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Hydroxyl radical and singlet oxygen are the key role factors involved in the photodynamic biocidal activity of methionine-riboflavin mixture

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Abstract. The possible involvement of various activated oxygen species in the photodynamic biocidal reaction of methionine-riboflavin mixture (MR) was investigated. Large amount of superoxide free radicals ($O_2^{-1}\cdot$) were detected from MR during the reaction process. At pHs ranged from 4 to 8, the production of $O_2^{-1}\cdot$ increased with the increase of pH. Although as high as 560 μM $O_2^{-1}\cdot$ were detected from test mixture at pH 8 after 2 hours light exposure, the high pH dependence appeared to downplay its direct participation in the biocidal reaction which was known to be greatly favored by low pH condition. Using *Agrobacterium tumefaciens* as a test organism, it was shown that supplementation of superoxide dismutase, ferrous or ferric iron, and sublethal amount of hydrogen peroxide, all enhanced the biocidal activity significantly. On the contrary, addition of desferal, thiourea, and sodium azide, all reduced the biocidal activity. The better stimulatory effect of ferrous iron than that of ferric iron, and the inhibitory effect of desferal on the biocidal activity seemed to depict the critical importance of involvement of iron catalyzed Haber-Weiss reaction and the resulted generation of hydroxyl free radical (·OH). Reduction of the biocidal activity by addition of thiourea, the efficient ·OH scavenger, seemed to also support this view. The similar efficacy of sodium azide, on the other hand, further suggested the contribution from singlet oxygen ($^1O_2$). It is clear that fast killing activity of MR to various microbial cells in an illuminated condition was due primarily the generation of $O_2^{-1}\cdot$, H_2O_2, ·OH, and $^1O_2$. In regard to their individual contribution to the studied biocidal activity, ·OH and $^1O_2$ seemed to be of upmost importance; whereas $O_2^{-1}\cdot$ and H_2O_2 appeared to serve as main reaction intermediates which led to ·OH radical production through the Haber-Weiss process.

Key words: *Agrobacterium tumefaciens;* Hydrogen peroxide; Hydroxyl radical; Methionine-riboflavin mixture; Photodynamic biocidal activity; Singlet oxygen; Superoxide anion.

Introduction

The unusual photodynamic biocidal activity of methionine-riboflavin mixture (MR) was discovered in our previous investigation on the photooxidative generation of ethylene by various phytopathogenic fungi (Tzeng and DeVay 1985). The fast killing activity of MR to various phytopathogens suggested the potential of using it as a chemical agent for plant disease control. One of the main advantages of such application is that both constituents are essential compounds required by most living entities and the field application would involve no risk of the environmental safety. Attempts to control powdery mildews and some

other plant pests by various MR-formulations have been proved to be effective by a series greenhouse as well as field trials (DeVay *et al.*, 1987; Tzeng, 1988).

As regards to the biocidal activity of MR, it was evident that certain toxic photodynamic reaction products were generated. The likely toxigenic photochemical reaction products in a MR reaction system, as revealed by studies in organic chemistry and some other related fields, appeared to be either various activated oxygen species or certain methionine breakdown products (Alarcon, 1976; Foote, 1976; Hemmerich and Wessiak, 1976; Ito, 1983; Sysak *et al.*, 1977; Tzeng *et al.*, 1988; Yang *et al.*, 1967). By high performance liquid chromatography, it was demonstrated that methionine sulfoxide, methional and acrolein were the predominant methionine derived products present in the reaction mixture (Tzeng *et al.*, 1988). The toxicity of the detected methionine derived products, however, was shown to be rather mild as compared to that of MR (Tzeng *et al.*, 1988). The main toxigenic compound generated in MR thus appeared to be various activated oxygen species. Our previous findings on the enhancement of biocidal activity of MR by aeration of the reaction system or by supplementation of certain transition metals or ascorbic acid, all appeared to support this view (Tzeng *et al.*, 1988). The possible involvement of the hydroxyl radical generating Haber-Weiss reaction was suggested based on these observations. As outlined in the following, availability of superoxide free radical ($O_2^{-1}\cdot$) and free ferrous iron (Fe^{2+}) were two critical factors known to assure the continuation of Haber-Weiss reaction (Baker and Gebicki, 1986; Halliwell and Gutteridge, 1984).

