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Production of hydroxyl radicals in photodynamic action of methionine riboflavin mixture: a consequence of iron catalyzed Haber-Weiss reaction

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Abstract. Production of · OH radical in photodynamic effect of methionine riboflavin mixture (MR) and its possible correlation to the associated biocidal activity were studied. By the method developed by Baker and Gebicki, we found that 10 mM sodium benzoate were needed to obtain a maximum production of fluorescent hydroxybenzoates for reflecting · OH formation in the test system. In MR, a much greater rate of · OH production was detected at pHs 4.0-5.0 than that at pHs 6.0-8.0. The low pH dependence not only indicated the importance of Haber-Weiss reaction, it also suggested the possible involvement of iron contaminant in MR in which iron was not supplied. Supplementation of exogenous iron enhanced · OH formation of MR at pHs higher than 6.0. On the contrary, supplementation of desferal and phenanthroline greatly reduced the radical forming activity and further emphasized the involvement of iron contaminants. Besides iron chelators, amendment of thiourea was found to have great scavenging effect on the · OH production. The · OH scavenging efficacy of sodium formate was rather poor. At pH 4.0, it was also noted that addition of ascorbic acid or hydrogen peroxide at certain concentrations greatly stimulated the generation of the test radical. The rapid increament of · OH formation via the iron catalyzed Haber-Weiss reaction was apparently a major factor which directly contributed to the strong biocidal activity of MR.

Key words: Biocidal activity; Haber-Weiss reaction; Hydroxyl radical; Methionine riboflavin mixture; Photodynamic effect.

Introduction

Application of suitable farm chemicals was one of the main contributing factors which assured the high quality and high productivity of today's agriculture. The intensive use of various pesticides, however, have led to quite a few deleterious effects on our living environment. Among them, most frequently encountered were the development of chemical resistance of various plant pests; the need of new chemicals to substitute the old ones which have lost their effectivity; and the increasingly endangered environmental safety due to the undesired residual effect of the applied chemicals. To solve these problems, the development of effective and environmently safe alternative pesticides was ergently in need.

Biological damages due to a dye sensitized photodynamic effect was known for many years (Ito, 1983; Spikes *et al.*, 1969). The term "photodynamic pesticide" however, has never earned its publicity until the recent success of using δ-amino levulinic acid as a photodynamic herbicide by researchers in Urbana, Illinois (Chen, 1986). Among the light activated pesticides known to date, most of the attention seemed to be centered on the control of insects and weeds (Chen,

1986; Pimprikar *et al.*, 1979). The use of photodynamic agents for the control of plant diseases has never been attempted, although photodynamic damages to microorganisms were quite occasionally encountered (Chelala *et al.*, 1983; Nakamura *et al.*, 1983; Ramsey *et al.*, 1957; Slotnick *et al.*, 1965).

The unusual photodynamic biocidal activity of methionine riboflavin mixture (MR) was discovered in our previous investigation on the photodynamic generation of ethylene by various phytopathogenic fungi (Tzeng and DeVay, 1985). Since then, a series of experiments have been conducted to elucidate the possible mode of action and to explore its potential use on controlling plant diseases (DeVay, *et al.*, 1987; Tzeng, 1988 and 1989; Tzeng, *et al.*, 1985). In regard to the mode of action of the photodynamic biocidal activity, we have shown in our previous study that production of various activated oxygen species such as superoxide anion ($O_2^{-1}\cdot$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$), were the principle toxigenic factors involved (Tzeng and DeVay, 1988; Tzeng, *et al.*, 1985). Using nitroblue tetrazolium chloride (NBT) as a radical trapping agent, production of significant amounts of $O_2^{-1}\cdot$ were detected (Tzeng, 1989). The direct involvement of this radical in the concerned biocidal activity was downplayed, however, by the evidence that its generation was high pH dependent. The inhibitory effect of thiourea on the biocidal activity of MR, on the other hand, implicated the great importance of hydroxyl radical (Tzeng, 1989). In order to understand how this highly reactive oxygen radical was involved in the biocidal activity, its generation during the course of reaction was further explored. The possible mechanism of $\cdot OH$ formation and its likely participation in the photodynamic biocidal activity of MR are herein discussed.

