Studies on Energy-Linked Reactions

ENERGY-LINKED REDUCTION OF OXIDIZED NICOTINAMIDE-ADENINE DINUCLEOTIDE BY SUCCINATE IN *ESCHERICHIA COLI*

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1. A method for the preparation of small particles from *Escherichia coli* is described. 2. These particles catalyse the ATP-driven reduction of NAD⁺ by succinate. 3. ATP utilized/NADH produced ratios below 2.0 were measured and a stoicheiometry of 1 molecule of ATP utilized per molecule of NADH produced is proposed. 4. The reaction is not inhibited by 2,4-dinitrophenol or by oligomycin but is inhibited by other uncouplers such as pentabromophenol and dicoumarol. 5. The specificity of the energy source, the specificity of the electron acceptor, the effects of pH, Mg²⁺, P_i and temperature have been studied.

Information on bioenergetic systems in *Escherichia coli* is needed if genetic techniques that have been so successful in other areas are to be applied to the study of membrane-bound energy-conservation systems. Oxidative phosphorylation in *E. coli* extracts has been described by a number of workers (Kashket & Brodie, 1963a; Bragg & Hou, 1968) but little is known of the energy-linked reactions such as the energy-linked reductions of NAD⁺ by succinate and the energy-linked transhydrogenase reaction although these reactions have been demonstrated in other bacteria (Asano, Imai & Sato, 1967; Kiesow, 1967; Keister & Yike, 1967). Kashket & Brodie (1963a) found that NAD⁺ was reduced at a low rate (8 nmol/min per mg of protein) when incubated with *E. coli* small particles and succinate under anaerobic conditions and that the rate was increased fourfold on addition of ATP. No further studies on the energy-linked nature of the reaction were reported.

The present work extends the studies of Kashket & Brodie (1963a,b) to a demonstration of the energy requirement for the reduction of NAD⁺ by succinate, together with the specificity of the energy source, the specificity of the electron acceptor, the effects of pH, Mg²⁺ and P_i, the effects of temperature and of electron-transfer inhibitors and uncouplers of oxidative phosphorylation and also the stoicheiometry of the reaction. A method for the preparation of *E. coli* small particles is also described.

MATERIALS AND METHODS

Chemicals. Oligomycin and 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Tetrachlorotri-

fluorobenzimidazole was a gift from Dr R. B. Beechey, Shell Research Ltd., Sittingbourne, Kent, U.K. Piericidin A was a gift from Dr N. Takahashi, University of Tokyo. Pyridine nucleotide analogues were obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A. The source of other chemicals and enzymes used was as described elsewhere (Griffiths & Robertson, 1966).

Growth of bacteria. Wild-type *E. coli* K12 was grown on a minimal medium, with succinate as the sole energy source, containing (per litre): 18.9 g of Na₂HPO₄, 6.39 g of KH₂PO₄, 0.2 g of MgSO₄·7H₂O, 2.0 g of (NH₄)₂SO₄ and 5 g of sodium succinate. The pH of the medium was adjusted to 7.6 with 10% (w/v) KOH. The bacteria were then grown aerobically in 5-litre flasks containing 2 litres of medium, with vigorous shaking on a rotary shaker at 37°C. The amount of growth was assessed with an EEL photoelectric colorimeter, a neutral-density filter being used.

Preparation of bacterial particles. Cells were harvested in the exponential phase of growth and collected by centrifugation at 4000g; 4 litres of bacterial suspension was normally processed at any one time. The cells were then washed with cold water and centrifuged at 4000g at 0°C and the packed cells were suspended in water at a concentration of 0.5 g wet wt./ml and the pH was adjusted to 7.4 with 40 mM-tris-HCl buffer, pH 8.2. Samples (11 ml) of this suspension were sonicated at 2°C for 3.5 min by using the maximum output from the large probe of the MSE 60W cell disintegrator. To counteract the heat generated by the sonicator the probe was precooled in ice and the bacterial suspension was kept on ice during the ultrasonic treatment. After removal of cell debris and large bacterial particles by centrifuging at 30000g for 15 min the small-particle fraction was sedimented at 140000g for 3 h. The pellet was finally suspended in 0.15 M-KCl containing 10 mM-MgCl₂ and the pH was adjusted to 7.4 with 40 mM-tris-HCl buffer, pH 8.2. Particles were normally used immediately after preparation but storage overnight did not affect the activity adversely. The protein concentration of the suspension was adjusted to fall in the range 30–50 mg/ml.

