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Original Article

Effect of Organophosphorus Insecticides on Hill Reaction

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Inhibition of Hill reaction by organophosphorus insecticides was examined to clarify their mechanism of phytotoxic action on Chinese cabbage leaves. Among organophosphates tested, those which caused no phytotoxic symptom did not affect Hill reaction at concentrations below 10^{-4} M. Degree of inhibition of Hill reaction varied dependent on the organophosphates in the case of those causing necrotic symptoms on Chinese cabbage leaves. Dimethoate did not affect Hill reaction, while fenthion and fenitrothion inhibited reaction by 50% at a concentration of 10^{-5} M. Organophosphates causing chlorotic symptoms were strong inhibitors and suppressed the reaction at 10^{-5}–10^{-6} M. All the inhibitors were hydrophobic. Even in the case of a series of compounds belonging to straight-chain alcohols, fatty acids, and polychlorobenzenes, the increase in their molecular weight and concomitant decrease in water solubility apparently were in parallel with their ability to increase inhibitory activity. Thus, hydrophobic compounds are considered to be inhibitory for Hill reaction. These compounds may have high affinity to chloroplasts resulting in inhibition of the electron transport in them and causing decomposition of chlorophyll.

INTRODUCTION

Organophosphates, one of the major insecticide groups, comprise about forty compounds and account for 20% of all the pesticides used in Japan. Seedlings of Chinese cabbage applied with organophosphorus insecticides easily show phytotoxic symptoms under the conditions of pot tests.[1] Changes in the content of carbohydrates and nitrogen in the treated plant are related to the appearance of phytotoxic symptoms.[2,3] Decrease of carbohydrates and the relative increase of total nitrogen seemed to depend on the suppression of photosynthesis by the inhibition of light reaction in photosynthesis or by the decrease of photosynthetic sites resulting from chlorosis. Most of the known photosynthetic herbicides are inhibitors of light reaction, especially of photosynthetic electron flow at photosystem II.

In order to ascertain whether the suppression of photosynthesis was caused by the inhibition of electron transport reaction by organophosphates, effect of these insecticides on Hill reaction was examined on chloroplast prepared from spinach leaves. Since organophosphates of low water solubility inhibited Hill reaction more strongly than water soluble ones, a series of homologous compounds of various water solubility other than organophosphate were examined to clarify a possible relationship between water solubility of chemicals and degree of inhibition of Hill reaction. The results are discussed in relation to the hydrophobicity of the compounds.

MATERIALS AND METHODS

1. Chemicals

Chemicals used as inhibitors of Hill reaction were pure organophosphorus insecticides, normal acids from capric (C_{10}) to palmitic (C_{16}), normal alcohols from octyl (C_8) to myristyl (C_{14}), and mono-, di- and trichlorobenzenes. Organophosphates were supplied by manufac-

turing companies concerned and other chemicals were purchased from Tokyo Kasei Chemicals Co., Ltd. These chemicals were extra pure and dissolved in acetone.

2. Preparation of Chloroplast

Spinach leaves without midrib were cut and homogenized in a Waring blender with 5 parts of a chilled medium containing 50 mM phosphate buffer (pH 7.5), 10 mM NaCl and 0.4 M sucrose. The homogenate was filtered through six layers of cheesecloth. The filtrate was centrifuged at 2,000 *g* for 5 min and the precipitate suspended in 10 mM of NaCl solution. After 20 min the suspension was centrifuged at 500 *g* for 5 min, and the supernatant was centrifuged at 2,000 *g* for 5 min. The precipitate was suspended again in 10 mM NaCl solution and this suspension was used in the test of Hill reaction.

3. Estimation of Hill Reaction

Degree of decoloration of blue 2,6-dichlorophenol indophenol (DCPIP), a Hill oxidant, was measured by a Shimadzu double beam photometer type UV-210. Optical density (600 nm) was measured before and after the illumination of 40,000 lux for 30 sec on 3 ml reaction mixture which consists of 50 mM phosphate buffer (pH 7.5, containing 10 mM NaCl and 10 mM methylamine·HCl), 10 μM DCPIP, 0.1 ml of acetone solution of an organophosphorus insecticide, and 0.1 ml of chloroplast suspension (containing 5–10 μg chlorophyll). Difference of optical density before and after the illumination was considered to be the activity of Hill reaction. Inhibitory activity of the added insecticide was compared to the control value of acetone. Inhibitory activities on the Hill reaction of 1×10^{-7} M diuron were 40–45% of control under these conditions.

