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Photoreduction of Parathion by Spinach Chloroplasts. II.[1] Ferredoxin-Independent Photoreduction of Parathion by Heated Chloroplasts with an Artificial Electron Donor System

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Parathion (O,O-diethyl-O-*p*-nitrophenyl phosphorothioate) was reduced to hydroxylaminoparathion and aminoparathion by heated spinach chloroplasts with an artificial electron donor system in the light. Spinach ferredoxin was not required for this reaction, the rate of which increased as the heating time of chloroplasts became longer, at least up to 3 min at 50°. In these respects, the present photoreduction of parathion is very different from the ferredoxin-dependent one by unheated chloroplasts. It is presumed that parathion directly accepts electrons from the primary electron acceptor of photosystem I in heated chloroplasts.

It is generally accepted that the physiological Hill reaction in whole chloroplasts is the photoreduction of $NADP^+$. Numerous artificial compounds have been found to serve as an electron acceptor in Hill reaction instead of $NADP^+$. Such artificial electron acceptors may be reduced at different points along the photosynthetic electron transport chain, the point of reduction being determined by the redox potential and the accessibility of the acceptor. Similarly, non-physiological electron donors donate electrons into the electron transport chain at various points in place of water.

In the previous paper,[1] we have reported that parathion (O,O-diethyl-O-*p*-nitrophenyl phosphorothioate) was photoreduced mainly to hydroxylaminoparathion by isolated spinach chloroplasts and that spinach ferredoxin was essential for the photoreduction. This paper describes that parathion can be reduced independently of ferredoxin by heated chloroplasts with an artificial electron donor system on illumination.

Experimental

Materials——Technical grade of parathion (more than 98.0% pure) was used throughout this investigation. Nicotinamide-adenine dinucleotide phosphate ($NADP^+$), flavin mononucleotide (FMN), phenazine methosulfate (PMS) and spinach ferredoxin were purchased from Sigma Chemical Co. Other chemicals were reagent grade.

Procedures for Parathion Photoreduction——Once-washed chloroplasts (defined as P_{1S1}) were prepared from spinach leaves by the method of Whatley and Arnon.[3] The chlorophyll content of chloroplasts was determined by the method of Arnon.[4] Unless otherwise specified, the reaction mixture contained 0.4 ml of chloroplast suspension (equivalent to 200 μg of chlorophyll), 50 μg of ferredoxin, 0.2 ml of 0.5M Tris-HCl buffer (pH 7.8), 0.1 μmole of parathion and deionized water to give a final volume of 3 ml. Heated chloroplasts were prepared by heating at 50° for 3 min. For heated chloroplasts, 0.2 μmole of 2,6-dichlorophenolindophenol (DCPIP) and 20 μmoles of sodium ascorbate were added in the reaction mixture instead of ferredoxin. The reaction was carried out in Thunberg-type cuvettes under nitrogen gas. Cuvettes were illuminated with 150 W tungsten lamp at room temperature and the reaction was terminated after 20 min

1) Part I: T. Suzuki and M. Uchiyama, *Chem. Pharm. Bull.* (Tokyo), **23**, 2175 (1975).
2) Location: *a) Aobayama, Sendai; b) 1-18-1, Kamiyoga, Setagaya, Tokyo.*
3) F.R. Whatley and D.I. Arnon, "Methods in Enzymology," Vol. 6, ed. by S.D. Colowick and N.O. Kaplan, Academic Press, New York and London, 1963, pp. 308—318.
4) D.I. Arnon, *Plant Physiol.*, **24**, 1 (1949).

by turning off the light. Two ml of reaction mixture was introduced into 10 ml of test tube containing 2 ml of *n*-hexane and 0.1 ml of Tollens' reagent. After vigorous shaking and centrifugation, portions of *n*-hexane phase were subjected to gas–liquid chromatography. Parathion and its reductive products were quantitatively analyzed as previously reported.[1)]

Procedures for NADP$^+$ Photoreduction——The reaction mixture in a quartz cuvette contained 1.0 μmole of NADP$^+$, 0.1 ml of chloroplast suspension (equivalent to 50 μg of chlorophyll), 50 μg of ferredoxin and 0.2 ml of 0.5M Tris-HCl buffer (pH 7.8) in a final volume of 3 ml. Cuvettes were illuminated for 10 min in the same manner as parathion photoreduction. NADP$^+$ reduced was determined from an increase of absorbance at 340 mμ against a blank which contained the complete reaction mixture but kept in the dark.