$$2O_2^{-1}\cdot + 2H^+ \longrightarrow O_2 + H_2O_2 \quad \cdots\cdots 1.$$

$$O_2^{-1}\cdot + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \quad \cdots\cdots 2.$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + \cdot OH \quad \cdots\cdots 3.$$

To further prove the above stated inference, the role of superoxide free radical and ferrous iron in the photodynamic biocidal reaction of MR were examined. The possible involvement of various oxygen free radicals in the photodynamic biocidal reaction of MR are herein discussed.

Materials and Methods

Biological Materials

Agrobacterium tumefaciens (isolate AT-2) which was obtained from the stock culture collection of the Department of Plant Pathology at the University of California, Davis, USA was used as a test microorganism. The culture was maintained on 523 (Kado and Heskett, 1970) agar slants in the dark at 25℃ and transferred once every week. Cells harvested from 2 days old slant cultures were used for the biocidal activity assay.

Chemicals and Reagents

Riboflavin, L-methionine, nitro blue tetrazolium chloride (NBT, sodium salt), ethylenediaminetetraacetic acid (EDTA, disodium salt), thiourea, sodium formate and sodium azide were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). Superoxide dismutase (from bovine erythrocyte; EC 1.15.1.1) and catalase (from beef liver; EC 1.11.1.6) were obtained from Boehringer Mannheim Chemical Co. (Mannheim, Germany). Dimethyl formamide (DMF) was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ., USA). While desferal (desferrioxamin B methanesulfonate) was a gift from Dr. DeVay of U. C. Davis which was originally obtained from Ciba Geigy. The rest of chemicals used were supplied by Merck Chemical Co. (Darmstadt, Germany). All the chemicals used were reagent grade or equivalent in purity. Freshly made glass double distilled water were used for the preparation or dilution of all the reaction mixtures. In all

tests, MR denoted for a 26.6 μM riboflavin and 1 mM methionine containing solution. Unless specified, pH of MR used for the experiment was adjusted to 4.0 with 0.1 N HCl.

Determination of Superoxide Free Radical

The method used by Korycka-dahl and Richardson (1978) was adapted. To a 1.5 ml MR–*A. tumefaciens* (approximately 1×10^8 CFU/ml) reaction mixture in disposable pyrex test tubes (13×100 mm), NBT was added to make a final concentration of 3×10^{-4} M at the beginning of the light illumination (using two pairs of Philips TLD 36W/33 fluorescent tubes, light intensity was approximately 1 $W\cdot m^{-2}$) to trap the superoxide anions generated. After certain period of light treatment, the reaction mixtures were centrifuged by microcentrifuge (Sigma 202 MC model, rotor No. 12042) at 10 krpm for 5 min. The purplish pellets obtained from the reaction mixtures were then washed once with 1 ml glass double distilled water. The resulted reaction product diformazan contained in the pellet were then extracted with DMF and measured by a spectrophotometer (Beckmen model 25) at 560 nm. For each molecule of diformazan generated, presumably 4 molecules of superoxide anion were consumed (Korycka-dahl and Richardson, 1970).

Assay of the Biocidal Activity

About 19 ml of MR were put in each Wheaton serum bottle (125 ml) and autoclaved. After the test solutions were cooled down to room temperature, 1 ml bacterial suspension (approx. 10^8 to 10^9 CFU as indicated in each individual experiment) was then inoculated to each bottle and then subjected to light treatment (the same light condition as above stated). Initial bacterial concentration was determined by dilution plates. After certain period of light treatment, 200 μl of cell suspension were then withdrawn from each replicate and plated on 523 agar plate for total colony forming units (CFU) to reflect the lethal effect of the test solution. All experiments were repeated at least three times and the data presented were obtained from representative individual experiments.

