PP. Further investigation by us showed that LOOH could replace both lipoxygenase and linoleic acid. Since the yield of ethylene and the effect of inhibitors in our experiments were essentially identical as those observed by Bousquet and Thimann [23], we concluded that their results also were dependent upon the product of lipoxygenase action, namely LOOH.

EDTA and dithiothreitol were potent inhibitors of EFA, both in Bousquet and Thimann's [23] method, and in our modification of their method. Since Mn^{2+} was an essential ingredient of EFA, it was not surprising that the metal chelator, EDTA, completely inhibited the reaction. The effect of dithiothreitol was more complex. In the present study dithiothreitol at 1 mM concentration did not inhibit EFA in the presence of 4 mM LOOH; however, dithiothreitol (6 mM) in molar excess of LOOH (4 mM) inhibited EFA completely. This observed stoichiometry suggests the decomposition of LOOH by the thiols. Thiols in alkaline pHs convert LOOH into hydroxyoctadecadienoic acid by nucleophilic attack on the hydroperoxide by thiolate anion [28]. Because 2 mM LOOH saturated EFA (Fig. 1), no inhibition of EFA would be expected by 1 mM dithiothreitol in the presence of 4 mM LOOH. Inasmuch as Bousquet and Thimann [23] used equimolar concentrations of both dithiothreitol and linoleic acid, it follows that their concentration of dithiothreitol (1 mM) was sufficient to react with any hydroperoxide that formed from linoleic acid (1 mM) by lipoxygenase action. Additionally, lipoxygenase activity is inhibited by cysteine [29], and possibly other thiols may inhibit as well.

Several lines of evidence suggested that EFA was dependent on a radical chain. Inhibition by antioxidants, a lag in EFA at low concentrations of LOOH, and inhibition by oxygen. Bousquet and Thimann [23] found that the antioxidant, *n*-propylgallate, was an efficient inhibitor of EFA, and likewise, the α-tocopherol analogue, PMHC, was an inhibitor of EFA in this study. Furthermore, the inhibition by PMHC in the presence of LOOH implied that this antioxidant inhibited radical formation from LOOH, and the absence of lipoxygenase in our preparation showed that the primary effect of antioxidant is not necessarily inhibition of lipoxygenase activity. The lag in EFA, especially at lower LOOH concentration, is indicative of a requirement for an initiation phase. Partial elimination of the lag by increased LOOH concentration demonstrates that increasing this radical precursor permits a more rapid transition into propagation chains. The response of EFA to oxygen is yet another indicator of the free radical character of the reaction. Although the ACC to ethylene transformation is known to require oxygen, the present study not only showed that LOOH can supplant oxygen, but demonstrated that the presence of oxygen actually can be inhibitory to EFA. Since oxygen is a scavenger of radicals, it is plausible that oxygen may compete with the ACC to ethylene conversion.

Free radicals are probably initiated by the well-known one electron redox cycle of transition metals with organic

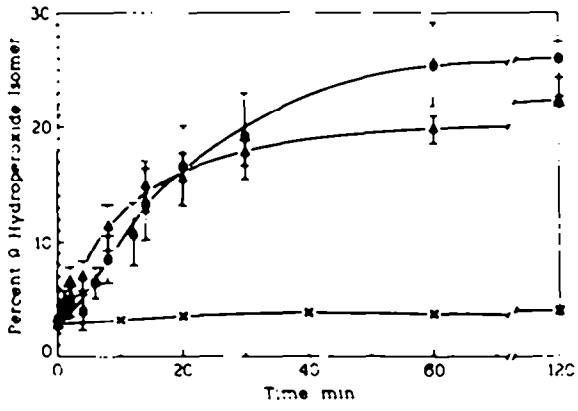


Fig. 3. Isomerization of the 13-hydroperoxide of LOOH (initially 97–98%) to the 9-hydroperoxide as a function of LOOH concentration and Mn^{2+}. (▲— -▲) 4 mM LOOH, (■ —■) 0.67 mM LOOH, (× – ×) 4 mM LOOH minus Mn^{2+}. Included in the reaction mixtures were 0.1 mM $MnSO_4$, 1 mM ACC, 0.1 mM PP and 10 mM NaHEPES (pH 8), except as noted. Values are means ± s.d. (n = 2–3).

hydroperoxides.

