Ethylene Production by Isolated Chloroplasts

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Ethylene Biosynthesis, Chloroplasts, Superoxide Free Radical

Isolated chloroplast lamellae from spinach or sugar-beet leaves produce ethylene from methylmercaptopropanal (MMP) or 2-keto-4-methylmercaptobutyrate (KMB) in the light. The pH-optimum for ethylene production from MMP is 6.3; ethylene production from KMB has an apparent pH-optimum at about pH 5.

Ethylene formation in red light (90 $\text{kerg}\cdot\text{cm}^{-2}\cdot\text{sec}^{-1}$) from the above substrates is stimulated by ferredoxin and inhibited by either DCMU (10^{-5} M), ferredoxin together with NADP, catalase, superoxide dismutase or under anaerobic conditions. From the inhibition by either DCMU, NADP or anaerobic conditions it is concluded, that an intact electron transport system from water as electron donor to oxygen as electron acceptor is necessary for ethylene formation. H_2O_2 alone does not stimulate ethylene formation from MMP or KMB. Inhibition by both catalase and superoxide dismutase support the view, that ethylene formation from MMP or KMB is driven by the OH-radical, which is formed from H_2O_2 and the superoxide free radical ion.

The presented data suggest that in addition to ferredoxin another membrane-bound factor is involved in photosynthetic oxygen reduction and ethylene formation. This factor (ORF = oxygen reducing factor) stimulates photosynthetic oxygen reduction in the presence of ferredoxin yielding H_2O_2 in addition to $O_2^{\cdot -}$, which is the product of the autooxidation of reduced ferredoxin.

During photosynthetic ethylene formation from MMP or KMB, the production of the OH-radical from H_2O_2 and $O_2^{\cdot -}$ according to

$$H_2O_2 + O_2^{\cdot -} \rightarrow OH^{\cdot} + OH^- + O_2$$

(Haber and Weiss, Proc. Roy. Soc. Ser. A **147**, 332 [1934], Beauchamp and Fridovich, J. Biol. Chem. **245**, 4641 [1970]) seems to be the rate-limiting step.

Introduction

The plant hormone ethylene is produced by certain bacteria, certain fungi and higher plants [1] and its biosynthesis has been studied by many workers [1].

Of the many possible precursors for ethylene synthesis which have been proposed, only α-ketoglutarate in fungi [2] and methionine, or its derivatives MMP or KMB (*cf.* ref. 3) and eventually α-linolenic acid [4] for higher plants are still being discussed. Peroxidase in the presence of several cofactors (as sulfite, Mn^{2+}, monophenoles) has been proposed as an enzymic system involved in ethylene biosynthesis from methionine derivatives [5, 6]. Recently the peroxidase system has been questioned, however, to function as the sole catalytic system responsible for ethylene formation in higher plants (*cf.* ref. 1 and 3). The site of ethylene formation in the plant cell is another open question. If methionine or its derivatives (MMP and/or KMB) are the precursors for ethylene in higher plants, peroxides and/or oxygen radicals are certainly involved in the process of conversion [5–7]. Since isolated chloroplasts are able to produce the superoxide free radical ion [8, 9] as well as H_2O_2 in the presence of ferredoxin [10–12], we investigated the possibility of the production of ethylene from the above substrates by illuminated chloroplast lamellae.

The conclusion that the capacity for ethylene production by the chloroplast might possibly be of physiological importance can be drawn from the following observations:

1. Subcellular particles seem to be required for ethylene biosynthesis in higher plants [1]; these particles are apparently not identical with mitochondria [13, 14].
2. The most likely substrates for ethylene formation in higher plants (methionine and/or α-linolenic acid) are synthesized in the chloroplasts [15, 16].
3. Ethylene excretion by isolated wheat chloroplasts has been observed; this ethylene production is stimulated after spraying the chloroplasts with a source for ionizing radiation (^{32}Phosphorus) [17].

Abbreviations: AQ, anthraquinone-2-sulfonic acid; MV, methylviologen; TQ, I,I′-trimethylene-2,2′-bipyridylium dibromide (Triquat); DCMU, 3-(3,4-dichlorophenyl-)I,I-dimethylurea; SOD, superoxide dismutase; Fd, ferredoxin; MMP, methylmercaptopropanal; KMB, 2-keto-4-methylmercaptobutyrate.

