# Energy-linked Functions of Submitochondrial Particles Prepared from Mung Bean Mitochondria[1]

S. B. WILSON AND W. D. BONNER, JR.
*Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania 19104*

## ABSTRACT

Submitochondrial particles prepared from mung bean mitochondria (*Phaseolus aureus*) are able to catalyze an energy-linked reduced nicotinamide adenine dinucleotide-nicotinamide adenine dinucleotide phosphate transhydrogenase reaction supported by ATP or by aerobically generated high energy intermediates. The energy transfer pathway appears to differ from that utilized for oxidative phosphorylation.

Mung bean submitochondrial particles will also reduce nicotinamide adenine dinucleotide by reversed electron transport from succinate or ascorbate tetramethyl-*p*-phenylenediamine. The energy requirement can be met by ATP or by aerobically generated high energy intermediates.

A scheme for the energy transduction pathway in mung beans is postulated from the effects of inhibitors and uncouplers of energy transfer on transhydrogenase and reversed electron transfer reactions.

There are two energy-linked reactions that have been widely studied in animal mitochondria (4–7, 11, 12) and in microorganisms (1). These reactions are the transhydrogenase and the reduction of NAD$^+$ by reversed electron transport. The following sequence has been postulated for the energy-linked transhydrogenase reaction (11, 12):

$$\text{NADH} + \text{NADP}^+ + I \sim X \rightarrow \text{NAD}^+ + \text{NADPH} + I + X$$
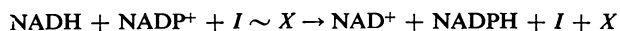
where $I \sim X$ is a high energy intermediate of the phosphorylation system and can be derived either from respiratory chain oxidations or from ATP. This reaction has also been observed in a non-energy-linked form in which $I \sim X$ is not involved, and both reactions are apparently catalyzed by the same enzyme. Similarly, the reversed electron transport reduction of NAD$^+$ requires high energy intermediates to overcome the energy gradient of the process.

The study of these two reactions in animal mitochondria and in submitochondrial particles derived from these mitochondria has helped in the elucidation of energy transport pathways; unfortunately, these reactions have not been studied in plant mitochondrial systems.

In an earlier paper of this series (13) it was shown (*a*) that succinate oxidation by submitochondrial particles prepared from mung bean mitochondria was stimulated by ATP and (*b*) that

both succinate and NADH oxidation could be inhibited by octylguanidine. These observations suggest that the particles have retained some of the energy-linked functions of the intact mitochondria and are suitable for a study of some of the energy transfer mechanisms. In this paper the energy-linked transhydrogenase and energy-driven reversed electron transport are demonstrated in submitochondrial particles prepared from mung bean hypocotyl (*Phaseolus aureus* var. jumbo) mitochondria, and the effects of inhibitors of energy transfer and electron transport on these reactions are detailed.

## METHODS

Submitochondrial particles were prepared from tightly coupled mung bean mitochondria, as described earlier (13), and were stored at $-15$ C until required. Particles stored in this way retained some of their energy-linked functions up to 2 weeks after preparation. The particles were resuspended with a Teflon homogenizer in a medium containing 0.3 M mannitol and 0.1% BSA (pH 7.2 with KOH) to give a SMP[2] protein content of 5 to 20 mg/ml and used at once.

The energy-linked transhydrogenase was studied (11) by measuring the rate of oxidation of NADH in a double beam spectrophotometer at 340 to 374 nm (3); the extinction coefficients at these wave lengths were assumed to be 6 cm$^{-1}$ mM$^{-1}$ (3). The reaction mixture, final volume 1.5 ml, consisted of 0.3 M mannitol, 10 mM KCl, 5 mM MgCl$_2$ adjusted to pH 7.2 with KOH (in some cases 0.05 M TES tris(hydroxymethyl) methylaminoethyl sulfonic acid buffer, pH 7.2, was included), 0.13 mM NADH, 0.13 mM NADP, 1.3 mM oxidized glutathione, 0.07 mM KCN, 5 µg of yeast glutathione reductase (30 units/mg), and submitochondrial particles equivalent to about 1 mg of protein. MgCl$_2$ was omitted when the non-energy-linked transhydrogenase was studied. The reaction was started by the addition of 0.6 mM ATP. If the reaction was to be supported by high energy intermediates, KCN was replaced by 1.7 µg of freshly prepared amytal or 5 µg of antimycin A and 17 mM succinate, or a freshly prepared mixture of 10 mM ascorbate and 1 mM TMPD was added to initiate the reaction.

