Proton Translocation Coupled to Quinone Reduction by Reduced Nicotinamide–Adenine Dinucleotide in Rat Liver and Ox Heart Mitochondria

By H. G. LAWFORD and P. B. GARLAND
*Department of Biochemistry, Medical Sciences Institute,
University of Dundee, Dundee DD1 4HN, U.K.*

(Received 17 July 1972)

Measurements were made of the stoichiometry of proton-translocation coupled to NAD(P)H oxidation by several quinones (duroquinone, ubiquinone₀, ubiquinone₁, ubiquinone₂) in mitochondria from rat liver and ox heart. Observed stoichiometries of protons translocated per mol of NADH oxidized ($\rightarrow\text{H}^+/2\text{e}^-$ ratios; Mitchell, 1966) ranged from 0.75 (rat liver mitochondria with ubiquinone₁) to 1.55 (ox heart mitochondria with ubiquinone₁ or ubiquinone₂). Only the rotenone-sensitive pathway of NADH oxidation by quinone was able to support proton translocation. Correction of the observed $\rightarrow\text{H}^+/2\text{e}^-$ ratios for the loss of reducing equivalents to the rotenone-insensitive pathway increased their value to approx. 2.0. It is concluded that the rotenone-sensitive NADH–ubiquinone reductase activity of the respiratory chain may be organized in the mitochondrial membrane as a proton-translocating oxidoreduction loop. The number of such loops between NADH and ubiquinone is one, and not two, as initially proposed by Mitchell (1966).

Respiration-driven proton translocation has been measured by Mitchell & Moyle (1967*b,c*) using oxygen or ferricyanide as the respiratory acceptor. The experimental observations were interpreted in favour of the previously postulated arrangement of the respiratory carriers whereby alternating hydrogen and electron carriers are oriented in the mitochondrial membrane in such a manner that proton translocation is an inevitable consequence of the transfer of reducing equivalents along the respiratory chain (Mitchell, 1966). The uncertainties about the sequence of carriers in the respiratory chain from NADH to the cytochromes (Slater, 1966) inevitably extended to any detailed proposals of how the carriers might be arranged as proton-translocating loops, and the scheme suggested by Mitchell (1966; see Scheme 1*a* below) should be viewed in that light. Setting aside the identity of the carriers, the scheme made the prediction that the ratio of protons translocated to reducing-equivalent pairs transferred to ubiquinone₁₀ (i.e. $\rightarrow\text{H}^+/2\text{e}^-$ ratio, in the terminology of Mitchell & Moyle, 1967*b*) would be 4.0 for NADH and 2.0 for succinate as the donor.

An alternative and currently perhaps more acceptable sequence of carriers such as that of Scheme 1(*b*) (Skulachev, 1971) can be folded between NADH and ubiquinone₁₀ to yield a proton-translocating loop

that would exhibit a $\rightarrow\text{H}^+/2\text{e}^-$ value of 2.0. Although the similar sequence of carriers from succinate to ubiquinone₁₀ could also be written as a proton-translocating loop leading to ubiquinone₁₀, they are shown in Scheme 1(*b*) in a non-looped arrangement that would yield a $\rightarrow\text{H}^+/2\text{e}^-$ value of zero.

A direct approach to the stoichiometry of proton translocation coupled to ubiquinone₁₀ reduction would be provided if ubiquinone₁₀ added to mitochondria acted as a carrier in the same fashion as endogenous ubiquinone₁₀. Unfortunately, this is not the case (Ziegler, 1961), and only the lower homologues such as ubiquinone₀, ubiquinone₁ and ubiquinone₂ react rapidly with the respiratory chain as do certain benzoquinones and naphthaquinones (Ernster, 1961; Ruzicka & Crane, 1971*a,b*). The behaviour of analogues and lower homologues of ubiquinone₁₀ can be used as a model for that of ubiquinone₁₀ itself, but with the serious reservation that they may differ from ubiquinone₁₀ in their chemical reactivities and membrane-modifying properties. Nevertheless, we have investigated the behaviour of a number of quinones in experiments designed to measure proton translocation coupled to the respiratory-chain-catalysed reduction of quinone by NADH or succinate. The results are consistent

Scheme 1. Folding of the respiratory chain into proton-translocating loops terminating in ubiquinone

Scheme 1(a) is based on Mitchell (1966), and Scheme 1(b) is suggested in this paper. Fe/S stands for iron-sulphur proteins; FMN and FAD are the prosthetic groups of the NADH dehydrogenase and succinate dehydrogenase (EC 1.3.99.1) respectively. 'Outer' and 'inner' refer to the phases on the outside and inside respectively of the inner mitochondrial membrane in intact mitochondria. Abbreviations: UQ and DQ, ubiquinone and duroquinone respectively in tables and figures.

with Scheme 1(b) and not 1(a). A preliminary account of these studies has been published (Lawford & Garland, 1972).

Materials and Methods

Preparation of mitochondria

Rat liver mitochondria were prepared as described by Garland *et al.* (1965) except that the homogenizing and washing medium was 0.25 M-sucrose saturated with N₂. Ox heart mitochondria were prepared by procedure 3 of Smith (1967) with the exception that the medium used for the final wash and resuspension was N₂-saturated 0.25 M-sucrose.

