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Involvement of Haber-Weiss reaction in the photochemical production of ethylene from methionine riboflavin mixture

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Abstract. Ethylene production from photodegradation of methionine with riboflavin as a photosensitizer was investigated. At pHs ranging from 4.0-8.0, rate of ethylene production of the methionine -riboflavin mixture (MR) appeared to be a function of the increase of pH. In the reaction mixture, methional was the likely intermediate for ethylene production. Addition of acrolein or methionine sulfoxide to the reaction mixture was not as effective in inducing ethylene production. At pH 4.0, addition of superoxide dismutase (SOD) and/or catalase was only slightly inhibitory to the reaction. When pH was raised to 8.0, the presence of SOD showed no obvious effect on the rate of ethylene production. In contrast to this, addition of catalase significantly enhanced the detected reaction. The lack of effectiveness of SOD suggested the participation of factors other than superoxide radicals in ethylene production; whereas the stimulatory activity of catalase further implicated the inhibitory effect due to hydrogen peroxide accumulation. The presence of antioxidants like ascorbate or α-tocopherol, free radical scavengers like thiourea, and singlet oxygen quenchers like β-carotene or Na-azide, all appeared to be greatly inhibitory to ethylene production. In addition, the supplementation of iron chelating agents such as desferal or phenanthroline also retarded the reaction greatly. The importance of hydroxyl free radical and singlet oxygen production was thus implicated. The accumulated evidence seemed to indicate that the superoxide driven Haber-Weiss reaction was a critical factor contributing to the photochemical production of ethylene from MR especially in an acidic condition.

Key words: Ethylene production; Haber-Weiss reaction; Methionine-riboflavin mixture; Oxygen radical involvement.

Introduction

Ethylene is an important growth hormone of higher plants being widely used in many studies in agricultural research and applications (Abeles, 1973; Adam and Yang, 1979; Yang and Hoffman, 1984). In industry, ethylene is also an important raw material for many organic synthesis reactions. For certain industrialized countries, biological sources of ethylene production have become one of the prime research topics in biotechnology during the last decade (Sato *et al.*, 1987). The chemical process related to ethylene production has been an actively researched subject in agriculture as well as industry for several decades. In biological system, methionine appears to be the main precursor for ethylene production in most higher plants and the "methionine --- > S - adenosyl methionine --- > 1 - aminocyclopropane - 1 - carboxylic acid (ACC) --- > ethylene" biosynthetic pathway proposed by Yang *et al.* has been well recognized (Adam and Yang, 1979; Yang and Hoffman, 1984). In addition, methionine also is a precursor for ethylene production in microorganisms through the α-keto-γ-methylthiobutyric acid intermediate pathway by either catalysis of peroxidase or a flavin-sensitized photochemical reaction (Billin-

gton *et al.*, 1979).

The flavin-mediated ethylene production was first observed by Abeles and Rubinstein (1964) in their early work on ethylene production using pea seedling extracts as an assay system. The active substrate leading to ethylene production in this reaction system was later identified by Yang *et al.* (1967a) as methionine. Continued study by these workers provided further evidence which indicated that methional was the likely intermediate in the process; a reaction scheme was proposed from the data that involved a free radical (Yang *et al.*, 1967b). While studying the photochemical production of ethylene by various phytopathogenic fungi, we found that in the presence of light, methionine -riboflavin (MR) mixture was strongly biocidal to a fairly wide spectrum of microorganisms including fungi, bacteria, and algae (Tzeng and DeVay, 1985). The discovery has led to the development of a biocidal concoction which appeared to be effective for use in controlling certain plant diseases (Tzeng, 1988). The unusual toxicity of MR mixture was due primarily to the complex effect of various activated oxygens and the associated methionine breakdown products (Tzeng and DeVay, 1989; Tzeng *et al.*, 1990a). In order to understand the involvement of methionine in the biocidal activity, photochemical degradation of methionine in the reaction system was investigated. Ethylene production was closely associated with the production of hydroxyl radical and singlet oxygen. The involvement of Haber-Weiss reaction is herein discussed.

Materials and Methods

Reagents and Chemicals

Riboflavin, L-methionine, methional, acrolein, methionine sulfoxide, β-carotene, desferal (desferoxamine methyl ester) and ethylenediaminetetraacetic acid (EDTA, disodium salt) were obtained from Sigma Chemical Co. (St. Louis, MO). Superoxide dismutase (from bovine erythrocyte; EC 1.15.1.1) and catalase (from beef liver; EC 1.11.1.6) were purchased from Boehringer Mannheim Chemical Co. (Mannheim, Germany). DL-α-tocopherol was obtained from Serva Feinbiochemica GmbH & Co. (Heidelberg, Germany); and 1-haxenesulfonic acid (PIC-B6, sodium salt, HPLC grade) was from Eastmant Kodak (Rochester, NY). Compressed air, hydrogen, and nitrogen used for gas chromatography analysis were supplied by Ho-Tsun Gas Co. (Taiwan, Republic of China); and all other chemicals or reagents were obtained from Merck Chemical Co. (West Germany). All the chemicals used were reagent grade or equivalent in purity. Freshly made glass double distilled water with electric-conductivity less than 1.5 umho/cm was used for the preparation or dilution of test reagents. Glasswares and plastic wares used were thoroughly cleaned and rinsed with distilled water. In all tests, MR denoted for a 26.6 μM (10 ppm) riboflavin and 1 mM L-methionine containing solutions were prepared using 0.1 M citrate phosphate buffer (pH 4.0 to 6.0) or 0.1 M phosphate buffer (pH 7.0 to 8.0).