Reagents were dissolved in double distilled water or in a small volume of alcohol and stored at $-15$ C prior to use unless otherwise stated.

The ATP-supported reversed electron transport reaction was studied by measuring the formation of NADH in a double beam spectrophotometer. The basic reaction mixture contained 0.3 M mannitol; 10 mM KCl; 5 mM MgCl$_2$; 50 mM TES tris(hydroxymethyl)methylaminoethyl sulfonic acid buffer, pH 7.2; 0.3 M ATP; 16 mM succinate; 0.3 mM NAD$^+$. The reaction was started

[2] Abbreviations: SMP: submitochondrial particles; 1799: bis(hexafluoroacetonitryl)acetone; DNP: 2,4-dinitrophenol; TES: tris(hydroxymethyl)methylaminoethyl sulfonic acid; TMPD: $N,N,N^1,N^1$-tetramethyl-*p*-phenylenediamine.
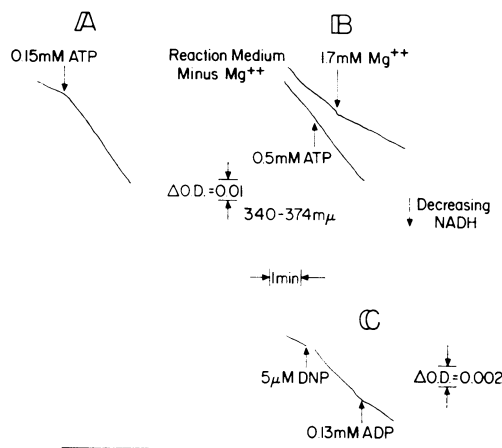
FIG. 1. Transhydrogenase reaction, conditions as described in the text, the traces show rates of NADH oxidation. A: The effect of ATP in supporting the energy-linked transhydrogenase (70% increase in NADH oxidation rate). B: The absence of ATP influence and the effect of $Mg^{2+}$ (50% inhibition) on the non-energy-linked reaction. $Mg^{2+}$ is omitted from the basic reaction mixture for this trace. C: The effect of DNP in releasing the non-energy-linked reaction (60% increase in reaction rate) and the effect of ADP on the DNP-stimulated rate (50% inhibition).

Table I. *Substrate Requirements for the Energy-linked Transhydrogenase*

| Reaction Mixture | Substrate Requirement |
|---|---|
|  | $\mu mole\ NADH/min\cdot mg\ protein$ |
| Complete | 0.008 |
| -ATP | 0.004 |
| -NADH | 0 |
| -NADP | 0.001 |
| -SMP | 0 |
| -Glutathione reductase | 0 |

by the addition of 0.035 mM KNC. The ATP-supported reaction was also carried out with 5 mM ascorbate and 0.5 mM TMPD as electron donor to supply electrons at the cytochrome $c$ level.

The reversed electron transport from succinate to $NAD^+$ could also be supported by high energy intermediates generated by the oxidation of 5 mM ascorbate and 0.5 mM TMPD in the presence of antimycin A. KCN was omitted from the mixture.

Recrystallized octylguanidine was kindly supplied by Dr. B. Pressman of the Johnson Research Foundation, and 1799 was supplied by Dr. P. Heytler of DuPont de Nemours, Wilmington, Delaware.

## RESULTS

**Energy-linked Transhydrogenase.** Figure 1A shows the effect of ATP in supporting the transhydrogenase reaction. In contrast, the energy-linked transhydrogenation which is observed in the same particles in the absence of magnesium is not influenced by ATP and is inhibited by magnesium ions (Fig. 1B). The action of an uncoupler, DNP, in releasing the non-energy-linked transhydrogenase in the presence of $Mg^{2+}$ is shown in Figure 1C, which also illustrates the partial inhibition of the non-energy-linked reaction by ADP.

