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The Proton-Translocating Nicotinamide–Adenine Dinucleotide (Phosphate) Transhydrogenase of Rat Liver Mitochondria

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1. The NAD(P) transhydrogenase activity of the soluble fraction of sonicated rat liver mitochondrial preparations was greater than the NAD-linked isocitrate dehydrogenase activity, and the NAD-linked and NADP-linked isocitrate dehydrogenase activities were not additive. The NAD-linked isocitrate dehydrogenase activity was destroyed by an endogenous autolytic system or by added nucleotide pyrophosphatase, and was restored by a catalytic amount of NADP. 2. We concluded that the isocitrate dehydrogenase of rat liver mitochondria was exclusively NADP-specific, and that the oxoglutarate/isocitrate couple could therefore be used unequivocally as redox reactant for NADP in experiments designed to operate only the NAD(P) transhydrogenase (or loop 0) segment of the respiratory chain in intact mitochondria. 3. During oxidation of isocitrate by acetoacetate in intact, anaerobic, mitochondria via the rhein-sensitive, but rotenone- and arsenite-insensitive, NAD(P) transhydrogenase, measurements of the rates of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone-sensitive and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone-insensitive pH change in the presence of various oxoglutarate/isocitrate concentration ratios gave an $\rightarrow H^+/2e^-$ quotient of 1.94 ± 0.12 for outward proton translocation by the NAD(P) transhydrogenase. 4. Measurements with a K^+-sensitive electrode confirmed that the electrogenicity of the NAD(P) transhydrogenase reaction corresponded to the translocation of one positive charge per acid equivalent. 5. Sluggish reversal of the NAD(P) transhydrogenase reaction resulted in a significant inward proton translocation. 6. The possibility that isocitrate might normally be oxidized via loop 0 at a redox potential of −450mV, or even more negative, is discussed, and implies that a P/O quotient of 4 for isocitrate oxidation might be expected.

Some years ago we reported (Mitchell & Moyle, 1965) that protons were translocated inwards through the NAD(P) transhydrogenase (NADPH–NAD^+ oxidoreductase, EC 1.6.1.1) system of sonically prepared particles from ox heart mitochondria during forward redox activity (i.e. oxidation of $NADPH+H^+$ by NAD^+), and that protons were translocated outwards during redox activity in the reverse or 'energy-consuming' direction. Very low, and presumably suboptimum, $\rightarrow H^+/2e^-$* quotients were observed in these preliminary studies. The activity of the NAD(P) transhydrogenase system was later shown to cause changes of distribution of phenyl dicarbaundecaborane anion (Skulachev, 1970; Dontsov *et al.*, 1972) and changes of fluorescence of anilinonaphthalenesulphonate (Dontsov *et al.*, 1972; Van de Stadt *et al.*, 1971) across the membrane of submitochondrial particles from ox heart, consistent with the generation of membrane potentials of appropriate sign by the translocation of protons inwards during forward redox activity, and outwards during reverse activity. But, since our preliminary work (Mitchell & Moyle, 1965) was published, no further direct observations on the translocation of acid equivalents across the mitochondrial cristae membrane by the NAD(P) transhydrogenase nor estimates of the value of the $\rightarrow H^+/2e^-$ quotient of the transhydrogenase reaction have been reported.

The fact that $\rightarrow H^+/O$ quotients close to 8 were observed during respiratory pulses in suspensions of *Micrococcus denitrificans* without added substrate (Scholes & Mitchell, 1970) suggested that, under certain conditions, the reductant for respiration could be $NADPH+H^+$, giving an $\rightarrow H^+/2e^-$ quotient of 2 for the NAD(P) transhydrogenase (loop 0), whereas the $\rightarrow H^+/2e^-$ quotient for the respiratory chain oxidizing $NADH+H^+$ (loops 1, 2 and 3) was 6 (see Mitchell, 1966). We recently observed (P. Mitchell,

* Abbreviations: $\rightarrow H^+/2e^-$, proton-translocation quotient giving protons translocated per formal bivalent reducing equivalent; $\rightarrow H^+/O$, proton-translocation quotient giving protons translocated per oxygen atom reduced; $\rightarrow H^+$ and $\rightarrow K^+$, quantity of protons and of K^+ ions translocated respectively; ΔH_o^+ and ΔK_o^+, quantity of protons and of K^+ ions added to (or entering) the outer aqueous phase respectively; pH_o and pK_o, −log (chemical activity of H^+ and K^+ ions respectively in the outer aqueous phase).

R. Mitchell & J. Moyle, unpublished work) that, when treated so as to cause NADPH+H^+ to act as the main respiratory reductant, rat liver mitochondria likewise gave $\rightarrow H^+/O$ quotients near 8, adding further indirect support to the suggestion that the $\rightarrow H^+/2e^-$ quotient for loop 0 may be 2.

These qualitative or relatively indirect observations on the proton-translocating property of the NAD(P) transhydrogenase indicated that it was desirable to determine the $\rightarrow H^+/2e^-$ quotient of the NAD(P) transhydrogenase directly in intact rat liver mitochondria.

Materials and Methods

General rationale and procedures

The $\rightarrow H^+/2e^-$ quotient of the NAD(P) transhydrogenase has been measured in intact rat liver mitochondria under anaerobic conditions by reducing $NADP^+$ with one substrate and oxidizing NADH with another substrate. After equilibration of the mitochondria with the first substrate, the NAD(P) transhydrogenase reaction and the associated proton translocation were initiated by adding the second substrate. Because of the need to obtain a rapid turnover of the NAD(P) transhydrogenase, the quantity of the second substrate required to initiate the transhydrogenase reaction was relatively large (i.e. 0.3mM final concentration) and the $\rightarrow H^+/2e^-$ quotient was therefore determined from the ratio of the maximal initial rate of H^+ translocation to the maximal initial rate of oxidoreduction through the NAD(P) transhydrogenase reaction after addition of the second substrate. We also used a similar method to operate the NAD(P) transhydrogenase in the reverse direction, but the reaction proved to be too sluggish to permit estimation of the $\rightarrow H^+/2e^-$ quotient.

To proceed with this work, it was necessary to show that certain substrate couples could be used specifically to react either with NAD or with NADP but not with both. The acetoacetate/β-hydroxybutyrate couple was the obvious choice for reaction with NAD through the mediation of the mitochondrial β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) (Lehninger *et al.*, 1960), but the literature indicated that there was no exclusively NADP-specific substrate couple in rat liver mitochondria. Williamson (1969) calculated from the observed total concentration ratios of substrate couples in whole freeze-clamped rat liver and from other related facts that the oxoglutarate/isocitrate concentration ratio in the mitochondria indicated a $NAD(P)^+/NAD(P)H$ ratio 1000 times smaller than the $NAD^+/NADH$ ratio obtained from the acetoacetate/β-hydroxybutyrate concentration ratio. He inferred that the oxoglutarate/isocitrate couple in the mitochondria of rat liver was near equilibrium with the $NADP^+/NADPH$ couple. These observations appeared to be consistent with the work of Stein *et al.* (1967), who found that only about 5% of the total isocitrate dehydrogenase activity of acetone-dried powders of mitochondria from rat liver was NAD-linked. On the other hand, it was claimed by Ernster & Navazio (1956, 1957), Ernster & Glasky (1960), Goebell & Klingenberg (1963, 1964), and Garland (1968) that the NAD-linked isocitrate dehydrogenase activity observed in the soluble fraction of isolated mitochondria from rat liver could represent as much as 35% of the total isocitrate dehydrogenase activity, and it was suggested that the activity should be attributed to an NAD-specific isocitrate dehydrogenase (EC 1.1.1.41), whose activity tended to be underestimated because this enzyme was relatively labile unless special precautions were taken during fractionation (Klingenberg, 1967). A re-examination of this question in the present paper has enabled us to conclude that there is no NAD-specific isocitrate dehydrogenase in mitochondria from rat liver. It has therefore been possible to use the oxoglutarate/isocitrate couple unequivocally for reaction with NADP through the mediation of the mitochondrial NADP-linked isocitrate dehydrogenase (EC 1.1.1.42).

