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NICOTINAMIDE-ADENINE DINUCLEOTIDE PHOTOREDUCTION IN *RHODOSPIRILLUM RUBRUM* CHROMATOPHORES*

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SUMMARY

1. The rates of succinate and ascorbate–*N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD)-supported NAD^+ photoreduction by *Rhodospirillum rubrum* chromatophores were approximately one-half and one-quarter, respectively, of the photophosphorylation rates.

2. All four reactions showed a very similar sensitivity to the uncoupler *m*-chlorocarbonyl cyanide phenylhydrazone.

3. NAD^+ photoreduction was strongly inhibited by the presence of an active phosphorylation system, whereas photophosphorylation showed no net decrease in activity in the presence of an active NAD^+ photoreduction system.

4. When both NAD^+ photoreduction and photophosphorylation were allowed to occur simultaneously, there appeared to be an increased utilization of high-energy intermediate.

5. At concentrations of antimycin A which strongly inhibited succinate and ascorbate–TMPD-associated photophosphorylation, succinate-supported NAD^+ photoreduction was also strongly inhibited, whereas NAD^+ photoreduction from ascorbate–TMPD was not affected.

6. These results are discussed in relation to pathways of electron transfer to NAD^+ and to the interaction of a high-energy intermediate with several energy-dependent reactions in *R. rubrum* chromatophores.

INTRODUCTION

In 1958 FRENKEL[1] demonstrated that *Rhodospirillum rubrum* chromatophores catalyse photoreduction of NAD^+ from succinate. This reaction has since received considerable attention and has been observed in several other photosynthetic bacteria *viz. Rhodopseudomonas spheroides*[2], *Rhodopseudomonas capsulata*[3] and *Chromatium*[4,5]. The photoreduction of NAD^+ from succinate by *R. rubrum* chromatophores was later confirmed and also shown to occur with a variety of electron donors[6–8]. More recently

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; TMPD, *N,N,N'N'*-tetramethyl-*p*-phenylenediamine; CCCP, *m*-chlorocarbonyl cyanide phenylhydrazone.

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KEISTER AND YIKE investigated the nature of the succinate-linked photoreduction of NAD^+ in detail and on the basis of uncoupler and inhibitor studies concluded that the reaction, unlike that leading to the photoreduction of $NADP^+$ in green plants (see review by SAN PIETRO AND BLACK[9]), was energy dependent and probably driven by a high-energy intermediate of photophosphorylation as shown below:

$$\text{Succinate} + NAD^+ + X\sim I \longrightarrow \text{fumarate} + NADH + H^+ + X+I$$

They demonstrated that the postulated high-energy intermediate $X\sim I$ could be generated either *via* a light-driven reaction or *via* the dark hydrolysis of several energy-rich compounds, *e.g.*, ATP and pyrophosphate[10].

This paper describes further studies on the relationship between photophosphorylation and NAD^+ photoreduction in chromatophores of *R. rubrum*.

METHODS

Cells of *R. rubrum* strain S-I were grown photosynthetically on the malate medium of NEWTON[11]. Chromatophores were prepared by differential centrifugation following sonication of the washed cells[12].

NAD^+ photoreduction was measured anaerobically under argon at 25° in all-glass Thunberg cuvettes. The standard reaction mixture consisted of 50 mM Tris–HCl buffer (pH 8.0), 1 mM $MgCl_2$, 0.67 mM NAD^+, 1 mg/ml bovine serum albumin, 16–18 μg/ml chromatophore bacteriochlorophyll and either 3.3 mM succinate or 1.67 mM sodium ascorbate (0.1 mM 2,6-dichlorophenolindophenol (DCIP) or 0.1 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) was also present when ascorbate was used).

After making the mixture anaerobic the chromatophores were tipped from the side-arm into the main cuvette, allowed to equilibrate for 2 min and then illuminated. The photoreduction was assayed discontinuously, by measuring the absorbance at 340 mμ before and after 3 min illumination (the reaction was linear with time under these conditions). Illumination was with 880 mμ light, obtained with a narrow band interference filter blocked to infinity. Incident-light intensity was measured using a YSI-Kettering radiometer and absorbed light was calculated from these data and absorption spectra and verified using the radiometer (light scattering was negligible in these chromatophores).

The dark, ATP-driven reduction of NAD^+ was measured using the same reaction mixture as described above but supplemented with 2 mM ATP or 2 mM sodium pyrophosphate and, where indicated, 6.7 mM creatine phosphate *plus* 0.1 mg/ml creatine kinase.

The dark oxidation of NADH was also assayed in the same reaction mixture except that NAD^+ and substrate were replaced by 0.15 mM NADH. The absorbance at 340 mμ was followed either aerobically (NADH oxidase) or anaerobically *plus* 3.3 mM sodium fumarate (NADH–fumarate reductase).

ATP formation was assayed anaerobically in a 3-ml reaction mixture containing 30 mM Tris–HCl buffer (pH 8.0), 3.3 mM P_i *plus* $^{32}P_i$ (approx. $5\cdot10^5$ counts/min) 0.25 mM ADP, 8 mM glucose, 1 mM $MgCl_2$, 1 mg/ml hexokinase, 1 mg/ml bovine serum albumin and 16–18 μg/ml chromatophore bacteriochlorophyll. As indicated the mixture was supplemented with 3.3 mM succinate or 1.67 mM ascorbate (*plus* 0.1 mM

TMPD). After making the system anaerobic the chromatophores were tipped into the main cuvette, allowed to equilibrate in the dark for 3 min, illuminated for a further 3 min, after which the reaction was terminated by the addition of 1 ml of 10% (v/v) trichloroacetic acid. [^{32}P]ATP, as glucose 6-[^{32}P]phosphate was assayed following isobutanol–benzene extraction by the method of AVRON[13]. Dark incorporation of $^{32}P_i$ under these conditions was very low, indicating low levels of chromatophore myokinase.