Detection of Ethylene by GC

The MR solutions were prepared fresh, adjusted to desired pH, and then dispensed into Wheaton serum bottles for ethylene production analysis. Each bottle contained 20 ml of test solution. The bottles were flushed with fresh air, sealed with rubber caps and then illuminated under two pairs of Philips TLD 36W/33 fluorescent tubes; the light intensity was approximately 1 $W \cdot m^{-2}$. After designated time of reaction, 1 ml gas samples were withdrawn from the head space of each bottle by a gas-tight syringe. The accumulated ethylene contents were determined by a Shimadzu GC-14A gas chromatograph using an alumina column and a flame ionization detector (Tzeng and DeVay, 1985; Tzeng *et al.*, 1990b). Calibrated ethylene gas at 1.006 ppm in concentration was obtained from Matherson Gas Products (New Jersey, U.S.A.) and used as the standard for quantification of ethylene generated in test samples.

Detection of Methional, Acrolein and Methionine Sulfoxide by HPLC

A Bio-Rad isocratic HPLC system equipped with a variable UV monitor was used for the detection of methional, acrolein and methionine sulfoxide in the MR reaction system. The test solutions exposed to light were filtered with 0.45 μm Millipore membranes. Components in the solution were then separated on a Merck Lichrosorb RP-18 reversed phase column (150 $\times$ 4 mm), using 5 mM PIC-B6 containing methanol (25%) as a mobile phase. Presence of methional, acrolein and methionine sulfoxide were detected by UV monitor (Bio-Rad model 1305A) at 220 nm wavelength

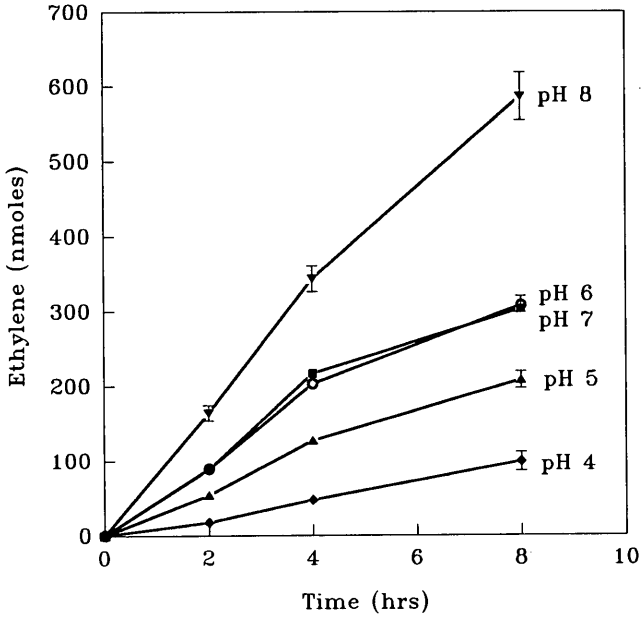

Fig. 1. Effect of pH on ethylene production from methionine riboflavin mixture under continuous illumination

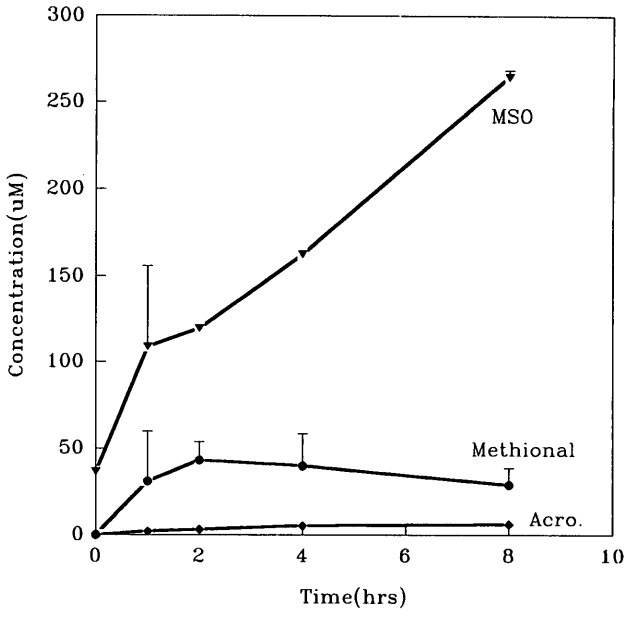

Fig. 2. Production of various methionine-derived products from methionine riboflavin mixture at pH 8.0 under continuous illumination. (MSO, methionine sulfoxide; Acro., acrolein).

(Tzeng *et al.*, 1990a).

Results

Effect of pH on Ethylene Production

At pHs from 4.0 to 8.0, the photochemical production of ethylene from MR appeared to be a function of the increase of pH. As indicated in Figure 1, the amount of ethylene produced from the reaction system at pH 8.0 after 8 h reaction was approximately 585.5 nmoles. With the decrease of reaction pH, the amount of ethylene produced was greatly reduced. Accumulated ethylene production from the reaction system at pHs 7.0, 6.0, 5.0, and 4.0 was 302.4, 307.2, 207.6, and 99.0 nmoles, respectively, after 8 h reaction.