Results

Photoreduction of Parathion by Heated Chloroplasts

Mild heating, *e.g.* 3 min at 50° or 5 min at 45°, is effective in selectively destroying the water oxidation step in the photosynthetic electron transport chain[5)] and a number of artificial electron donor systems can restore the Hill activity of heated chloroplasts. The same trend was observed in parathion photoreduction by spinach chloroplasts, as presented in Table I.

Table I. Restoration of Parathion Photoreducing Activity of Heated Chloroplasts by DCPIP-Ascorbate

Heating[a)]	DCPIP ascorbate[b)]	Nanomoles of parathion		
		Disappeared	Reduced to HAP[c)]	Reduced to AP[c)]
−	−	57.0	43.6	5.8
+	−	0.5	0.0	0.0
+	+	55.2	31.4	13.5

a) Chloroplasts were heated for 3 min at 50°.
b) Sixty-seven μM of DCPIP and 6.7 mM of ascorbate were added.
c) HAP, hydroxylaminoparathion; AP, aminoparathion

Heat treatment for 3 min at 50° destroyed the ability of chloroplasts to reduce parathion, and the ability was almost completely restored by adding DCPIP-ascorbate as an artificial electron donor system. Table I also indicates that the ratio of aminoparathion formed from parathion *via* hydroxylaminoparathion was higher in heated chloroplasts-ferredoxin-DCPIP-ascorbate system than unheated chloroplasts-ferredoxin system. These facts suggest that the mechanism of parathion photoreduction by heated chloroplasts may differ from that by unheated chloroplasts, not only in respect to the electron donor but also in some other factors. In order to elucidate the above-mentioned possibility, the following investigations were carried out.

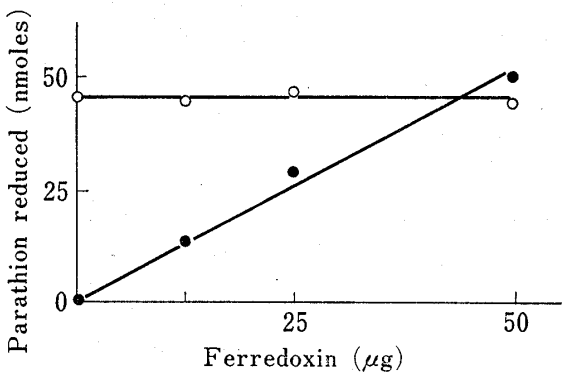

Fig. 1. Photoreduction of Parathion by Heated or Unheated Chloroplasts as a Function of Ferredoxin Concentration

—●—: unheated chloroplasts
—○—: heated chloroplasts with DCPIP-ascorbate

Ferredoxin Requirement

As already described in our previous paper,[1)] the photoreduction of parathion by unheated chloroplasts strictly depends on ferredoxin. Fig. 1 shows a linear relationship

5) T. Yamashita and W.L. Butler, *Plant Physiol.*, **43**, 2037 (1968); S. Katoh and S. A. Pietro, *Arch. Biochem. Biophys.*, **128**, 378 (1968).

between ferredoxin concentration and the parathion photoreduction rate, expressed by the sum of hydroxylaminoparathion and aminoparathion, in unheated chloroplasts. On the other hand, heated chloroplasts could reduce parathion independently of ferredoxin in the presence of DCPIP-ascorbate.

Some Characteristics of Parathion Photoreducing System in Heated Chloroplasts

Since parathion was not reduced at all in the dark or in the absence of artificial electron donor system, it is apparent that the present nitro reduction is a photochemical reaction, which can be effected by photosystem I only. The requirement of chlorophyll content for producing the maximum activity to reduce parathion was fairly different between ferredoxin-dependent and -independent systems. In the former, 30—70 μg of chlorophyll per ml of reaction medium was optimum concentration, whereas in the latter 100—130 μg of chlorophyll per ml gave the highest reduction rate.

