Herbicide Activities in Relation to Oxygenase and Active Oxygens

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Herbicides show many kinds of mode of action and are metabolized in many ways. These biochemical patterns are closely related to the efficacy or mammalian toxicity and also to the selectivity of environmental persistance of herbicides, respectively. In this short review, oxygenase will be discussed as to the metabolisms of monuron, EPTC and bensulfuron methyl, and active oxygens will be reviewed from the standpoint of the mode of action of paraquat and diphenylether herbicides.

Oxygenase and Herbicides

Mixed Function Oxygenase

The oxidation in biological systems are performed by dehydrogenase, oxidase and oxygenase as shown in Table 1.

In the case of both dehydrogenase and oxidase, the mode of action is the transfer of hydrogen atom, and the physiological meaning is mainly the production of energy or constitutional basic metabolisms. Dehydrogenase can transfer hydrogen from substrate to coenzyme (NAD or NADP), and reduced coenzyme will be oxidized through electron transport system, for instance cytochrome c oxidase, by molecular oxygen. Oxidase will pick up hydrogen from substrate and transfer it to oxygen molecule, producing hydrogen peroxide. Some of oxidase, for instance xanthine oxidase, the product is superoxide radical O_2^-, which will be explained later.

Table 1. Oxidation in biological systems

Dehydrogenase	$AH_2+2NAD(P)=A+2NAD(P)H$
Oxidase	$AH_2+O_2=A+H_2O_2$
Oxygenase	
Monooxygenase*	$A+O_2+2NADPH=AO+2NADP+H_2O$
Dioxygenase	$A+O_2=AO_2$

* Mixed-function oxidase

Oxygenase, the present target, is and enzyme system which oxidize substrate introducing the atom(s) of molecular oxygen O_2 into substrate. Oxygenase is classified into mono-oxygenase and dioxygenase. The latter can introduce two atoms of oxygen into the substrate molecule, for instance tryptophan oxygenase. On the other hand, the former can transfer only one atom of oxygen into the substrate, and is also called mixed function oxygenase (MFO).

MFO is playing an improtant role in the metabolism, activation or inactivation of xenobiotics, for instance drugs, insecticides or herbicides. Figure 1 shows the participation of MFO in the metabolism of insecticides.

As shown in Table 2, MFO can be classified into three types ; microsomal, mitochondria, and bacteria types. However in higher plants there is almost no information about this subject.

In MFO system, the enzyme reaction proceeds with consumption of molecular oxygen, and one atom of oxygen is used for hydroxylation etc., of the substrate and the other for oxidation of NADH or NADPH. Then although this reaction is an oxidizing process, reduced form of the coenzyme is required. The oxidizing activity of this enzyme system is mediated by a special cytochrome, P-450, and in animal system it will

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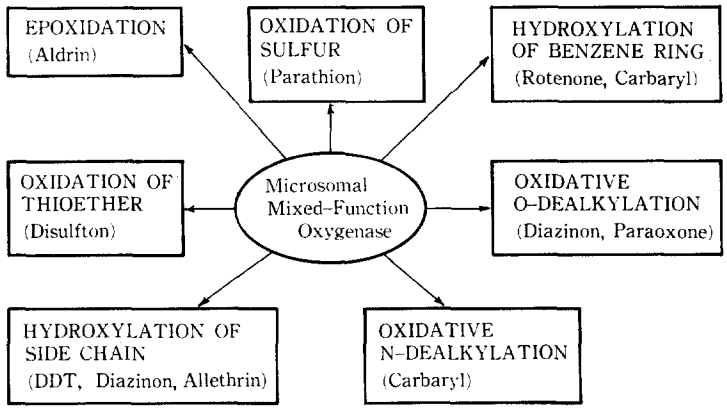

