FLAVIN NUCLEOTIDES AND LIGHT-INDUCED PHOSPHORYLATION IN CELL-FREE EXTRACTS OF *RHODOSPIRILLUM RUBRUM*

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SUMMARY

Light-induced phosphorylation (LIP) in extracts of the photosynthetic bacterium *Rhodospirillum rubrum* can be stimulated by addition of FAD, but not by addition of FMN. Atebrin, a flavin antagonist, strongly inhibits LIP. This inhibition can be completely overcome with FAD but only partially with FMN.

The inhibition of LIP by atebrin is equally strong in the presence of phenazine methosulfate, which stimulates LIP, as in its absence. Thus, in contrast to the case when antimycin A or 2-*n*-heptyl-4-hydroxyquinoline-N-oxide (HOQNO) are used as inhibitors, phenazine methosulfate does not provide a "by-pass" around the point of action of atebrin.

Antimycin A and HOQNO inhibit LIP almost completely both in the presence and in the absence of added FAD. This indicates that any electrons passing through FAD also pass through the site which is inhibited by antimycin A and HOQNO.

It is suggested that FAD is an electron transport carrier in LIP in cell-free extracts of *Rhodospirillum rubrum*.

INTRODUCTION

The light-induced phosphorylation (LIP) reactions in bacterial[1] and in plant[2] systems are assumed to be linked to electron transport. The main experimental reasons for this assumption are: (1) addition of various known electron carriers stimulate LIP[2-4], (2) typical respiratory chain inhibitors inhibit LIP[5-7] and, in extracts of a bacterium, the facultative phototroph *Rhodospirillum rubrum*, (3) results from spectrophotometric studies indicate the participation of cytochromes in LIP[8].

Knowledge about the details of electron transport in bacterial LIP is very limited. FRENKEL's[1] original scheme gives the sequence (H) $\rightarrow$ C $\rightarrow$ (OH), where C is the oxidized form of hypothetical electron carriers, which are coupled to LIP. (H) and (OH) are the symbols for the reduced and the oxidized product, respectively, of the bacterial equivalent to the Hill reaction. SMITH AND M. BALTSCHEFFSKY[5] have shown that HOQNO in low concentrations strongly inhibits LIP in *Rhodospirillum rubrum*, and GELLER[6] has obtained the same effect with antimycin A. As SMITH AND M.

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

BALTSCHEFFSKY[8] have demonstrated, C in FRENKEL's scheme appears to include a *b*-type cytochrome and cytochrome c_2. In the presence of HOQNO they observed that the *b*-type cytochrome became reduced and cytochrome c_2 oxidized, indicating that the inhibitor blocked electron transport from the *b*-type cytochrome to cytochrome c_2. CHANCE[9] has shown that both antimycin A and HOQNO block the respiration in animal mitochondria between cytochrome *b* and cytochrome c_1, and the assumption has been made[7] that the point of action of the two inhibitors is the same in the two electron transport systems.

No data have been found in the literature about a possible role of flavin compounds in bacterial LIP. The present paper is centered on the role of flavin nucleotides in LIP in cell-free extracts of *Rhodospirillum rubrum*. FAD but not FMN stimulates this LIP. Low concentrations of atebrin, a flavin inhibitor, inhibit LIP. FAD and to a lesser degree FMN counteract this inhibition. The above findings support the conclusion that flavin is involved in the electron transport of LIP in extracts of *R. rubrum*, and that the flavin in question is FAD.

MATERIALS AND METHODS

Materials

ATP, FAD and FMN were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. FAD was of the 80–90 % type. HOQNO was an appreciated gift from Dr. J. W. CORNFORTH, National Institute for Medical Research, London, England.

Culture of bacteria and preparation of cell-free extracts

Rhodospirillum rubrum, van Niel Strain S1, were obtained from Dr. J. M. OLSON at Brandeis University, Waltham, Mass., U.S.A. The bacteria were grown in completely filled glass-stoppered bottles under illumination from incandescent, 150-W Philips lamps, at 30°, in essentially the medium described by GEST, KAMEN AND BREGOFF[10]. The only differences were that the concentrations in mg/l of Difco yeast extract and of biotin were 500 and 0.010 instead of 250 and 0.005 respectively, in agreement with the advice of Dr. M. D. KAMEN at Brandeis University, Waltham, Mass., U.S.A. The bacteria were harvested in the resting phase.

