Energy-Linked Reactions in Photosynthetic Bacteria. III. Further Studies on Energy-Linked Nicotinamide–Adenine Dinucleotide Reduction by *Rhodospirillum rubrum* Chromatophores*

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ABSTRACT: The effect of uncouplers of photophosphorylation on the reduction of oxidized nicotinamide–adenine dinucleotide in chromatophore preparations of *Rhodosprillum rubrum* was that the inhibitory effect of these compounds increased as the light intensity and rate of oxidized nicotinamide–adenine dinucleotide reduction was decreased. Inhibitors of electron transport such as rotenone and 2-heptyl-4-hydroxyquinoline *N*-oxide had the same effect at all light intensities. These results are interpreted to indicate that all of the oxidized nicotinamide–adenine dinucleotide reduction observed in this system is an energy-driven reaction utilizing a high-energy intermediate (I∼X) of phosphorylation. The rate of the adenosine triphosphate driven reaction in these chromatophores was generally 15–25% the rate of the light-driven reduction. Several experiments indicated that oxidized nicotinamide–adenine dinucleotide reduction had a greater requirement for I∼X than the energy-linked transhydrogenase also found in these preparations. Therefore, the rate-limiting step was postulated to be the formation of I∼X. Low concentrations of quinacrine were found to stimulate oxidized nicotinamide–adenine dinucleotide reduction when reduced 2,6-dichloroindophenol was the electron donor but not with succinate or *N,N,N',N'*-tetramethyl-*p*-phenylenediamine as the electron donor. Other studies indicated that quinacrine was stimulating the photooxidation of reduced 2,6-dichloroindophenol.

In a recent publication (Keister and Yike, 1967a), we demonstrated that NAD^+ reduction in chromatophores of *Rhodospirillum rubrum* could be driven by ATP and PP_i as well as light and that the reaction could be described best by the equation

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

where I∼X represents a high-energy intermediate of phosphorylation which can be formed by light, ATP, GTP, and at lower levels by PP_i and ITP (Keister and Yike, 1967b).

However, the rate we observed for the succinate-linked ATP-driven reaction was only about 25% the rate of the light-supported reaction. To account for this discrepancy in rates, we proposed that the rate-limiting reaction was the formation of the intermediate I∼X from ATP since the rate of ATP hydrolysis by chromatophores was lower than the maximum rate of photophosphorylation. A further indication that the level of I∼X was the limiting factor was the observation that the addition of PP_i, which is an energy source in *R. rubrum*, plus ATP increased the rate of NAD^+ reduction over that obtained with ATP alone. Oligomycin, which inhibited the utilization of ATP, had no effect on PP_i utilization. Thus the pathway of I∼X formation from these compounds may be different and lends support to the postulation that the level of I∼X was increased by adding both ATP and PP_i. These observations did not eliminate the possibility that part of the NAD^+ reduction in *R. rubrum* occurs by a direct reduction of NAD^+ from a low-potential electron donor (such as occurs in green plants) as has been previously proposed (Nozaki *et al.*, 1961; Vernon, 1963).

The studies presented in this paper lead to the conclusion that all of the light-driven NAD^+ reduction is an energy-linked reaction and that the enzyme complex which catalyzes NAD^+ reduction is similar to the NADH dehydrogenase of mitochondria in its sensitivity to inhibitors.

Experimental Procedure

R. rubrum S-1 was grown and chromatophores were prepared as previously described (Keister and Yike, 1966). NAD^+ reduction was determined as previously described except that the normal intensity of illumination was 2.6×10^5 ergs cm^{-2} sec^{-1} filtered through 1 in. of water. Bacteriochlorophyll was determined using the *in vivo* extinction coefficient reported by Clayton (1963).

Photophosphorylation and the energy-linked transhydrogenase were measured as previously described (Keister and Yike, 1967b). The reaction mixture for the determination of NADH oxidation contained the following: 50 mM Tris-Cl (pH 8), 1 mM $MgCl_2$, 0.83 mM ADP, 3.3 mM P_i, 0.1 mM NADH, and 3.3 mM potassium fumarate in a total volume of 3 ml.

