Origin of the ATP Formed during the Light-Dependent Oxygen Uptake Catalyzed by *Rhodospirillum rubrum* Chromatophores

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(Z. Naturforsch. **30 c**, 46–52 [1975]; received October 7, 1974)

Photophosphorylation, Photooxidase, *Rhodospirillum rubrum*

The oxygen uptake which is observed when *Rhodospirillum rubrum* chromatophores are illuminated under air and in the presence of reduced 2,6-dichlorophenolindophenol (DCIP), 2,3,5,6-tetramethyl-*p*-phenylenediamine (diaminodurene, DAD) or N,N′-tetramethyl-*p*-phenylenediamine (TMDP) depends on the electron-donor concentration according to the equation of Michaelis-Menten. The apparent K_m for the donor is lowered by the electron-transfer inhibitor 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO) which causes therefore a stimulation of the rate of the reaction at non-saturating concentrations of the donors. In contrast, the ATP formation which takes place simultaneously to oxygen uptake does not show an enzyme-like dependence on donor concentration. Moreover it is inhibited by HQNO to a variable extent, depending on the particular donor present and on its concentration. Therefore it appears that the HQNO-sensitive phosphorylation is coupled to a cyclic flow which coexists and competes with the non-cyclic flow from donor to oxygen.

In the presence of HQNO, substrates and uncouplers of ATP formation accelerate somewhat the rate of the oxygen uptake supported by reduced DCIP and DAD. Thus part of the HQNO-resistant phosphorylation seems to be associated with the non-cyclic flow from those two donors to oxygen. The lack of stimulation by phosphorylation or by uncoupling of the TMPD-supported oxygen uptake does not permit a conclusion as to whether this reaction is coupled to ATP formation or not.

Another part of the HQNO-resistant ATP formation is independent of the presence of oxygen and appears to be associated to cyclic flows which bypass the HQNO site. This type of phosphorylation is most important in the presence of TMPD.

Introduction

Membrane preparations (chromatophores) of the non-sulfur purple bacterium *Rhodospirillum rubrum* catalyze a light-dependent electron transport from exogenous donors to oxygen [1]. The reaction requires to some degree the structural integrity of the chromatophore – as evidenced by its sensitivity to mild-heat treatment – and seems to be a valid measurement for part of the photochemical process even though oxygen is not a natural acceptor for photosynthetic electrons within the intact cell [2]. The light-dependent oxygen uptake – or the concomitant photooxidation of the exogenous donor – is accompanied by ATP formation when ADP and orthophosphate are included in the reaction mixture [3–5]. Using ferrocytochrome c as electron donor, Zaugg *et al.* [6] concluded that the phosphorylation was not associated with electron transfer from donor to oxygen but with a simultaneous cyclic flow stimulated by ferrocytochrome c. However, more recent reports have proposed that the ATP formation observed during the aerobic photooxidation of TMPD [4] and reduced DCIP [5] is coupled to non-cyclic electron flow.

The present study concerns the nature of the photophosphorylation which accompanies the oxygen-uptake catalyzed by illuminated *R. rubrum* chromatophores and supported by reduced DCIP, DAD or TMPD as electron donors. A substantial part of the formation of ATP appears to be coupled to cyclic types of electron flow which coexist and compete with the non-cyclic electron transport from donor to oxygen. This fact makes it difficult to estimate unequivocally the stoichiometry of the phosphorylation coupled to oxygen uptake which seems to be lower than 1 mol ATP per 6 mol of oxygen when DAD or reduced DCIP are the electron donors. It is not clear whether the TMPD-supported oxygen uptake is coupled to ATP formation or not.

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; DCIP, 2,6-dichlorophenolindophenol; HQNO, 2-heptyl-4-hydroxyquinoline-N-oxide; TMPD, N,N′-tetramethyl-*p*-phenylenediamine; Tricine, N-tris(hydroxymethyl)methylglicine; Tris, tris-hydroxymethyl-aminomethane.