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If the chloroplasts are indeed potential sites for ethylene formation in higher plant cells, this capacity should also be observed in the dark. This communication deals only with the conditions for the light-dependent ethylene production (a preliminary report has been published [18]); ethylene production by isolated chloroplast lamellae in the dark has also been observed under certain conditions and will be described elsewhere.

Materials and Methods

Chloroplast lamellae and ferredoxin were isolated from spinach [19, 20]. Methylmercaptopropanal (MMP) and 2-keto-4-methylmercaptobutyrate (KMB) were synthesized and the purity of the compounds were tested as described [18]. Superoxide dismutase was prepared from dried green peas [21, 9] and the specific activity was tested according to McCord and Fridovich [22]. Catalase was purchased from Boehringer, Mannheim. The SOD used had no catalase activity and the catalase had no SOD activity at the applied concentrations.

The reactions were carried out with Fernbach flasks (with a side arm) in a Photo-Warburg thermostate for 40 min at 20 °C with illumination from the bottom either with white light (20000 lx) or red light (90 $\text{kerg} \cdot \text{cm}^{-2} \cdot \text{sec}^{-1}$), as indicated in the Tables and Figures.

The test system for photosynthetic ethylene production contained in 1 ml: chloroplast lamellae with 50 µg chlorophyll, 25 µmol phosphate buffer pH 6.3; 2.5 µmol NH_4Cl, 2.5 µmol $MgCl_2$, 4 µmol MMP or 2 µmol KMB as substrate, and the indicated additions. The vessels were closed with a rubber-stopper and the reactions were terminated with 0.1 ml of 0.5 N H_2SO_4. Ethylene was determined in a Varian Aerograph model 1400, with a flame ionization detector, equipped with a Poropak R column (1/8, 80/10). The temperature of the column oven was 50 °C.

Decarboxylation of [1-^{14}C]KMB was determined as described [12]. [1-^{14}C]Methionine was purchased from Amersham-Buchler, Braunschweig.

Results

Conditions for photosynthetic ethylene formation

Illuminated chloroplast lamellae produce ethylene from either MMP or KMB, but not from Methionine or α-ketoglutarate [18]; ethylene production under the described conditions (see Methods) is increased linearly with increasing amounts of chloroplast lamellae up to 80 µg chlorophyll-content (Fig. 1 a) and is inhibited by more than 90% by 10^{-5} M DCMU (Fig. 1 b). The reaction is saturated with either 15 µmol MMP or 3 µmol KMB as substrates for ethylene formation (Fig. 2). Although the pH-optimum for photosynthetic electron transport is around 8.0 (the water-splitting system is already partially inhibited at pH 7.4 [23]), the pH-optimum for the ethylene production from MMP is at 6.3, and even lower with KMB as substrate (Fig. 3). This result indicates that photosynthetic electron transport is not the limiting step in the light-dependent ethylene production from MMP or KMB by isolated chloroplast lamellae.

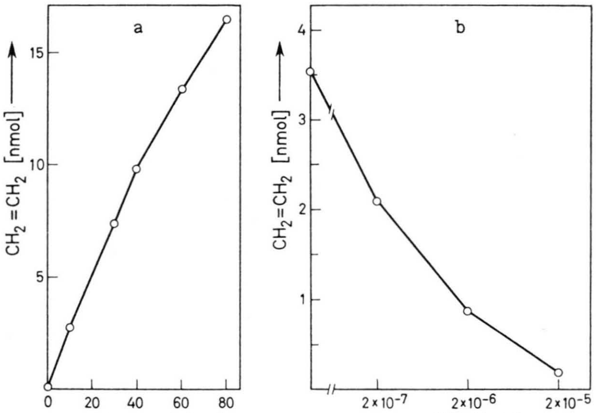


Fig. 1. a. For experimental conditions see Material and Methods, chlorophyll as indicated, 4 µmol MMP (red light). b. For experimental conditions see Fig. 2, 2 µmol KMB, DCMU as indicated.

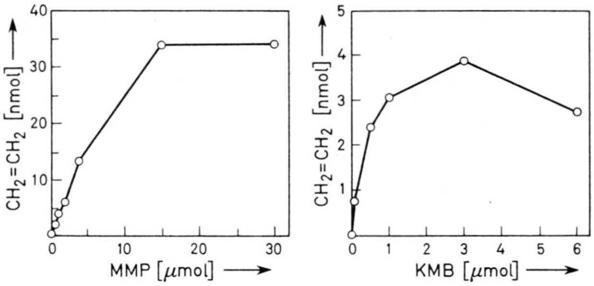


Fig. 2. The reaction mixture contained in 1 ml: 25 µmol phosphate-buffer, pH 6.3, 50 µg chlorophyll, 2.5 µmol NH_4Cl, 2.5 µmol $MgCl_2$, 30 nmol Fd, MMP and KMB as indicated, 40 min at 20 °C, red light.