ATPase activity was assayed by measuring the dark release of $^{32}P_i$ from [^{32}P]ATP over a period of 10 min. The standard reaction mixture contained 50 mM Tris–HCl buffer (pH 8.0), 1 mM $MgCl_2$, 2 mM ATP *plus* [^{32}P]ATP (approx. $8 \cdot 10^6$ counts/min), 1 mg/ml bovine serine albumin and 16–18 μg/ml chromatophore bacteriochlorophyll. The reaction was terminated by the addition of 1 ml 10% (v/v) trichloroacetic acid and the ^{32}P released was extracted and assayed by the isobutanol–benzene method[13]. [^{32}P]ATP used in these experiments was prepared by the method of AVRON[14].

The bacteriochlorophyll content of the chromatophores was determined by the method of VAN NIEL AND ARNOLD[15].

General chemicals were obtained from Fischer Scientific Company, Fair Lawn, N.J. (U.S.A.), and fine chemicals and enzymes from the Sigma Chemical Company, St. Louis, Mo. (U.S.A.).

RESULTS

The ability of various electron donors to reduce NAD+ in the presence of *R. rubrum* chromatophores is shown in Table I. No reduction of NAD+ occurred in the dark in the absence of an added energy source such as ATP or pyrophosphate. The rates were stimulated slightly in the ATP-driven system by the presence of an ATP-regenerating system (*viz.* creatine phosphate *plus* creatine kinase).

Succinate, ascorbate–TMPD and ascorbate–DCIP all supported a photoreduction of NAD+; the rate from succinate being approximately double that from the ascorbate-linked substrates. Substantial dark, ATP-driven reduction of NAD+

TABLE I

NADH/NAD+ OXIDATION–REDUCTION REACTIONS OF *R. rubrum* CHROMATOPHORES

The reactions were assayed as described in METHODS. Activities are expressed as μmoles NAD+ reduced or NADH oxidized/h·mg bacteriochlorophyll. All reactions except NADH oxidase were assayed anaerobically. Light-driven reactions were carried out at 880 mμ at an absorbed light intensity of $1.6 \cdot 10^4$ ergs·cm^{-2}·sec^{-1}.

Expt. No.	*Donor*	*Acceptor*	*Activity*			
			Light	*Dark*		
				—	*+ATP*	*+P~P*
1	Succinate	NAD+	56	0	17	8
	Ascorbate–TMPD	NAD+	26	0	3	0
	Ascorbate–DCIP	NAD+	27	0	4	0
2	NADH	Oxygen		13		
	NADH	Fumarate		13		

was observed only with succinate as the electron donor, in which case the rate regularly reached 30–40 % of the photoreduction rate. The reaction from ascorbate–TMPD was very much slower, rarely exceeding 15 % of the light-driven rate, and the rate from ascorbate–DCIP was extremely slow and not consistently demonstrable. Pyrophosphate-driven dark reduction of NAD^+ could only be detected when succinate was used as the electron donor, and the rate was considerably lower than that of the ATP-driven reaction.

NADH was readily oxidized in the dark, either by oxygen or anaerobically by fumarate. The NADH oxidase was sensitive to light ($1.6 \cdot 10^4$ ergs absorbed $\cdot cm^{-2} \cdot sec^{-1}$ at 880 mμ caused 32 % inhibition). The aerobic oxidation of NADH by fumarate was approximately equal in rate to the sum of the NADH oxidase and anaerobic NADH–fumarate reductase activities.

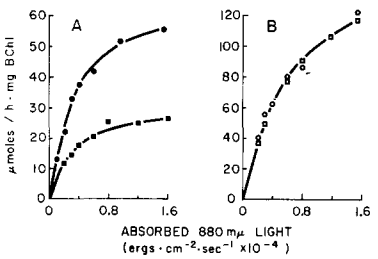

Fig. 1. 880 mμ light saturation curves for (A) NAD^+ photoreduction from succinate (●—●) or ascorbate–TMPD (■—■); (B) photophosphorylation supported by succinate (○—○) or ascorbate–TMPD (□—□). Reactions were assayed as described in METHODS. Bacteriochlorophyll (BChl) concn., 17.2 μg/ml.

A comparison of the donor-supported NAD^+ photoreduction and photophosphorylation is shown in Figs. 1A and 1B. Over a wide range of absorbed light intensities the rate of NAD^+ photoreduction from succinate was consistently double the rate from ascorbate–TMPD (Fig. 1A). The latter rate could not be stimulated by increasing either the concentration of TMPD above that routinely used (100 μM; K_m for TMPD = 6.5 μM) or the concentration of ascorbate (routinely 1.67 mM; 10 mM partially inhibited NAD^+ photoreduction). On the other hand, the rate of photophosphorylation in the presence of added electron donors was not significantly changed when succinate was replaced by ascorbate–TMPD (Fig. 1B). Expressed as conventional Lineweaver–Burk reciprocal plots, all four light saturation curves yielded straight lines and very similar half-saturation values with respect to light ($0.60 \cdot 10^4$–$0.95 \cdot 10^4$ erg absorbed $\cdot cm^{-2} \cdot sec^{-1}$ at 880 mμ). A comparison of the NAD^+ photoreduction and photophosphorylation rates, when assayed separately over a wide range of absorbed light intensities using the same chromatophore preparation (as was the case in Figs. 1A, 1B) consistently indicated that at any given light intensity the rate of ATP synthesis was approximately twice (1.5–2.5) that of NAD^+ photoreduction from succinate and approx. 4-fold (3.4–4.6) that of NAD^+ photoreduction from ascorbate–TMPD.

The ATPase activity of freshly prepared chromatophores (Table II) was very low (approx. 18 μmoles ATP hydrolysed/h $\cdot$ mg bacteriochlorophyll) compared with the rates of 880 mμ light-driven photophosphorylation (v_{max} approx. 200 μmoles

TABLE II

STOICHIOMETRY OF DARK ATP-DRIVEN NAD+ REDUCTION IN *R. rubrum* CHROMATOPHORES

ATPase and dark ATP-driven NAD+ reduction were assayed as described in METHODS. Bacteriochlorophyll concn., 15.8 μg/ml.

Donor	*Acceptor*	*ATP**	*NAD+**	*ATP consumed (net)* / NAD+ reduced*	*ATPase***
—	—	0.139	—	—	17.6
Succinate	NAD+	0.258	0.065	1.84	
Ascorbate–TMPD	NAD+	0.210	0.012	5.17	

* μmoles/10 min.
** μmoles/h·mg bacteriochlorophyll.