Materials and Methods

Reagents and Chemicals

Riboflavin, L-methionine, ethylenediamine tetraacetic acid (EDTA, disodium salt), thiourea, phenanthroline, sodium formate, and ascorbic acid (sodium salt) were obtained from Sigma Chemical Co. (St, Louis, Mo). Desferal (desferrioxamin B methane sulfonate) was kindly provided by Dr. DeVay of U. C. Davis which was originally obtained from Ciba Geigy. The rest of chemicals used were purchased from Merck Chemical Co. (Darmstadt, 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 μ mho/cm were used for the preparation or dilution of test reagents. All the glasswares and plastic wares used were thoroughly cleaned and rinsed twice with the glass double distilled water. In all tests, MR denoted for a 26.6 μM riboflavin and 1 mM L-methionine containing solution. Unless specified, pHs of MR used for the experiment were adjusted to 4.0 with 0.1 N HCl.

Determination of $\cdot OH$ Production

The method recently developed by Baker and Gebicki which used sodium benzoate as the $\cdot OH$ radical trapping agent was adapted (Baker and Gebicki, 1984 & 1986). In tested MR solution, amount of sodium benzoate suitable for the radical detection was determined in the first experiment. About 10 mM of this radical trapping agent appeared to be most suitable for the reaction and were thus applied in the proceeded experiments. For the determination of $\cdot OH$ production, 1.5 ml 10 mM sodium benzoate containing MR solution in disposable pyrex tubes (13 × 100 mm) were placed under a continuously illuminated condition as previously described (Tzeng and DeVay, 1985). During the course of reaction, fluorescence of resulted hydroxylated benzoates were measured by Aminco SPF-125 spectrofluorometer at 300 nm excitation and 410 nm emission. A 0.2 μg/ml quinine sulfate (dissolved in 0.1 M H_2SO_4) was used to calibrate and standardize the instrument (Baker and Gebicki, 1984). All the experiments were performed at room temperature (approximately 28°C).

Results

Effect of Sodium Benzoate Concentration on Detection of $\cdot OH$

The basis of the analytical method developed by Baker and Gebicki for detection of $\cdot OH$ resides principly on the potency of sodium benzoate to react with the radical molecule and by which the fluorescent hydroxylated benzoates were generated. In order to know what amount of sodium benzoate is appropriate for running the test in MR, 0.1, 1, 5, 10, 50, and 100 mM of sodium benzoate were applied respectively to the reaction system. As shown in Fig. 1, the formation of fluorescent hydroxylated benzoate generally increased

with the increases of sodium benzoate addition. For tested MR, the maximum production of fluorescent hydroxylated benzoates was detected by the addition of 10 mM sodium benzoate. The further increament of this radical trapping agent then appeared to have no more additive effect on the hydroxylation reaction. In the rest of this study, 10 mM of sodium benzoate were thus applied for the detection of • OH generation.

Effect of pH on • OH Formation

PHs of the tested MR were adjusted to 4.0, 5.0, 6.0, 7.0 and 8.0 respectively by 0.1 N HCl and 0.1 N NaOH. The followed time course study after light exposure indicated that • OH production in MR was greatly favored at pHs lower than 5.0 (Fig. 2). At pH 4.0 to 5.0, rapid increases of • OH production were detected about 15 minutes after light exposure. Whereas at pHs higher than 6.0, production of • OH was not significant until 1 hour after light treatment; and the • OH production rate were all much less than that at pH 4.0 and 5.0. The rate of • OH production throughout the 6 hours experimental period appeared to be highest at pH 4.0 and then followed the order at pH 5.0, 7.0, 6.0, and 8.0.

Effect of Iron on • OH Formation of MR at Different pHs

For this experiment, one set of MR were supplemented with 0.1 mM $FeCl_2$ and 10 ppm EDTA and then had the pHs adjusted to 4.0 to 8.0 as above stated. Detection of the radical generation by the same method indicated that presence of ferrous iron greatly enhanced • OH production of MR at pHs higher than 6.0 (Fig. 3). The rate of • OH generation in the presence of ferrous iron throughout the 6 hours experimental period appeared to be highest at pH 4.0 and 5.0, then followed by that at pH 7.0 and 8.0, and lowest at pH 6.0. At a low pH range (4.0-5.0), no stimulatory effect of • OH production by iron supplementation was observed.