As already shown, photosynthetic ethylene production by isolated chloroplast lamellae is stimulated by ferredoxin [18]. It has been shown, on the other

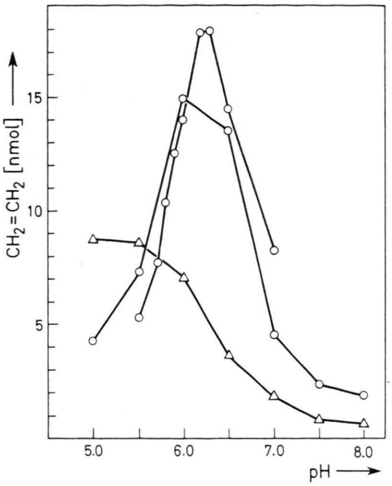


Fig. 3. For experimental conditions see Fig. 2, 25 μmol phosphate-buffer, pH as indicated, △, with 2 μmol KMB, ○, with 4 μmol MMP.

hand, that ferredoxin upon autooxidation yields in the production of H_2O_2 [10–12]. The autooxidation of reduced ferredoxin seems to be a rather slow process [24], compared to the autooxidation of low potential dyes like MV, TQ or AQ, however. The above findings can be taken as reasons, why photosynthetic ethylene formation depends on

a. aerobic conditions (Fig. 4), and is

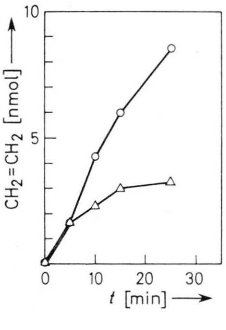
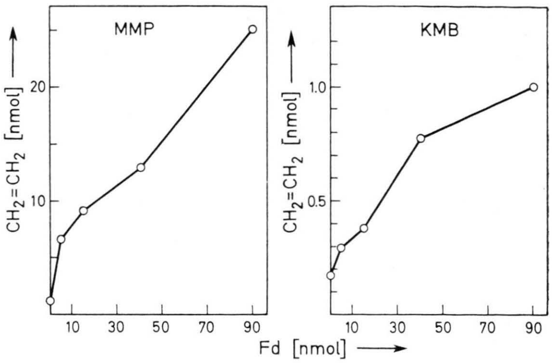


Fig. 4. For experimental conditions see Fig. 2, 4 μmol MMP; ○, in air; △, in argon.

b. increased by addition of increasing amounts of ferredoxin (Fig. 5).

As shown in Fig. 5, ethylene production from both MMP and KMB is stimulated by ferredoxin up to 90 nmol Fd added. Both curves show a shoulder (at approx. 10 nmol Fd in the KMB-system and 30 nmol Fd in the MMP-system) which seems to be followed by a new, but slower increase of ethylene production upon increasing amounts Fd.

Ferredoxin can be substituted for by low potential electron acceptors like MV, or TQ, although the rates of ethylene production are lower compared to the ones with Fd as electron acceptor (Table I).



5 a 5 b

Fig. 5. For experimental conditions see Fig. 2, Fd as indicated; 5 a with 4 μmol MMP, 5 b with 2 μmol KMB.

Table I. Comparison of the influence of different electron acceptors on photosynthetic ethylene production by isolated chloroplast lamellae. The reaction mixture contained in 1 ml: 40 μmol Tris buffer pH 8.0, 5 μmol NH_4Cl, 5 μmol $MgCl_2$ chloroplast lamellae with 0.1 mg chlorophyll, 3 μmol KMB and the inticated additions; 20 °C, white light (20000 lx), 40 min.

Electron acceptor	Ethylene produced [rel. units] *
none	16.7
plus 0.3 μmol dopamine	16.6
plus 0.2 μmol TQ	74.3
plus 0.2 μmol MV	66.6
plus 30 nmol Fd	100.0

* The system with 30 nmol Fd added was set as 100, according to 780 pmol ethylene produced in 40 min.

Dopamine has also been shown to act as an electron acceptor for photosystem I and to stimulate oxygen uptake as well as H_2O_2 formation by illuminated chloroplast lamellae [25, 24]. Dopamine does not stimulate photosynthetic ethylene formation, however.