Reagents

Antimycin A, oligomycin and rotenone were purchased from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. Carbonyl

cyanide *m*-chlorophenylhydrazine (Heytler, 1963) and valinomycin were from Calbiochem Ltd., London W.1, U.K. Duroquinone (2,3,4,5-tetra-methyl-1,4-benzoquinone) was from Ralph N. Emanuel Ltd., Wembley, Middx., U.K.; juglone (5-hydroxy-1,4-naphthaquinone), 1,2-naphthaquinone and 1,4-naphthaquinone were from Koch-Light Ltd., Colnbrook, Bucks., U.K., and menadione (2-methyl-1,4-naphthaquinone) was from British Drug Houses Ltd., Poole, Dorset, U.K. Gifts of ubiquinone₀, ubiquinone₁ and ubiquinone₂ were from Hoffmann-La Roche, Basle, Switzerland, or Merck, Sharp and Dohme Research Laboratory, Rahway, N.J., U.S.A. The disodium salt of NADH was purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. All other reagents were of AnalaR grade where obtainable. Water distilled twice from an all-glass apparatus was used for all aqueous solutions. Standard solutions of HCl for calibration of pH-electrode responses were obtained from British Drug Houses Ltd., and diluted to 50 or 5 mM before use.

Media for mitochondrial incubations

For measurement of proton translocation, the incubation medium at 25°C consisted of N₂-saturated 150 mM-KCl containing 3.3 mM-glycylglycine and either 2.0 mM-DL-3-hydroxybutyrate or 2.0 mM-succinate as their potassium salts at pH 7.2. Spectroscopic or fluorimetric studies of mitochondria were done in 150 mM-KCl-20 mM-tris-HCl-1 mM-EDTA (pH 7.4) at 25°C.

Apparatus and procedure for the measurement of proton translocation by mitochondria

Incubations of mitochondria for the measurement of pH changes were done in a cylindrical chamber drilled in a block of Perspex. The block was nearly submerged in a water bath maintained at 25°C, and an immersible magnetic stirrer (Rank Bros., Bottisham, Cambs., U.K.) rotated a small glass-covered magnetic follower-bar in the chamber. The open upper end of the cylindrical chamber was sealed with a Perspex plug in which was fixed a combined glass microelectrode (type M27DP, *E*₀ value of zero at pH 7.0; from Activation Glass Ltd., Kinglassie, Fife, U.K.). The plug was also drilled to permit the insertion of a micro-syringe needle (Shandon-Terumo microsyringes; Shandon Southern Instruments Ltd., Camberley, Surrey, U.K.). The glass electrode was connected to a Pye model 290 pH-meter (Pye Unicam Ltd., Cambridge, U.K.) and the recorder output was taken to a Heath model IRM-18 pen recorder (Daystrom Ltd., Gloucester, U.K.). The recorder sensitivity was 0-1 mV for full-scale response of 25.4 cm and the use of a 100 Ω resistor across the

recorder terminals converted the current output of the pH meter into a voltage at a sensitivity of 0.1 pH unit/mV. A 2500 μ F electrolytic capacitor in parallel with the 100 Ω resistor removed unacceptable noise arising from the pH-electrode and meter. The rise time of the complete system (i.e. the time taken for the recorder reading to change from 10 to 90% of any given total change after the injection of acid or alkali into incubation medium in the chamber) was between 1.5 and 2.0 s. This time includes the time taken for injection, mixing, electrode response, meter response and recorder response. The peak-to-peak noise level recorded was 0.001 pH. The diffusion of oxygen from the air into the injection hole in the plug sealing the chamber was minimized by its length (2.0 cm), narrow diameter (0.05 cm), and by flow of nitrogen continuously directed at its top. The working volume of the chamber was 1.0 ml.

At the beginning of each experiment with anaerobic mitochondria, the incubation chamber was filled with N_2 -saturated medium containing DL-3-hydroxybutyrate or succinate. The stirrer was off. About 10 mg of mitochondrial protein in 0.2 ml of 0.25 M-sucrose were added, and this addition formed a layer at the bottom of the chamber. The sealing plug containing the electrode was inserted, displacing air and excess of incubation medium. The stirrer was then started. The pH was adjusted to 7.2 by the addition of 50 mM-KOH. All solutions, except where stated, were previously saturated with N_2 and added with a microsyringe. After incubation for 10 min 1 μ l of a valinomycin solution in ethanol (1 mg/ml) was added. After a further 5 min various experimental additions of inhibitor and quinones were made as described in the Tables and legends to the Figures.

Calibration of the pH scale was made by additions of 5 mM-HCl in 150 mM-KCl. It is assumed that in the absence of uncoupling agents the pH changes measured referred to the bulk phase of the medium and inter-membrane space of the mitochondria (Mitchell & Moyle, 1967a). The change of H^+ concentration measured by the glass electrode was calculated on the assumption that over the small pH increments measured (less than 0.05 pH unit), the pH scale was linearly proportional to H^+ . After addition of a pulse of an oxidant to anaerobic mitochondria under appropriate conditions there is a rapid fall in pH within 4 s or less, followed by an exponential decay back to the initial value (Mitchell & Moyle, 1967b). The peak value for H^+ concentration was measured directly from the recorder tracing, and correction for decay of this peak during its formation was not made.

