PHOTOPHOSPHORYLATION COUPLED TO THE REDUCTION OF INDOPHENOL DYES*

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Indophenol dyes have long been known to be reduced photochemically by chloroplasts at the highest observable rate (1, 2). However, unlike other Hill reagents (e.g. NADP or ferricyanide), the photoreduction of indophenol dyes was not found to be coupled to ATP synthesis (3-5). It had been, therefore, accepted that the reduction of indophenol dyes by chloroplasts is not accompanied by phosphorylation (cf. 6-9).

In experiments to be reported in detail elswhere (10) we concluded that oxidized indophenol dyes, at concentrations exceeding 1×10^{-4} M, uncoupled all photophosphorylations in chloroplasts. This conclusion prompted us to consider the possibility that the failure of previous workers to observe photophosphorylation accompanying the photoreduction of indophenol dyes was due to the use of uncoupling concentrations of indophenol.

Table 1 summarizes measurements of ATP formation accompanying the reduction of different initial concentrations of oxidized 2,6-dichlorophenol indophenol (DPIP). Under the conditions employed less than half of the DPIP was reduced at its lowest concentration. It can be seen that ATP was formed during the photoreduction of DPIP at concentrations below 1×10^{-4} M under air as well as under N_2. Similar results were obtained when 2,3',6-trichlorophenol indophenol substituted DPIP.

Photophosphorylation could be sustained in this system only during the reduction of the low concentrations of DPIP. Therefore, only very short periods with low chlorophyll concentrations could be used. It was thought that the photoreduction and coupled phosphorylation might be maintained for longer periods if the reduced dye could be reoxidized in the reaction mixture by the addition of an inert terminal electron acceptor. Manganese dioxide

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was suggested for such a function by Hochster and Quastel (12), and used with chloroplasts by Lumry and Spikes (13).

Table 1

PHOTOPHOSPHORYLATION COUPLED TO DPIP REDUCTION

Initial concentration of oxidized DPIP	Specific activity	
	Under N_2	Under air
(M)	µmoles ATP formed/mg chlorophyll/hr	
0	5	16
5×10^{-6}	163	186
1×10^{-5}	108	163
2×10^{-5}	105	186
5×10^{-5}	69	138
1×10^{-4}	49	99
2×10^{-4}	16	36

The experiments were performed with once washed swiss-chard chloroplasts prepared according to Avron (11), but washed in a medium without ascorbate. ATP formation was assayed by the method of Avron (11).

The reaction system contained in µmoles: Tris-HCl, pH 7.8 - 45; NaCl - 60; $MgCl_2$ - 12; Na,K phosphate - 12; P^{32} - 1.9×10^7 c/m; ADP - 4; chloroplasts containing 5 µg chlorophyll, and water to a total volume of 3.0 ml. The flasks were allowed to equilibrate in an Aminco photosynthetic Warburg apparatus at 20°C for 10 min., before illumination. During the equilibration and reaction periods, water saturated N_2 was continuously flushed through the "under N_2" flasks. The tank N_2 used was analysed by mass spectrophotometry and found to contain no less than 99.95% N_2. Reactions were run for 20 sec. in the light (50,000 lux), in 25 ml Erlenmeyer flasks.

When used at the proper concentration, manganese dioxide was found to reoxidize reduced DPIP without by itself inducing photophosphorylation. Fig. 1 illustrates typical time courses of ATP formation induced by DPIP in the presence or absence of manganese dioxide. It can be seen that manganese dioxide lengthened the period of ATP formation from one to more than six minutes. Since the manganese dioxide continually reoxidized the indophenol dye, (as indicated also by the blue color), it is evident that in this case the production of ATP was coupled to the reduction of the indophenol dye.

If photophosphorylation was indeed coupled to the reduction of indophenol dyes, it may be expected that the addition of phosphorylating reagents or an uncoupler should stimulate the rate of indophenol reduction, as had been observed with ferricyanide (14, 15). Table 2 illustrates that this indeed was the case. In several experiments the rate of DPIP reduction was stimulated up to

two fold by the addition of the phosphorylating reagents. For maximal stimulation, all three factors were necessary. In the experiment summarized in Table 2 the rate of ferricyanide reduction was stimulated 2.7 fold. Ammonium chloride and methylamine hydrochloride, both known to uncouple photophosphorylation (15, 17), highly stimulated the rate of indophenol reduction. Also atebrin, at a concentration which virtually completely uncoupled all known types of photophosphorylation (18), highly stimulated the rate of DPIP reduction.

