Biochem. J. (1968) **107**, 659
Printed in Great Britain

The Permeability of Mitochondria to Oxaloacetate and Malate

By J. M. HASLAM* and H. A. KREBS†
Department of Biochemistry, University of Oxford, and School of Molecular Sciences, University of Warwick, Coventry

(*Received* 22 *November* 1967)

1. A spectrophotometric assay of the rates of penetration of oxaloacetate and L-malate into mitochondria is described. The assay is based on the measurement of the oxidation of intramitochondrial NADH by oxaloacetate and of the reduction of intramitochondrial NAD^+ by malate. 2. The rate of entry of both oxaloacetate and L-malate into mitochondria is restricted, as shown by the fact that disruption of the mitochondrial structure can increase the rate of interaction between the dicarboxylic acids and intramitochondrial NAD^+ and NADH by between 100- and 1000-fold. 3. The rates of entry of oxaloacetate and malate into liver, kidney and heart mitochondria increased by up to 50-fold on addition of a source of energy, either ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine aerobically, or ATP anaerobically. 4. In the absence of a source of energy the changes in the concentrations of intramitochondrial NAD^+ and NADH brought about by the addition of L-malate or oxaloacetate were followed by parallel changes in the concentrations of $NADP^+$ and NADPH, indicating the presence in the mitochondria of an energy-independent transhydrogenase system. 5. The results are discussed in relation to the hypothesis that malate acts as a carrier of reducing equivalents between mitochondria and cytoplasm.

Several investigators (Bücher & Klingenberg, 1958; Borst, 1963; Sacktor & Dick, 1964; Lardy, Paetkau & Walter, 1965; Krebs, Gascoyne & Notton, 1967; Krebs, 1967) have come to the conclusion that malate and oxaloacetate may act as carriers of hydrogen equivalents between mitochondria and cytoplasm. NADH in one compartment is taken to react with oxaloacetate; the malate formed traverses the mitochondrial membranes and reacts in the other compartment with NAD^+ to generate NADH and oxaloacetate. When carbohydrate or lactate undergo oxidation NADH arises in the cytoplasm and must be transferred to the mitochondria. When carbohydrate is synthesized from pyruvate, serine or alanine, NADH must be transferred in the opposite direction. This postulated function of malate and oxaloacetate requires that the two dicarboxylic acids can readily pass through the mitochondrial membrane. No direct measurements of the rates of passage of oxaloacetate and malate through the mitochondrial membrane have so far been reported. The present work is an attempt to supply information on the permeability of mitochondria for the dicarboxylic acids.

* Present address: Department of Biochemistry, Monash University, Clayton, Vic., Australia.

† Present address: Metabolic Research Laboratory, Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford.

Following a suggestion made by Dr J. B. Chappell, the rates of entry of oxaloacetate and L-malate into isolated washed mitochondria of rat liver, kidney and heart were measured spectrophotometrically by following the effect of oxaloacetate or malate on the concentration of intramitochondrial NADH. As the activity of the mitochondrial malate dehydrogenase under the test conditions was very high in relation to the rates of penetration of oxaloacetate or L-malate, the initial rate of oxidation of NADH was taken to be a measure of the rate of penetration of oxaloacetate, and the initial rate of reduction of NAD^+ as a measure of the rate of penetration of the L-malate.

MATERIALS AND METHODS

Chemicals and enzymes. Oxaloacetic acid, NAD^+, NADH, lactate dehydrogenase, yeast alcohol dehydrogenase and malate dehydrogenase were obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany), tris base and L-malic acid were from Sigma Chemical Co. (St Louis, Mo., U.S.A.), rotenone was from Aldrich Chemical Co. (Milwaukee, Wis., U.S.A.), TMPD‡ was from Eastman Kodak Co. (Rochester, N.Y., U.S.A.), tricine was from General Biochemicals Inc. (Chagrin Falls, Ohio, U.S.A.) and

‡ Abbreviations: TMPD, *NNN'N'* - tetramethyl - *p* - phenylenediamine dihydrochloride; tricine, tris(hydroxymethyl)methylglycine.

antimycin A and oligomycin A were from Mann Research Laboratories (New York, N.Y., U.S.A.). ATP and ADP were added as neutral sodium salts, and ascorbate, oxaloacetate and malate as tris salts.

Measurement of the kinetics of reduction of intramitochondrial NAD+. Two methods of measuring changes in the state of reduction of NAD^+ were used. The main method was the spectrophotometric procedure of Chance (1951, 1957) with the Aminco–Chance dual-wavelength spectrophotometer (see also Klingenberg & Slenczka, 1959). Changes in the state of NAD^+ reduction were detected by measuring $E_{340}-E_{374}$, assuming ϵ_{mM} to be 4·4 cm.$^{-1}$ (Chance & Hollunger, 1961).

This method is not accurate if the mitochondria undergo extensive swelling or shrinkage during the experiment, because these affect the extinction especially at lower wavelengths (see Lehninger, 1962). Swelling can be detected in the dual-wavelength spectrophotometer by measuring E_{340} and E_{374} independently. For the purpose of the calculations the ϵ_{mM} values of NADH were taken to be 6·2 cm.$^{-1}$ at 340 mμ and 1·8 cm.$^{-1}$ at 374 mμ. Thus if the mitochondria did not undergo volume changes during the oxidation of NADH the $\Delta E_{340}/\Delta E_{374}$ ratio would be 6·2/1·8 = 3·4. If swelling or shrinkage occurred during the experiment the ratio would change, and in both cases would decrease. Tests by this criterion showed that liver and heart mitochondria did not swell or shrink in tris–HCl- or tricine–KOH-buffered media under the conditions investigated. Kidney mitochondria swelled in tris–HCl-buffered medium but not in tricine–KOH-buffered medium in the presence of a source of energy, and hence the latter medium was employed.