Material and Methods

Rhodospirillum rubrum strain S-1 was grown in the medium of Lascelles [7] at 30 °C till the late exponential phase. The cultures were incubated in completely filled, stoppered tubes under illumination from a bank of incandescent lamps. The cells were collected by centrifugation at $8000 \times g$ for 10 min, washed with 50 mM Tricine-NaOH (pH 8.0) and centrifuged again. The cell paste was ground with alumina and extracted with the same buffer. Alumina and large cell debris were removed by centrifugation at $27\,000 \times g$ for 10 min and the supernatant was centrifuged at $80\,000 \times g$ for 35 min. The sediment was suspended in Tricine and centrifuged again at $80\,000 \times g$. The final residue suspended in Tricine was the chromatophore preparation used throughout this work. The grinding of the cells and all the subsequent steps were carried out near 0 °C. A fresh batch of chromatophores was prepared every day. Bacteriochlorophyll concentration was estimated using the extinction coefficient *in vivo* given by Clayton [8].

The reaction mixture for the assay of oxygen uptake contained: 47 mM Tricine-NaOH (pH 8.0), 1.7 mM sodium ascorbate, 1 mM KCN, 10 – 14 μM bacteriochlorophyll (as chromatophores) and variable additions which are indicated under the particular experiment, to a final volume of 3 ml. KCN was included to inhibit the dark oxygen uptake catalyzed by the respiratory chain [1]. The samples were kept at 25 °C and the rate of oxygen consumption was determined polarographically (Biological Oxygen Monitor, model 53, Yellow Springs Instrument Co., Yellow Springs, Ohio). Illumination was provided by a slide projector equipped with a 150-W tungsten-halide lamp and an interference filter (IR-total, Balzers, Liechtenstein). After additional filtering through 10 cm of water, the light intensity reaching the samples was 79 kerg · cm^{-2} · sec^{-1}.

The reaction mixture for the assay of photophosphorylation contained: 0.1 mM ADP, 5 mM KH_2PO_4, 5 mM $MgCl_2$, 26.7 mM glucose and 25 units of hexokinase (E.C. 2.7.1.2) in addition to the components of the reaction mixture for oxygen uptake. The samples, contained in open test-tubes (11-mm diameter), were placed in a water bath at 25 °C and illuminated as in the assay for oxygen uptake. After 3 min in the light, 0.2 ml of 10 M HCl was added to each sample and the mixture was shaken vigorously and centrifuged at 0 °C (10 min at $17\,000 \times g$). 1 ml of the clear supernatant was brought to about pH 7.5 with 1.0 ml of 0.5 M Tris-HCl (pH 7.5) and an appropriate amount of 10 M KOH in a 1-cm spectrophotometer cell. After the addition of 0.6 μmol of NADP and water to complete a final volume of 3 ml the absorbance at 340 nm was measured. 10 μl (2.5 units) of glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) were added to the mixture and the increase in absorbance at 340 nm after 3 min was used to estimate the rate of ATP formation during the illumination period. When anaerobic conditions were desired during the assay of phosphorylation, 10 units of glucose oxidase (E.C. 1.1.3.4) and 2 ml of paraffin oil were included in the reaction mixture which was then kept in the dark at 25 °C for 3 min before illumination. The oxygen content of the sample decreased to less than 1% of saturation during this dark preincubation.

Enzyme preparations and biochemicals were obtained from Sigma Chemical Co. (Saint Louis, Missouri). DAD was a kind gift of Dr. A. Trebst (Bochum, Germany). All other chemicals were of analytical grade.

Results

A light-dependent oxygen uptake is observed when *R. rubrum* chromatophores are illuminated in the presence of any of the three redox com-