Determination of protein content. After solubilization of the pellet with sodium deoxycholate (0.33%, w/v), protein

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was determined by the method of Gornall, Bardawill & David (1949) with bovine serum albumin as standard.

Determination of NAD^+ and $NADH$. Both compounds were determined by using the alcohol dehydrogenase assay (Colowick, Kaplan & Ciotti, 1951) with ethanol as substrate for NAD^+ and acetaldehyde as substrate for $NADH$ determination.

Energy-linked reduction of NAD^+ by succinate. A Beckmann DK 2A recording spectrophotometer was used to determine the increase in extinction at 340nm. Blank and experimental cuvettes of 1cm light-path contained 375 μ mol of sucrose, 75 μ mol of tris-HCl buffer, pH 8.0, 10 μ mol of $MgCl_2$, 3 μ mol of NAD^+ , 50 μ mol of Na_2S , 20 μ mol of succinate and bacterial particles (2–5mg of bacterial protein). The extinction was recorded until a steady trace was obtained, when the reaction was started by the addition of 6 μ mol of ATP to the experimental cuvette to give a final volume of 2ml. The reaction was measured at 30°C and water-insoluble inhibitors were added as their methanolic solutions to both experimental and reference cuvettes and suitable controls with equivalent amounts of methanol alone were carried out. When ethanolic solutions of inhibitors were used an ATP-independent increase of extinction at 340nm was observed which was suspected to be due to the action of a particle-bound alcohol dehydrogenase.

Determination of nucleotide triphosphatases. Nucleotide triphosphatase activity was measured by determining the P_i released (Fiske & Subbarow, 1925) from the nucleotide triphosphate in 0.1ml samples removed from the reaction medium at appropriate time-intervals. The reaction medium contained 650 μ mol of nucleotide triphosphate (ATP, GTP, ITP) and bacterial particles (6mg of bacterial protein) in a final volume of 3ml at 30°C.

RESULTS

Demonstration of the ATP-dependent reduction of NAD^+ by succinate. A simple procedure for isolating a subcellular fraction from *E. coli* that contained the enzyme system catalysing the ATP-dependent reduction of NAD^+ by succinate was developed (see the Materials and Methods section). Fig. 1 shows the relative rates of the reaction in the presence and absence of ATP. The bacterial particles were incubated with succinate and NAD^+ in the presence of sulphide. Addition of ATP to the incubation medium caused an increase in the rate of reduction of NAD^+ , after a short lag time (20–30s). The reaction product was shown to be $NADH$ both by its specific extinction at 340nm and by the rapid decrease in extinction brought about by the addition of acetaldehyde plus alcohol dehydrogenase.

The reaction was dependent on the presence of Mg^{2+} and succinate. Fumarate, added in the same concentrations as succinate, did not result in any reduction of NAD^+ , in the presence or absence of ATP (Table 1). The activity sometimes observed in the absence of Mg^{2+} was probably due to the

Fig. 1. Time-course of NAD^+ reduction by succinate. The incubation medium contained 375 μ mol of sucrose, 75 μ mol of tris-HCl buffer, pH 8.0, 10 μ mol of $MgCl_2$, 3 μ mol of NAD^+ , 5 μ mol of Na_2S , 20 μ mol of sodium succinate and 3.5mg of bacterial protein (small particles). The energy-dependent reaction was started by the addition of 6 μ mol of ATP to the experimental cuvette (final volume 2.0ml). The temperature of the incubation was 30°C. At point A on the graph 10 μ mol of acetaldehyde and 20 μ g of yeast alcohol dehydrogenase were added to the incubation medium.