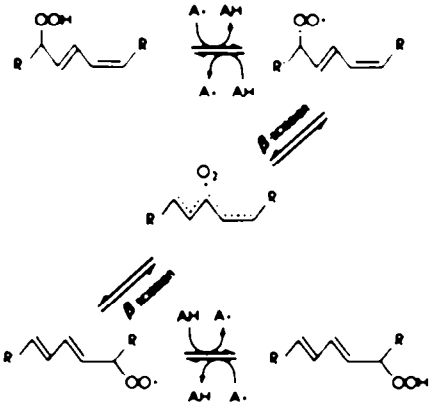
$$Mn^{2+} + LOOH \rightarrow Mn^{3+} + LO^{\cdot} + OH^{-} \quad (1)$$

$$Mn^{3+} + LOOH \rightarrow Mn^{2+} + LOO^{\cdot} + H^{+} \quad (2)$$

In neutral or acidic aqueous solution, Mn^{2+} is very resistant to oxidation into Mn^{3+} (+1.4 V), but in basic media $Mn(OH)_2$ is much more easily oxidized into Mn_2O_3 and MnO_2 [30] suggesting that LOOH-metal redox reactions may be favoured at pH 8.0. Peroxy or alkoxy radicals formed by such redox reactions will initiate radical chains. The apparent polymeric material from TLC of reaction products thus could result from radical reactions.

Previous investigations offered a potential method of testing for the presence of peroxy radicals. According to Chan *et al.* [25], peroxy radicals undergo β-scission and subsequent isomerizations (Scheme 1). This β-scission reaction has been characterized thoroughly by Porter *et al.* [31], who have derived a kinetic expression for this phenomenon. Thus, isomerization of the 13-hydroperoxide isomer of LOOH into a mixture of 9- and 13-hydroperoxides should constitute a measure of peroxy radicals. Derivatization of the hydroperoxides remaining in the reaction to methyl 9- and 13-trimethylsilyloxystearates followed by their separation via GC afforded a practical means of measuring the cumulative effect of peroxy radicals at any time. It was determined that the isomerization of LOOH under an air atmosphere paralleled EFA, including a probable lag at the 0.67 mM concentration of LOOH. Since there was a considerable concentration difference between 0.67 mM and 4 mM LOOH, the effective peroxy radical concentration at these two levels of LOOH cannot be strictly compared from the data in Fig. 3. If this factor was taken into account, undoubtedly the lag at 0.67 mM LOOH would be more accentuated. As with EFA, the absolute requirement for Mn^{2+} in isomerization (Fig. 3) reflects the role of Mn^{2+} in radical initiation.

An observed inhibition of isomerization under an Ar atmosphere did not, however, correlate with enhanced EFA under those conditions (data not given). This seemingly unexpected result is readily explained by examination of Scheme 1. Absence of oxygen would hinder recombination of oxygen with the pentadiene radical, and



Scheme 1. Isomerization of LOOH via β-scission of peroxy radicals according to Chan *et al.* [25]. R' = $CH_3(CH_2)_4$-, R = -$(CH_2)_7CO_2H$

thus radical would then be scavenged by another available radical. Under an inert atmosphere the isomerization method for assessing peroxy radicals appeared to be of limited utility, but does not necessarily indicate a diminished rate of peroxy radical formation.

Since peroxy radicals were present during EFA, the possible existence of other active oxygen species was considered. Bousquet and Thimann [23] showed that superoxide dismutase had no effect on EFA, which eliminated superoxide as a causative agent. Because superoxide is a reductive species, one would not expect its presence under the oxidizing conditions imposed by the presence of LOOH. Contrary to this conclusion, Legge and Thompson [14] surmised that superoxide was formed from hydroperoxides and that superoxide was responsible for conversion of ACC to ethylene. As evidence they showed that superoxide-scavenging reagents interfered with the process, but the reagents tested by them also should be sensitive to lipid peroxy radicals. Bousquet and Thimann [23] also discounted the presence of hydroxyl radicals by demonstrating no effect with mannitol, a hydroxyl radical scavenger. On theoretical grounds alone, hydroxyl radicals do not appear to be possible. Metal-catalysed reduction of organic hydroperoxides affords reduction of the hydroxyl portion of the hydroperoxide to yield a hydroxide ion and an alkoxy radical. However, the formation of singlet oxygen from peroxy radicals of LOOH has been documented [32]. Accordingly, the singlet oxygen scavengers, DABCO and histidine, were tested and found to be ineffective as inhibitors of EFA. The cause of stimulation of EFA by histidine was probably due to a prooxidant effect. Tjhio and Karel [33] reported that histidine markedly stimulated the autoxidation of methyl linoleate in the presence of Mn^{2+} at pH 8.0.