Fig. 1. Metabolism of insecticides by microsomal mixed-function oxygenase

Table 2. Mixed-function oxygenase systems

Microsome Type	
NADPH→NADPH-P450⟶P450	RH+O_2 / ROH+H_2O
Mitochondria Type	
NADPH→NADPH-Ferredoxin→Ferredoxin⟶P450 (Reductase)	RH+O_2 / ROH+H_2O
Bacteria Type	
NADH→NADH-Ferredoxin→Ferredoxin⟶P450 (Reductase)	RH+O_2 / ROH+H_2O

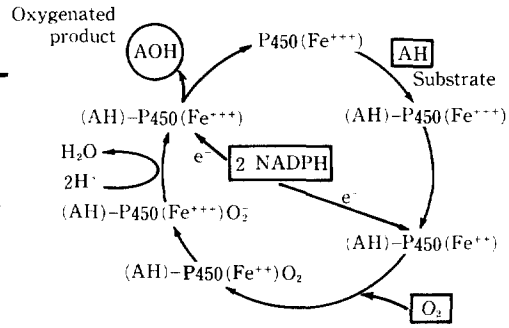

Fig. 2. Monooxygenase reaction of P450

be specifically inhibited by piperonyl butoxide or SKF 525A.

Cytochrome P-450

Here cytochrome P-450 should be explained. In 1964 Ohmura and Sato[1] found a curious carbon monooxide difference spectrum in a suspension of lever microsome. The absorption curve shows a distinguished peak at 450 nm. Then this special component is called cytochrome P-450, or simply P-450.

P-450 content in the organ will be increased after application of drugs or chemicals, and there found many kinds of P-450 molecular species.

The most important role of P-450 in cells is the contribution in the oxidation of substrates as a component of MFO. A part of the mechanism is not clear, but it may be illustrated as shown in Figure 2. The substrate AH will be combined with P-450 in ferric state. After oxygen binding, AH will be changed to AOH, oxidized product. One atom of molecular oxygen was introduced directly to this product AOH.

Contributions of MFO in Herbicide Metabolisms

Old story of MFO-related reaction in herbicides was reported in monuron metabolism by Frear and others[2] in 1969 as shown in Figure 3. In cotton plants, *N*-dealkylation reaction was catalyzed by an MFO system that required molecular oxygen and reduced pyridine nucleotides, NADH or NADPH, as cofactors. When monuron was the substrate, the reaction products were monomethyl monuron and formaldehyde. An unstable *N*-hydroxymethyl intermediate was detected as a reaction product when monomethyl monuron was the substrate. This intermediate was not isolated, but it was degraded stoichiometrically to *p*-chlorophenylurea and formaldehyde. The *N*

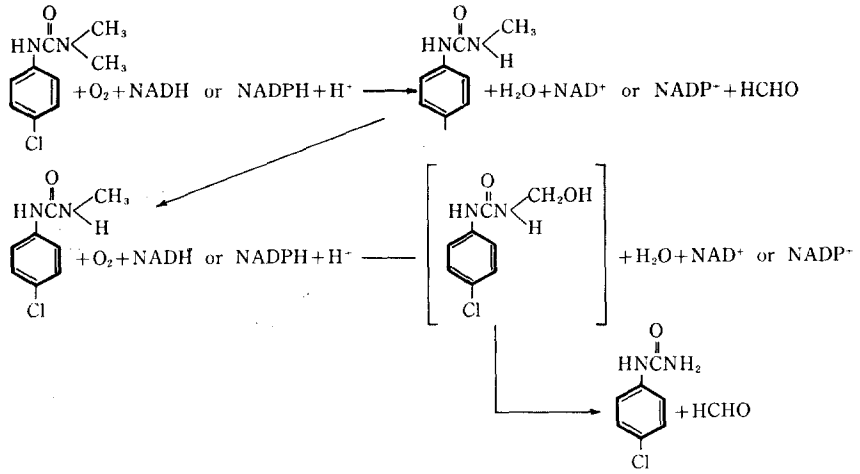

Fig. 3. Mechanism of monuron *N*-demethylation by microsomal MFO from cotton (Frear *et al*, 1969)

-demethylation system had limited substrate specificity for dimethyl and monomethyl phenylurea herbicides and was strongly inhibited by carbamate insecticides, but not by piperonyl butoxide or SKF 525A, the typical MFO inhibitors in animal system.