Results

*Production of Superoxide in a MR–**A. tumefaciens** Reaction System*

For this experiment, pHs of the tested MR solution were maintained at 4.0, 5.0, 6.0, 7.0 and 8.0 by 0.1 M citrate-phosphate (pH 4.0–7.0) and 0.1 M phosphate (pH 8.0) buffer, respectively. The control treatments contained only buffers. In one set of MR mixture, approximately 1 mg of superoxide dismutase (SOD) were included in each replicate as an additional control to assure the observed reaction a superoxide anion ($O_2^{-1}\cdot$) effect. As shown in Table 1, after light exposure, production of $O_2^{-1}\cdot$ increased with the time in MR solution at all pHs tested. At pHs ranged from 4 to 8, the amount of $O_2^{-1}\cdot$ production increased greatly with the increase of solution pH. With the addition of SOD, as expected, the activity of $O_2^{-1}\cdot$ production in these solution was greatly reduced, although certain amount of $O_2^{-1}\cdot$ was still detected from the solution. The best performance of SOD was observed at pH 8. Effectiveness of this enzyme to quench $O_2^{-1}\cdot$ production in MR was greatly affected with the decrease of pH. The production of $O_2^{-1}\cdot$ in all compared control treatments was very low although considerable higher background was observed at high pH range.

Effect of SOD and Catalase on Biocidal Activity of MR

In this experiment, pHs of tested MR were maintained at 4.0 and 7.0 with 0.01 M citrate-phosphate buffer and 0.01 M phosphate buffer

respectively. Both SOD and catalase were prepared with the same buffer and added to MR solution aseptically right before inoculation of test bacteria. Data in Table 2 showed the survived colony forming units of test bacteria after designated time of light exposure. At pH 4.0, both enzymes rendered no help to survival of test bacteria. On the contrary, slight enhancement of lethal effect of MR was observed with the addition of SOD. At pH 7.0, the

Table 1. *Production of superoxide anion in MR-treated cell suspension of* ***Agrobacterium tumefaciens*** *(AT-2 isolate, approx. 10^8 CFU for each test solution) under continuous illumination*

pH of the test solution was maintained at the denoted level with 0.1 M citrate-phosphate (pH 4.0-7.0) and 0.1 M phosphate (pH 8.0) buffer, respectively. SOD (3000 U) was included in one set of the MR-treatment as a special control to indicate the presence of tested free radical. Control (CK) cells were treated with only buffers. Data presented were amount of superoxide anion (μM) produced.

Treatments	pH				
	4.0	5.0	6.0	7.0	8.0
2 h in					
MR	88.8±6.3	142.3±40.4	330.4±27.6	523.2±56.9	560.1±129.1
MR+SOD	47.4±6.2	91.8± 5.9	115.2± 2.5	140.0±22.7	91.1± 35.6
CK	0	0	0	2.0+ 2.8	0
4 h in					
MR	119.0±5.6	288.9± 7.1	488.8± 3.6	685.4±61.2	829.5±121.7
MR+SOD	63.2±6.6	88.1± 8.6	79.7±19.7	48.0±13.4	61.6± 3.4
CK	0	0	0	0	0

Table 2. *Effect of superoxide dismutase (3000 units) and catalase (26000 units) supplementation on photodynamic biocidal activity of MR on* ***Agrobacterium tumefaciens*** *(AT-2 isolate, 2.2×10^9 CFU at 0 h) at pH 4.0 and 7.0, respectively*

pH of tested solution was maintained at 4.0 and 7.0 with 0.01 M citrate-phosphate buffer and 0.01 M phosphate buffer, respectively. The enzyme which had been prepared with the respective buffers was added to the test solution aseptically right before inoculation. The control (CK) treatments were amended with only buffer. Data presented were survived CFU after light treatment.

Treatment	Treatment time (h)					
	1	2	4	8	12	46
pH 4.0						
SOD	$>10^5$	9.6×10^3	0	0	0	0
Catalase	$>10^5$	2.2×10^4	5.0×10^1	0	0	0
CK	$>10^5$	2.3×10^4	4.5×10^2	0	0	0
pH 7.0						
SOD	$>10^5$	$>10^5$	$>10^5$	$>10^5$	3.4×10^3	0
Catalase	$>10^5$	$>10^5$	$>10^5$	$>10^5$	$>10^5$	$>10^5$
CK	$>10^5$	$>10^5$	$>10^5$	$>10^5$	$>10^5$	0

adverse effect of SOD supplementation on bacterial survival seemed still exist. However, the addition of catalase at this pH apparently offered the test bacteria great protection against photodynamic damage of MR.