Assay of quinones

The quinones used in this work were reducible by the combination of NADH and submitochondrial particles, and they could therefore be assayed by

measuring the decrease in E_{340} resulting from the oxidation of NADH. A 1:1 stoichiometry between quinone reduced and NADH oxidized was assumed (Ruzicka & Crane, 1971a), as was complete inhibition by antimycin and cyanide of the re-oxidation of reduced quinone by oxygen. This latter assumption was not true for naphthaquinones, and the concentrations of their solutions were calculated from the weight of naphthaquinone dissolved. Stock solutions of duroquinone, ubiquinone₆, ubiquinone₁, and ubiquinone₂ in ethanol were assayed spectrophotometrically as follows. Both a reference and a test cuvette were prepared, each containing at 25°C, 3.0 ml of 10 mM-tris-HCl buffer, pH 7.4, antimycin A (0.25–0.5 μ g), KCN (0.4 μ mol), NADH (0.5 μ mol) and submitochondrial particles (about 1 mg of protein) from ox heart or rat liver. The light-path of the cuvette was 1.0 cm, and extinction measurements of the test minus reference difference at 340 nm were made with a split-beam spectrophotometer (Haddock & Garland, 1971). The oxidation of NADH by quinone was initiated by the addition of the quinone (about 0.1 μ mol in 10 μ l of ethanol) to the test cuvette. The reduction of quinone was completed within 2–3 min, and the fall in E_{340} was proportional to the amount of quinone added over the range 0.01–0.4 μ mol.

Measurement of the fluorescence of intramitochondrial NADH and NADPH

Fluorescence measurements do not distinguish between NADH and NADPH, and for convenience the two dinucleotides will be collectively referred to as NAD(P)H when reduced and NAD(P)⁺ when oxidized. The fluorimeter used in these studies utilized a 55 W quartz-iodide car headlamp bulb (Philips 12258/99 HI) powered by a 12 V, 5 A stabilized d.c. supply (Coutant Electronics Ltd., Reading, Berks., U.K.). Exciting light centred at 340 nm was selected with an interference filter of 17 nm band-width at half-peak height (type R-UV from Balzers High Vacuum Ltd., Berkhamsted, Herts., U.K.), and focused with a quartz lens on to the optical surface of a quartz spectrophotometer cuvette of either 1.0 cm light-path or, when used with a rapid-mixing accessory, 0.2 cm light-path. Fluorescence was measured from the front face of the cuvette, by using a 3 mm-thick secondary filter of WG405 glass or KV408 plastic from Jena Glaswerk, Schott und Gen, Mainz, Germany, a side-window photomultiplier type 9781B (E.M.I. Ltd., Valve Division, Hayes, Middx., U.K.) and a current-to-voltage amplifier with zero-offset controls and a time-constant (expressed as the resistance $\times$ capacitance product in s) of 0.3 s for conventional mixing and 5.0 ms for rapid mixing. The amplifier output was displayed on a Heath EU-20B pen recorder, or for rapid mixing, a storage

oscilloscope (model 564; Tektronix Inc., Guernsey, C.I., U.K.). Photographic records of the oscilloscope trace were made with a type P.L.5 Polaroid camera (D. Shackman and Sons Ltd., Chesham, Bucks., U.K.).

Rapid mixing for fluorescence studies

Rapid mixing of mitochondrial suspensions with simultaneous measurement of NAD(P)H fluorescence was accomplished with a moving mixing chamber constructed as described by Strittmatter (1964). The reactants were contained in two 1.0 ml glass syringes mounted in a pushing block fitted with a calibrated and adjustable stop. The stop was set before each discharge to provide a mixed volume of 0.4 ml, each syringe contributing equally. Because of the rather large illuminated height of the fluorimeter cuvette and therefore the large mixed volume, the time from the initiation to completion of the mixing flow was about 50 ms, and not 5 ms as described by Strittmatter (1964) for the original apparatus. However, this mixing time was sufficiently fast to observe the oxidation of mitochondrial NAD(P)H by added quinones, since the half-time for oxidation was 0.7–2.0 s. Such rates cannot be accurately observed by using conventional mixing by hand into a cuvette, but do not require a more elaborate technique than that just described.

Direct spectroscopic measurement of quinone reduction by mitochondria

The reduced-minus-oxidized difference spectrum of duroquinone and ubiquinone₀ has a trough at 265 nm, and that of ubiquinone₁ and ubiquinone₂ a trough at 275 nm ($\epsilon = -12500 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$). Although severe technical difficulties are met in the direct spectroscopic measurement of intramitochondrial ubiquinone₁₀ (Chance, 1961) they do not arise when measuring the reduction of relatively large concentrations of added quinones provided that precautions such as the use of low mitochondrial concentrations and a stray-light filter are taken. Thus, for making such measurements, the split-beam spectrophotometer (Haddock & Garland, 1971), equipped with a 25 W deuterium arc or 100 W high-pressure mercury arc, was fitted with a 3 mm thickness of UG5 glass (Jena Glaswerk) at the entrance slit, and the concentration of mitochondrial protein was kept to 0.5 mg/ml or less. Measurements of extinction differences between test and reference samples were made at 25°C in cuvettes of 1.0 cm light-path. Each cuvette contained in a final volume of 2.6 ml tris-HCl buffer, pH 7.4 (50 μmol), KCl (375 μmol), EDTA (2.5 μmol), antimycin A (0.25 μg), KCN (2.5 μmol) and rat liver or ox heart mitochondria (usually 1–2 mg of protein). Either succinate (2.5 μmol) or L-glutamate

(2.5 μmol) with L-malate (2.5 μmol) as their tris salts (pH 7.4) were also present. Quinone reduction was initiated by adding quinone to the test cuvette in sufficient amounts to give an instantaneous increase of extinction of 0.3–0.5 at the appropriate measuring wavelength (265 or 275 nm). The subsequent rate of decrease of extinction was recorded and equated with the quinone reduction rate.