ATP/h·mg bacteriochlorophyll) although the ATPase rate could be increased up to 8 fold by the addition of an uncoupler (*e.g.*, 1 μM desaspidin). These results indicate the presence of a fairly tightly coupled photophosphorylation system in these chromatophores. Neither succinate, ascorbate–TMPD nor NAD+ alone significantly altered the ATPase activity. However, in the presence of a complete NAD+ photoreduction system (NAD+ *plus* either succinate or ascorbate–TMPD) ATPase activity was consistently stimulated. Simultaneous determination of the amounts of NAD+ reduced and ATP hydrolysed (after correcting for the endogenous ATPase activity) allowed calculation of the stoichiometry of these dark reactions. As shown in Table II, ratios for ATP consumed (net)/NAD+ reduced of 1.8 and 5.2 were obtained for succinate and ascorbate–TMPD, respectively. The presence in the reaction mixture of 2.5 μmoles ADP inhibited ATPase activity by only 5%, indicating that the very much lower

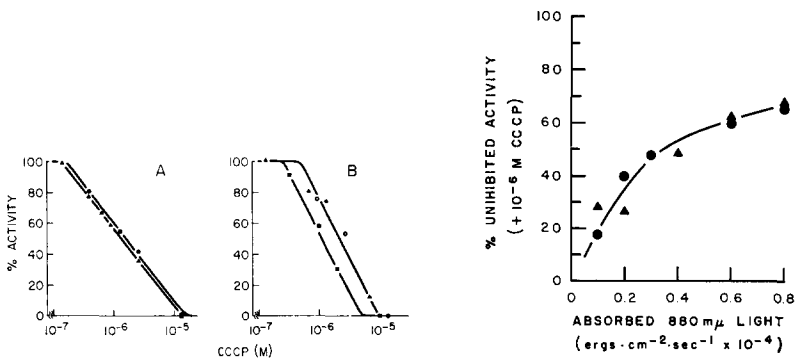

Fig. 2. The sensitivity of various reactions to CCCP. A. NAD+ photoreduction from succinate (●—●) or ascorbate–TMPD (▲—▲). B. Dark ATP-driven NAD+ reduction from succinate (■—■) and photophosphorylation supported by succinate (○—○) or ascorbate–TMPD (▲—▲). Reactions were assayed as described in METHODS. A concentrated solution of CCCP was prepared in methanol and then diluted as required with 1 mM Tris–HCl buffer (pH 7.4). The exact concentration was determined in this aqueous solution from the absorption at 378 mμ (ε = 23700). Illumination: 880 mμ. Intensity: $0.6 \cdot 10^4$ erg absorbed·cm^{-2}·sec^{-1}. Bacteriochlorophyll concn., 17.7 μg/ml.

Fig. 3. The effect of increasing absorbed light at 880 mμ on the inhibition by CCCP ($1.0 \cdot 10^{-6}$ M) of succinate photophosphorylation (▲—▲) and NAD+ photoreduction from succinate (●—●). Bacteriochlorophyll concn., 17.0 μg/ml.

levels of ADP liberated by the ATPase during the assay (no creatine phosphate *plus* creatine kinase was present) were insufficient to significantly inhibit ATPase activity. These dark ratios for ATP consumed (net)/NAD^+ reduced were higher than expected, either by analogy with similar energy-linked reactions in mitochondria or from thermodynamic considerations.

The sensitivities of the donor-supported photophosphorylation, dark ATP-driven NAD^+ reduction and NAD^+ photoreduction to the classical uncoupler *m*-chlorocarbonyl cyanide phenylhydrazone (CCCP) are shown in Figs. 2A and 2B. All five reactions showed closely similar sensitivities to this uncoupler; 50% inhibition of activity was observed with $1.1 \cdot 10^{-6}$–$2.2 \cdot 10^{-6}$ M CCCP, with photophosphorylation being slightly less sensitive than NAD^+ photoreduction. These reactions also exhibited similar sensitivities to desaspidin (not shown in Fig. 2); 50% inhibition was obtained with $5.8 \cdot 10^{-6}$–$6.8 \cdot 10^{-6}$ M desaspidin.

The results obtained when the concentration of CCCP was kept constant at $1.0 \cdot 10^{-6}$ M and the light intensity was varied are shown in Fig. 3. The percentage activity did not remain constant over the range of light intensities employed, but rather increased as the light intensity increased. This light-induced alleviation of inhibition followed a closely similar course for both succinate photophosphorylation and the succinate-linked photoreduction of NAD^+.

Figs. 4A and 4B show the effect of an active phosphorylation system (ADP, P_i, glucose and hexokinase) on the photoreduction of NAD^+ from succinate, over a wide range of absorbed light intensities at 880 mμ. The presence of such a phosphorylating system, which would compete for and utilize any high-energy intermediate, strongly inhibited NAD^+ photoreduction, especially at low light intensities (Fig. 4A). Plotted on the conventional Lineweaver–Burk basis (Fig. 4B), a mixed inhibition was obtained; the v_{max} for photoreduction decreased from 83 to 61 μmoles NAD^+/h·mg bacteriochlorophyll and the half-saturation value for absorbed light increased from $0.82 \cdot 10^4$ to $1.35 \cdot 10^4$ ergs·cm^{-2}·sec^{-1}. Figs. 5A and 5B present data similar to Figs. 4A and 4B but show the effect of an active phosphorylation system on the rate of NAD^+ photoreduction from ascorbate–TMPD. Again NAD^+ photoreduction was strongly inhibited, especially at low light intensities (Fig. 5A), but on the reciprocal