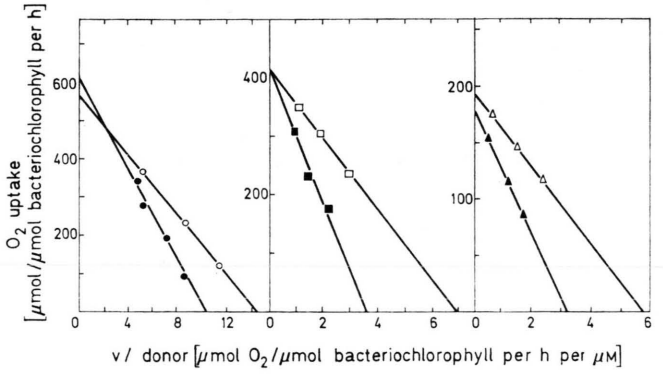

Fig. 1. The stimulatory effect of HQNO on light-dependent oxygen uptake. The assays were performed as described under Methods with DCIP (●, ○), DAD (■, □) and TMPD (▲, △) as electron donors, both in the absence (●, ■, ▲) and in the presence (○, □, △) of 1.7 μM HQNO. The results were represented in the form of Eadie-Hofstee plots.

pounds, DCIP, DAD or TMDP kept in the reduced state by an excess of sodium ascorbate. The rate of the reaction, which is zero when DCIP, DAD and TMPD are omitted from the reaction mixture, depends on the concentration of the donor according to the equation of Michaelis-Menten, as it is shown in the linear plots of Fig. 1. The differences among the three donors lie mainly in the saturation rates which, depending on the particular chromatophore preparation, range from 150 – 250 μmol of O_2 taken up per μmol of bacteriochlorophyll per hour for TMPD to 550 – 800 for DCIP. The apparent half-saturating concentrations (K_m) have values from 50 to 100 μM.

The ATP formation observed under the same conditions shows a completely different dependence on donor concentration (Fig. 2 a). Only TMPD – the less effective donor for oxygen uptake – causes a significant increase of the rate of phosphorylation above that observed with ascorbate alone and all three donors become inhibitory at concentrations which start to saturate the uptake of oxygen. It is obvious, therefore, that a straightforward relationship between the rates of electron flow from donor to oxygen and those of the simultaneous phosphorylation cannot be established.

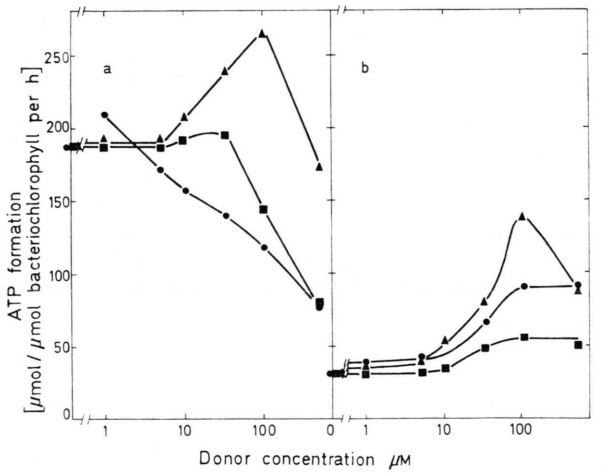

Fig. 2. Effect of the concentration of DCIP (●), DAD (■) and TMPD (▲) on the rate of phosphorylation under aerobic conditions: a. in the absence of HQNO; b. in the presence of 3.3 μM HQNO. The assays were performed as described under Methods.

A further difference between oxygen uptake and ATP formation is the effect of the electron-transport inhibitor, HQNO [9], on both reactions. HQNO decreases the rate of phosphorylation under the conditions for oxygen uptake. The extent of the inhibition depends on the particular electron donor used and on its concentration, as seen from the comparison of the experimental data of Figs 2 a and 2 b. In contrast, HQNO causes a stimulation of the rate of oxygen uptake with all the electron donors (Fig. 1) and in the three cases the effect is due mainly to a lowering of the apparent K_m for the donor, while the saturation rates of the reactions are little or not affected. In agreement with these results, Govindjee *et al.* [10] have also observed recently that HQNO stimulates the rate of the light-dependent oxygen uptake sustained by reduced DCIP.