A second method for measuring the state of oxidation of the dinucleotides was required as a check because the spectrophotometric method fails to distinguish between NADH and NADPH. NAD^+, $NADP^+$, NADH and NADPH were determined separately by the enzymic methods described by Klingenberg (1963).

Chemical determinations. Oxaloacetate was assayed by the method of Hohorst & Reim (1963), and L-malate by the method of Hohorst (1963). Mitochondrial protein was measured by the method of Gornall, Bardawill & David (1949).

Preparation of mitochondria. Twice-washed mitochondria were prepared from the organs of 250g. female rats by a modification of the method of Hogeboom (1955). After removal of connective tissue, liver, heart and kidney cortex were homogenized in 0·25 M-sucrose–10 mM-tris–HCl–0·1 mM-tris–EDTA medium, pH 7·6. The homogenates were centrifuged twice for 7 min. at 500g and the pellets discarded. Mitochondria were then separated by centrifugation for 15 min. at 5400g, washed by resuspension in 0·25 M-sucrose–10 mM-tris–HCl medium, pH 7·6, and recentrifuged. Mitochondria were finally suspended in 0·25 M-sucrose–10 mM-tris–HCl medium, pH 7·6. The protein concentration of the suspension was 15–30 mg./ml. These mitochondria gave P/O ratios 2·5–2·8 with pyruvate plus L-malate as substrate, and showed good respiratory control, as indicated by a large increase in the O_2 uptake on addition of ADP. There was no significant leakage of NAD^+, as tested by addition of ethanol plus yeast alcohol dehydrogenase, or of NADH, as tested by the addition of pyruvate plus lactate dehydrogenase.

Disrupted mitochondria required for the assay of mitochondrial malate dehydrogenase were prepared either by incubation with 0·1% Triton X-100 at 0° for 30 min., or by subjecting mitochondria to ultrasonic oscillation for 2 min. with the MSE Ultrasonic power unit.

Measurement of oxygen uptake. The respiration of mitochondria was measured polarographically in the Clark-electrode cell as modified by Chappell (1964).

Assay of malate dehydrogenase. The malate dehydrogenase activity of disrupted mitochondria was determined spectrophotometrically by measuring E_{334} with the Eppendorf photometer or by measuring $E_{340}-E_{374}$ with the Aminco–Chance dual-wavelength spectrophotometer. The basal medium was 0·25 M-sucrose–20 mM-tris–HCl, pH 7·6. The formation of malate was measured with 0·1 mM-NADH, 0·5–1·0 μg. of mitochondrial protein and various amounts of oxaloacetate. The reverse reaction was measured with 0·26 mM-NAD^+, 2–10 μg. of mitochondrial protein and various amounts of L-malate.

Conditions of incubation for the study of the oxidation of intramitochondrial NADH by oxaloacetate. The measurement of the oxidation of NADH by oxaloacetate requires a relatively high initial concentration of NADH in the mitochondria. This can be achieved by the addition of 0·5–2·0 mM-succinate, which is known to cause an extensive reduction of NAD^+ (Chance & Hollunger, 1957). When this reduction was maximal, after a few minutes, rotenone and antimycin A, which block the reoxidation of NADH and its regeneration by succinate, were added, and the addition of oxaloacetate now produced a rapid oxidation of NADH (plus NADPH). However, high concentrations of succinate or a long period of succinate oxidation decreased the rate and extent of the subsequent oxidation of NADH (plus NADPH) by oxaloacetate. Two factors were responsible for these complications: fumarate and malate arising from succinate caused a regeneration of NADH and, secondly, succinate is a competitive inhibitor of the penetration of oxaloacetate in the mitochondria (D. E. Griffiths & J. M. Haslam, unpublished work).

This approach was therefore abandoned in favour of the following procedure. Freshly prepared mitochondria were preincubated with rotenone plus antimycin A for 2–3 min. to inhibit NADH oxidase, and NADH was generated from endogeneous substrate. Addition of 2–20 μM-oxaloacetate caused the rapid oxidation of up to 4 mμmoles of NADH/mg. of protein and the initial rates of oxidation of intramitochondrial NADH in such experiments did not vary very much from preparation to preparation.

Conditions of incubation for the study of the reduction of intramitochondrial NAD+ by L-malate. The measurement of the reduction of NAD^+ by L-malate requires a relatively high NAD^+/NADH ratio in the mitochondria. It is not sufficiently high in freshly prepared mitochondria, so that addition of malate has little effect on the concentration of NADH. The ratio can be raised by preincubation of mitochondria at 0° for 8–18 hr. in 0·25 M-sucrose. The reduction of intramitochondrial NAD^+ by L-malate could not be measured without this prolonged preincubation of the mitochondria at 0°, and, though not ideal, this material yields reproducible values.

RESULTS

Oxidation of intramitochondrial NADH and NADPH by oxaloacetate. A comparison of the rates

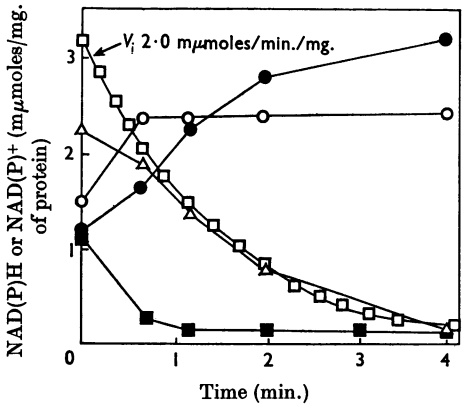

Fig. 1. Spectrophotometric and enzymic assays of the oxidation of intramitochondrial NADH and NADPH by 20 μM-oxaloacetate in rat liver mitochondria. Rat liver mitochondria (2·5 mg. of protein) were incubated at 30° in the presence of rotenone and antimycin A and the oxidation of the sum of intramitochondrial NADH plus NADPH by oxaloacetate was measured spectrophotometrically under the conditions stated in Table 1. A series of parallel incubations were stopped at different times by the addition of 0·6 ml. of 3 M-$HClO_4$ for the determination of NAD^+ and $NADP^+$ or of 1·5 ml. of N-KOH in 50% (v/v) ethanol for the determination of NADH and NADPH as described by Klingenberg (1963). V_i is the initial rate of oxidation of intramitochondrial NADH. Spectrophotometric assay: □, NADH+NADPH. Enzymic assays: ■, NADH; △, NADPH; ○, NAD^+; ●, $NADP^+$.

