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Inhibitor Studies on Light-Induced Phosphorylation in Isolated Spinach Chloroplasts

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Light-induced phosphorylation in isolated chloroplasts is greatly stimulated by the addition of several types of compounds capable of undergoing reduction and oxidation [1-3], among them flavin nucleotides. Whether the stimulatory agent is a flavin or not, 4×10^{-5} M atebrin * completely or almost completely inhibits the phosphorylation, indicating that endogenous chloroplast flavin is an obligatory participant in this system. The inhibition is not reversed by high concentrations of flavin nucleotide. In fact, flavin adenine dinucleotide (2×10^{-3} M) gives nearly complete and flavin mononucleotide (5×10^{-3} M) partial inhibition of phosphate esterification.

Amytal ** gives only slight inhibition.

Low concentrations of antimycin A, 2-*n*-heptyl-4-hydroxyquinoline-N-oxide and oligomycin A have no inhibitory effect on light-induced phosphorylation in isolated spinach chloroplasts comparable to the effects on light-induced phosphorylation in a bacterial *(Rhodospirillum rubrum)* extract and respiration and phosphorylation in animal mitochondria. The hypothesis [2] that added compounds effective as cofactors, such as FMN, menadione and phenazine methosulfate, may bridge over a gap in electron transport of the chloroplasts as they are presently isolated, is discussed.

Light-induced phosphorylation † (LIP) in isolated spinach chloroplasts is stimulated by both physiological electron carriers and non-physiological redox-compounds [1-3]. This and other evidence indicates that the phosphorylation process is linked to electron transport. Several schemes for the electron transport have been suggested [4-8]. In the present paper results of inhibitor studies on LIP in isolated spinach chloroplasts are presented and discussed.

Some data have already been given in an abstract [9].

* 3-Chloro-7-methoxy-9-(1-methyl-4-diethylamino-butylamino) acridine.

** 5-Ethyl-5-*iso*amylbarbiturate.

† This term as used here is equivalent to "cyclic photophosphorylation" [4].

MATERIALS AND METHODS

ATP *, FAD, FMN, PMS and antimycin A were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. The FAD was of the 80–90 % pure type. Gramicidin D was obtained from Mann Research Laboratories, New York, N.Y., U.S.A. HOQNO was obtained from Dr. J. W. Cornforth, National Institute for Medical Research, London, England, and oligomycin A from Dr. F. M. Strong, Department of Biochemistry, University of Wisconsin, Madison, Wisc., U.S.A.; these gifts are gratefully acknowledged. Hexokinase was prepared and stored as in Ref.[10]. Antimycin A, HOQNO, gramicidin and oligomycin A were dissolved in ethanol. The stock solutions were kept in a deep-freeze. In the reaction medium for LIP the ethanol content was kept below 1 % in order not to inhibit the LIP. In such a medium, antimycin A occasionally did not keep solubilized when used in high concentrations, in which case the medium was discarded. The molecular weight of antimycin A was taken as 548 (*cf.* Ref.[11]).

Spinach was obtained as whole spinach plants from local growers. The chloroplasts were isolated according to Allen, Whatley and Arnon [12], with one washing and subsequent fragmentation of the whole chloroplasts by addition of hypotonic (0.035 M) NaCl. The chlorophyll content was determined according to Arnon [13]. All experiments were performed immediately after the preparation was finished. The medium employed for the phosphorylation experiments contained, unless otherwise noted, 1.0 ml 0.08 M tris-(hydroxymethyl)-aminomethane pH 8.3, 8 μmoles $K_2H^{32}PO_4$, 10 μmoles ATP, 30 μmoles $MgCl_2$, 60 μmoles glucose and an excess of yeast hexokinase. The final volume was 3.0 ml. The experiments were performed under aerobic conditions in test tubes which were immersed in a water-bath of 20°C. The light source consisted of a row of incandescent 150 W Philips lamps, adjusted to give a light intensity of about 30 000 lux at the reaction tubes. The reaction was stopped with 1 ml of 2 M perchloric acid.

Phosphorylation was measured by the ^{32}P method recommended by Lindberg and Ernster [14]. In all experiments two controls were run. In one of them the reaction was stopped at zero time. The other one was a dark control which was wrapped in aluminium foil and run parallel with the other tubes. The low amount of phosphate esterified in the dark was used as a zero for the LIP values.