Effect of Methional, Acrolein and Methionine Sulfoxide

In an attempt to elucidate the photochemical reaction products relating to the biocidal activity of MR, the production of methional, acrolein, and methionine sulfoxide in the reaction system at pH 4.0 has been analyzed by HPLC in our previous work (Tzeng *et al.*, 1990a). The amount of methional, acrolein, and methionine sulfoxide detected from this reaction system after 8 h reaction was 39.0, 6.9 and 225.9 μM, respectively. In the present investigation, the production of these three components from MR at pH 8.0 was analyzed by the same method. The amount of methional, acrolein, and methionine sulfoxide in the reaction mixture after 8 h reaction was 28.6, 6.0 and 264.8 μM, respectively (Fig. 2). To further illustrate their role in ethylene production, 1 mM of each component was amended to MR individually right before light treatment. The addition of methional greatly stimulated ethylene production from the reaction system at either pH 4.0 or 8.0 (Figs. 3 and 4). At pH 4.0, accumulated ethylene production from methional amended treatment after 8 h reaction reached 548.9 nmoles, which was more than 5 times greater than that from the control (Fig. 3). In comparison, at pH 8.0 the amount of ethylene produced from the methional amended treatment during the 8 h reaction period was approximately 1704.7 nmoles which was more than twice of that from the compared control (Fig. 4). In contrast to this, the addition of acrolein or methionine sulfoxide did not enhance ethylene production from the reaction system at either pH 4.0 or 8.0. The addition of 1 mM acrolein or methionine sulfoxide to MR at pH 8.0 was in fact slightly inhibitory to the ethylene production (Fig. 4).

Effect of Hydrogen Peroxide

The addition of 100 to 1000 ppm of hydrogen peroxide to MR at pH 4.0 was slightly stimulatory to the rate of ethylene production during the first 2 to 4 h reaction period (Fig. 5). However, the reaction rate then declined, and the amount of ethylene produced from the hydrogen peroxide amended solutions after 8 h was either not different or was greatly lowered as compared to the control treatment. At pH 8.0, the addition of hydrogen peroxide at 100 ppm did not lead to a significant change of ethylene production throughout the 8 h reaction period (Fig. 6), whereas the addition at 1000 ppm greatly reduced ethylene production.

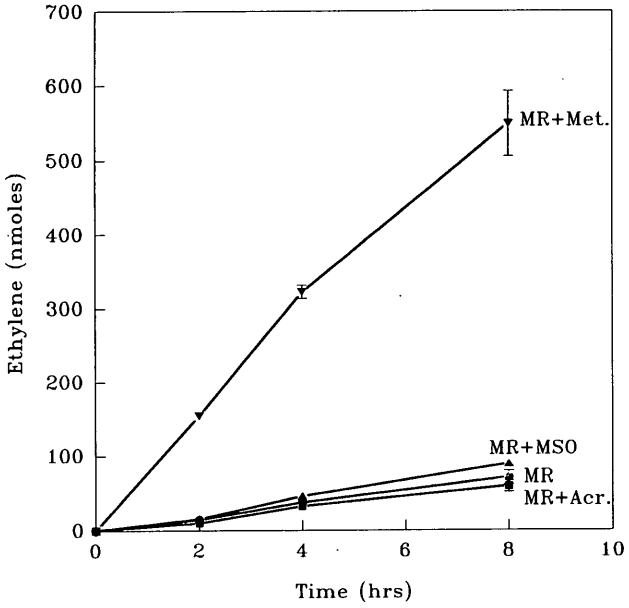

Fig. 3. Effect of methionine-derived-products supplementation (each at 1 mM) on photodynamic ethylene production from methionine riboflavin mixture at pH 4.0.

Effect of Antioxidants and free Radical Scavengers

The addition of both superoxide dismutase (SOD) at 150 units/ml or catalase at 1300 units/ml to MR at pH 4.0, slightly inhibited the photodynamic production of ethylene (Fig. 7). When applied to MR at pH 8.0, addition of catalase at the same concentration was greatly stimulatory to ethylene production; whereas addition of SOD at the same concentration by itself or together with catalase had no effect on the detected reaction (Fig. 8). In contrast, the presence of antioxidants like ascorbic acid or α-tocopherol, and the commonly used hydroxyl radical scavenger, thiourea, were all inhibitory to ethylene production from MR at either pH 4.0 or 8.0 (Figs. 9 and 10). Thiourea was among the most effective; total amount of ethylene detected from 1 mM thiourea amended MR at either pH 4.0 or 8.0

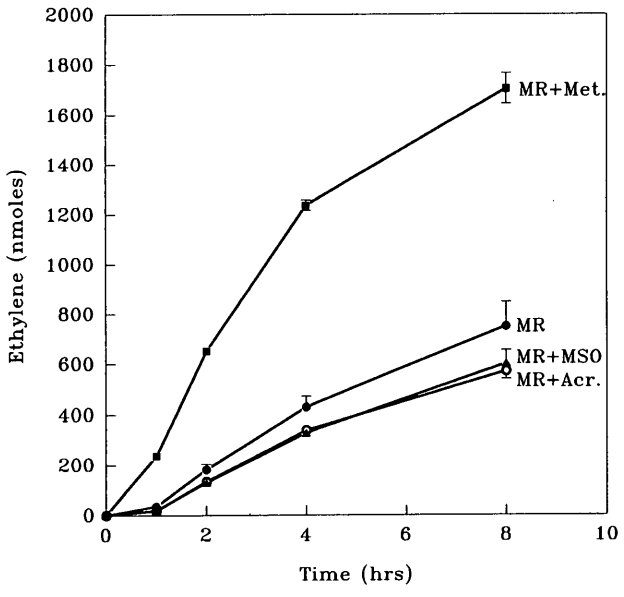

Fig. 4. Effect of methionine-derived-products supplementation (each at 1 mM) on photodynamic ethylene production from methionine riboflavin mixture at pH 8.0.