It is known that HQNO blocks the photosynthetic electron transfer of *R. rubrum* between cytochrome b (or cc′) and cytochrome c_2. As a consequence it inhibits the return of electrons from the acceptor to the donor of the photochemical reaction center during the "endogenous" cyclic electron flow in illuminated chromatophores [9]. Our interpretation of the results presented up to here is that, under conditions for oxygen uptake, this cyclic pathway is responsible for part of the phosphorylation and is competitive with the artificial electron donor for the reduction of the photooxidized reaction center. According to this proposal, the expected effect of the inhibitor would be the one found experimentally: A partial inhibition of ATP formation (Fig. 2) and a "competitive stimulation" of the rate of oxygen uptake (Fig. 1).

Other authors [5] have observed that the light-dependent oxygen uptake supported by reduced DCIP is stimulated by 2-nonyl-4-hydroxyquinoline-N-oxide and antimycin A, inhibitors which block the photosynthetic electron transport of *R. rubrum* at the same site as HQNO [9]. The stimulation was attributed to a possible uncoupling effect of the inhibitors since the acceleration of coupled electron flows is a well-known property of uncouplers of phosphorylation [9]. However two differences between the behaviour of HQNO and that of typical uncouplers do not favour this alternative interpretation of our results: First, typical uncouplers inhibit the phosphorylation to about the same extent either in the presence or in the absence of electron donors while the inhibition by HQNO is variable (Fig. 3); second, the stimulatory effect of HQNO on the rate of oxygen uptake using any of the three electron donors is maintained or even enhanced by the previous presence of the uncouplers gramicidin D or

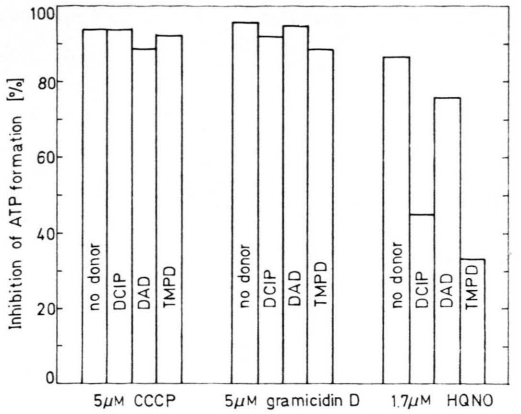

Fig. 3. Comparative effects of uncouplers and HQNO on rates of photophosphorylation observed in the absence and in the presence of the electron donors for oxygen uptake. The assays were performed under aerobic conditions as described under Methods. DCIP, DAD and TMPD at 33 μM were included in the reaction mixture where indicated. For control rates see Fig. 2 a.

CCCP at concentrations which abolish about 90% of the formation of ATP (Table I). These results are not consistent with the proposal that HQNO acts as a typical uncoupler and that the stimulation of oxygen uptake is the consequence of its uncoupling properties.

Table I. Effect of HQNO on the rate of the light-dependent oxygen uptake in the presence and in the absence of uncouplers of phosphorylation. The assays were performed as described under Methods. 33 μM DCIP, 33 μM DAD, 33 μM TMPD, 5 μM gramicidin D and 5 μM CCCP were present where indicated. The rate of oxygen uptake in the light was measured before and 2 min after the addition of 1.7 μM HQNO.

System	μmol O_2/ μmol bacteriochlorophyll per h	Effect of HQNO [% stimulation]
Experiment 1		
DCIP	153	16
DCIP + gramicidin D	198	47
DAD	102	40
DAD + gramicidin D	118	39
TMPD	73	28
TMPD + gramicidin D	61	38
Experiment 2		
DCIP	189	14
DCIP + CCCP	205	25
DAD	125	38
DAD + CCCP	123	49
TMPD	85	33
TMPD + CCCP	77	41

It has been reported that several redox compounds, including DCIP [11] and TMPD [12], are able to carry electrons around the HQNO-sensitive site and, as a consequence, to catalyze "artificial" cyclic electron flows which are coupled to the formation of ATP. Therefore the phosphorylation which accompanies light-dependent oxygen uptake in the presence of HQNO (Fig. 2 b) may be supported by the non-cyclic electron flow from donor to oxygen, by the cyclic flow catalyzed by the donor or by both cyclic and non-cyclic flows. To investigate these possibilities we have tested the ability of reduced DCIP, DAD and TMPD to stimulate the HQNO-resistant phosphorylation under anaerobic conditions. Since oxygen is not present, non-cyclic flow cannot take place and the stimulation is an estimation of the ability of the donors to catalyze phosphorylating bypasses of the HQNO-sensitive site. Fig. 4 shows that all three donors stimulate the rate