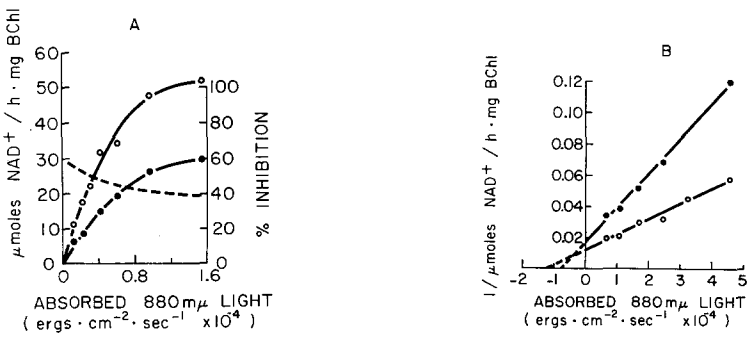

Fig. 4. The effect of a phosphorylation system (ADP, P_i, glucose and hexokinase; concentrations as described in METHODS) on the rate of photoreduction of NAD^+ by succinate as a function of absorbed light intensity. O—O, no additions; ●—●, *plus* phosphorylation system. — — —, percentage inhibition. Bacteriochlorophyll (BChl) concn., 16.9 μg/ml.

plot (Fig. 5B) a competitive rather than a mixed inhibition was observed. The half-saturation value for absorbed 880 mμ light was increased considerably by the presence of the phosphorylating system (from $0.52 \cdot 10^4$ to $2.56 \cdot 10^4$ ergs·cm^{-2}·sec^{-1}), whereas

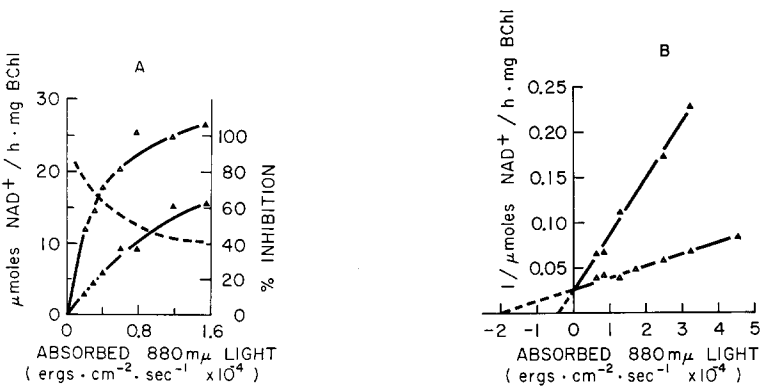

Fig. 5. The effect of a phosphorylation system (ADP, P_i, glucose and hexokinase; concentrations as described in METHODS) on the rate of photoreduction of NAD+ by ascorbate–TMPD as a function of absorbed light intensity. △—△, no additions; ▲—▲, *plus* phosphorylation system. – – –, percentage inhibition. Bacteriochlorophyll (BChl) concn., 16.7 μg/ml.

the v_{max} remained unchanged (38 μmoles NAD+/h·mg bacteriochlorophyll). In the experiments shown in Figs. 4 and 5 neither phosphate nor ADP alone showed any inhibitory effect; the presence of both was required for inhibition. The glucose *plus* hexokinase merely enhanced this inhibition by recycling the ADP. Thus, as regards the nature of the inhibition of NAD+ photoreduction by an active phosphorylation system, a significant difference was observed between the succinate and ascorbate–TMPD-linked systems. Such a difference could be related to the manner in which electrons from these two electron donors enter the cyclic flow; in the former case the entry is enzyme mediated (*via* succinate dehydrogenase), while in the case of ascorbate–TMPD, entry is *via* a purely chemical reaction between the electron donor and a *c*-type cytochrome.

The effect of an active succinate-linked NAD+ photoreduction system on photophosphorylation at a series of light intensities is shown in Fig. 6. Unlike the results shown in Figs. 4 and 5, where the presence of an active phosphorylation system strongly inhibited NAD+ photoreduction, the presence of an active NAD+ photoreduction system had no effect on the v_{max} of photophosphorylation (and only very small effects at measurable light intensities). The half-saturation value for absorbed light was not significantly increased above the value for endogenous photophosphorylation ($0.71 \cdot 10^4$ erg·cm^{-2}·sec^{-1}).

Succinate (and ascorbate–TMPD) alone caused a slight (0–20 %) decrease in the phosphorylation rate from the endogenous level at low light intensities, which was probably due to a slight change in the redox state of the chromatophores. NAD+ alone on the other hand caused no significant change in the rate of photophosphorylation. When the complete system of donor *plus* NAD+ was present, no further significant inhibition of photophosphorylation below the rate with succinate (or ascorbate–TMPD) alone was observed; quite often the rate in fact increased almost to

the endogenous level, presumably due to a 'rebalancing' of the redox state of the chromatophores by the mild oxidizing agent, NAD^+. Thus, there appeared to be no net inhibition of photophosphorylation during active NAD^+ photoreduction, even

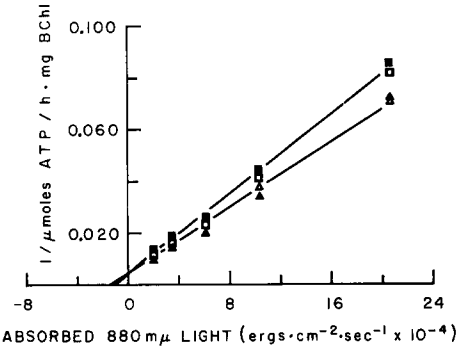

Fig. 6. The effect of an NAD^+ photoreduction system (succinate *plus* NAD^+; concentrations as described in METHODS) on the rate of photophosphorylation as a function of absorbed light intensity. △—△, endogenous; ▲—▲, *plus* NAD^+; □—□, *plus* succinate; ■—■, *plus* succinate + NAD^+. Bacteriochlorophyll (BChl) concn., 18.0 μg/ml.

though the latter reaction was still occurring at approximately one-half the uninhibited rate. Hence it appeared that when photophosphorylation and NAD^+ photoreduction were proceeding simultaneously, there was an increase in the efficiency of utilization of high-energy intermediate. This is clearly shown in Table III, where the photoformation of ATP and NADH was assayed simultaneously in the same reaction mixture. The rate of formation of total ~ was calculated from the rates of ATP *plus* NADH synthesis using two different values for the stoichiometry of ~ utilized/NAD^+ reduced (column a, 1 and 2 ~/NAD^+; column b, 2 and 4 ~/NAD^+, respectively, for succinate and ascorbate–TMPD-linked NAD^+ photoreduction). It can be seen that whichever of the two pairs of ratios were used, total ~ utilization was higher in the

TABLE III

A CALCULATION OF TOTAL~ UTILIZATION BY PHOTOPHOSPHORYLATION AND NAD^+ PHOTOREDUCTION

Photophosphorylation and NAD^+ photoreduction were assayed as described in METHODS. Total~ was computed by adding the rate of photophosphorylation (1 ~/ATP) in the presence of donor *plus* NAD^+, to the rate of NAD^+ photoreduction in the presence of an active phosphorylation system. Column (a) used 1 and 2 ~/NAD^+ and Column (b) 2 and 4 ~/NAD^+, respectively, for succinate and ascorbate–TMPD-supported NAD^+ photoreduction. Illumination: $0.8 \cdot 10^4$ erg absorbed·cm^{-2}·sec^{-1} at 880 mμ. Bacteriochlorophyll concn., 17.8 μg/ml. Data are expressed as μmoles/h·mg bacteriochlorophyll.