Table 1. Requirements for energy-linked NAD^+ reduction by succinate

Experimental conditions were as described for Fig. 1. Additions and omissions were made as indicated: 3 μ mol of ADP and 10 μ mol of fumarate were added as shown and 2.2mg of small-particle protein was present in all assays.

Conditions	$NADH$ formed (nmol/min per mg of protein)
Complete	10.41
– Mg^{2+}	1.86
–Succinate	0
+Fumarate	0
–ATP + fumarate	0
– NAD^+	0
–ATP	0.73
–ATP + ADP	0.5

small amount of Mg^{2+} in the suspension medium, corresponding to a final concentration of 0.25–0.5mM. When the particles were thoroughly washed and suspended in a medium deficient in Mg^{2+} no reaction was observed and an absolute requirement for Mg^{2+} was demonstrated (Fig. 2), with maximum activity at a concentration of 4mM- Mg^{2+} .

Specificity for ATP. As replacement of ATP with ADP resulted in loss of the reaction it seemed un-

Fig. 2. Effect of Mg^{2+} on the energy-linked reduction of NAD^+ by succinate. Conditions were as described for Fig. 1, except that 2.5 mg of bacterial protein (small particles) was added. Mg^{2+} was added to give the concentrations shown.

Fig. 3. Effect of ATP on the energy-linked reduction of NAD^+ by succinate. Conditions were as described for Fig. 1 except that 2.0 mg of bacterial protein (small particles) was added. ATP was added to give the concentrations shown.

likely that regeneration of ATP via the adenylate kinase reaction was playing any part in this particular system. The reaction rate was found to be dependent on the amount of ATP added, indicating that ATP was acting as a substrate in the reaction. The K_m for ATP was $470 \mu M$ and maximum activity was observed in the region of 2–3 mM-ATP (Fig. 3).

Other nucleotides were tested for their ability to drive the energy-linked reduction of NAD^+ by

Table 2. Effect of various nucleotides on the reduction of NAD^+ by succinate

Experimental conditions were as described for Fig. 1. Nucleotides were added to the incubation medium at the concentrations shown and 1.8 mg of small-particle protein was present in all assays.

Nucleotide added	Concentration (mM)	NADH formed (nmol/min per mg of protein)
ATP	1.5	4.67
ATP	3.0	5.42
ADP	3.0	0.30
AMP	3.0	0.34
ITP	3.0	1.02
GTP	3.0	0.41
CTP	1.5	0.17
UTP	3.0	0.28

Fig. 4. Energy-linked reduction of NAD^+ by succinate and nucleotide triphosphatase activity. The energy-linked reduction of NAD^+ by succinate was measured as described in Fig. 1 except that $3 \mu mol$ of ATP, ITP or GTP was added as indicated. The amount of protein added was 2 mg (small particles). Phosphatase activity was determined by measuring the P_i released from $6 \mu mol$ of ATP, ITP or GTP in a medium containing $650 \mu mol$ of sucrose, $130 \mu mol$ of tris-HCl buffer, pH 8.0, and $15 \mu mol$ of $MgCl_2$ in a final volume of 3.0 ml. $\blacktriangle$, Energy-linked reduction of NAD^+ by succinate: (a) ATP-dependent reaction; (b) ITP-dependent reaction; (c) GTP-dependent reaction. $\bullet$, Nucleotide triphosphatase activity: (d) adenosine triphosphatase; (e) inosine triphosphatase; (f) guanosine triphosphatase.

succinate. Table 2 shows that only ITP produced any significant reduction of NAD^+ . It was found that the ability of the various nucleotides to drive the reaction was related to the presence of the corresponding nucleotide triphosphatases in the preparation. Adenosine triphosphatase was the

Table 3. *Reduction of NAD⁺ and NAD⁺ analogues by succinate*

Experimental conditions were as described for Fig. 1. Various analogues were substituted for NAD⁺ in the reaction as shown. The analogues were obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A. NADH formed is given as nmol/min/mg of protein, measured as the rate after the lag period, and 2.5mg of small-particle protein was present in all the assays.