TABLE II. Effect of Electron Acceptors for Photosystem I on Parathion Photoreduction by Heated Chloroplasts-DCPIP-Ascorbate System

Addition (μmole)	Nanomoles of parathion		
	Disappeared	Reduced to HAP[a)]	Reduced to AP[a)]
—	46.0	28.5	14.6
$NADP^+$(0.5) & ferredoxin (50 μg)	24.6	11.8	5.5
PMS (0.1)	0.0	0.0	0.0
FMN (0.1)	28.5	18.7	6.3

a) HAP, hydroxylaminoparathion; AP, aminoparathion

Addition of electron acceptor for photosystem I resulted in a strong inhibition as presented in Table II. However, $NADP^+$ inhibition was much less than that on ferredoxin-dependent photoreduction, which was almost completely inhibited.[1)]

Other Electron Donor Systems

The ability of heated chloroplasts to reduce parathion in the presence of other electron donor system is compared in Table III. At a concentration of 10^{-4}M and above, *p*-phenylenediamine can serve as an electron donor for photosystem I,[6)] like DCPIP. At lower concentration (10^{-5}M), it is an electron donor for photosystem II.[6,7)] Furthermore, *p*-hydroquinone[7)] and a higher concentration (more than 10^{-2}M) of hydroxylamine[8)] are known to be electron donors for photosystem II.

All electron donor systems used here could donate electrons to heated chloroplasts for parathion photoreduction. From the results in Table III, the electron donor systems for photosystem I could restore the ability of heated chloroplasts to reduce parathion more efficiently than those for photosystem II.

Alteration of Parathion Photoreducing Activity by Heating Chloroplasts for Various Periods

Chloroplast suspension was heated at 50° for 0.5, 1, 2 or 3 min and parathion photoreducing activities were determined in the presence of ferredoxin or DCPIP-ascorbate. $NADP^+$ photoreducing activities were also assayed in the presence of ferredoxin.

Fig. 2 shows that the activity of ferredoxin-dependent parathion photoreduction was lost by heating in the same manner as that of $NADP^+$ photoreduction. In both reactions, the activity temporarily increased and then rapidly decreased by heating chloroplasts. Such

6) T. Yamashita and W.L. Butler, *Plant Physiol.*, **43**, 1978 (1968).
7) T. Yamashita and W.L. Butler, *Plant Physiol.*, **44**, 435 (1969).
8) S. Izawa, R.L. Heath, and T. Hind, *Biochim. Biophys. Acta*, **180**, 388 (1969).

TABLE III. Parathion Photoreducing Activities of Heated Chloroplasts in the Presence of Various Electron Donor Systems

Electron donors	Nanomoles of parathion		
	Disappeared	Reduced to HAP[a)]	Reduced to AP[a)]
67 μM DCPIP+6.7 mM ascorbate	63.8	36.9	17.5
670 μM *p*-phenylenediamine+67 mM ascorbate	54.4	19.4	23.1
33 μM *p*-phenylenediamine+330 μM ascorbate	18.2	11.9	8.7
200 μM *p*-hydroquinone+330 μM ascorbate	21.9	9.4	6.3
10 mM hydroxylamine	16.0	4.4	3.0

a) HAP, hydroxylaminoparathion; AP, aminoparathion

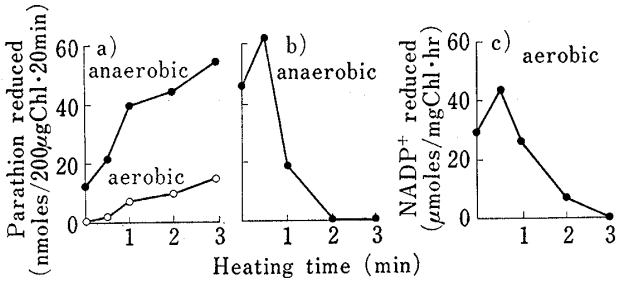

Fig. 2. Alteration of Parathion and NADP+ Photoreducing Activities by Heating Chloroplasts for Various Periods

a) parathion photoreduction in the presence of DCPIP-ascorbate
b) parathion photoreduction in the presence of ferredoxin
c) NADP+ photoreduction in the presence of ferredoxin