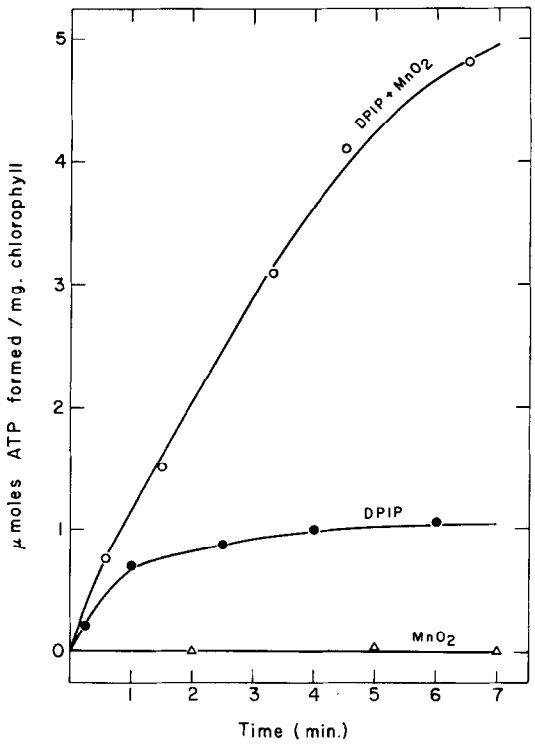

Fig. 1. The effect of manganese dioxide on photophosphorylation couples to DPIP reduction.

The reaction system was as in Table 1, but with 22 μg chlorophyll, and, when added, 2×10^{-5} M DPIP and or 0.2 mg manganese dioxide prepared according to Hochster and Quastel (11).

Similar, but much smaller, stimulations of indophenol dye reduction were previously observed (2, 14). The degree of stimulation achieved in different experiments seemed to be dependent on the degree of uncoupling caused during the preparation of the chloroplasts. This may account for the rapid rates of reduction and low stimulation reported for indophenol dyes by previous workers (2, 14).

Additional evidence that the photophosphorylation described in this communication was strictly of a "non-cyclic" type (7), was its extreme sensi-

Table 2

THE EFFECT OF PHOSPHORYLATING REAGENTS OR UNCOUPLERS ON DPIP REDUCTION

Additions	DPIP reduction
	%
-	100
Mg	107
ADP	83
Phosphate	100
Mg + ADP	100
Mg + phosphate	93
ADP + phosphate	93
Mg + ADP + phosphate	190
NH_4Cl - 5×10^{-4} M	207
$CH_3NH_2.HCl$ - 1.2×10^{-2} M	282
Atebrin - 5×10^{-5} M	300
Atebrin + Mg + ADP + phosphate	268

The reaction system contained in umoles: Tris-HCl, pH 7.8 - 45; NaCl - 60; DPIP - 0.06; chloroplasts containing 10 µg of chlorophyll; and when added, $MgCl_2$ - 12; Na,K phosphate - 12; ADP - 2; and water to a total volume of 3.0 ml. The assays were run in cuvettes for 20 sec. with 80,000 lux of light at 20°C. A value of 100 corresponds to 170 µmoles of indophenol reduced per mg. chlorophyll/hr. DPIP reduction was followed by the change in optical density at 620 mµ (16).

tivity to 3-(3,4-dichlorophenol)-1,1-dimethylurea (DCMU) (Table 3, lines 1,2). In this respect, it was similar to ferricyanide dependent photophosphorylation (19), and different from the indophenol dependent phosphorylation described by krogmann (2, 5, 20), Avron (21) and Trebst and Eck (8). The data also illustrate that the optimum indophenol dye concentration was markedly different in the DCMU sensitive and DCMU insensitive (actually - stimulated) phosphorylations. Stimulation of cyclic photophosphorylation by DCMU (Table 3, lines 3-6) have been noted previously in the phenazine methosulphate catalyzed system (19).

In conclusion it is believed, despite previous indications (6-9), that the results presented strongly support the inclusion of indophenol dyes with the other electron acceptors whose photoreduction is coupled to phosphorylation. However, since high concentrations of indophenol dyes uncouple photophosphorylation (10), the coupling can be observed only under controlled conditions.

Table 3

THE EFFECT OF DCMU ON PHOTOPHOSPHORYLATION INDUCED BY OXIDIZED OR REDUCED DPIP

Additions	DCMU	Specific activity
		(μmoles ATP formed/mg chlorophyll/hr)
1. DPIP (2×10^{-5} M) + MnO_2	-	87
2. DPIP (2×10^{-5} M) + MnO_2	+	1
3. DPIP (2×10^{-5} M) + NADH	-	1
4. DPIP (2×10^{-5} M) + NADH	+	14
5. DPIP (2×10^{-4} M) + NADH	-	29
6. DPIP (2×10^{-4} M) + NADH	+	54

Conditions as in Table 1, but with P^{32} - 4.8×10^{6} c/m, 27 μg chlorophyll, and when indicated 0.2 mg MnO_2, 4 μmoles NADH, and 5×10^{-7} M DCMU. The reactions were run for 3 min., under N_2.

This interpretation provides an explanation for several previous observations on the effect of DPIP in chloroplast reactions. For example, Witt et al. (22, 23) found that the rate of disappearance of a light-induced absorption increase at 515 mμ was greatly enhanced by a high concentration of DPIP (2×10^{-4} M , which is uncoupling), but not by ferricyanide, benzoquinone, or low concentrations of DPIP (below 10^{-5} M, which mediates ATP formation). Also, Whittingham and Bishop (9) observed that ferricyanide in the presence of an uncoupler behaved similarly to a high concentration of DPIP (2×10^{-4} M).

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