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Photodynamic Action of Phloxine on Mitochondrial Respiration

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Photodynamic action of phloxine on mitochondrial respiration was examined, using rat liver mitochondria. Rat liver mitochondria were suspended in a medium containing phloxine, and irradiated with visible light in the presence of oxygen. The respiratory activities of the photooxidized mitochondria were assayed. The results are summarized as follows.

(1) Phloxine-sensitized photooxidation of mitochondria inhibited 2,4-dinitrophenol (2,4-DNP)-released respiration. It can be deduced, from an approximate calculation, that the released respiration was inhibited by 10% as every 3 nmoles of oxygen per mg of mitochondrial protein was consumed by photooxidation.

(2) Phloxine-sensitized photooxidation of mitochondria released the suppressed respiration. The supernatant obtained by centrifugation of photooxidized mitochondria also released the suppressed respiration of intact mitochondria. This suggests that certain uncoupling factors were released from photooxidized mitochondria. Uncoupling factor in question may be unsaturated fatty acids or U-factor, because bovine serum albumin abolished the respiration-releasing effect of the supernatant of photooxidized mitochondria.

Photodynamic action of phloxine (disodium salt of 9-(3,4,5,6-tetra-chloro-O-carboxyphenyl)-6-hydroxy-2,4,5,7-tetra-bromo-3-isoxanthone; C.I.45410; C.I.Acid Red 92 (778); Schultz 890, Hecht) on living organism has been reported by several investigators.

Kada, *et al.* have confirmed the lethal activity of phloxine on mutant cells of Bacillus subtilis and the evident mutagenic action of phloxine on *E. coli*, using their newly developed procedure for screening chemical mutagens.[2] Inada, *et al.* have demonstrated that phloxine has lethal effect on bacteria, especially on Flabobacterium aquatile, in the presence of oxygen and visible light.[3] Concerning higher organisms, a teratogenic action of phloxine in mice has been reported by Uchida, *et al.*[4] However, photodynamic action of phloxine on subcellular organella has not been examined enough. In this experiment, photodynamic action of phloxine on respiratory activity of isolated mitochondria was examined.

Experimental

Chemicals and Materials——Phloxine was purchased from Tokyo Chemical Industry Co., Ltd. Aspartic acid, cysteine, glycine, histidine, leucine, methionine, phenylalanine and serine were obtained from Nippon Rikagakuyakuhin Co., Ltd., and arginine, lysine hydrochloride, tryptophan and tyrosine were from Tokyo Chemical Industry Co., Ltd. Adenosine, guanosine and thymidine were the products of Tokyo Chemical Industry Co., Ltd. Uridine was obtained from Daiichi Pure Chemical Co., Ltd. Vitamins B_1, B_2, B_6, B_{12}, C, K_1, and K_5 were purchased from Tokyo Chemical Co. Ltd., and vitamin A acetate and vitamin D_2 were from Wako Pure Chemical Industries, Ltd. and Daiichi Pure Chemical Co., Ltd., respectively. Oleic acid and triolein were the products of Tokyo Chemical Industry Co., Ltd., and linoleic acid the product of Sigma Chemical Co.

Rat liver mitochondria were prepared, according to the method of Schneider,[5] using 0.25 M sucrose containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM Tris-HCl (pH 7.5) as a preparation medium. Mitochondria obtained were washed twice with 0.25 M sucrose by centrifugation at $7000 \times g$ for

1) Location: *Hatanodai, 1-5-8, Shinagawa-ku, Tokyo.*
2) T. Kada, K. Tuchikawa, and Y. Sadaie, *Mutation Res.*, **16**, 165 (1972).
3) K. Inada, F. Miyazawa, and A. Tanimura, *J. Food Hyg. Soc. Japan*, **11**, 238 (1970).
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5) W.C. Schneider, *J. Biol. Chem.*, **176**, 250 (1958).

10 min. The washed mitochondria were suspended in the preparation medium at a concentration of 12 mg protein/ml, and stocked in an ice bath.

Protein concentration was determined by the method of Folin and Ciocalteu.[6]

Determination of Photodynamic Action of Phloxine——Several experimental procedures were involved in this study. Phloxine-sensitized photooxidation of various biological substances was examined according to the procedure I.