To obtain the extracts the bacteria were first centrifuged down in an International Refrigerated Centrifuge, Model PR 1 (Head No. 840), for 10 min at 4,100 × *g**. The pellet was washed once with distilled water and recentrifuged as above. These operations were repeated once with 0.02 *M* glycylglycine pH 7.4 instead of distilled water. The pellet so obtained was weighed and ground in a porcelain mortar with a 2- to 3-fold excess of alumina (Alcoa A-301). After 3 min grinding at 0° the material was taken up in 0.2 *M* glycylglycine buffer (about 3 ml buffer per g pellet) and centrifuged in the Spinco refrigerated centrifuge, for 10 min at 10,000 × *g*, in rotor 40, which also was used for the subsequent centrifugations. The supernatant from this centrifugation** was recentrifuged for 60 min at 25,000 × *g*. The sediment was

* The values given are for the bottom of the tube with the International Refrigerated Centrifuge and the middle of the tube with the Spinco Refrigerated Centrifuge.

** This material had been used in the work described in a preliminary, short communication on LIP in *R. rubrum*[7]. In contrast to the other inhibitors, atebrin did not in the long run give reproducible effects with this "crude extract". Thus the value for atebrin inhibition in the above-mentioned communication is not valid.

then washed once in 0.2 *M* glycylglycine and recentrifuged for 60 min at 25,000 × *g*. This sediment was taken up in a small volume of 0.2 *M* glycylglycine and stored in the dark at 0° as the "chromatophores" containing fraction.

The supernatant from the first centrifugation at 25,000 × *g* was centrifuged for 90 min at 100,000 × *g*. The sediment from this centrifugation was washed once in 0.2 *M* glycylglycine and recentrifuged for 90 min at 100,000 × *g*. This sediment was taken up in a small volume of 0.2 *M* glycylglycine and stored in the dark at 0° as the "chromatophore fragments" containing fraction. (The names of the fractions should be regarded as functional only; they do not imply any degree of homogeneity.)

Measurement of LIP

The amount of phosphate esterified in the LIP experiments was measured by the ^{32}P method recommended by LINDBERG AND ERNSTER[11]. FRENKEL[1] has shown that the ATP formed in LIP is equivalent to the amount of inorganic phosphate that has disappeared. This was confirmed by paper chromatography* employing the method of KREBS AND HEMS[12].

The efficiency of LIP was measured and related to the concentration of chlorophyll according to the method used by FRENKEL[1] as μmoles of phosphate esterified per hour per unit absorbancy at 800 mμ ("OD_{800}").

The medium employed for the LIP measurements contained, unless otherwise noted, 1.5 ml 0.2 *M* glycylglycine pH 7.4, 8 μmoles $K_2H^{32}PO_4$, 10 μmoles ATP, 30 μmoles $MgCl_2$, 1 μmole sodium succinate, 60 μmoles glucose and an excess of yeast hexokinase**. The volume during the experiment was 3 ml. The reaction was stopped with 1 ml 2 *M* perchloric acid.

The "chromatophore fragments" fraction was used in all the experiments reported here. The temperature of the water-bath which contained the test-tubes with the reaction mixture was 30°. A saturating light-intensity was always used. Since the efficiency of LIP was roughly the same under aerobic as under anaerobic conditions[14], aerobic conditions were chosen. In order to "avoid complications due to light-induced reactions involving molecular oxygen" FRENKEL[1] used anaerobiosis for his investigations. The possibility that O_2 might serve as an alternative to (OH) as the oxidizing agent in LIP cannot be excluded when aerobic conditions are used.

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. Their % P_{org} was often negligible and seldom exceeded 1.5.

RESULTS

Fig. 1 shows that FAD stimulates LIP in washed chromatophore fragments from *R. rubrum*, whereas FMN does not. Here, as in most of the experiments, the stimulation reaches a value of between 50 and 100 %, but occasionally less than 50 or more than 100 % stimulation has been obtained. FMN has an inhibitory effect on the LIP. The effect is small and usually more pronounced at lower concentrations of FMN than at higher, as can be seen from Fig. 1.

* Dr. T. E. CONOVER kindly guided and assisted in these experiments.

** The hexokinase was obtained from Dr. L. ERNSTER and Dr. H. LÖW. It had been prepared and stored as described in ref.[13].

