Energy-Linked Reactions in Photosynthetic Bacteria. II. The Energy-Dependent Reduction of Oxidized Nicotinamide–Adenine Dinucleotide Phosphate by Reduced Nicotinamide–Adenine Dinucleotide in Chromatophores of *Rhodospirillum rubrum**

Donald L. Keister and Norma Jean Yike

ABSTRACT: Chromatophore preparations of *Rhodospirillum rubrum* contain a particle-bound transhydrogenase which catalyzes the energy-dependent reduction of oxidized nicotinamide–adenine dinucleotide phosphate ($NADP^+$) by reduced nicotinamide–adenine dinucleotide (NADH). The energy requirement can be supplied by light, adenosine triphosphate (ATP), guanosine triphosphate (GTP), or inorganic pyrophosphate. The enzyme is fairly specific for NADH and $NADP^+$, as the hydrogen donor and acceptor, respectively. The Michaelis constants for NADH, $NADP^+$, ATP, GTP, and inorganic pyrophosphate were determined. The stoichiometry of reduced nicotinamide–adenine dinucleotide phosphate (NADPH) formed to ATP utilized was 1:1. Uncoupling of phosphorylation from light-induced electron transport inhibited the energy-linked transhydrogenase when light or ATP was used as an energy source. Inhibitors of electron transport did not effect the ATP-driven reaction, whereas energy-transport inhibitors (*i.e.*, oligomycin) had no effect on the light-driven reaction but inhibited the ATP-driven reaction. Pyrophosphate utilization was not affected by this class of inhibitors. From this data, the energy source for the enzyme appears to be a high-energy intermediate (or state) or phosphorylation ($I\sim X$) as has been proposed previously for a similar enzyme found in submitochondrial particles. Light saturation curves for the transhydrogenase and photophosphorylation were determined and at low light intensity the rates of ATP formation and $NADP^+$ reduction were the same. However, little competition between the two energy-requiring processes could be demonstrated. The significance of these results is discussed.

The electron-transport system of *Rhodospirillum rubrum* is localized in membraneous structures distributed throughout the cytoplasm in cells grown photosynthetically. These membranes are apparently invaginations of the cell membrane. Rupture and comminution of them result in particles which vesiculate and are called chromatophores and contain the photosynthetic and electron-transport apparatus of the cell. These particles contain an energy-linked transhydrogenase which is tightly bound to the chromatophore.

The first observation that an energy-linked transhydrogenase might exist was in 1958, when Klingenberg and Slenczka (1959) observed that in isolated liver mitochondria incubated with NAD[1]-specific substrates or succinate (minus P_i), the rapid and almost complete reduction of intramitochondrial $NADP^+$. These and related observations led Klingenberg and co-workers to postulate the existence of an asymmetric transhydrogenase which used the energy derived from oxidative phosphorylation to drive the reaction. This transhydrogenase was demonstrated in submitochondrial particles by Danielson and Ernster (1963a,b), who proposed that the mechanism of the reaction was

$$NADH + NADP^+ + \sim X \longrightarrow NAD^+ + NADPH + X$$

where $\sim X$ (or $I\sim X$) is a high-energy intermediate

* From the Charles F. Kettering Research Laboratory, Yellow Springs, Ohio 45387. *Received August 28, 1967.* Contribution No. 286 of this laboratory.

[1] Abbreviations used: NAD^+, oxidized nicotinamide–adenine dinucleotide; NADH, reduced NAD^+; $NADP^+$, oxidized nicotinamide–adenine dinucleotide phosphate; NADPH, reduced $NADP^+$; thio-$NADP^+$, oxidized thionicotinamide adenine–dinucleotide phosphate; HHNQ, 2-hydroxy-3-(ω-cyclohexyloctyl)-1, 4-naphthoquinone; DCCD, *N,N'*-dicyclohexylcarbodiimide; *m*-Cl-CCP, *m*-chlorocarbonyl cyanide phenylhydrazone; $AcPyAD^+$, 3-acetylpyridine–adenine dinucleotide; $NHyD^+$, nicotinamide–hypoxanthine dinucleotide (commonly known as the deamino analog of $NAD(P)^+$); $PyAlAD^+$, 3-pyridinealdehyde–adenine dinucleotide; $AcPyDHy^+$, 3-acetylpyridine–hypoxanthine dinucleotide; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole; HOQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; Bchl, bacteriochlorophyll; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; DCIP, 2,6-dichloroindophenol; AMP, ADP, and ATP, adenosine mono-, di-, and triphosphates; TCA, trichloroacetic acid; GTP, UTP, CTP, ITP, guanosine, uridine, cytidine, and inosine triphosphates; PMS, *N*-methylphenazinium methyl sulfate.

TABLE I: Substrate Specificity and Michaelis Constants for Reaction 1.[a]

Hydrogen Donor	Hydrogen Acceptor	Rel Max Velocity	K_m (M)
NADH		100	8.3×10^{-6}[b]
AcPyADH		48	
NHyDH		45	
PyAlADH		1[c]	
Thio-NADH		1[c]	
AcPyHyDH		1[c]	
	$NADP^+$	100	3.3×10^{-5}
	Thio-$NADP^+$	75[d]	
	$AcPyADP^+$	17	
	$NHyDP^+$	7	

[a] These reactions were performed using ATP as the energy source and substituting the appropriate analog in the reaction mixture for NADH or $NADP^+$. A mixture of yeast and horse liver alcohol dehydrogenase was used to provide a broad substrate specificity for the reduction of the NAD^+ analogs. [b] K_m was determined with 6.7 mM $MgCl_2$ and 10^{-3} M rotenone present to suppress the endogenous reaction (see Figure 3). The K_m of the endogenous reaction in the absence of $MgCl_2$ was 1.4×10^{-6} M. The K_m of $NADP^+$ was the same for the endogenous and ATP-driven reaction. [c] Very slow reaction that may be due to endogenous NADH or NAD^+ contamination. [d] Thio-$NADP^+$ cannot be used for light-driven reaction. The kinetics of the reaction in the light appear to be a rapid light-driven reduction which stops after 4–6 min, followed by a photooxidation.

(or state) of oxidative phosphorylation, and can also be generated from ATP.