* Contribution No. 329 from the Charles F. Kettering Research Laboratory, Yellow Springs, Ohio 45387. *Received August 19, 1968.*

TABLE I: Effect of Uncouplers and Inhibitors on NAD^+ Reduction.[a]

Compound (μM)	Electron Donor (% inhibition)							
	Succinate			2,5-Diaminotoluene			*N,N,N',N'*-Tetramethyl-*p*-phenylenediamine	Reduced 2,6-Dichloroindophenol
	Light Intensity (ergs cm^{-1} sec^{-1} $\times$ 10^{-3})							
	100	24	7.4	100	24	7.4	100	100
m-Chlorocarbonyl cyanide phenylhydrazone (4)	32	75	90	28	83	87	45	76
Desaspidin (0.04)	28	52	83	41	84	98	40	52
Dicumarol (100)	40	48	79	38	69	80	20	25
Gramicidin (0.02)	31	57	67	38	64	66	17	12
Quinacrine (20)	40	36	39	18	22	36	14	*b*
Pentachlorophenol (15)	42	35	28	10	20	49		
Chlorpromazine (30)	36	32	25				20	12
Linolenate (10)	41	43	39	37	35	35	25	40
Rotenone (0.2)	42	50	50	50	47	50	35	37
2-Heptyl-4-hydroxyquinoline *N*-oxide (0.67)	63	59	61					

[a] These results were compiled from a group of experiments. Typical rates for the succinate-linked reaction were 26, 17, and 7 μmoles per mg of bacteriochlorophyll per hr for 100×10^3, 24×10^3, and 7.4×10^3 ergs cm^{-2} sec^{-1}, respectively. Relative rates of the 2,5-diaminotoluene, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, and reduced 2,6-dichloroindophenol linked reactions can be found in Table II. The concentration of the artificial electron donor for 2,5-diaminotoluene, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, and reduced 2,6-dichloroindophenol was 2×10^{-3}, 1.5×10^{-4}, and 6×10^{-5} M, respectively. The reaction also included 3.3×10^{-3} M ascorbate instead of succinate. [b] Stimulates 60%.

Enzymes and nucleotides were obtained from the Sigma Chemical Co. and P–L Biochemicals, Inc. Desaspidin was obtained from Ox Medica AB Helsingfors, Finland, and 2,5-diaminotoluene from Aldrich Chemical Co.

Results

The Effect of Uncouplers and Inhibitors. The effect of uncoupling agents and inhibitors as a function of the light intensity is presented in Table I. The first four compounds are well-known uncouplers in mitochondria and appear to be uncouplers in chromatophores based on their effect on the energy-linked transhydrogenase and photophosphorylation (Keister and Yike, 1967b). These compounds (*m*-chlorocarbonyl cyanide phenylhydrazone, desaspidin, gramicidin, and dicoumarol) all inhibited NAD^+ reduction more at low light intensity than at higher intensities and almost 100% inhibition was observed with *m*-chlorocarbonyl cyanide phenylhydrazone and desaspidin at the low intensity. Essentially the same results were observed whether the electron donor was succinate or a nonphysiological compound such as 2,5-diaminotoluene. These results are compatible with and expected for an energy-linked reduction of NAD^+.

The mechanism of inhibition of the second group of compounds (quinacrine, pentachlorophenol, and chlorpromazine) is ambiguous. With succinate as the electron donor, variation in the light intensity had no effect on the degree of inhibition. They had a lesser effect when artificial electron donors were used but showed a response to the light intensity typical of an uncoupling agent. These results could be explained by postulating that these compounds are inhibitory to the succinate-oxidizing system in addition to having an uncoupling effect. However, they had no effect on succinate–cytochrome *c* reductase activity. Quinacrine actually stimulated NAD^+ reduction with reduced 2,6-dichloroindophenol as the electron donor, and this phenomenon will be described more completely below.

The last two compounds (rotenone and 2-heptyl-4-hydroxyquinoline *N*-oxide) are well-known inhibitors of electron transport, rotenone in the NADH–cytochrome *b* region and 2-heptyl-4-hydroxyquinoline *N*-oxide in the cytochrome *b*–cytochrome *c* region. Thus rotenone is a specific inhibitor of NAD^+ reduction while 2-heptyl-4-hydroxyquinoline *N*-oxide inhibits photosynthetic electron transport. Both of these compounds have the same effect at all light intensities. We are not sure of the inhibitory mechanism of linolenate. This type of fatty acid is known to be an uncoupling agent in mitochondria (Slater, 1963) and an inhibitor

of electron transport in chloroplasts (Krogmann and Jagendorf, 1959). In chromatophores, it has a greater inhibitory effect on NAD^+ reduction than on NADH oxidation or photophosphorylation.[1] Thus we think the primary action of linolenate is an uncoupling effect specific for the NAD^+ coupling site.