Pirrung [24] recently suggested that PP functioned by shifting the redox potential of Mn^{2+} by chelation into the range needed to reduce LOOH into an alkoxy radical and a hydroxide ion. According to him, H-abstraction by alkoxy radicals were responsible for the initial oxidation of the amino group of ACC. We also considered this possibility, as well as H-abstraction by peroxy radicals, but our data did not support this possibility. Since peroxy radicals were produced as readily with or without PP while ethylene was not, this suggests that peroxy radicals are not directly involved in H-abstraction of ACC. Replacement of the $MnSO_4$ catalyst with an $FeCl_3$-cysteine redox couple, known to generate alkoxy radicals [28], was inefficient in EFA, as well as similar experiments with hematin, haemoglobin and cytochrome *c* catalysts. Rather than abstract hydrogen, alkoxy radicals generated from LOOH have a propensity to undergo intramolecular cyclization into epoxides, and this rearrangement is preferred even in the presence of readily abstractable sulphydryl hydrogens [28].

The data suggest that PP is required for the action of peroxy radicals during EFA. A plausible explanation was peroxy radical oxidation of a PP-ACC Schiff base. Baldwin *et al.* [34] oxidized the imine of a *p*-methoxybenzaldehyde-ACC Schiff base with organic peroxides into an intermediate oxazinidine derivative, which decomposed into ethylene. In their system *cis*-[2,3-$^2H_2$]ACC resulted in only *cis*-[1,2-$^2H_2$]ethylene, whereas, Pirrung [24] found that the Bousquet and Thimann method [23] afforded a mixture of *cis*- and *trans*-[1,2-$^2H_2$]ethylene from *cis*-[2,3-$^2H_2$]ACC. The

latter conversion is also the one observed *in vivo*, and was proposed to be the result of carbon–carbon bond rotation of a free radical intermediate in the ACC to ethylene conversion [35]. Thus, the stereochemistry does not support the mechanism of Baldwin *et al.* [34] in this study. A second possibility involving a metal oxidation of ACC via an ACC PP-Mn^{3+} complex was considered. As shown by equation (1), Mn^{3+} could be derived via oxidation by LOOH. It is known that metal–amino acid–PP Schiff base complexes can readily catalyse diverse reactions, such as decarboxylation, transamination, racemization and elimination [36], but Pirrung's data [24] convincingly argued against the involvement of Schiff base chemistry in the Bousquet and Thimann [23] reaction. Pirrung [24] showed that the oxidation potential of Mn^{3+} was shifted by complex formation with PP; however, the oxidation potential of the PP-Mn^{3+} complex appeared to be out of the range of the +0.8 V required for direct oxidation of ACC [35]. This led to the conclusion that the PP-Mn^{3+} complex was further converted into a higher oxidation state via oxidation by peroxy (or oxy) radicals, like the reaction reported for oxidation of Co^{2+} by peroxy radicals [37]:

$$PP\text{-}Mn^{3+} + LOO\cdot \rightarrow PP\text{-}Mn^{4+}LOO^{-} \quad (3)$$

Possibly, this oxidation state could complete the two one-electron oxidations of ACC required by the Pirrung mechanism [35].

$$PP\text{-}Mn^{4+}LOO^{-} + \tfrac{1}{2}ACC \rightarrow PP\text{-}Mn^{3+}LOO^{-} + \tfrac{1}{2}\,CH_2{=}CH_2 \quad (4)$$