The reactions investigated in this study are the following:

$$NADH + NADP^+ \longrightarrow NADPH + NAD^+ \quad (1)$$

and the reverse reaction

$$NADPH + NAD^+ \longrightarrow NADH + NADP^+ \quad (2)$$

Reaction 1 can also be measured using the thio-$NADP^+$ analog of $NADP^+$.

$$NADH + \text{thio-}NADP^+ \longrightarrow \text{thio-}NADPH + NAD^+ \quad (3)$$

In chromatophores of *R. rubrum*, I~X can be generated by photosynthetic electron transport, from ATP, from inorganic pyrophosphate, and at low levels, by oxidative electron transport. The results presented in this paper indicate that the properties of the enzyme are similar to the properties of an energy-linked transhydrogenase in submitochondrial particles that has been studied by Danielson and Ernster (1963-a,b), Lee and Ernster (1966), Griffiths and Roberton (1966), and by van Dam and ter Welle (1966). These elegant studies have indicated that the same enzyme or enzyme system can catalyze both an energy-linked and a nonenergy-requiring transhydrogenation. The same conclusion was reached by Kawasaki *et al.* (1964) based on antienzyme studies. While the transhydrogenase is apparently tightly bound to the electron-transport-containing particles, the structural relationship of this enzyme(s) to the electron-transport system has not been elucidated.

Preliminary observations on the transhydrogenase in *R. rubrum* have been reported (Keister and Yike, 1966; Keister, 1966). Orlando *et al.* (1966) have demonstrated the occurrence of a similar enzyme in *Rhodopseudomonas spheroides*. Nozaki *et al.* (1963) reported that *R. rubrum* contained a transhydrogenase that catalyzed reaction 1 in the dark and is probably the same as the endogenous activity of our preparations.

Experimental Procedure

R. rubrum, S-1, obtained from Dr. H. Gest, was grown and chromatophores were prepared as previously described (Keister and Yike, 1966). The energy-linked transhydrogenase can be assayed in whole cell homogenates since the cells have very little capacity for NADPH oxidation. The specific activity of the energy-linked enzyme on a bacteriochlorophyll basis was approximately the same throughout the purification of chromatophores.

We have used two assays for transhydrogenase. (1) With $NADP^+$ as the hydrogen acceptor, the reaction mixture contained in 3 ml the following: 50 mM Tris (pH 8), 1 mM $MgCl_2$, 0.09 mM NADH, 0.13 mM $NADP^+$, 0.2 M ethanol, 30 μg of alcohol dehydrogenase (an excess), and chromatophores as noted. ATP and PP_i when added were 0.33 mM. Control cuvets contained no $NADP^+$. The increase in A_{340} was a direct measure of the enzyme activity. (2) With thio-$NADP^+$ as the hydrogen acceptor, the reaction mixture contained: 50 mM Tris (pH 8), 1 mM $MgCl_2$, 0.12 mM NADH,

0.067 mM thio-NADP+, chromatophores, and ATP or PP_i as above. The increase in A_{395} due to reduction of thio-NADP+ is a direct enzyme assay. This assay is useful since the reduction of thio-NADP+ can be measured directly without the addition of a NADH-generating system. However, it cannot be used with light as the source of energy (Table I, footnote d). The millimolar extinction coefficient at 395 mμ was taken as 11.3.

Incandescent light at an intensity of 10^5 ergs cm^{-2} sec^{-1}, filtered through water, was the source of illumination. The reactions were measured aerobically since there was little effect of anaerobicity. Red and white light at the same intensity were equally effective. The standard reaction mixture for photophosphorylation contained in 3 ml: 50 mM Tris (pH 8), 1 mM $MgCl_2$, 1.67 mM sodium succinate, 3.3 mM $^{32}P_i$, 0.83 mM ADP, 20 mM glucose, and 0.67 unit of hexokinase. The reaction was illuminated with a light intensity of 2.6×10^5 ergs cm^{-2} sec^{-1} for 6 min. The reaction products were fixed by adding TCA to 5% and assaying for organic ^{32}P as previously described (Keister, 1965).

Enzymes and nucleotides were obtained primarily from the Sigma Chemical Co. thio-NADP+ was obtained from Boehringer Mannheim Corp. Desaspidin was obtained from Ox Medica AB Helsingfors, Finland. HHNQ was a generous gift of Dr. S. Archer. DCCD was obtained from the Aldrich Chemical Co.

Results

Time Course of the Reaction. In a preliminary publication (Keister and Yike, 1966) we reported that PP_i and ATP were equally effective in driving the reaction and that the rate was approximately 50% that of the light-driven reaction. Since then, our cumulative experiments have indicated that the time course presented in Figure 1 is more representative. The rate of the light-driven reaction in this experiment was 62 μmoles of NADPH/mg of Bchl per hr while ATP and PP_i supported the reaction 70 and 56% as well as light, respectively. The addition of both ATP and PP_i stimulated the rate more than either alone, and in some experiments the addition of both was almost as good as light. However, the addition of ATP and PP_i plus light was no more effective than light alone. It should be pointed out that the rate of these reactions on a bacteriochlorophyll basis is very dependent on the growth conditions of the organism since the protein:Bchl ratio of the preparation varies with the light intensity and aerobicity of the culture (Cohen-Bazire and Kunisawa, 1963). The protein (milligrams):Bchl (milligrams) ratio of our chromatophore preparations averaged 14. Thus the rate of the ATP-driven reaction was 0.052 μ mole/mg of protein per min which was approximately the rate reported by Danielson and Ernster (1963b) for the reaction in submitochondrial particles.

The endogenous transhydrogenation was inhibited approximately 50% by uncoupling reagents such as

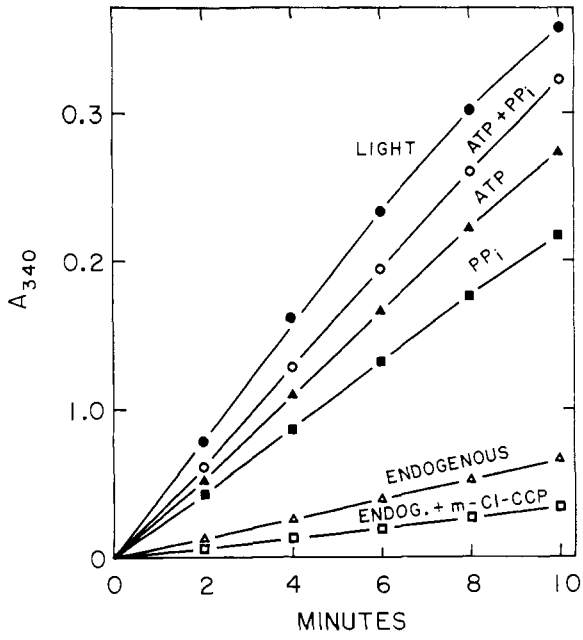

FIGURE 1: Time course of energy-linked NADP+ reduction. The reaction mixtures contained 18.8 μg of Bchl and other components as described for the standard assay with NADP+.

m-Cl-CCP, desapidin, and gramicidin and by the NADH oxidase inhibitor, rotenone. These results indicate that the inhibited portion of the endogenous reaction was due to the energy generated by the oxidation of NADH by the chromatophore preparation. That chromatophores are capable of low levels of oxidative phosphorylation has been reported by Geller (1962) and confirmed during this investigation. Inorganic phosphate had little affect on any of the reactions.