Procedure I——Photooxidation was performed in a reaction cell (of about 3.5 ml capacity), which was devised for determination of dissolved oxygen. Hydrophilic test compounds were dissolved in 0.1 M phosphate buffer (pH 7.0). Hydrophobic test compounds, such as vitamins A, D_2, E, K_1, and K_5, and linoleate, oleate and triolein, were emulsified in 0.1 M phosphate buffer containing 0.1% sodium dodecyl sulfate by means of sonication. Sonic irradiation was carried out for 2 min in a Tomy model UR-150P (Tominaga Works, Ltd.). A reaction mixture, which contained 1.97 mM test compound and 1.5×10^{-6} M phloxine, was illuminated with visible light at a room temperature. The visible light was obtained from a 300 W slide projector Canon 302, to which a Corning color filter (C.S. No. 4.72., the transmittance was less than 10% below 355 nm and above 565 nm) was attached. The intensity of illumination was about 100 klux at the side surface of the reaction cell (without the color filter). Oxygen consumption due to photooxidation was followed by a Clark oxygen electrode (Yellow Spring Instrument Co., Inc.) connected through a voltage divider to a Hitachi recorder QPD 53. The rate of photooxidation was determined from the slope of O_2-decrease at the initial period of the reaction, and expressed as nmoles of oxygen consumed per min.

Photodynamic action of phloxine on mitochondrial electron transport was examined according to the procedure II.

Procedure II——To 0.2 ml of the mitochondrial suspension, was added 3.3 ml of an assay medium, which contained 0.25 M sucrose, 10 mM K-phosphate, 5 mM $MgCl_2$, 10 mM KCl, 0.2 mM EDTA and 5 mM Tris-HCl (pH 7.2). The medium has been recommended by B. Hagiwara[7] for measurement of respiratory activity. The mitochondrial suspension was illuminated in the presence of phloxine for selected period, as described in the procedure I, and various respiratory activities of the photooxidized mitochondria were measured polarographically in the dim light. Succinate oxidation activity was measured in the presence of 9.7 μM rotenone and 9.7 mM succinate. Succinate dehydrogenase activity was determined in the presence of 9.7 μM rotenone, 1 mM KCN, 1.5 mM $CaCl_2$, 9.7 mM succinate and 250 μM phenazine methosulfate, essentially according to P. Bernath and T.P. Singer.[8] Cytochrome c oxidase activity was measured in the presence of 3.75 mM Na-ascorbate and 0.3 mM N,N,N′,N′-tetramethyl-*p*-phenylene-diamine (TMPD), as described by C.A. Schneitman, *et al.*[9]

Procedure III——Quantitative relation between the degree of photooxidation and the degree of inactivation of mitochondrial respiratory activity was examined as follows. Oxygen consumption during the course of photooxidation was determined polarographically, and expressed as nmoles of oxygen consumed per mg of protein. After the suspension of photooxidized mitochondria was saturated with air, the respiratory activity was determined in the dim light.

Procedure IV——Effect of photooxidized mitochondria on respiratory activity of intact mitochondria was examined in the following manner. Mitochondrial suspension (about 12 mg protein per ml) containing 1.75×10^{-5} M phloxine was illuminated with the visible light for 10 min, and totally photoinactivated. An aliquot of the totally photoinactivated mitochondria was added to intact mitochondria, and the volume of the mixture was adjusted to 3.5 ml by addition of the assay medium. Respiratory activity of the mixture was determined as described in procedure II.

Result and Discussion

(1) Phloxine-sensitized Photooxidation of Various Biological Compounds

Susceptibility of various biological compounds to phloxine-sensitized photooxidation was as follows. Of all the amino acids tested, histidine, tryptophan, cysteine and tyrosine were photooxidized. Rates of photooxidation of all the amino acids tested are listed in Table I. In the lower part of the table, are also shown the rates of photooxidation of nucleosides and vitamins. Among the four nucleosides tested, guanosine and adenosine were susceptible to the dye-sensitized photooxidation. Of all the vitamins tested, vitamins C, E, B_6, K_5, K_1, A and D_2 were photooxidized with the rates indicated in Table I. Oleate, linoleate or triolein