Table I shows that the omission of any of the components of the reaction mixture inhibits the rate of the reaction of the ATP-supported transhydrogenase. Both $P_i$ and uncouplers of energy transfer inhibit the ATP-supported reaction (Fig. 2). Oligomycin

at various levels results in the effects shown in Figure 3 which also indicate the lack of response of the aerobically driven reaction to oligomycin inhibition, the half-maximal inhibition occurring at 2.8 $\mu$g/mg protein and maximal stimulation being shown at 0.82 $\mu$g/mg protein. The non-energy-linked transhydrogenase reaction initiated by DNP or 1799 was not affected by oligomycin at any level.

Figure 4 illustrates the effects on the transhydrogenase of high energy intermediates generated either by the oxidation of succinate in the presence of amytal (Fig. 4A), or the ascorbate-TMPD mixture in the presence of antimycin A (Fig. 4B). KCN inhibits
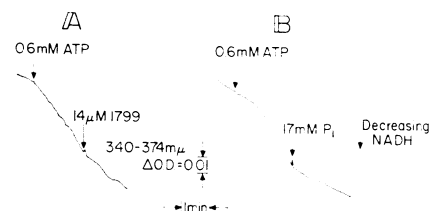


FIG. 2. The effects of 1799 and $P_i$ on the ATP-supported transhydrogenase reaction. At the indicated concentrations 1799 and $P_i$ cause 60 and 79% inhibition, respectively, of the rate of NADH oxidation.
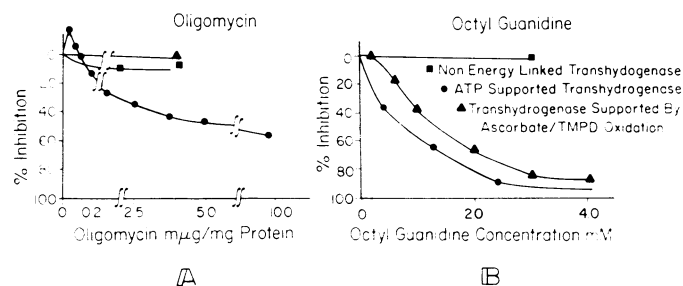


FIG. 3. The effects of oligomycin (A) and octylguanidine (B) on the transhydrogenase reactions.
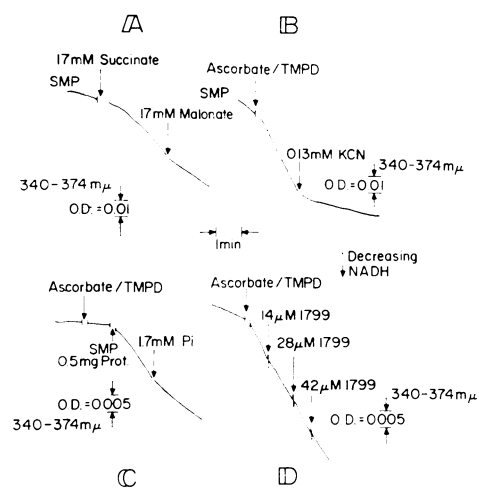


FIG. 4. The transhydrogenase reaction supported by high energy intermediates. Conditions of assay as outlined in the text, the traces show rates of NADH oxidation. A: Intermediates generated by succinate oxidation. The inhibition (50%) caused by adding 17 mM malonate to the system is also shown. B: Intermediates generated by ascorbate-TMPD oxidation. The inhibition (83%) caused by adding 0.13 mM KCN to the system is also shown. C: Dependence of the ascorbate-TMPD-supported transhydrogenase on SMP. The inhibition (55%) caused by adding 1.7 mM $P_i$ to the system is also shown. D: The effects of 1799 on the ascorbate-TMPD-supported transhydrogenase.