Effect of Iron and H_2O_2 on Biocidal Activity of MR

Ferric and ferrous chloride, and H_2O_2 were introduced aseptically into MR right before inoculation. To assure the free distribution of iron, approximately 100 ppm of EDTA were included in the test solution. As shown in Table 3, addition of ferrous iron greatly enhanced the biocidal activity of MR to *A. tumefaciens* as compared to that of ferric iron. As for H_2O_2 supplementation, presence H_2O_2 by itself appeared not lethal at 10 ppm in concentration (Table 4). However, supplementation of 0.1 to 1 ppm of H_2O_2 enhanced the biocidal activity of MR greatly as that by ferric or ferrous iron.

Effect of Desferal on Biocidal Activity of MR

In contrast to the effect of iron and H_2O_2, addition of desferal appeared to provide the test bacteria certain degree of protection against the damaging effect of MR (Tables 5 and 6). In MR solution, although cells of the test bacteria were eventually all killed within 24 hours, it was evident that addition of increasing amount of desferal helped the bacterial cells to prolong their life in the test condition (Table 5). With the presence of exogenous

Table 3. *Effect of ferric and ferrous iron supplementation on photodynamic biocidal activity of MR to* ***Agrobacterium tumefaciens*** *(AT-2 isolate, 9.1×10^8 CFU at 0 h) under continuous illunation*

To assure the well distribution of iron, 100 ppm of EDTA were included in all test solution. Data presented were numbers of survived CFU after light treatment.

Treatment	Treatment time (h)			
	1	2	4	8
MR+$FeCl_2$ (1 mM)	4.0×10^2	0	0	0
MR+$FeCl_3$ (1 mM)	1.0×10^4	6.5×10^2	0	0
MR	6.2×10^4	5.5×10^3	4.5×10^2	0

Table 4. *Effect of hydrogen peroxide (H_2O_2) on the photodynamic biocidal activity of MR to* ***Agrobacterium tumefaciens*** *(AT-2 isolate, 2.9×10^9 CFU at 0 h) under continuous illumination.*

H_2O_2 was added to the test solution aseptically by millipore filtration right before inoculation. Data presented were numbers of survived CFU after light treatment.

Treatment	Treatment time (h)			
	1	2	4	8
Without MR				
H_2O_2 0.1 ppm	$>10^5$	$>10^5$	$>10^5$	$>10^5$
H_2O_2 1 ppm	$>10^5$	$>10^5$	$>10^5$	$>10^5$
H_2O_2 10 ppm	$>10^5$	$>10^5$	$>10^5$	$>10^5$
H_2O_2 100 ppm	8.5×10^2	0	0	0
With MR				
H_2O_2 0 ppm	$>10^5$	5.7×10^5	0	0
H_2O_2 0.1 ppm	$>10^5$	5.5×10^3	0	0
H_2O_2 1 ppm	$>10^5$	2.1×10^3	0	0
H_2O_2 10 ppm	3.2×10^4	0	0	0
H_2O_2 100 ppm	0	0	0	0

Table 5. *Effect of desferal supplementation on photodynamic biocidal activity of MR to* ***Agrobacterium tumefaciens*** *(AT-2 isolate, 1.3×10^9CFU at 0 h) under continuous illumination.*

Data presented were numbers of survived CFU after light treatment.

Desferal (mM)	Treatment time (h)				
	1	2	4	8	24
0	$>10^5$	1.4×10^3	0	0	0
1	1.3×10^4	2.5×10^2	1.5×10^2	0	0
5	$>10^5$	2.5×10^2	4.5×10^2	0	0
10	$>10^5$	$>10^5$	5.0×10^2	5.0×10^1	0
20	$>10^5$	$>10^5$	$>10^5$	0	0

Table 6. *Effect of desferal supplementation (20 mM) on photodynamic biocidal activity of 1 mM $FeCl_2$ or $FeCl_3$ amended MR to* ***Agrobacterium tumefaciens*** *(AT-2 isolate, 1.5×10^9 CFU at 0 h) under continuous illumination*

Data presented were numbers of survived CFU after light treatment.