A thiocarbamate herbicide, EPTC, shows its herbicidal activity after the oxidation of the herbicide molecule to sulfoxide[3]. The oxidation of thiocarbamates in plants could occur either by MFO, as it does in mammals, or by peroxidase. The sulfoxide seems to be the active form of the herbicide.

The oxidation process will be done as shown in Figure 4, and produced sulfoxide may be conjugated with glutathione by glutathione-*S*-transferase into inactive one, then the oxidation process by MFO seems to be activation and the beginning of detoxification.

In the case of thiobencarb(benthiocarb), one of thiocarbamates, sulfoxide was not detected in rice plant metabolism.[4]

Recent example of oxidation of herbicide by MFO is the case of bensulfuron in rice plant, a tolerant plant to this herbicide. Takeda and others[5] showed that the reaction will proceeds as shown in Figure 5. At first the methoxy carbon on hetero-ring will be hydroxylated and, after dealkylation,

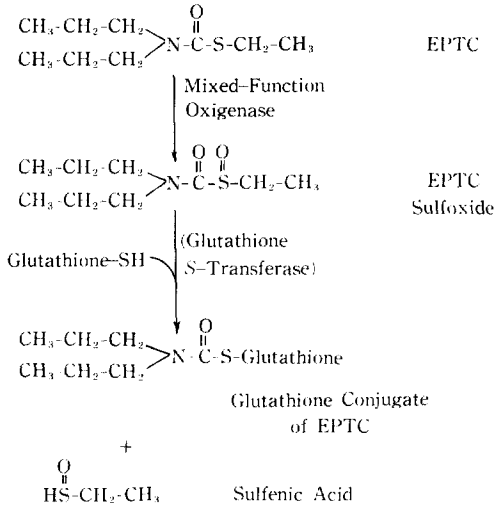

Fig. 4. Metabolism of EPTC to glutathione conjugate (Fuerst, 1987)

hydroxylated ring were produced. This process is really detoxification one in rice plants, causing high selectivity of this herbicide in rice culture.

This detoxification process is accelerated by some kind of thiocarbamate herbicides as like as thiobencarb, dimepiperate (MY-93), and isopropylate (CH-83), and actually these thiocarbamates can be the safener for rice plants under the over dosage application of bensulfuron methyl. In Table 3, the additions of thiobencarb or dimepiperate were found to decrease the half life

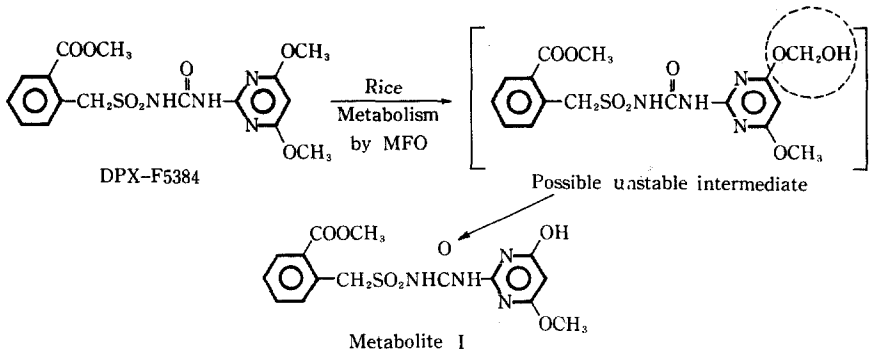

Fig. 5. Detoxification of bensulfuron by rice plants(Takeda *et al.*, 1986).