Table II. *Half-maximal Inhibitory Concentrations of*
*Octylguanidine and Oligomycin for Energy-linked*
*Functions by Submitochondrial Particles*

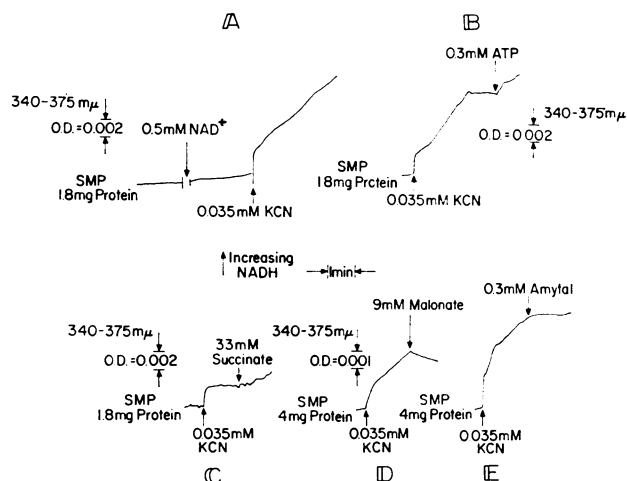| System | Energy Source | Octyl-guani-dine Concn | Oligomycin Concn |
|---|---|---|---|
| | | *mM* | *mμmoles/mg protein* |
| Transhydrogenase | ATP | 0.61 | $2.8 \times 10^{-3}$ |
| Transhydrogenase | Ascorbate oxidation | 1.08 | NI[1] |
| Transhydrogenase | Non-energy-linked | NI | NI |
| Reversed electron transport | | | |
| Succinate to NAD | ATP | 0.38 | 0.9 |
| Succinate to NAD | Ascorbate oxidation | 0.68 | NI |
| Ascorbate to NAD | ATP | 0.52 | 1.3 |

[1] Not inhibited.



FIG. 5. The reduction of $NAD^+$ by succinate. The traces show rates of $NAD^+$ reduction under the assay conditions described in the text. A: Supported by ATP; B: supported by a limiting amount of ATP, after utilization of the added ATP the reaction ceases but continues after a further ATP addition; C: supported by ATP, showing the effect of omitting succinate; D: the inhibiting effect of malonate on the ATP-supported reaction; E: the inhibiting effect of amytal on the ATP-supported reaction.

the latter reaction by inhibiting the oxidation of ascorbate-TMPD (Fig. 4B), while the malonate inhibits the reaction supported by succinate (Fig. 4A). The reaction is not a result of the nonenzymic oxidation of NADH by TMPD but requires the presence of submitochondrial particles (Fig. 4C). Unlike the ATP-supported transhydrogenase, oligomycin did not affect the transhydrogenase driven by high energy intermediates generated by ascorbate-TMPD (Figs. 3A and 4D). Inorganic phosphate, however, inhibited both ATP and high energy intermediate-supported transhydrogenase (Figs. 2B and 4C). Both transhydrogenases were also inhibited by octylguanidine, an inhibitor of energy transfer (Fig. 3B). The non-energy-linked transhydrogenase was not inhibited by either oligomycin or octylguanidine. Table II lists the concentration of oligomycin and octylguanidine for half-maximal inhibition of the transhydrogenase reaction.

**Reduction of $NAD^+$ by Reversed Electron Transport.** Submitochondrial particles catalyze the reduction of $NAD^+$ by succinate when KCN is added to prevent the oxidation of succinate (Fig. 5A). The reaction is dependent on the presence of both ATP

and succinate (Fig. 5, B, C, and D) and induces transfer of electrons through an amytal-sensitive site (Fig. 5E).

The ATP-supported succinate reduction of $NAD^+$ is inhibited by an uncoupler, 1799 (Fig. 6A), $P_i$ (Fig. 6B), and oligomycin or octylguanidine (Fig. 7, A and B).

Similarly, ascorbate-TMPD can serve as electron donor for $NAD^+$ reduction in a manner akin to the succinate reduction of $NAD^+$ (Fig. 8). Antimycin A inhibits the ascorbate-TMPD reduction of $NAD^+$. 1799, $P_i$, and ADP all inhibit the ascorbate-TMPD reduction of $NAD^+$ (Fig. 9). The reduction is inhibited by oligomycin and octylguanidine (Fig. 7, A and B).

