

## Uncoupling Agents and Photophosphorylation in Chromatophores and Chloroplasts

HERRICK BALTSCHIEFFSKY

*Wenner-Gren Institute and Department of Biochemistry, University of Stockholm, Stockholm, Sweden*

Photophosphorylation in chromatophores from *Rhodospirillum rubrum* and in spinach chloroplasts was inhibited by two agents which are known to uncouple oxidative phosphorylation in mitochondria, namely 2,6-dinitro-4-isooctylphenol (octyl-DNP) and desaspidin.

In bacterial chromatophores both uncoupling agents inhibited photophosphorylation at comparatively low concentrations. This was the case both in the "physiological system", where no electron carrier had been added to the reaction mixture, and in the "phenazine methosulfate-system", after inhibition of the "physiological system" with 2-heptyl-4-hydroxyquinoline-N-oxide (HOQNO) and "bypassing" this inhibition by addition of phenazine methosulfate.

In chloroplasts, cyclic photophosphorylation in the presence of added phenazine methosulfate and "pseudocyclic" photophosphorylation in the presence of added flavin mononucleotide (FMN) were inhibited by comparatively low concentrations of octyl-DNP. An uncoupling action by this agent was demonstrated with the non-cyclic system for electron transport from water to ferricyanide. Cyclic photophosphorylation in the presence of phenazine methosulfate was inhibited by low concentrations of desaspidin. "Pseudocyclic" photophosphorylation in the presence of FMN required about a 100 times as high concentrations of desaspidin for inhibition as cyclic photophosphorylation. This indicates that desaspidin may specifically uncouple only the latter system and that the two systems may contain different coupling sites with at least partly different energy transfer reactions.

It is well known that electron transport phosphorylation in isolated sub-cellular structures can be acted upon in a specific manner by different kinds of interfering agents such as 1) inhibitors of electron transport, 2) inhibitors of energy and phosphate transfer, and 3) uncoupling agents. By systematic use of several agents, which taken together give a broad spectrum of inhibitory or uncoupling effects, a possibility might be opened to identify common denominators of essential reactions in biological electron transport phosphorylation and to separate them from such reactions, which in any given system

are essential only for that system. Obviously, certain differences may be expected in systems with such grossly different functions as oxidative phosphorylation and photophosphorylation. It is thus not surprising, that in response to some specific interfering agents such differences indeed appear to exist and to reside in regions of the light-reactions in the photosynthetic systems, whereas the same agents have been found to interact in a similar manner on oxidative phosphorylation and the regions of the dark reactions in photophosphorylation.<sup>1,2</sup>

The effects of two agents, desaspidin (a phlorobutyrophenone derivative) and 2,6-dinitro-4-isooctylphenol (octyl-DNP), which both uncouple oxidative phosphorylation in animal mitochondria,<sup>3,4</sup> have been tested on photophosphorylation in spinach chloroplasts and chromatophores from the photosynthetic bacterium *Rhodospirillum rubrum*. In this paper the results which have been obtained are presented and discussed. Brief accounts of the work<sup>5,6</sup> and an extension of it<sup>7</sup> have been given.

Avron and Shavit<sup>8</sup> have recently discussed uncoupling of photophosphorylation in chloroplasts in some detail.

#### MATERIALS AND METHODS\*

Octyl-DNP was obtained from Dr. Hemker, Laboratory of Physiological Chemistry, University of Amsterdam, The Netherlands, and desaspidin from Dr. Runeberg, Department of Medical Chemistry, University of Helsinki, Finland. These gifts are gratefully acknowledged. The phenol was dissolved in ethanol and desaspidin in alkaline solution,

*Table 1.* Effects of octyldinitrophenol on photophosphorylation in chloroplasts and chromatophores. *Chloroplasts.* Each tube contained 1 ml 0.8 M tris(hydroxymethyl)-aminomethane pH 8.0, 10  $\mu$ moles  $K_2H^{32}PO_4$ , 10  $\mu$ moles ATP, 30  $\mu$ moles  $MgCl_2$ , 60  $\mu$ moles glucose, an excess of hexokinase, and, where added, 0.4  $\mu$ moles of FMN and 0.06  $\mu$ moles of phenazine methosulfate. Time of experiment: 6 min. 0.12 mg chlorophyll/3 ml (final volume). *Chromatophores.* Each tube contained 1.5 ml 0.2 M glycylglycine pH 7.5, 10  $\mu$ moles  $K_2H^{32}PO_4$ , 10  $\mu$ moles ATP, 30  $\mu$ moles  $MgCl_2$ , 60  $\mu$ moles glucose and an excess of hexokinase. "OD<sub>800</sub>" was 0.1 for 0.1 ml "chromatophore fragments" in the final volume of 3.0 ml. The "PMS-system" (phenazine methosulfate-system) contained 10<sup>-6</sup> M HOQNO and 10<sup>-4</sup> M phenazine methosulfate. Time of experiment: 20 min in the "physiological system" and 6 min in the "phenazine methosulfate-system". All the values given in the table were obtained after subtraction with the dark controls, which were always very low.