Table 3. Effect of thiocarbamate herbicides on the metabolism of bensulfuron by excided rice leaves (Yuyama *et al.*, 1986)

Foliar spray*	Half-life of bensulfuron methyl*** in excised leaves (hours)
Solvent control**	8.4
Thiobencarb(2,000g a.i./ha)	1.9
MY-93(2,000g a.i./ha)	2.6
Molinate(2,000g a.i./ha)	7.9

* Foliar spray of rice plants was made 24 hours prior to excised leaf uptake of bensulfuron methyl.

** Solvent : 20% acetone plus 0.1% "Tween 20"

*** Bensulfuron methyl was taken up through cut ends of excised leaves (one hour uptake from 2 ppm solution of the herbicide).

of bensulfuron to one quater, means acceleration of detoxification.[6]

These thiocarbamates can contribute not only to intensify the herbicidal activity of bensulfuron broading the weed spectrum which can be controlled, but also decreasing the phytotoxocoty of the main herbicide to rice plants by this safening process. Weeds have not such detoxification system for the herbicide, then almost no adverse effect is observed by the addition of such thiocarbamates. Although molinate is one of thiocarbamate herbicides, it can not accelerate the detoxification of bensulfuron and does not show the safening effect on rice plants.

As to the mixed function oxygenase (MFO), we can understand the importance in herbicide metabolisms. However fundamental works on MFO in higher plants are still now insufficient. These fields should be clarified by younger weed scientists.

Active Oxygens and Herbicides

Active Oxygens

In general, the presence of green, oxygen and sunlight is assumed very healthy condition. However the combination is always facing to very dangerous reactions, producing active oxygens. Higher plants have evolved special mechanisms to cope with the active oxygens, and usually are kept to be a balanced status to make them safe by many enzyme systems. However, at once the balance may be broken for instance by the application of paraquat as described below, abnormal phenomenon will occur in the treated plants.

As shown on the left side of Figure 6, four electrons are required for the reduction of one molecule of oxygen to water. Molecular oxygen, which is called triplet oxygen, is the most oxidized but stable form owing to the biradical electron arrangement.

In the process of four electron reduction, the first one produces the superoxide radical (O_2^-) which is a product of univalent reduction of O_2. Next, by one more electron reduction, hydrogen peroxide (H_2O_2) is formed. H_2O_2 is a product of two electron reduction from O_2.

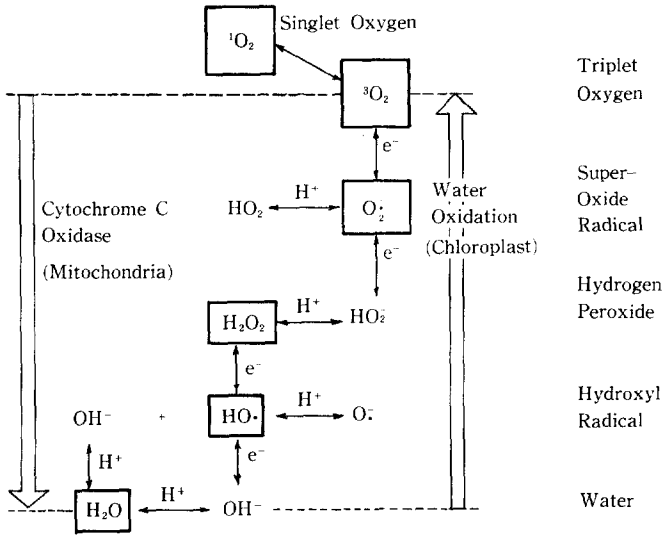

Fig. 6. Mutual relationships among oxygens and their reduced products.

Three electron reduction of O_2 produces hydroxyl radical ($OH\cdot$). These reduced molecules of oxygen may be protonized or deprotonized depending upon the surrounding pH. Usually at the neutral pH, they will be O_2^-, H_2O_2 and $OH\cdot$, as shown by the retangles in Figure 6.

Although these three reduced molecules of oxygen are at somewhat lower oxidized level than molecular oxygen itself, their oxidizing power is very high and they have high activity to biological targets.

