Studies on Energy-Linked Reactions

ENERGY-LINKED TRANSHYDROGENASE REACTION IN *ESCHERICHIA COLI*

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1. Small particles from *Escherichia coli* catalyse an ATP-dependent reduction of NADP$^+$ by NADH. 2. The reaction was stimulated by Mg^{2+} and only ATP and ITP could serve as energy donors. 3. Studies of the stoicheiometry of the reaction indicate that 1–2 mol of ATP are utilized/mol of NADH produced. 4. The reaction is inhibited by uncoupling agents such as 2,4-dinitrophenol (200 μM) and tetrachlorotrifluorobenzimidazole (2 μM) but oligomycin does not inhibit. 5. The reaction is inhibited by relatively high concentrations (200 μM) of piericidin A.

The energy-dependent reduction of NADP$^+$ to NADPH by NADH (energy-linked transhydrogenase) was first described in mammalian submitochondrial particles by Danielson & Ernster (1963). It was shown that energy for the reaction could be supplied either by added ATP or by the aerobic oxidation of respiratory substrates. The sensitivity of the reaction to uncouplers and inhibitors of oxidative phosphorylation implies the participation of high-energy intermediates of oxidative phosphorylation.

Measurements of the energy requirement of the reaction demonstrated that one ATP molecule was utilized/NADPH molecule formed. The submitochondrial particles contained a non-energy-dependent transhydrogenase, a reaction that could proceed in the absence of added ATP. It was shown that the equilibrium constant ($K = $[NAD$^+$][NADPH]/[NADH][NADP$^+$]) was 0.79 for the non-energy-linked reaction. In the energy-dependent reaction there was a strong displacement of the equilibrium towards the formation of NAD$^+$ and NADPH with an equilibrium constant of 480 (Lee & Ernster, 1964).

Studies with inhibitors such as rotenone and Amytal and studies on the stereospecificity of hydrogen transfer (Griffiths & Roberton, 1966) indicate that the NADH dehydrogenase–flavoprotein system is not involved but little is known of the reaction in *Escherichia coli*.

The energy-linked transhydrogenase has been demonstrated in certain bacterial preparations (Aleem, 1966*a,b*; Keister & Yike, 1967; Murthy & Brodie, 1964), but studies have been limited

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because of technical difficulties. The present work describes the characterization of the reaction in small particles derived from *E. coli* so that further experiments could be carried out on the mechanism of the reaction and its relation to the electron-transport system in *E. coli*.

METHODS

Methods for the growth of *E. coli* K12, preparation of particles from *E. coli*, determination of protein and nucleotide triphosphatase activity and the sources of chemicals and enzymes are described in the preceding paper (Sweetman & Griffiths, 1971). Further details are given in the legends to the table and figures.

Measurement of ATP-dependent reduction of NADP$^+$ by NADH. NADPH production was determined essentially as described by Danielson & Ernster (1963) by using a Beckman DK 2A recording spectrophotometer to measure the increase in extinction at 340 nm. Blank and experimental cuvettes of 1 cm light-path contained 375 μmol of sucrose, 75 μmol of tris–HCl buffer, pH 8.0, 10 μmol of MgCl$_2$, 0.05 μmol of NAD$^+$, 200 μg of yeast alcohol dehydrogenase [Sigma (London) Chemical Co., London S.W.6, U.K.], 180 μmol of ethanol, 5 μmol of Na$_2$S and bacterial particles (2–5 mg of bacterial protein).

The non-energy-linked reaction was initiated by the addition of 1 μmol of NADP$^+$. After 2–3 min the energy-linked reaction was started by the addition of 3–6 μmol of ATP to give a final volume of 2 ml. All incubations were carried out at 30°C.

Ethanol and alcohol dehydrogenase were used as an NADH-regenerating system. Inhibitors and uncoupling agents were added to both blank and experimental cuvettes.

Non-energy-linked reductions. The non-energy-linked reduction of NADP$^+$ by NADH was measured as described above. The non-energy-linked reduction of NAD$^+$ by NADPH was determined by the method of Murthy & Brodie (1964) or Bragg & Hou (1968).