Methods

Measurement of the NAD(P) transhydrogenase reaction rate and of the H^+ and K^+ translocation rates in intact mitochondria. Mitochondrial suspensions (6mg of protein/ml) were preincubated anaerobically at 25°C at approx. pH7 in the closed reaction vessel for 20min with various ratios of oxoglutarate/isocitrate at a total concentration of 2mM or 3mM in the presence of rotenone (25μg/g of protein) and arsenite (1mM), and the NAD(P) transhydrogenase reaction was initiated by injecting 5μl of anaerobic acetoacetate solution (200mM), giving a final concentration of 0.3mM.

In experiments designed to measure the $\rightarrow H^+/2e^-$ quotient, the medium used was 150mM-KCl containing 25mM-sucrose and 3.3mM-glycylglycine. Valinomycin (10μg/g of protein) was also added to this medium to permit the measurement of the rate of proton translocation from the rate of change of pH_o on initiating the NAD(P) transhydrogenase reaction. The rate of the NAD(P) transhydrogenase reaction was equal to the rate of reduction of acetoacetate by NADH under appropriate conditions described in the Results and Discussion section, and was measured by including both valinomycin (10μg/g of protein) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (1μM) in the medium and observing the rate of net alkalinization, represented by the rate of increase of pH_o, owing to the oxidation of NADH+H^+ to NAD^+.

In experiments designed to measure the electrogenicity of proton translocation by comparing changes of pH_o with those of pK_o, it was necessary to employ a relatively low K^+ concentration in the medium to obtain sufficient sensitivity of the K^+-responsive electrode, and it was also necessary to immobilize endogenous Ca^{2+} by adding EDTA. The medium used was 250mM-sucrose containing 10mM-choline chloride, 1mM-EDTA (choline salt), 3.3mM-glycylglycine–choline hydroxide buffer, pH7.0, and 100μg of valinomycin/g of mitochondrial protein. Sufficient anaerobic KCl solution was added from a microsyringe to adjust pK_o to 3.0 at the end of the preincubation period.

Measurement of NAD^+ and $NADP^+$ reduction in mitochondrial fractions. The reduction of NAD^+ or of $NADP^+$ by β-hydroxybutyrate or isocitrate in the sonic-particle fraction or soluble enzyme fraction (dialysed or undialysed) from the mitochondria was measured by the increase in absorbance at 340nm, observed in a Pye Unicam SP.800B or SP.1800B ultraviolet spectrophotometer by using the general methods of Ochoa (1955). Isocitrate dehydrogenase activity was measured in a medium (2.5ml) containing 40mM-Tris–HCl buffer, pH7.5, 2mM-$MnCl_2$ (or 6mM-$MgCl_2$), 2mM(or 10mM)-DL-isocitrate, 0.4mM-KCN and 0.2ml of soluble enzyme fraction (corresponding to 3mg of mitochondrial protein). The concentration of NAD^+ or $NADP^+$ added to initiate isocitrate dehydrogenase activity was 0.4mM. β-Hydroxybutyrate dehydrogenase activity was measured in a medium (2.5ml) containing 150mM-KCl, 40mM-Tris–HCl buffer, pH7.5, 4mM-β-hydroxybutyrate, 0.4mM-KCN, 0.4μM-rotenone and 1mg of particle protein (corresponding to 6.3mg of mitochondrial protein). The concentration of NAD^+ or $NADP^+$ added was 0.4mM.

Measurement of oxidation of β-hydroxybutyrate, NADH and NADPH in mitochondrial fractions. The rate of oxidation of β-hydroxybutyrate, NADH and NADPH in the sonic-particle fraction was determined by measuring the rate of decrease of the concentration of dissolved O_2 with a Clark O_2 electrode in the closed reaction vessel used as a routine in these studies. These measurements gave minimum values for the activities of the corresponding substrate- or NAD(P)-linked dehydrogenases because some rate-limiting effect may have been due to the cytochrome oxidase and respiratory-chain system (including the transhydrogenase) which was involved in the overall oxidoreduction process. The medium (3.3ml) contained 150mM-KCl, 3.3mM-glycylglycine–KOH buffer, pH7.0–7.1 at 25°C, 0.3μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, and 16mg of particle protein (corresponding to 100mg of mitochondrial protein). The various reductants were added at the following concentrations: NADPH, 1–4mM; NADH, 1mM; β-hydroxybutyrate, 2–4mM. In some experiments, NAD^+ (30μM–0.4mM) was added as a cofactor.

Measurement of the malate dehydrogenase and NAD(P) transhydrogenase reaction rates in the soluble fraction of mitochondria. The activities of the malate dehydrogenase (EC 1.1.1.37) and NAD(P) transhydrogenase in the soluble fraction were measured by the rates of oxidation by oxaloacetate of NADH and NADPH respectively, by using the change of absorbance at 340nm, as for the measurements of NAD^+ and $NADP^+$ reduction described above. The medium (2.5ml) contained 40mM-Tris–HCl buffer, pH7.5, 0.4mM-KCN and 0.2ml of soluble enzyme fraction (corresponding to 3mg of mitochondrial protein). The concentration of oxaloacetate was 4mM, and that of NADH or NADPH was 0.25mM. The malate dehydrogenase activity was sufficiently high (Table 1) not to limit the NAD(P) transhydrogenase activity observed as described here. However, the latter was presumably dependent on NAD that was present in the soluble fraction of the mitochondria. In practice, the observed NAD(P) transhydrogenase activities were not enhanced by increasing the NAD concentration in these experiments.

Measurement of substrate permeation by apparent absorbance changes of mitochondrial suspensions. The entry of β-hydroxybutyrate and acetoacetate through the cristae membrane of the mitochondria was measured by the changes of apparent absorbance at 800nm, by using a cuvette of 1cm light-path in the cuvette position near the photocell in a Pye Unicam SP.800B recording spectrophotometer. The methods were essentially similar to those employed previously (Chappell & Crofts, 1966; Mitchell & Moyle, 1969*b*), but the use of the 'near' cuvette position and light of longer wavelength than that previously used gave relatively less apparent light absorbance and permitted the use of mitochondrial suspensions corresponding to a protein concentration of some 5mg/ml, whereas the previous technique was not applicable at mitochondrial protein concentrations much above 0.5mg/ml. As originally shown by Chappell & Crofts (1966), at the lower mitochondrial concentrations, the presence of EDTA was essential to maintain stability of the mitochondria. As the presence of EDTA affects the permeability of mitochondrial cristae membranes (Azzi & Azzone, 1967; Mitchell & Moyle, 1969*b*), the technique described here, with similar mitochondrial concentrations to those employed in the respiratory and other metabolic studies, was preferable to the earlier method because EDTA was not required.

Reagents

Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, valinomycin and rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid) were gifts from Dr. P. G.

Heytler of E. I. du Pont de Nemours and Co. Inc. (Wilmington, Del., U.S.A.), from Dr. J. C. MacDonald of Prairie Research Laboratory (Saskatoon, Sask., Canada), and from Dr. H. U. Balthasar, Dr. H. Friedli and Dr. M. Taeschler of Sandoz Ltd. (Basle, Switzerland) respectively. Nucleotide pyrophosphatase (EC 3.6.1.9) from *Crotalus adamanteus* venom was obtained from Sigma (London) Chemical Co. (Kingston upon Thames, Surrey, U.K.), and a stock solution was made by dissolving 10mg in 1ml of water.

Simple organic and inorganic reagents were of AnalaR grade where available, or otherwise of the highest purity obtainable commercially.

Standard aqueous acid and alkali solutions, aqueous solutions of rhein (5mM), and ethanolic solutions of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and valinomycin were prepared and freed of O_2 in the usual way (Mitchell & Moyle, 1967*a*,*b*, 1969*a*).