Along with these three, usual triplet oxygen, expressed as $^3O_2$, is excited to the singlet oxygen ($^1O_2$) which also shows high activity in biological reactions. Singlet oxygen is formed for instance by the photodynamic action as shown in the mode of action of diphenylether herbicides later. Singlet oxygen is also produced by the oxidation of superoxide, hydrogen peroxide or lipid peroxide which will be explained next.

These oxygen molecules, O_2^-, H_2O_2, $OH\cdot$ and singlet oxygen at highly reactive state are called active oxygens. During the oxidation process of unsaturated lipids by singlet oxygen, lipoxigenase or autooxidation, hydroperoxide (ROOH) is produced. ROOH is also assumed as one of active oxygens.

Almost all electron transfers in the respiration of aerobic organisms are mediated by chytochrome oxidase in mitochondria and no intermediate formation of active oxygens is observed in this four electron reduction from $^3O_2$ to H_2O. Also in the process of photosynthesis shown on the right side of Figure 6, the oxidation of H_2O to O_2 does not produce appearently any active oxygen. Both processes are very fine and elegant ones which can avoid the dangerous intermediary production of active oxygens.

Although aerobic organisms have obtained these safe biochemical processes during their evolutionary history, some kind of active oxygen may be produced by biochemical or physical reactions. Active oxygens except H_2O_2 produced in cells,

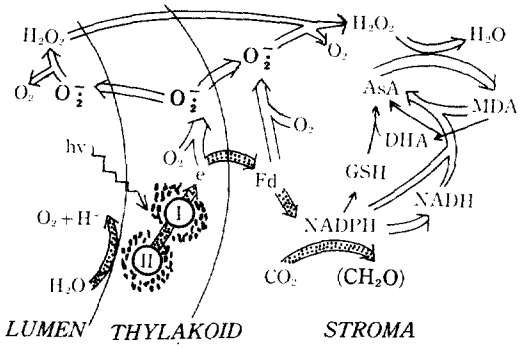

Fig. 7. Production and scavenging of superoxide and hydrogen peroxide in chloroplasts (Hossain, Nakano & Asada, 1984)

have a very short life, and normally are converted to the other molecular structures which are inactive to cells. The mediator of the conversion are called scavengers or quenchers of active oxygens.

Figure 7 shows the production and scavenging of superoxide and hydrogen peroxide in chloroplasts under illuminated condition.[7]

Paraquat and Active Oxygen

The left half of Figure 8 is the electron transport system in photosynthesis. In this process the solar energy will be changed into both biochemical energy ATP and reducing power NADPH. The process consists of photosystem I and II. After the first excitation process of electron by sunlight, the transport can be inhibited by many herbicides belong to Hill reaction inhibitors. After the second excitation, the electron will be accepted by P430, different one from above-described P-450.

A famous herbicide, paraquat can cross the chloroplast envelope and accepts one electron probably from P430 in photosystem I in this figure. Then it will be reduced to its free radical by the one electron. Usual $^3O_2$ at ground state has low activities in chemical reactions, but against free radicals it shows high reactivity. Then the paraquat free radical react with $^3O_2$ very rapidly and reoxidized to the original paraquat molecule. In this process at the same time univalent reduction of $^3O_2$ occurs, and the $^3O_2$ molecule will be changed to superoxide radical, which plays the herbicidal role of paraquat.

Paraquat is inactive in darkness, in coexistence with the Hill reaction inhibitors and the absence of O_2. These phenomena are easily understood by this mode of action of paraquat.

The usual concentration of superoxide radical in chloroplasts is assumed at 6.0×10^{-9}M, but in the presence of paraquat it increases 10 to 20 times, about 6 to 12×10^{-8}M. Thus chloroplasts can tolerate up to 10^{-9}M superoxide radical, but will be damaged by 10^{-8} to 10^{-7}M. Even without paraquat, the process of photosynthetic electron transport system may produce a small amount of superoxide radical at 6×10^{-9}M as described just before, in chloroplasts under illuminated condition, but the active oxygen can be scavenged by superoxide dismutase (SOD), ascorbate peroxidase and glutathione reductase. Produced superoxide will also inhibit these scavenging systems.