Effect of Desferal on • OH Formation

The production of • OH in MR supplemented with 0.1, 1, 5, and 10 mM of desferal respectively were tested. Presence of desferal appeared to reduce the radical generating activity greatly (Fig. 4). In an 8 hours reac-

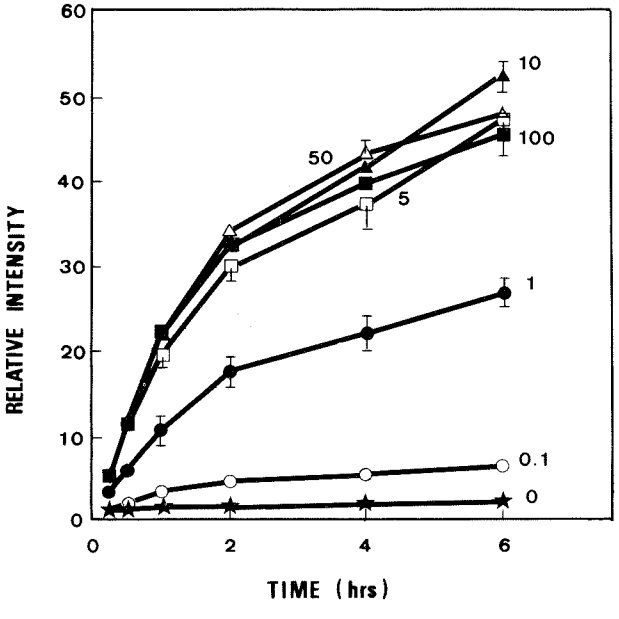

Fig. 1. Effect of Na-benzoate concentration on detection of hydroxyl radical from methionine riboflavin mixture by spectrofluorometry. Numbers in the figure indicate concentration (mM) of Na-benzoate amended to tested methionine riboflavin mixture right before light treatment. Bars indicate standard deviation.

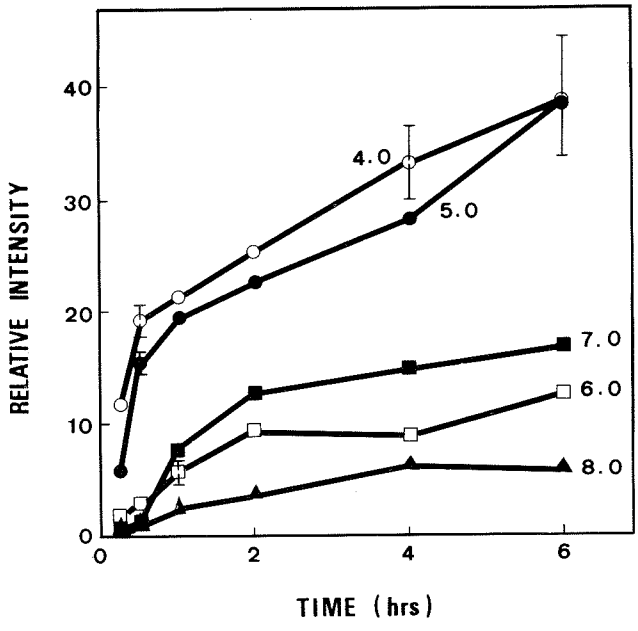

Fig. 2. Effect of pH (4.0~8.0) on production of hydroxyl radical in methionine riboflavin mixture. Bars indicate standard deviation.

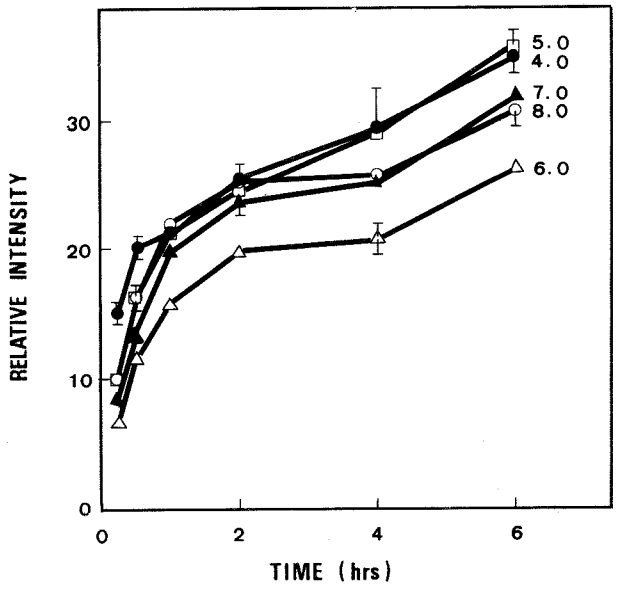

Fig. 3. Effect of pH (4.0~8.0) on production of hydroxyl radical in a Fe^{++}-EDTA amended methionine riboflavin mixture. Bars indicate standard deviation.