Mitochondrial preparations

Mitochondria were isolated from the livers of male Wistar rats as previously described (Mitchell & Moyle, 1967*a*).

According to our normal routine, a cristae membrane fraction and a soluble enzyme fraction were prepared by sonicating mitochondrial suspensions (30mg of protein/ml) at 4°C in 250mM-sucrose containing 20mM-glycylglycine–KOH buffer, pH7.5, by using an MSE 60W sonicator operated at full power for 2min. Fractionation of the sonicate was done at 4°C. Unbroken mitochondria and debris were removed from the sonic particle and soluble enzyme fractions by centrifugation for 10min at 17000*g*, and the sonic particles were separated from the soluble enzyme fraction by centrifuging the supernatant for 60min at 75000*g*. The pellet of sonic particles was further freed of soluble enzyme fraction by redispersing it in sucrose–glycylglycine medium of the same composition as that used for the sonication, and centrifuging again for 60min at 75000*g*. The pellet of sonic particles was finally redispersed in 250mM-sucrose at a concentration corresponding to about 80mg of protein/ml. In some experiments, the soluble enzyme fraction prepared in the sucrose–glycylglycine sonication medium (called S–G) was dialysed in Cellophane dialysis tubing against 100mM-Na_2SO_4 containing 50mM-Tris–HCl buffer, pH7.3, for 18h. The yield of sonic-particle protein obtained from the mitochondria normally corresponded to about 16% of the original mitochondrial protein. Protein was determined by the method of Itzhaki & Gill (1964).

Since Goebell & Klingenberg (1963, 1964) claimed that the NAD-linked isocitrate dehydrogenase required special extraction conditions to prevent inactivation, we also followed their methods of preparing the soluble enzyme fraction of the mitochondria. The sonication medium in this case (called GSH–P_i–ADP) contained 100mM-potassium phosphate buffer, pH7.2, 10mM-GSH and 1mM-ADP. Soluble enzyme fractions of mitochondria were also prepared in sonication media containing 100mM-potassium phosphate buffer, pH7.2, and 1mM-ADP (called P_i–ADP), or 250mM-sucrose, 20mM-glycylglycine–KOH buffer, pH7.5, and 1mM-ADP (called S–G–ADP).

Use of closed reaction vessel and systems for measuring pH, pK and O_2 consumption

Our routine procedures employing the closed temperature-controlled reaction vessel of 3.3ml capacity, and the recording systems for measuring O_2 consumption and for measuring changes of pH_o and pK_o in submitochondrial enzyme preparations and in mitochondrial suspensions, were similar to those described previously (Mitchell & Moyle, 1967*a*,*b*, 1969*a*).

Results and Discussion

Substrates reacting specifically with NAD or NADP

Table 1 confirms that the β-hydroxybutyrate dehydrogenase of rat liver mitochondria is specifically NAD-linked (Lehninger *et al.*, 1960). We have therefore used β-hydroxybutyrate as a reductant and acetoacetate as an oxidant for the $NAD^+/(NADH+H^+)$ couple. Following Chappell (1969), we have alternatively used aspartate+oxoglutarate to generate oxaloacetate as an oxidant for the $NAD^+/(NADH+H^+)$ couple. The results in Table 1 show that the NAD-linked isocitrate dehydrogenase activity of the soluble fraction from the mitochondria may represent some 10% of the NADP-linked activity.

As indicated in Table 1, we confirmed the observations of Goebell & Klingenberg (1964) that the NAD-linked isocitrate dehydrogenase activities were relatively high when P_i and ADP were present in the preparation and storage media, but we did not confirm that GSH affected the initial NAD-linked isocitrate dehydrogenase activity or its subsequent stability. It was, of course, essential to bear in mind that the soluble fraction from the mitochondria contained a considerable number of enzymes and cofactors, and it was significant that the NAD(P) transhydrogenase activities, given in Table 1, were always higher than the corresponding NAD-linked isocitrate dehydrogenase activities.

As illustrated in Fig. 1, further observations on the relationships between the rate of the NAD(P) transhydrogenase reaction and the rates of oxidation of isocitrate by added NAD^+ or $NADP^+$ in soluble

enzyme fractions showed that: (1) the NAD(P) transhydrogenase reaction rate (curve *a*) was faster than the apparent NAD-linked isocitrate dehydrogenase reaction rate (curve *b*); (2) the rate of the total isocitrate dehydrogenase reaction in the presence of both NAD^+ and $NADP^+$ (curve *d*) was identical with that in the presence of $NADP^+$ alone (curve *c*); (3) in a preparation aged in the absence of ADP (curve *f*) the apparent NAD-linked isocitrate dehydrogenase rate declined to about 30% of that of a similar preparation aged in the presence of ADP (curve *e*), but the original activity of the preparation aged in the absence of ADP was restored (curve *g*) by adding a catalytic concentration (80μM) of NADPH to the assay medium. Addition of 80μM-NADPH to the preparation corresponding to curve (*b*) did not increase the NAD-linked isocitrate dehydrogenase activity.

These observations indicated that the NAD-linked isocitrate dehydrogenase activity was due, not to an NAD-specific isocitrate dehydrogenase, but to the coupling of the NADP-specific isocitrate dehydrogenase through a NAD(P) transhydrogenase activity that was dependent on the presence of NADP in the preparation, or on its addition to the assay medium. It seemed likely that the loss of the NAD-linked isocitrate dehydrogenase activity during aging was due to the destruction of NADP by an endogenous nucleotidase, which was inhibited by the presence of ADP. This interpretation was confirmed by the finding that the NAD-linked isocitrate dehydrogenase activity of the freshly prepared soluble fraction was decreased by preincubation with nucleotide pyrophosphatase in the assay medium before the addition of the NAD^+. The rate of loss of the NAD-linked isocitrate dehydrogenase activity varied with the quantity of nucleotide pyrophosphatase added, and it was found that 0.1ml of the stock nucleotide pyrophosphatase solution (in the 2.5ml of assay medium) decreased the half-life of the NAD-linked isocitrate dehydrogenase activity to about 1h. The original activity of the NAD-linked isocitrate dehydrogenase was restored after treatment with nucleotide pyrophosphatase by adding 80μM-NADPH to the assay medium, as it was after aging.

Translocation of feeder substrates (oxoglutarate+CO_2)/isocitrate and acetoacetate/β-hydroxybutyrate across the mitochondrial membrane system

To estimate the stoicheiometry of the translocation of acid equivalents and of charge associated directly with the NAD(P) transhydrogenase reaction in the intact mitochondria, it was necessary to allow for any translocation of acid equivalents or of charge associated with the translocation of the feeder substrates between the outer aqueous medium and their respective dehydrogenases in the inner aqueous medium.

In rat liver mitochondria, specific oxoglutarate/dicarboxylate and isocitrate/dicarboxylate antiporters permit the non-electrogenic exchange of the fully protonated acid species (or species in equivalently deprotonated states) across the cristae membrane (Papa *et al.*, 1971; Robinson *et al.*, 1971). Presumably, CO_2 diffuses non-electrogenically across the cristae membrane and its rapid equilibration with H_2O, H_2CO_3 and HCO_3^- is catalysed by carbonic anhydrase in the aqueous phases on either side (Klingenberg, 1970). At pH7, oxoglutarate is present almost entirely as the bivalent anion, and likewise the first two acidic groups of isocitrate are virtually completely ionized. However, the pK_a of the third acidic group of isocitrate is 5.8, whereas the apparent pK_a for the ionization of total dissolved CO_2 to give HCO_3^- is 6.35 (*Handbook of Chemistry and Physics*, 1962–63). At pH7, the third acidic group of isocitrate would therefore be 93% ionized, whereas 83% of the total CO_2 would be ionized as HCO_3^-. It follows that the overall antiport reaction isocitrate/(oxoglutarate $+CO_2$) is non-electrogenic, but involves an alkalinization of the outer medium corresponding to the disappearance of 0.1 acid equivalent from the outer medium per mol of antiport when isocitrate is the species passing inward. Similarly, the overall reaction isocitrate $\rightarrow$ oxoglutarate$+CO_2$ involves a net alkalinization corresponding to the disappearance of 0.1 acid equivalent per mol.