Compound	Lag time (min)	NADH formed (nmol/min per mg of protein)
NAD ⁺	3	11.35
Nicotinamide-hypoxanthine dinucleotide	4	10.02
[3-Acetylpyridine]-NAD ⁺	8	8.00
[3-Pyridinealdehyde]-NAD ⁺	13	7.35
3-Acetylpyridine-hypoxanthine dinucleotide	8	5.34
3-Pyridinealdehyde-hypoxanthine dinucleotide	20	2.20

most active triphosphatase and ATP was the most efficient at driving the reaction. GTP, on the other hand, was split only to a small extent by the preparation and the resulting stimulation of NADH formation was minimal. An inosine triphosphatase, operating at about 25–35% of the rate of the adenosine triphosphatase, resulted in energy-linked NADH formation at 20% of the ATP-stimulated rate. These results are summarized in Fig. 4. ADP added to the incubation medium, at concentrations equal to the amount of ATP present, caused about 50% inhibition of the reaction.

Specificity of the electron acceptor. Several NAD⁺ analogues were tested for their ability to act as electron acceptors in the system. The specificity for NAD⁺ was relatively low (Table 3), although the lag period of the reaction was generally lengthened by the analogues. Substitution at the 6-position of the adenine ring with a hydroxyl group to give nicotinamide-hypoxanthine dinucleotide did not result in any significant change in activity. Alterations of the 3-position of the pyridine ring to give [3-acetylpyridine]-NAD⁺ and [3-pyridinealdehyde]-NAD⁺ caused a 30–40% decrease in the rate of the reaction and a two- to three-fold increase in the lag time. Interference with both the 6-position of the adenine ring and the 3-position of the pyridine ring to give 3-acetylpyridine-hypoxanthine dinucleotide and 3-pyridinealdehyde-hypoxanthine dinucleotide resulted in further decreases in activity, and for the latter compound a considerable increase in the lag time. The K_m for NAD⁺ in the reaction was found to be very high (4.3mM).

Relationship between NADH formed and ATP added. Adenosine triphosphatase activity, measured by using relatively high concentrations of ATP (3mM), was five times greater than the rate of NAD⁺ reduction by succinate. No significant

Table 4. *Stoichiometry of the energy-linked reaction*

Reaction conditions were as described for Fig. 1 and 2.5mg of bacterial protein (small particles) was present. ATP was added at the concentrations shown and the reaction was allowed to go to completion, when the total amount of NADH formed was determined from the increase in E_{340} .

ATP (μ M)	NADH formed (μ M)	ATP/NADH ratio
1500	122.7	12.23
300	61.8	4.85
150	45.1	3.33
60	30.8	1.94

difference between adenosine triphosphatase activity in the presence and absence of NAD⁺ reduction could be detected. In fact, the adenosine triphosphatase activity decreased after 3–5min, but NADH formation proceeded at a linear rate for 15–20min. It was not possible, under these conditions, to determine any relationship between ATP hydrolysed and NADH formed. To overcome these difficulties lower concentrations of ATP were used in the hope that all the ATP that was hydrolysed might be used to drive the energy-linked reduction of NAD⁺ by succinate. The reactions were allowed to go to completion and the amount of NADH formed was calculated. The ATP/NADH ratio was decreased by decreasing ATP concentration (Table 4). When equilibrium was established, 0.062 μ mol of NADH was formed with 0.12 μ mol of ATP, giving an ATP/NADH ratio of 1.94:1. This was the lowest practicable value obtainable since further decreases in ATP concentration gave reaction rates that were difficult to measure. However, extrapolation of the results obtained to negligible ATP concentration gave a value for the ratio of 1.3

and indicated that 1–2 ATP molecules were consumed per molecule of NADH formed.

Temperature dependence of the energy-linked reduction of NAD⁺ by succinate. Haslam (1967) showed that energy-dependent reactions in submitochondrial particles have characteristic Arrhenius plots, which have a sharp break in them.