RESULTS

Demonstration of the ATP-dependent reduction of NADP+ by NADH. In the presence of an active-NADH-regenerating system *E. coli* small particles catalysed the reduction of NADP+ (non-energy-linked transhydrogenase) as shown by an increase in extinction at 340nm. The reduction of NADP+ was increased four- to five-fold by the addition of ATP after a lag time of about 1min (Fig. 1).
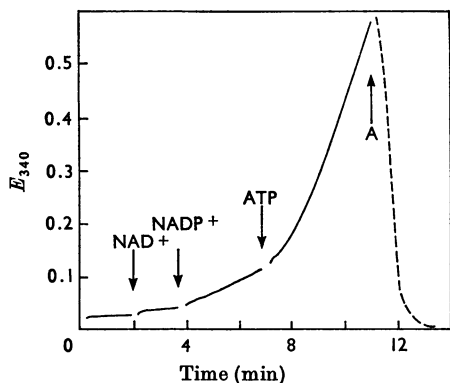

Fig. 1. Time-course of ATP-dependent and ATP-independent reduction of NADP+ by NADH. Blank and experimental cuvettes contained 375μmol of sucrose, 75μmol of tris–HCl buffer, pH8.0, 10μmol of $MgCl_2$, 0.05μmol of NAD+, 200μg of yeast alcohol dehydrogenase, 180μmol of ethanol, 5μmol of Na_2S and bacterial particles (3.5mg of protein). The temperature was 30°C. The non-energy-linked reaction was initiated by the addition of 1μmol of NADP+ at the point indicated. After 3min the energy-linked reaction was started by the addition of 6μmol of ATP to give a final volume of 2ml. At point A, 5μmol of GSSG and 5μg of glutathione reductase were added.

Table 1. *Requirements for reduction of NADP+ by NADH*

Experimental conditions were as described for Fig. 1. Additions and omissions were made as indicated; 6μmol of AMP and 6μmol of ADP were added as shown; 2.5mg of small-particle protein was used in each assay.

Conditions	NADPH formed (nmol/min per mg of protein)
Complete	14.5
−NADH	0
−NADP+	0
−ATP	3.2
−ATP, +ADP	5.3
−ATP, +AMP	3.2
−ATP, −Mg^{2+}	7.2
−Mg^{2+}	8.1

The formation of NADPH under these conditions was demonstrated by the oxidation of the product by glutathione reductase, as shown by a rapid drop in extinction at 340nm on addition of GSSG and the NADPH-specific glutathione reductase.

An absolute requirement for both NADH and NADP+ was demonstrated for the energy-linked reaction (Table 1). In the absence of ATP a slower rate of NADPH formation was observed; this rate was unaffected by AMP, but ADP produced a slight stimulation, indicating that ADP can act as an energy donor in this system, probably by the action of an adenylate kinase system. In the absence of ATP and Mg^{2+} and in the absence of Mg^{2+} alone the reaction was decreased by about 50%.

Effect of Mg^{2+}. Mg^{2+} had different effects on the energy-dependent and the energy-independent reactions. It was found to inhibit the non-energy-linked reaction going from NADH to NADPH and from NADPH to NADH (Fig. 2). Maximum inhibition of both reactions was achieved by 5mM-Mg^{2+}. The energy-linked reaction, however, was stimulated by Mg^{2+}, optimum activity occurring at about 2.5mM-Mg^{2+}. The activity was not completely abolished in the absence of Mg^{2+}, probably due to the presence of some endogenous Mg^{2+} in the bacterial particles.

Specificity for ATP. The extent of the energy-linked transhydrogenase was found to be dependent on the amount of ATP added (Fig. 3) and the apparent K_m for the reaction was 277μM. Apart from ATP the only nucleotide triphosphates capable
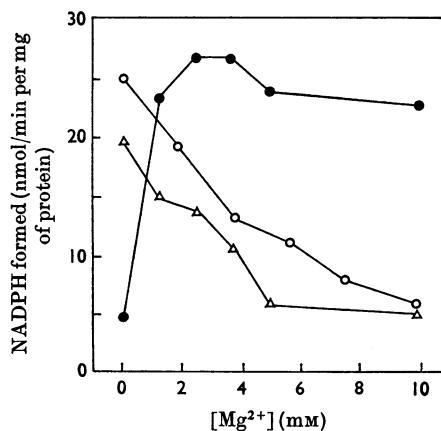