In the absence of published experimental data on translocation of acetoacetate and β-hydroxybutyrate [but see Klingenberg (1970)], we have measured the relative rates of permeation into rat liver mitochondria of the free acid and anionic species by using the osmotic-swelling technique (Chappell & Crofts, 1966; Mitchell & Moyle, 1969*b*), as shown in Fig. 2. Applying the usual rationale, the fact that rapid mitochondrial swelling in the iso-osmotic potassium salts occurred only when both valinomycin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone were present shows that acetoacetate and β-hydroxybutyrate permeate effectively only as the protonated acid species. The presence of 0.3mM-*N*-ethylmaleimide had no effect on the rate of mitochondrial swelling in the iso-osmotic potassium salts when valinomycin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone were added. Since the pK_a values of acetoacetate and β-hydroxybutyrate are 3.58 and 4.70 respectively, so that both are more than 99% ionized at pH7, the overall acetoacetate/β-hydroxybutyrate antiport is practically pH-neutral as well as non-electrogenic.

The translocation reactions associated with the use of oxoglutarate+aspartate as oxidant for the $NAD^+/(NADH+H^+)$ couple are incompletely understood, and we have used this substrate mixture only in

Table 1. *Enzyme activities of mitochondrial fractions*

Enzyme activities are expressed as μmol of oxidant reduced (or reductant oxidized) or μg-atom of O consumed/s per g of mitochondrial protein (no allowance being made for losses during isolation of fractions). The values in parentheses are for fractions that had been stored at 4°C for 24h before measurement of enzyme activity. Inhibitors were used at the following concentrations: *N*-ethylmaleimide, 0.3mM (with a 15 min preincubation period); dicoumarol, 1μM; arsenite, 1mM; rotenone, 0.4μM. The general methods of preparation of fractions and of measurement of enzyme activity are described in the Methods and Materials section, and appropriate details are specified, where required, in Table 1.

Mitochondrial fraction and medium used	Reductant and concn. (mM)		Cofactor added	Inhibitor	Oxidant	Activity
Soluble S–G (dialysed)	Isocitrate	2	Mn^{2+}	—	$NADP^+$	1.13
		2	Mn^{2+}	*N*-Ethylmaleimide	$NADP^+$	1.09
		2	Mn^{2+}	Arsenite	$NADP^+$	1.08
	NADH	1	—	—	Oxaloacetate	4.35
		1	—	*N*-Ethylmaleimide	Oxaloacetate	4.25
Soluble S–G	Isocitrate	10	Mn^{2+}	—	NAD^+	0.029
		10	Mn^{2+}	*N*-Ethylmaleimide	NAD^+	0.026
		10	Mg^{2+}	—	$NADP^+$	0.60 (0.41)
		10	Mg^{2+}	—	NAD^+	0.032 (0.007)
	NADPH	1	—	—	Oxaloacetate	0.13 (0.13)
Soluble S–G–ADP	Isocitrate	10	Mg^{2+}	—	$NADP^+$	0.63 (0.63)
		10	Mg^{2+}	—	NAD^+	0.042 (0.016)
	NADPH	1	—	—	Oxaloacetate	0.086 (0.09)
Soluble GSH–P_i–ADP	Isocitrate	10	Mn^{2+}	—	$NADP^+$	1.06
		10	Mn^{2+}	—	NAD^+	0.07
		10	Mg^{2+}	—	$NADP^+$	0.60 (0.42)
		10	Mg^{2+}	—	NAD^+	0.07 (0.06)
	NADPH	1	—	—	Oxaloacetate	0.11
Soluble P_i–ADP	Isocitrate	10	Mg^{2+}	—	$NADP^+$	0.55 (0.48)
		10	Mg^{2+}	—	NAD^+	0.08 (0.06)
		2	Mg^{2+}	—	$NADP^+$	0.68 (0.57)
		2	Mg^{2+}	—	NAD^+	0.04 (0.03)
	NADPH	1	—	—	Oxaloacetate	0.10
Sonic particle	β-Hydroxy-butyrate	2	—	—	O_2	0.006
		2	NAD^+ [30μM]	—	O_2	0.042
		2	NAD^+ [30μM]	*N*-Ethylmaleimide	O_2	0
		2	NAD^+ [30μM]	Arsenite	O_2	0.042
		4	NAD^+ [0.2mM]	—	O_2	0.210
		4	NAD^+ [0.4mM]	—	O_2	0.230
		4	—	—	NAD^+	0.40
		4	—	*N*-Ethylmaleimide	NAD^+	0.006
		4	—	—	$NADP^+$	0

NADH	1	—	—	O_2	0.34	
	1	—	—	O_2	0.32	
	1	*N*-Ethylmaleimide	—	O_2	0.061	
	1	Rotenone	—	O_2	0.040	
NADPH	1	—	—	O_2	0.085	
	1	—	NAD^+ [30 μM]	O_2	0.066	
	1	*N*-Ethylmaleimide	NAD^+ [30 μM]	O_2	0.085	
	1	Dicoumarol	NAD^+ [30 μM]	O_2	0.084	
	1	Arsenite	NAD^+ [30 μM]	O_2	0.205	
	2	—	NAD^+ [0.2 mM]	O_2	0.225	
	4	—	NAD^+ [0.4 mM]	O_2		

qualitative studies of proton translocation by the NAD(P) transhydrogenase.

Outward proton translocation associated with NAD(P) transhydrogenase activity in intact mitochondria

The translocation of protons during the activity of the NAD(P) transhydrogenase system in intact mitochondria is shown in the typical recording in Fig. 3(*a*). An anaerobic mitochondrial suspension was preincubated with isocitrate in the presence of rotenone, arsenite and valinomycin, and the NAD(P) transhydrogenase reaction was initiated by injection of acetoacetate. The rotenone and arsenite were present to inhibit respiratory-chain activity and oxoglutarate dehydrogenase activity respectively, and the valinomycin was present to permit K^+ ions to equilibrate across the membrane and minimize the electrogenic effect of proton translocation across the membrane. As shown in Fig. 3(*b*), when the membrane was made permeable to H^+ ions by adding carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, the acidification of the outer medium accompanying the NAD(P) transhydrogenase activity rapidly subsided, confirming that this acidification was attributable to the translocation of H^+ ions from the inner aqueous medium of the mitochondria to the outer aqueous medium. Fig. 3(*c*) shows that when rhein, an inhibitor of the NAD(P) transhydrogenase reaction (Kean *et al.*, 1971), was present under conditions otherwise the same as in the experiment of Fig. 3(*a*), the proton translocation initiated by adding acetoacetate was almost completely abolished. However, the rhein did not prevent the oxidation of the intramitochondrial NADH by the β-hydroxybutyrate dehydrogenase since, as illustrated in Fig. 3(*d*), alkalinization attributable to the oxidation of $NADH+H^+$ was observed when carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and valinomycin were present to equilibrate the pH changes across the cristae membrane. It is noteworthy that the acetoacetate used in these experiments corresponded to an initial concentration of 0.3 mM in the mitochondrial suspensions, and only a small proportion of the acetoacetate was reduced during the proton translocation recorded in Fig. 3. Lower rates of proton translocation were obtained with smaller concentrations of acetoacetate, presumably because the rate-limiting processes were the diffusion of the acetoacetate across the membrane and the reduction of the acetoacetate by the β-hydroxybutyrate dehydrogenase.