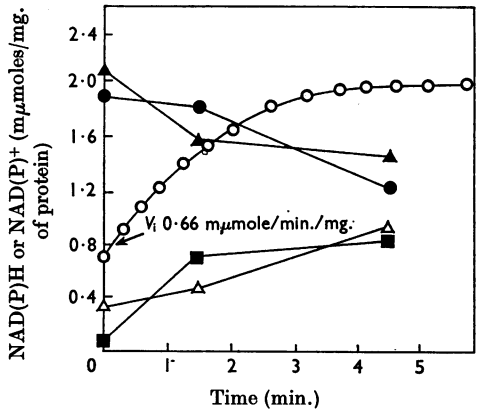

Fig. 2. Spectrophotometric and enzymic assays of the reduction of intramitochondrial NAD^+ and $NADP^+$ by L-malate in rat liver mitochondria. Incubation conditions and the spectrophotometric and enzymic assays were as described in Fig. 1. The reaction was initiated by addition of 1·67 mM-L-malate. V_i is the initial rate of reduction of intramitochondrial NAD^+ by L-malate. Spectrophotometric assay: ○, NADH+NADPH. Enzymic assays: ■, NADH; △, NADPH; ▲, NAD^+; ●, $NADP^+$.

of oxidation of intramitochondrial NADH plus NADPH, as measured spectrophotometrically, with the rate of oxidation of NADH and NADPH, as measured separately by the enzymic methods, is shown in Fig. 1. The curve obtained on addition of oxaloacetate, representing the oxidation of NADH plus NADPH, closely corresponds to the sum of the two curves for NADH and NADPH obtained separately. In the initial period the velocities of the oxidation of NADH plus NADPH and of NADH alone are almost identical. The oxidation of NADPH lagged behind by less than 1 min. and during the later period of incubation (after 1 min.) the rate of NADPH oxidation was almost identical with that of the sum of NADH plus NADPH. As expected, the oxidation of the nucleotides on addition of excess of oxaloacetate was virtually complete and the sum of NAD^+, $NADP^+$, NADH and NADPH remained constant. As malate dehydrogenase does not react with NADP and as there was no source of energy under the test conditions, the oxidation of NADPH must have been due to transhydrogenation catalysed by one of the non-energy-requiring transhydrogenase systems such as the glutamate dehydrogenases, isocitrate dehydrogenases or NAD(P) transhydrogenase (EC 1.6.1.1). The activity of these transhydrogenase systems is obviously sufficient to establish equilibrium between the NAD and NADP systems.

Reduction of intramitochondrial NAD^+ and $NADP^+$ by L-malate. An analogous investigation on the reduction of intramitochondrial NAD^+ and $NADP^+$ by malate is shown in Fig. 2. The rate of reduction of NAD^+ plus $NADP^+$, as measured spectrophotometrically, again tallied with the sum of the rates of reduction of NAD^+ and $NADP^+$, as measured by enzymic assays. Again NAD^+ reacted initially more rapidly but $NADP^+$ soon followed. Owing to the position of the equilibrium in the malate dehydrogenase system the reaction did not go to completion but ceased when about 40% of the intramitochondrial NAD^+ plus $NADP^+$ had been reduced.

Factors limiting the rate of intramitochondrial oxidation of NADPH by oxaloacetate and of reduction of NAD^+ by L-malate. The rates of reaction in the presence of various inhibitors and substrates are shown in Table 1. Antimycin A was added to the system to block the respiratory chain between cytochrome *b* and cytochrome *c*. Rotenone alone inhibited the oxygen uptake due to endogenous substrates by 85–95% and the further addition of antimycin A abolished this residual respiration. This inhibition of respiration by antimycin A was accompanied by inhibitions of both the oxidation of

Table 1. *Effects of ATP and TMPD plus ascorbate on the rates of oxidation of NADH by oxaloacetate and the rates of reduction of NAD+ by L-malate in intact mitochondria*

The rates of reaction were measured spectrophotometrically as described in the Materials and Methods section. Mitochondria were preincubated with rotenone (0·5 μM), antimycin A (1·2 μM) and ATP (2·8 mM) as indicated before ascorbate (2·8 mM) and TMPD (0·13 mM) were added. The preincubation period was 5 min. when ATP was present and 2–3 min. otherwise. The reaction was initiated by adding either 10 μM-oxaloacetate or 1·7 mM-L-malate.

	Rate of oxidation of NADH by oxaloacetate (mμmoles/min./mg. of protein)			Rate of reduction of NAD+ by L-malate (mμmoles/min./mg. of protein)		
Source of mitochondria	Liver	Kidney	Heart	Liver	Kidney	Heart
Additions during preincubation						
Rotenone	1·54	1·05	2·4	1·48	3·6	1·3
Rotenone, antimycin A	1·08	0·22	1·7	0·71	2·2	0·7
Rotenone, antimycin A, TMPD + ascorbate	3·40	10·2	26·7	2·41	14·3	4·9
Rotenone, antimycin A, ATP	2·92	2·72	3·3	1·82	6·0	2·3

Table 2. *Rates of oxidation of NADH by oxaloacetate in intact and disrupted mitochondria*

The oxidation of NADH was detected spectrophotometrically by measuring $E_{340}-E_{374}$ as described in the Materials and Methods section and in Table 1. Ascorbate (2·8 mM) plus TMPD (0·13 mM) were added to intact mitochondria (see the text), and 0·1 mM-NADH was added to disrupted (Triton X-100-treated) mitochondria.