Fig. 2. Effect of Mg^{2+} on transhydrogenase reactions. The reactions were measured as described for Fig. 1, except that 2mg of bacterial protein (small particles) was used. ●, ATP-dependent reduction of NADP+ by NADH; ○, ATP-independent reduction of NAD+ by NADPH; △, ATP-independent reduction of NADP+ by NADH.
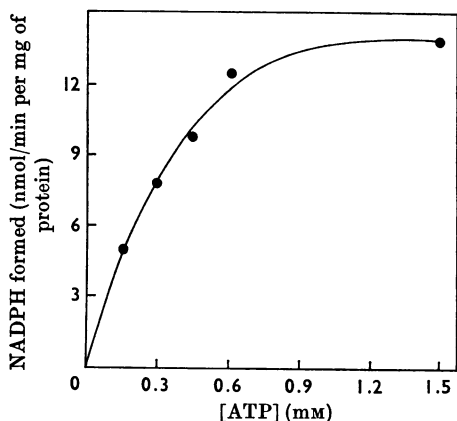
Fig. 3. Effect of ATP on energy-dependent reduction of $NADP^+$ by NADH. Conditions were as described for Fig. 1, except that 3.0mg of protein (small particles) was present. ATP was added to give the concentrations shown.

Table 3. *Ability of NAD^+ and NAD^+ analogues to act as donors for energy-linked $NADP^+$ reduction*

Experimental conditions were as described for Fig. 1. Various analogues (obtained from Pabst Biochemicals, Milwaukee, Wis., U.S.A.) were substituted for NAD as shown and 2.5mg of small-particle protein was present in all assays.

Compound	NADPH formed (nmol/min per mg of protein)	
	Non-energy-linked	Energy-linked
NAD^+	4.1	12.42
Nicotinamide-hypoxanthine dinucleotide	1.95	0
[3-acetylpyridine]-NAD^+	1.72	1.18
[3-pyridinealdehyde]-NAD^+	2.25	0
3-acetylpyridine–hypoxanthine dinucleotide	2.4	0
3-pyridinealdehyde–hypoxanthine dinucleotide	1.9	0

Table 2. *Effect of various nucleotides on $NADP^+$ reduction by NADH*

Experimental conditions were as described for Fig. 1; 2.0mg of small-particle protein was present in all assays. The table shows the rate of NADPH formation observed after the addition of 3.0μmol of the various nucleotides indicated. Corrections have been made for the non-energy-linked reaction. Similar relative rates with ATP, ITP and GTP were obtained with measurements of nucleotide triphosphatase activity.

Nucleotide added	NADPH formed (nmol/min per mg of protein)
ATP	9.68
ADP	2.0
AMP	0
ITP	3.67
GTP	1.66
CTP	0
UTP	0

Table 4. *Relationship between ATP added and NADPH formed*

Conditions were as described for Fig. 1; 2.7mg of small-particle protein was present. ATP was added at the concentrations shown and the reaction was allowed to go to completion, when the total amount of NADPH formed was determined from the increase in E_{340}. Extrapolation to low ATP concentrations gives a ATP/NADPH ratio of 1.55:1.

ATP (μM)	NADH formed (μM)	ATP/NADPH ratio
600	51.4	11.67
300	33.5	8.95
150	28.7	5.23
60	18.8	3.19

of driving the reaction were ITP and to a lesser extent GTP (Table 2). The pattern was similar to that observed for the energy-linked reduction of NAD^+ by succinate (Sweetman & Griffiths, 1971): i.e. ability to drive the reaction was related to the corresponding nucleotide triphosphatase activity, indicating that this reaction is involved in the energy-linked reaction mechanism.

Specificity of the electron donor. The specificity for NAD^+ analogues in the non-energy-linked transhydrogenase was relatively broad (Table 3) as all the analogues tested were capable of supporting at least 50% of the control rate. However, the

energy-linked reaction was specific for NAD^+; the only analogue tested that produced any measureable response was [3-acetylpyridine]-NAD^+.

Relationship between NADPH formed and ATP added. Direct phosphorylation of NADH by ATP to give NADPH is a possible pathway for the reaction. This can be excluded since the amount of NADPH formed exceeded the total amount of NADH in the system.