Highly larger production of superoxide by paraquat application can not be compensated by these detoxification systems, and the lipid peroxidation and disruption of chloroplast membranes will occur, and the cells are damaged. Recently

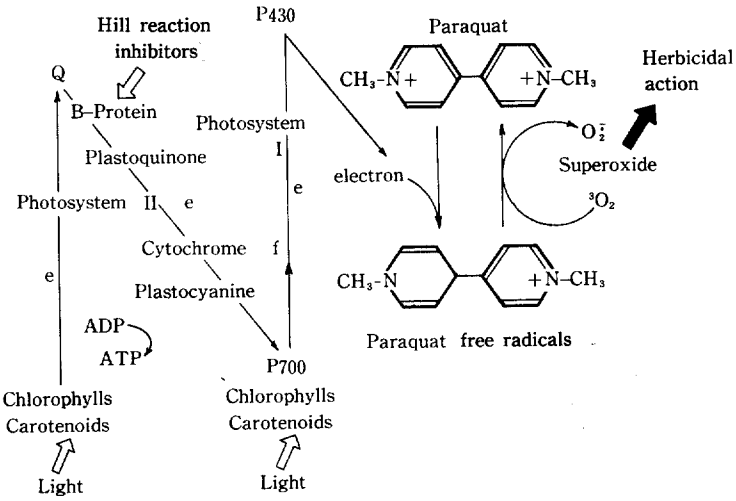

Fig. 8. Photosynthetic electron transfer and mode of action of paraquat

the appearance of paraquat tolerant weeds are reported in several places, and some of the tolerant mechanisms is explained by the increase of the scavenging system, superoxide dismutase only or a combination of it with ascorbate peroxidase and glutathione reductase in tolerant plants.

Diphenyl Ether Herbicides and Active Oxygen

Diphenyl ether herbicides have been contributing in the practical weeding in the farms these twenty years. In 1969, the author reported that diphenyl ether herbicides required for light in their herbicidal action, and normal green plants and yellow mutants are more susceptible than the white mutants[8]. After then many researchers have been studied the mode of action and the kinds of target herbicides were expanded to oxadiazone[9] or phthalimides[10,11] and others.

Very recently Matringe and Scalla[12] reported that "The phytotoxicity of diphenyl ether herbicides should be explained by their ability to cause accumulations of tetrapyrroles, mainly protoporphyrin IX, Which in turn induce lethal photooxidative reactions producing singlet oxygen".

Also very recently Haworth and Hess[13] reported that a diphenyl ether herbicide, oxyfluorfen, caused a light induced consumption of oxygen which resembled the electron acceptor reaction of paraquat. However, this reaction is not linked to the electron transport system through photosystem I. And using the bleaching of *N*, *N*-dimethyl *p*-nitrosoaniline (RNO) as a specific detector of singlet oxygen, they demonstrated that oxyfluorfen is a potent generator of this toxic compound. The production of singlet oxygen occurs in the presence of inhibitors of photoxynthetic electron transport and also under low temperature condition (3℃) which prevent the electron transport. This light induced reaction results in oxygen consumption and is the primary action of oxyfluorfen.

Also in my laboratory, following results were obtained[14]. Homogenate of nonchlorophyllous

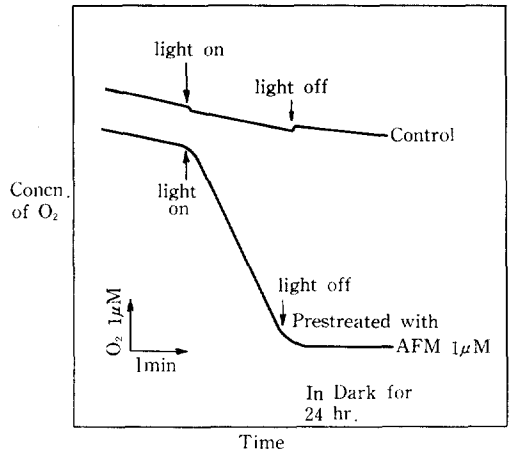

Fig. 9. Effects of AFM on light-dependent O_2 comsumption in tobacco cell homogenate (Kouji, Masuda & Matsunaka, 1988)