Treatment	Treatment time (h)			
	1	2	4	8
MR+$FeCl_2$	8.6×10^4	5.5×10^2	0	0
MR+$FeCl_2$+desferal	$>10^5$	$>10^5$	$>10^5$	$>10^5$
MR+$FeCl_3$	$>10^5$	7.7×10^4	0	0
MR+$FeCl_3$+desferal	$>10^5$	$>10^5$	$>10^5$	$>10^5$

ferrous or ferric iron, the observed protective role of desferal was even more prominent (Table 6). In the ferrous or ferric iron amended MR solution, most of the bacterial cells were killed within 1 to 2 hours after light exposure. However, with the supplementation of 20 mM desferal, more than 10^5 bacterial cells have survived an eight-hour long light treatment.

Effect of Oxygen Radical Scavengers on Microbial Toxicity of MR

Thiourea, sodium formate and sodium azide each at 1 mM in concentration were added to MR solution before autoclaving. Among them, sodium azide and thiourea were in the order effective in reducing the microbial toxicity of MR (Table 7). Whereas the supplementation of sodium formate, on the contrary, had an apparent stimulatory effect on the micobicidal activity.

Discussion

Oxygen is needed by a great majority of living entities; however, oxygen tends to be very toxic when its amount exceeds ambient level (Fridovich, 1977; Mark, 1987). Toxicity of oxygen to living cells has been an issue of great concern in biological as well as medical fields for many years (Halliwell and Gutteridge, 1986; Hoffmann and Meneghini, 1978; Mello Filho *et al.*, 1984). In biological system, oxygen toxicity was so common and devastating, a lot of efforts have been directed to understand the generation and the mode of action of the toxigenic oxygen species during the past 30 years. Papers of related studies were voluminous. One of the most thoroughly investigated areas in this aspect was the toxigenic oxygens involved in photodynamic damages of living cells or certain biomolecules (Foote, 1981; Ito, 1983; Rabinowitch and Fridovich, 1983; Spikes and Livingston, 1969).

In a photodynamic reaction of methionine with riboflavin as a photosensitizer, it was

Table 7. *Effect of supplementation of oxy-radical scavengers on the photodynamic biocidal activity of MR on* ***Agrobacterium tumefaciens*** *(AT-2 isolate, 1.2×10^9 CFU at 0 h) under continuous illumination*

Data presented were numbers of survived CFU after light treatment.

Supplements	Treatment time (h)				
	1	2	4	6	8
Thiourea (1 mM)	$>10^5$	$>10^5$	$>10^5$	9.7×10^4	3.0×10^3
Na-formate (1 mM)	3.2×10^3	0	0	0	0
Na-azide (1 mM)	$>10^5$	$>10^5$	$>10^5$	$>10^5$	$>10^5$
H_2O (CK)	9.3×10^4	4.5×10^2	0	0	0