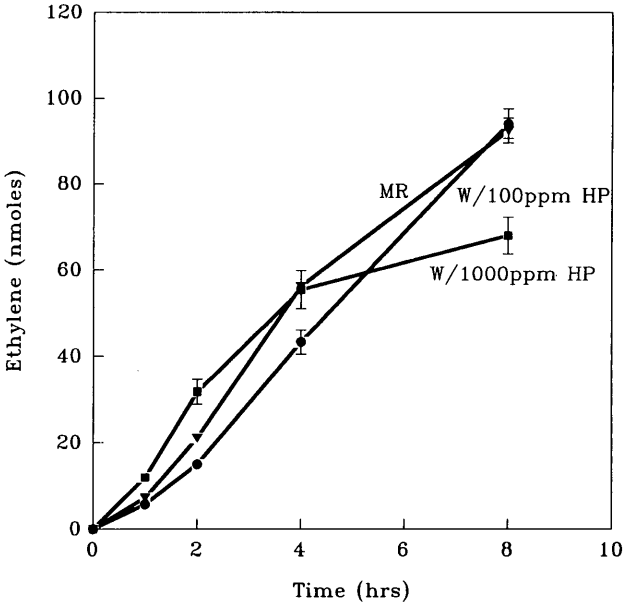

Fig. 5. Effect of hydrogen peroxide (HP) supplementation on photodynamic ethylene production from methionine riboflavin mixture at pH 4.0.

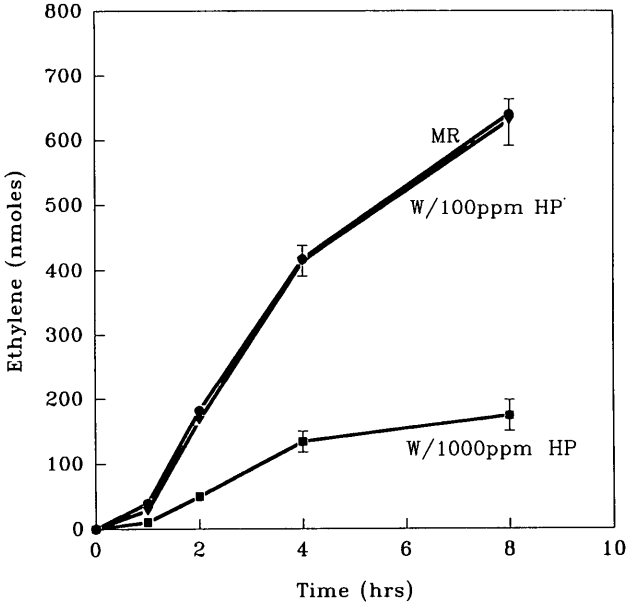

Fig. 6. Effect of hydrogen peroxide (HP) supplementation on photodynamic ethylene production from methionine riboflavin mixture at pH 8.0.

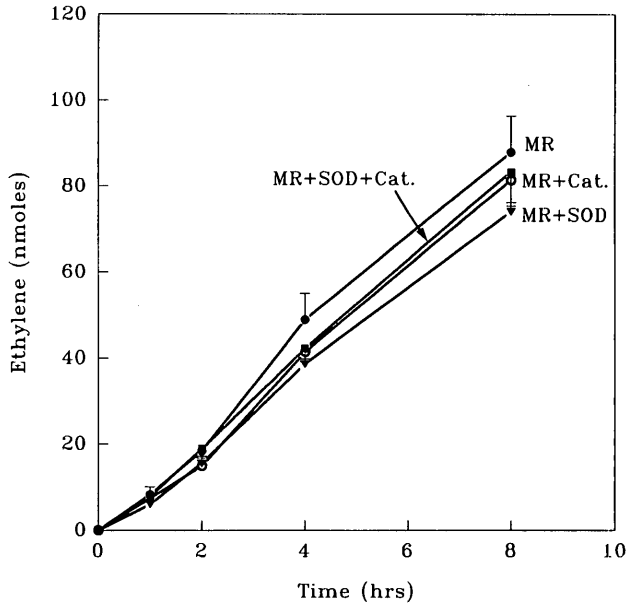

Fig. 7. Effect of superoxide dismutase (SOD, 150 units/ml) and catalase (CAT, 1300 units/ml) on photodynamic ethylene production from methionine riboflavin mixture at pH 4.0.