Figure 10A shows that high energy intermediates generated by the oxidation of ascorbate-TMPD in the presence of antimycin A can be utilized to support the reduction of $NAD^+$ by succinate. KCN, amytal, and malonate all inhibit this reaction by inhibition of ascorbate-TMPD oxidation, electron transport from succinate to $NAD^+$, and succinic dehydrogenase, respectively.

The reduction of $NAD^+$ supported by aerobically generated high energy intermediates differs from the ATP-supported reaction by being only slightly or not inhibited by 1799 and oligomycin (Figs. 7 and 10). Octylguanidine could, however, inhibit this reaction. Table II lists the half-maximal inhibitory concen-
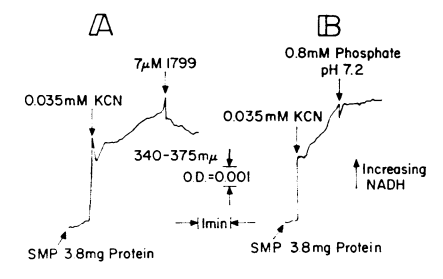


FIG. 6. The inhibition of the ATP-supported succinate reduction of $NAD^+$ by 1799 (A) and $P_i$ (B). The traces show rates of $NAD^+$ reduction as measured under the assay conditions described in the text.
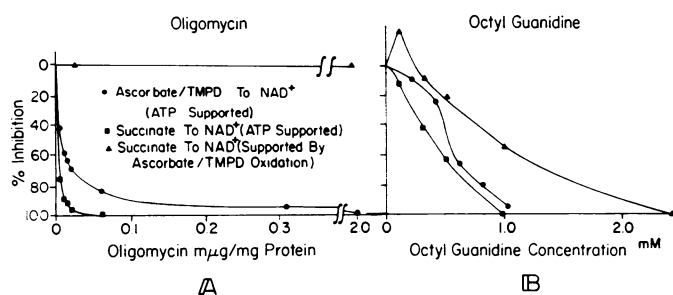


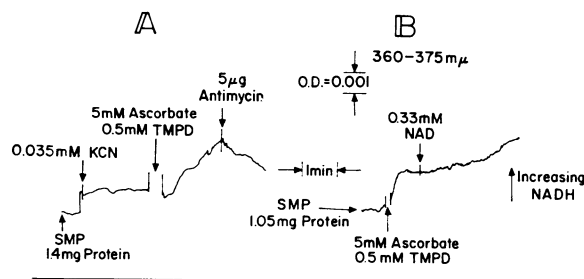FIG. 7. The effect of oligomycin (A) and octylguanidine (B) on the energy-linked reduction of $NAD^+$.



FIG. 8. The reduction of $NAD^+$ by ascorbate-TMPD driven by ATP. Shown also are the inhibition of the reaction by antimycin A (A) and the requirement for $NAD^+$ (B). Assay conditions as described in the text.
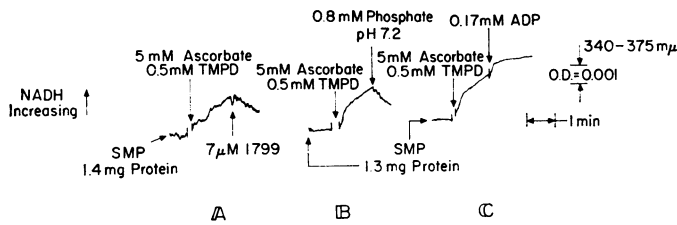
FIG. 9. The reduction of NAD⁺ by ascorbate-TMPD. The effect of 1799 (A), $P_i$ (B) and ADP (C). Assay conditions as described in the text.
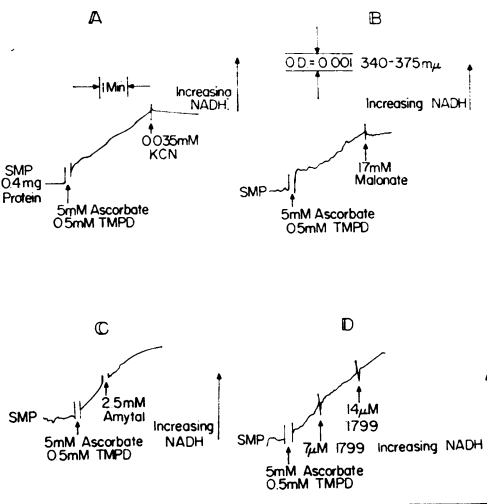


FIG. 10. The reduction of NAD⁺ by succinate driven by high energy intermediates generated through the oxidation of ascorbate-TMPD.

trations of octylguanidine for the reduction of NAD⁺ in various systems described above.