RESULTS

1. Degree of Inhibition on Hill Reaction

Figures 1 and 2 show inhibition value of the insecticides on Hill reaction with concentrations from 10^{-4} M to 10^{-6} M. Acephate, dichlorvos (DDVP), dimethoate, trichlorfon (DEP) and vamidothion did not affect the Hill reaction. About 10% inhibition was noticed with 10^{-4} M of methidathion (DMTP), salithion, chlorfenvinphos (CVP), mecarbam and malathion. The activity was inhibited with 10^{-5} M of diazinon, fenitrothion (MEP), fenthion (MPP) and phenthoate (PAP), and with 10^{-6} M of isoxathion, EPN, chlorpyriphos-

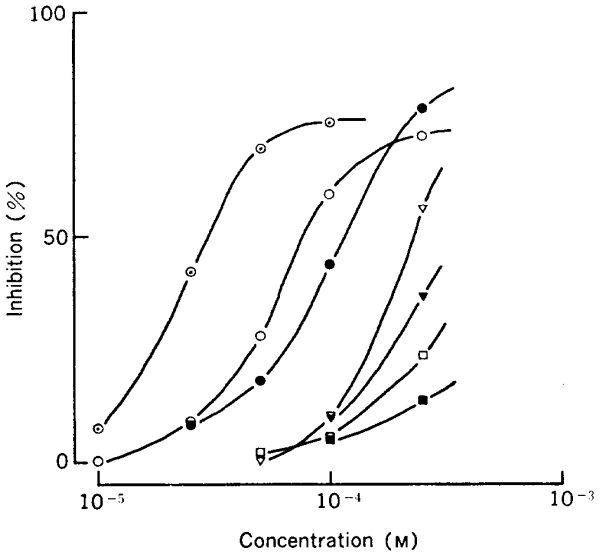

Fig. 1 Inhibition of Hill reaction by organophosphates causing necrotic symptom on Chinese cabbage.
⊙: fenthion, ○: fenitrothion, ●: diazinon, ▽: chlorfenvinphos, ▼: mecarbam, □: salithion, ■: methidathion.

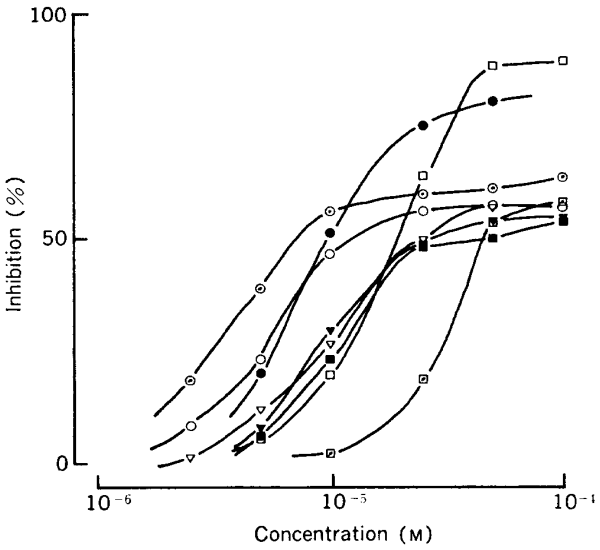

Fig. 2 Inhibition of Hill reaction by organophosphates causing chlorotic symptom on Chinese cabbage.
⊙: EPN, ○: dialifor, ●: chlorpyriphos, ▽: phosalone, ▼: isoxathion, □: chlorpyriphos-methyl, ■: cyanofenphos, ▣: phenthoate.

Table 1 Phytotoxic symptoms on Chinese cabbage and inhibition of Hill reaction by organophosphates.

Organophosphate	Molar concentration for 20% inhibition (I_{20}) of Hill reaction	Symptoms of Chinese cabbage
Acephate	$>10^{-4}$	Normal
Dichlorvos	$>10^{-4}$	
Trichlorfon	$>10^{-4}$	
Vamidothion	$>10^{-4}$	
Dimethoate	$>10^{-4}$	Leaf necrosis
Methidathion	3.2×10^{-4}	
Diazinon	5.5×10^{-5}	
Salithion	2.2×10^{-4}	Necrosis on young leaves
Chlorfenvinphos	1.3×10^{-4}	Leaf burn
Mecarbam	1.5×10^{-4}	
Fenitrothion	3.9×10^{-5}	
Fenthion	1.6×10^{-5}	
Malathion	3.7×10^{-4}	Chlorosis on young leaves
Isoxathion	7.4×10^{-6}	Chlorosis along leaf vein
EPN	2.6×10^{-6}	
Phenthoate	2.6×10^{-5}	Chlorosis
Chlorpyriphos-methyl	1.0×10^{-5}	
Cyanofenphos	8.7×10^{-6}	
Phosalone	7.3×10^{-6}	
Chlorpyriphos	5.0×10^{-6}	
Dialifor	4.4×10^{-6}	