Photophosphorylation system plus	*ATP*	*NAD^+*	*Total*	
			(a)	*(b)*
—	111			
Succinate	94			
NAD^+	112			
Succinate + NAD^+	99	22	121	143
Ascorbate–TMPD	86			
Ascorbate–TMPD + NAD^+	109	11	131	153

presence of both energy-dependent systems than in the presence of either system alone. Similar results were obtained over a wide range of absorbed light intensities ($0.1 \cdot 10^4$–$1.6 \cdot 10^4$ ergs·cm^{-2}·sec^{-1} at 880 mμ).

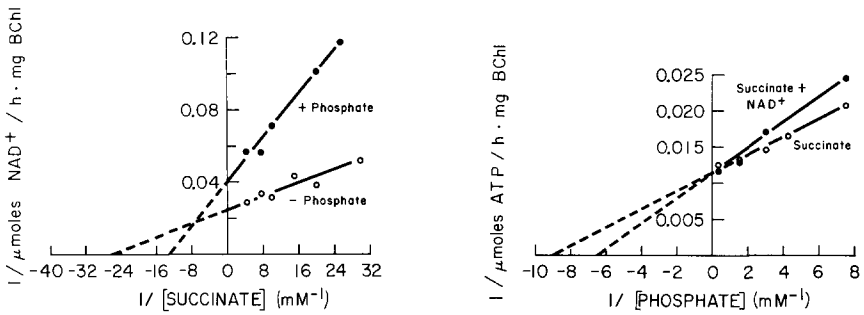

Fig. 7. The influence of succinate concentration on NAD+ photoreduction in the presence and absence of an active phosphorylating system. The reaction vessels contained the complete system for NAD+ photoreduction and photophosphorylation (●—●) or lacked phosphate (○—○). Illumination: $0.6 \cdot 10^4$ erg absorbed·cm^{-2}·sec^{-1} at 880 mμ. Bacteriochlorophyll (BChl) concn., 17.0 μg/ml.

Fig. 8. The influence of phosphate concentration on succinate supported photophosphorylation in the presence and absence of an active NAD+ photoreduction system. The reaction vessels contained the complete system for photophosphorylation and NAD+ photoreduction from succinate (●—●) or lacked NAD+ (○—○). Illumination: $0.6 \cdot 10^4$ erg absorbed·cm^{-2}·sec^{-1} at 880 mμ. Bacteriochlorophyll (BChl) concn., 16.3 μg/ml.

A further investigation into the nature of the competition between NAD+ photoreduction and photophosphorylation was carried out by keeping the intensity of illumination constant ($0.6 \cdot 10^4$ erg absorbed·cm^{-2}·sec^{-1} at 880 mμ) and by varying the concentrations of some of the reactants of the two photosystems. It was found that the concentration of succinate required to saturate NAD+ photoreduction (Fig. 7) was higher in the presence than in the absence of an active phosphorylation system (K_m for succinate increased from 38 to 76 μM), whereas the v_{max} of the photoreduction with respect to succinate concentration was lowered (from 41 to 25 μmoles NAD+/h·mg bacteriochlorophyll). The concentration of phosphate routinely employed for photophosphorylation (3.3 mM) was sufficient to give maximal inhibition of NAD+ photoreduction (in the presence of the other phosphorylation cofactors); higher concentrations had no further inhibitory effect, whilst substantially lower concentrations (<2 mM) were less inhibitory. Phosphate or ADP alone caused no inhibition.

A small increase in the K_m for phosphate of the photophosphorylation system (from 112 to 155 μM) was observed in the presence of an active NAD+ photoreduction system (Fig. 8) under similar conditions of illumination to those described for Fig. 7. However, no decrease was observed in the v_{max} for photophosphorylation (with respect to phosphate concentration) which remained at 89 μmoles/h·mg bacteriochlorophyll. It can be clearly seen that even when the phosphate concentration was as low at 5 % of that routinely used (165 μM *cf.* 3.3 mM), the presence of an active NAD+ photoreduction system inhibited photophosphorylation by only 16 %. This latter value may perhaps be a slight underestimate, since the addition of NAD+, apart from completing the requirements for an active photoreduction system, has

also on occasion (see Table III) been shown to slightly restimulate photophosphorylation by 'rebalancing' the redox state of the chromatophores.

The effects of antimycin A on succinate and ascorbate–TMPD-supported NAD^+ photoreduction and photophosphorylation are shown in Figs. 9A and 9B. The photoreduction of NAD^+ from succinate was much more sensitive to antimycin than was the ascorbate–TMPD-supported reaction (Fig. 9A); 50% inhibition occurred at

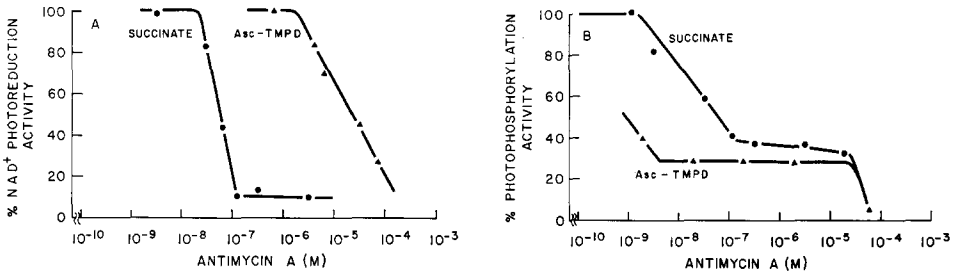

Fig. 9. The effect of antimycin A on succinate and ascorbate–TMPD-supported photophosphorylation and NAD^+ photoreduction. The reactions were assayed as described in METHODS. A. NAD^+ photoreduction from succinate (●—●) and ascorbate–TMPD (▲—▲). B. Photophosphorylation supported by succinate (●—●) and ascorbate–TMPD (▲—▲). Illumination: $0.6 \cdot 10^4$ erg absorbed$\cdot cm^{-2} \cdot sec^{-1}$ at 880 mμ. Bacteriochlorophyll concn., 17.6 μg/ml.