Another possibility is that ATP was acting as a catalyst in the reaction. Although control experiments showed that the particulate adenosine triphosphatase was not stimulated in the presence of the ATP-dependent transhydrogenase it was possible to show a relationship between the ATP split and the NADPH formed in the presence of low concentrations of ATP (Table 4). These results
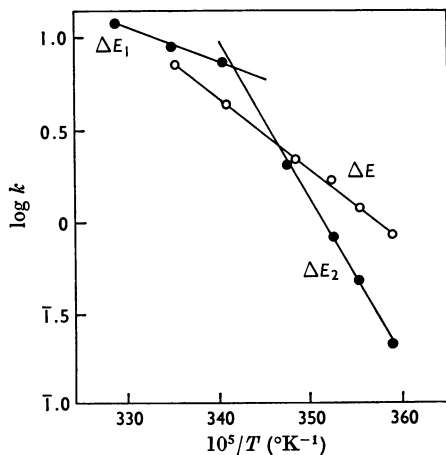
Fig. 4. Arrhenius plots showing the effect of temperature on the bacterial ATP-dependent and ATP-independent reduction of $NADP^+$ by NADH. Reactions were measured as described for Fig. 1, except that 2.5mg of bacterial protein (small particles) was added. $\bigcirc$, ATP-independent reaction: $\Delta E = 16.5\,cal/mol$; $\bullet$, ATP-dependent reaction: $\Delta E_1 = 8.4\,cal/mol$, $\Delta E_2 = 38.5\,cal/mol$, transition temperature $= 19°C$.

Table 5. *Effect of inhibitors and uncouplers on $NADP^+$ reduction*

Experimental conditions were as described for Fig. 1; 2.5mg of small particle protein was present in each assay. Water-insoluble inhibitors were added as their ethanolic solutions and suitable controls were carried out with equivalent amounts of ethanol alone.

Compound	Concentration (μM)	% Inhibition
Dicoumarol	33	70
	66	97
Pentabromophenol	25	70
	50	95
Tetrachlorotrifluoro-benzimidazole	1	42
	2	73
2,4-Dinitrophenol	90	50
	180	66
Piericidin A	100	28
	200	83
2-n-Heptyl-4-hydroxy-quinoline N-oxide	125	10
	250	92
Thyroxine	50	56
	100	77
Oligomycin	12.5μg	0

suggested that it is unlikely that ATP is catalytic in function and that there is a stoicheiometric utilization of ATP. The ATP/NADPH ratio decreased with decreasing ATP concentration to a minimum experimentally observed value of 3.19:1. By extrapolation to low ATP concentration an ATP/NADPH ratio of 1.55:1 was obtained. Therefore it is concluded that 1–2ATP molecules were consumed/NADPH molecule formed (cf. energy-linked reduction of NAD^+ by succinate; Ernster & Lee, 1964; Sweetman & Griffiths, 1971).

Effect of temperature. Sweetman & Griffiths (1971) showed that the energy-linked reduction of NAD^+ by succinate and the adenosine triphosphatase of the bacterial particles were characterized by Arrhenius plots that had a sharp discontinuity. It was concluded that the discontinuity was due to a conformational change in the adenosine triphosphatase, and that this enzyme was taking part in both reactions. The effect of temperature on the energy-linked and non-energy-linked transhydrogenases was measured (Fig. 4). The results for the energy-linked reaction showed a sharp break in the Arrhenius plots at 19°C, with activation energies of 8.4 and 38.5cal/mol above and below that temperature respectively. These values are in good agreement with Arrhenius plots for the adenosine triphosphatase and ATP-dependent reduction of NAD^+ by succinate (Sweetman & Griffiths, 1971), indicating that the enzyme is involved in all three reactions.

The non-energy-linked reaction had an activation energy of 16.5cal/mol and did not exhibit a break in the Arrhenius plot, which indicates that the reaction was not dependent on the particulate adenosine triphosphatase.

Effect of inhibitors of electron transport and uncouplers of oxidative phosphorylation. A variety of compounds were shown to inhibit the energy-dependent reduction of $NADP^+$ by NADH (Table 5). The mitochondrial uncouplers 2,4-dinitrophenol and tetrachlorotrifluorobenzimidazole inhibited the reaction by 50% at concentrations of 90 and 1μM respectively, and appear to act as true uncoupling agents. The concentrations for dicoumarol, pentabromophenol and thyroxine inhibition are lower than the corresponding concentrations for the energy-linked reduction of NAD^+ by succinate (Sweetman & Griffiths, 1971).

Also, piericidin A (50% at 150μM) and 2-n-heptyl-4-hydroxyquinoline N-oxide (50% at 190μM) both inhibited the energy-linked transhydrogenase, which is in contrast with their stimulatory effect on the energy-linked reduction of NAD^+ by succinate (Sweetman & Griffiths, 1971).