## DISCUSSION

The data presented in this paper clearly show that mung bean submitochondrial particles are able to perform transhydrogenase

reactions and catalyze the reduction of NAD⁺ by reversed electron transport in a manner similar to that shown for mammalian mitochondria (cf. 4-7, 11, and 12). The properties of the mung bean transhydrogenase are likewise similar to those of the beef heart particles and Micrococcus denitrificans membrane fragments (1).

Both the transhydrogenase and reversed electron transport reactions were inhibited by oligomycin, octylguanidine, and uncouplers, showing the dependence of these reactions on ATP as an energy source and indicating a pathway of energy transfer similar to that postulated (12) for forward phosphorylation reactions in mammalian mitochondria.

The ATP-supported transhydrogenase is stimulated by low levels of oligomycin in a manner analogous to that observed for beef heart particles, inhibition only occuring at a high oligomycin concentration. Both stimulating and inhibiting oligomycin concentrations are in excess of those required to inhibit either the forward (9) or the reversed phosphorylation. Similarly, the half-maximal inhibition concentration of octylguanidine required for the ATP-supported transhydrogenase differs from that required by any of the forward or reverse phosphorylations. Since it has been shown that octylguanidine inhibition of the various coupling sites occurs at concentration levels which are characteristic of the coupling sites, it must be concluded that the energy-linked transhydrogenase utilizes a quantitatively different pathway of energy transduction from ATP when compared with phosphorylation linked to the respiratory chain.

Lee and Ernster (12) reported that several energy-linked functions of nonphosphorylating beef heart particles could be stimulated by low levels of oligomycin and attributed their results to the inhibition of energy loss by ATPase activity. Such an explanation seems likely for the oligomycin-stimulated transhydrogenase reported in this paper. Lack of stimulation when high energy intermediates are utilized reflects an apparent greater phosphorylating efficiency of the mung bean particles which thus resemble the phosphorylating beef heart particles rather than the nonphosphorylating EDTA particles.

Consideration of the level of energy carrier connecting the transhydrogenase energy transduction pathway to the electron transfer pathway and enabling high energy intermediates to be utilized indicates that transfer occurs at a level close to but not on
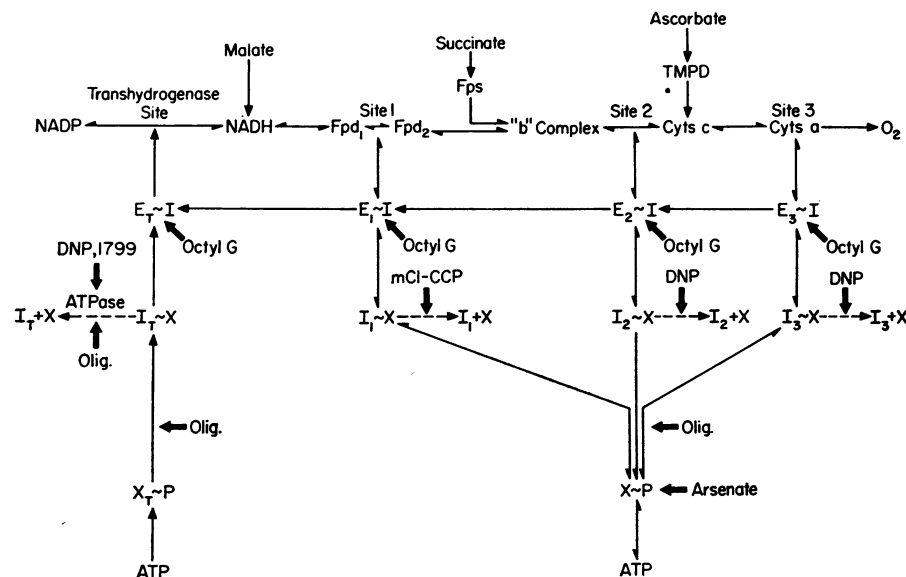