The formation of a brown, inorganic precipitate after extended incubation of the reaction mixtures indicated the formation of MnO_2, which validated the formation of Mn^{4+}. In theory at least 4 mol of LOOH would be required to generate one mol of ethylene:

$$2\,Mn^{2+} + 2\,LOOH \rightarrow 2\,Mn^{3+} + 2\,LO\cdot + 2\,OH^{-} \quad (5)$$ (catalytic initiation)

$$2\,Mn^{3+} + 2\,LOOH \rightarrow 2\,Mn^{2+} + 2\,LOO\cdot + 2\,H^{+} \quad (6)$$

Same as (5) (7) (cyclic)

$$2\,Mn^{3+} + 2\,LOO\cdot \rightarrow 2\,Mn^{4+} + 2\,LOO^{-} \quad (8)$$

$$2\,Mn^{4+} + ACC \rightarrow 2\,Mn^{3+} + CH_2 = CH_2. \quad (9)$$

Reaction 5, a catalytic oxidation needed for initiation, should not greatly affect the actual consumption of hydroperoxide in the process. The stoichiometry outlined by reactions 6–9 may explain the high molar ratio of LOOH required for ethylene production.

What is the physiological significance of the data presented here? Pirrung [24] noted that the Bousquet and Thimann method [23] afforded both CN and *cis*- and *trans*-[1,2-$^2H_2$]ethylene from *cis*-[2,3-$^2H_2$]ACC, like the natural EFA; however, the method failed the third test for stereoselectivity between diastereomers. That is, there was a lack of selectivity for the natural (1*R*,2*S*)-2-methyl-1-aminocyclopropane-1-carboxylic acid isomer [24]. Other *in vitro* systems of EFA also failed to exhibit stereoselectivity in reacting with AEC diastereomers [5–7]. Thus, it is unlikely that the Bousquet and Thimann method [23] represents the normal physiological production of ethylene by plants [24]. Although certain research results suggest that a connection may exist between the 'wound ethylene' response and *in vitro* EFA, there are also arguments against this contention. Like *in vitro* EFA, wound ethylene was inhibited by EDTA [38], but *in vivo* EFA was not [3]. A role for lipoxygenase has been established for *in vitro* EFA, and likewise, lipoxygenase activity has been correlated with wound ethylene [17]. It is well known that wounding triggers the action of lipoxygenase in plants [39]. The case against an *in vitro* mechanism regards the fact that increased EFA from wounding originated from newly synthesized ACC rather than from decompartmentalized ACC, and the lack of effect on the ACC to ethylene conversion by the PP inhibitor, aminoethoxyvinylglycine, after wounding showed that PP is not required in the wound response [38]. The stereospecificity for the (1*R*,2*S*)-AEC diastereomer has not been tested with wounded plants, and thus remains the crucial experiment to determine if wound ethylene originates by either an *in vitro* or *in vivo* mechanism.

EXPERIMENTAL

Ethylene-forming activity (EFA). Ethylene was generated from ACC by the method of ref [23], except that $MnCl_2$, sodium 4(2-hydroxyethyl)-1-piperazinepropanesulphonate buffer (pH 8), linoleic acid, and lipoxygenase were replaced with $MnSO_4$, sodium *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonate (NaHEPES) buffer (pH 8), and LOOH. Thus, the reaction mixture was composed of 1 mM ACC, 0.1 mM $MnSO_4$, 0.1 mM PP, 10 mM NaHEPES (pH 8) and LOOH (0.25–8 mM). Before addition of $MnSO_4$ catalyst, the reaction mixtures were transferred into 15 ml flasks fitted with Teflon-lined septa, and those flasks requiring inert atmospheres were evacuated and exchanged with Ar for 3 cycles. At zero time 5 μl of 0.1 M $MnSO_4$ was injected into a final reaction vol. of 5 ml at 25°. Ethylene was sampled by withdrawal of *ca* 0.5 ml from the 10 ml head space by a gas-tight syringe, and this sample was analysed by a GC fitted with FID and a 2.74 m × 6.4 mm Porapak R (80–100 mesh) column (Applied Science Laboratories, State College, PA). Elution was with a flow of about 25 ml/min of He carrier gas at room temp. Standard mixtures formulated from high purity ethylene and air permitted quantitation of the ethylene peak. EFA was inhibited by PMHC synthesized by the procedure of ref [40].