The electrogenicity of the outward proton translocation accompanying the oxidation of NADPH by NAD^+ was examined by driving the NAD(P) transhydrogenase reaction with isocitrate and acetoacetate, as in the experiments of Fig. 3, but with a medium

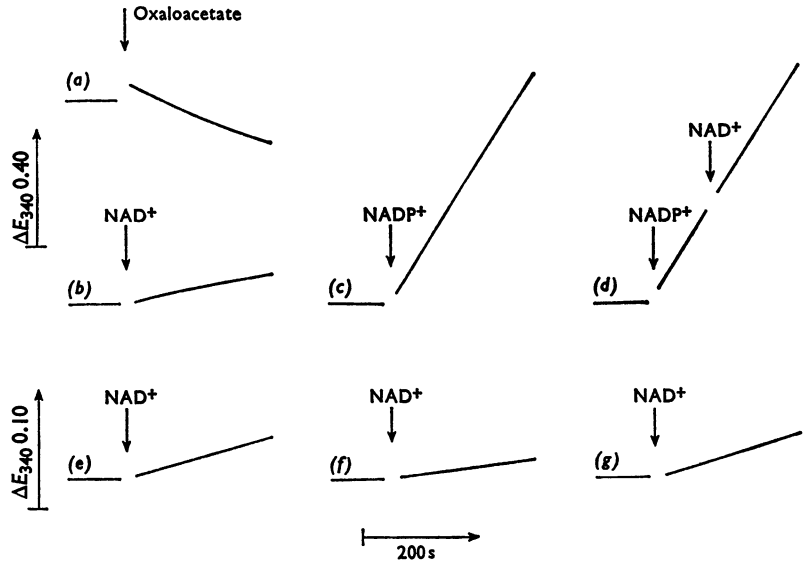

Fig. 1. *Reduction of NAD+ and NADP+ by isocitrate, and the transhydrogenase-mediated oxidation of NADPH by oxaloacetate, in soluble enzyme fractions from rat liver mitochondria*

Details of methods are given in the Methods and Materials section. (*a*), Time-course of the decrease in E_{340} on addition of 4mM-oxaloacetate to soluble enzyme fraction (prepared in S–G sonication medium and stored at 4°C for 24h) in an assay medium containing 0.25mM-NADPH; (*b*), (*c*) and (*d*), time-course of the increase in E_{340} on addition of 0.4mM-NAD^+ or 0.4mM-$NADP^+$ (as indicated) to soluble enzyme fraction (prepared in GSH–P_i–ADP sonication medium) in an assay medium containing 10mM-DL-isocitrate and 6mM-$MgCl_2$; (*e*), as (*b*), but soluble enzyme fraction was prepared in S–G–ADP sonication medium and stored at 4°C for 24h; (*f*), is as (*e*), but soluble enzyme fraction was prepared in S–G sonication medium and stored at 4°C for 24h; (*g*), is as (*f*), but the assay medium contained 80μM-NADPH in addition to routine constituents.

containing only 1mM-K^+, and following pK_o changes with a K^+-sensitive electrode. In this case, EDTA was required to immobilize the endogenous Ca^{2+} and enable the K^+ ion translocation to be used as an indicator of the electrogenicity of the proton-translocation reaction. The results of a typical experiment, described in Fig. 4(*a*), show that the NAD(P) transhydrogenase-linked proton translocation is electrogenic. As illustrated in Fig. 4(*b*), when the NAD(P) transhydrogenase reaction was initiated in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, there was virtually no net H^+ or K^+ translocation. In 11 experiments corresponding to Fig. 4(*a*), the observed $\rightarrow K^+/\leftarrow H^+$ translocation quotient had a mean value (±S.D.) of 0.80±0.19. Since it would be expected that at the rather slow translocation rates obtained in this type of experiment, some charge-carrying particles other than K^+ would be able to diffuse across the membrane, the results indicate that the NAD(P) transhydrogenase causes the translocation of one positive charge with each acid equivalent. In other words, H^+ is the species that is effectively translocated outwards (i.e. either H^+ goes outwards or OH^- goes inwards) during the forward NAD(P) transhydrogenase reaction.

Comparison of the rate of proton translocation in Figs. 3 and 4 shows that translocation was slower by a factor of about 2 in the medium containing the low K^+ concentration. This difference is attributable to the fact that the internal pH of the mitochondria is considerably lower under the latter conditions (Mitchell & Moyle, 1969*a*), and the rate of the NAD(P) transhydrogenase reaction is depressed either directly, since it has an alkaline pH optimum (Rydström *et al.*, 1970), or indirectly because of a lower internal concentration of isocitrate and acetoacetate.

Qualitatively similar results to those shown in Fig. 3 were obtained by injecting 0.33mM-aspartate after preincubating the mitochondria with 2mM-isocitrate or 2mM-isocitrate+0.6mM-oxoglutarate. As illustrated by the experiments of Figs. 5(*a*) and 5(*b*), the proton translocation observed under these conditions

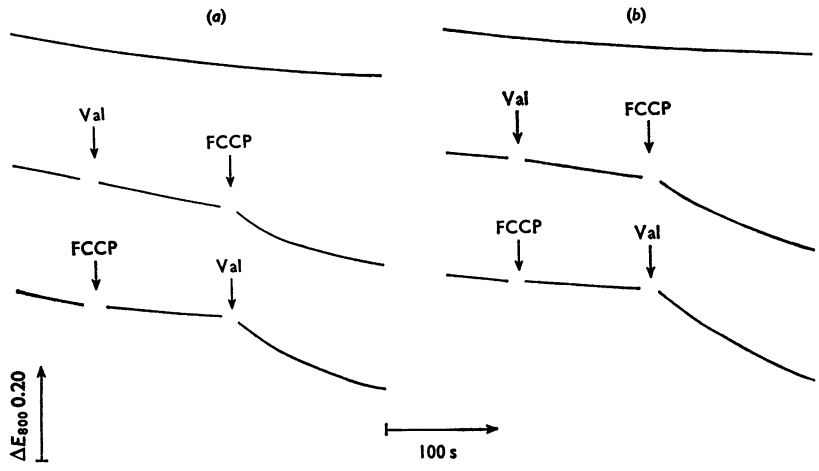

Fig. 2. *Permeation of acetoacetate and β-hydroxybutyrate into rat liver mitochondria*

Mitochondria [3.7 (*a*) or 3.5 (*b*) mg of protein/ml] were suspended in a medium (2.5 ml) containing 20 mM-Tris–HCl buffer, pH 7.0, 0.2 mM-KCN and 140 mM-potassium acetoacetate (*a*) or 140 mM-potassium β-hydroxybutyrate (*b*). The potassium acetoacetate was prepared from acetoacetate ethyl ester by the method of Ljunggren (1924). The mitochondrial stock suspensions (60 mg of protein/ml in 250 mM-sucrose) had previously been treated with 1.3 mg of rotenone/g of mitochondrial protein, and were kept at 4°C. Where indicated, 1 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and 20 μg of valinomycin/g of mitochondrial protein (Val) were added. (*a*) and (*b*) Time-courses of changes of apparent absorbance at 800 nm. The initial apparent absorbance in (*a*) was 0.98, and that in (*b*) was 0.94.