methyl, cyanofenphos (CYP), phosalone, chlorpyriphos and dialifor. In the case of strong inhibitors, except chlorpyriphos and chlorpyriphos-methyl, the inhibitory activity reached an equilibrium at about the 50% level (Fig. 2).

2. Relations between Inhibition of Hill Reaction and Phytotoxicity

Table 1 lists phytotoxic symptoms on Chinese cabbage leaves and degree of inhibition of Hill reaction with organophosphates. The insecticides without any phytotoxic symptoms did not affect Hill reaction at concentrations below 10^{-4} M. Fenthion and fenitrothion, which caused severe necrotic symptoms on Chinese cabbage leaves, strongly inhibited the reaction. Other organophosphates causing mild necrotic symptoms weakly inhibited it. Mecarbam weakly inhibited the reaction despite severe necrosis but dimethoate which caused marginal necrosis did not inhibit at all. Almost all the insecticides tested which caused chlorotic symptoms strongly inhibited Hill reaction. Inhibition occurred at a concentration of 10^{-6} M. Malathion caused mild chlorosis on young leaves alone and scarcely inhibited.

3. Relation between Water Solubility and Inhibition of Hill Reaction

EPN, phosalone, dialifor etc., which strongly inhibited Hill reaction and caused chlorosis on Chinese cabbage, are hardly soluble in water while insecticides such as acephate, dichlorvos, dimethoate, trichlorfon and vamidothion, which did not inhibit, are highly soluble in water. Therefore, homologous compounds of various water solubility such as normal acids, normal alcohols, and chlorinated benzenes were tested to determine the relation between the water solubilities and degree of inhibition of Hill reaction. The results are shown in Figs. 3, 4 and 5. Degree of the inhibition with normal acids increased with length of the carbon chain. The inhibitory activities appeared at a lower concentration of alcohols with longer chain length. However, the inhibition value reached a maximum; degree of the inhibition

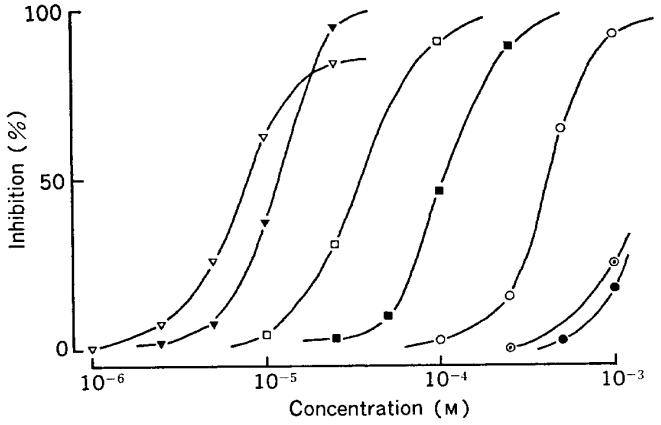

Fig. 3 Inhibition of Hill reaction by normal fatty acids in isolated chloroplasts.
●: C_{10}, ⊙: C_{11}, ○: C_{12}, ■: C_{13}, □: C_{14}, ▼: C_{15}, ▽: C_{16}.

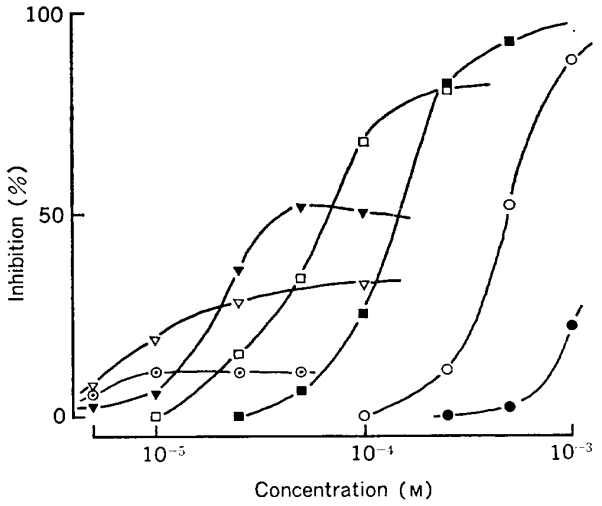

Fig. 4 Inhibition of Hill reaction by normal alcohols in isolated chloroplasts.
●: C_8, ○: C_9, ■: C_{10}, □: C_{11}, ▼: C_{12}, ▽: C_{13}, ⊙: C_{14}.