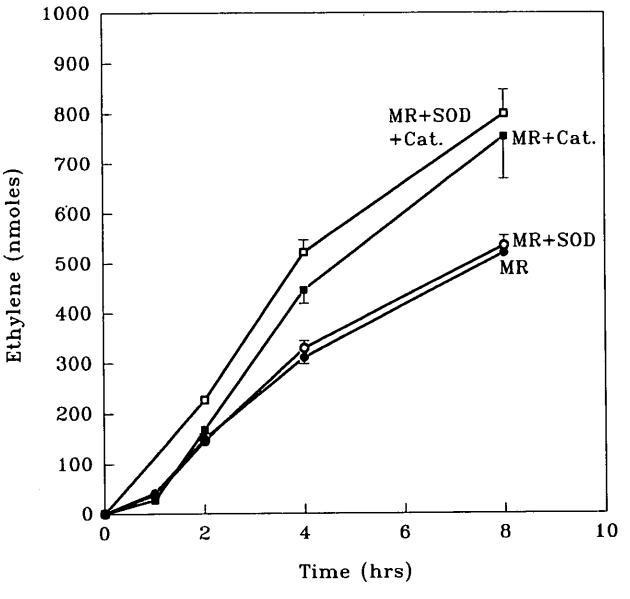

Fig. 8. Effect of superoxide dismutase (SOD, 150 units/ml) and catalase (CAT, 1300 units/ml) on photodynamic ethylene production from methionine riboflavin mixture at pH 8.0.

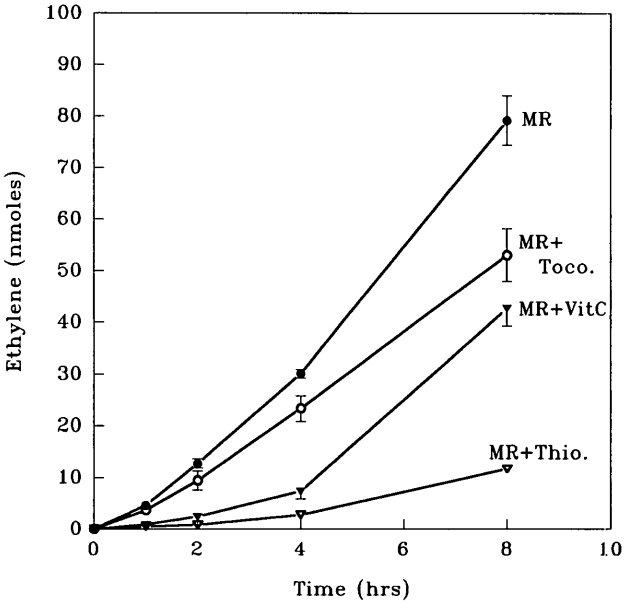

Fig. 9. Effect of α-tocopherol (Toco), ascorbate (VitC), and thiourea (Thio) each at 1 mM on photodynamic ethylene production from methionine riboflavin mixture at pH 4.0.

after 8 h reaction was reduced to less than 10% of the non-treated controls. The inhibitory effect of ascorbic acid at pH 8.0 was somewhat comparable to that of thiourea treatment throughout the 8 h reaction period (Fig. 10); however, at pH 4.0, this strong inhibitory effect was greatly reduced after the reaction had proceeded for 4 h (Fig. 9).

Effect of Singlet Oxygen Quenching Agents

Singlet oxygen quenching agents like β-carotene or sodium azide were similar to the activity of antioxidants and hydroxyl radical scavengers, and also appeared to be strongly inhibitory to ethylene production from MR (Figs. 11 and 12). The inhibitory effect of sodium azide was apparently a function of pH. At pH 8.0, the amount of ethylene produced from 1 mM sodium azide amended MR after an 8 h reaction period was about 17% of the non-treated control (Fig. 12). To achieve the same inhibitory action at pH 4.0, 10 mM of this supplement was required (Fig. 11). The inhibitory action of 1 mM β-carotene amended to MR at pH 4.0 was much higher than that of sodium azide at the same concentration (Fig. 12). At pH 8.0, however, sodium azide appeared to be a superior inhibitor of ethylene

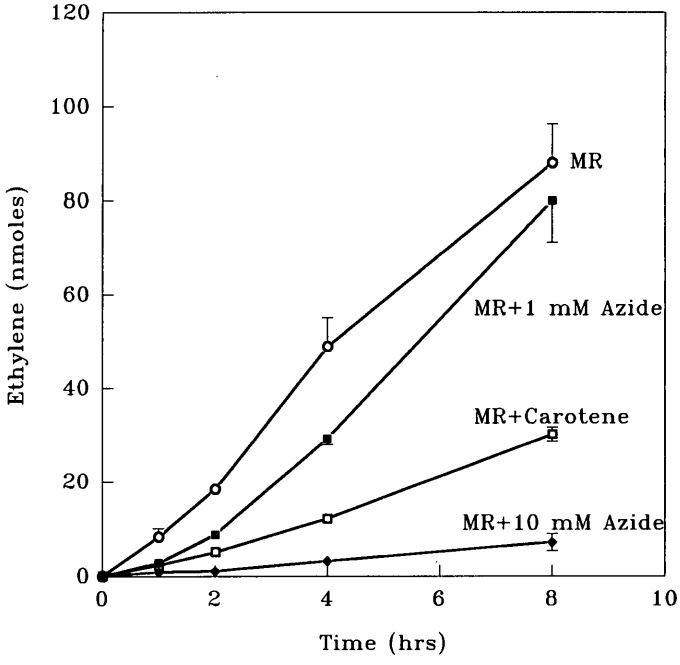

Fig. 11. Effect of Na-azide (1 and 10 mM) and β-carotene (1 mM) on photodynamic ethylene production from methionine riboflavin mixture at pH 4.0.