Other assays

Protein was assayed by the method of Lowry *et al.* (1951); NADH was assayed spectrophotometrically at 340 nm. The oxygen concentration of air-saturated 150 mM-KCl at 25°C was taken to be 0.47 $\mu\text{g-atom/ml}$ (Chappell, 1964).

Results

Suitability of quinones as respiratory-chain acceptors for studies of proton translocation

Proton translocation experiments as described by Mitchell & Moyle (1967*b*) were based on the ability of anaerobic mitochondria to respond to the addition of a small amount of oxygen with a burst of respiratory activity, followed by a return to anaerobic conditions within 1 or 2 s of adding the oxygen. Under such conditions the sudden acidification of the bulk phase of the incubation medium could be accurately measured and related in a stoichiometric manner to the amount of oxygen added without marked interference from the relatively slower return of protons back across the mitochondrial membrane. The high activity of cytochrome oxidase and its low apparent K_m for oxygen are essential for obtaining short-lived bursts of oxygen uptake. Similar considerations apply for any other acceptor irrespective of its point of interaction with the respiratory chain and, if they are not met, then it is unlikely that $\rightarrow \text{H}^+ / 2\text{e}^-$ ratios can be accurately measured. A further requirement for artificial acceptors is that they should not by-pass respiratory inhibitors used with the intention of isolating for study particular segments of the respiratory chain. It is not necessary at the outset of an investigation such as this one to ensure that the artificial acceptors do not short-circuit a part of the respiratory chain with the loss of energy conservation. Indeed, it was decided to investigate for a given acceptor the correlation between support of proton translocation and previously documented support of ATP synthesis from ADP and P_i (Ernster, 1961).

We have made measurements of the rate of quinone reduction by mitochondria, of the K_m for quinone, of the mitochondrial quinone reductase activities and of the effectiveness of antimycin A and cyanide in block-

ing mitochondrial reoxidation of reduced quinones by oxygen. Fig. 1 shows spectrophotometer recorder tracings of the reduction of ubiquinone₀ and ubiquinone₂ by ox heart mitochondria measured at 275 nm. The dependence of the rate of quinone reduction on the quinone concentration was calculated by drawing tangents to the reaction progress curves. The V_{max} for the reaction was equated with

the initial linear part of the reaction curve and the apparent K_m was equated with the remaining quinone concentration at which the rate had declined to 50% of the V_{max} . The apparent K_m value obtained in this way may have been influenced by the accumulation of reduced quinone but is nevertheless the relevant value in the context of these studies. The maximal activities and apparent K_m values measured in this way are listed in Table 1.

The oxidation of intramitochondrial NAD(P)H by added quinone was investigated by measuring the response of NAD(P)H fluorescence to additions of quinones in the presence of antimycin A and DL-3-hydroxybutyrate. Such experiments are shown in Fig. 2. The addition of quinone caused a temporary oxidation of NAD(P)H, there being a fast initial oxidation followed within a few seconds by a slower return to the reduced state. In further experiments of this type, it was found that the duration of this cycle of oxidation and reduction increased as the concentration of quinone relative to mitochondria increased. Exact quantitation was not possible owing to the relatively slow rate of the reduction phase of the oxidation-reduction cycle but we estimate that, at the relative concentrations of quinone and mitochondria (up to 6 nmol of quinone/mg of protein) used in proton translocation experiments described below, the time taken for the reduction of added duroquinone, ubiquinone₁ and ubiquinone₂ was less than 3–5 s. This was confirmed by direct experiment where the kinetics of oxidation and reduction were too fast after hand-mixing to permit more than a semi-quantitative estimate of the cycle time, but nevertheless shared an upper limit of 5 s. Similar results were obtained with ox heart mitochondria.

More direct measurements of the speed of quinone reduction were made with a simple rapid-mixing fluorimeter. The mixing time of this apparatus, as used by us, was about 50 ms and filled the gap between the mixing time of hand-stirring (1–2 s) and more sophisticated stop-flow methods (1–4 ms).

Fig. 1. Spectrophotometric recordings of ubiquinone₀ and ubiquinone₂ reduction by ox heart mitochondria

Details of the experimental conditions are described in the Materials and Methods section. Ox heart mitochondria (1.2 mg of protein) were suspended in 2.5 ml of 150 mM-KCl – 20 mM-tris – HCl – 1 mM-EDTA, pH 7.4 at 25°C. L-Malate (2.5 μmol), L-glutamate (2.5 μmol), antimycin A (0.25 μg) and KCN (2.5 μmol) were added. The arrows indicate the addition of ubiquinone₀ (79 nmol) and ubiquinone₂ (70 nmol). The reduction of quinone corresponds to a decrease in extinction at 275 nm. (a) and (b) were separate experiments. At ‘reset’, the recorder pen was returned to the zero position electronically. Note that in (b) the second addition of ubiquinone₂ is reduced at a significantly slower rate than the first.

Table 1. NADH-quinone oxidoreductase activity in rat liver and ox heart mitochondria

The reduction of ubiquinone₁ and ubiquinone₂ by intact rat liver or ox heart mitochondria was followed spectrophotometrically at 275 nm or, for ubiquinone₀ and duroquinone at 265 nm. Details of the experimental procedure and conditions are given in the legend to Fig. 1. The method of calculating V_{max} and apparent K_m is outlined in the text.