Addition Octyl-DNP (moles/l)	Chloroplasts		Chromatophores	
	% P <sub>i</sub> esterified		% P <sub>i</sub> esterified	
	FMN	phenazine methosulfate	"physiological system"	"PMS- system"
—	10.3	12.7	8.8	22.2
10 <sup>-5</sup>	0.7	1.6	1.4	8.1
10 <sup>-4</sup>	0	0	0	0

\* Abbreviations: ATP, adenosine triphosphate; FMN, flavin mononucleotide; PMS, phenazine methosulfate; DNP, 2,4-dinitrophenol; octyl-DNP, 2,6-dinitro-4-isooctylphenol; HOQNO, 2-heptyl-4-hydroxyquinoline-N-oxide.

which was subsequently neutralized to a pH of about 8.5 (turbidity occurred at lower pH-values). Care was taken to avoid effects of ethanol or pH-changes.

Standard methods were used for preparation of chloroplast fragments from spinach,<sup>9</sup> which was obtained from local growers, and chromatophores<sup>10</sup> (the "chromatophore fragments" fraction) from *Rhodospirillum rubrum*, Strain 1. The bacteria were grown in 2 l bottles in synthetic medium,<sup>11</sup> at a light intensity of about 30 000 lux and a temperature of 30°C. The photophosphorylation experiments were performed in the same media and under the same conditions as in earlier studies, both with chloroplasts<sup>12</sup> and chromatophores,<sup>10</sup> unless otherwise mentioned.

Non-cyclic photophosphorylation in the chloroplast system with electrons transported from water to added ferricyanide was performed as in Ref. 13.

### RESULTS

As is shown in Table 1, octyl-DNP at a final concentration of  $10^{-5}$  M strongly inhibited photophosphorylation in the chloroplast and chromatophore systems tested. Complete inhibition was obtained at  $10^{-4}$  M concentration of the uncoupling agent, which thus acts on these systems at much lower concentration than DNP.<sup>14-18</sup> As the pathways for electrons in the systems tested do not allow measurements of rates of electron transport and a distinction between inhibition of the total process of photophosphorylation and uncoupling, the effect of octyl-DNP on photophosphorylation coupled to the non-cyclic transport of electrons from water to ferricyanide was investigated. It was found that an uncoupling action existed in this system,