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7) B. Hagiwara, *Biochem. Biophys. Acta*, 46, 134 (1961).
8) P. Bernath and T.P. Singer, "Succinic Dehydrogenase, Methods in Enzymology," Vol. V, ed. by S.P. Colowick and N.O. Kaplan, Academic Press, Inc., New York, 1962, p. 579.
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Table I. Phloxine-sensitized Photooxidation of Various Biological Compounds

Compound	Oxygen consumed (nmoles/min)	Compound	Oxygen consumed (nmoles/min)
Arginine	—	Thymidine	—
Cysteine	38	Uridine	—
Glycine	—	Vitamin A	17
Histidine	242	B_1	—
Leucine	—	B_2	6
Lysine	—	B_6	125
Methionine	76	B_{12}	—
Phenylalanine	—	C	1170
Serine	—	D_2	13
Tyrosine	13	E	150
Tryptophan	125	K_1	38
Adenosine	7	K_5	76
Guanosine	49		

was not photooxidized. The photooxidation described above was observed only in the presence of phloxine, but not in the absence of the dye.

(2) Phloxine-sensitized Photoinactivation of 2,4-DNP-released Respiration

Effect of phloxine-sensitized photooxidation on 2,4-DNP-released respiration (succinate-O_2) is shown in Table II. As seen in the table, 2,4-DNP-released respiration was inhibited by illumination in the presence of phloxine. But, in the case of various control systems indicated in Table II, the inhibition was not observed.

Table II. Phloxine-sensitized Photoinactivation of 2,4-DNP-released Respiration

System	2,4-DNP-released respiration ($-O_2$ nmoles/min/mg)
Mitochondria alone	62
Mitochondria+phloxine	59
Mitochondria illuminated	62
Mitochondria+phloxine alone illuminated	55
Mitochondria alone illuminated+phloxine	57
Mitochondria alone illuminated+phloxine alone illuminated	59
(Mitochondria+phloxine)mixture illuminated	6

Experiments were carried out according to the procedure II: phloxine: 1.5×10^{-6}M, mitochondria: 0.7 mg/ml, illumination time: 5 min

Dependency of the photoinactivation on phloxine concentration is shown in Fig. 1. The mitochondrial photoinactivation was observed even in a diluted solution of phloxine, which was not more than 1.5×10^{-8}M.

Quantitative relation between the degree of photooxidation of mitochondria and the degree of inactivation of 2,4-DNP-released respiration is shown in Fig. 2. From an approximate calculation, it can be estimated that 2,4-DNP-released respiration decreased by 10%, as every 3 nmoles of oxygen per mg of protein was consumed during the course of photooxidation.

(3) Effects of Photooxidation on Various Respiration Activities

Effects of photooxidation on various respiration activities of isolated mitochondria are shown in Fig. 3, where per cent of control activity is plotted against illumination time.

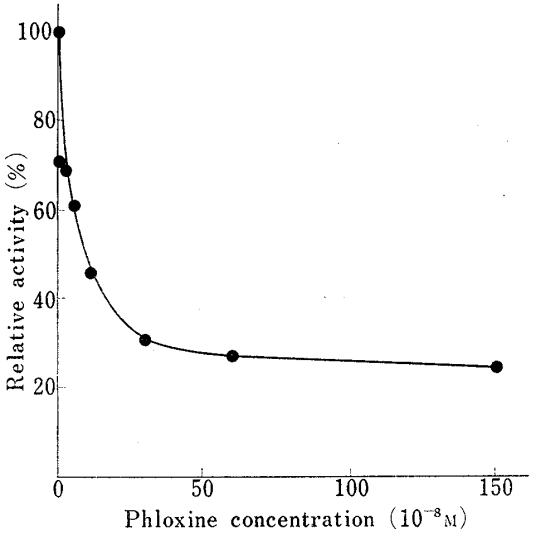

Fig. 1. Effect of Phloxine Concentration on Photoinactivation of 2,4-DNP-released Respiration

Experimental conditions were the same as described in the footnote of Table II, except that phloxine concentration was 1.5 to 150×10^{-8}M. Control activity (without phloxine) was taken to be 100%.