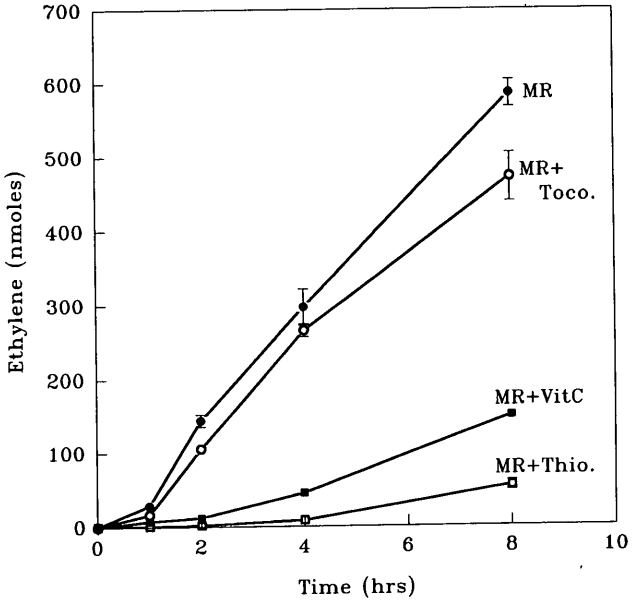

Fig. 10. Effect of α-tocopherol (Toco), ascorbate (VitC), and thiourea (Thio) each at 1 mM on photodynamic ethylene production from methionine riboflavin mixture at pH 8.0.

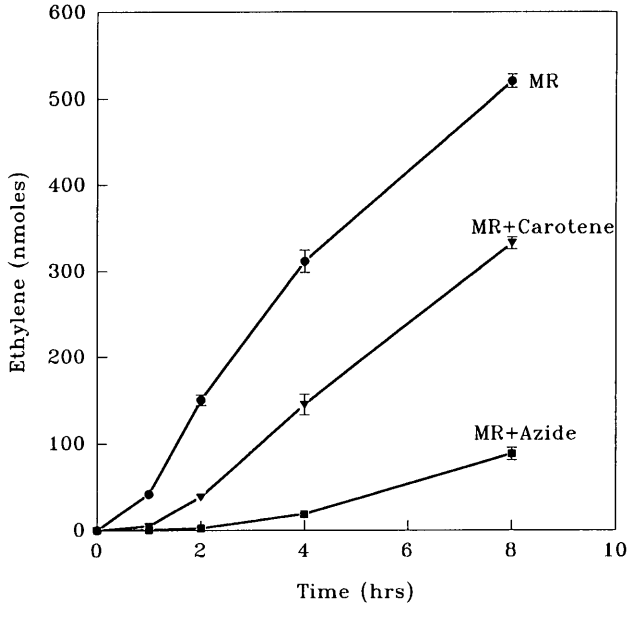

Fig. 12. Effect of Na-azide and β-carotene each at 1 mM in concentration on photodynamic ethylene production from methionine riboflavin mixture at pH 8.0.

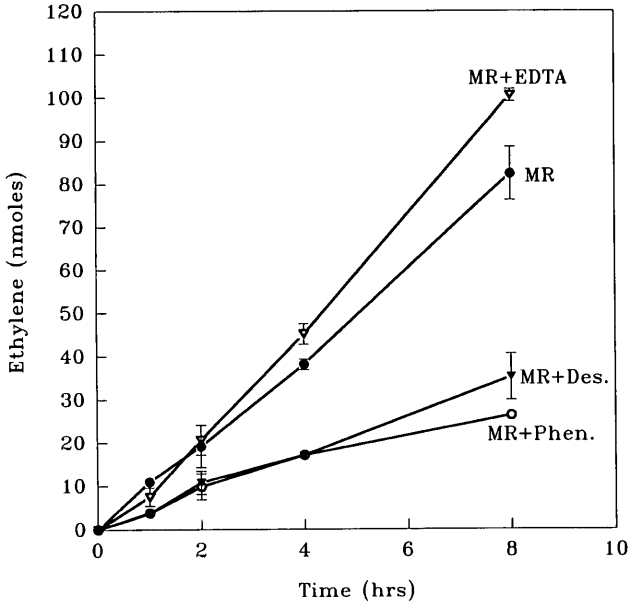

Fig. 13. Effect of EDTA, desferal (Des), and phenanthroline (Phen) supplementation each at 1 mM on photodynamic ethylene production from methionine riboflavin mixture at pH 4.0.