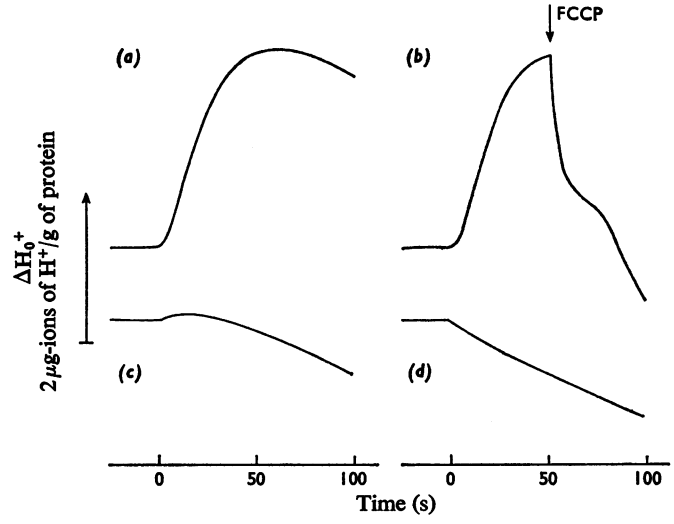

Fig. 3. *Proton translocation associated with forward NAD(P) transhydrogenase activity in intact rat liver mitochondria*

The mitochondria (5.8 mg of protein/ml) were suspended at 25°C and at pH_o 7.0–7.1 in an anaerobic medium (3.3 ml) containing 150 mM-KCl, 25 mM-sucrose, 3.3 mM-glycylglycine–KOH buffer, 2 mM-potassium DL-isocitrate, 25 μg of rotenone/g of mitochondrial protein, 1 mM-potassium arsenite and 10 μg of valinomycin/g of mitochondrial protein. In (*a*) there were no other additions; in (*b*), 0.3 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was injected at the arrow; in (*c*), 100 μM-rhein was present; and in (*d*), 100 μM-rhein and 1 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone were present. At zero time (after a 20-min preincubation period), 5 μl of anaerobic 0.2 M-potassium acetoacetate solution adjusted to pH 7.05 was injected. The time-course of ΔH_o^+ is derived directly from the recording of changes of pH_o, corrected for a slow base-line drift of 0.003 pH unit/100 s.

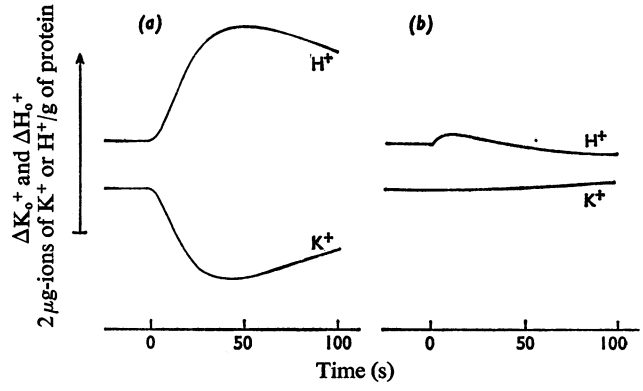

Fig. 4. *Electrogenicity of proton translocation associated with forward NAD(P) transhydrogenase activity in intact rat liver mitochondria*

The mitochondria (7.2mg of protein/ml) were suspended at 25°C and at pH_o 7.0–7.1 in an anaerobic medium (3.3ml) containing 250mM-sucrose, 15mM-choline chloride, 3.3mM-glycylglycine – choline hydroxide buffer, 1mM-EDTA (choline salt), 2mM-DL-isocitrate (choline salt), 25μg of rotenone/g of mitochondrial protein, and 1mM-choline arsenite; and the pK_o was adjusted to 3.0 by adding a small volume of anaerobic KCl solution after 15min. In (*a*), 100μg of valinomycin/g of mitochondrial protein was present; in (*b*), 1 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was present. At zero time (after a 20min preincubation period), 5μl of anaerobic 0.2M-choline acetoacetate solution adjusted to pH7.05 was injected. The time-courses of ΔH_o^+ and ΔK_o^+ are derived directly from recordings of changes of pH_o and pK_o, corrected for a slow base-line drift of 0.004pH unit and 0.002pK unit per 100s respectively.

subsided rapidly after addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. It was also possible to obtain proton translocation by adding isocitrate, as reductant for $NADP^+$, after preincubating the mitochondria with aspartate+oxoglutarate, as oxidant for NADH [Fig. 5(*c*)]; but, as shown in Fig. 5(*d*), there was very little proton translocation if the oxoglutarate was omitted.

The proton translocation obtained by injecting isocitrate after preincubating the mitochondria with acetoacetate was relatively slow, and this may have been due either to the relatively slow entry of isocitrate through the membrane in the absence of a source of L-malate, or to the relatively reducing midpoint potential of the acetoacetate/β-hydroxybutyrate couple compared with that of the oxaloacetate/L-malate couple, or to both factors.

Inward proton translocation associated with NAD(P) transhydrogenase activity

It was difficult to operate the NAD(P) transhydrogenase reaction backwards (i.e. reducing $NADP^+$ with NADH) in the intact mitochondria because the oxoglutarate/isocitrate feeder couple used for reducing the $NADP^+$ in the forward reaction had too reducing a midpoint potential [–414mV (Krebs & Veech, 1969)] to be employed as an oxidant feeder couple. Moreover, the high concentration of isocitrate in the mitochondria provided an endogenous source of reducing power that had to be removed before an alternative oxidant for the NADPH could be used effectively to drive the transhydrogenase reaction backwards. However, we succeeded in obtaining a sluggish reversal of the NAD(P) transhydrogenase after partial oxidation of the NAD(P), as follows. Mitochondrial suspensions were preincubated in air-saturated medium in the usual closed reaction vessel at pH7 with the addition of 1mM-NH_4Cl to convert the endogenous isocitrate into glutamate and CO_2. After exhaustion of the O_2, the anaerobic mitochondria were equilibrated for 20min in presence of arsenite, valinomycin and rotenone at the usual concentrations (see the Methods and Materials section), and the transhydrogenase reaction was initiated by injecting 5μl of anaerobic 200mM-β-hydroxybutyrate solution. Fig. 6 shows that a significant inward proton translocation, prevented by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, followed the injection of the β-hydroxybutyrate. The net acidification observed in the presence of valinomycin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone indicated that the addition of the β-hydroxybutyrate caused a net reduction of NAD^+ and $NADP^+$, and thus con-

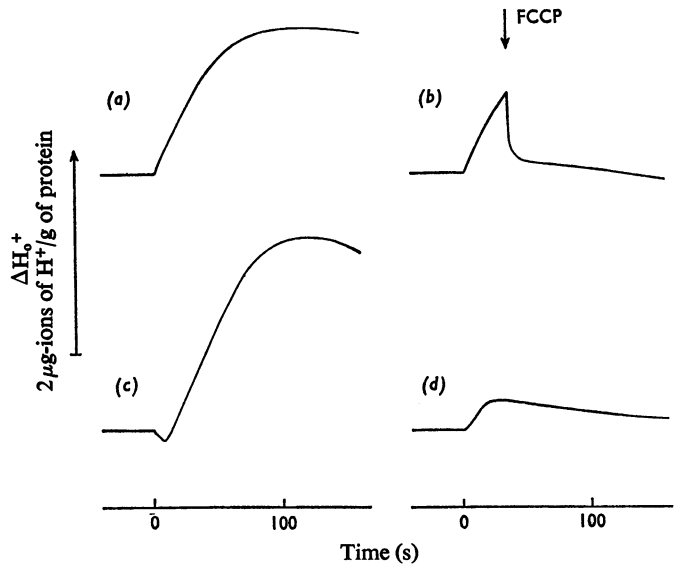

Fig. 5. *Proton translocation associated with forward NAD(P) transhydrogenase activity in intact rat liver mitochondria*

The general experimental conditions were as for Fig. 3(*a*), except that the protein content of the mitochondrial suspension was 6.0mg/ml. In (*a*), there were no other additions; in (*b*), 0.3μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added at the arrow. In (*a*) and (*b*), 10μl of anaerobic 0.1M-aspartate solution adjusted to pH7.05 was injected at zero time (after a 20-min preincubation period). In (*c*), the 2mM-DL-isocitrate in the medium was replaced by 1mM-aspartate+1mM-oxoglutarate; in (*d*), the 2mM-DL-isocitrate was replaced by 1mM-aspartate. In (*c*) and (*d*), 5μl of anaerobic 0.2M-DL-isocitrate solution adjusted to pH7.05 was injected at zero time (after a 20-min preincubation period). The time-course of ΔH_o^+ is derived directly from the recording of changes of pH_o, corrected for a slow base-line drift of 0.003pH unit/100s.

firmed that reversal of the transhydrogenase reaction occurred in this type of experiment.