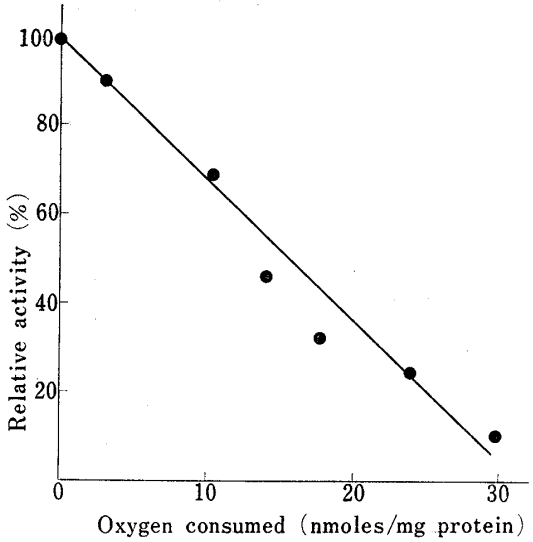

Fig. 2. Quantitative Relation between the Degree of Photooxidation of Mitochondria and the Degree of Photoinactivation of 2,4-DNP-released Respiration

phloxine: 1.5×10^{-6}M, mitochondria: 0.7mg/ml, medium: as described in Experimental
Experiments were carried out according to the procedure III. Control activity (without illumination) was taken to be 100%.

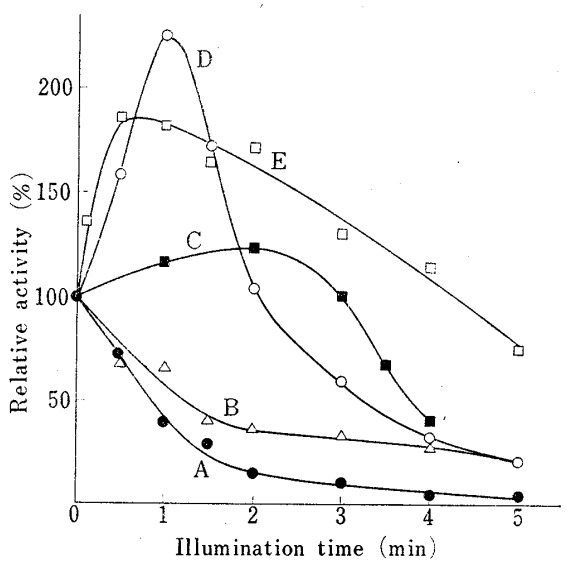

Fig. 3. Effects of Photooxidation on Various Respiratory Activities

Experimental conditions were the same as described in the footnote of Table II, except that samples were illuminated for selected period. curve A: succinate-O_2 (+DNP), curve B: succinate-phenazine methosulfate, curve C: TMPD-O_2(+DNP), curve D: succinate-O_2 (−DNP), curve E: TMPD-O_2 (−DNP)

Succinate oxidation (succinate-O_2) in the presence of 2,4-DNP, DNP-released respiration, decreased with increasing illumination time (curve A). Succinate dehydrogenase activity (curve B) also decreased in parallel with the decrease of succinate oxidation (succinate-O_2). Cytochrome c oxidase activity (TMPD-O_2) in the presence of 2,4-DNP was not so much changed by short illumination (within 2 min), and then, decreased by longer illumination (curve C).

On the other hand, in the absence of 2,4-DNP, both succinate oxidation activity (succinate-O_2) (curve D) and cytochrome c oxidase activity (TMPD-O_2) (curve E) increased initially, and then, decreased.

Thus, phloxine-mediated photooxidation of mitochondria was found to release the suppressed respiration and, on the other hand, to inhibit 2,4-DNP-released respiration. Photoinactivation of 2,4-DNP-released respiration (succinate-O_2) may be caused mainly by inactivation of succinate dehydrogenase.

The extent of the photooxidation-induced release of the suppressed respiration was examined with mitochondria illuminated in the presence and the absence of succinate. As seen in Table III, a, respiration rate (succinate-O_2) was intensified about 4-fold by illumination in the presence of succinate, and 2.5-fold by illumination in its absence.