Fig. 5. Arrhenius plots showing the effect of temperature on the bacterial adenosine triphosphatase and energy-linked reduction of NAD⁺ by succinate. (a) Adenosine triphosphatase measured as described for Fig. 4 with 8.7mg of bacterial protein and 12 μ mol of ATP. (b) Energy-linked reduction of NAD⁺ by succinate, measured as described for Fig. 1 with 2.5mg of bacterial protein (small particles). Adenosine triphosphatase: $\Delta E_1 = 9.7$ kcal/mol, $\Delta E_2 = 33.3$ kcal/mol, transition temperature = 18.8°C. Reduction of NAD⁺: $\Delta E_1 = 9.3$ kcal/mol, $\Delta E_2 = 31.8$ kcal/mol, transition temperature = 19.5°C.

Reactions not dependent on oxidative phosphorylation, such as the non-energy-linked transhydrogenase activity, did not exhibit these sharp breaks. These studies have been extended here to include the energy-linked reduction of NAD⁺ by succinate in bacterial particles. The results obtained (Fig. 5) were in agreement with those of the mammalian system. There was a sharp break in the line at 19.5°C; for temperatures above this the activation energy was calculated to be 9.3 kcal/mol, and below 19.5°C a higher value (31.8 kcal/mol) was obtained. It was postulated that at the transition temperature there was a conformational change in one of the enzymes involved in the reaction sequence. Haslam (1967) suggested that it was the adenosine triphosphatase of the submitochondrial particles that exhibited this temperature effect. The effect of temperature on the adenosine triphosphatase of the bacterial particles was determined (Fig. 5) and a transition temperature of 18.8°C was calculated, with activation energies of 9.7 kcal/mol and 33.3 kcal/mol above and below this temperature respectively. The values for the adenosine triphosphatase reaction are in good agreement with those for the energy-dependent reduction of NAD⁺ by succinate, indicating that the adenosine triphosphatase reaction is involved in the reduction of NAD⁺ by succinate.

Effect of inhibitors of electron transport and uncouplers of oxidative phosphorylation. (a) Electron-transport inhibitors. A variety of electron-transport inhibitors were found to inhibit the energy-linked reduction of NAD⁺ by succinate (Table 5).

Table 5. *Effect of inhibitors and uncouplers on NAD⁺ reduction*

Experimental conditions were as described for Fig. 1 and 2.5mg of small-particle protein was present in all assays. Inhibitors that were insoluble in water were added as their methanolic solutions and suitable controls were carried out with equivalent amounts of methanol alone.

Compound	Concentration (μM)	% Inhibition
Malonate	2500	49
	6250	66
Thenoyltrifluoroacetone	100	60
	150	93
2- <i>n</i> -Heptyl-4-hydroxyquinoline <i>N</i> -oxide	50	+45
	80	17
Piericidin A	100	+35
	250	17
Rotenone	50	0
Thyroxine	100	23
Dicoumarol	50	31
	67	47
Pentabromophenol	84	47
	168	90
2,4-Dinitrophenol	180	0
Tetrachlorotrifluorobenzimidazole	5	50
Oligomycin	12.5 μg	0

Malonate, an inhibitor of the succinate dehydrogenase complex, inhibited NADH formation in a competitive manner, 50% inhibition occurring at about 3 mM. This characterizes the reaction as one involving the succinate dehydrogenase flavoprotein system of *E. coli* particles. A similar result was obtained with thenoyltrifluoroacetone, which is believed to interact with the non-haem-iron component of the succinate dehydrogenase. At low concentrations both 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide and piericidin stimulated the reduction of NAD⁺ by about 50%. These compounds inhibit NADH and succinate oxidation in *E. coli* at a point where their electron-transfer pathways converge, i.e. beyond the primary dehydrogenase level. Stimulation of the reaction could be due to a change in steady-state redox levels of respiratory-chain components or could be due to incomplete inhibition of the oxidation of NADH by sulphide, completed by the presence of piericidin or 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide. At high concentrations the latter compound caused inhibition of NADH formation because of its interaction with the NADH dehydrogenase-flavoprotein system of the particles. Rotenone, a specific inhibitor of the NADH dehydrogenase-flavoprotein system in mammalian mitochondria, was without effect on the bacterial reaction. This indicates that a rotenone- and piericidin-sensitive site is not present in the NADH dehydrogenase system of *E. coli*.