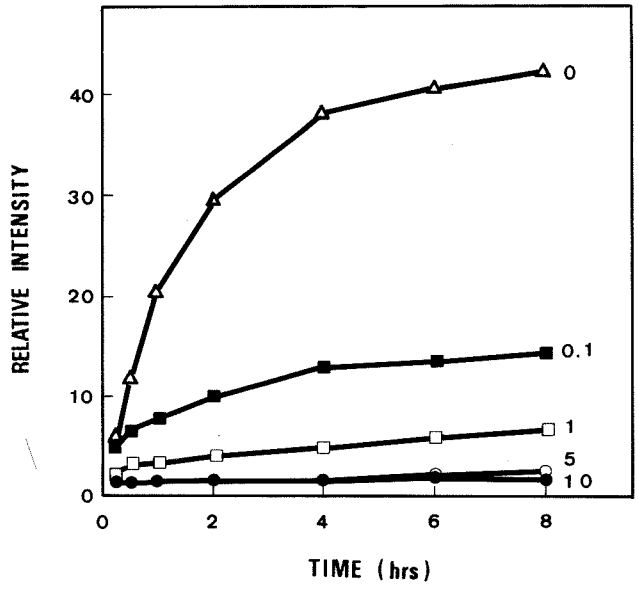

Fig. 4. Effect of desferal on production of hydroxyl radical in methionine riboflavin mixture. Numbers in the figure indicate the amount (mM) of desferal added. Bars indicate standard deviation.

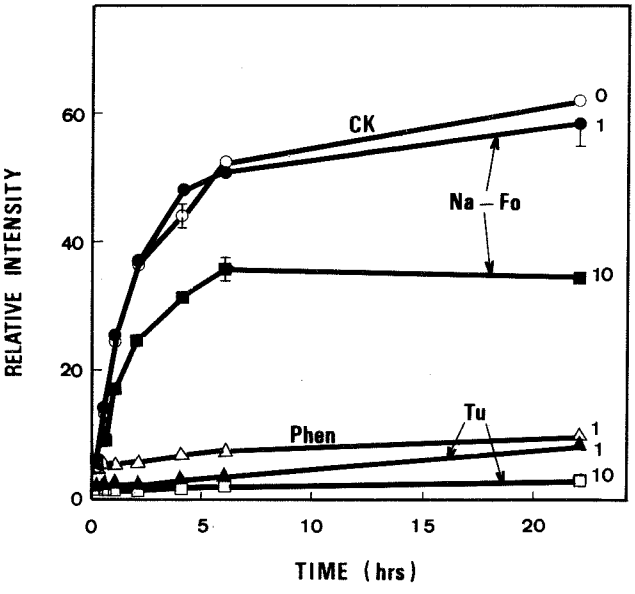

Fig. 5. Effect of hydroxyl radical scavengers on production of hydroxyl radicals in methionine riboflavin mixture. Thiourea (Tu) and Na-formate (Na-Fo) both at 1 and 10 mM respectively were added to the test solution right before light treatment. Phenanthroline (Phen) at 1 mM in concentration were included in this experiment to further indicate the involvement of iron in the detected reaction. Bars indicate standard deviation.

tion period, presence of 0.1 mM desferal reduced • OH production of the reaction mixture to much less than half of that in a control treatment. The production of • OH appeared to be totally inhibited with the addition of 5 to 10 mM desferal.

Effect of Radical Scavengers on • OH Formation

Two known • OH radical scavengers—thiourea and sodium formate, each at 1 and 10 mM concentrations were tested. Thiourea appeared to be a much better • OH scavenger than sodium formate in MR solution (Fig. 5). The tested sodium benzoate hydroxylation was nearly completely anihilated with the addition of just 1 mM of thiourea. In contrast to this, the addition of 1 mM sodium formate showed no preventive effect at all on the tested hydroxylated benzoate formation. To achieve half of the effectivity of that by 1 mM thiourea, more than 10 mM sodium formate seemed to be required. In this experiment, phenanthroline—an efficient iron chelator was included as an additional control to further illustrate the importance of iron contaminant in the studied reaction. The effectivity of this iron chelating agent in preventing • OH generation in MR appeared to be similary to that of desferal (Figs.