Ascorbate gives a very low stimulation of LIP under aerobic conditions [15] but has nevertheless been used as a stimulatory agent in some of the experiments with inhibitors. It is also a stabilizer for the isolated chloroplasts [2,6] and has thus been added in some experiments along with other stimulating agents.

RESULTS

To obtain conveniently measurable rates of LIP in isolated spinach chloroplasts it is necessary to add one of a number of suitable stimulatory agents to the reaction medium. The following stimulatory agents have been employed in the present work: phenazine methosulfate [3], FMN [15], FAD, menadione [1], DNP [16] and ascorbate [15].

In Fig. 1 a typical time curve for LIP is given. It shows that 6 min, which has also been used by Jagendorf and Avron [3], is a suitable reaction time.

Amytal gave only a weak inhibition of LIP in chloroplasts with any of the stimulating agents used, even at concentrations several times higher than those which strongly inhibit the oxidation of DPN-linked substrates in animal mitochondria [17] (Fig. 2).

* Abbreviations: P, orthophosphate; ATP, adenosine triphosphate; FAD, flavinadenine dinucleotide; FMN, flavin mononucleotide; PMS, phenazine methosulfate; HOQNO, 2-*n*-heptyl-4-hydroxyquinoline-N-oxide; DNP, 2,4-dinitrophenol; M, moles per liter; % P_{org}, percentage ortophosphate esterified.

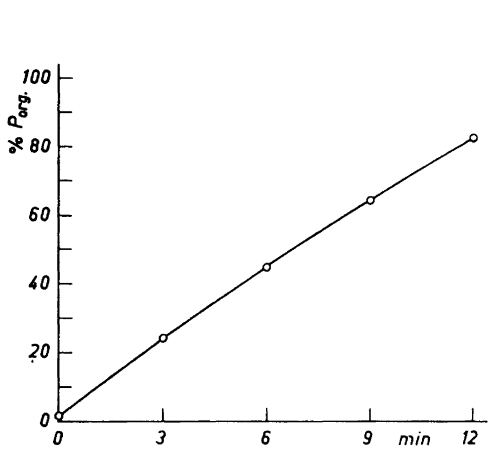


Fig. 1. Time curve of LIP stimulated by FMN. All tubes contained 30 μmoles ascorbate, 0.4 μmoles FMN and 0.16 mg chlorophyll.

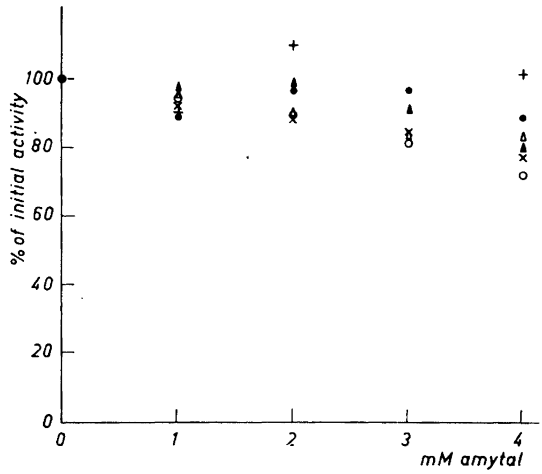


Fig. 2. The effect of amytal on LIP in the presence of various stimulatory agents. × = FMN, △ = PMS, ▲ = DNP, ○ = FAD, ● = menadione and + = ascorbate. The PMS- and menadione-series represent mean values of three, respectively two, different experiments. With the exception of the PMS-series all tubes contained 30 μmoles of ascorbate.

Atebrin, a flavoenzyme antagonist [18], inhibited LIP strongly (Fig. 3) regardless of the stimulatory agent used. The inhibition occured at concentrations of atebrin which were of the same order of magnitude as those required for inhibition of LIP in extracts of *Rhodospirillum rubrum* [19].

In extracts of *R. rubrum* the inhibition obtained with atebrin could be overcome completely with FAD and partially with FMN [19]. With chloroplasts such an effect did not appear. On the contrary it was found that the corres-

Table 1. Effects of FMN and FAD on LIP. All tubes contained 30 μmoles ascorbate. The chlorophyll content in each tube was with FAD 0.066 mg., with FMN 0.072 mg. (Repeated experiments confirmed the inhibition at 2×10^{-3} and 5×10^{-3} M FMN.)