Effect of pH. Using the thio-NADP+ assay (2) which eliminated pH effects on the NADH-generating system, the pH optimum of the ATP-driven reaction was

TABLE II: The Effect of Adenine Nucleotides.

		% Inhibn of Transhydrogenase			
		Energy Source			
Nucleotide	Concn (mM)	ATP	Light	PP_i	Endogenous
ADP	1	47	0	0	0
	5	82	35[a]	22	<10
2′-AMP	1.7	17	18		16
	5	39	37		39
5′-AMP	1.7	<5	<9		<10
	5	12	18		29

[a] This effect was not reversed by oligomycin.

TABLE III: Specificity of Phosphate Ester Hydrolysis.[a]

Phosphate Ester	μmoles/mg of Bchl per hr			K_m for Transhydrogenase Reaction (M)
	NADPH	P_i Liberated	P_i Liberated (−$NADP^+$)	
None	6			
ATP	32	44	37	2.4×10^{-5}
GTP	37	46	36	1.8×10^{-5}[b]
ITP	24	30	30	
UTP	9	<3	<3	
CTP	<8	0[c]	0[c]	
PP_i[d]	23	185[e]	185[e]	6.7×10^{-5}[f]
Tripolyphosphate	6		5	

[a] Conditions for the experiments were as described in Experimental Procedures. Nucleotides were added to a concentration of 0.67 mM. The reactions mixtures were fixed with TCA to a final concentration of 5% and analyzed for P_i by the Fiske–Subbarow method. [b] Value obtained using low concentrations. A reciprocal plot gives a hyperbolic curve that may indicate substrate activation at higher concentrations. [c] Undetectable using Fiske–Subbarow method. [d] The ratio of pyrophosphatase to ATPase varied considerably between preparations whereas, the activity of transhydrogenase driven by ATP and PP_i was more constant. [e] Rate of PP_i hydrolysis, rather than P_i appearance. [f] The rapid rate of hydrolysis made accurate readings of NADPH at low PP_i concentrations difficult, thus this is an approximation.

between 8.0 and 8.5 while the optimum for the endogenous reaction was 7.3.

Pyridine Nucleotide Specificity. The pyridine nucleotide specificity and K_m's for the energy-linked reaction are presented in Table I. The enzyme was relatively specific for the naturally occurring cofactors, NADH and $NADP^+$, and the K_m's were appropriate for a physiologically active compound.

Inhibition by Adenine Nucleotides. Adenine nucleotides have been demonstrated to affect transhydrogenase enzymes in various ways. A bacterial transhydrogenase from *Pseudomonas* was stimulated by 2′-AMP (Kaplan *et al.*, 1953a), whereas this nucleotide had no effect on an enzyme isolated from beef heart (Kaplan *et al.*, 1953b). This compound has been demonstrated to inhibit specifically some $NADP^+$-linked enzymes (Neufeld *et al.*, 1955) and to be a competitive inhibitor of NADP(H) with some other transhydrogenases (Keister *et al.*, 1960; Keister and Hemmes, 1966). Other adenine nucleotides also inhibited but were less effective. The results presented in Table II illustrate that 2′- and 5′-AMP inhibited the endogenous transhydrogenase activity equally or greater than the energy-linked reactions. This inhibition was probably by competition with a pyridine nucleotide substrate. Conversely, ADP inhibited the energy-linked reactions but had little effect on the endogenous activity. The greatest effect of ADP was on the ATP-driven reaction where ADP was competitive with ATP. However, at higher concentrations ADP also inhibited the light and PP_i-driven reactions. This inhibition was not reversed by oligomycin. We interpret this effect of ADP as an inhibition of the utilization of some intermediate in the energy-linked reaction prior to the site of oligomycin inhibition.

Phosphate Ester Specificity. The specificity of the reaction for nucleotide triphosphates and PP_i as an energy source is presented in Table III. ATP and GTP were equally effective in driving the reaction whereas ITP and UTP were 75 and 28% as effective as ATP, respectively. This specificity is closely correlated with nucleoside triphosphatase activity of the preparation as is illustrated in column 4 of Table III. In contrast, the pyrophosphatase activity was much greater than the ATPase. The inverse of this relationship is found in mitochondria where ATPase is much greater than pyrophosphatase activity. This is in accord with the observations of Baltscheffsky (1964).

Stimulation of Nucleotide Triphosphate Hydrolysis by Transhydrogenase. In an attempt to determine the stoichiometry of high-energy bond utilization for $NADP^+$ reduction we measured P_i formation from various triphosphates during NADPH formation. The results presented in Table IV illustrate that $NADP^+$ reduction stimulated P_i appearance but that the increase in P_i was never equal to the NADPH formed as would be expected if one high-energy bond was utilized per $NADP^+$ reduced. At least two interpretations are possible. One is that one high-energy bond catalyzes the reduction of more than one $NADP^+$ molecule. The average number from these experiments was 3.6. The second interpretation which we favor and is substantiated in the next section is that the endogenous hydrolysis of the nucleotide triphosphates was due to an energy-conserving ATPase. When an energy-requiring reaction was present, part of the energy of

TABLE IV: Stimulation of Nucleoside Triphosphate Hydrolysis by Transhydrogenase.[a]

Expt	Nucleotide	Reaction Time (min)	P_i Liberated (μmoles)		NADPH Formed (−endogenous) (μmoles)	P_i:NADPH	
			Endogenous	+NADP$^+$		Using Total P_i	−Endogenous P_i
1	ATP	6	0.203	0.221	0.148	1.49	0.12
	ITP	12	0.173	0.242	0.148	1.64	0.46
2	ATP	6	0.160	0.182	0.118	1.54	0.19
	GTP	8	0.244	0.303	0.187	1.62	0.31
3	ATP	12	0.317	0.373	0.184	2.04	0.30
				Thio-NADP$^+$	Thio-NADPH		
	ATP	12	0.277	0.342	0.167	2.04	0.38
	ITP	12	0.154	0.178	0.115	1.54	0.21
					Av	1.67	0.28

[a] Conditions were as described in Table III.

ATP which was dissipated otherwise, was utilized to drive the reaction. Thus little "extra" hydrolysis was required. This latter interpretation is also in accord with that put forward by Danielson and Ernster (1963b).