$5.9 \cdot 10^{-8}$ M and $3.1 \cdot 10^{-5}$ M antimycin A, respectively—a greater than 500-fold difference in concentration. The concentration required to inhibit the ascorbate–TMPD reaction was so high as to suggest that this was a general inhibition rather than a specific inhibition at the antimycin A-sensitive site. 50% inhibition of succinate and ascorbate–TMPD-supported photophosphorylation (Fig. 9B) was brought about by $5.5 \cdot 10^{-8}$ M and $1 \cdot 10^{-9}$ M antimycin A, respectively. The reason for the lower sensitivity of the ascorbate–TMPD-linked reaction was not clear. NAD^+ had no alleviatory effect upon antimycin inhibition of photophosphorylation and hence the argument put forward by BOSE AND GEST[16,17] that the insensitivity to antimycin A of the ascorbate–TMPD-supported NAD^+ photoreduction was due to a TMPD-mediated bypass of electron flow around the antimycin-sensitive site appeared, under these assay conditions, to be invalid.

TABLE IV

THE EFFECT OF ANTIMYCIN A ON PHOTOPHOSPHORYLATION AND NAD^+ PHOTOREDUCTION SUPPORTED BY SUCCINATE AND ASCORBATE–TMPD

Photophosphorylation and NAD^+ photoreduction were assayed as described in METHODS. Illumination: $0.6 \cdot 10^4$ erg absorbed$\cdot cm^{-2} \cdot sec^{-1}$ at 880 mμ. Bacteriochlorophyll concn., 17.6 μg/ml. $1.0 \cdot 10^{-6}$ M antimycin A was present where indicated. Data are expressed as μmoles/h$\cdot$mg bacteriochlorophyll.

Electron donor	*No inhibitor*			*+ Antimycin A*		
	ATP	*NAD^+*	*$\frac{ATP}{NAD^+}$ ratio*	*ATP*	*NAD^+*	*$\frac{ATP}{NAD^+}$ ratio*
Succinate	47.9	28.0	1.72	17.2	2.8	6.14
Ascorbate–TMPD	47.8	13.0	3.68	13.3	13.0	1.02

It can be seen from Fig. 9 that complete inhibition by antimycin A was not obtained for any of the four reactions assayed; an antimycin-insensitive plateau region was consistently observed at antimycin A concentrations between 10^{-7} M and 10^{-5} M. Table IV compares the rates of NAD^+ photoreduction and photophosphorylation in this region with those observed in the absence of antimycin A. It was clear that, with succinate as electron donor, NAD^+ photoreduction was more sensitive to antimycin A than was photophosphorylation; hence an increase in the ATP/NAD^+ ratio was observed. On the other hand, with ascorbate–TMPD as electron donor, photophosphorylation was more sensitive to antimycin than was NAD^+ photoreduction; then the ATP/NAD^+ ratio fell from approx. 3.7 to almost unity. However, the NAD^+ photoreduction was still fully sensitive to CCCP.

Finally, NADH oxidase, NADH–fumarate reductase (anaerobic) and the dark, ATP-driven reduction of NAD^+ by succinate were all insensitive to $1.0 \cdot 10^{-6}$ M antimycin A, whereas the dark, ATP-driven reduction of NAD^+ by ascorbate–TMPD appeared to be completely inhibited at this concentration.

DISCUSSION

The results reported above appear to confirm previous suggestions[10,18] that the photoreduction of NAD^+ by succinate in chromatophores of *R. rubrum* occurs *via* an energy-requiring electron flow, presumably driven by high-energy intermediates of photophosphorylation. The results in this paper suggest that the photoreduction of NAD^+ from a second donor system, *viz.* ascorbate–TMPD, is also energy dependent.

The 880-mμ light saturation curves for NAD^+ photoreduction and photophosphorylation showed similar half-saturation values with respect to absorbed light for each of the four photoreactions assayed. This suggested that the photosystem responsible for the formation of NADH or ATP contained a common limiting step(s). The high and very similar sensitivity of the photophosphorylation and NAD^+ photoreduction systems to uncouplers such as CCCP and desaspidin strongly indicated that such a common step existed at the level of a high-energy intermediate of photophosphorylation (probably nonphosphorylated) which we shall call, for the sake of scientific continuity and the lack of anything better, X ~ I. This view was further supported by the observation that NAD^+ photoreduction from either electron donor was strongly inhibited in the presence of an active photophosphorylation system, presumably as a result of a competition for this high-energy intermediate.

(*a*) *Pathways of electron flow in NAD^+ photoreduction*

R. rubrum may be grown either aerobically in the dark with the resultant formation of an electron transport system capable of catalysing oxidative phosphorylation, or anaerobically in the light with the resultant formation of a photosynthetic electron transport system capable of catalysing cyclic photophosphorylation. In the latter case a small residual oxidative activity can also be observed.