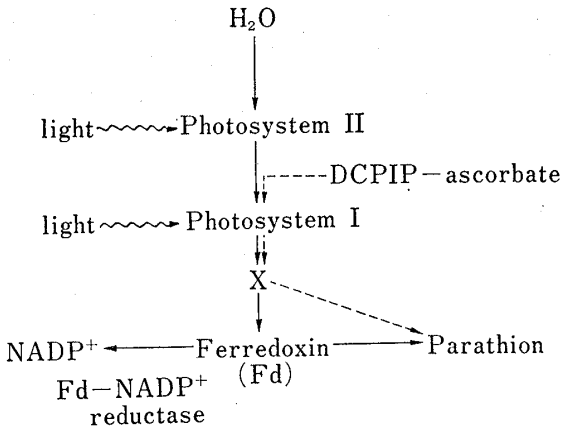

Fig. 3. Diagrammatic Representation of the Photoreduction of Parathion by Spinach Chloroplasts

Solid arrows represent natural pathways. Broken arrows represent the pathways of parathion photoreduction by heated chloroplasts-DCPIP-ascorbate system.

a change of Hill activity caused by the heating of chloroplasts has been reported by Mukohata, *et al.*,[9] and Oku and Tomita.[10] It was also recognized that parathion photoreduction was more easily inactivated than NADP+ photoreduction by the heating.

In the presence of DCPIP-ascorbate, parathion photoreducing activity underwent a change with an entirely different pattern from ferredoxin-dependent photoreductions. As shown in Fig. 2-a, the longer heating caused the higher activity of chloroplasts to reduce parathion. In consequence of the heating of chloroplasts, parathion was reduced even under aerobic condition. These results indicate that parathion photoreduction by heated chloroplasts with an artificial electron donor system may be closely related to the loss of ability of chloroplasts to evolve oxygen.

Discussion

The experimental results obtained from this investigation show that parathion is photoreduced independently of ferredoxin by heated chloroplasts with an artificial electron donor system. It is presumed that a final electron carrier of parathion photoreducing system in heated chloroplasts is a primary electron acceptor (X) of photosystem I, like the photoreduction of viologen dyes. The ferredoxin-dependent and -independent photoreductions of parathion are diagrammatically represented in Fig. 3.

9) Y. Mukohata, M. Mitsudo, S. Kakumoto, and M. Higashida, *Plant and Cell Physiol.*, **13**, 287 (1972).
10) T. Oku and G. Tomita, *Biochem. Biophys. Res. Commun.*, **44**, 958 (1971).

In this scheme, we have not an answer why electrons do not flow from X to parathion in unheated chloroplasts. However, it may be possible that the redox potential of X alters to the appropriate one to reduce parathion by the heating of chloroplasts or by the addition of artificial electron donor. Such a phenomenon is often observed in mammalian mitochondrial electron transfer components. Furthermore, it may be related to various changes which are brought about by the heating of chloroplasts, *e.g.* structural modification of chloroplast membrane.

The ability of chloroplasts to reduce parathion in the presence of DCPIP-ascorbate is adversely related to the ability of them to evolve oxygen (Fig. 2). The reaction system, which requires an artificial electron donor, seems to be biologically insignificant, at least, in higher plants; however, it may be possible that the nitro-containing organophosphorus insecticides are reduced by such a type of photochemical reaction in the other kinds of photosynthetic organisms. For example, a group of photosynthetic bacteria, *purple bacteria*, do not produce oxygen during photosynthesis. Instead of using water as an electron donor, they use H_2S and produce elementary sulfur. Such photosynthetic reactions, in which H_2S, H_2, $H_2S_2O_3$, or certain organic molecules are used as an electron donor, are generally observed in photosynthetic bacteria (Chlorobacteriaceae, Thiorhodaceae and Athiorhodaceae).