Rates of Light and ATP-Driven NAD^+ Reduction. The rates of NAD^+ reduction with various electron donors are presented in Table II. The best electron donor that we have found for the light-driven reaction is diaminodurene as reported recently by Trebst *et al.* (1967). Phenol blue and 2,5-diaminotoluene were also found to be very efficient electron donors. For the ATP-driven reaction succinate was the best electron donor and in this experiment was 20% the rate of the light-driven reaction. 2,5-Diaminodurene was a fair electron donor while little reaction was found with the other compounds. There appears to be little correlation of the effectiveness of the various electron donors to promote the light or ATP-driven reaction. The best rate of ATP-driven NAD^+ reduction that we have observed using chromatophores prepared from normal photosynthetic cells (Keister and Yike, 1966) was 18 μmoles/mg of bacteriochlorophyll per hr compared with a light-driven rate of 49.

Antimycin a was found to inhibit the ATP-driven reaction with the artificial electron donors but had no effect on the succinate-linked reaction. This indicates that the electrons from these compounds enter the electron transport chain beyond the antimycin a site (possibly through cytochrome *c*) and must traverse the antimycin a sensitive site which is presumably within the cytochrome *b*–cytochrome *c* region of the electron transport chain. Succinate probably enters at the quinone–cytochrome *b* level. This observation gives a clue to the low efficiency of the artificial electron donors for the ATP-driven reaction. We have found that there is little ATP formation coupled with the oxidation of succinate in our chromatophore preparations ($P/O \leq 0.1$) whereas there is ATP formation coupled to the oxidation of NADH ($P/NADH \geq 0.4$) in agreement with the results of Geller (1962) and Yamashita *et al.* (1967). Either there is no coupling site between cytochrome *b* and oxygen or it has been destroyed during preparation of the chromatophores. Since there is apparently no energy-coupling site between cytochrome *b* and the site where the artificial electron donors enter the chain, there is no effective way to drive the electrons from the cytochrome *c* level ($E'_0 \cong 0.32$) to the cytochrome *b* ($E'_0 \cong 0.05$) level in the dark. We believe that this is the probable explanation for the lower rates of NAD^+ reduction with the artificial electron donors than with succinate. This problem does not exist with succinate and we must look for another explanation for the low rates with this electron donor.

The effect of antimycin a on the light-driven reduction of NAD^+ using the artificial electron donors was dependent upon the concentration of the electron donor used. Strong inhibition was observed using suboptimal concentrations whereas little effect was observed at higher concentrations. The optimal concentration of the electron donors for NAD^+ reduction generally corresponded closely with the concentration required to optimally restore antimycin a inhibited photophosphorylation. We interpret these results to indicate that low concentrations of the donors can supply electrons for NAD^+ reduction at a site beyond the antimycin a sensitive site provided cyclic electron flow is unimpeded, whereas higher concentrations are required to effectively restore cyclic electron transport, thus providing the $I\sim X$ required to drive NAD^+ reduction.

Effect of Inhibitors on NAD^+ Reduction and NADH Oxidation. The curves presented in Figure 1a–1c were designed to demonstrate that the same enzyme or enzyme complex is involved in both the light and ATP-driven NAD^+ reduction. Within the concentrations used in these experiments these inhibitors have little effect on photophosphorylation or the energy-linked transhydrogenase (Keister and Yike, 1967a). Rotenone is a well-known inhibitor of NADH oxidation in mitochondria and apparently inhibits in the flavoprotein region of the electron transport chain (Chance *et al.*, 1967). In *R. rubrum* this compound inhibits both the ATP and light-driven reduction of NAD^+ and NADH oxidation to approximately the same extent. Amytal has a dual effect in mitochondria, inhibiting both electron and energy transfer at site I (Chance and Hollunger, 1963). In *R. rubrum*,

TABLE II: Rates of NAD^+ Reduction with Various Electron Donors.[a]

Electron Donor	Energy Source (μmoles/mg of Bacteriochlorophyll per hr)		
	Light	ATP	Inhibn by Antimycin a
2,5-Diaminodurene	80	4.5	+
2,5-Diaminotoluene	66	2.2	+
Phenol blue	60	1.5	+
Succinate	40	7.8	−
N,N,N',N'-Tetramethyl-*p*-phenylenediamine	21	2.7	+
Reduced 2,6-dichloroindophenol	20	1.3	

[a] The concentration of 2,5-diaminodurene and phenol blue was 3.3×10^{-4} and 1.3×10^{-4} M, respectively, and included 3.3×10^{-3} M ascorbate instead of succinate. For the ATP-driven reactions, a generating system was used consisting of 1.5×10^{-3} M phosphocreatine, 5–10 units of creatine phosphokinase, and 3.3×10^{-3} M ATP. The ATP-driven reactions were carried out in anaerobic cuvets under argon. Antimycin a concentration was 10^{-6} M.