Concentration of FMN or FAD M	% P_{org} FMN	FAD
—	5	5
5×10^{-5}	53	30
1.5×10^{-4}	51	29
6×10^{-4}	57	17
2×10^{-3}	47	7
5×10^{-3}	44	5

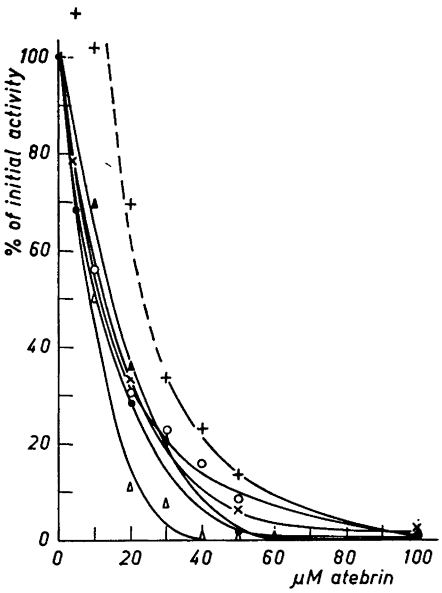


Fig. 3. The effect of atebrin on LIP in the presence of various stimulatory agents. × = FMN, △ = PMS, ▲ = DNP, O = FAD, ● = menadione and + = ascorbate. With the exception of the FAD- and PMS-series all tubes contained 30 μmoles of ascorbate.

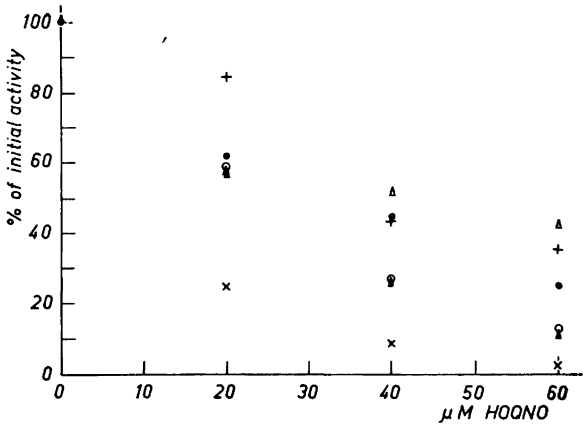


Fig. 4. The effect of HOQNO on LIP in the presence of various stimulatory agents. × = FMN, △ = PMS, ▲ = DNP, O = FAD, ● = menadione and + = ascorbate.

ponding concentrations of the flavin nucleotides inhibited the LIP in chloroplasts, the inhibition due to FAD being very strong (Table 1). The strong inhibitory effect of 2 mM FAD (Table 1) was obtained also when the LIP had been stimulated with PMS or menadione. The inhibition in one experiment was more than 95 % in the former and more than 75 % in the latter case.

Table 2. Effect of antimycin A on LIP, stimulated by FMN, PMS or menadione. With the exception of the PMS-series all tubes contained 30 μmoles ascorbate. Where added: 0.4 μmoles FMN, 0.06 μmoles PMS or 0.3 μmoles menadione. In the FMN-, PMS- and menadione-series, respectively, values for 100 % initial activity was 18, 33 and 56 % P_{org} and the chlorophyll content 0.044, 0.044 and 0.105 mg.

Concentration of antimycin A M	Percentage of initial activity		
	FMN	PMS	menadione
—	100	100	100
2×10^{-5}	83	78	—
5×10^{-5}	24	19	32

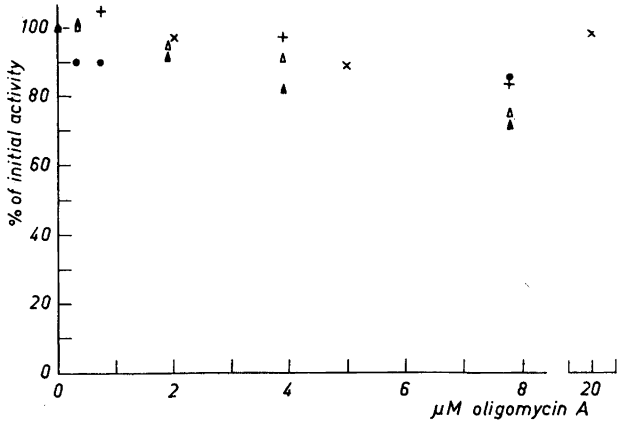


Fig. 5. The effect of oligomycin A on LIP in the presence of various stimulatory agents. △ = PMS, ▲ = DNP, ● = menadione, X = FMN and + = ascorbate. With the exception of the PMS-series all tubes contained 30 μmoles ascorbate. The chlorophyll content in each tube was for the different series taken in the above order, 0.12, 0.28, 0.10, 0.13 and 0.35 mg. The 100 % initial activity was, in the same order, 42, 48, 54, 57 and 9 % P_{org}. These values are included as examples of the rates of LIP obtained and may be regarded as representative, although the rate with menadione was unusually high and although considerable variations occured. (FAD gave rates similar to or somewhat lower than FMN.)