		Intact mitochondria			Triton X-100-treated mitochondria		
Source of mitochondria	Temp.	Apparent K_m (μM)	Apparent $V_{max.}$ (mμmoles/min./mg. of protein)	Oxidation of NADH by 10 μM-oxaloacetate (mμmoles/min./mg. of protein)	Apparent K_m (μM)	Apparent $V_{max.}$ (mμmoles/min./mg. of protein)	Oxidation of NADH by 10 μM-oxaloacetate (mμmoles/min./mg. of protein)
Kidney	30°	⩾20	—	12	3·5	2200	1550
Heart	30	⩾20	—	27	6	6900	5050
Liver	5	40	5·1	1·2	2	1700	1500
	13	40	7·6	1·5	3	2600	2300
	22	40	15	2·7	3	3400	2900
	30	⩾20	—	4·0	3	5000	4600

NADH by oxaloacetate and the reduction of NAD+ by L-malate, which suggests that the residual oxygen uptake remaining in the presence of rotenone (which is not due to the oxidation of NADH) may provide energy facilitating the penetration of the dicarboxylic acids into the mitochondria. This assumption is borne out by the effects of the addition of ascorbate (2·8 mM) plus TMPD (0·14 mM), a substrate combination that, by by-passing the antimycin A block, allowed a rapid oxygen consumption (Packer & Jacobs, 1962). With the liver mitochondria, the oxygen uptake was 100 mμg.atoms/min./mg. of protein at 30°. On addition of ascorbate plus TMPD the rate of oxidation of NADH by oxaloacetate and of reduction of NAD+ by L-malate was greatly increased. The largest absolute effect was in the heart mitochondria, where the increase of the rate of oxidation of NADH was 16-fold. The rate of reduction of NAD+ by malate was increased 6·5-fold. Addition of ATP also increased the reaction rates but much less effectively than the addition of ascorbate plus TMPD.

Effect of disruption of mitochondria. To establish whether the entry of oxaloacetate or L-malate or the activity of malate dehydrogenase was the rate-limiting factor in the oxidation and reduction of the intramitochondrial nucleotides, the rates of reaction in intact and disrupted mitochondria were compared. With disrupted kidney mitochondria the rate of oxidation of NADH on addition of 10 μM-oxaloacetate was more than 100-fold greater than in intact mitochondria, even under conditions optimum for the intact mitochondria, i.e. in the presence of ascorbate plus TMPD (Table 2). With heart mitochondria the rate was 200-fold and with liver mitochondria 1000-fold greater after disruption.

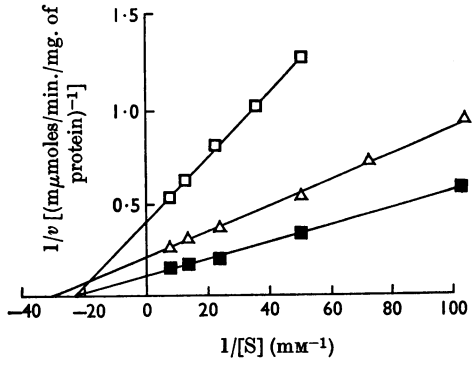

Fig. 3. Lineweaver–Burk plots of the rate of oxidation of the intramitochondrial NADH of liver mitochondria by oxaloacetate at 13° in the presence and absence of an energy supply. Rates of penetration were measured as described in Table 1. The temperature was 13°. □, Rotenone, antimycin A: apparent K_m, 40 μM; apparent $V_{max.}$ 2·5 mμmoles/min./mg. of protein. △, Rotenone, antimycin A, ATP: apparent K_m, 30 μM; apparent $V_{max.}$, 4·6 mμmoles/min./mg. of protein. ■, Rotenone, antimycin A, TMPD+ascorbate: apparent K_m, 40 μM; apparent $V_{max.}$, 7·6 mμmoles/min./mg. of protein.

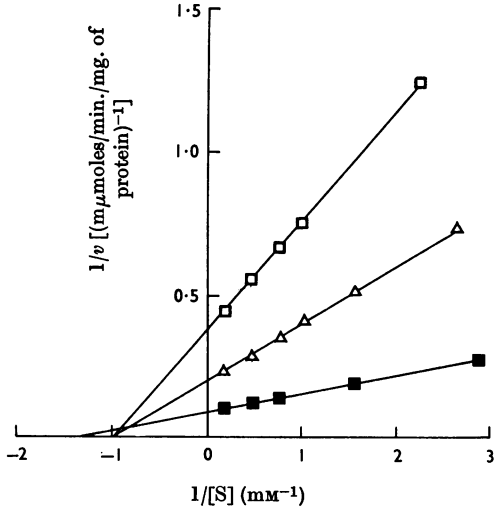

Fig. 4. Lineweaver–Burk plots of the rates of reduction of the intramitochondrial NAD^+ of kidney mitochondria by L-malate at 30° in the presence and absence of an energy supply. Rates of penetration were measured as described in Table 1. □, Rotenone, antimycin A: apparent K_m, 1·0 mM; apparent $V_{max.}$, 2·9 mμmoles/min./mg. of protein. △, Rotenone, antimycin A, ATP: apparent K_m, 1·0 mM; apparent $V_{max.}$, 5·2 mμmoles/min./mg. of protein. ■, rotenone, antimycin A, TMPD+ascorbate: apparent K_m, 0·8 mM; apparent $V_{max.}$, 14·3 mμmoles/min./mg. of protein.