The effect of an alkoxy radical-generating system [28] was tested for EFA efficiency. The soln contained 1 mM ACC, 0.1 mM PP, 4 mM LOOH, 16 mM cysteine and 0.1 mM $FeCl_3$, MeOH–H_2O (4:1).

Preparation of linoleic acid hydroperoxide (LOOH). Linoleic acid was oxidized by soybean lipoxygenase (EC 1.13.11.12) as described previously [41]. The extracted crude product of the reaction (*ca* 800 mg) was slurried in hexane with 2 g Silic AR CC-4 (Malinckrodt, St. Louis, MO), and the slurry was applied to the

top of a column (2.5 cm i.d.) containing 50 g Silic AR CC-4 packed with hexane. Stepwise elution was performed with 50 ml hexane followed by acetone in hexane in the proportions of 0.25 1 5%, 0.31 7.5%, 0.3 L, 10%. When the LOOH peak started to elute as detected by A at 234 nm, the next 150 ml of eluant was collected (about 0.48–0.63 l total eluant). Generally, a 350 mg sample of 97–98% (9Z,11E)-13-hydroperoxy-9,11-octadecadienoic acid was obtained by this procedure. Later fractions contained increasing percentages of the 9-hydroperoxide isomer and were discarded.

Determination of hydroperoxide isomers. Hydroperoxide isomer composition was analysed in aliquots taken from a scaled-up ethylene reaction mixture (25–70 ml). Samples equivalent to 1–2.5 mg LOOH were extracted with the following mixture (per ml aliquot): 3 ml $CHCl_3$–CH_3OH (2:1), 40 μl 0.1 M Na_2EDTA and 60 μl 1 M citric acid. The collected $CHCl_3$ layer was washed twice with 3 ml H_2O, and the $CHCl_3$ subsequently was evaporated under a N_2 stream. The residue immediately was dissolved in 0.5 ml CH_3OH and hydrogenated with 10% Pd on charcoal catalyst for 0.5 hr. Recovered hydrogenation products were esterified with CH_2N_2. The methyl esters subsequently were treated with 100 μl HMDS–TMCS–pyridine (2:1:1). After the reagent was permitted to react a few hours, it was evaporated with a stream of dry N_2, and the residue was redissolved in 50 μl $CHCl_3$ for capillary GC using FID and a (12 m × 0.22 mm) WCOT column coated with SE-30. The carrier gas (He) flow rate was about 1 ml/min, and temp was programmed from 200° to 250° at 3°/min. TMSi derivatives of methyl 9- and 13-hydroxystearates (from R. G. Powell of this laboratory) served to determine their elution times (6.2 and 6.6 min, respectively) and response factor (equivalent). Early-eluting peaks were largely methyl 9- and 13-trimethylsilyloxystearates and the hydrogenolysis products, methyl stearate and methyl 9- and 13-oxostearates, whereas, later-eluting materials presumably were further oxidation products of LOOH.

TLC. $CHCl_3$–CH_3OH (2:1) extracted products of the reaction were separated by TLC on precoated plates (Silica Gel 60 F-254, EM Science, Cincinnati, OH) using a hexane–Et_2O–HOAc (50:50:1) solvent system. Spots were made visible either by UV illumination or by charring after spraying with 50% aq H_2SO_4.