Antimycin A and HOQNO have been shown to inhibit LIP in extracts of *R. rubrum* [20] at similar concentrations as they inhibit electron transport in mitochondrial systems [21,11]. According to Arnon, Allen and Whatley [22] antimycin A does not inhibit LIP in spinach chloroplasts (in anaerobic experiments). No studies with HOQNO have been reported. Also under aerobic conditions LIP was unaffected by low concentrations of antimycin A as well as of HOQNO. As Table 2 and Fig. 4 show, both antimycin A and HOQNO inhibited LIP in chloroplasts at concentrations only as high as between 10^{-5} and 10^{-4} M.

Oligomycin A, which inhibits oxidative phosphorylation in animal mitochondria and the respiration connected with it, but not the respiration induced by addition of DNP [23], gave no significant inhibition of LIP in spinach chloroplasts (Fig. 5). As has been reported, oligomycin A inhibits LIP in extracts of *R. rubrum* [20].

Whatley, Allen and Arnon [24] have shown that gramicidin, which is an uncoupler of oxidative phosphorylation, strongly inhibits LIP under anaerobic conditions. Table 3 shows that inhibition occurs also under aerobic conditions.

DISCUSSION

The path of electron transport in LIP of plant chloroplasts is still essentially unknown. A comparison of various schemes which have been proposed [4-8] shows that no general agreement has been reached in this field. In a recent scheme for LIP in green plants Arnon [4] proposes three different electron trans-

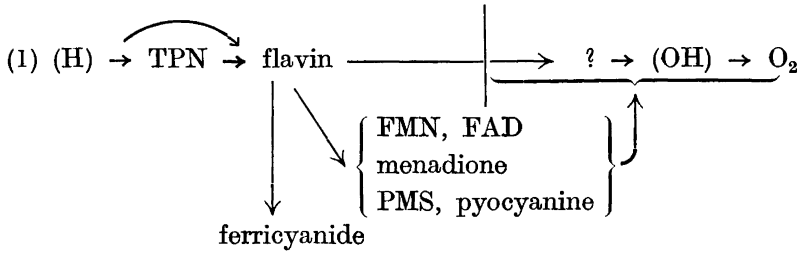
Table 3. The inhibition of LIP by gramicidin. All tubes contained 30 μmoles ascorbate. Where added: 0.4 μmoles FMN or 0.3 μmoles menadione. The chlorophyll content in each tube and the values for 100 % initial activity were as follows: In the FMN-series, experiment 1, 0.090 mg chlorophyll, 46.5 % P_{org}; experiment 2, 0.116 mg chlorophyll, 36.3 % P_{org}. In the menadione-series, experiment 1, 0.089 mg chlorophyll, 15.7 % P_{org}; experiment 2, 0.116 mg chlorophyll, 16.3 % P_{org}.

Concentration of gramicidin M	Percentage of initial activity FMN		menadione	
	expt. 1	expt. 2	expt. 1	expt. 2
—	100	100	100	100
10^{-7}	64	67	54	33
10^{-6}	36	32	9	12

port pathways, all coupled to phosphorylation: (1) the cyclic electron transport system involving vitamin K and a cytochrome, (2) the cyclic electron transport system involving TPN, FMN and cytochromes and (3) the "open" non-cyclic mechanism, which transports electrons from chlorophyll to TPN. According to Wessels [6] TPN is not a member of the cyclic electron carrier chain(s). Both Wessels [25] and Whatley, Allen and Arnon [24] have reported that FMN and menadione are involved in separate pathways of electron transport in LIP under aerobic conditions. Marré, Servettaz and Forti [7] have presented a scheme with five electron carriers, among them cytochrome c, in a single electron transport chain joining the oxidizing and reducing equivalents produced by the photolysis of water. Jagendorf and Avron [3] have pointed out that FMN and menadione as well as PMS may act by bridging a gap in electron transport of the chloroplasts as they are presently isolated.

The absence of a strong inhibition by amytal indicates that no electron transport step identical with that in phosphorylating mitochondria between DPNH and flavin exists in the system studied. A similar lack of inhibition by amytal has been found for bacterial LIP [20]. Arnon, Whatley and Allen [2] have earlier shown that DPN does not seem to be involved in LIP of chloroplasts.