The values for the apparent K_m and $V_{max.}$ given in Tables 2 and 3 were obtained from Lineweaver–Burk plots (see Figs. 3 and 4). The rate of oxidation of intramitochondrial NADH was proportional to the concentration of oxaloacetate at 30° throughout the concentration range that could be tested, so that no K_m values could be calculated, but at lower temperatures values for the apparent K_m and $V_{max.}$ were obtained for liver mitochondria (see Fig. 3). The apparent K_m for the oxidation of NADH by oxaloacetate was much higher in intact mitochondria and the apparent $V_{max.}$ more than 200-fold higher in disrupted mitochondria. These experiments leave no doubt that the rate-limiting step was the entry of oxaloacetate into the mitochondria and that the initial rates of oxidation are a measure of the rates of entry of oxaloacetate.

The rates of reduction of NAD^+ in the presence of 50 μM-malate were 100–200-fold greater in disrupted mitochondria than in the intact mitochondria (Table 3) under optimum conditions for the mitochondrial reaction, i.e. in the presence of TMPD and ascorbate. The apparent $V_{max.}$ was 100-fold greater in disrupted heart and liver mitochondria and 10-fold greater in disrupted kidney mitochondria. The apparent K_m values were similar for intact and disrupted heart and liver mitochondria, but were higher in intact than in disrupted kidney mitochondria. Thus the rate-limiting step in the reduction of intramitochondrial NAD^+ by malate was also the rate of entry of the substrate.

Respiration of mitochondria in the presence of L-*malate.* As a further check on the validity of the spectrophotometric method of measuring the rates of penetration of L-malate into mitochondria, the rate of respiration of mitochondria in state 3 (Chance & Williams, 1956), where the availability of substrate within the mitochondria limits the rate of respiration, was measured in the presence of L-malate. Kidney and liver mitochondria oxidized L-malate, but the rate was low at the start. It increased over a 30 min. period and the fixed steady rate was 5–6-fold greater than the initial rate (Fig. 5). Apparent K_m and $V_{max.}$ values for the initial and final rates of respiration were calculated from Lineweaver–Burk plots (Fig. 6), and Table 4 compares these with the apparent K_m and $V_{max.}$ values for the reduction of intramitochondrial NAD^+ by L-malate in mitochondria respiring in the presence of rotenone and antimycin A with ascorbate plus TMPD as substrate. In liver and kidney mitochondria apparent K_m values closely agreed for both reactions. The apparent $V_{max.}$ values for the initial rates of respiration also agreed with the apparent $V_{max.}$ for the reduction of intramitochondrial NAD^+, but the apparent $V_{max.}$ values for the final rates of respiration were 5–6-fold

Table 3. *Rates of reduction of NAD+ by* L-*malate in intact and disrupted mitochondria*

The reduction of NAD+ was detected spectrophotometrically by measuring $E_{340}-E_{374}$ as described in the Materials and Methods section and in Table 1. The conditions were as described in Table 1.

	Intact mitochondria			Triton X-100-treated mitochondria		
Source of mitochondria	Apparent K_m (mM)	Apparent $V_{max.}$ (mμmoles/min./mg. of protein)	Reduction of NAD+ by 50μM-L-malate (mμmoles/min./mg. of protein)	Apparent K_m (mM)	Apparent $V_{max.}$ (mμmoles/min./mg. of protein)	Reduction of NAD+ by 50μM-L-malate (mμmoles/min./mg. of protein)
Kidney	0·80	14·3	0·6	0·16	230	50
Heart	0·40	4·9	0·4	0·12	475	114
Liver	0·13	1·8	0·6	0·21	310	60

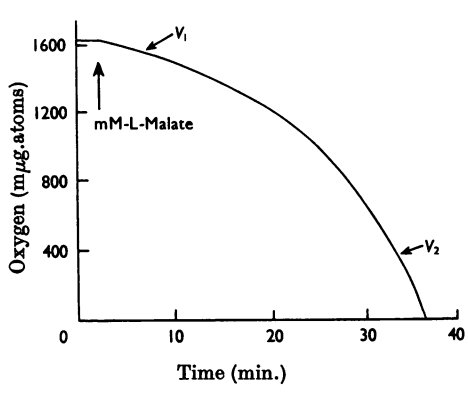

Fig. 5. Polarographic recording of respiration of kidney mitochondria in the presence of L-malate. Respiration was measured in the Clark-electrode cell described by Chappell (1964) in 3·60ml. containing 0·25M-sucrose, 20mM-tris–HCl buffer, pH7·6, 2·8mM-potassium phosphate buffer, pH7·6, 2·8mM-$MgCl_2$, 2mM-ADP and rat kidney mitochondria (3·5mg. of protein). V_1 (initial rate of respiration), 6·2mμg.atoms/min./mg. of protein; V_2 (final steady-state rate of respiration), 29mμg.atoms/min./mg. of protein.

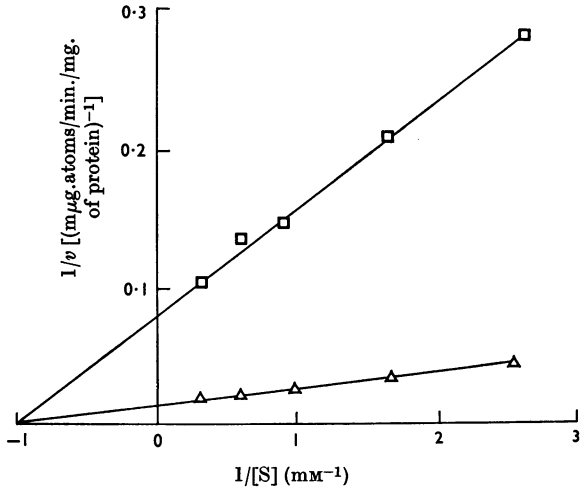

Fig. 6. Lineweaver–Burk plots of the experiment recorded in Fig. 5. □, Initial rate of respiration: apparent K_m, 1·1mM; apparent $V_{max.}$, 15mμg.atoms/min./mg. of protein. △, Final steady-state rates of respiration: apparent K_m, 1·1mM; apparent $V_{max.}$, 73mμg.atoms/min./mg. of protein.

higher. This is the value expected if malate undergoes complete oxidation, as one malate molecule corresponds to six pairs of hydrogen atoms.