The inhibition of LIP by atebrin is demonstrated in Fig. 2. At a concentration of $4 \cdot 10^{-5}$ M atebrin 50 % inhibition is obtained. This value is not entirely constant from preparation to preparation. The highest concentration of atebrin required for 50 % inhibition has been $1.2 \cdot 10^{-4}$ M and the lowest $2 \cdot 10^{-5}$ M.

The effect of FAD and FMN on atebrin-inhibited LIP is shown in Fig. 3. Both of the flavin nucleotides can restore LIP, but FAD is more efficient than FMN. Only with FAD has a complete restoration been obtained. For example, in an experiment

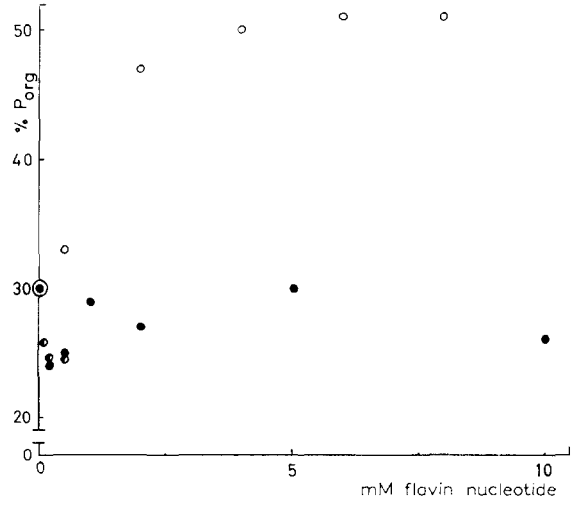


Fig. 1. Effects of FAD and FMN on LIP. In each tube "OD_{800}" = 0.21. 30 min experiment. ○ = FAD, ● and ◐ = FMN. Two separate experiments with FMN are included in order to show the limit of inhibition obtained at low concentrations. In the ◐-series the % P_{org} was 34 without added FMN. For the sake of clarity this was reduced to 30 in the figure and the other values were multiplied by the same factor.

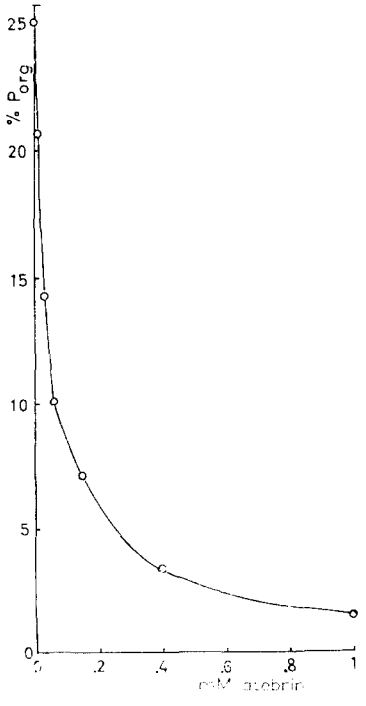


Fig. 2. Inhibition of LIP with atebrin. In each tube "OD_{800}" = 0.19. 30 min experiment.

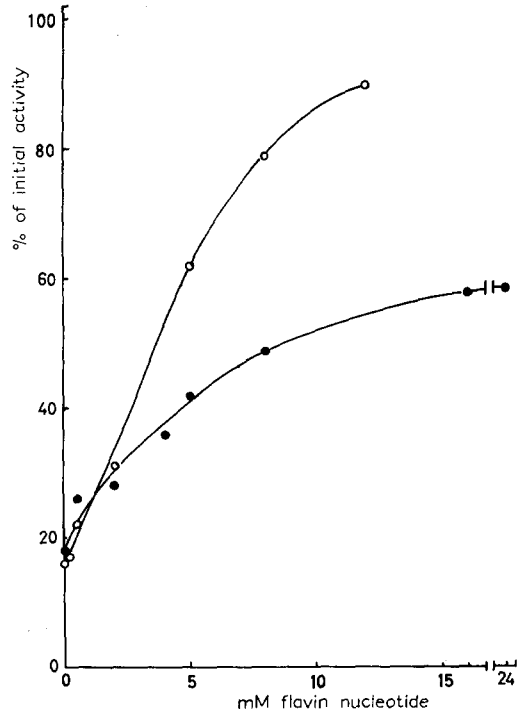


Fig. 3. Restoration of LIP by FAD and FMN in an atebrin-inhibited system. 30 min experiment. The value obtained with no atebrin added corresponds to 100% initial activity. In all other tubes the atebrin concentration was $2 \cdot 10^{-4}$ *M*. ○ = FAD, ● = FMN.