The fact that atebrin inhibited LIP not only when FMN or FAD were added but also when menadione, PMS, DNP or ascorbate were used indicates that chloroplast flavin participated in the electron transport regardless of the nature of the stimulatory agent. When considered together with other results listed below, this finding supports scheme (1) for representation of electron transport in LIP of isolated spinach chloroplasts:



Cyclic electron transport in LIP is in this paper written in its classical form, combining reducing (H) and oxidizing (OH) equivalents from the Hill-reaction (cf Ref.[4]). The stimulatory agents in this scheme may be reoxidized by (OH) or, at least under aerobic conditions, O_2, or by any agent between these and the indicated block in the electron transport chain. Differences between the response of LIP towards various inhibitors when different stimulatory agents are employed may be due to different sites of reoxidation of the stimulatory agents (*cf.* Ref.[4]).

The following information is relevant for the scheme presented. (a) Light induces a phosphorylation concomitantly with the reduction of TPN, or of ferricyanide [26]. In both cases one molecule of ATP is reported to be formed during the transport of two electrons. For the reduction of TPN (but not for that of ferricyanide) an enzyme, photosynthetic pyridine nucleotide reductase [27,28], is necessary. This enzyme is isolated and purified from the supernatant of the chloroplast preparation. (b) Oxidation of photo-reduced TPN in chloroplasts is accomplished when any of the compounds FMN, menadione and pyocyanine is added [29]. (c) A TPNH-specific diaphorase, containing FAD [30], has been isolated from spinach chloroplasts [31]. Electrons from this diaphorase may be accepted by compounds such as potassium ferricyanide, menadione and FMN [32]. (d) High rates of LIP in spinach chloroplasts are obtained by addition of a stimulatory agent with oxidation-reduction properties, such as FMN, FAD, menadione, PMS or pyocyanine [32].

If electron transport between flavin and (OH) is blocked immediately on the oxidizing side of flavin in the isolated chloroplasts, then any physiological electron carrier between the point of inhibition and the point at which the added stimulatory agent is oxidized will remain undetected by inhibitor or spectrophotometric experiments. Four electron carriers which could be so situated have been found in chloroplasts: cyctochrome b_6[33], cytochrome f[34] (with a cytochrome c-like spectrum), vitamin K_1[35] and plastoquinone (Q_{254})[36,37] which is a quinone closely related to coenzyme Q. It is possible that such a by-pass might explain the resistence of LIP in isolated chloroplasts towards antimycin A and HOQNO. Another possibility is that the compound on which these inhibitors act in mitochondria and chromatophores is lacking in chloroplasts or has a structure modified so as to make it less sensitive to the inhibitors. They appear to act in the b-type cytochrome or coenzyme Q region in mitochondria and chromatophores, where they are effective in concentrations of less than one thousandth of what is needed to inhibit LIP in chloroplasts.

Hill [33] has reported an experiment which may support the concept of a block in electron transport of isolated chloroplasts on the reducing side of cytochrome b_6. With illuminated leaves of the "golden varieties" of certain plants he could observe the sharp absorption band of reduced cytochrome b_6 at 563 mμ. With chloroplasts prepared from such leaves, however, no reduced cytochrome b_6 was found, until the preparation was reduced with sodium dithionite.

It is to be observed that not only with respect to the effect of the electron transport inhibitors antimycin A and HOQNO does the electron transport phosphorylation system in isolated chloroplasts show differences from those

of bacterial chromatophores and of animal mitochondria. Low concentrations of oligomycin A, which is reported to block a reaction involved in phosphate transfer in mitochondrial oxidative phosphorylation [23] and inhibit LIP in bacterial chromatophores [20], have no or very small influence on LIP in isolated chloroplasts. These facts are the more remarkable as the two former systems have a similar sensitivity to these inhibitors. On the basis of present evidence it is not possible to decide whether the findings reflect inherent differences between the systems, or whether the sensitivity towards the inhibitors is lost in spinach chloroplasts during the preparation. However, the fact remains that the system for LIP in isolated plant chloroplasts differs in its response to inhibitors both of electron transport and of phosphate transfer from the system for LIP in bacterial chromatophores and the system for oxidative phosphorylation in animal mitochondria. It still seems necessary to strongly consider the possibility that the system for LIP in spinach chloroplasts is partially destroyed, as they are presently prepared.

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