Stoichiometry of ATP and PP_i Hydrolyzed to Reduced NADP$^+$. In the experiments reported in the previous section, saturating quantities of ATP were used to estimate the consumption of ATP during the reduction of NADP$^+$. It has been reported (Danielson and Ernster, 1963b) that the P_i:NADPH ratio in submitochondrial particles was 10–20 using large amounts of ATP. However, the ratio decreased to less than 2 when the ATP level was decreased. The stoichiometry obtained in this manner was criticized (Slater, 1963) since the assumption was made that the nonspecific ATPase was decreased more than the energy-coupling ATPase. Haas (1964) independently found an ATP:NADPH ratio of about 2 and concluded that the correct ratio was one since ratios of less than 2 had been previously reported.

Chromatophores contain much less ATPase activity than mitochondria and in fact the endogenous ATPase activity approximates the rate of the transhydrogenase as shown in Tables III and IV, even with an excess of ATP present. In Table V we used small amounts of ATP and measured the total NADPH formation and found that when corrected for the small endogenous NADP$^+$ reduction and myokinase activity that the ATP:NADPH ratio averaged one. The highest ratios that we obtained even with large excesses of ATP were 1.5. We also measured the stoichiometry using the lower potential nucleotide, thio-NADP$^+$, as the hydrogen acceptor and again found a stoichiometry of one. Although not illustrated in the table we also measured the stoichiometry of the reaction using AcPyADH as the hydrogen donor and found a stoichiometry of about one using low ATP concentrations. In contrast the stoichiometry of PP_i:NADPH was much higher. This appears reasonable since the pyrophosphatase activity of the preparation was high.

We believe that these data provide strong support for the postulate that one high-energy bond is required for each molecule of NADP$^+$ reduced at least when that high-energy bond is formed from ATP.

Inhibitor Studies. We have studied the effect of a

TABLE V: Requirement of ATP and PP_i for NADP$^+$ Reduction.[a]

ATP (μmoles)	NADPH (μmoles)	ATP:NADPH	Thio-NADPH (μmoles)	ATP:Thio-NADPH
0.020	0.020	1.00		
0.040	0.044	0.91	0.032	1.25
0.070	0.076	0.92	0.064	1.10
0.100	0.089	1.12	0.095	1.05
0.150	0.125	1.20	0.170	1.41
PP_i (μmoles)		PP_i:NADPH		
0.40	0.035	8.8		
0.60	0.047	12.8		
0.80	0.063	12.7		
1.20	0.089	13.5		
1.60	0.115	13.9		

[a] Assay 1 was used with the following changes: $MgCl_2$ (3.3 mM) and rotenone (10^{-5} M) (to suppress endogenous activity). Thio-NADP$^+$ was substituted for NADP$^+$ in that assay. Reaction mixtures contained 34 μg of Bchl and were followed until all ATP or PP_i was utilized. Total NADPH was corrected for non-energy-linked reduction and a small myokinase activity which was determined by using ADP in place of ATP.

TABLE VI: Effect of Inhibitors.[a]

	Compounds (μM)	Photophosphorylation (% inhibn) Light Intensity (ergs cm^{-2} sec^{-1}) 10^4	5×10^4	Transhydrogenase (% inhibn) Light	ATP	PP_i	Endogenous
1	*m*-Cl-CCP (2)	83	53	32	52	79	25 (max)[b]
2	Desaspidin (0.1)	87	60	34	60	80	30 (max)
3	Gramicidin (0.03)	64	39	34	60	72	31 (max)
4	Dicumarol (150)	34	46	59	56	62	30 (→)
5	Quinacrine (27)	68	73	39	45	45	27 (→)
6	Chlorpromazine (30)	49	64	52	36	49	53 (→)
7	TTFB (0.5)	51	44	18	20	69	30 (max)
8	HHNQ (0.33)		98	94	7	0	0
9	HOQNO (30)		95[c]	67	0	0	0
10	HOQNO (0.25)		53	23	0	0	0
11	Antimycin a (0.1)		95[c]	68	0	0	0
12	Oligomycin (8.3)		100	10[d]	100	16[d]	30
13	DCCD (3)		51	0	65	16	22

[a] The reaction conditions were as described in Experimental Procedures. The water-insoluble inhibitors were added in ethanol. These data were necessarily the result of many experiments. The average rates in micromoles per milligram of Bchl per hour for photophosphorylation were 53 and 137 for 10^4 and 5×10^4 ergs cm^{-1} sec^{-1}. Transhydrogenase rates were light (42), ATP (30), and PP_i (21), respectively. [b] (max) indicates that this is the maximal inhibition observed even with higher concentration and was due to inhibition of the energy-linked portion of the reaction. (→) indicates that higher concentrations produce greater inhibition. [c] This is maximum inhibition observed with this compound. The residual electron transport is enough to drive the transhydrogenase to 33% of the maximum rate. [d] Stimulates slightly at lower concentration.

large number of inhibitors on photophosphorylation and the transhydrogenase reaction. These reagents can be grouped into the following four classes. (1) uncouplers: Compounds that have been demonstrated to inhibit phosphorylation but not electron transport in mitochondria. These compounds inhibit photophosphorylation and the transhydrogenase driven by light, ATP, or PP_i but have little effect on the nonenergy-linked enzyme. (2) electron-transport inhibitors: These compounds inhibit the light-driven reactions but not the ATP- or PP_i-driven reactions. (3) energy-transport inhibitors: Compounds which inhibit photophosphorylation and the ATP-driven but not the light- or PP_i-driven transhydrogenase. (4) enzyme inhibitors: Compounds which inhibit both the endogenous and energy-linked transhydrogenase reactions. Some of the compounds as would be expected fall into more than one group.

The effect of some of these compounds on transhydrogenase and photophosphorylation is presented in Table VI. The first three compounds have been demonstrated to be uncouplers of phosphorylation from electron transport in mitochondria and appear to be uncouplers in chromatophores also. They inhibited the energy-linked transhydrogenase 30–50% maximally. The effect on the endogenous observed was due to the inhibition of the energy-coupled portion of the endogenous reaction produced by the oxidation of NADH. These compounds had no effect on the endogenous reaction when NADH oxidation was inhibited by rotenone. Higher concentrations of inhibitor had no greater effect. Dicumarol, quinacrine, and chlorpromazine were probably uncouplers, also, but in addition, they inhibited the endogenous nonenergy-linked enzyme as measured by reaction 1 or 2 at higher concentrations. These compounds are noted as flavoprotein antagonists and this inhibition may indicate that a flavoprotein is involved in the nonenergy-linked enzyme reaction. Rotenone which is also a flavoprotein inhibitor had no effect on the nonenergy-linked reaction.