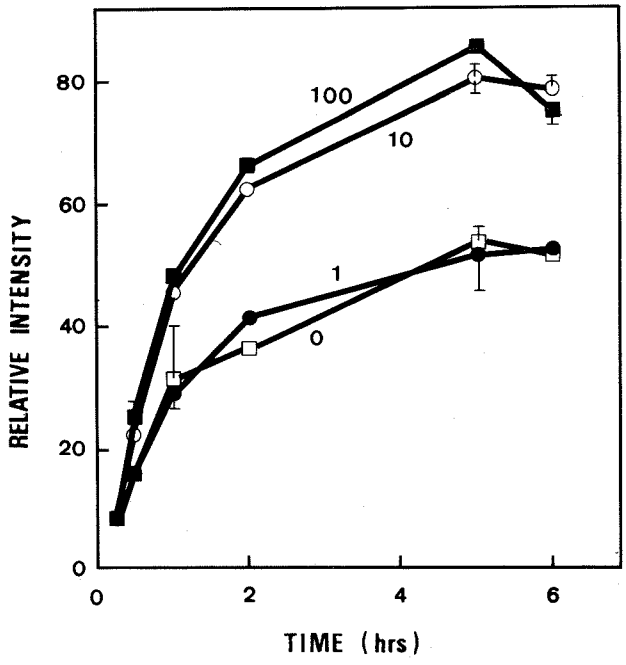

Fig. 6. Effect of hydrogen peroxide on production of hydroxyl radical in methionine riboflavin mixture. Numbers in the figure indicate the amount (ppm, W/V) of hydrogen peroxide used. Bars indicate standard deviation.

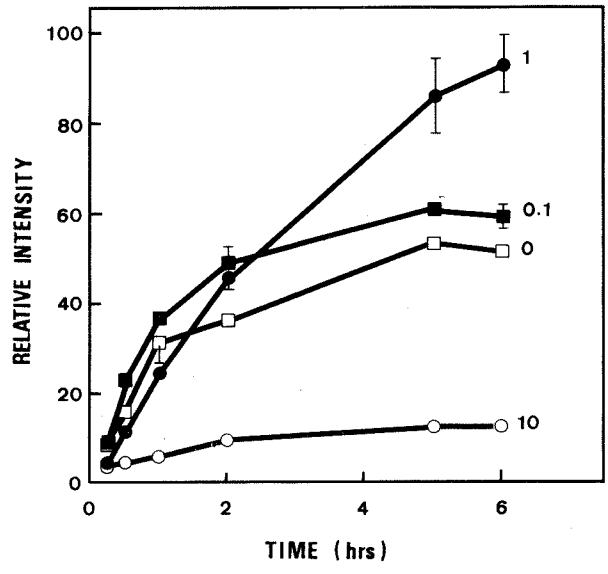

Fig. 7. Effect of ascorbic acid on production of hydroxyl radical in methionine riboflavin mixture. Numbers in the figure indicate the amount (mM) of ascorbic acid used. Bars indicate standard deviation.

4 & 5).

Effect of Hydrogen Peroxide and Ascorbic Acid on • OH Formation

Hydrogen peroxide were added to MR at the concentration of 29.4, 294 and 2940 μM (i.e. 1, 10, and 100 ppm, W/V) respectively. Significant enhancement of • OH production was observed with the supplementation of 294 and 2940 μM H_2O_2; whereas the treatment at 29.4 μM in concentration was not effective (Fig. 6). The effectiveness of ascorbic acid appeared to resemble that of H_2O_2 in that supplementation at 0.1 and 1 mM in concentration greatly enhanced • OH formation of the reaction system (Fig. 7). However, addition of ascorbic acid at 10 mM in concentration on the contrary, greatly inhibited the radical formation as that by 1 mM thiourea supplementation (Figs. 5 & 7).