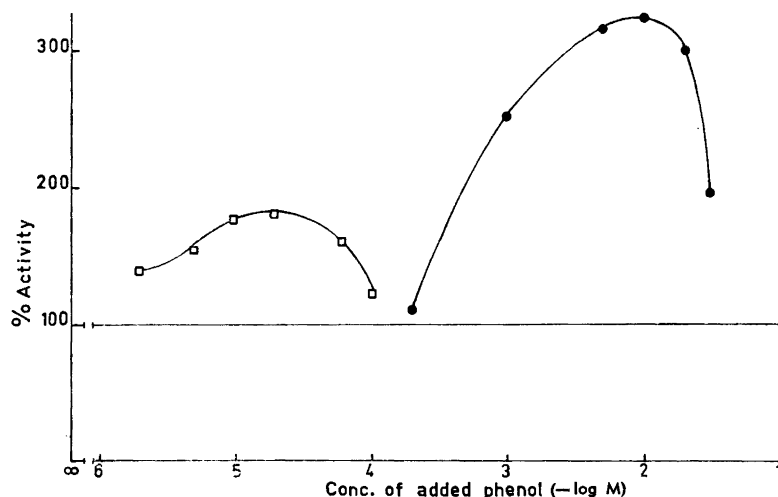


Fig. 1. Effects of DNP and octyl-DNP on ATP-ase activity in chromatophores. ● = DNP; □ = octyl-DNP. Each tube contained 0.3 ml 0.1 M tris(hydroxymethyl)-amino-methane pH 7.5, 10  $\mu$ moles ATP, 8  $\mu$ moles  $MgCl_2$ , and chromatophores to give, in final dilution, an absorbancy at 800  $m\mu$  of 0.625 in the experiment with DNP and 0.850 in the experiment with octyl-DNP. The experiment was performed in the dark. Reaction time: 20 min. Temperature: 30°C. 100 % activity (obtained with no phenol added) in the experiment with DNP was 1.48 and with octyl-DNP 1.65  $\mu$ moles ATP hydrolyzed/OD<sub>800</sub>/h. Orthophosphate was analyzed with the standard molybdate-method, using  $SnCl_2$  and reading the absorbancy at 660  $m\mu$  in a Beckman Model B spectrophotometer.<sup>23</sup>

as  $10^{-4}$  M octyl-DNP caused a decrease of about 90 % in the rate of phosphate esterification but only about 50 % in the rate of electron transport.

In order to obtain more information about the actions of DNP and octyl-DNP on photophosphorylation systems, these agents were tested on the dark ATP-ase reaction in chromatophores. The result is shown in Fig. 1, where a marked stimulation of the ATP-ase activity is seen at the concentration ranges where the agents act on photophosphorylation. This pattern thus appears to be analogous to the well-known DNP-induced ATP-ase reaction in phosphorylating mitochondria.

The effects of desaspidin on cyclic (PMS added) and "pseudocyclic" (FMN added) photophosphorylation in chloroplasts is shown in Fig. 2. A drastic difference between the sensitivities of the two tested systems is seen. The unusual situation that the phenazine methosulfate-system, on which low concentrations of desaspidin are active, is the most sensitive one, will be further discussed below. As Fig. 3 shows, low concentrations of desaspidin inhibit both the chromatophore systems, of which the "phenazine methosulfate system" is the less sensitive one.

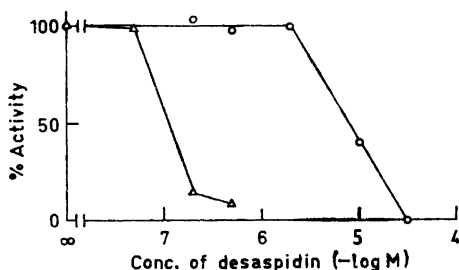
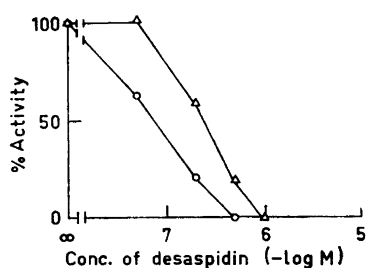


Fig. 2. Effect of desaspidin on photophosphorylation in chloroplasts. O = "pseudocyclic" photophosphorylation in the presence of FMN;  $\Delta$  = cyclic photophosphorylation in the presence of phenazine methosulfate. Conditions were as in the experiment with chloroplasts in Table 1, except that the chlorophyll concentration per tube was 0.05 mg/3 ml.



speculations that the mechanisms for electron transport coupled energy transfer in oxidative phosphorylation and photophosphorylation are basically different.<sup>19</sup> Also in recent studies, where a stronger effect of this agent has been reported to occur in certain chloroplast systems,<sup>20</sup> the concentrations required have been higher than those which completely uncouple oxidative phosphorylation in mitochondria. Our data show that in agreement with the situation found in mitochondria, octyl-DNP is acting upon photophosphorylation in the systems tested at concentrations which are two to three orders of magnitude lower than those of DNP necessary, and that, on the other hand, the required concentration of octyl-DNP in the photophosphorylation systems tested is even somewhat higher than that of DNP in oxidative phosphorylation. On the basis of this over-all picture the concept may be advanced that differences in solubilization or distribution between phases may be important factors to consider when comparing the effects of nitrophenols on oxidative phosphorylation and photophosphorylation. The data from the ATP-ase experiments, which show a stimulation of ATP-ase activity by the nitrophenols at concentrations affecting photophosphorylation, provide indirect but independent support for this view.