TTFB has been demonstrated to be an uncoupling agent in mitochondria (Beechey, 1965) but is primarily an inhibitor of electron transport in chloroplasts although higher concentrations had an uncoupling effect (Bückel *et al.*, 1965). This compound appears to be primarily an inhibitor of photosynthetic electron transport in chromatophores also since most of the inhibition can be overcome by adding PMS or TMPD which bypasses the site of inhibition. However, the inhibition is not completely overcome by these com-

pounds indicating that the TTFB is also an uncoupler. This inhibition is reflected by the effect on the ATP- and PP_i-driven transhydrogenase.

HHNQ, HOQNO, and antimycin a are electron-transport inhibitors which blocked the light-catalyzed reactions but had no effect on the ATP- or PP_i-driven reaction. TMPD or DCIP and ascorbate can restore photophosphorylation and the light-driven transhydrogenase in these chromatophores by forming a shunt around the site at which these compounds inhibit.

The last two compounds, oligomycin (Lardy *et al.*, 1958) and DCCD (Beechey *et al.*, 1966), are energy-transport inhibitors. They have little effect on the light- or PP_i-driven transhydrogenase but inhibited the ATP-driven reaction and photophosphorylation. Although not listed in the table 3,3′,5-triiodo-L-thyronine inhibited the endogenous and energy-linked enzyme in reactions 1 and 2 in agreement with the observations of Estabrook *et al.* (1963), Stein *et al.* (1959), and Ball and Cooper (1957).

In general, the compounds that are uncouplers inhibited the ATP- and PP_i-driven more than the light-driven reactions. We think that this probably is a reflection of the rate of formation of a high-energy intermediate (I~X). This intermediate (or state) is formed at a greater rate by the light than from ATP. Evidence for this is that the rate of ATPase activity was two to three times less than the rate of photophosphorylation (see Table III). The degree of inhibition of photophosphorylation by these compounds was also increased by lowering the light intensity. The rate of ATP formation at the lower intensity was decreased by 60% and the per cent inhibition was effectively increased. Although not indicated in the table, the same effect can be demonstrated on the ATP-driven transhydrogenase by using rate-limiting concentrations of ATP. In contrast, the compounds that are both uncouplers of phosphorylation and inhibitors of the transhydrogenase, dicumarol, quinacrine, and chlorpromazine, inhibited the light-driven reaction equally or greater than the ATP- and PP_i-driven reactions. Also they inhibited photophosphorylation at the higher light intensity to a greater extent than at lower intensities. One explanation of these results is that these compounds exerted their effect on a different intermediate than one affected by the first four compounds in Table VI.

Effect of Divalent Cations. Hommes (1963) has observed that Mg^{2+} inhibited the nonenergy-linked transhydrogenase in submitochondrial particles of beef heart in both the forward (reaction 1) and reverse directions. Mg^{2+} was competitive with NAD^+ in the reverse direction while a mixed type of inhibition was found with NADH in the forward direction. These and other observations on the kinetics of inhibition led him to postulate that Mg^{2+} was part of an enzyme–NAD(H)–Mg^{2+} intermediate in both the transhydrogenase and succinate-linked NAD^+ reduction. In Figure 2, data are presented which illustrates that Mg^{2+} inhibited the nonenergy-linked enzyme in *R. rubrum* chromatophores also, while stimulating the

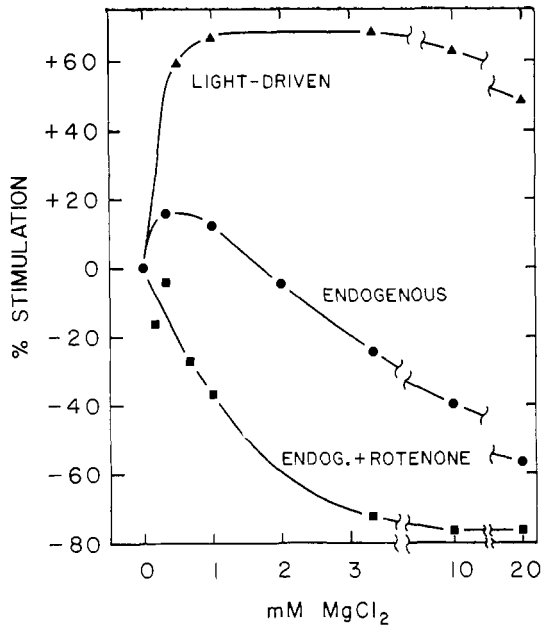

FIGURE 2: Effect of magnesium chloride. The assay using thio-$NADP^+$ as hydrogen acceptor was used. Chromatophores containing 35 μg of Bchl were used. Rotenone was added to 10^{-5} M.

light-driven reaction. In the presence of rotenone to inhibit the NADH dehydrogenase, a greater inhibition by Mg^{2+} was observed. This result illustrates that the effect on the endogenous reaction (minus rotenone) was due to an inhibition of the endogenous and a stimulation of the energy-linked portion of the reaction [Mg^{2+} or Mn^{2+} is required for the utilization of ATP or PP_i as an energy source (Keister, 1966)]. Mg^{2+} can be replaced by Ca^{2+}, Ba^{2+}, and Sr^{2+}. Indeed Ba^{2+} was frequently more effective than Mg^{2+} in stimulating the light-catalyzed reaction. The divalent cations (Cu^{2+}, Co^{2+}, Cd^{2+}, and Zn^{2+}) inhibited both the endogenous and energy-linked reaction, while the monovalent ions (Na^+, K^+, and NH_4^+) had little effect. Mg^{2+} also inhibited the nonenergy-linked enzyme in the reverse direction according to reaction 2.

We have previously reported that Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+} stimulated the succinate-linked photochemical reduction of NAD^+ in *R. rubrum* which also appears to be an energy-linked process (Keister and Yike, 1967). Thus, these ions may be stimulating by effecting the energy-transfer process and/or by influencing structure.

Equilibrium Constant. Kaplan *et al.* (1953b) reported an equilibrium constant [K = (NADH)($NADP^+$)/(NADPH)(NAD^+)] of 1.43 for the transhydrogenase from beef heart. This value was confirmed by Lee and Ernster (1964) who reported a K of 1.23 and also obtained a value of 0.79 for [K = (NADPH)(NAD^+)/(NADH)($NADP^+$)]. Lee and Ernster (1964) also measured the equilibrium constant of the reaction in sonicated beef heart mitochondria with succinate