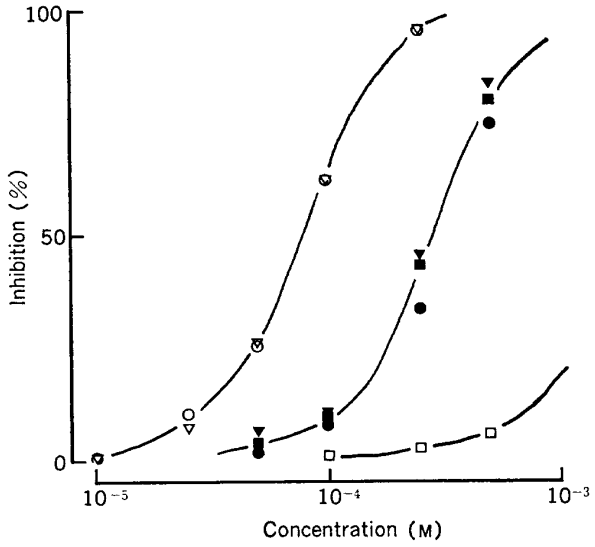

Fig. 5 Inhibition of Hill reaction by chlorinated benzenes in isolated chloroplasts.
□: monochloro-, ▼: 1,2-dichloro-, ■: 1,3-dichloro-, ●: 1,4-dichloro-, ○: 1,2,4-trichloro-, ▽: 1,3,5-trichloro-.

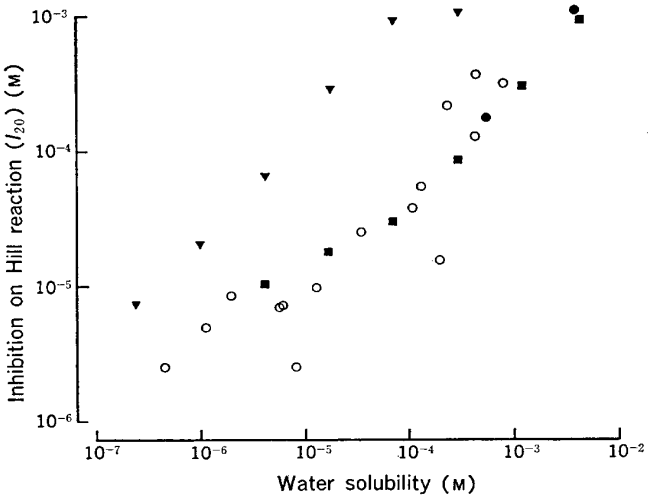

Fig. 6 Water solubilities of various compounds and their molar concentrations for 20% inhibition.
○: organophosphate, ▼: normal acid, ■: normal alcohol, ●: chlorinated benzene.

did not exceed a definite value in the case of alcohols with eleven or more carbon atoms. The maximum inhibition values were about 85, 55, 30, and 10% for the alcohols of C_{11}, C_{12}, C_{13}, and C_{14}, respectively. Of chlorinated benzenes, the highest inhibition was observed on trichlorobenzenes followed by dichloro-compounds. Degree of the inhibition was almost the same among respective isomers of dichloro- or trichlorobenzenes. In every case, the concentration to inhibit Hill reaction was lower with higher molecular weight and lower water solubility. Namely, chemicals hardly soluble in water strongly inhibited Hill reaction.

The concentrations which reduced reaction activity to 80%, that is, a concentration of 20% inhibition (I_{20}), may be seen in Figs. 1–5. In Fig. 6, the toxic concentrations (I_{20}) of the compounds were plotted against their water solubilities.[4–9] The water solubilities of normal acids and alcohols were calculated by the method of Pierotti *et al.*[10] Concentrations of

I_{20} were highly correlated with the water solubility of compounds. Inhibition by acids was weaker than that by other compounds of comparable water solubility.