[1] Unpublished results.

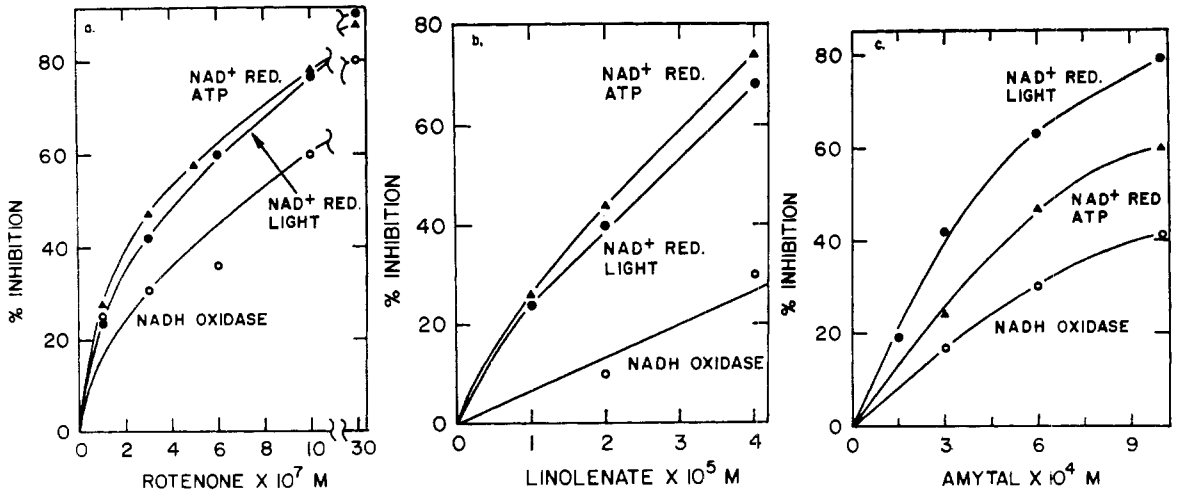

FIGURE 1: Effect of rotenone (a), linolenate (b), and amytal (c) on NAD^+ reduction and NADH oxidation.

amytal apparently is primarily an energy-transfer inhibitor since it has a greater effect on NAD^+ reduction than on NADH oxidation. As previously noted, linolenate inhibited somewhat specifically the reduction of NAD^+. We cannot distinguish from these studies whether it is an inhibitor or uncoupler, but it is more likely to be acting as an uncoupler since there was much less effect on NADH oxidase than on the reduction.

These results support the hypothesis that both the light and ATP-driven reduction of NAD^+ occur by the same mechanism although the mechanism of inhibition by amytal and linolenic acid is unclear. We believe that amytal and linolenate are acting as either energy-transfer inhibitors or uncouplers in this system since they have much less effect on the oxidase than on the reduction of NAD^+. If this is true, they are site specific since they have little effect on photophosphorylation or the energy-linked transhydrogenase at these concentrations.

Effect of Quinacrine. From our previous studies, quinacrine appeared to be an uncoupling agent in *R. rubrum* chromatophores since it inhibited both ATP and light-driven energy-linked reactions (Keister and Yike, 1967a). Therefore, we were surprised to find that it stimulated the light-driven reduction of NAD^+ when we used reduced 2,6-dichloroindophenol and ascorbate as the electron donor system (Table I).[2] The maximum rate of NAD^+ reduction with reduced 2,6-dichloroindophenol was about 50% that observed with succinate, and this rate was stimulated 85% by 1 $\times 10^{-5}$ M quinacrine. This same concentration of quinacrine was inhibitory when succinate, 2,5-diaminotoluene, or *N,N,N',N'*-tetramethyl-*p*-phenylenediamine was the electron donor. Stimulation was also observed when compounds related to 2,6-dichloroindophenol, *i.e.*, 2,3',6-trichloroindophenol and phenol blue, were used as electron donors. In addition, chlorpromazine, which is related structurally to quinacrine, had a slight stimulatory effect at 1 $\times 10^{-5}$ M. These results are presented in Figure 2. Increasing the concentration of quinacrine above 1 $\times 10^{-5}$ M produces inhibition and if the inhibition is plotted using the rate obtained with 1 $\times 10^{-5}$ M as the 100% level the curve parallels the *N,N,N',N'*-tetramethyl-*p*-phenylenediamine curve of Figure 2. The stimulatory effect of quinacrine is dependent upon the reduced 2,6-dichloroindophenol concentration and increases considerably when lower donor (suboptimal) concentrations were used. Thus, we have observed stimulations of almost 200% with a low (1.6 $\times 10^{-5}$ M) level of reduced 2,6-dichloroindophenol. The maximum rate of NAD^+ reduction that we have observed, however, has never exceeded that obtained with succinate as the electron donor. Quinacrine alone does not act as an electron donor and red light was as effective as white light in supporting this reduction.