**𝄡 FIG. 11.** Hypothetical scheme of the respiratory chain-linked energy transfer system in mung bean mitochondria and submitochondrial particles with the proposed sites of action of octylguanidine (Octyl. G.); oligomycin (olig.); dinitrophenol (DNP); carbonyl cyanide m-chlorophenylhydrazone (m-Cl-CCP); and bis(hexafluoroacetonitryl) acetone (1799). E, I, and X denote high energy intermediates belonging to the four coupling sites, T (transhydrogenase), 1, 2, and 3. The three b cytochromes, two c cytochromes and two a cytochromes are designated as "b" Complex Cyts c, and Cyts a, respectively.

the respiratory chain. These exchangeable energy carriers have been designated $E$ to differentiate them from the carriers $C$ and $I$ discussed by Lee and Ernster (cf. 12). Unlike the beef heart particles the mung bean transhydrogenase driven by high energy intermediates was not inhibited by oligomycin or uncouplers, only by octylguanidine and at a concentration required for inhibition of site III forward phosphorylation (14). Inorganic phosphate was not required when high energy intermediates were utilized as an energy source.

The ATP-supported $NAD^+$ reduction closely resembles the reversal of oxidative phosphorylation. Thus, the ATP-driven succinate reduction of $NAD^+$ is sensitive to octylguanidine with a half-maximal level similar to that observed for the forward reaction when NADH is substrate. The ATP-supported reversal from ascorbate is sensitive to a level of octylguanidine intermediate between those observed for sites I and II. The reverse reaction can also be inhibited by oligomycin, although at lower levels than the forward reaction. This change in sensitivity may, however, represent the smaller amounts of protein present in the particles and a greater accessibility of the active sites to the inhibitor. The great difference in oligomycin levels required to inhibit the transhydrogenase compared with the reversed electron transport reaction indicates that the pathways to ATP are not interchangeable, energy transfer only occurring from respiratory chain to transhydrogenase and not in the reverse direction.

In a manner analogous to the transhydrogenase, $E \sim I$ also appears to be the carrier responsible for energy transduction between the various energy conservation sites of the electron transport chain. Thus, with succinate as electron donor $NAD^+$ reduction was driven by the oxidation of ascorbate-TMPD in a reaction not influenced by oligomycin, only slightly affected by 1799 but inhibited by octylguanidine at a level intermediate between the sensitivity (14) of the forward phosphorylation reaction of sites I and III. The involvement of $E \curvearrowleft I$ in the interchange of energy between the various coupling sites is also demonstrated by the sensitivity of the ATP-driven ascorbate-TMPD reduction of $NAD^+$ to octylguanidine which is intermediate between the reported sensitivities of coupling sites I and II. If interchange of energy at the $E \sim I$ level did not occur, then the sensitivity observed would be that of site I alone, the most sensitive step of the reaction.

Phosphorylation has not previously been demonstrated in submitochondrial particles prepared from mung bean mitochondria, but the reactions described in this paper suggest that they are capable of energy conservation to ATP. The arguments outlined above and the observation that both $P_i$ and ADP inhibit the high energy intermediate-driven reactions strongly imply that the particles from mung bean mitochondria are phosphorylating particles. Subsequent experiments with mitochondria have confirmed that ATP can be produced by SMP prepared as described in this paper.

The above considerations are summarized in schematic form in Figure 11, which shows, by analogy with the hypothetical schemes for phosphorylation in beef heart, a postulated mechanism for energy transfer in mung bean mitochondria. The scheme differs from that described for beef heart mitochondria in the site of action of uncouplers. In the mung bean particles the results presented here show that this occurs closer to the formation of ATP than the site of interaction for high energy intermediate transfer.

Octylguanidine appears to inhibit transfer of energy in the region of the exchangeable energy carriers. Inhibition by octylguanidine at a site between $E \sim I$ and $X \sim I$ or between $E \sim I$ and the electron transfer chain would not result in the sensitivities of the energy-linked functions observed in these experiments.