Acceptor	Rat liver mitochondria		Ox heart mitochondria	
	V_{max} (nmol/min per mg of protein)	K_m (μM)	V_{max} (nmol/min per mg of protein)	K_m (μM)
Duroquinone	30.4	3.6	32.7	3.9
Ubiquinone ₀	23.5	3.2	39.6	5.8
Ubiquinone ₁	27.5	3.9	79.2	5.0
Ubiquinone ₂	26.9	3.9	82.4	5.5

Fig. 2. Fluorimetric recordings of the oxidation of rat liver mitochondrial NAD(P)H by added quinones

Fluorescence was measured as described in the Materials and Methods section. (a), (b), (c) and (d) represent separate experiments and in all cases a downward deflexion corresponds to oxidation of intramitochondrial NAD(P)H. The vertical bar labelled '10%' corresponds to 10% of the total photomultiplier signal at the beginning of the experiment. In each experiment the reaction cuvette contained 2.5 ml of 150 mM-KCl-20 mM-tris-HCl-1 mM-EDTA buffer, pH 7.4; rat liver mitochondria (2.9 mg of protein); L-malate (2.5 μ mol); L-glutamate (2.5 μ mol) and antimycin A (0.25 μ g). The arrows indicate the addition of duroquinone (2,3,4,5-tetramethyl-1,4-benzoquinone) (35 nmol), ubiquinone₀ (158 nmol), ubiquinone₁ (55 nmol) and ubiquinone₂ (70 nmol). Note the prolonged cycle time in (b) and (d) with the second addition of quinone.

Fig. 3. shows that, on rapidly mixing duroquinone with antimycin-treated rat liver mitochondria, there is a rapid oxidation of NAD(P)H proceeding with a half-time of 1.0 s in the absence of rotenone and 1.8 s in its presence. (Note that the time scales for the two traces are different.) This experiment was performed with a final (mixed) mitochondrial protein concentration of 4 mg/ml, and the oxidation phase was completed within 4 s. At the protein concentration used in proton-translocation experiments (10 mg/ml), the oxidation phase would be complete in less than 2 s. The relative concentration of quinone to mitochondria in the experiment of Fig. 3 was 10.4 nmol/mg of protein, and the quinone used (duroquinone) was the most slowly reduced quinone of the series duroquinone, ubiquinone₀, ubiquinone₁ and ubiquinone₂ (Table 1). Thus the kinetics of NAD(P)H oxidation shown in Fig. 3 represent the slowest and, for our purposes, worst case; the oxidation phase was prolonged by the higher concentration and lower reactivity of duroquinone.

Measurements of the sensitivity to antimycin A of DL-3-hydroxybutyrate oxidation by mitochondria were made by using an oxygen electrode. Fig. 4 shows that, with rat liver mitochondria, oxygen uptake was completely inhibited by antimycin A and was not restored by the addition of duroquinone or ubiquinone₂ or (in other experiments) ubiquinone₀ or ubiquinone₁. By contrast, the addition of naphthaquinone (1,2-naphthaquinone, 1,4-naphthaquinone, juglone or menadione) caused a slight relief of the

inhibition of oxygen uptake by antimycin A. The respiration of ox heart mitochondria was partially insensitive to antimycin A (Brown *et al.*, 1965), but was completely inhibited by the further addition of KCN. Antimycin A insensitivity was not increased by duroquinone, ubiquinone₀, ubiquinone₁ or ubiquinone₂.

It can be concluded from these observations that low concentrations of duroquinone, ubiquinone₀, ubiquinone₁ and ubiquinone₂ are all rapidly reduced by mitochondrial NAD(P)H and can be used to provide short bursts of NAD(P)H oxidation in otherwise non-respiring mitochondria. In the presence of antimycin A alone (rat liver mitochondria) or with cyanide, the activity of the respiratory chain is restricted to the segment from NAD(P)H to the point(s) of interaction with added quinone, and this segment is confined to the carrier between NADH and the site of inhibition by antimycin A. Thus the criteria outlined above for satisfactory use of duroquinone, ubiquinone₀, ubiquinone₁ and ubiquinone₂ in proton-translocating experiments of the type described by Mitchell & Moyle (1967b) were met.

The oxidation of mitochondrial NAD(P)H by naphthaquinone was not investigated in any detail other than showing rapid cycles of NAD(P)H oxidation and reduction analogous to those of Fig. 2. Our interest in the naphthaquinones declined when we found that their reduction by rat liver mitochondrial NAD(P)H was not associated with proton translocation (see below).

Fig. 3. Oscilloscope recordings of fluorescence measurements of rat liver mitochondrial NAD(P)H oxidation by added duroquinone

Measurement of mitochondrial NAD(P)H fluorescence was accomplished with a Strittmater moving mixing chamber. Details of the apparatus and experimental procedure are given in the Materials and Methods section. Two separate experiments (a) and (b) are shown. One syringe contained rat liver mitochondria (concentration 8 mg of protein/ml) suspended in 150 mM-KCl–20 mM-tris–HCl–1 mM-EDTA buffer, pH 7.4, at 25°C to which antimycin A (0.1 µg/ml) was added; the other syringe contained duroquinone (83.5 nmol/ml) in 150 mM-KCl–20 mM-tris–HCl–1 mM-EDTA buffer, pH 7.4. Each syringe delivered 0.2 ml to the reaction cuvette. In (b), rotenone (0.2 µg/ml) was added to the mitochondrial suspension. The vertical bar labelled '10%' corresponds to 10% of the total photomultiplier signal at the beginning of the experiment. The signal was recorded on a storage oscilloscope. A downward deflexion of the oscilloscope trace represents a decrease in fluorescence and corresponds to an oxidation of intramitochondrial NAD(P)H. Note the difference in time-scale when comparing (a) and (b).