If on the other hand the ratio of decarboxylation of [$1\text{-}^{14}C$]KMB is compared with ethylene formation, dopamine shows a stimulation of $^{14}CO_2$-formation. H_2O_2 in the absence of chloroplast lamellae, like dopamine, only shows decarboxylation of KMB, but no ethylene formation [18] (Table II). By comparing Table I with Table II it is evident, that Fd stimulates both ethylene formation from and decarboxylation of KMB, while dopamine only stimulates decarboxylation of KMB.

Mechanism of photosynthetic ethylene formation

As shown by Fig. 4 photosynthetic ethylene formation is only observed under aerobic condi-

Table II. Influence of Fd, dopamine and H_2O_2 on the ratio of $^{14}CO_2$ formation and ethylene production from [1-^{14}C]-KMB. The reaction mixture contained in 2 ml: 80 μmol Tris buffer pH 8.0, 5 μmol NH_4Cl, 5 μmol $MgCl_2$, chloroplast lamellae with 0.2 mg chlorophyll and 6 μmol [1-^{14}C]-KMB (0.028 Ci/mol).

Electron acceptor	CO_2/C_2H_4 [Molar ratio]
none	1.3
60 nmol Fd	1.8
0.3 μmol dopamine	2.4
10 μmol H_2O_2 (in the absence of chloroplasts)	18.2

tions (*cf.* ref. 1). Beauchamp and Fridovich showed that, in the presence of canthine and xanthine oxidase as an oxygen reducing system, the OH-radical seems to be responsible for the production of ethylene from MMP[7]. The following experiments try to provide evidence that the OH radical is also formed during photosynthetic oxygen reduction and seems to be responsible for photosynthetic ethylene formation. As shown by Fig. 6, photosynthetic ethylene production is inhibited by the addition of 4 μmol NADP in addition to 30 nmol Fd. If only 2 μmol of NADP are added, similar inhibition kinetics are obtained up to 20 min as in the presence of 4 μmol; from 20 to 40 min of illumination, however, the same rates of ethylene formation with 2 μmol NADP added are obtained as in the absence of NADP. As shown in an earlier paper[12], photosynthetic oxygen reduction in the presence of NADP and Fd is only observed, when the available NADP is reduced. The result presented in Fig. 6 is in agreement with these findings.

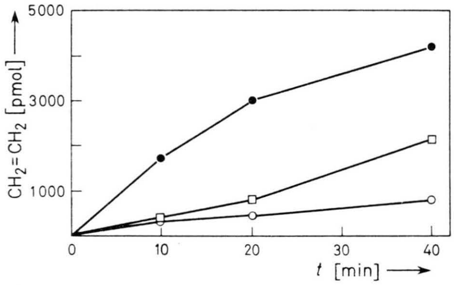
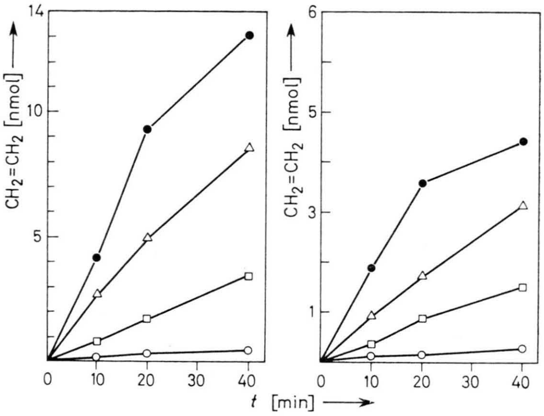


Fig. 6. For experimental conditions see Fig. 2, 2 μmol KMB; ●, without NADP, □, 2 μmol, ○, 4 μmol NADP added.

Although H_2O_2 in the absence of illuminated chloroplast lamellae is not active in the production of ethylene from MMP or KMB (Table II), catalase strongly inhibits ethylene formation from both these substrates in the presence of illuminated chloroplast lamellae (Fig. 7). This result implies that H_2O_2 is necessary for ethylene formation from both MMP and KMB, although yet another compound seems to be involved, which together with H_2O_2 is forming the final oxidant. This compound seems to be the superoxide free radical ion, since photosynthetic ethylene formation from KMB (similar results are obtained with MMP as the substrate) is inhibited by superoxide dismutase. Very low concentrations of SOD show no influence on the kinetics of ethylene formation up to 20 min of illumination, a higher rate of ethylene formation is retained at longer illumination-times, however (Fig. 8).