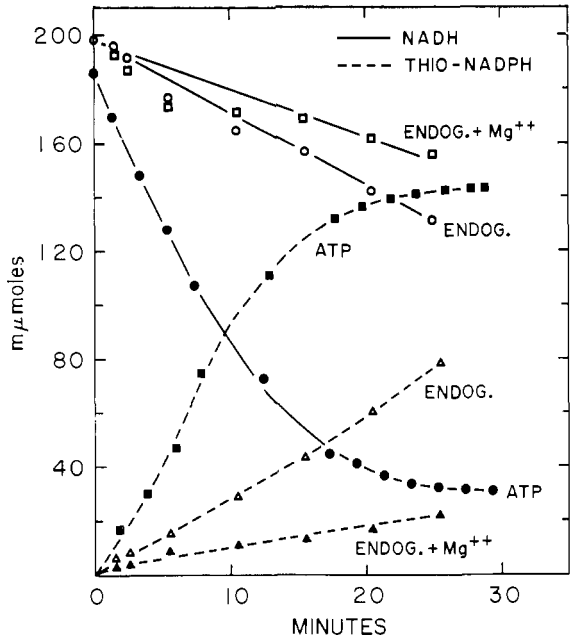

FIGURE 3: Time course of NADH oxidation by thio-NADP+. The reaction mixture for the ATP-driven reaction contained 186 mμmoles of NADH, 143 mμmoles of thio-NADP+, 10^{-5} M rotenone, 50 mM Tris (pH 8), 3.3 mM $MgCl_2$, and 39 μg of Bchl. The reaction mixture for the endogenous reaction contained 323 mμmoles of thio-NADP+ and $MgCl_2$ was omitted. The oxidation of NADH and reduction of thio-NADP+ were followed at 340 and 395 mμ, respectively.

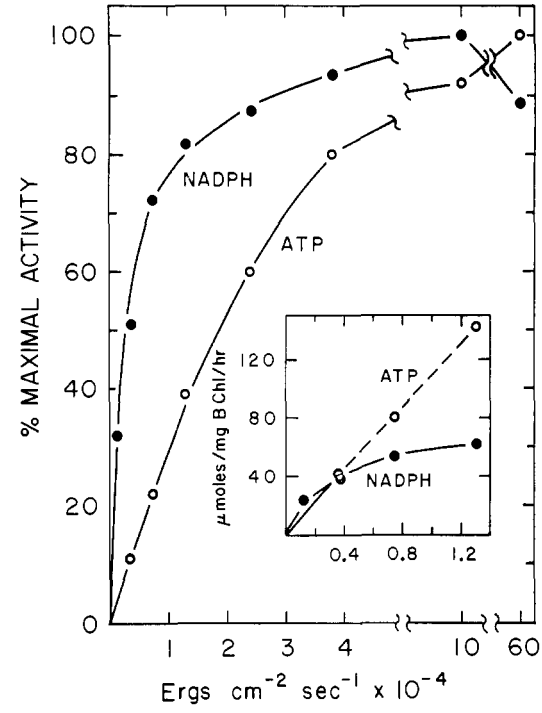

FIGURE 4: Effect of light intensity. Incandescent light filtered through water was varied in intensity by increasing the distance from the source. The rates were corrected for endogenous activity. Chromatophores were prepared by grinding the whole cells with double the wet weight of Alumina A-305, diluting with 10% sucrose containing 0.05 M Tris (pH 8). Alumina and whole cells were removed by centrifugation at 10,000*g* for 10 min. The remaining procedure was as previously described for cells broken in the French press (Keister and Yike, 1966). The maximum rates of ATP formation and NADP+ reduction were 365 and 76 μmoles per mg of Bchl per hr, respectively.

present as an energy source and found that *K* of the latter reaction was shifted to 480. These results were indicative that the requirement for high-energy intermediates was stoichiometric rather than catalytic. van Dam and ter Welle (1966) obtained a *K* value of 78 for the transhydrogenase in intact mitochondria by measuring the endogenous nucleotide concentration with acetoacetate present, while Klingenberg (1966) obtained a maximum value of 22 for the same reaction using an unreported energy source.

We have measured *K* for this reaction, and found a constant of about 28 in chromatophores using light as the energy source and rotenone to inhibit NADH oxidation. Rotenone did not completely inhibit NADH oxidation (90%) in this system and a slight nonspecific oxidase would tend to increase the apparent *K*. Thus 28 probably represent a maximum value for *K* in this system. This corresponds to a difference in redox potential of 43 mv and a free-energy change of 1970 cal.

Since about 7000 cal are available from one ATP, the largest part of the energy of a high-energy bond is wasted if the stoichiometry of the system is one high-energy bond hydrolyzed per mole of NADP+ reduced and this appears to be the correct stoichiometry.

We have also attempted to measure the equilibrium constant of the reaction using thio-NADP+ in place of NADP+. The time course of the reaction can be followed directly in a spectrophotometer using this analog since the maximum absorption of the reduced form occurs at 395 mμ. This experiment is illustrated in Figure 3. With a slight excess of NADH present, the reduction of thio-NADP+ goes to completion. The potential of thio-NAD+ has been reported as −0.285 v (Anderson and Kaplan, 1959) and thio-NADP+ would be expected to approximate this value. Thus the equilibrium constant of the reaction would be expected to be greater than with NADP+.

The Effect of Light Intensity. The effect of light intensity on the transhydrogenase as compared with photophosphorylation is presented in Figure 4. The slope of the curve of transhydrogenase activity *vs.* light intensity was greater than was the curve for photophosphorylation and consequently when compared on a per cent maximal activity basis, the transhydrogenase appeared to be more sensitive to light than was photophosphorylation. However, when

TABLE VII: Competition between NADPH Formation and Photophosphorylation.[a]

Expt	Light Intensity (ergs cm^{-2} sec^{-1})	Additions	NADPH Reduction[d]		ATP Formation[d]	
			$-ADP, P_i$	$+ ADP, P_i$	$-NADP^+$	$+NADP^+$
1	1.3×10^3		21	14	17	16
	3.6×10^3		27	18	34	35
	1.3×10^4		41	27	118	118
	1×10^5		47	34	302	321
2	3.8×10^4			30	83	81
	3.8×10^4	*m*-Cl-CCP (4 μM)		14	16	15
	3.8×10^4	Gramicidin (0.06 μM)		10	20	22
3	6×10^3	P_i concentration decreased		17	17	15
	1.3×10^4	to 0.33 mM		25	33	33
	1.0×10^5			33	64	63
4	5×10^4	HOQNO (27 μM)	11[b]	10	17[c]	15
5	1×10^5	Oligomycin (6 μg/ml)	25	24		

[a] Standard assay for photophosphorylation was used except ethanol, alcohol dehydrogenase and NADH were included as for the transhydrogenase assay. [b] Inhibited by 67%. [c] Inhibited by 95%. [d] Micromoles per hour per milligram of Bchl.

the actual rates of the reaction are compared on a molar basis (see insert in Figure 4) at the low light intensities, the rates are approximately the same. In this experiment we used chromatophores prepared from cells broken by grinding with Alumina and these chromatophores are somewhat more active than cell preparations which are ruptured in the French pressure cell. With less active preparations the rate of the transhydrogenase at the lower light intensities was up to double the rate of ATP formation.