$\rightarrow H^+/2e^-$ ratios for the oxidation of 3-hydroxybutyrate or succinate by oxygen

To compare our pH-measuring system with that used by Mitchell & Moyle (1967b), we first repeated certain of their experiments. We confirmed that, on addition of oxygen pulses to anaerobic suspensions of either rat liver or ox heart mitochondria, $\rightarrow H^+/2e^-$ ratios of close to 6.0 and 4.0 were observed with DL-3-hydroxybutyrate or succinate respectively as substrates. We also observed, in agreement with Mitchell & Moyle (1967b), that respiration-driven proton translocation (i.e. acidification of the medium sampled by the pH electrode) was abolished by 1 µM-carbonyl cyanide chlorophenylhydrazone and, with rat liver mitochondria, by antimycin A. With ox heart mitochondria the inhibition of proton translocation by antimycin A was incomplete whereas 1 mM-KCN caused complete inhibition.

$\rightarrow H^+/2e^-$ ratios for the reduction of quinones by mitochondria

The effects of adding duroquinone to an anaerobic suspension of antimycin-treated mitochondria in the presence of DL-3-hydroxybutyrate are shown in Fig. 5. The rapid acidification observed on adding duroquinone was complete within 2–3 s and the response of the instrument was probably rate-limiting in following this change. The acidification phase was followed by an alkalization phase that returned exponentially towards the original pH value. The half-time for this decay of the acidification was about 40 s for the experiment shown. Shorter half-times (20–30 s) were usually observed in these studies. Fig. 5 shows that the extent of acidification after additions of duroquinone increased with the amount of duroquinone. However, at higher concentrations of duroquinone the acidification became biphasic, there being a fast and extensive initial fall in pH followed by a slower and further fall before the alkalization phase started. Fig. 5(b) shows this effect with ox heart mitochondria. The $\rightarrow H^+/2e^-$ ratios were about 1.0 for the lower concentrations of duroquinone (<4–5 nmol/mg of protein) and fell to 0.6 or lower as the duroquinone concentration was further increased (>6 nmol/mg of protein). A similar fall in the $\rightarrow H^+/2e^-$ ratio was also observed for higher concentrations of ubiquinone₁ and ubiquinone₂, and this is shown graphically for ubiquinone₁ and rat liver mitochondria in Fig. 6. Determinations of $\rightarrow H^+/2e^-$ ratios for rat liver and ox heart mitochondria by using duroquinone, ubiquinone₀, ubiquinone₁ and ubiquinone₂ at concentrations in the range 0.25–5.0 nmol of quinone/mg of protein are listed in Table 2. The behaviour of ubiquinone₀ with rat liver mitochondria was very inconsistent, and the changes of pH after additions of ubiquinone₀ to antimycin-treated anaerobic mitochondria varied

Fig. 4. Polarographic recordings of oxygen uptake by rat liver and ox heart mitochondria

The experiments were done in a water-jacketed chamber at 30°C by using a Clark electrode to measure oxygen consumption. In (a), 18 mg of rat liver mitochondrial protein (rat liver mito.) and in (b) 7 mg of ox heart mitochondrial protein (ox heart mito.) were added to 3.0 ml of tris-HCl buffer, pH 7.4, (60 μmol), KCl (450 μmol), K₂HPO₄ (6.0 μmol) and EDTA (3.0 μmol). Further additions were: DL-3-hydroxybutyrate (3-HO-Bu, 3.0 μmol), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (0.5 nmol), antimycin A (AA, 0.25 μg), KCN (3.0 μmol), duroquinone (158 nmol), ubiquinone₂ (140 nmol), 5-hydroxy-1,4-naphthaquinone (juglone, 80 nmol). Two experiments (a) and (b) are shown.

between different batches of mitochondria. All possible changes (acidification, alkalization, none) were observed, and a similar inconsistency of the rotenone sensitivity of ubiquinone₀ reduction by rat liver mitochondria was also met (see below). The $\rightarrow\text{H}^+/2\text{e}^-$ values for ubiquinone₀ and rat liver mitochondria are therefore omitted from Table 2. The behaviour of ubiquinone₀ with ox heart mitochondria was consistent and similar to that of duroquinone, ubiquinone₁ and ubiquinone₂.

Our interpretation of the fall in $\rightarrow\text{H}^+/2\text{e}^-$ value when the concentration of added quinone exceeded about 6 nmol/mg of protein is that the extent of the rapid initial phase of acidification is limited by the maximal concentration of mitochondrial NAD(P)H which is 5.3 nmol/mg of protein for rat liver (Klingenberg & Slenczka, 1959). This interpretation assumes that added quinones oxidize both NADH and NADPH (reaction 1) and that this rate is considerably faster than the rate of NAD(P) reduction by D-3-hydroxybutyrate or endogenous substrates.

In the fast phase of acidification, reaction (1) would be rate-limiting until NAD(P) was largely oxidized. Reaction (2) would then become rate-limiting and give a proton-translocation rate that was slow relative to the back flow of protons across the mitochondrial membrane. The $\rightarrow\text{H}^+/2\text{e}^-$ value would therefore be underestimated. It is immaterial for this interpretation whether the reactivity of NAD(P) was direct or via transhydrogenation with NAD.