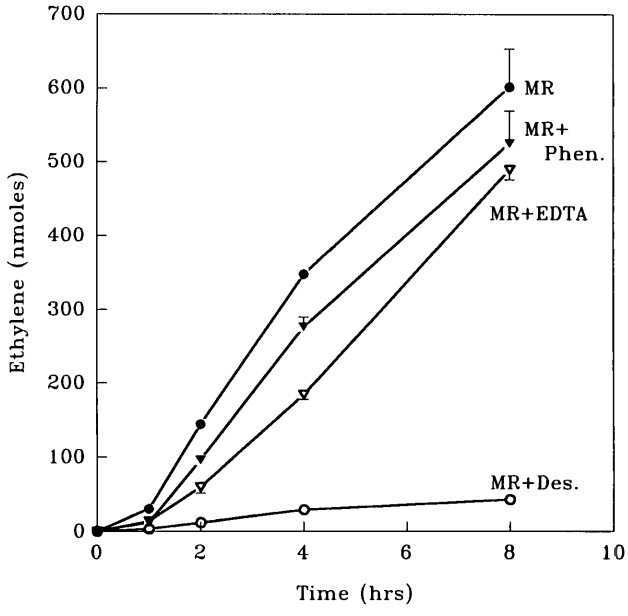

Fig. 14. Effect of EDTA, desferal (Des), and phenanthroline (Phen) supplementation each at 1 mM on photodynamic ethylene production from methionine riboflavin mixture at pH 8.0.

production than β-carotene during the 8 h reaction period.

Effect of Iron Chelating Agents

The changes of rate of ethylene production due to the presence of iron chelating agents EDTA, desferal and phenanthroline were examined at pH 4.0 and 8.0 of MR. At pH 4.0, the addition of 1 mM desferal or phenanthroline to MR both greatly reduced ethylene production (Fig. 13). The addition of EDTA to the same reaction system, however, was slightly stimulatory to ethylene production. When the pH of the test solution was raised to 8.0, addition of desferal completely inhibited ethylene production; while addition of either phenanthroline or EDTA only slightly inhibited ethylene production (Fig. 14).

Discussion

Photochemical production of ethylene from methionine degradation was first explored in early works by Yang and his colleagues using flavin mononucleotide (FMN) as a photosensitizer (Yang *et al.*, 1967a, 1967b). Although a novel reaction scheme for methionine degradation by photoactivated-FMN has been proposed by these authors, other characteristics of the system involving free radical chemistry remained to be further explored. By comparing the effect of different substrates on ethylene production, it was suggested by previous workers that methional was the likely reaction intermediate for ethylene production while the photodegradation of methional might proceed through the abstraction of an electron from the sulfur atom of methional by the photoactivated flavin molecule (Yang *et al.*, 1967b). The detection of methional from MR solution by HPLC analysis (Fig. 2) and the immediate stimulatory effect of methional supplementation on ethylene production (Figs. 3 and 4) were both supportive of the view that methional was the likely intermediate involved in the flavin sensitized photochemical production of ethylene from methionine. During the 8 h reaction period, the amount of methional detected from the reaction system at either pH 4.0 (Tzeng *et al.*, 1990a) or pH 8.0 (Fig. 1) was consistently low; suggesting that methional produced during the reaction was further converted into ethylene and other methionine breakdown products. In addition to increase of ethylene production, a parallel increase of methanethiol and dimethyl disulfide was detected by gas chromatography (data not shown). The production of methionine sulfoxide apparently resulted from the oxygenation of methionine (Foote, 1976); whereas acrolein is a product

from methional decomposition by hydrogen peroxide (Alarcon, 1976). Detection of these components reflected the presence of singlet oxygen and hydrogen peroxide during the reaction process.

The effect of pH on ethylene production as shown in Fig. 1 was similar to Yang's previous results in which pH 8.5 was optimum for the reaction (Yang *et al.*, 1967b). In regard to the involvement of oxygen radicals in methionine photodegradation, we have shown that in MR at pH 8.0, superoxide anion was the predominant oxygen radical generated (Tzeng, 1989). Whereas at pH 4.0, the amount of superoxide was greatly reduced with a corresponding increase in concentration of hydroxyl radical and singlet oxygen predominantly through the superoxide driven Haber-Weiss reaction (Tzeng, 1989; Tzeng and Lee, 1989). The effect of pH on ethylene production suggested the involvement of superoxide anions in the methionine degradation. In a photochemical reaction, the presence of superoxide anion generally leads to further production of hydrogen peroxide via autodismutation. In MR at pH 4.0, in which an iron contaminant was available in free form, hydrogen peroxide molecules might react with superoxide anion and ferrous iron leading to an increase in hydroxyl radical and singlet oxygen production. Our results have indicated that amendment of MR with hydrogen peroxide greatly enhanced hydroxyl radical production at pH 4.0 (Tzeng and Lee, 1989). The transient increase of ethylene production at early stages of reaction (Fig. 5) could be due to an enhanced Haber-Weiss reaction process. On the contrary, by itself, hydrogen peroxide was inhibitory to the ethylene production. In the presence of a substantial amount of hydrogen peroxide, methional was degraded into acrolein and no ethylene was produced (Alarcon, 1976; Tzeng *et al.*, 1990a). It was also noted by Yang *et al.* (1967b) that hydrogen peroxide might lead to oxygenation of methionine causing an inhibition of ethylene production in the FMN-methionine photoreaction system. This explanaiton could also apply to the observed inhibitory effect of hydrogen peroxide on the ethylene production from MR at either pH 4.0 or pH 8.0 during the 8 h reaction period (Figs. 5 and 6). The adverse effect of hydrogen peroxide was further supported by the evidence that addition of catalase to MR greatly stimulated the ethylene production at pH 8.0 (Fig. 8). We have recently analyzed the production of hydrogen peroxide in MR during the reaction; at pHs 4.0 to 8.0, production of this reactive oxygen derivative appeared to be a function of increasing pH (data not shown).