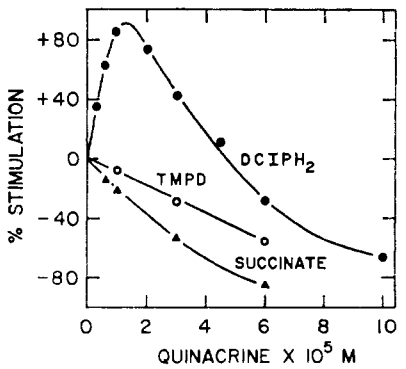

FIGURE 2: Effect of quinacrine on NAD^+ reduction. Concentrations of 2,6-dichloroindophenol and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine were as in Table I.

Quinacrine has no stimulatory effect on photophosphorylation which has been stimulated with reduced 2,6-dichloroindophenol nor on the photophosphorylation catalyzed by reduced 2,6-dichloroindophenol in the presence of 2-heptyl-4-hydroxyquinoline *N*-oxide or antimycin a. In addition, we have not found any effect on the dark oxidation of succinate or NADH using 2,6-dichloroindophenol as the electron acceptor.

Ash *et al.* (1961) have reported that quinacrine stimulated the photoreduction of methyl red and tetrazolium

[2] Similar results have been found by J.-H. Klemme, personal communication.

blue using reduced 2,6-dichloroindophenol as the electron donor. Stimulations of up to eightfold were observed with 1×10^{-4} M quinacrine. Bose and Gest (1962) have found that quinacrine stimulated the reduction of fumarate using H_2 as the electron donor and 2,6-dichloroindophenol as the electron carrier. In addition, Vernon (1963) has reported that quinacrine stimulated the photooxidation of reduced 2,6-dichloroindophenol using fumarate as the electron acceptor. This is essentially the same reaction studied by Bose and Gest. The only common component in these reactions is 2,6-dichloroindophenol, and it appears as if quinacrine is acting to facilitate the photooxidation of reduced 2,6-dichloroindophenol. We confirmed this by studying the photooxidation of reduced 2,6-dichloroindophenol using oxygen as the electron acceptor and found that 1×10^{-4} M quinacrine stimulated this oxidative process by sevenfold but had no effect on the dark oxidation. In addition, the photooxidation of reduced phenol blue was stimulated threefold.

Thus it appears as if quinacrine has a dual effect on the reduction of NAD^+ linked to reduced 2,6-dichloroindophenol oxidation. The initial stimulatory effect on NAD^+ reduction is probably due to a stimulation of the oxidation of reduced 2,6-dichloroindophenol followed by inhibition due to the interference with the energy-coupling system.

Discussion

In mitochondria, the energy-linked reduction of NAD^+ involves a reversal of oxidative phosphorylation at coupling site I which implies a stoichiometric requirement for I∼X and reduced flavoprotein as the electron donor for NAD^+ reduction at this site. In mitochondria, this process is not likely to be an important source of NADH for cellular reactions. Krebs (1967) has estimated that when carbohydrate is oxidized in the cell, a maximum of one-ninth and, during fatty acid oxidation, two-ninths of the potential NADH could be generated by energy-linked reduction. Furthermore, he concluded that this contribution was likely to be zero. The situation is entirely different with chemoautotrophic microorganisms which obtain their energy by oxidizing substrates with redox potentials more positive than NAD^+–NADH. Examples of these organisms which have been demonstrated to couple the oxidation of inorganic substrates with NAD^+ reduction are *Nitrosomonas* which oxidizes ammonia (Aleem, 1966a), *Nitrobacter* which oxidizes nitrite (Kiesow, 1963; Aleem *et al.*, 1963), and *Thiobacillus* which oxidizes thiosulfate (Aleem, 1966b). The only mechanism available to these organisms to produce NADH for the reduction of CO_2 apparently is the energy-linked reversal of oxidative electron transport.