DISCUSSION

Herbicides of photosynthetic inhibitor inhibit Hill reaction at concentrations of 10^{-5}–10^{-7} M. For example, concentrations of 50% inhibition are 10^{-7} M for diuron[11] and 10^{-5} M for bentazon,[12] while in this study organophosphorus insecticides causing chlorotic symptoms on Chinese cabbage leaves inhibited Hill reaction to 50% at about 10^{-5} M, about 100 times higher than the concentration of diuron and almost the same as that of bentazon. The concentration of leptophos, an organophosphate, in Chinese cabbage leaves applied at a solution of 1,000 ppm, was about 80 ppm one day after the application and about 100 ppm two days afterward.[13] Since the molecular weight of leptophos is 412, 100 ppm of leptophos corresponds to 2.4×10^{-4} M. If other organophosphates should persist in a crop for the same period as leptophos and uniformly distribute in Chinese cabbage leaves, the organophosphates in the leaves would exceed the concentration of 50% inhibition of Hill reaction for several days. Therefore, the inhibitory concentration of many organophosphates on Hill reaction, 10^{-4}–10^{-5} M, suggests the possibility that these insecticides applied on leaves inhibit the electron transport reaction on chloroplast.

Inhibition of Hill reaction is different from the cause of chlorosis, although both actions cause reduction of photosynthesis. However the organophosphates, which caused the chlorotic symptom on Chinese cabbage, strongly inhibited Hill reaction. Therefore it is considered that there are some relations between the reduction of chlorophyll and the inhibition of Hill reaction. The organophosphates which cause chlorotic symptom on the leaves and strongly inhibit Hill reaction, are hardly soluble in water, that is, hydrophobic. Logarithmic values of *n*-octanol/water partition coefficient of various pesticides, an index of lipophilic property, are inversely proportional to logarithmic water solubilities of the pesticides.[8,14] It could be said that the pesticides causing chlorosis are highly lipophilic.

Krogmann *et al.*[15] reported that normal fatty acids longer than 12 carbon atoms were potent inhibitors of Hill reaction, while caproic (C_6 member) and caprylic acids (C_8 member) were not inhibitory, suggesting the presence of a critical chain length. Present results indicating a positive correlation between the degree of inhibition of Hill reaction and number of carbon atoms in normal fatty acids, agree with the results of Krogmann *et al.*[15] Hydrophobicity of the fatty acid increases with the length of the chain.[16] Therefore, the hydrophobic property apparently correlates with the inhibition of Hill reaction by fatty acids. The inhibitory action of normal alcohols and chlorinated benzenes also increased with the increase of hydrophobicity.

Biological responses are considered to be regulated by hydrophobicity, electronic factor and/or steric factors of the compounds.[17] Hansch *et al.*[18] reported that the lipophilic nature of the substituent in the phenyldimethylureas and anilides of isobutylic acid was of primary importance. A correlation between the inhibition of photosynthesis and lipophilicity was also found in the case of substituted diphenyl ethers.[19] Thus, the inhibition of Hill reaction seems to be related with hydrophobicity in many groups of compounds. In the case of organophosphates, which caused the chlorotic symptom, hydrophobic property appeared to be the most important factor in the inhibition of Hill reaction.

Okamoto *et al.*[20] described a hydrophobic interaction in the binding of linolenic acid with chloroplasts. Linolenic acid associates first with the membrane surface, then it moves into the interior of the membrane. Disorganization of the functional integrity of the membrane thus induced would cause the inactivation of electron transport reaction. It is supposed that hydrophobic organophosphates similarly cause disorganization of chloroplasts.

Degree of the inhibition of Hill reaction did not exceed a definite value even in higher concentrations in the case of particular organophosphates and alcohols. Maximum percentage of inhibition decreases with the increase in length of alkyl chain in the case of normal alcohols. Albert[21] described the degree of tox-

icity of the primary aliphatic amines on various kinds of bacteria as being higher with longer length of molecular chain. A maximum toxicity was observed on dodecylamine (C_{12} member) and the toxicity rapidly fell when one or two more carbon atoms were added to the carbon chain. Falling-off of toxicity depended on the decrease of monomer in the solution due to micelle formation. Similarly, the inhibition of organophosphates appeared to reach a maximum value with the decrease of monomer in the reaction solution due to low solubility.