The differential effects of inhibitors and uncoupling agents on the energy-linked transhydrogenase reaction and oxidative phosphorylation have been pointed out by Bragg & Hou (1968), who showed that dicoumarol, pentachlorophenol and 2-n-heptyl-4-hydroxyquinoline N-oxide also inhibited NADH–cytochrome b_1 reductase and the non-

energy-linked transhydrogenase reaction. It is difficult to evaluate the role of these compounds as energy-transfer inhibitors in view of their additional inhibitory effects on electron-transport pathways in *E. coli* and further information on the site of action of these inhibitors is required before any comments can be made on the pathway of electron transfer in energy-linked reactions. Oligomycin, a mitochondrial energy-transfer inhibitor, had no effect on the reaction at the concentrations tested. The effects of uncouplers on the reaction characterize it as one involving high-energy intermediates of oxidative phosphorylation. 2,4-Dinitrophenol and tetrachlorotrifluorobenzimidazole did not affect the non-energy-linked reduction of $NADP^+$ by NADH, confirming that this reaction does not involve the participation of high-energy intermediates.
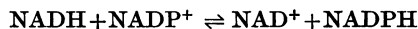
DISCUSSION

Many of the anabolic reactions in a cell require NADPH as a source of reducing equivalents and this requirement could possibly be satisfied by $NADP^+$-linked oxidations, but as most of the cellular dehydrogenases are NAD^+-linked other mechanisms may be utilized for NADPH production. In green plants and algae $NADP^+$ is reduced by a light-dependent process (San Pietro & Black, 1965), providing a source of NADPH for synthetic reactions. In non-photosynthetic tissues NADPH can be generated by an energy-linked transhydrogenase reaction, which is dependent on oxidative phosphorylation as the source of energy (Ernster & Lee, 1964).

The results presented here show that *E. coli* small particles are capable of catalysing two kinds of transhydrogenase reaction. The reactions observed were the reduction of $NADP^+$ by NADH and the ATP-dependent reduction of $NADP^+$ by NADH. Both reactions resulted in the generation of reducing power in the form of NADPH.

The energy requirement for the energy-dependent reaction could be supplied by added ATP and to a lesser extent ITP and GTP. There was a relationship between the amount of ATP hydrolysed and the amount of NADPH formed. The minimum value determined for the ATP/NADPH ratio was 3.19:1, but extrapolation to negligible ATP concentration gave a value of 1.55:1. It is concluded that for the reaction in *E. coli* one molecule of ATP is hydrolysed/molecule of NADPH formed. However, in view of the fact that no direct evidence for ATP/NADPH ratios below 2.0:1 has been obtained this conclusion should be treated with caution. Nevertheless, good evidence has been obtained for a relationship between ATP concentration and NADPH production that is in agreement with results obtained for the energy-linked reduction of NAD^+ by succinate

(Sweetman & Griffiths, 1971) and the mammalian energy-linked transhydrogenase reaction (Ernster & Lee, 1964). Equations for the two reactions may be written as follows:

(i) Non-energy-linked transhydrogenase

$$NADH + NADP^+ \rightleftharpoons NAD^+ + NADPH$$

(ii) Energy-linked transhydrogenase

$$NADH + NADP^+ + ATP \rightleftharpoons$$
$$NAD^+ + NADPH + ADP + P_i$$
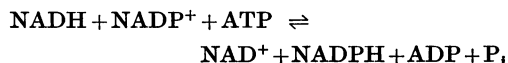
It has been proposed that the two reactions in mammalian submitochondrial particles involve the same hydrogen-transfer enzyme (Griffiths & Roberton, 1966) because both reactions show similar sensitivities towards inhibitors such as tri-iodothyronine (Hommes & Estabrook, 1964) and to specific antibodies prepared against the purified non-energy-linked transhydrogenase (Kawasaki, Satoh & Kaplan, 1964). Both reactions involve a direct hydrogen transfer from the A-locus of NADH to the B-locus of $NADP^+$, providing circumstantial evidence in support of the concept that both reactions use the same enzyme. Evidence against this concept is that the two reactions have different sensitivities to Mg^{2+}, different equilibria, and energy is required in one case and not in the other (Ernster & Lee, 1964).

In the bacterial preparation described here there are several differences between the two reactions that suggest they are catalysed by different enzymes or different forms of the same enzyme. ATP shifts the equilibrium towards NADPH formation with the result that the ATP-dependent reaction is 4–5 times faster than the non-energy-linked transhydrogenase. The activation energy of the non-energy-linked transhydrogenase is twice that of the ATP-dependent reaction between 20° and 30°C. At 19°C the energy-dependent reaction shows a sharp break in the Arrhenius plot and below this temperature the activation energy is increased to twice that of the non-energy-linked reaction, which has no break in the Arrhenius plot. The temperature effects on the energy-linked transhydrogenase are identical with those of the particulate adenosine triphosphatase and the energy-linked reduction of NAD^+ by succinate (Sweetman & Griffiths, 1971). It is concluded that these three reactions have some reaction pathway in common, probably the adenosine triphosphatase or ATP synthetase complex. This pathway is not involved in the non-energy-dependent transhydrogenase.