Discussion

The production of various activated oxygens like O_2^{-1} •, $^1O_2$, H_2O_2, and • OH, was generally believed to be the main factors which contributed to biological damages caused by photodynamic action. (Foote, 1976 & 1981; Hoffmann *et al.*, 1978; Ito, 1983; Spikes *et al.*, 1969). In biological system, oxygen toxicity was so common and devastating that the related mechanismal study have been a very actively researched area for long time in biological as well as medical science (Fridovich, 1977 & 1986; Halliwell *et al.*, 1986; Krinsky, 1979; Marx, 1987). Among the mentioned toxigenic oxygen species, • OH was considered by great majority of workers to be most toxic (Halliwell and Gutteridge, 1984 & 1986). The production of this oxygen radical in biological system was known to be primarily by the iron dependent Haber-Weiss reaction as shown below (Baker and Gebicki, 1986):

$$O_2^{-1} \cdot + H^+ \text{-----} > HO_2 \quad [1]$$

$$HO_2 + O_2^{-1} \cdot + H^+ \text{-----} > H_2O_2 + O_2 \quad [2]$$

$$X\text{-}Fe^{+++} + O_2^{-1} \cdot \text{-----} > X\text{-}Fe^{++} + O_2 \quad [3]$$

$$X\text{-}Fe^{++} + H_2O_2 \text{-----} > X\text{-}Fe^{+++} + \cdot OH + OH^- \quad [4]$$

The method herein applied for detection of • OH was adapted from that of Baker and Gebicki (1984 & 1986). As indicated by these authors, in a low pH condition

like that of MR at 4.0, the method detected mainly the generation of 2-hydroxylated benzoate. Since the detection of 3- or 4-hydroxylated benzoates— the two other likely products resulted from the radical scavenging reaction, was not possible at the given pH condition, calculation of the absolute concentration of ·OH was not attempted. However, in a 53.4 Gy/min Co^{60} gamma ray irradiated 16 mM phosphate buffer solution, it was noted by Baker and Gebicki that presence of sodium benzoate at 2 mM in concentration were in well excess to scavenge all the ·OH formed. The need of 10 mM sodium benzoate to obtain a maximum scavenging effect on ·OH formation in MR suggested that production of this radical in MR was much higher than that in the Co^{60} system of previous authors.

As above indicated (reactions [1] to [4]), provision of sources of O_2^{-1}· and availability of appropriate iron containing compounds were the two critical factors which might assure the continuation of Haber-Weiss reaction. In the presence of available iron, autodismutation of O_2^{-1}· (i.e. reaction [1] & [2]) appeared to be the rate limiting step for the overall process. In the Co^{60} gamma ray irradiated system in which iron was exogenously provided, Baker and Gebicki found that maximum production of ·OH was achieved by lowering down pH of reaction mixture to 4.8—the pKa of reaction [2] (Baker and Gebicki, 1984). The detection of higher yields of ·OH at pH 4.0 and 5.0 than that at pH 6.0, 7.0 and 8.0, 6 hours after light exposure (Fig. 2) seemed to agree with the conclusion of these workers. In MR, iron was not supplemented. Detection of ·OH generation suggested the presence of iron contaminants. It was known from medical research that lowering down pH to 5.6 or lower would lead to the increasing releases of iron from their binding ligands and thus was dangerous to living cells. In MR, should there be iron contaminants, enhanced release of this metal ion with the decrease of solution pH was foreseeable. This might serve to explain why at the early stages of reaction, higher rate of ·OH production was observed at pH 4.0 in stead of pH 5.0; although the later pH was closer to the optimum condition for dismutation of O_2^{-1}· . In the case of iron supplementation, a higher rate of ·OH production than that without iron supplementation was expected. However, this appeared to be true only when pHs of test solution were greater than 6.0 (Fig. 3). At pH 4.0 to 5.0, no stimulatory effect of ·OH formation by iron addition were observed. The stimulatory effect of iron addition to biocidal activity of MR at the low pH range as seen in our previous work (Tzeng, *et al.* 1985) was thus not due to enhancement of ·OH production. In regards to ·OH toxicity, it was emphasized by Halliwell and Gutteridge (1986) that chemistry of ·OH formation is not chemistry of bulk solution, but chemistry at the specific sites where metal catalysts are located. With the provision of exogenous iron, it can be envisioned that certain portion of added iron would bind to negatively charged biomolecules like proteins, phospholipids and nucleic acids; and thus led to the formation of ·OH right on these critical biomolecules. This seemed to also explain why addition of iron at pH 4.0 enhanced biocidal activity of MR although rate of ·OH formation at this pH was not changed.

The participation of iron contaminant in MR was clearly shown in this study by the evidence that addition of both desferal (Fig. 4) and phenanthroline (Fig. 5) —the specific iron chelating agent, greatly reduced ·OH production. In clinical research, desferal was generally applied to patients with iron-overloading resulted illness (Halliwell and Gutteridge, 1986). It was also noted in our previous works that presence of desferal effectively reduced biocidal activity of MR to *Agrobacterium tumefaciens* (Tzeng, 1989). The great reduction of rate of ·OH formation by supplementation of desferal (Fig. 4) further indicated that the protective effect observed was due primarily to the interference of Haber-Weiss process.