Heart mitochondria consumed oxygen for a short time on addition of malate and then stopped respiring. Addition of cysteinesulphinate, which removes oxaloacetate by transamination (Singer & Kearney, 1956), promoted a continuing oxygen consumption by heart mitochondria, which indicates that oxaloacetate accumulated in the mitochondria and inhibited malate dehydrogenase in the absence of systems that generate acetyl-CoA. Cysteinesulphinate had no effect on the oxidation of malate in liver and kidney mitochondria.

DISCUSSION

Restricted permeability of mitochondria for oxaloacetate and malate. The present experiments confirm the view expressed by Lardy *et al.* (1965) that the permeability of mitochondria for oxaloacetate is restricted and that the rapid entry of oxaloacetate into the mitochondria requires special mechanisms. Borst (1963) and Lardy *et al.* (1965) suggested that oxaloacetate may undergo a transamination reaction and that the carbon skeleton of oxaloacetate traverses the mitochondria in the form of aspartate. This possibility is supported by experimental evidence, though not yet conclusively established. Transamination cannot have

Table 4. *Comparison of the kinetics of oxygen uptake of mitochondria in the presence of* L-*malate with the kinetics of the reduction of intramitochondrial NAD*$^+$ *by* L-*malate*

The rates of O_2 uptake of mitochondria at 30° were measured as described in Fig. 5, and apparent K_m and $V_{max.}$ values for the initial and final rates of the O_2 uptake were calculated from Lineweaver–Burk plots. The data on the rates of reduction of intramitochondrial NAD^+ by L-malate are taken from Table 3.

	O_2 uptake in the presence of L-malate			Reduction of NAD^+ by L-malate	
		Apparent $V_{max.}$ (mμmoles/min./mg. of protein)			
Source of mitochondria	Apparent K_m (mM)	Initial	Final	Apparent K_m (mM)	Apparent $V_{max.}$ (mμmoles/min./mg. of protein)
Liver	0·15	2	13	0·13	1·8
Kidney	1·1	15	73	0·80	14·3
Heart	—	—	—	0·40	4·9

played a major role in the present experiments because the extramitochondrial transaminase was removed during the preparation of the mitochondria. The experiments demonstrate for all three types of mitochondria investigated (liver, kidney and heart) that energy supplied either in the form of oxygen consumption, with ascorbate as substrate, or in the form of ATP, promoted the entry of both oxaloacetate and malate. It should be mentioned that Tager (1966) has already considered the possibility that the translocation of oxaloacetate within mitochondrial compartments might be energy-dependent.

Correlation of measurements on isolated mitochondria to metabolic processes in vivo. The observed rates of penetration of oxaloacetate and malate into mitochondria and the rates of reaction of the dicarboxylic acids in disrupted mitochondria must be correlated to the maximal rates postulated on the assumption that the oxaloacetate–malate couple is the only carrier of reducing equivalents between mitochondria and cytoplasm. When gluconeogenesis from lactate takes place (or from substrates of the same redox level as lactate) no transfer of reducing equivalents is required. When pyruvate is the gluconeogenic precursor the maximal rates of gluconeogenesis recorded for rat liver are 1·5 μmoles/min./g. at 37° (Ross, Hems, Freedland & Krebs, 1967). With serine and alanine the maximal rates are somewhat lower. For each molecule of glucose formed from pyruvate two pairs of hydrogen atoms have to be transferred from the mitochondria to the cytoplasm, but as oxaloacetate is continuously generated in the mitochondria, possibly at a rate equivalent to that of gluconeogenesis, no transfer of oxaloacetate into the mitochondria is to be postulated. This depends on whether pyruvate carboxylase is in rat liver predominantly located in the mitochondria, a view widely held (see Lardy *et al.* 1965) but contested by Henning, Stumpf, Ohly & Seubert (1966). If oxaloacetate is formed from pyruvate only in the mitochondria its rate of reduction should be at least 3 μmoles/min./g., and the postulated rate of passage of malate out of the mitochondria into the cytoplasm is the same.

The measurements in the present paper all refer to rates per mg. of mitochondrial protein. About 20% of the liver fresh weight is protein, and according to Price, Miller & Miller (1948) 30% of rat liver protein is mitochondrial protein. Thus 1 g. of fresh liver contains about 60 mg. of mitochondrial protein. It follows from the data in Table 2 that the rate of reduction of oxaloacetate (10 μM) at 30° was $4·6 \times 60 = 275$ μmoles/min./g. Thus it was even at 30° 92-fold greater than the maximal postulated rate for 37°. In view of the low apparent K_m (3 μM) the activity of the enzyme cannot be appreciably lower at physiological oxaloacetate concentrations. By contrast, the highest rate of intramitochondrial reduction of external oxaloacetate in intact mitochondria (in the presence of ascorbate plus TMPD) was only 4·0 mμmoles/min./mg. of protein or 0·24 μmole/min./g. at 30° (Table 2).

The fact that no major concentration gradients of malate between mitochondria and cytoplasm have ever been recorded (see Gamble, 1965) indicates that the passage of malate out of the mitochondria is of the same order of magnitude as its formation from oxaloacetate.