Further differences are seen with the effect of Mg^{2+} on the two transhydrogenase reactions. The non-energy-dependent reaction is inhibited by Mg^{2+} in both directions, whereas the energy-linked reaction is completely dependent on Mg^{2+} for

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activity. The selectivity towards Mg^{2+} argues against the identity of the two enzymes as does the specificity towards nicotinamide nucleotides. Six analogues of NADH were tested for their ability to act as electron donors in the two reactions. All the analogues were able to form the corresponding NADPH analogue with the non-energy-linked enzyme at about 50% of the control rate. The energy-linked transhydrogenase, on the other hand, was active only towards [3-acetylpyridine]-NADH and then at only 10% of the control rate, indicating that this enzyme was specific for NADH.

The action of uncouplers of oxidative phosphorylation characterizes the energy-linked reaction as one involving the participation of high-energy compounds derived from ATP hydrolysis. These reagents were without effect on the non-energy-linked reaction. The evidence is in favour of the hypothesis that in *E. coli* there is more than one hydrogen-transfer enzyme responsible for the formation of NADPH from NADH. It is suggested that there are two enzymes, one of which requires ATP and the other not. However, there is no evidence to favour this hypothesis over the alternative hypothesis that we are dealing with different conformational states, induced by ATP, of the same enzyme. Further information on the electron-transfer pathway in *E. coli* is required.

The energy-transfer pathway for the ATP-dependent reaction appears to be identical with that of the ATP-dependent reduction of NAD^+ by succinate. However, the hydrogen-transfer pathway of the two reactions is quite different. The reduction of NAD^+ involves the NADH and succinate dehydrogenase–flavoprotein systems, but there is no evidence to suggest that these enzymes also participate in the transhydrogenase. Further, the transhydrogenase is selectively inhibited by piericidin and 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide, compounds that stimulate the energy-linked reduction of NAD^+ by succinate. If the electron-transport chain is not involved in the transhydrogenase then there are obviously other sites of action of piericidin in the bacterial particles. These results are in agreement with studies on submitochondrial particles (A. J. Sweetman & D. E. Griffiths, unpublished work), which showed that piericidin inhibited the mitochondrial transhydrogenase. It seems likely that piericidin could be a useful tool in the study of transhydrogenase reactions.

The energy-linked transhydrogenase in *E. coli* was first described by Murthy & Brodie (1964) although they could not find the reaction in *Mycobacterium phlei*, and a report describing the effect of inhibitors and uncouplers on oxidative phosphorylation and the energy-dependent transhydrogenase in *E. coli* was published by Bragg & Hou (1968). These investigators concluded that non-phosphorylated high-energy intermediates were involved and Asano, Imai & Sato (1967) concluded that the transhydrogenase in *Micrococcus denitrificans* was similar to that found in mammalian preparations. An energy-linked transhydrogenase has also been reported in a photosynthetic bacterium *Rhodospirillum rubrum* (Keister & Yike, 1967).

The present work shows that the energy-linked transhydrogenase in *E. coli* exhibits the same general properties as found in other bacterial systems and also in a mammalian mitochondria.

REFERENCES

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

Aleem, M. I. H. (1966b). *J. Bact.* **91,** 729.

Asano, A., Imai, I. & Sato, R. (1967). *Biochim. biophys. Acta,* **143,** 477.

Bragg, P. D. & Hou, C. (1968). *Can. J. Biochem.* **46,** 631.

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

Ernster, L. & Lee, C. P. (1964). *A. Rev. Biochem.* **33,** 729.

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

Hommes, F. A. & Estabrook, R. W. (1964). *Biochem. biophys. Res. Commun.* **11,** 1.

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

Keister, D. L. & Yike, N. J. (1967). *Biochemistry, Easton,* **6,** 3847.

Lee, C. P. & Ernster, L. (1964). *Biochim. biophys. Acta,* **81,** 187.

Murthy, P. S. & Brodie, A. F. (1964). *J. biol. Chem.* **239,** 4292.

San Pietro, A. & Black, A. A. (1965). *A. Rev. Pl. Physiol.* **16,** 155.

Sweetman, A. J. & Griffiths, D. E. (1971). *Biochem. J.* **121,** 117.