REFERENCES

1. Adams, D. O. and Yang, S. F. (1979) *Proc. Natn. Acad. Sci. U.S.A.* **76**, 170.
2. Lurssen, K., Naumann, K. and Schroder, R. (1979) *Z. Pflanzenphysiol.* **92**, 285.
3. Yang, S. F. and Hoffman, N. E. (1984) *Annu. Rev. Plant Physiol.* **35**, 155.
4. Hoffman, N. E., Yang, S. F., Ichihara, A. and Sakamura, S. (1982) *Plant Physiol.* **70**, 195.
5. Venis, M. A. (1984) *Planta* **162**, 85.
6. McKeon, T. A. and Yang, S. F. (1984) *Planta* **160**, 84.
7. Guy, M. and Kende, H. (1984) *Planta* **160**, 281.
8. Rohwer, F. and Mader, M. (1981) *Z. Pflanzenphysiol.* **104**, 363.
9. Konze, J. R. and Kwiatkowski, G. M. K. (1981) *Planta* **151**, 320.
10. Shimokawa, K. (1983) *Phytochemistry* **22**, 1903.
11. Vinkler, C. and Apelbaum, A. (1983) *FEBS Letters* **162**, 252.
12. Vinkler, C. and Apelbaum, A. (1984) *FEBS Letters* **167**, 64.
13. Vioque, A., Albi, M. A. and Vioque, B. (1981) *Phytochemistry* **20**, 1473.
14. Legge, R. L. and Thompson, J. E. (1983) *Phytochemistry* **22**, 2161.
15. Lynch, D. V., Sridhara, S. and Thompson, J. F. (1985) *Planta* **164**, 121.
16. Legge, R. L., Thompson, J. E. and Baker, J. E. (1982) *Plant Cell Physiol.* **23**, 171.
17. Kacperska, A. and Kubacka-Zebalska, M. (1985) *Physiol. Plant.* **64**, 333.
18. McRae, D. G., Baker, J. E. and Thompson, J. E. (1982) *Plant Cell Physiol.* **23**, 375.
19. Mattoo, A. K., Achilea, O., Fuchs, Y. and Chalutz, E. (1982) *Biochem. Biophys. Res. Commun.* **105**, 271.
20. Konze, J. R. and Kende, H. (1979) *Planta* **146**, 293.
21. Mayak, S., Legge, R. L. and Thompson, J. F. (1981) *Planta* **153**, 49.
22. Boller, T., Herner, R. C. and Kende, H. (1979) *Planta* **145**, 293.
23. Bousquet, J.-F. and Thimann, K. V. (1984) *Proc. Natn. Acad. Sci. U.S.A.* **81**, 1724.
24. Pirrung, M. C. (1986) *Biochemistry* **25**, 114.
25. Chan, H. W.-S., Levett, G. and Matthew, J. A. (1978) *J. Chem. Soc. Chem. Commun.* 756.
26. Ouannes, C. and Wilson, T. (1968) *J. Am. Chem. Soc.* **90**, 6527.
27. Nilsson, R., Merkel, P. B. and Kearns, D. R. (1972) *Photochem. Photobiol.* **16**, 117.
28. Gardner, H. W. and Jursinic, P. A. (1981) *Biochim. Biophys. Acta* **665**, 100.
29. Roza, M. and Francke, A. (1973) *Biochim. Biophys. Acta* **327**, 24.
30. Cotton, F. A. and Wilkinson, G. (1962) *Advanced Inorganic Chemistry. A Comprehensive Text.* Interscience, New York.
31. Porter, N. A., Lehman, L. S., Weber, B. A. and Smith, K. J. (1981) *J. Am. Chem. Soc.* **103**, 6447.
32. Nakano, M., Takayama, K., Shimizu, Y., Tsuji, Y. and Inaba, H. (1976) *J. Am. Chem. Soc.* **98**, 1974.
33. Tjhio, K. H. and Karel, M. (1969) *J. Food Sci.* **34**, 540.
34. Baldwin, J. E., Jackson, D. A., Adlington, R. M. and Rawlings, B. J. (1985) *J. Chem. Soc. Chem. Commun.* 206.
35. Pirrung, M. C. (1983) *J. Am. Chem. Soc.* **105**, 7207.
36. Metzler, D. E., Ikawa, M. and Snell, E. E. (1954) *J. Am. Chem. Soc.* **76**, 648.
37. Lande, S. S. and Kochi, J. K. (1968) *J. Am. Chem. Soc.* **90**, 5196.
38. Konze, J. R. and Kwiatkowski, G. M. K. (1981) *Planta* **151**, 327.
39. Vick, B. A. and Zimmerman, D. C. (1982) *Plant Physiol.* **69**, 1103.
40. Smith, L. I., Ungnade, H. E., Hoehn, H. H. and Wawzonek, S. (1939) *J. Org. Chem.* **4**, 311.
41. Gardner, H. W. (1975) *Lipids* **10**, 248.