The rate of entry of malate into mitochondria is to be correlated to the rate of oxygen consumption when carbohydrate is oxidized, either to serve as a fuel of respiration or as a precursor in the synthesis of fatty acids. In both situations the breakdown of each molecule of glucose yields two molecules of pyruvate and of NADH in the cytoplasm and these must be transferred to the mitochondria. In liver the rate of the oxygen consumption is between 2 and 8 μmoles/min./g. depending on the presence of substrates (Hems, Ross, Berry & Krebs, 1966). When glucose is completely oxidized one molecule of NADH is to be transferred into the mitochondria for three molecules of oxygen consumed. Thus between 0·7 and 2·4 μmoles of NADH have to enter

per min./g. This is the rate at which malate, if it is the sole carrier of reducing equivalents, must enter the mitochondria. The maximal observed rate at 30° (Table 1) was $2{\cdot}4 \times 60 \times 10^{-3} = 0{\cdot}14$ μmole/min./g. or 20% of the lowest postulated rate. Considering the temperature difference and the uncertainty of the assumption that carbohydrate or lactate is the sole fuel of respiration, it can be argued that the observed rate of malate entry approaches the order of magnitude of the postulated one. Moreover the rate under the test conditions, i.e. in the presence of and the absence of possible accessory mechanisms, especially of the intramitochondrial transaminase, may have been sub-maximal, but it is nevertheless probable that in the liver another carrier, the α-glycerophosphate system (Bücher & Klingenberg, 1958), is the major agent in the transfer of reducing equivalents from the cytoplasm to the mitochondria.

In heart muscle the rate of penetration of malate into mitochondria must also be correlated to the rate of oxygen consumption. Maximal rates of oxygen consumption in the working rat heart are 13 μmoles/min./g. fresh weight (Neely, Liebermeister, Battersby & Morgan, 1967). Thus when glucose or lactate are the fuels of respiration malate must penetrate into the mitochondria at a rate of 4·5 μmoles/min./g. and oxaloacetate must be discharged by the mitochondria at the same rate. Assuming that in heart the mitochondrial protein constitutes 3% of the fresh weight of the whole tissue, the rate of entry of malate into the mitochondria should be maximally 140 μmoles/min./g. of protein. The observed rate (at 30°) was 4·9 μmoles/min./g. (Table 1) when measured by the reduction of NAD^+. This is hardly more than 3·5% of the postulated rate. It is possible that under the test conditions the rate was less than *in vivo*, as mentioned above with reference to the liver. The α-glycerophosphate shuttle cannot play a major role in heart muscle because the activity of the enzyme in this tissue is too low.

Nature of energy-aided transport of oxaloacetate. Under the test conditions, entry of oxaloacetate was in the direction of the concentration gradient and, though in terms of absolute concentration differences the gradient was relatively low, because of the low external oxaloacetate concentration (0·01 mM) the gradient was maintained by the rapid reduction of oxaloacetate after entry. This might lead to the idea that energy is needed for overcoming barriers other than concentration gradients, but this is not necessarily correct. To preserve electro-neutrality the movement of oxaloacetate must be accompanied by a parallel movement of cations, or by an opposite movement of anions. The energy-aided transport is likely to effect primarily one category of ions only, the electrochemical equivalent being moved passively. The question thus arises of which of the ions is primarily moved by the energy-aided mechanisms. Experiments by Chappell & Haarhoff (1967) suggest that there are different mechanisms for the various intermediates of the tricarboxylic acid cycle, which could make it possible that it is the transport of anions that is promoted actively. On the other hand, there is evidence indicating the presence in mitochondria of energy-linked cation transport tightly coupled to the synthesis and fission of ATP (see Cockrell, Harris & Pressman, 1966, 1967). Fission of ATP promotes the accumulation of K^+ and, as the system is reversible, the movement of K^+ in the opposite direction brings about ATP synthesis (see also Garrahan & Glynn, 1967), but whether K^+ or Ca^{2+} or H^+ is the primarily moving ion is an open question. The attractive aspect of the assumption that cations are the primary agents (see also Gamble, 1965; Harris, Höfer & Pressman, 1967*a*; Robinson & Chappell, 1967) is the close and direct relation that has been established between oxidative phosphorylation and cation movements.

If the transport of oxaloacetate is in the direction of the concentration gradient, this does not mean that the parallel movement of cations, or the opposite movement of anions, is also in the direction of the concentration gradient. For this reason it is understandable that the transport of oxaloacetate is facilitated by the supply of energy. Consideration of a Donnan system illustrates the point that, even where concentration gradients exist, energy is nevertheless needed to transport ions in the direction of the gradient.

Energy-dependence of the mitochondrial uptake of succinate. The rate of oxidation of succinate by heart mitochondria is inhibited by 2,4-dinitrophenol and addition of ATP counteracts this inhibition (Krebs, 1962). This may be taken to indicate that the uptake of succinate by mitochondria, like that of malate and oxaloacetate, is facilitated by the supply of energy (see also Harris *et al.* 1967*a*; Harris, Van Dam & Pressman, 1967*b*; Palmieri, Cisternino & Quagliariello, 1967).

Effectiveness of energy-independent intramitochondrial transhydrogenase. It was recently shown (Williamson, Lund & Krebs, 1967) that the glutamate dehydrogenase of liver mitochondria rapidly reacts with NAD, contrary to the views expressed by Klingenberg & Slenczka (1959), Klingenberg & Pette (1962) and Tager & Papa (1965), who came to the conclusion that glutamate dehydrogenase of intact mitochondria reacts with NADP only. Williamson *et al.* (1967) pointed out that if glutamate dehydrogenase in the intact liver reacts with both dinucleotides the NAD and NADP systems in the matrix of liver mitochondria would be in equilibrium.