Theoretically in photosynthetic microorganisms, NAD^+ could be reduced by a low-potential compound produced by light such as occurs in green plants and algae, or by reversed electron transport utilizing the energy produced by photophosphorylation. The mechanism of reduction of pyridine nucleotide has been established for several years in green plants (see review by San Pietro and Black, 1965) and involves the reduction of $NADP^+$ by a flavoprotein enzyme using ferredoxin ($E'_0 = -0.42$ V) as the electron donor. It has been postulated that chromatophores utilized a similar process primarily, we believe, to preserve the idea of biochemical unity among photosynthetic cells. Only recently has it been established that chemoautotrophic microorganisms were capable of reducing NAD^+ by an energy-linked process. Thus it is now possible to preserve the idea of biochemical unity by postulating that purple nonsulfur photosynthetic bacteria resemble the chemoautotrophes rather than green plants.

We believe that we have presented strong evidence that all of the NAD^+ reduction observed in *R. rubrum* is by an energy-linked process. While it can be argued that the inhibitory effect of uncoupling agents could be due to their accelerating cyclic electron flow and thus diverting electrons from the NAD^+ pathway, the increasing inhibition observed with *m*-chlorocarbonyl cyanide phenylhydrazone, desaspidin, dicoumarol, and gramicidin as the light intensity is decreased (Table I) is not compatible with this mechanism. This argument implies that the rate-limiting step in cyclic electron flow is the site of coupling of electron flow to phosphorylation. Otherwise, uncouplers would have no effect. As the light intensity is lowered, the photoreaction would become rate limiting rather than the coupling site. If the coupling site is not the rate-limiting step, uncoupling agents should have less effect at low light intensities. Since we observe the opposite situation, this cannot be the mechanism of inhibition by the uncouplers. The more likely effect, therefore, is that these compounds dissipate a high-energy state (I∼X) which is required to drive NAD^+ reduction. Since less I∼X would be present when the light intensity is low, greater inhibition is observed. In our earlier paper (Keister and Yike, 1967a,b) we observed that these uncoupling agents inhibited the ATP-driven NAD^+ reduction more than the light-driven reaction and postulated that this was due to the lower level of I∼X formed by ATP than by light which is in agreement with the mechanism proposed above.

Requirement for I∼X. Normally, the rates of ATP-driven NAD^+ reduction are 15–25% the rate observed with a saturating light intensity. We believe that these low rates can be explained on the basis of the requirement for I∼X. There are five observations listed below that indicate a high level of I∼X is required for NAD^+ reduction as contrasted with another energy-linked reaction, transhydrogenation.

A. LIGHT INTENSITY. The light saturation curve for NAD^+ reduction as compared with the transhydrogenase is presented in Figure 3. The K_m's for light as taken from these curves are 2.8×10^3, 1.5×10^4, and 2.1×10^4 ergs cm^{-2} sec^{-1} for the transhydrogenase, NAD^+ reduction, and photophosphorylation, respectively. This is a fivefold higher requirement for light for NAD^+ reduction as contrasted with the transhydrogenase.

B. ATP CONCENTRATION. We have determined the K_m for ATP required to drive NAD^+ reduction using succinate as the electron donor to be 1.3×10^{-4} M by the typical graphical method. With the same preparations we found the K_m for ATP hydrolysis to be identical (1.4

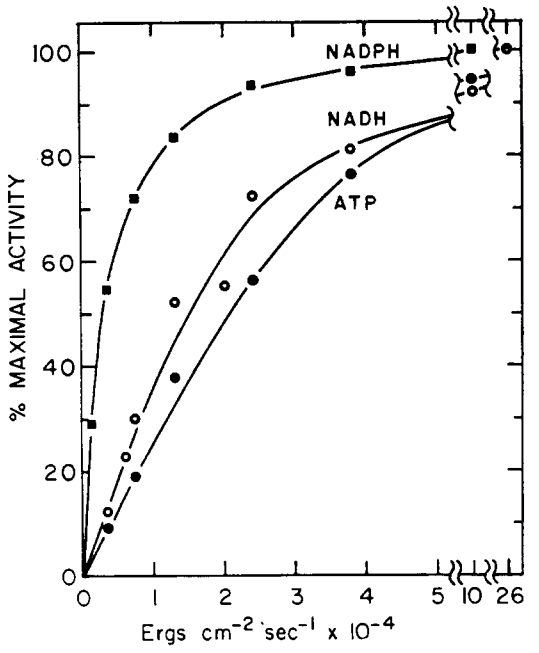

FIGURE 3: Effect of light intensity on the transhydrogenase (NADPH), succinate-linked NAD^+ reduction, and photophosphorylation (ATP). Incandescent light filtered through water was varied in intensity by increasing the distance from the source. The rates were corrected for any endogenous activity. The maximum rates for the transhydrogenase, NAD^+ reduction and photophosphorylation were 50, 33, and 207 μmoles, per mg of bacteriochlorophyll per hr, respectively.