*On the effects of desaspidin on photophosphorylation.* Desaspidin inhibited photophosphorylation in chromatophores in both the "physiological system" and the "phenazine methosulfate-system" in the same concentration range as was earlier found by Runeberg<sup>3</sup> to uncouple oxidative phosphorylation. On the other hand, in chloroplasts a factor of about 100 separated the concentrations required for 50 % inhibition of phosphate uptake in the presence of phenazine methosulfate and those required in the presence of FMN. The phenazine methosulfate-system was the more sensitive one, and was inhibited at concentrations similar to those acting on bacterial photophosphorylation. Avron and Shavit<sup>8</sup> reported that ferricyanide-dependent photophosphorylation in chloroplasts from Swiss-chard is more sensitive to carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone than PMS-dependent photophosphorylation, and suggested that different coupling sites may be involved in the two systems. The difference between the sensitivities of the two chloroplast systems to desaspidin was tentatively assumed to reflect a basic difference in the energy transfer mechanisms of ATP-formation in these systems. Much stronger experimental evidence had, however, to be obtained in order to give any satisfactory weight to this assumption, which implies that the two chloroplast systems under investigation contain different coupling sites. Such evidence was indeed obtained in subsequent studies in collaboration with Miss D.Y. de Kiewiet.<sup>7,13,21</sup>

The unusual situation in chloroplasts, that the "phenazine methosulfate-pathway" is particularly sensitive to an added interfering agent, has been observed also with another agent. Burris and Plengvidhya<sup>22</sup> reported that valacidin inhibits photophosphorylation in their phenazine methosulfate system but has little effect on their system with 2,6-dichlorophenol indophenol as cofactor.

## REFERENCES

1. Baltscheffsky, H. and Arwidsson, B. *Biochim. Biophys. Acta* **65** (1962) 425.
2. Baltscheffsky, H. *Federation of European Biochemical Societies, 2nd Meeting, Abstracts*, Vienna 1965, p. 110.
3. Runeberg, L. *Societas Scientiarum Fennica. Commentationes Biologicae XXVI, 7. Diss.*, Helsinki 1963.
4. Hemker, H.C. *Biochim. Biophys. Acta* **63** (1962) 46.
5. Baltscheffsky, H. *Federation of European Biochemical Societies, 1st Meeting. Abstracts*, London 1964, p. 66.
6. Baltscheffsky, H. and Baltscheffsky, M. *Sixth International Congress of Biochemistry, New York, Abstracts, X*. 1964, p. 773.
7. Baltscheffsky, H. and de Kiewiet, D.Y. *Acta Chem. Scand.* **18** (1964) 2406.
8. Avron, M. and Shavit, N. In *Photosynthetic Mechanisms of Green Plants*, NAS. NRC Publication 1145, Library of Congress 63 - 65396, Washington 1963, p. 611.
9. Allen, M.B., Whatley, F.R. and Arnon, D.I. *Biochim. Biophys. Acta* **27** (1958) 16.
10. Baltscheffsky, H. *Biochim. Biophys. Acta* **40** (1960) 1.
11. Bose, S.K., Gest, H. and Ormerod, J.G. *J. Biol. Chem.* **236** (1961) PC 13.
12. Baltscheffsky, H. *Acta Chem. Scand.* **14** (1960) 264.
13. Baltscheffsky, H. and de Kiewiet, D.Y. *Biochim. Biophys. Acta. In press.*
14. Baltscheffsky, H. and Baltscheffsky, M. *Acta Chem. Scand.* **14** (1960) 257.
15. Arnon, D.I., Allen, M.B. and Whatley, F.R. *Biochim. Biophys. Acta* **20** (1956) 449.
16. Ohmura, T. *J. Biochem. (Tokyo)* **45** (1958) 319.
17. Krogmann, D.W., Jagendorf, A.T. and Avron, M. *Plant Physiol.* **34** (1959) 272.
18. Avron, M. *Biochim. Biophys. Acta* **40** (1960) 257.
19. Jagendorf, A.T. *Brookhaven Symp. Biol.* **11** (1959) 236.
20. Tagawa, K., Tsujimoto, H.Y. and Arnon, D. I. *Proc. Natl. Acad. Sci. U.S.* **50** (1963) 544.
21. de Kiewiet, D.Y. and Baltscheffsky, H. *Biochim. Biophys. Acta. In press.*
22. Burris, R.H. and Prachote Plengvidhya, *Sixth International Congress of Biochemistry, New York, Abstracts, X*. 1964, p. 775.
23. Siekewitz, P., Löw, H., Ernster, L. and Lindberg, O. *Biochim. Biophys. Acta* **29** (1958) 378.

Received June 22, 1965.