known from photochemistry studies that O_2^{-1}• and $^1O_2$ would be the two predominant activated oxygen species resulted from interaction of the light activated triplet photosensitizer and molecular oxygen with methionine as a mediator (Foote, 1976 and 1981). Using nitro blue tetrazolium (NBT) as a trapping agent, Korycka-dahl and Richardson (1976) were able to detect the production of O_2^{-1}• from a riboflavin-methionine containing system under fluorescent light illumination. They also demonstrated that presence of both riboflavin and methionine in milk was the main factor which caused the production of sunlight flavor when milk was exposed to light. As expected, production of O_2^{-1}• was detected from MR-treated cell suspension of *A. tumefaciens* (Table 1). Data obtained in this study seemed to resemble that of previous authors in that production of O_2^{-1}• in the riboflavin initiated photodynamic reaction was greatly favored by the increase of pH. In a milk simulated model system which contained 3.2 μM riboflavin and 2 mM L-methionine, Korycka-dahl and Richardson noted that approximately 97 μM of O_2^{-1}• were generated within one hour under continuous illumination. The rates of O_2^{-1}• production in MR as that shown in Table 1 were evidently much greater than this. Since O_2^{-1}• is a highly reactive oxygen species, production of it in great quantities (e.g., approx. 560 μM within 2 hours at pH 8.0) would lead one to anticipate the participation of this oxygen species in the photodynamic biocidal reaction of MR-mixture. However, this seemed to be contradictory to the low pH dependence of photodynamic biocidal activity of MR-mixture as we have previously reported (Tzeng and DeVay, 1988). The direct involvement of O_2^{-1}• in the concerned biocidal activity thus appeared to be unlikely. This view was further supported by the evidence that supplementation of SOD to the reaction system offered no protection to the test bacteria at either pH 4.0 or 7.0 (Table 2). The slight enhancement of biocidal activity by SOD at both pHs further implicated that production of other activated oxygen species such as H_2O_2 could be of greater importance than that of O_2^{-1}• *per se*. It was known from organic chemistry studies that presence of O_2^{-1}• would lead to generation of H_2O_2, •OH and $^1O_2$—the main activated oxygen species seen in most biological systems (Fridovich, 1986; Halliwell and Gutteridge, 1986; Krinsky, 1979). Among these oxygen species, H_2O_2 was the only stable form which might persist in an aqueous solution for long time. It's biotoxicity was well recognized (Chelala and Margolin, 1983; McCormick *et al.*, 1976; Zika, 1983). And as indicated in our previous paper, in an acidic condition which favors the biocidal activity of MR-mixture, a high rate of O_2^{-1}• autodismutation and production of H_2O_2 would be encountered (Baker and Gebicki, 1984; Tzeng *et al.*, 1988). Involvement of H_2O_2 in biocidal activity of MR-mixture thus appeared to be natural and the protective effect of catalase at pH 7 seemed to also support this view (Table 2). However, the role of H_2O_2 in tested biocidal activity was downplayed by the evidence that supplementation of catalase offered no protection to test bacteria at pH 4.0 which favored the killing activity.

In regard to damages caused by oxygen toxicities, it was generally believed that •OH is the most powerful oxidants among various oxygen radicals. The biological importance of this radical was thoroughly discussed by Halliwell and Gutteridge (1986) in their recent review article. In biological system, •OH radical was known to be generated mainly through the above stated Haber-Weiss reaction. As the reaction was in essence a O_2^{-1}• initiated homolysis of H_2O_2 by ferrous iron, the whole process was also named as a O_2^{-1}• driven Fenton reaction (Baker and Gebicki, 1986). We have suggested in our previous paper the

possible participation of this reaction process based on the evidence that amendment of copper, iron and ascorbic acid all significantly enhanced the biocidal activity of MR mixture (Tzeng *et al.*, 1988). In the proceeded experiment, involvement of Haber-Weiss reaction in the studied biocidal reaction was clearly shown by several lines of evidence. For the formation of ·OH through this reaction process, presence of ferrous iron and H_2O_2 was of critical importance (reaction 3). Better enhancement of the tested biocidal activity by supplementation of ferrous iron than that by ferric iron seemed to fit right into the scheme. In the H_2O_2 supplementation experiment, by itself H_2O_2 below 10 ppm appeared not lethal to the test bacteria. To achieve the same toxicity of MR-mixture, about 10 to 100 ppm of H_2O_2 would be needed. The synergistic effect of H_2O_2 at a sublethal dose seemed to also implicate the importance of reaction 3. In a living cell, it was depicted by Halliwell and Gutteridge (1984) that presence of free iron was essential for the iron dependent ·OH radical generation and the resulted oxygen toxicities. To avoid such kind of disaster, almost all irons entering a living cell were instantly bound to certain biomolecules (Baker and Gebicki, 1986; Halliwell and Gutteridge, 1986). In medical research, desferal is a clinical preparation generally administered to patients with iron-overload syndromes primarily due to its extraordinary ability to chelate ferric iron and scavenge ·OH radical (Halliwell and Gutteridge, 1986). The protective effect of desferal to bacteria in the MR-mixture without the addition of iron salts seemed to suggest the availability of free iron in the test system. The release of iron from its binding biomolecules like ferritin was rather common in an acidic condition (Baker and Gebicki, 1986) which favored the biocidal activity of MR mixture. As in the case of iron supplementation, it was known that irons bound to desferal were very hard to be reduced by O_2^{-1}· (Halliwell and Gutteridge, 1986). Very likely the iron-desferal complex outside the tested bacterial cells would serve as cationic center which attracted most O_2^{-1}· generated and thus prevented the iron catalyzed Haber-Weiss reaction. Should this be the case, it then became understandable why desferal provided the test bacteria better protection in an iron-supplemented MR-mixture than that without iron amendment (Tables 5 and 6).