Stoicheiometry of outward proton translocation by the NAD(P) transhydrogenase

To determine the $\rightarrow H^+/2e^-$ quotient for the forward NAD(P) transhydrogenase reaction, it was necessary to measure the ratio of the rate of proton translocation to the rate of transfer of bivalent reducing equivalents (formally represented as $2e^-$) from $NADPH+H^+$ to NAD^+ in the transhydrogenase reaction. As shown in Table 1, the isocitrate dehydrogenase activity in the soluble fraction and the forward NAD(P) transhydrogenase activity in the sonic-particle fraction were equivalent to throughputs of 1.2 and 0.23μequiv./s per g of mitochondrial protein respectively. These are minimum values because no corrections were made for preparative losses. The corresponding rate of acetoacetate reduction by the whole mitochondria given by experiments like that of Fig. 3(*a*) is not greater than 0.07μequiv./s per g of mitochondrial protein. Thus in these experiments, the rate-limiting process in the oxidation of isocitrate by acetoacetate [via isocitrate dehydrogenase, NAD(P) transhydrogenase and β-hydroxybutyrate dehydrogenase] is the oxidation of $NADH+H^+$ by acetoacetate, provided that the redox potential of the (oxoglutarate$+CO_2$)/isocitrate couple remains sufficiently reducing to prevent inhibition of the NAD(P) transhydrogenase by $NADP^+$. During the transhydrogenase reaction initiated by addition of acetoacetate after preincubating the mitochondria with isocitrate, the $NAD^+/(NADH+H^+)$ and $NADP^+/(NADPH+H^+)$ couples should therefore be close to equilibrium with each other, and the very active isocitrate dehydrogenase should rereduce the $NADP^+$ practically as fast as it is produced by the transhydrogenase. It follows that the rate of the transhydrogenase reaction should be almost equal to the rate of acetoacetate reduction determined by the β-hydroxybutyrate dehydrogenase, and the corresponding rate of proton translocation should be given by the maximal initial rate of acidification of the medium in the presence of valinomycin, as shown in

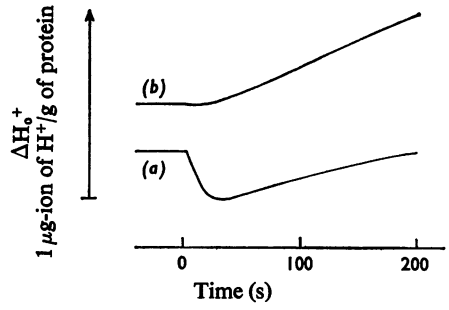

Fig. 6. *Proton translocation associated with backward NAD(P) transhydrogenase activity in intact rat liver mitochondria*

The mitochondria (6.5mg of protein/ml) were preincubated at 25°C and at pH_o 7.0–7.1 in an air-saturated medium (3.3ml, in the usual closed reaction vessel) containing 150mM-KCl, 25mM-sucrose, 3.3mM-glycylglycine–KOH buffer and 1mM-NH_4Cl. When the O_2 had been exhausted, the mitochondria were equilibrated anaerobically for a further 20min in presence of 1mM-arsenite, 10μg of valinomycin and 25μg of rotenone/g of mitochondrial protein. In (*a*), there were no other additions; in (*b*), 1μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was added during the anaerobic equilibration; 5μl of anaerobic 0.2M-potassium β-hydroxybutyrate solution adjusted to pH7.05 was then injected (zero time). The time-course of ΔH_o^+ is derived directly from the recording of changes of pH_o, corrected for a slow base-line drift of about 0.003pH unit/100s.

Fig. 7(*a*). The rate of the rate-limiting β-hydroxybutyrate dehydrogenase reaction was determined by measuring the rate of alkalinization produced by oxidation of NADH and NADPH when acetoacetate was added to mitochondria preincubated with oxoglutarate in the presence of valinomycin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, as shown in Fig. 7(*d*). Under these relatively oxidizing conditions, the $NADP^+$ produced by the transhydrogenase should not be initially re-reduced rapidly by the isocitrate dehydrogenase. The validity of this procedure was confirmed by a set of experiments, similar to those of Figs. 7(*a*) and 7(*d*), in which the maximal initial rate of proton translocation and the net rate of NADH+NADPH oxidation were measured after preincubation of the mitochondria in the presence of various added isocitrate/oxoglutarate concentration ratios, as summarized in Table 2. The results show that proton translocation was fastest and was relatively independent of the isocitrate/oxoglutarate concentration ratio when this ratio was high and the conditions were strongly reducing for NADP, and conversely the net rate of NADH+NADPH oxidation was greatest and was relatively independent of the isocitrate/oxoglutarate concentration ratio when this ratio was low and the conditions were not strongly reducing for NADP. Figs. 7(*c*) and 7(*b*) show the time-courses of proton translocation and of net NADH+NADPH oxidation respectively under conditions corresponding to the lowest and highest isocitrate/oxoglutarate concentration ratios shown in Table 2.

The maximal initial rate of proton translocation in experiments like that of Fig. 7(*a*) was not significantly affected by the presence of 1mM-EDTA, and was depressed by only 7% when 5mM-$MgCl_2$ was included in the medium.

Table 2 shows that, in practice, the rate of re-reduction of the $NADP^+$ by the isocitrate dehydrogenase did not quite keep pace with its rate of oxidation by the transhydrogenase when only isocitrate was added to the preincubation medium, and there was virtually no re-reduction of $NADP^+$ when the concentration ratio of added isocitrate/oxoglutarate was 0.5 or less. Thus, the maximum value of the net rate of oxidation of NADH+NADPH probably gives a correct estimate of the rate-limiting acetoacetate reduction rate, which we shall call v_A. It was possible to estimate the actual rate of re-reduction of the $NADP^+$ (which we shall call v_B) after addition of the acetoacetate, from the extent to which the rate of net NADH+NADPH oxidation fell short of the maximum, on the assumption that the velocity of the rate-limiting acetoacetate reduction (controlled by β-hydroxybutyrate dehydrogenase) was independent of the isocitrate/oxoglutarate concentration ratio. The values of v_B are shown in Table 2. Assuming that the NAD(P) transhydrogenase maintained the NAD and NADP pools near equilibrium, the rate of the NAD(P) transhydrogenase reaction v_T, after adding the acetoacetate, is given by:

$$v_T = v_A \frac{NADP}{NAD+NADP} + v_B \frac{NAD}{NAD+NADP} \quad (1)$$

where NAD and NADP stand for the total amounts of NAD and NADP in the mitochondria. From the amounts of NAD and NADP estimated in rat liver mitochondria by Williamson (1969), eqn. (1) can be written as:

$$v_T = 0.58 v_A + 0.42 v_B \quad (2)$$

The $\rightarrow H^+/2e^-$ values obtained from the ratio of the observed rate of proton translocation to the rate of the transhydrogenase reaction given by v_T are listed in Table 3. The $\rightarrow H^+/2e^-$ quotient of 1.53 shown in Table 3 is probably an underestimate because proton translocation was comparatively slow in this case and there was a significant loss of H^+ ions back through the membrane by the time the maximal initial rate of proton translocation was recorded. At isocitrate/

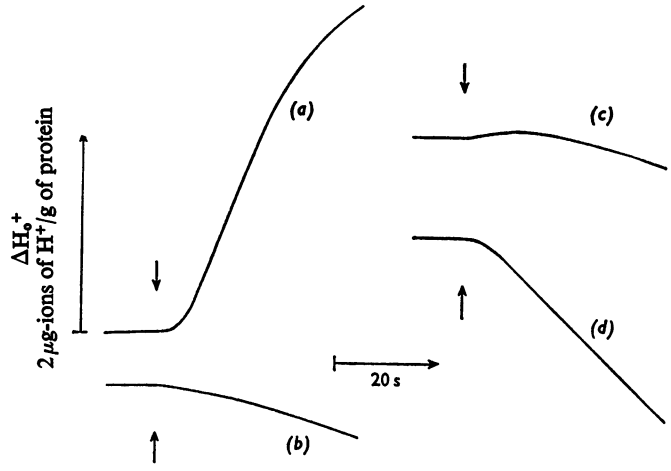

Fig. 7. *Proton translocation and net (NADH+NADPH) oxidation on injection of acetoacetate into anaerobic mitochondrial suspensions preincubated with isocitrate or oxoglutarate*

The general experimental conditions were as for Fig. 3(*a*), except that the protein content of the mitochondrial suspension was 6.2mg/ml. In (*a*), there were no other additions; in (*b*) and (*d*), 1 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was present; in (*c*) and (*d*), the DL-isocitrate of the medium was replaced by 2mM-potassium oxoglutarate. Acetoacetate (5 μl of anaerobic 0.2M-potassium acetoacetate at pH7.05) was injected at the arrows. The time-course of ΔH_o^+ is derived directly from the recording of changes of pH_o, corrected for a slow base-line drift of 0.003pH unit/100s.