7 a 7 b

Fig. 7. For experimental conditions see Fig. 2, 7 a with 4 μmol MMP, 7 b with 2 μmol KMB; ●, without catalase, △, 5 units, □, 50 units, ○, 500 units catalase added.

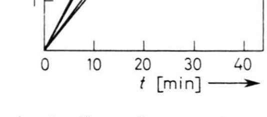


Fig. 8. For experimental conditions see Fig. 2, 2 μmol KMB; ●, without SOD, ○, 2 units, □, 100 units, △, 1000 units SOD.

A similar observation can be made, if ethanol is added to the reaction mixture; although the inhibition of ethylene formation is not as severe as with catalase or SOD, a stimulating influence of the lowest concentration applied can be shown (Fig. 9).

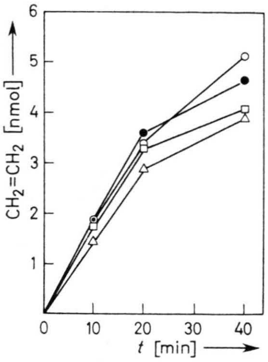


Fig. 9. For experimental conditions see Fig. 2, 2 μmol KMB; ●, without ethanol, ○, 1×10^{-3} M, □, 1×10^{-2} M, △, 5×10^{-2} M ethanol.

The effect of ethanol on photosynthetic ethylene formation is not as strong as observed for the xanthine-xanthineoxidase system described by Beauchamp and Fridovich[7]. The superimposed "preserving" effect of ethanol may partly be responsible for this difference.

Ascorbate has been shown to inhibit photosynthetic oxygen reduction with Fd as the electron acceptor, but has no effect if low potential electron acceptors are used[24, 26]. The inhibition by acsorbate has been shown to specifically inhibit the function of an oxygen reducing factor (ORF) involved in photosynthetic oxygen reduction in the presence of ferredoxin. Photosynthetic ethylene formation shows a similar behaviour; while ethylene formation in the presence of MV or TQ is stimulated in the presence of ascorbate, the reaction is inhibited by ascorbate if ferredoxin is used as the electron acceptor (Table III).

Table III. Influence of ascorbate on ethylene formation in the presence of either MV, TQ or Fd as electron acceptors. For experimental conditions see Table I: ascorbate 10 μmol, further additions as indicated.

	Ethylene produced [pmol] Minus ascorbate	Plus ascorbate
+0.2 μmol TQ	460	552
+0.2 μmol MV	400	726
+30 nmol Fd	900	526

Discussion

As already communicated[18], ethylene formation by isolated chloroplast lamellae is dependent on light, and an intact electron transport system. The intent of this communication is to present more details and kinetic data of this light-dependent ethylene production.

Ethylene production is an oxidative process, as shown *in vivo* as well as by *in vitro* experiments (*cf.* ref. 1). All the *in vitro* systems, capable of ethylene production from certain substrates described so far are known to produce peroxides and/or oxygen radicals[27, 5, 6, 28].

Photosynthetic oxygen reduction, which in contrast to the above mentioned systems is observed in the absence of artificial supplements, has been shown to produce H_2O_2[10, 11] as well as $O_2^{\cdot -}$[8, 9] in a reaction which is light dependent and stimulated by ferredoxin.

Photosynthetic ethylene production in the presence of NADP is only observed when photosynthetic electron transport is forced to reduce oxygen, *e. g.* when the available NADP is reduced and the favoured electron transport with NADP as the final acceptor is blocked (Fig. 6). Exactly the same conditions have been established for photosynthetic decarboxylation of α-keto acids[12].

As demonstrated by Figs 7 and 8, photosynthetic ethylene production seems to be dependent on the presence of both H_2O_2 and $O_2^{\cdot -}$. These findings are in agreement with the results of Beauchamp and Fridovich[7] who used xanthine and xanthine oxidase as a generator for H_2O_2 and $O_2^{\cdot -}$. These authors suggested, that the $OH^{\cdot}$ radical, which is formed from H_2O_2 and $O_2^{\cdot -}$ according to

$$H_2O_2 + O_2^{\cdot -} \rightarrow O_2 + OH^- + OH^{\cdot}\ ^{29} \qquad (1)$$

is the oxidizing species for MMP as substrate. This suggestion was confirmed by the finding that ethanol as a scavenger for OH-radicals was also an inhibitor of the ethylene formation[7].