Effects of rotenone on $\rightarrow\text{H}^+/2\text{e}^-$ ratios

The pH recording of Fig. 7 shows that if rotenone was added to the incubation medium, the addition of ubiquinone₁ to cyanide-treated ox heart mitochondria caused a brief acidification that was only one-tenth as extensive as that observed in the absence of rotenone. A further difference is that the brief acidification phase was followed by alkalization to a pH higher than the starting value. Finally, the pH slowly fell to the starting value. Similar observations were made

Fig. 5. pH recordings of proton translocation by rat liver and ox heart mitochondria with duroquinone as respiratory acceptor

The apparatus, experimental procedure and conditions are outlined in detail in the Materials and Methods section. An upward deflexion corresponds to an acidification of the suspending medium. Two experiments are shown: the top trace (a) represents proton translocation by rat liver mitochondria (13 mg of protein) and the bottom trace (b) represents ox heart mitochondria (10 mg of protein). Initially the mitochondria were added to 150 mM-KCl-3.3 mM-glycylglycine-HCl-2 mM-DL-3-hydroxybutyrate medium, pH 7.2 at 30°C. This medium had been previously saturated with N₂; the total volume of the anaerobic mitochondrial suspension was 1.0 ml. Antimycin A (0.2 µg) was added (with ox heart mitochondria, addition of 1.0 µmol of KCN was also necessary), the chamber was sealed and the pH adjusted to 7.2 with either 50 mM-HCl or -KOH. After 10 min, valinomycin (1 µg) was added and after a further 5 min additions of quinones were made. The amount of duroquinone added (nmol) is indicated by the arrows.

with duroquinone, ubiquinone₀, ubiquinone₂, and with rat liver mitochondria, duroquinone, ubiquinone, and ubiquinone₂.

Although rotenone completely inhibits the oxidation of mitochondrial NAD(P)H by oxygen (Ernster *et al.*, 1963), it inhibits the oxidation of NAD(P)H by duroquinone, ubiquinone₀, ubiquinone₁ and ubiquinone₂ by not more than 60% with rat liver mito-

Fig. 6. Relationship between protons translocated by rat liver mitochondria and the amount of ubiquinone added

The experimental conditions for pH measurements are described in the legend to Fig. 5. Rat liver mitochondria (approx. 10 mg of protein) were used in this experiment. The value for →H⁺ was determined by measuring the maximal ΔpH after addition of quinone.

chondria and 90% with ox heart mitochondria (see below). Thus it seems that there are two main modes of quinone reduction by mitochondrial NAD(P)H. One is rotenone-sensitive and proton-translocating, the other is rotenone-insensitive and non-proton-translocating.

*Effects of carbonyl cyanide *m*-chlorophenylhydrazone on →H⁺/2e⁻ ratios*

When carbonyl cyanide *m*-chlorophenylhydrazone was added to a suspension of rat liver mitochondria shortly after a proton-translocating addition of duroquinone, the pH reverted rapidly to its original value and then commenced a slow downward drift that was generally observed when mitochondria were treated with carbonyl cyanide *m*-chlorophenylhydrazone (Fig. 8). Further additions of duroquinone caused abrupt increases in pH that returned to the previous value in about 30–60 s. Although there was a slow and continuous drift in the base-line, it was clear that a definite stoichiometry existed

Table 2. Observed $\rightarrow H^+/2e^-$ ratios with exogenous quinones as terminal respiratory acceptors and values of $\rightarrow H^+/2e^-$ ratios which have been corrected for the non-proton-translocating rotenone-insensitive NADH-quinone oxidoreductase activity in intact rat liver and ox heart mitochondria

The rotenone sensitivity of the NADH-quinone oxidoreductase in intact rat liver and ox heart mitochondria was determined by two different methods. Method A: inhibition by rotenone of exogenous quinone reduction was measured spectrophotometrically at either 265 nm or 275 nm. Details of the experimental conditions are described in the legend to Fig. 9. Method B: inhibition by rotenone of mitochondrial NAD(P)H oxidation by added quinone. Details of the rapid mixing fluorescence measurements are described in the legend to Fig. 3. The values of $\rightarrow H^+/2e^-$ ratio are presented as the mean $\pm$ S.E.M. with the number of observations in parentheses.

	Rotenone sensitivity (% inhibition)			$\rightarrow H^+/2e^-$ ratio	
	Method A	Method B	Mean	Observed	Corrected
Rat liver mitochondria with					
Duroquinone	44	38	41.0	0.85 ± 0.14 (21)	2.08 ± 0.34
Ubiquinone ₀	20	16	18.0	—	—
Ubiquinone ₁	51	56	53.5	0.75 ± 0.11 (22)	1.40 ± 0.21
Ubiquinone ₂	60	49	54.5	1.09 ± 0.16 (16)	2.00 ± 0.30
Ox heart mitochondria with					
Duroquinone	56	50	53.0	1.26 ± 0.18 (16)	2.38 ± 0.34
Ubiquinone ₀	66	59	62.5	1.20 ± 0.34 (18)	1.78 ± 0.33
Ubiquinone ₁	90	88	89.0	1.56 ± 0.14 (20)	1.75 ± 0.16
Ubiquinone ₂	82	79	79.0	1.55 ± 0.22 (29)	1.96 ± 0.28

Fig. 7. Effect of rotenone on $\rightarrow H^+/\text{ubiquinone}_1$ with ox heart mitochondria

The experimental conditions for pH measurements are described in the legend to Fig. 5. An upward deflexion corresponds to an acidification of the suspending medium. Ox heart mitochondria (approx. 10 mg of protein) were used in this experiment. Additions, as indicated by the arrows, were: air-saturated 150 mM-KCl (O, 9.4 ng-atoms of oxygen); ubiquinone₁ (nmol added are appropriately indicated) and rotenone (Rot., 0.2 μ g).