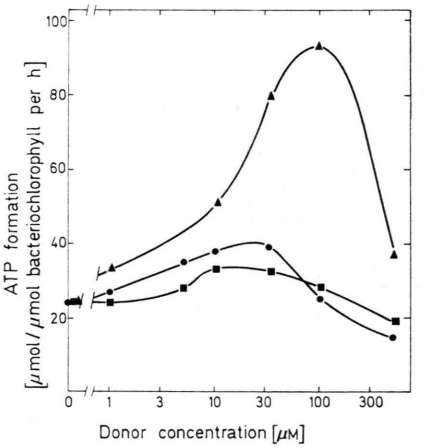

Fig. 4. Effect of the concentration of DCIP (●), DAD (■) and TMPD (▲) on the rate of phosphorylation under anaerobic conditions and in the presence of 3.3 μM HQNO. Assays were performed as described under Methods.

of phosphorylation at different optimum concentrations and to different extents. TMPD is by far the most effective catalyst of artificial cyclic phosphorylation among the compounds tested here. Besides, the similar shapes of the TMPD curves of Figs 2 b and 4 suggest that part of the ATP formation observed in the presence of TMPD and HQNO under aerobic conditions may be also supported by the artificial cyclic flow.

The comparison of Figs 2 b and 4 shows that the rate of phosphorylation is higher when the reaction is carried out under oxygen, particularly at donor

concentrations above 50 μM. This result may be interpreted as an indication that non-cyclic flow from donor to oxygen is coupled to ATP formation. In fact a similar observation led Isaev *et al.* [4] to propose that the aerobic photooxidation of TMPD was associated to the simultanous phosphorylation. However the presence of oxygen could also facilitate the ocurrence of cyclic pathways through a modification of the redox state of the electron carriers – endogenous and added – of the chromatophore [11, 13]. Because of the possible existence of these artificial cycles in the presence of exogenous donors, direct measurement of the rates of the HQNO-resistant photophsophorylation provides only an indication that the non-cyclic flow from donor to oxygen is coupled to ATP formation, but not a definitive proof. A different approach to the problem of whether the electron-transport system responsible for oxygen uptake involves an (some) energy-conserving step(s) is to test the ability of substrates and uncouplers of photophosphorylation to accelerate electron flow, a property of coupled systems which has been already referred to [9]. Such a stimulation has been previously detected during the photooxidation of ferrocytochrome c [3] and reduced DCIP [5, 14] but not during that of TMPD [14]. We have reinvestigated this effect and have found that under our experimental conditions the stimulation is absent or small for reduced DCIP and DAD and non-existant for TMPD. In addition we have observed that the presence of HQNO causes always an enhancement of the stimulation by substrates of ATP formation and by uncouplers in the case of DAD or reduced DCIP (Table II). If the stimulations are actually the consequence of the removal of rate-limiting coupling sites, we may conclude that electron flow from DAD to oxygen is coupled to ATP formation as it had been proposed for reduced DCIP [5, 14]. Besides, the enhancement by HQNO of the stimulation is consistent with our interpretation that this inhibitor blocks a simultaneous and phosphorylating cyclic flow: uncouplers and substrates of phosphorylation would stimulate both the cyclic and the non-cyclic flow but, as the flows compete with each other, the expected stimulation of either of them when both are operative would be lower than the stimulation of one of the flows when the other is inhibited.

The acceleration of a simultaneous cyclic flow would also explain the small inhibition produced by uncouplers on the TMPD-supported oxygen uptake. The fact that no stimulation is observed even in the presence of HQNO may be due to the occurrence of an intense HQNO-resistant cyclic flow (Fig. 4) and/or merely to the non-existence of coupling sites along the transfer of electrons from TMPD to oxygen. At this moment we cannot offer a satisfactory solution to this problem.