The major consequence of Haber-Weiss reaction was of course the production of ·OH radical—the most reactive radical species known to date. In the studied biocidal activity, the determinative role of ·OH was clearly indicated by protective effect provided by thiourea supplementation (Table 7). Both thiourea and sodium formate were known to be efficient ·OH radical scavengers. The contradictory effect of sodium formate might be due to its proness of forming sodium formate radical which was known to be toxic to living cells (Halliwell and Gutteridge, 1986). The effectiveness of sodium azide, moreover, provided further evidence for the direct involvement of $^1O_2$. It thus appeared safe to conclude from the present study that photodynamic toxicity of MR to microbial cells was due primarily to the generation of O_2^-·, H_2O_2, ·OH, and $^1O_2$. Among these oxygen species, ·OH and $^1O_2$ appeared to be of upmost importance in regard to the direct contribution to the biocidal activity. Whereas for O_2^{-1}· and H_2O_2, their primary role in the tested low pH condition appeared to be mainly serving as Haber-Weiss intermediates which led to ·OH production.

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羥基自由基與單重氧為甲硫氨酸與核黃素光動殺生效應之主要因子

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本研究之主要目的在探討各種激活態氧與甲硫氨酸-核黃素混合物（以下簡稱 MR）光動殺生效應之關係。於 MR 發生作用過程中反應溶液裏可測到很快有超氧自由基（$O_2^{-1}\cdot$）之形成；在 pH 4～8 範圍內，MR 中 $O_2^{-1}\cdot$ 產生量隨 pH 增加而顯著提高，以 pH 8 爲例，照光 2 小時後，$O_2^{-1}\cdot$ 產生量高達 560 μM，惟由於 MR 之殺生效應係隨 pH 降低而提高，因而 $O_2^{-1}\cdot$ 直接參與殺生作用之可能性似不大。利用冠頭癌腫病菌（*Agrobacterium tumefaciens*）作爲供試菌，本試驗發現超氧物歧化酶（Superoxide dismutase），二價或三價鐵離子以及低於致死量的過氧化氫（H_2O_2）等之添加，均有顯著提高 MR 光動殺生作用之效果；相反的，添加 Desferal，硫脲（Thiourea）以及疊氮化鈉（Sodium azide）等，則均使殺生作用效果大爲降低，其中由所見二價鐵較三價鐵爲優的殺生促進效果，以及 Desferal 對殺生作用之抑制效果，似皆明白顯示，由鐵所催化的 Haber-Weiss 反應及其所導致產生之羥基自由基（·OH），對所探討的殺生作用，具有決定性的影響。另外，添加硫脲所見對殺生作用之抑制效果，亦支持此一觀點，而添加疊氮化鈉之類似效果則進一步顯示單重氧（$^1O_2$）在殺生作用中之重要性。綜合上述結果不難看出，MR 於光照下對多數微生物之致死作用，主要係由於 $O_2^{-1}\cdot$，H_2O_2，·OH 及 $^1O_2$ 等激活態氧種類之產生，這些活性氧中，就生物毒性而言，當以 ·OH 及 $^1O_2$ 爲最，而 $O_2^{-1}\cdot$ 及 H_2O_2 則爲 MR 光反應過程中，導致 ·OH 產生之主要中間產物。