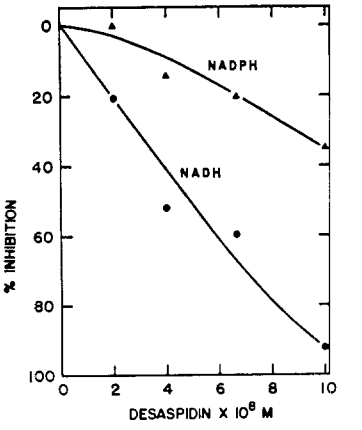

FIGURE 4: Effect of desaspidin on the transhydrogenase (NADPH) and NAD^+ reduction. Reactions were performed by the standard assays using a light intensity of 2.4×10^4 ergs cm^{-2} sec^{-1}. The reaction mixture for the transhydrogenase and NAD^+ reduction contained 15.2 and 22.8 μg of bacteriochlorophyll per ml, respectively. No bovine serum albumin was included in the reaction mixture.

$\times 10^{-4}$ M). Earlier we reported that the K_m for ATP required to drive the transhydrogenase was almost sixfold lower (2.4×10^{-5} M; Keister and Yike, 1967b). In addition, the theoretical maximum velocity, V_{max}, of NAD^+ reduction as determined by the graphic method was about 50% higher than the observed maximum rate. This may indicate that the level of I~X formed from ATP is too low to support a higher rate of NAD^+ reduction.

C. EFFECT OF DESASPIDIN. Figure 4 illustrates the relative effectiveness of desaspidin in inhibiting NAD^+ reduction and the energy-linked transhydrogenase. This experiment was performed at a light intensity of 2.4×10^4 ergs cm^{-2} sec^{-1} which amplifies somewhat the differential inhibition. This differential effect can also be observed using ATP as the energy source. Our interpretation of these results is that desaspidin had a lesser effect on the transhydrogenase since a lower level of this energized intermediate was required than for NAD^+ reduction.

D. EFFECT OF PP_i PLUS ATP. We have reported previously (Keister and Yike, 1967a) that PP_i can be used as an energy source for driving NAD^+ reduction and the transhydrogenase. In agreement with the results of Baltscheffsky *et al.* (1966) on the formation of PP_i, the utilization of PP_i was insensitive to oligomycin, indicating that the hydrolysis of this compound occurred *via* a pathway different from the utilization of ATP. The rate of NAD^+ reduction was stimulated more with both ATP and PP_i present than with either alone, and these results were interpreted to indicate that a higher level of I~X can be maintained with both energy sources present than with either alone.

E. EFFECT OF OLIGOMYCIN. We have reported that this energy transfer inhibitor stimulated the light and PP_i-driven reduction of NAD^+ by over 60% in some experiments (Keister and Yike, 1967a). In contrast, this antibiotic had little effect on the light or PP_i-driven transhydrogenase (Keister and Yike, 1966). Lee and Ernster (1966) have reported that low concentrations of oligomycin stimulated the succinate-linked NAD^+ reduction in mitochondria and have interpreted their results to indicate that oligomycin inhibited an endogenous nonspecific hydrolysis of I~X, thus providing a higher level of this intermediate to support energy-linked reactions. We believe that the same interpretation applies to chromatophores and that this observation provides additional evidence that a high level of I~X is required to drive NADH formation. Alternatively, the lack of a stimulatory effect on the transhydrogenase indicates a lower requirement of I~X for this reaction.

Light can probably provide a high level of I~X (the rate of photophosphorylation in a typical chromatophore preparation is about 250 μmoles of ATP/mg of bacteriochlorophyll per hr) whereas the level provided by ATP is relatively lower (typical unstimulated rates of ATP hydrolysis are about 40 μmoles/mg of bacteriochlorophyll per hr). This difference is probably amplified by the poor coupling between NADH oxidation and ATP formation (P/NADH $\cong$ 0.4). Thus, the above group of experiments provides a possible explanation for the low efficiency of ATP-driven NAD^+ reduction.