As stated above, some organophosphorus insecticides clearly inhibited Hill reaction. Hydrophobic organophosphates causing chlorosis seem to have an affinity to lipid rich chloroplast. The affinity of organophosphates to chloroplast is considered to cause an inhibition in the electron transport reaction occurring on thylakoid membrane and to cause decomposition of chlorophyll resulting in chlorosis. Chinese cabbage leaves appear light green suggesting their concentration of chlorophyll is lower than in other vegetables. Therefore, chlorosis is supposed to be appeared easily on Chinese cabbage leaves due to the disorganization of chloroplast. On the other hand, appearance of the necrotic symptom on Chinese cabbage leaves could not be explained by inhibition of Hill reaction due to hydrophobicity. Further investigations are necessary to elucidate the phytotoxic effect of organophosphates in plants from other than a hydrophobic viewpoint.

REFERENCES

1) A. Ishitani, M. Yukimoto & K. Yoshida: *Bull. Agric. Chem. Insp. Stn.* **15**, 92 (1975)
2) M. Yukimoto: *J. Pesticide Sci.* **6**, 51 (1981)
3) A. Ishitani & M. Yukimoto: *Bull. Agric. Chem. Insp. Stn.* **19**, 35 (1979)
4) E. Y. Spencer: "Guide to the Chemicals Used in Crop Protection," Agriculture Canada, Ottawa, p. 542, 1973
5) M. Eto: "Organophosphorus Pesticides," CRC Press, Ohio, p. 387, 1974
6) K. Fukunaga: "Pesticide Handbook," Jpn. Plant Prot. Assoc., Tokyo, p. 356, 1981
7) V. H. Freed, D. Schmedding, R. Kohnert & R. Haque: *Pestic. Biochem. Physiol.* **10**, 203 (1979)
8) J. Kanazawa: *Ecol. Chem.* **2**, 105 (1979)
9) K. Ando: *Jpn. Pestic. Inf.* No. 35, 27 (1978)
10) G. J. Pierotti, C. H. Keal & E. L. Derr: *Ind. Eng. Chem.* **51**, 95 (1959)
11) S. Matsunaka: "Pesticide Design," ed. by I. Yamamoto & J. Fukami, Soft Science, Tokyo, p. 359, 1979
12) A. Mine & S. Matsunaka: *Pestic. Biochem. Physiol.* **5**, 444 (1975)
13) M. Yukimoto, A. Ishitani, K. Yoshida & N. Kobayashi: *J. Pesticide Sci.* **3**, 243 (1978)
14) C. T. Chiou, V. H. Freed, D. W. Schmedding & R. L. Kohnert: *Environ. Sci. Technol.* **11**, 475 (1977)
15) D. W. Krogmann & A. T. Jagendorf: *Arch. Biochem. Biophys.* **80**, 421 (1959)
16) R. B. Simpson, J. D. Ashbrook, E. C. Santos & A. A. Spector: *J. Lipid Res.* **15**, 415 (1974)
17) T. Fujita: "Pesticide Design," ed. by I. Yamamoto & J. Fukami, Soft Science, Tokyo, p. 801, 1979
18) C. Hansch & E. W. Deutsch: *Biochim. Biophys. Acta* **112**, 381 (1966)
19) G. van den Berg & J. Tipker: *Pestic. Sci.* **13**, 29 (1982)
20) T. Okamoto & S. Katoh: *Plant Cell Physiol.* **18**, 539 (1977)
21) A. Albert: "Selective Toxicity," 6th edition, Chapmann & Hall Ltd., London, p .42, 1979

要　　約

有機リン系殺虫剤による Hill 反応阻害

行本峰子

有機リン系殺虫剤のハクサイに対する薬害作用を解明するため，光合成反応のうち Hill 反応に対する阻害について調べた．薬害を起こさなかった有機リン剤は，10^{-4} M 以下の濃度で Hill 反応をまったく阻害しなかった．ハクサイ葉にえ死症状を表わした有機リン剤の場合は，dimethoate のようにまったく阻害しなかったもの，fenthion (MPP) や fenitrothion (MEP) のように 50% 阻害濃度が 10^{-5} M オーダーのものなど，Hill 反応阻害の程度はさまざまであった．クロロシスを起こす有機リン剤は，強く Hill 反応を阻害し，10^{-6} M のオーダーで阻害が見られた．さらにこのような有機リン剤は，いずれも水溶解度が低いものであった．Hill 反応阻害の程度と疎水性との間には相関関係が見られ，直鎖第1級アルコールおよび酸，およびポリクロルベンゼンを含め，疎水性の高い化合物ほど，Hill 反応を強く阻害する傾向がみられた．疎水性の化合物は葉緑体に結合しやすく，その結果，電子伝達反応の阻害およびクロロフィルの破壊が起こったと推測した．