Competition of NADPH Formation and Photophosphorylation. If I~X is the high-energy intermediate that is used to drive the transhydrogenase and is also on the pathway of ATP formation it should be possible to demonstrate competition between the transhydrogenase and ATP formation since the stoichiometry of $NADP^+$ reduced to ATP utilized is apparently one. Also in the prior section, it was demonstrated that at low light intensity, the rates of ATP formation and $NADP^+$ reduction were essentially equal. This led us to predict that a strong competition should be observed at low light intensity.

First attempts to demonstrate competition in submitochondrial particles were unsuccessful (van Dam and ter Welle, 1966) but Ernster *et al.* (1967) were successful later in demonstrating competition. They found that it was necessary to use large amounts of enzyme (submitochondrial particles) and limit the formation of I~X by inhibiting the oxidation of succinate with malonate. Under these conditions both $NADP^+$ reduction and ATP formation were suppressed from control values but in addition the efficiency of oxidation as measured by NADPH:O plus P:O was double the P:O ratio in the absence of transhydrogenation.

The experiments presented in Table VII indicate that significant competition did not occur even when conditions were adjusted so that the rates of $NADP^+$ reduction and ATP formation were equal.

We have altered the rate of $NADP^+$ reduction and photophosphorylation by lowering light intensity, limiting P_i concentration, and including uncouplers and inhibitors. These conditions generally effect phosphorylation to a greater extent than $NADP^+$ reduction.

In general, the addition of ADP and P_i inhibited the rate of the light-driven transhydrogenase up to 35% and this inhibition was reversed by oligomycin indicating that photophosphorylation could compete with the transhydrogenase to a limited degree but conversely no significant effect of transhydrogenation was observed on ATP formation. If one high-energy bond is required per molecule of $NADP^+$ reduced, the efficiency of light utilization can be increased by almost 100% at the lower light intensities. This agrees with the increase in efficiency observed in submitochondrial particles. (van Dam and ter Welle, 1966; Ernster *et al.*, 1967).

Two interpretations of these results are apparent. (1) Since chromatophores catalyze "cyclic" electron transport there is no good way to measure the efficiency of coupling of photosynthetic electron transport to phosphorylation. Therefore, if the coupling between electron transport and ATP formation is poor, sufficient I~X may be formed to supply both ATP formation and the transhydrogenase and thus little competition would be observed. (2) The stoichiometry of $NADP^+$ reduced to I~X utilized may be greater than one. However, this does not appear probable since the stoichiometry was one when ATP was the energy source.

We feel that the first interpretation is probably correct although even a third possibility exists; namely that the intermediate utilized by the transhydrogenase is not on the pathway to ATP formation (and may be

poorly or not at all linked with ATP formation). In this case, to explain the stoichiometry using ATP as the energy source, we have to postulate that the pathway of ATP utilization is not the reverse of ATP formation. The recent paper by Mitchell *et al.* (1967) summarizes evidence that such a pathway may exist. We presently do not know of any good evidence which excludes the latter postulate in chromatophores or in mitochondria.

Transhydrogenase in Other Organisms. In addition to *R. rubrum*, *R. spherioides* (Orlando *et al.*, 1966), and mitochondria from mammalian sources, we have found evidence for an energy-linked enzyme in *Rhodopseudomonas viridis*, *Rhodospirillum molischianum*, and *Rhodopseudomonas palustris*. We have not found the enzyme in *Chromatium*, *Rhodomicrobium vannielli*, *Chloropseudomonas ethylicium*, chloroplasts of spinach, or the blue-green algae, *Nostoc* and *Anabaena variablis*. However, the Hill reaction activities of the algae preparations were very low.

Discussion

The requirement of NADPH for many anabolic reactions by the cell has been well established. While this requirement could conceivably be provided by oxidative reactions, the cellular dehydrogenases are predominately NAD^+ linked. Thus other mechanisms are required for providing NADPH. In green plants and many algae, $NADP^+$ is reduced by a photochemical process that has been established fairly conclusively (see review by San Pietro and Black, 1965). This reaction provides a direct supply of NADPH for cellular synthesis. In chromatophores of the photosynthetic bacteria, *R. rubrum*, NAD^+ is photochemically reduced (Frenkel, 1958) and this reduction appears to be an energy-linked process probably *via* reversed electron transport (Keister and Yike, 1967). However, the mechanism of this reaction has not been firmly established (see review by Vernon, 1964). Thus a mechanism for providing NADPH is probably required. The energy-linked transhydrogenase may provide such a mechanism if it is functional *in vivo*.

The conclusion that $I\sim X$ was the source of energy for the transhydrogenase was based primarily on the oligomycin inhibition of the reaction when ATP was the energy source but not when electron transport was used to generate the energy (Danielson and Ernster, 1963a). $I\sim X$ may be a chemical intermediate or another high-energy state such as an ion gradient as proposed by Mitchell (1966) (the data do not exclude either). Our data are in agreement with this conclusion but in addition in *R. rubrum* chromatophores, PP_i can be used as an energy source and as previously reported (Keister and Yike, 1966) PP_i utilization was not inhibited by oligomycin. This result indicates that a different pathway exists for energy transfer from PP_i than from ATP. This observation concurs with previous observations (Baltscheffsky and von Stedingk, 1966; Baltscheffsky *et al.*, 1966) which demonstrated the light-induced formation of PP_i in the absence of ADP by *R. rubrum*. The formation of PP_i was not inhibited by oligomycin either, thus PP_i may be an alternative to ATP as an energy-storage compound in this organism. The transhydrogenase utilizes the energy to increase the rate and extent of the reduction of $NADP^+$. Whereas, the equilibrium constant (K) of the nonenergy-linked transhydrogenase approximates one as would be predicted from the potential of the substrates, a source of energy displaces this equilibrium toward the formation of NADPH.

Two mechanisms for this reaction have been postulated (see review by Ernster and Lee, 1964). One involves the compartmentation of pyridine nucleotides (Klingenberg, 1963), whereby the movement of pyridine nucleotide from one compartment to another would proceed by active transport. Since the transhydrogenase is tightly bound to the cristae membrane (and chromatophore), it may catalyze preferentially, the reaction in one direction. Spatial movement of the pyridine nucleotides would also account for the shift in equilibrium effected by an energy source.

Another postulate is that an energized form of a pyridine nucleotide is an intermediate in the reaction. Such an intermediate if utilized stoichiometrically during the reaction would also account for the shift in equilibrium. However, there is no good evidence yet available that relates to the mechanism of the reaction.