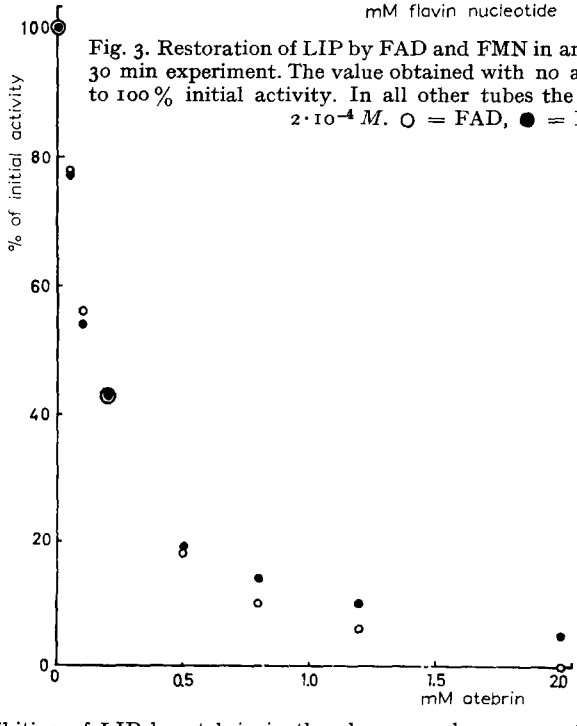


Fig. 4. Inhibition of LIP by atebrin in the absence and presence of PMS. 6 min experiment. ○ = without PMS (100% initial activity = 13 μmoles P esterified per hour per "OD_{800}"), ● = with PMS (100% initial activity = 54 μmoles P esterified per hour per "OD_{800}"). The final concentration of PMS, where added: 0.01 %.

in which LIP was inhibited by atebrin to 63 % of its original value, 12 mM FAD in the atebrin-containing system increased the LIP to a value that was 25 % above the original, uninhibited value.

PMS stimulates the LIP in extracts of *R. rubrum*, as was first shown by GELLER AND GREGORY[15]. Fig. 4 shows that the atebrin inhibition of LIP is the same whether PMS is added or not. PMS gave in this experiment a more than 4-fold stimulation, as is indicated in the figure legend.

As is shown in Table I, both antimycin A and HOQNO almost completely inhibit LIP, not only in the absence of FAD but also in the presence of stimulating amounts of this compound. Thus the electrons which pass over FAD also pass over the site that is inhibited by antimycin A and HOQNO, which is to be expected if FAD is a member of the electron transport chain for LIP in *R. rubrum*.

TABLE I

THE INHIBITION OF LIP BY HOQNO AND ANTIMYCIN A IN THE ABSENCE AND THE PRESENCE OF A STIMULATING CONCENTRATION OF FAD

Conditions	*Additions*	*LIP (% P_{org})*
0 min, light		1.2
30 min, dark		1.1
30 min, light		16
30 min, light	FAD	23
30 min, light	HOQNO	1.5
30 min, light	Antimycin A	2.6
30 min, light	FAD + HOQNO	2.6
30 min, light	FAD + Antimycin A	1.9

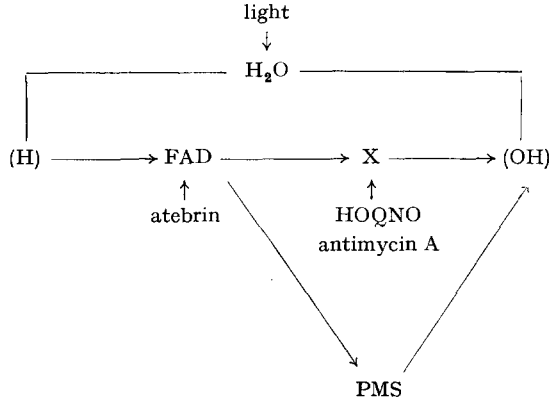
The final concentrations of the following compounds, where added, were: FAD 3 mM, HOQNO $2 \cdot 10^{-6}$ M and antimycin A $2 \cdot 10^{-6}$ M.