(b) Uncouplers of oxidative phosphorylation. The involvement of high-energy intermediates of oxidative phosphorylation in the reaction is supported by the action of tetrachlorotrifluorobenzimidazole, dicoumarol and pentabromophenol (Table 5). These compounds inhibited the reduction of NAD⁺ by about 50% at concentrations of 5, 70 and 80 μ M respectively. Oligomycin and 2,4-dinitrophenol had no effect on the reaction. This was in agreement with the results of Kashket & Brodie (1936b) who found no effect with these compounds on oxidative phosphorylation by *E. coli* small particles. The ineffectiveness of uncoupling agents has been noted by other workers (Bragg & Hou, 1968), who found that energy-linked reactions seem sensitive to uncoupling agents such as 2,4-dinitrophenol whereas oxidative phosphorylation is unaffected.

DISCUSSION

Oxidative phosphorylation in *E. coli* extracts has been described by a number of workers (Kashket & Brodie, 1963a,b; Bragg & Hou, 1968). These studies showed that the mechanism of coupling of phosphorylation to oxidation, as well as the sequence of respiratory carriers, was markedly similar to that found in mitochondrial systems. The bacteria lack a definite organelle comparable with

the mitochondrion in which energy production is isolated from the many other reactions of the cell and for this reason particulate fractions, presumably membrane fragments, prepared from bacteria frequently require the addition of a soluble component for coupled activity. It was of particular interest when Kashket & Brodie (1963b) described the isolation of a small-particle fraction from *E. coli* that was capable of catalysing phosphorylation of ADP coupled to the oxidation of NAD-linked substrates and succinate.

It has been known for some time that particles prepared from mitochondria are capable of catalysing oxidative phosphorylation. Later it was shown that these particles could drive certain energy-dependent reactions such as the energy-dependent reversal of electron transport and the energy-linked transhydrogenase (Ernster & Lee, 1964). These reactions were shown to be closely integrated with the oxidative phosphorylation machinery of the particles and have proved to be important tools in the study of the mechanism of oxidative phosphorylation. In particular they have led to the concept that the mitochondrion is able to utilize high-energy intermediates of oxidative phosphorylation as a source of energy for driving endergonic reactions. Knowledge of the occurrence and mechanism of these reactions in bacteria was very limited when the present studies were initiated.

The present results show that a small-particle fraction prepared from *E. coli* catalyses an energy-dependent reversal of electron transport and oxidative phosphorylation. The energy for the reaction was supplied by ATP. The bacterial particles were incubated under non-respiring conditions in the presence of sulphide with succinate as the electron donor and NAD⁺ as the electron acceptor. The reaction observed was the ATP-dependent reduction of NAD⁺ by succinate.

The effects of electron-transport inhibitors support the conclusion that the succinate dehydrogenase-flavoprotein system and the NADH dehydrogenase-flavoprotein system are involved. The electron-transfer pathway between the two flavoprotein-linked dehydrogenases is not elucidated by the present studies, but the involvement of non-haem iron is inferred by the action of the chelating agent thenoyltrifluoroacetone. However, the possible involvement of ubiquinone, vitamin K and cytochrome *b* are not established. The electron-transfer pathway in *E. coli* is being further investigated.

The action of uncouplers indicates that high-energy intermediates of oxidative phosphorylation provide the necessary energy supply. In this connexion the inhibitory effect of ADP + P_i shows that the 'phosphate potential', i.e. the ATP/(ADP + P_i) ratio, is an important factor. These reagents may be acting by competing for a non-phosphorylated

high-energy intermediate required for NAD⁺ reduction. This conclusion is supported by the finding that P_i, added alone, has no effect on the reaction rate. Unfortunately the mitochondrial energy-transfer inhibitor oligomycin, which has been used to show that a non-phosphorylated high-energy intermediate is utilized for driving the energy-dependent reversal of electron transport in submitochondrial particles, has no effect on oxidative phosphorylation in *E. coli*.