One of the interesting properties of *R. rubrum* is its ability to grow either oxidatively or photosynthetically when oxygen tension is limited. Photosynthetically grown organisms contain bacteriochlorophyll-containing membranes throughout the cytoplasm of the cell while aerobically grown organisms contain little or no chlorophyll and few intercytoplasmic membranes. The electron-transport system, both oxidative and photosynthetic, is contained in these membranes and it is unclear whether they are entirely different systems or intimately interrelated. We have proposed (Keister and Yike, 1967) that the oxidative and photosynthetic electron-transport systems are interrelated *via* the succinic dehydrogenase system and by the interaction or formation of a high-energy intermediate such as $I\sim X$ with both the oxidative and photosynthetic systems. We have observed that when photosynthetically grown cells are transferred to aerobic growth conditions and allowed to grow for several generations oxidatively (Bchl synthesis is suppressed and the protein:Bchl ratios of the chromatophores increases), that the rate of the energy-linked transhydrogenase, photophosphorylation, succinate-linked energy-driven NAD^+ reduction, and NADH oxidase activity increases several-fold on a Bchl basis while changing less or not at all on a protein basis. We interpret these observations to mean that new protein (maybe new membranes) is being synthesized at sites that can interact with the previously formed photosynthetic apparatus (which was obviously not rate limiting). This may also indicate that the photosynthetic and oxidative electron-transport chains have a common electron carrier(s). However it is also possible that the interaction is only *via* $I\sim X$. The energy-linked transhydrogenase is a common

component of both types of electron-transport chain and thus offers a tool for studying their interaction, for the rate of the ATP-driven enzyme on a protein basis is the same for both photosynthetically and aerobically grown cells. We are currently investigating the interaction and synthesis of the electron-transport pathways.

References

Anderson, B. M., and Kaplan, N. O. (1959), *J. Biol. Chem. 234*, 1226.

Ball, E. G., and Cooper, O. (1957), *Proc. Natl. Acad. Sci. U. S. 43*, 357.

Baltscheffsky, M. (1964), *Abstr. 1st Meeting European Biochem. Soc., London*, 67.

Baltscheffsky, H., and von Stedingk, L. (1966), *Biochem. Biophys. Res. Commun. 22*, 722.

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

Beechey, R. B. (1965), *Biochem. J. 98*, 284.

Beechey, R. B., Holloway, C. T., Knight, I. G., and Roberton, A. M., (1966), *Biochem. Biophys. Res. Commun. 23*, 75.

Bückel, K. H., Korte, F., Trebst, A. and Pistorius, E. (1965), *Angew. Chem. Intern. Ed. Engl. 4*, 789.

Cohen-Bazire, G., and Kunisawa, R. (1963), *J. Cell Biol. 16*, 401.

Danielson, L., and Ernster, L. (1963a), *in* Energy-Linked Functions of Mitochondria, Chance, B., Ed., New York, N. Y., Academic, p 157.

Danielson, L., and Ernster, L. (1963b), *Biochem. Z. 338*, 188.

Ernster, L., and Lee, C. P. (1964), *Ann. Rev. Biochem. 33*, 729.

Ernster, L., Lee, C. P., and Janda, S. (1967), *in* Biochemistry of Mitochondria, Slater, E. C., Kaniuga, Z., and Wojtczak, L., Ed., London, Academic, p 29.

Estabrook, R. W., Hommes, F., and Gonze, J. (1963), *in* Energy-linked Functions of Mitochondria, Chance, B., Ed., New York, N. Y., Academic, p 143.

Frenkel, A. W. (1958), *Brookhaven Symp. Biol. 11*, 276.

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

Griffiths, D. E., and Roberton, A. M. (1966), *Biochim. Biophys. Acta 118*, 453.

Haas, D. W. (1964), *Biochim. Biophys. Acta 82*, 200.

Hommes, F. A. (1963), *in* Energy-linked Functions of Mitochondria, Chance, B., Ed., New York, N. Y., Academic, p 39.

Kaplan, N. O., Colowick, S. P., and Neufeld, E. F. (1953a), *J. Biol. Chem. 205*, 1.

Kaplan, N. O., Colowick, S. P., Neufeld, E. F., and Ciotti, M. M. (1953b), *J. Biol. Chem. 205*, 17.

Kawasaki, T., Satoh, K., and Kaplan, N. O. (1964), *Biochem. Biophys. Res. Commun. 17*, 648.

Keister, D. L. (1965), *J. Biol. Chem. 240*, 2673.

Keister, D. L. (1966), *Brookhaven Symp. Biol. 19*, 255.

Keister, D. L., and Hemmes, R. B. (1966), *J. Biol. Chem. 241*, 2820.

Keister, D. L., San Pietro, A., and Stolzenbach, F. E. (1960), *J. Biol. Chem. 235*, 2989.

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

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

Klingenberg, M. (1963), *in* Energy-linked Functions of Mitochondria, Chance, B., Ed., New York, N. Y., Academic, p 121.

Klingenberg, M. (1966), *in* Regulation of Metabolic Processes in Mitochondria, Tager, J. M., Papa, S., Quagliariello, E., and Slater, E. C., Ed., Amsterdam, Elsevier, p 246.

Klingenberg, M., and Slenczka, W. (1959), *Biochem. Z. 331*, 486.

Lardy, H. A., Johnson, D., and McMurray, W. C. (1958), *Arch. Biochem. Biophys. 78*, 587.

Lee, C. P., and Ernster, L. (1964), *Biochim. Biophys. Acta 81*, 187.

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.

Mitchell, P. (1966), *Biol. Rev. 41*, 455.

Mitchell, R. A., Hill, R. D., and Boyer, P. D. (1967), *J. Biol. Chem. 242*, 1793.

Neufeld, E. F., Kaplan, N. O., and Colowick, S. P. (1955), *Biochim. Biophys. Acta 17*, 525.

Nozaki, M., Tagawa, K., and Arnon, D. I. (1963), *in* Bacterial Photosynthesis, Gest, H., San Pietro, A., and Vernon, L. P., Ed., Yellow Springs, Ohio, Antioch, p 175.

Orlando, J. A., Sabo, D., and Curnyn, C. (1966), *Plant Physiol. 41*, 937.

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

Slater, E. C. (1963), *in* Energy-linked Functions of Mitochondria, Chance, B., Ed., New York, N, Y., Academic, p 176.

Stein, A. M., Kaplan, N. O., and Ciotti, M. M. (1959), *J. Biol. Chem. 234*, 979.

van Dam, K., and ter Welle, H. F. (1966), *in* Regulation of Metabolic Processes in Mitochondria, Tager, J. M., Papa, S., Quagliariello, E., and Slater, E. C., Ed., Amsterdam, Elsevier, p 235.

Vernon, L. P. (1964), *Ann. Rev. Plant Physiol. 15*, 73.