Experimental proof for the presence in liver mito-

chondria of a transhydrogenase system (which is not dependent on the supply of energy) is provided by the demonstration that experimentally produced changes of the intramitochondrial concentrations of NAD^+ and NADH are followed by parallel changes in the concentrations of $NADP^+$ and NADPH (Figs. 1 and 2). Similar observations under different conditions have been reported by Klingenberg & Slenczka (1959).

The work described in this paper was supported by U.S. Public Health Service Research Grant no. GM11275 and Department of Scientific and Industrial Research Grant no. B/SR/917 to Dr D. E. Griffiths. The authors thank Dr D. E. Griffiths for his help in this work, and Mr J. R. P. O'Brien for the temporary use of an Aminco–Chance spectrophotometer. J.M.H. held a Medical Research Council Scholarship during this work.

REFERENCES

Borst, P. (1963). In *Funktionelle und morphologische Organisation der Zelle*, p. 137. Ed. by Karlson, P. Berlin: Springer-Verlag.

Bücher, Th. & Klingenberg, M. (1958). *Angew. Chem.* **70**, 552.

Chance, B. (1951). *Rev. sci. Instrum.* **22**, 634.

Chance, B. (1957). In *Methods in Enzymology*, vol. 4, p. 273. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Chance, B. & Hollunger, G. (1957). *Fed. Proc.* **16**, 163.

Chance, B. & Hollunger, G. (1961). *J. biol. Chem.* **236**, 1534.

Chance, B. & Williams, G. R. (1956). *Advanc. Enzymol.* **17**, 65.

Chappell, J. B. (1964). *Biochem. J.* **90**, 225.

Chappell, J. B. & Haarhoff, K. (1967). In *Biochemistry of Mitochondria*, p. 75. Ed. by Slater, E. C., Kaniuga, Z. & Wojtczak, L. London: Academic Press (Inc.) Ltd.

Cockrell, R. S., Harris, E. J. & Pressman, B. C. (1966). *Biochemistry*, **5**, 2326.

Cockrell, R. S., Harris, E. J. & Pressman, B. C. (1967). *Nature, Lond.*, **215**, 1487.

Gamble, J. L., jun. (1965). *J. biol. Chem.* **240**, 2668.

Garrahan, P. J. & Glynn, I. M. (1967). *Nature, Lond.*, **211**, 1414.

Gornall, A. G., Bardawill, G. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.

Harris, E. J., Höfer, M. P. & Pressman, B. C. (1967*a*). *Biochemistry*, **7**, 1348.

Harris, E. J., Van Dam, K. & Pressman, B. C. (1967*b*). *Nature, Lond.*, **213**, 1126.

Hems, R., Ross, B. D., Berry, M. N. & Krebs, H. A. (1966). *Biochem. J.* **101**, 284.

Henning, H. V., Stumpf, B., Ohly, B. & Seubert, W. (1966). *Biochem. Z.* **344**, 274.

Hogeboom, G. H. (1955). In *Methods in Enzymology*, vol. 1, p. 16. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Hohorst, H.-J. (1963). In *Methods of Enzymatic Analysis*, p. 328. Ed. by Bergmeyer, H. U. Weinheim: Verlag Chemie; New York: Academic Press Inc.

Hohorst, H.-J. & Reim, M. (1963). In *Methods of Enzymatic Analysis*, p. 335. Ed. by Bergmeyer, H. U. Weinheim: Verlag Chemie; New York: Academic Press Inc.

Klingenberg, M. (1963). In *Methods of Enzymatic Analysis*, pp. 528, 531. Ed. by Bergmeyer, H. U. Weinheim: Verlag Chemie; New York: Academic Press Inc.

Klingenberg, M. & Pette, D. (1962). *Biochem. biophys. Res. Commun.* **7**, 430

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

Krebs, H. A. (1962). In *Horizons in Biochemistry*, p. 285. Ed. by Kasha, M. & Pullman, B. New York: Academic Press Inc.

Krebs, H. A. (1967). In *Biochemistry of Mitochondria*, p. 105. Ed. by Slater, E. C., Kaniuga, Z. & Wojtczak, L. London: Academic Press (Inc.) Ltd.

Krebs, H. A., Gascoyne, T. & Notton, B. M. (1967). *Biochem. J.* **102**, 275.

Lardy, H. A., Paetkau, V. & Walter, P. (1965). *Proc. nat. Acad. Sci., Wash.*, **53**, 1410.

Lehninger, A. L. (1962). *Physiol. Rev.* **42**, 467.

Neely, J. R., Liebermeister, H., Battersby, E. J. & Morgan, H. E. (1967). *J. biol. Chem.* **236**, 262.

Packer, L. & Jacobs, E. E. (1962). *Biochim. biophys. Acta*, **57**, 371.

Palmieri, F., Cisternino, M. & Quagliariello, E. (1967). *Biochim. biophys. Acta*, **143**, 625.

Price, J. M., Miller, E. C. & Miller, J. A. (1948). *J. biol. Chem.* **173**, 345.

Robinson, B. H. & Chappell, J. B. (1967). *Biochem. J.* **105**, 18P.

Ross, B. D., Hems, R., Freedland, R. A. & Krebs, H. A. (1967). *Biochem. J.* **105**, 869.

Sacktor, B. & Dick, A. R. (1964). *Science*, **145**, 606.

Singer, T. P. & Kearney, E. B. (1956). *Arch. Biochem. Biophys.* **61**, 397.

Tager, J. M. (1966). In *Biochim. biophys. Acta Library Vol. no. 7: Regulation of Metabolic Processes in Mitochondria*, p. 202. Ed. by Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. Amsterdam: Elsevier Publishing Co.

Tager, J. M. & Papa, S. (1965). *Biochim. biophys. Acta*, **99**, 570.

Williamson, D. H., Lund, P. & Krebs, H. A. (1967). *Biochem. J.* **103**, 514.