Table 2. *Rate of proton translocation and net rate of NADH+NADPH oxidation on injection of acetoacetate into anaerobic suspensions of rat liver mitochondria preincubated with isocitrate/oxoglutarate mixtures*

General experimental conditions were as for Fig. 3(*a*), but 2mM-DL-isocitrate was replaced by DL-isocitrate/oxoglutarate mixtures (total concentration 3mM), and the protein content of the mitochondrial suspension was 5.8mg/ml. The proton-translocation rates were obtained from experiments similar to those illustrated in Figs. 7(*a*) and 7(*c*). The net rates of NADH+NADPH oxidation were obtained from experiments done in presence of 1 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, similar to those of Figs. 7(*b*) and 7(*d*), the values having been appropriately corrected for net alkalinization caused by conversion of isocitrate into oxoglutarate+CO_2. The maximal initial rates of change of pH_o after injection of the acetoacetate were used to calculate the proton-translocation rate and the net rate of NADH+NADPH oxidation. The rate of re-reduction of $NADP^+$, v_B, is given by the maximum value for the net NADH+NADPH oxidation rate minus that observed in the presence of the various isocitrate/oxoglutarate mixtures. The [isocitrate]/[oxoglutarate] ratios refer to added substrate.

[Isocitrate]/[Oxoglutarate]	Proton-translocation rate (μg-ion/s per g of protein)	Net rate of NADH+NADPH oxidation (μmol/s per g of protein)	v_B
0	0.002	0.064	0
0.5	0.058	0.065	0
2.0	0.081	0.049	0.016
3.3	0.089	0.039	0.026
10.0	0.108	0.036	0.028
∞	0.113	0.006	0.058

oxoglutarate concentration ratios of 2.0 or more, the values in Table 3 give an average $\rightarrow H^+/2e^-$ quotient (±S.D.) of 1.94±0.12.

The observation that the $\rightarrow H^+/2e^-$ stoicheiometry of the NAD(P) transhydrogenase is close to 2.0 is in agreement with the observation that the overall

Table 3. *$\rightarrow H^+/2e^-$ quotient for the forward NAD(P) transhydrogenase reaction in intact rat liver mitochondria*

The rates of the NAD(P) transhydrogenase reaction and of proton translocation are described as v_T and $v_{\rightarrow H+}$ respectively. The methods of calculation of v_T and $\rightarrow H^+/2e^-$ from the data in Fig. 7 and Table 2 are given in the text.

$\frac{[\text{Isocitrate}]}{[\text{oxoglutarate}]}$	v_T	$v_{\rightarrow H+}$	$\rightarrow H^+/2e^-$
(From data in Table 2)			
0.5	0.038	0.058	1.53
2.0	0.045	0.081	1.80
3.3	0.048	0.089	1.86
10.0	0.050	0.108	2.16
∞	0.062	0.113	1.83
(From data in Fig. 7)			
∞	0.064	0.128	2.00
∞	0.064	0.127	1.98

$\rightarrow H^+/P$ quotient for hydrolysis of external ATP by the mitochondrial adenosine triphosphatase is close to 2.0 (Mitchell & Moyle, 1968), and that the reduction of one $NADP^+$ molecule by $NADH+H^+$ involves the equivalent of the hydrolysis of one ATP molecule (Lee & Ernster, 1966).

Conclusions

The quantitative measurements of the pH_o changes and pK_o changes accompanying the forward NAD(P) transhydrogenase reaction described in the present paper confirm our qualitative observations of proton translocation by the NAD(P) transhydrogenase in sonic-vesicle preparations from ox heart mitochondria (Mitchell & Moyle, 1965), and establish that the passage of each formal bivalent reducing equivalent from NADPH to NAD^+ via the transhydrogenase system is coupled to the effective outward translocation of two H^+ ions across the cristae membrane of intact rat liver mitochondria. Skulachev and coworkers (Dontsov *et al.*, 1972) showed that forward activity of the NAD(P) transhydrogenase of sonic vesicles from ox heart mitochondria caused an electric potential difference across the membrane that appeared to correspond to the translocation of about two positive charges from the aqueous phase on the outside to that on the inside per formal bivalent reducing equivalent passing through the transhydrogenase reaction. Our observations show that this electrogenic reaction is actually accounted for by the net translocation of two H^+ ions.

The observations described here do not favour the possibility, discussed previously (Mitchell, 1966), that the NAD(P) transhydrogenase might primarily translocate a specific cation species other than H^+, which was linked to H^+ by a specific H^+/cation antiporter. On the contrary, they strongly favour the primary translocation of two H^+ ions per formal bivalent reducing equivalent passing through the transhydrogenase system, and suggest that further attention should be given to the type of proton-translocation mechanism originally suggested for loop 1, assuming cyclic translocations of the nicotinamide nucleotides within (but not completely across) the coupling membrane in such a way as to cause their phosphate groups to act as carriers of protons across the M phase (Mitchell, 1966, 1972; Skulachev, 1970).

Our observations support the suggestion, from the work of Cohen & Kaplan (1970) on the kinetics of the isolated transhydrogenase of *Pseudomonas aeruginosa*, and from the work of Scholes & Mitchell (1970) on the proton-translocation stoicheiometry of the respiratory-chain system of *M. denitrificans* [and see Teixeira da Cruz *et al.* (1971) and Rydström *et al.* (1971)], that the NAD(P) transhydrogenase reaction proceeds readily in the same forward direction as that of the rest of the respiratory chain. In this context, it is difficult to reach definite conclusions about the general metabolic significance of the lack of NAD-linked isocitrate dehydrogenase in rat liver mitochondria. However, it seems necessary to consider the speculative possibility that oxidation of isocitrate in rat liver mitochondria may occur via the NADP-linked isocitrate dehydrogenase at a redox potential in the region of −450mV, or even more negative. This would presumably require the effective midpoint potential of NADP to be some 150mV more negative than that of the free dinucleotide; and, similarly, the effective midpoint potential of the NAD would presumably have to be some 100mV more positive than that of the free dinucleotide, to provide a redox span of some 250mV across the ('energy-linked') proton-translocating NAD(P) transhydrogenase reaction. The results of Williamson (1969) on the total and free nicotinamide–adenine dinucleotides in the mitochondria of perfused rat liver indicate that NADH and $NADP^+$ are relatively much more strongly bound in the mitochondria than NAD^+ and NADPH. As originally discussed by Theorell & Bonnichsen (1951), such preferential binding of one form of a nicotinamide–adenine dinucleotide couple has the effect of shifting the midpoint redox potential. It is not inconceivable, therefore, that a kinetically effective redox loop (loop 0) could operate forwards (rather than backwards) between NADP and NAD. This would, of course, imply that a P/O quotient of 4 should be obtained during isocitrate oxidation in rat liver mitochondria under appropriate conditions, a possibility that is amenable to experimental investigation.

We are indebted to Professor Peter Garland for suggesting the use of nucleotide pyrophosphatase to confirm our interpretation of the NAD-linked isocitrate dehydrogenase activity. We thank Miss Stephanie Phillips and Mr. Robert Harper for expert assistance, and Glynn Research Ltd. for general financial support.

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