Table II. Effect of gramicidin D, CCCP and subsrates of phosphorylation on the rate of the light-dependent oxygen uptake in the presence and in the absence of HQNO. The assays were performed as described under Methods. 33 μM DCIP, 33 μM DAD, 33 μM TMPD, 5 mM $MgCl_2$ and 1.7 μM HQNO were present where indicated. The rate of oxygen uptake in the light was measured before and 2 min after one of the following aditions: 5 μM gramicidin D, 5 μM CCCP or 1 mM ADP *plus* 4 mM potassium phosphate (pH 8.0).

System	μmol O_2/ μmol bacteriochlorophyll per h	Addition	Effect of the addition [% stimulation]
Experiment 1			
DCIP	146	gramicidin D	26
DCIP+HQNO	176	gramicidin D	56
DAD	107	gramicidin D	16
DAD+HQNO	148	gramicidin D	21
TMPD	79	gramicidin D	−14
TMPD+HQNO	100	gramicidin D	−9
Experiment 2			
DCIP	207	CCCP	3
DCIP+HQNO	240	CCCP	7
DAD	146	CCCP	0
DAD+HQNO	167	CCCP	24
TMPD	97	CCCP	−10
TMPD+HQNO	128	CCCP	−5
Experiment 3			
DCIP+Mg^{2+}	238	ADP+PO_4^{3-}	0
DCIP+Mg^{2+}+HQNO	250	ADP+PO_4^{3-}	16
DAD+Mg^{2+}	106	ADP+PO_4^{3-}	−1
DAD+Mg^{2+}+HQNO	123	ADP+PO_4^{3-}	14
TMPD+Mg^{2+}	65	ADP+PO_4^{3-}	1
TMPD+Mg^{2+}+HQNO	84	ADP+PO_4^{3-}	0

Discussion

Our interpretation of the experimental results just described is summarized in the scheme of Fig. 5. The cyclic electron-transfer system of *R. rubrum* chromatophores [15] is depicted as consisting of two parts: the first includes the photochemical reaction center and an unknown number of secondary donors and acceptors; the second one is the chain of redox carriers which returns electrons from the photoreduced acceptors to the photooxidized donors and

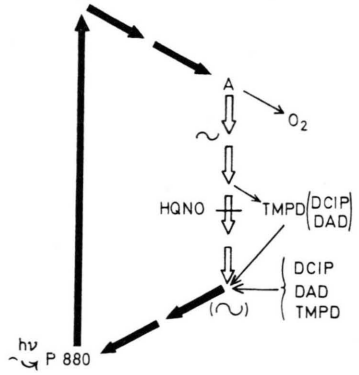

Fig. 5. Schematic representation of the electron-transport pathways operative during the light-dependent oxygen uptake catalyzed by *R. rubrum* chromatophores. A part of the cyclic system mediates the transfer of electrons from the exogenous donors to oxygen (black arrows). A part of the cyclic system which does not mediate this reaction (white arrows) contains the HQNO-sensitive site and a (some) site(s) of energy conservation. No attempts have been made to identify the endogenous electron-carriers except for the pigment of the photochemical reaction center (P 880). More details are given in the text.

includes the HQNO-sensitive site [9]. Artificial electron donors such as DAD, TMPD and reduced DCIP reduce also the photooxidized endogenous donors [16] interfering with the normal reoxidation of the chain of redox carriers and causing the accumulation of a (some) reduced autooxidizable component(s) of the chain which is (are) responsible for the observed oxygen consumption. At concentrations of artificial donors which do not saturate the rate of oxygen uptake, the cyclic flow still takes place and its inhibition by HQNO produces a stimulation of the rate of oxygen uptake. The effect of HQNO is thus that of removing an inhibitor (the redox-carrier chain) competitive with the artificial donors (Fig. 1). The alternative possibility that HQNO could stimulate the uptake of oxygen as the consequence of an uncoupling effect has been already discussed under Results and will not be considered here again.