tobacco cells treated with the diphenyl ether herbicides, mainly acifluorfen methyl (AFM) was used, still possesed high light-dependent oxygen consumption activities shown in Figure 9. This activity had been induced in the dark incubation with AFM. Another experiments showed that the lipid peroxide content was increased in the homogenate during such illumination after the dark incubation with AFM. Also these oxygen consumption of the treated cells was affected by the time length of dark incubation.

Table 4 shows that the light dependent oxygen consumption activity was closely related to both cell growth inhibition and herbicidal activities. Further more the increase of light-dependent oxygen consumption was also observed in the homegenate of velvet-leaf leaves.

These results suggest that either an enzyme or a substance which catalyze the photooxidation of membrane lipids was accumulated in the plant cells by the treatments with diphenyl ether herbicides in the dark.

Further experiments showed us that the light-dependent oxygen consumption acivity was not altered with heat treatment or pH change. Therefore, it was assumed that the oxygen consumption activity was caused not by an enzyme but also by a heat-stable substance located in the membrane. The activity could be

Table 4. Light dependent O_2 consumption and herbicidal activities

Diphenyl ethers	(μ M)	O_2 consumption	Growth inhibition* (pI_{50})	Herbicidal activity** (pI_{50})
Control	-	0.24	-	-
Acifluorfen-Methyl	1	6.49	7.38	7.00
Oxyfluorfen	1	6.33	6.64	-
Nitrofluorfen	10	3.62	5.83	5.67
Nitrofluorfen 2-Nitro isomer	10	<0.10	<4.00	<4.00

* Non-chlorophyllous tobacco cell. ** Tobacco seedling (Kouji, Masuda & Matsunaka, 1988)

transferred to an organic solvent layer by extraction. As a result of analysis of the organic layer by TLC, HPLC and fluorescence spectrophotometry, here also protoporphyrin IX was identified as the active substance. Its structure is shown in Figure 10. This compound itself with coexistence of lecithin showed O_2 consumption only under illuminated condition.

Our experiments of the effective tetrapyrrole biosynthetic pathway showed that diphenyl ether herbicides did not inhibit ferrochelatase activity but stimulated 5-aminolevulinic acid (ALA) synthesizing system.

These results suggests that the diphenyl ether herbicides stimulate ALA synthesizing enzymes and cause the accumulation of tetrapyrroles in plant cells, and that tetrapyrrole-sensitized photooxidation produce singlet oxygen to make the membrane disruption.

Active Oxygens and Development of New Herbicides

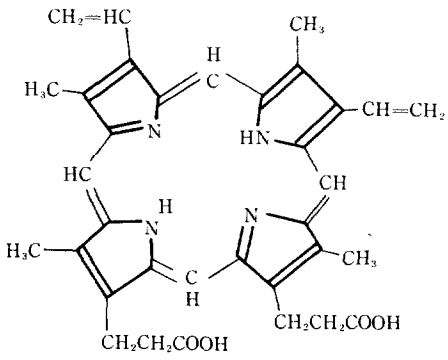

Fig. 10. Protoporphyrin IX

In the previous paper[15], "Active Oxygens and Development of New Herbicides" presented at the 7th Conference of Asian-Pacific Weed Science Society in 1979, the author proposed how to utilize the active oxygens for the development of new herbicides. In 1984, Hallwell[16] reviewed about "Toxic Oxygen Species and Herbicide Action", and proposed almost the same considerations.

Then, finally, in order to find out new herbicide candidates, we can propose to seek the chemicals which can increase active oxygens in weeds, can inhibit the scavenging activity in weeds, and can decrease scavenger content in weeds.

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