Both thiourea and sodium formate were known ·OH scavengers. However, in our previous works, we found that addition of thiourea greatly reduced toxicity of MR; whereas addition of sodium formate, on the contrary, tended to increase the toxicity of MR significantly (Tzeng, 1989). We have suggested the formation of biotoxic sodium formate radicals as the likely reason for this observed contradictory results. The much less efficacy of sodium formate to scavenge ·OH than that by thiourea shown in Fig. 5 further implicated a synergistic effect of ·OH and formate radical in the concerned enhancement of biocidal activity.

In the process of Haber-Weiss reaction, the major roles of O_2^{-1}· were to provide a continuous source of H_2O_2 (reaction [1] & [2]) and to keep iron in its ferrous state (reaction [3]). To the later function, ascorbic acid was also known to be effective either by itself or by the ascorbic acid sensitized photodynamic superoxide gen-

eration (Girotti *et al.*, 1985; Korycka-dahl *et al.*, 1978). The enhancement of • OH production by ascorbic acid (Fig. 6) at 0.1 and 1 mM in concentration appeared to due at least in part to these functions. The reason of the contradictory effect of ascorbic acid at 10 mM in concentration, however, remained to be investigated. In regard to H_2O_2 supplementation, by itself, H_2O_2 at 2940 μM (100 ppm) was toxic to microbial cells. Mello Filho *et al.* (1984) and Starke *et al.* (1985) have reported that toxicity of H_2O_2 to living cells was mainly due to its participation in Haber-Weiss reaction. In our previous work, we have noted that addition of sublethal amount of H_2O_2 significantly increased photodynamic toxicity of MR (Tzeng, 1989). Similary to that shown by the above authors, the enhanced rate of • OH formation by H_2O_2 supplementation (Fig. 7) also indicated the participation of the Haber-Weiss reaction.

Experimental results obtained in this investigation seemed to have provided a firm base to support our previous inference on the direct involvement of • OH in photodynamic biocidal activity of MR. Like that in biological systems (Halliwell and Gutteridge, 1984 & 1986), the formation of this radical in MR was obviously via a O_2^{-1} • and ferrous iron catalyzed Haber-Weiss process. In addition to • OH, our previous work also suggested the possibility of direct contribution of $^1O_2$ formation to the toxicity of MR (Tzeng, 1989). We have learned from Krinsky's work (1979) that $^1O_2$ was also a common product generated in Haber-Weiss reaction. The confirmation of • OH formation via Haber-Weiss reaction thus also supported our previous view on the direct contribution of $^1O_2$ to the studied toxicity of MR.

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甲硫氨酸與核黃素光動效應中之羥基自由基產生：哈伯-韋斯反應參與之作用

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本研究旨在探討甲硫氨酸與核黃素混合物(以下簡稱 MR)光動效應中之羥基自由基(・OH)產生及其與 MR 光動殺生效應之關係。利用 Baker 與 Gebicki 氏等方法，吾人發現於 MR 中添加10mM 左右苯甲酸鈉時，反應系統中・OH 生成作用的螢光性羥基苯甲酸衍生物生成量可達最高，利用此一最適苯甲酸添加濃度進一步測試發現，pH4.0～5.0左右之 MR，其・OH 生成率顯較 pH 6.0～8.0之 MR 要高得多，此顯示 MR 中有哈伯-韋斯(Haber-Weiss)反應過程之參與，且反應液中自由態鐵離子的存在對・OH 之生成作用似極爲重要。鐵離子的添加對 pH6.0以上 MR 中之・OH 生成有明顯的促進作用，添加 desferal 與 phenanthroline 等鐵嵌合劑(cheleting agent)則具相反效果，此似再次強調 MR 中鐵離子存在之重要性。另者，本研究並發現硫脲之存在，可明顯消除 MR 中產生之・OH，甲酸鈉對 MR 中・OH 之消除效果則遠遜於硫脲，而添加適量抗壞血酸及過氧化氫則對 MR 中之・OH 產生有明顯的促進作用，這些証據充分顯示，經由鐵所催化之哈伯-韋斯反應及其所致之・OH 快速產生，對 MR 之強力殺生效應具有決定性之直接關係。