A postulated scheme for electron transport in *R. rubrum* chromatophores from photosynthetically grown cells is shown in Fig. 10. Antimycin A at low concentrations inhibits only cyclic electron flow (hence photophosphorylation and dark, ATP-driven reduction of NAD^+ by ascorbate–TMPD) but has no effect on the oxidative systems as expressed by NADH oxidase, NADH–fumarate reductase and the dark ATP-driven

reduction of NAD^+ by succinate. Similarly, the photoreduction of NAD^+ by ascorbate–TMPD is also insensitive to concentrations of antimycin A which were sufficient to substantially inhibit ascorbate–TMPD-associated photophosphorylation. This latter observation suggests that although electrons originating from ascorbate–TMPD

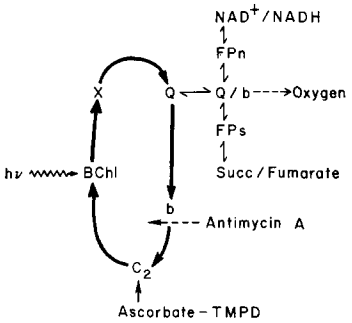

Fig. 10. A possible scheme for electron transport in *R. rubrum* chromatophores illustrating photosynthetic (➡) and oxidative electron transport (→). The open dotted arrow represents partial inhibition by antimycin A.

may normally pass backwards through the antimycin-sensitive site in order to photoreduce NAD^+ *via* a strongly energy-dependent route, it would appear that in the presence of antimycin A a thermodynamically more favourable type of electron flow may occur in which high-energy intermediates formed as a result of antimycin-insensitive cyclic flow now have to overcome only one thermodynamically unfavourable step, *viz.* at the level of NADH dehydrogenase.

A type of noncyclic electron flow between ascorbate–DCIP (which probably denotes electrons at the same point as ascorbate–TMPD) and NAD^+ in the presence of antimycin A (with the formation of ATP) has previously been claimed by ARNON *et al.*[19,20]. However, BOSE AND GEST[16,17] were able to show that this ATP formation was a result of the ability of certain redox dyes such as DCIP and TMPD to by-pass the antimycin-sensitive site and thus allow cyclic photophosphorylation to proceed at something approaching the uninhibited rate. This in turn could drive the energy-linked photoreduction of NAD^+ from ascorbate–TMPD, which would then appear to be insensitive to antimycin A.

Our results support the concept that high-energy intermediates of cyclic photophosphorylation provide the energy needed for the photoreduction of NAD^+ by ascorbate–TMPD even in the presence of antimycin A. However they also demonstrate that under the conditions of our assays ascorbate–TMPD ($\pm$ NAD^+) does not reactivate antimycin A-inhibited photophosphorylation by catalysing an electron by-pass around the antimycin-sensitive site (contrast BOSE AND GEST[16,17]) and that as a direct result of this, the ratio of the rate of photophosphorylation to NAD^+ photoreduction supported by ascorbate–TMPD severely decreases in the presence of antimycin A. Thus two mechanisms for the photoreduction of NAD^+ from ascorbate–TMPD may exist in these chromatophores; firstly a relatively inefficient reaction with respect to $\sim$ utilization, which occurs under uninhibited conditions (ATP/NAD^+ ratios 4–5:1) and a second reaction of apparently higher efficiency which occurs in the presence of low concentrations of antimycin A (ATP/NAD^+ ratios approx. 1:1).

Both reactions appear to be completely energy linked due to their complete sensitivity to CCCP.

The photoreduction of NAD^+ by succinate, on the other hand, exhibited a sensitivity towards antimycin A which was not unlike that of succinate-supported photophosphorylation, although the extent of inhibition was somewhat greater than for phosphorylation.

The measured stoichiometry for ATP consumed/NAD^+ reduced for the dark, ATP-driven reduction of NAD^+ by succinate (1.8) or ascorbate–TMPD (5.2) suggests the presence of more phosphorylation sites than hitherto contemplated. Owing to the difficulties in designating locations for the coupling sites within the limited framework of known electron carriers, it seems reasonable to suspect that these values may be high. One might reasonably have expected, from thermodynamic considerations and by analogy with results obtained with mitochondria, ratios of ATP consumed/NAD^+ reduced of approx. 1 and 2 for succinate and ascorbate–TMPD, respectively. However, it is possible that an as yet unexplained hydrolysis of $X \sim I$ (and thus ATP) may occur in the presence of substrate *plus* NAD^+ which does not occur in the presence of either substrate or NAD^+ alone, and which cannot be used to drive the reduction of NAD^+. Although CHANCE AND HOLLUNGER[21] originally obtained ATP consumed/NADH formed ratios of approx. 2 for the reduction of NAD^+ by succinate in intact mitochondria, the accepted stoichiometry is now 1 (see refs. 22–24) and 2 for the reduction of NAD^+ by ascorbate–TMPD[25].

The possibility that a direct (*i.e.*, nonenergy-dependent) photoreduction of NAD^+ may occur is unlikely, since low concentrations of uncouplers can effect complete inhibition of these reactions. However, if cyclic electron flow was so tightly coupled to phosphorylation, such that by the addition of an uncoupler (or of an active phosphorylation system) cyclic flow became considerably stimulated, then this could cause a draining away of electrons from a direct pathway of NAD^+ reduction. Under such conditions one might expect a partial inhibition of NAD^+ photoreduction following the addition of either an uncoupler or an active phosphorylation system but hardly the complete inhibition which was experimentally observed in the former case. Also there is no convincing evidence to suggest that the cyclic system is indeed coupled to phosphorylation with this degree of tightness, although electron flow in the oxidative system can be somewhat stimulated by the addition of phosphorylation cofactors.

(b) The interaction of the high-energy intermediate with the NAD^+ photoreduction and photophosphorylation system

LEE AND ERNSTER[26] have discussed the case of competition for available energy in sub-mitochondrial particles between the energy-linked transhydrogenase and oxidative phosphorylation in terms of classical enzyme kinetics. They postulated that a high-energy intermediate of phosphorylation, $X \sim I$, was able to combine either with NADH (as a substrate for the transhydrogenase) or P_i (as the primary substrate for phosphorylation) or both, and that the affinity of the $X \sim I$ for these substrates and the rates of formation of the products of these reactions were dependent upon which of the three possible enzyme–substrate complexes (NADH–$I \sim X$, $I \sim X$–P_i or NADH–$I \sim X$–P_i) were formed.