Our conclusion that all of the NAD^+ reduction in *R. rubrum* is energy linked has been strengthened by the recent reports of Beugeling (1968) and Ke *et al.* (1968) that ubiquinone is rapidly photoreduced and is coupled to the photooxidation of P_{890}. The quantum efficiency

of this process was reported to be about one and the reaction was independent of temperature down to 77° K. These observations are compatible with ubiquinone being the primary photoreductant of P_{890} and thus the strongest reductant in the cyclic electron-transfer system. While the reductant for NAD^+ is not known, ubiquinone is a likely candidate (Vernon, 1968). At physiological pH values the redox potential of ubiquinone (+98 mV, Moret *et al.*, 1961) is not low enough to permit the direct reduction of NAD^+ (−0.32 mV) to the degree we observe in *R. rubrum* chromatophores. Therefore, an energy-requiring reduction is the only mechanism readily apparent to account for NADH formation in this bacterium. Klemme (1968) has presented evidence that NAD^+ reduction in *Rhodopseudomonas capsulata* is *via* an energy-linked pathway, also. However, the mechanism for NAD^+ reduction in the purple and green sulfur bacteria remains to be established.

A good discussion of the comparative biochemistry of photosynthetic processes and the role of energy-linked reactions can be found in a recent article by Gest (1966).

References

Aleem, M. I. H. (1966a), *Biochim. Biophys. Acta 113*, 216.

Aleem, M. I. H. (1966b), *Biochim. Biophys. Acta 128*, 1.

Aleem, M. I. H., Lees, H., and Nicholas, D. J. D. (1963), *Nature 200*, 759.

Ash, O. K., Zaugg, W. S., and Vernon, L. P. (1961), *Acta Chem. Scand. 15*, 1629.

Baltscheffsky, H., von Stedingk, L.-V., Heldt, H.-W., and Klingenberg, M. (1966), *Science 153*, 1120.

Beugeling T. (1968), *Biochim. Biophys. Acta 153*, 143.

Bose, S. K., and Gest, H. (1962), *Nature 195*, 1168.

Chance, B., Ernster, L., Garland, P. B., Lee, C.-P., Light, P. A., Ohnishi, T., Ragan, C. I., and Wong, D. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 1498.

Chance, B., and Hollunger, G. (1963), *J. Biol. Chem. 278*, 418.

Clayton, R. K. (1963), *Biochim. Biophys. Acta 75*, 312.

Geller, D. M. (1962), *J. Biol. Chem. 237*, 2947.

Gest, H. (1966), *Nature 209*, 879.

Ke, B., Vernon, L. P., Garcia, A., and Ngo, E. (1968), *Biochemistry 7*, 311.

Keister, D. L., and Yike, N. J. (1966), *Biochem. Biophys. Res. Commun. 24*, 519.

Keister, D. L., and Yike, N. J. (1967a), *Arch. Biochem. Biophys. 121*, 415.

Keister, D. L., and Yike, N. J. (1967b), *Biochemistry 6*, 3847.

Kiesow, L. (1963), *Biochem. Z. 338*, 400.

Klemme, J.-H. (1968), *Intern. Congr. Photosyn. Res., Freudenstadt, Germany*.

Krebs, H. A. (1967), *in* Biochemistry of Mitochondria, Kaniuga, L., and Wojtczak, L., Ed., London, Academic, p 105.

Krogmann, D. W., and Jagendorf, A. T. (1959), *Arch. Biochem. Biophys. 80*, 421.

Lee, C.-P., and Ernster, L. (1966), *in* Regulation of Metabolic Processes in Mitochondria, Tager, J. M., Papa, S., Quagliariello, E., and Slater, E. C., Ed., Amsterdam, Elsevier, p 218.

Moret, V., Pinamonti, S., and Fornasari, E. (1961), *Biochim. Biophys. Acta 54*, 381.

Nozaki, M., Tagawa, K., and Arnon, D. I. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 1334.

San Pietro, A., and Black, C. C. (1965), *Ann. Rev. Plant Physiol. 16*, 155.

Slater, E. C. (1963), *in* Metabolic Inhibitors, Vol. III, Hochster, R. M., and Quastel, J. H., Ed., New York, N. Y., Academic, p 503.

Trebst, A., Pistorius, E., and Baltscheffsky, H. (1967), *Biochim. Biophys. Acta 143*, 257.

Vernon, L. P. (1963), *in* Bacterial Photosynthesis, Gest, H., San Pietro, A., and Vernon, L. P., Ed., Yellow Springs, Ohio, Antioch, p 235.

Vernon, L. P. (1968), *Bact. Rev.* (in press).

Yamashita, J., Yoshimura, S., Matuo, Y., and Horio, T. (1967), *Biochim. Biophys. Acta 143*, 154.