Table IIIa. Respiration-releasing Effect of Mitochondrial Photooxidation

Illumination time (sec)	Respiratory activity (succinate-O_2) (% of control)	
	Illuminated in the absence of succinate	Illuminated in the presence of succinate
30	160	232
60	251	294
90	203	379

Table IIIb. A Difference in Susceptibility of 2,4-DNP-released Respiration to the Photoinactivation

Illumlination time (sec)	2,4-DNP-released respiratory activity (% of control)	
	Illuminated in the absence of succinate	Illuminated in the presence of succinate
30	60	76
60	34	48
90	20	41

Mitochondrial suspension were photooxidized in the presence or absence of 3 mM, succinate. Then, their respiration activity was measured as described in Experimental. Respiratory activity of control (not photoxidized) sample was taken to be 100%. phloxine: 1.5×10^{-6}M, succinate: 3 mM, 2.4-DNP: 40 μM, mitochondria: 0:7 mg/ml

There was a difference in susceptibility to the phloxine-mediated photoinactivation of 2,4-DNP-released respiration (succinate-O_2) between mitochondria illuminated in the presence of succinate and those in the absence. As seen in Table IIIb, mitochondria in succinate-supplemented medium were less susceptible to the photoinactivation.

It can be deduced that, as electron transport system, probably succinate dehydrogenase system, is less susceptible in the presence of succinate to the photoinactivation, the photooxidation-induced release of the suppressed respiration is more clearly observed in the mitochondria illuminated in the presence of succinate.

(4) Effect of Photooxidized Mitochondria on Respiration of Intact Mitochondria

The photooxidized mitochondria were found to release the suppressed respiration (succinate-O_2) of intact mitochondria, but not remarkably effective on 2,4-DNP-released respiration, as shown in Table IV.

Table IV. Effect of Photooxidized Mitochondria on Respiration of Intact Mitochondria

Volume of photooxidized mitochondrial suspension added (ml)	Respiratory activity (% of control)	
	-2,4-DNP	+2,4-DNP
0.1	182	120
0.2	238	119
0.3	282	85

Experiments were carried out according to the procedure VI. Respiratory activity of intact mitochondria was taken to be 100%.

Supernatant obtained by centrifugation at $7000\times g$ for 10 min of the suspension of photoinactivated mitochondria was also found to release the suppressed respiration (succinate-O_2) of intact mitochondria, as seen in Table V. The respiration-releasing effect of the supernatant was abolished by the presence of bovine serum albumin (BSA).

Table V. Effect of Supernatant Obtained by Centrifugation of Photooxidized Mitochondria on Respiratory Activity of Intact Mitochondria

Volume of supernatant (ml)	Respiratory activity (% of control)	
	−2,4-DNP	+2,4-DNP
0.3	175	81
0.5	280	68
0.5+0.075%BSA[a)]	140	84
0.5+0.15 %BSA	125	76
0.5+0.79 %BSA	119	82

Respiratory activity of intact mitochondria was taken to be 100%.
a) bovine serum albumin

Undoubtedly, it can be deduced that the supernatant contained certain respiration-releasing factor (uncoupler). Calcium ion can not be expected as uncoupler in question, because the assay medium contained EDTA enough to chelate free calcium ion. Photooxidized amino acids or photooxidized nucleosides did not release the suppressed respiration of intact mitochondria. Considering that BSA abolished the respiration-releasing effect of the supernatant, endogenous uncouplers, such as unsaturated fatty acids and U-factor,[10–12] may be released from photooxidized mitochondria.

Pace, *et al.*[13] have reported that dihydroquinidine acts as uncoupling agent, when rabbit heart mitochondria were irradiated in the presence of the photo-sensitizer. In this case also, true uncoupler may be certain substances released from mitochondrial membranes, probably unsaturated fatty acids or U-factor. Haga[14] has studied photodynamic action of methylene blue, eosine Y and thiopyronine on paramecia mitochondria, and reported that photosensitized oxidation caused swelling of mitochondria. In this experiment, decrease in turbidity of mitochondria was caused by phloxine-mediated photooxidation. The decrease in turbidity might be due to swelling of mitochondria. It is possible that swelling of mitochondria may be induced by the endogenous swelling and uncoupling factors described above.

In parallel experiment, effect of succinate on the state of phloxine in mitochondria was examined by spectrophotometry. On addition of succinate, energy-dependent spectral change of phloxine was observed. Further study on this spectral change is now under investigation.

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