At this point it seems interesting to comment that the observed lack of inhibition by HQNO of oxygen uptake supported by DAD as electron donor (Fig. 1) does not agree with a previous conclusion of Trebst *et al.* [12]. These authors found that the photoreduction of NAD by DAD, catalyzed by *R. rubrum* chromatophores, was inhibited by antimycin A, which appears to act at the same site as HQNO [9]. It was proposed that DAD donated electrons at a site above that sensitive to the inhibitors. In view of our results and of the fact that NAD photoreduction by *R. rubrum* chromatophores appears to be a complex process involving an energy-linked reversed electron flow [17, 18], the inhibition by antimicyn A of the DAD-supported NAD reduction seems to result from the small ability of that phenylenediamine to catalyze an energy-conserving cyclic flow in the presence either of antimicyn A – as observed by Trebst *et al.* [12] themselves – or of HQNO – as appears from the data of Fig. 4.

The origin of the ATP formed during light-induced oxygen uptake is diverse. Some of the phosphorylation is inhibited by HQNO (Fig. 2) and therefore it seems to be coupled to the part of the cyclic system which does not mediate the transfer of electrons from added donors to oxygen (Fig. 5). At high donor concentrations electron flow through this part of the cyclic system seems to be competitively inhibited and the sensitivity of phosphorylation to HQNO is lower (Fig. 2). Therefore the ATP formation observed at donor concentrations which saturate the rate of oxygen uptake appears to have a different origin.

In the presence of HQNO, uncouplers and substrates of phosphorylation accelerate to some extent the rate of the non-cyclic flow from DAD and reduced DCIP to oxygen (Table II), suggesting that this flow is coupled to ATP formation. Since the ability of these two donors to catalyze HQNO-insensitive cyclic phosphorylation seems to be low (Fig. 4), we might assume that most of the phosphorylation observed at concentrations of the donors which saturate oxygen uptake and in the presence of HQNO originates during non-cyclic flow. The maximum yield of ATP, estimated from the data of Figs 1 and 2, would be 1 mol per 6 – 8 mol of oxygen taken up. These figures indicate a low efficiency of coupling for the light-induced oxygen uptake. An observation which may have some relevance in this respect is that oxygen uptake goes on in the dark for some time after a period of illumination (data not shown). A similar observation had been made by Good and Hill [19] during the study of ascorbate photooxidation by plant chloroplasts in the presence of quinone. Recently Elstner and Kramer [20] have confirmed these findings and concluded that the autooxidation of a quinone-type photoreduced acceptor in the presence of ascorbate induces a chain of dark reactions which results in the uptake of several moles of oxygen per equivalent of

photochemical electrons. The low ratio of ATP to O_2 observed during the chromatophore-catalyzed oxygen uptake could be easily explained by the operation of a mechanism similar to that proposed for the chloroplast reaction [20].

No stimulation of TMPD-supported oxygen uptake is produced by uncouplers and substrates of ATP formation even in the presence of HQNO (Table II). This lack of stimulation may be due to the absence of coupling sites in electron transport from TMPD to oxygen. If this were the case the situation would be very similar to that observed when DAD and TMPD function as electron donors for photoreductions catalyzed by System I of chloroplasts, which are coupled to ATP formation with DAD but not with TMPD [21]. Trebst and co-workers [22, 23] have explained recently those results on the basis that the oxidation and the reduction of the artificial electron carriers take place at opposite sides of the photosynthetic membrane. Those carriers which transfer hydrogen atoms (such as DAD and DCIP) transport protons across the membrane during their oxidation-reduction cycle and create artificial sites of phosphorylation. The carriers which transfer only electrons (such as TMPD) do not transport protons and do not create artificial coupling sites. An appropriate location of reducing and oxidizing sites in the chromatophore membrane would explain in the same way why light-dependent oxygen uptake does not appear to be coupled to phosphorylation with TMPD as electron donor.

This article has benefited from the critical comments of Dr. F. F. del Campo. We thank Dr. A. Trebst for DAD and for calling our attention to recent publications of his laboratory, Miss Eva V. Marín for technical assistance and the "Comisión Asesora de Investigación Científica y Técnica, III Plan de Desarrollo" for financial support.

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