DISCUSSION

Several compounds, such as TPN[16], FMN[17] and vitamin K_3[2], are assumed to be electron carriers in plant LIP. The experimental evidence for the participation of these agents is their stimulating effect on the rate of ATP-formation and, in the case of TPN, the phosphorylation which according to ARNON, WHATLEY AND ALLEN[18] is coupled to the reduction of the pyridine nucleotide. In LIP of photosynthetic bacteria (*R. rubrum*), results from spectrophotometric studies have permitted SMITH AND M. BALTSCHEFFSKY[8] to suggest a role for cytochrome c_2 and probably a *b*-type cytochrome as electron carriers. No evidence for participation of other compounds in bacterial LIP has so far been reported.

The results given in the present paper support the conclusion that flavin takes part in the electron transport in bacterial (*R. rubrum*) LIP. We regard the evidence presented as strong for the following reasons: (1) FAD (but not FMN) stimulates the LIP, (2) powerful inhibition is obtained with the flavin-antagonist atebrin and (3) FAD can completely (and FMN partially) restore the LIP in an atebrin-inhibited system. The above evidence supports the conclusion that FAD is the flavin involved. In this connection it might be mentioned that PEEL[19] has determined the content of flavins of light-grown *R. rubrum*, and found FAD, FMN and riboflavin, FAD being present in the highest concentration.

PMS provides a short-cut for the electron flow from (H) to (OH) around the site where antimycin A and HOQNO inhibit LIP in *R. rubrum*[7]. When the system is inhibited by atebrin, however, the inhibition is equally strong whether or not PMS is present. This shows that atebrin does not inhibit the same site as antimycin A and HOQNO. The fact that both the "basal" LIP and the additional phosphorylation that is obtained by addition of FAD are strongly inhibited by antimycin A and HOQNO, viewed in conjunction with the information from the experiments with PMS, supports the following schematic representation of an electron transport chain for LIP in *R. rubrum*:

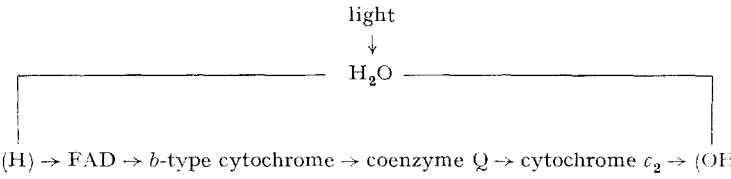


In this scheme, flavin has been put on the reducing side of the site "X", which is inhibited by HOQNO and antimycin A, in analogy with the known sequence in phosphorylating animal mitochondria. PMS will accept electrons from FAD, and this is in analogy with the situation for the succinic dehydrogenase of KEARNEY AND SINGER[20]. This enzyme, a flavoprotein, is reported to function optimally with PMS as acceptor of electrons from the flavin moiety of the enzyme[21,22].

The fact that atebrin inhibits LIP equally in the presence of PMS as in its absence seems to indicate that the component which atebrin acts upon takes part in the rate-limiting reaction in both cases. This is in agreement with the given scheme, where the rate-limiting reaction would be between FAD and "X" in the absence of PMS and between FAD and PMS in its presence.

The above scheme, which accounts only for results from inhibitor studies, can be combined with the one that is given by SMITH AND M. BALTSCHEFFSKY[8] on the basis of results obtained with difference spectrophotometry. These authors suggest that a "cytochrome chain including cytochrome c_2 and probably a *b*-type cytochrome" mediates the electron transport between the reductant and the oxidant from the photolysis of water. They also show that HOQNO inhibits electron transport from the *b*-type cytochrome to cytochrome c_2. It may be assumed that flavin is on the reducing side of the *b*-type cytochrome, in analogy with the sequence in the respiratory chain of phosphorylating animal mitochondria, and in microsomes. In the laboratory of GREEN[23] strong experimental evidence has been obtained that antimycin A acts on a rather ubiquitously occurring quinone, coenzyme Q, to which a role is ascribed in mitochondrial electron transport between cytochrome *b* and cytochrome *c*[24]. CRANE[25] has recently reported that coenzyme Q is present in large amounts in *R. rubrum*. It

seems reasonable to assume that antimycin A acts on this compound also in the LIP of this bacterium. The following tentative formulation for the electron transport chain in LIP of *R. rubrum* is given on the basis of the above information:



In this scheme, "X" from the first scheme has been changed to coenzyme Q. A possible role for other electron carriers in the chain, as for example pyridine nucleotide or vitamin K, is by no means excluded. Attention is called to the fact that it has been possible to derive a scheme, where the electron transport chain in LIP of *R. rubrum* shows a great similarity to the corresponding part of the electron transport chain of phosphorylating animal mitochondria.

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