The relative resistance of oxidative phosphorylation and energy-linked systems to uncoupling agents is a puzzling feature of these reactions in *E. coli* and may represent differences in membrane structure from mammalian systems.

The similar effects of temperature on both the ATP-driven reduction of NAD⁺ by succinate and the particulate adenosine triphosphatase provide further evidence for the participation of oxidative phosphorylation in the reaction. Both reactions give an Arrhenius plot with a sharp break at 19°C, possibly due to a conformation change in the adenosine triphosphatase which is probably involved in both reactions. Further, addition of fumarate to the reaction medium in place of succinate did not cause reduction of NAD⁺ under any of the experimental conditions used, showing that NAD⁺ reduction was not occurring via citric acid-cycle oxidations, such as malate dehydrogenase, as originally suggested by Krebs (1961).

It appeared that the energy-dependent reaction was associated with the phosphorylation process, since treatment of the particles with reagents and conditions that destroyed the capacity to couple phosphorylation to oxidation also resulted in loss of ability to carry out the ATP-dependent reduction of NAD⁺ by succinate.

The reaction was completely dependent on the presence of Mg²⁺. This is in agreement with previous results obtained with ox heart submitochondrial particles (Low & Vallin, 1963). The particles possessed nucleotide triphosphatase activity towards ATP and ITP and to a lesser extent GTP. No reaction was found with CTP and UTP. These activities correlated with the ability of the various nucleotide triphosphates to supply energy for NAD⁺ reduction. CTP and UTP gave no significant reduction of NAD⁺ but the other nucleotides were active in the order ATP > ITP > GTP. Similar results were obtained with mitochondria (Danielson & Ernster, 1963).

The energy requirement of the reaction was measured by comparing the amount of P_i released from ATP with the amount of NADH formed. The ATP/NADH ratio varied from 12 to 1.9, the values decreasing with the concentration of ATP used. By extrapolation to negligible ATP concentration a value of 1.3 was obtained. This indicates that

1–2 molecules of ATP are being consumed per molecule of NADH formed. It seems unlikely that all the ATP hydrolysed would be utilized for NAD⁺ reduction; if this is the case then the results obtained give an overestimate of the ATP/NADH ratio, so the ratio is more likely to be 1 than 2. Re-examination of the ATP/NADH ratio under conditions where NADH production is stimulated, as in the presence of piericidin and 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide, may lower the ATP/NADH ratio appreciably and ratios approaching unity may be observed experimentally. Thus the following equation could be used to describe the reaction in *E. coli* particles:

Ratios of 1 have also been calculated for mammalian reactions (see review by Ernster & Lee, 1964).

The ATP-dependent reduction of NAD⁺ by succinate has been reported in other bacterial preparations. These bacteria may be divided into two main types (Stanier, Doudoroff & Adelberg, 1966). (a) Chemotrophic: these organisms utilize either inorganic electron donors (lithotrophic) or organic compounds as electron donors (organotrophic). The reaction has been demonstrated in both types of organism. Lithotrophic bacteria such as *Thiobacillus novellus* and *Nitrosomonas europaea* (Aleem, 1966a,b) support the ATP-dependent reduction of NAD⁺ by succinate, and so do organotrophic bacteria such as *E. coli* (Kashket & Brodie, 1963, this paper) and *Micrococcus denitrificans* (Asano *et al.* 1967). (b) Phototrophic: chromatophores prepared from *Rhodospirillum rubrum* catalysed an ATP- or light-dependent reduction of NAD⁺ by succinate (Keister & Yike, 1967; Jackson & Crofts, 1968).

The significance of energy-linked reductions as indispensable elements of the assimilation of energy by chemoautotrophic organisms has been discussed by Kiesow (1967). It is, however, somewhat difficult to see the significance of these reactions in *E. coli* and also in mammalian mitochondria. The degree of energy-linked activities in *E. coli* appears to vary with the growth conditions and the carbon source and further studies of these variations may provide a clue to the significance of energy-linked reactions in the economy of the aerobic cell.

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