Oligomycin inhibits forward transfer from malate or succinate at the same half-maximal inhibitory concentration (9), an observation confirmed by the inhibition of ATP-driven reversed electron transport from succinate or ascorbate-TMPD. Thus, the energy transduction pathways from the coupling sites coincide at this point. Separate energy pathways, however, occur at the level of octylguanidine inhibition which occurs at different concentrations for each site and at the uncoupling site where carbonyl cyanide m-chlorophenylhydrazone and dinitrophenol have differential effects on malate and succinate oxidation (9).

Mitochondria from plants differ from those produced from mammalian tissues by the presence of an alternate cyanide-insensitive oxidase system. Inhibition of this pathway by octylguanidine (14), the reversal of electron transport in the presence of cyanide (2), and the generation of ATP in the presence of cyanide (8) suggest that the alternate oxidase pathway is capable of energy conservation, probably by a similar pathway to that presented in Figure 11 for the cyanide-sensitive respiratory chain.

## LITERATURE CITED

1. ASANO, A., K. IMAI, AND R. SATO. 1967. Oxidative phosphorylation in Micrococcus denitrificans. II. The properties of the pyridine nucleotide transhydrogenase. Biochim. Biophys. Acta 143: 477–486.
2. BONNER, W. D. AND D. S. BENDALL. 1968. Reversed electron transport in mitochondria from the spadix of Arum maculatum. Biochem. J. 109: 47.
3. CHANCE, B. 1957. Techniques for the assay of the respiratory enzymes. In: S. P. Colowick and N. O. Kaplan, eds., Methods in Enzymology, Vol. IV. Academic Press, New York. pp. 273–329.
4. DAM, K. VAN AND J. F. TER WELLE. 1966. The energy linked transhydrogenase of mitochondria. In: J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater, eds., Regulation of Metabolic Processes in Mitochondria. Elsevier, Amsterdam. pp. 235–45.
5. DANIELSON, L. AND L. ERNSTER. 1963. Demonstration of a mitochondrial energy dependent pyridine nucleotide transhydrogenase reaction. Biochem. Biophys. Res. Commun. 10: 91–96.
6. DANIELSON, L. AND L. ERNSTER. 1963. Energy dependent reduction of TPN by DPNH. In: B. Chance, ed., Energy Linked Functions of Mitochondria. Academic Press, New York. pp. 157–175.
7. DANIELSON, L. AND L. ERNSTER. 1963. Energy dependent reduction of triphosphopyridine nucleotide by reduced diphosphopyridine nucleotide coupled to the energy transfer system of mitochondria. Biochem. Z. 338: 188–205.
8. HACKETT, D. P., B. RICE, AND C. SCHMID. 1960. The partial dissociation of phosphorylation from oxidation in plant mitochondria by respiratory chain inhibitors. J. Biol. Chem. 235: 2140–2144.
9. IKUMA, H. AND W. D. BONNER. 1967. Properties of higher plant mitochondria. II. Effect of DNP, m-Cl-CCP, and oligomycin on respiration of mung bean mitochondria. Plant Physiol. 42: 1400–1406.
10. IKUMA, H. AND W. D. BONNER. 1967. Properties of higher plant mitochondria. III. Effects of respiratory inhibition. Plant Physiol. 42: 1535–1544.
11. LEE, C. P. AND L. ERNSTER. 1966. The energy linked transhydrogenase reaction, its characteristics and its use as a tool for the study of oxidative phosphorylation. In: J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater, eds., Regulation of Metabolic Processes in Mitochondria. Elsevier, Amsterdam, pp. 218–234.
12. LEE, C. P. AND L. ERNSTER. 1968. Studies of the energy transfer systems of submitochondrial particles. I. Competition between oxidative phosphorylation and the energy linked nicotinamide adenine nucleotide transhydrogenase reaction. Eur. J. Biochem. 3: 385–390.
13. WILSON, S. B. AND W. D. BONNER. 1970. Preparation and some properties of submitochondrial particles from tightly coupled mung bean mitochondria. Plant Physiol. 46: 25–30.
14. WILSON, S. B. AND W. D. BONNER. 1970. Effects of guanidine inhibitors on mung bean mitochondria. Plant Physiol. 46: 21–24.