The critical role of various oxygen radicals in the studied reaction was clearly indicated by the evidence that antioxidants like ascorbate or α-tocopherol (Figs. 9 and 10), free radical scavengers like thiourea (Figs. 9 and 10), and singlet oxygen quenchers like β-carotene and sodium azide (Figs. 11 and 12), all appeared to be greatly inhibitory to ethylene production (Kaiser *et al.*, 1990; Kelner *et al.*, 1990; Morehouse *et al.*, 1982). Among various oxygen radicals, superoxide anion was suggested as the main factor contributing to ethylene production in a sulfite-activated peroxidase system in which methionine was used as a reaction substrate (Yang *et al.*, 1967b). In MR, the role of superoxide was dependent on pH. The low activity of SOD in reducing ethylene production from MR at either pH 4.0 (Fig. 7) or 8.0 (Fig. 8), indicates a minor role for superoxide anion in methionine degradation. The inhibitory effect of ascorbate (Figs. 9 and 10) also supports this view. Ascorbate is a known photodynamic agent which upon illumination, leads to the formation of superoxide anion (Girotti *et al.*, 1985). By Electron Spin Resonance Spectroscopy, stimulated production of superoxide anion from MR amended with 1 mM ascorbate was detected (data not shown). These contradictory results clearly indicate the involvement of factors other than superoxide free radical. In a system wherein superoxide is generated, singlet oxygen and hydroxyl radical are generally associated products. For MR solution at pH 4.0, we have demonstrated that production of these highly reactive oxygen derivatives was a determinative factor contributing to its unusual photodynamic biocidal activity (Tzeng, 1989; Tzeng and DeVay, 1989). Moreover, in the presence of iron contaminants, the inhibitory effects of α-tocopherol, β-carotene, sodium azide and thiourea presented (Figs. 9 and 11) were strong indications that singlet oxygen and hydroxyl radical were both critical factors for the photochemical production of ethylene in an acidic medium (Burton and Ingold, 1984; Tzeng and Lee, 1989). The greatly reduced ethylene production by addition of desferal and phenanthroline to MR further indicated the participation of contaminant iron ions and the importance of the iron-catalyzed Haber-Weiss reaction. As for the opposite effect of EDTA, an increase in the Fenton reaction by EDTA-iron has been reported (Morehouse *et al.*, 1982); its stimulatory rather than diminishing

effect on ethylene production (Fig. 13) also supports the involvement of the Haber-Weiss reaction.

Results of this study do not exclude the possible involvement of superoxide anion radical in the activity of MR. In MR at pH 8.0, the production of hydroxyl radical was negligible (Tzeng and Lee, 1989) and superoxide anion was one of the main radicals associated with the photochemical production of ethylene. A plausible reason for the low activity or negligible effect of SOD is the possible toxicity of reaction products of SOD. Both hydrogen peroxide and singlet oxygen are additional reaction products of SOD.

In oxygen radical research, thiourea was frequently used as a scavenging agent to demonstrate the presence of hydroxyl radical. Kelner *et al.* (1990), however, showed evidence recently that thiourea is a direct scavenger of superoxide radical as well as hydroxyl radical and hydrogen peroxide. The strong reduction in toxicity of MR in the presence of thiourea at pH 8.0 (Fig. 10) may be related to the quenching of superoxide in addition to hydroxyl radical. The activity of desferal at the same reaction condition in reducing the toxicity of MR may also related to its quenching activity (Fig. 14); the possibility of desferal-iron complex as a center attracting superoxide anions was previously indicated (Tzeng and Lee, 1989).

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哈伯瑋斯效應在甲硫胺酸核黃素混合物光化反應產生乙烯過程之參與作用

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本研究旨在探討以核黃素作爲光敏感體情況下甲硫胺酸光分解產生乙烯之作用。在 pH 4.0 至 8.0 範圍，供試甲硫胺酸核黃素混合物(簡稱 MR)之乙烯形成作用明顯隨 pH 提高而增加。反應溶液中，甲硫醛爲其形成過程之可能中間產物，丙烯醛及甲硫胺酸亞碸則對乙烯形成不具影響。在 pH 4.0 時，反應液中添加超氧自由基歧化酶 (SOD) 與過氧化氫酶 (catalase) 對其乙烯形成僅有些許抑制作用；於 pH 8.0 時，SOD 之存在對乙烯產生亦不具影響，然而添加過氧化氫酶則對乙烯產生反有明顯促進作用，SOD 所表現的不具影響之特性顯示，MR 反應液中乙烯生成爲超氧自由基以外其他因子所參與之反應，而過氧化氫酶之促進效應則進而顯示，過氧化氫之存在對此乙烯形成作用有明顯的抑制作用。各種抗氧化物如抗壞血酸 (ascorbate) 與生育素 (tocopherol)，自由基清除劑 (scavenger) 如硫脲，以及單重氧消除劑 (quencher) 如類胡蘿蔔素與疊氮化鈉等，對乙烯形成反應均有極強之抑制作用。此外添加鐵離子之嵌合劑 (chelating agent) 如 desferal 或 phenanthroline 等，亦可使反應液之乙烯形成大爲降低。這些現象顯示羥基自由基與單重氧之產生，在此作用過程中相當重要。由本研究所獲悉之各種證據並顯示由超氧自由基驅動之哈伯-瑋斯反應過程，尤其是於酸性情形下，確爲參與 MR 光化反應產生乙烯之一主要關鍵性因子。