As shown by Fig. 9, an influence of ethanol is also observed in our system, but an inhibition over 20% was never observed, independent of the kind of substrate used (MMP or KMB).

H_2O_2 cannot substitute for illuminated chloroplast lamellae (Table II) or xanthine and xanthine oxidase[7], but photosynthetic ethylene production is nevertheless inhibited by catalase. This finding implies a formulation for photosynthetic ethylene production similar to the one presented by Beauchamp and Fridovich[7], namely the oxidation of MMP or KMB by the OH-radical to yield CO_2, ethylene, eventually formic acid and methylated sulfur-compounds (*cf.* ref. 1 and 3).

During the whole process of light-dependent ethylene production from the above substrates, the formation of the OH-radical seems to be the rate-limiting step. This is concluded from the following observations:

1. The low pH-optimum would favour the formation of OH-radical (*cf.* Eqn (1) and Fig. 3);

2. H_2O_2 seems to be needed in higher concentrations than produced by dismutation of $O_2^{\cdot -}$ in order to favour a rapid formation of the OH-radical (*cf.* Eqn (1) and also the strong inhibition by catalase as compared to SOD). This is also concluded by the finding, that in the presence of Fd, where apparently both H_2O_2 and $O_2^{\cdot -}$ are produced simultaneously by different mechanisms [26], more ethylene is produced than with MV or TQ as acceptors. A similar observation was made by Beauchamp and Fridovich, who found that an addition of H_2O_2 to their xanthine-xanthine oxidase system strongly stimulated ethylene synthesis.

The participation of an oxygen reducing factor (ORF) in photosynthetic ethylene formation can be concluded from ethylene production in the presence of different amounts of ferredoxin (Fig. 5) [26]. The shoulder at 10 to 30 nmol Fd added seems to represent the saturation of one Fd-requiring system (ORF), while at higher Fd-concentrations a second system seems to be superimposed (autooxidation of reduced Fd). The inhibition by ascorbate in the presence of ferredoxin but not in the presence of MV or TQ of photosynthetic ethylene formation is in agreement with this assumption (Table III).

The different activities of the various electron acceptors in stimulating photosynthetic ethylene formation are in good agreement with the expected products during their involvement in photosynthetic oxygen reduction:

1. Dopamine does not stimulate photosynthetic ethylene production but has been found to stimulate the photosynthetic decarboxylation of α-keto acids [26]. Photosynthetic oxidation of hydroxylamine, a reaction which is solely driven by the superoxide free radical ion is not stimulated by dopamine [30]. Dopamine as an electron acceptor for photosystem I apparently can only produce H_2O_2 and not $O_2^{\cdot -}$. An identical behaviour was found for the ORF from sugar beet leaves [30].

2. MV or TQ produce $O_2^{\cdot -}$ by autooxidation and H_2O_2 by dismutation of $O_2^{\cdot -}$. These low potential electron acceptors stimulate both decarboxylation of α-keto acids and oxidation of hydroxylamine in the presence of illuminated chloroplast lamellae at high rates.

3. Ferredoxin stimulates both decarboxylation of α-keto acids and oxidation of hydroxylamine, but yields the highest rates of photosynthetic ethylene production apparently by producing both H_2O_2 and $O_2^{\cdot -}$ by separate mechanisms. An optimal ratio of $H_2O_2/O_2^{\cdot -}$ which seems to be needed for the rapid formation of OH-radical is provided by cooperation of Fd and ORF. The data on the decarboxylation are in agreement with the above suggestion.

In the presence of Fd and illuminated chloroplast lamellae the ratio of decarboxylation of [1-^{14}C]-KMB compared to ethylene formation seems to be close to 2. This is a higher rate of decarboxylation of KMB than observed by Yang [31]. By using the peroxidase system, he observed a ratio of CO_2/ethylene-formation of $32/18 \times 10^{-3}$ dpm from [U-^{14}C]KMB. This is about half the decarboxylation we observed since C-2 of KMB seems also to be converted into CO_2.

The result that low concentrations as well of added SOD as of ethanol stimulate ethylene formation from MMP or KMB after longer illumination times (Figs 8 and 9) may indicate that both SOD and ethanol preserve the activities of the chloroplasts during illumination. Apparently the OH-radical is one of the harmful oxygen species causing damage to cell organelles [32-35] and whole organisms [36] during monovalent oxygen reduction.

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