Briefly the results which we have obtained with *R. rubrum* chromatophores per-

taining to the competition for available energy between energy-linked NAD^+ photoreduction and photophosphorylation are very different from those obtained by LEE AND ERNSTER for the competition between the energy-linked transhydrogenase and oxidative phosphorylation in submitochondrial particles. In our chromatophore preparations, photophosphorylation appeared to be the dominant energy-linked reaction, since the presence of an active phosphorylation system considerably lowered the v_{max} for NAD^+ photoreduction and at the same time increased the K_m for both succinate and light, whereas the presence of an active NAD^+ photoreduction system did not significantly affect either the v_{max} for photophosphorylation or the K_m for light, and only marginally increased the K_m for phosphate. In the submitochondrial particles used by LEE AND ERNSTER on the other hand, the energy-linked transhydrogenase appeared to be dominant over oxidative phosphorylation, since in the presence of an active transhydrogenase system, the v_{max} for ATP synthesis decreased whilst the K_m for phosphate (but not for ADP) increased. In the presence of an active phosphorylation system the v_{max} for transhydrogenase was not changed, whereas the K_m for NADH was increased.

However, in spite of the different experimental results obtained with submitochondrial particles and chromatophores, we feel that the postulate of LEE AND ERNSTER[26], that the transhydrogenase system and the phosphorylation system may react at two different sites on $X \sim I$, may also be applicable to *R. rubrum* chromatophores where one also has to implicate $X \sim I$ in a third energy-dependent system, *viz.* the photoreduction of NAD^+. Results expressed in Fig. 1 indicated that the rates of photoreduction of NAD^+ from succinate or ascorbate–TMPD were approximately one-half and one-quarter of the photophosphorylation rates, respectively, when assayed in parallel experiments. This suggested that either (a) the $X \sim I$ was being efficiently utilized in all the reactions, in which case the photoreduction of NAD^+ from succinate and ascorbate–TMPD required approx. 2 and 4 $\sim$/NAD^+, respectively, or (b) that approx. 50% of the $X \sim I$ was undergoing a useless hydrolysis to $X + I$ + heat and that only the remaining 50% of the total $X \sim I$ was able to drive NAD^+ photoreduction, now with $\sim$/NAD^+ ratios of approx. 1 and 2, respectively, for succinate and ascorbate–TMPD. The second of these possibilities appeared to be the more attractive, since it utilized $\sim$/NAD^+ ratios which are both thermodynamically and mechanistically more feasible than the higher values and which have been experimentally verified for mitochondria. Support for this mechanism was furnished by the observation of KEISTER AND YIKE[27] that when the succinate-linked photoreduction of NAD^+ at less than saturating light intensities was assayed by the rate of formation of NADH, the rates were slightly slower than when coupled to the energy-linked transhydrogenase and assayed as NADPH formation. Since the energy-linked transhydrogenase utilized 1 ATP/$NADP^+$ reduced from NADH[27] sufficient $X \sim I$ must have been generated by cyclic flow to drive both reactions simultaneously without any lowering of rates. This could not have occurred had the $\sim$/NAD^+ ratio for the photoreduction of NAD^+ from succinate been 2 rather than 1 as initially indicated by the results from Fig. 1, and strongly suggests that less than one-half of the $X \sim I$ formed could be used to drive NAD^+ photoreduction, whereas almost all of the $X \sim I$ formed appeared to be capable of driving ATP synthesis. This would mean that the rate of useless hydrolysis of $X \sim I$ to $X + I$ + heat is very much greater in the former case than in the latter. Thus, one might expect that when the NAD^+

photoreduction and photophosphorylation system are operating simultaneously, the useless hydrolysis of X ~ I would be virtually eliminated, *i.e.*, useful utilization of X ~ I would tend towards a maximum. This was indeed observed in Table III where the rate of total ~ formation (ATP + NADH) when both systems were operating simultaneously was greater than the sum of the rates of formation of ATP and NADH when either system was operating singly.

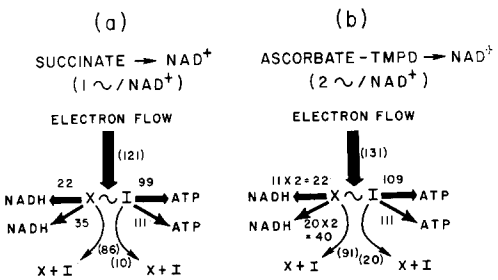

Fig. 11. Possible interaction of X ~ I with the photophosphorylation and NAD+ photoreduction systems supported by succinate and ascorbate–TMPD. The diagram shows the generation of X ~ I as a result of cyclic electron flow (▬▶), the utilization of X ~ I to form NADH and ATP when both systems are operating simultaneously (▬▶) or when either system is operating singly (⟶) and the useless hydrolysis of X ~ I when either system is operating singly (⟶). The numbers refer to the rates of each reaction expressed as μmoles/h per mg bacteriochlorophyll and were either taken from the results shown in Table III (open numbers) or were calculated from those results (bracketed numbers). In the latter case the rate of useless hydrolysis of X ~ I was calculated by subtracting the rate of utilization (*e.g.*, to form ATP or NADH) from the rate of generation. It was assumed that in the presence of both photophosphorylation and NAD+ photoreduction systems that the rates of useless hydrolysis were zero, *i.e.*, complete utilization. Illumination: $0.8 \cdot 10^4$ erg absorbed $\cdot cm^{-2} \cdot sec^{-1}$ at 880 mμ.

A postulated scheme for the involvement of X ~ I with succinate or ascorbate–TMPD-supported NAD+ photoreduction and photophosphorylation is shown in Fig. 11. The numbers refer to the rates of the various reactions, and are expressed as μmoles/h · mg bacteriochlorophyll and were either experimentally observed values (see Table III) or calculated from these values (bracketed numbers). It can be seen that when NAD+ photoreduction was assayed in the absence of an active photophosphorylation system, high rates of useless hydrolysis of X ~ I were calculated to have occurred (86 and 91), yet when photophosphorylation was assayed in the absence of an active NAD+ photoreduction system these rates were very much lower (10 and 20) and were in approximate agreement with the endogenous ATPase activity (18 μmoles/h · mg bacteriochlorophyll).

This scheme as it stands does not consider the active transhydrogenase system present in these chromatophores. The data of KEISTER AND YIKE[27] indicate that the light-driven transhydrogenase activity parallels that of photophosphorylation, when assayed separately at low light intensities, which suggests that the transhydrogenase is able to utilize X ~ I as efficiently as the phosphorylation system.

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