between the amount of duroquinone added and the extent of alkalization. This alkalization should be regarded as occurring both intra- and extra-mito-

chondrially since carbonyl cyanide *m*-chlorophenylhydrazine is one of a class of compounds (classical uncoupling agents) that conducts protons across

Fig. 8. Effect of carbonyl cyanide *m*-chlorophenylhydrazone on $\rightarrow H^+$ /duroquinone with rat liver and on $\rightarrow H^+$ /ubiquinone₂ with ox heart mitochondria

Conditions for pH measurements are described in the legend to Fig. 5. An upward deflexion corresponds to an acidification of the suspending medium. Rat liver mitochondria were used in (a) and ox heart mitochondria in (b). The amount of duroquinone (nmol) or ubiquinone₂ (nmol) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (nmol) is indicated by the arrows.

membranes (Mitchell & Moyle, 1967a). Changes of the pH of the medium in the presence of carbonyl cyanide *m*-chlorophenylhydrazone are therefore referred to as ΔH^+ rather than $\rightarrow H^+$. In the presence of carbonyl cyanide *m*-chlorophenylhydrazone the $\Delta H^+/2e^-$ value for duroquinone was -0.7 (Fig. 8). Similar observations were made with ubiquinone₁ and ubiquinone₂ with rat liver mitochondria, and duroquinone, ubiquinone₆, ubiquinone₁ and ubiquinone₂ with ox heart mitochondria.

The temporary alkalization occurring on the addition of quinone to mitochondria in the presence of carbonyl cyanide *m*-chlorophenylhydrazone is most likely due to fast completion of reaction (1) (see above) that consumes protons, and slow completion of reaction (2) that releases protons. The overall pH change of reactions (1) and (2) is zero around pH 7, a pH value that is well removed from the pK_a values for acetoacetate (3.62), 3-hydroxy-

butyrate (4.39) and reduced quinone (9.85; Dawson *et al.*, 1969).

$\rightarrow H^+/2e^-$ ratios with naphthaquinones as acceptors

Naphthaquinones are known to by-pass the rotenone-sensitive site between NADH and the cytochrome in mitochondria, and the naphthaquinone-mediated reduction of the cytochromes by NADH is not coupled to ATP synthesis from ADP and P_i (Ernster, 1961; Conover & Ernster, 1962). As expected, therefore, we were unable to detect proton translocation coupled to reduction of naphthaquinones (juglone, menadione, 1,2-naphthaquinone, 1,4-naphthaquinone) in experiments identical with those of Figs. 5 and 7 and Table 2, except that naphthaquinones were used in place of benzoquinones. Proton translocation with naphthaquinones was observed in the naphthaquinone solution was not fully anaerobic or if cyanide was not added; this proton translocation can be attributed to oxygen as the respiratory acceptor and partial by-pass of the antimycin A-sensitive site by naphthaquinones. These results need not conflict with those of Grinius *et al.* (1971), who studied submitochondrial particles rather than intact mitochondria.

Determination of rotenone sensitivity of quinone reduction by NAD(P)H

There are routes in intact mitochondria other than the respiratory-chain NADH dehydrogenase whereby NAD(P)H can reduce added quinones (Ernster, 1961). These other routes are rotenone insensitive and, because they do not have primary roles in the respiratory chain or in oxidative phosphorylation, would not be expected to be proton translocating. An important consequence of this is that the observed $\rightarrow H^+/2e^-$ ratios for quinones are lowered if some of the quinone reduction proceeds through a rotenone-insensitive and non-proton-translocating route. The true $\rightarrow H^+/2e^-$ ratios for reduction of quinone by the rotenone-sensitive respiratory chain can be calculated by making allowance for the relative contributions of the rotenone-sensitive and -insensitive pathways towards quinone reduction. Although the sensitivity to rotenone of NAD(P)H oxidation by quinones can easily be measured spectroscopically at 340 nm by using submitochondrial particles, there is no certainty that the rotenone sensitivity, measured in this manner, will correspond to that of intact mitochondria utilizing intramitochondrial NAD(P)H. Indeed, the observation that the matrix of rat liver mitochondria contains an active and rotenone-insensitive NAD(P)H-quinone oxidoreductase (Ernster, 1961; Conover & Ernster, 1962) indicates that if it is the sensitivity of intact mitochondria to

Fig. 9. Effect of rotenone on the reduction of duroquinone by ox heart mitochondria

The reduction of duroquinone was measured spectrophotometrically at 265 nm. (a) and (b) were separate experiments and the conditions were similar to those described in the legend to Fig. 1 except that in (b) rotenone ($5\mu\text{g}$) was added to the mitochondrial suspension 5 min before the addition of duroquinone. The arrows indicate the addition of duroquinone (70 nmol).

rotenone that is sought, then submitochondrial particles cannot be substituted for intact mitochondria. We therefore investigated three independent methods of measuring rotenone sensitivity of quinone reduction by NAD(P)H in intact mitochondria. The results are described below.

Rotenone sensitivity measured by direct spectroscopic observation of quinone reduction

For reasons of sensitivity in this assay it was necessary to use relatively low concentrations of mitochondrial protein. Thus, the ratio of quinone to mitochondrial protein was high and the measurements of reduction rates and rotenone sensitivity were representative of the steady-state condition when intramitochondrial NAD(P) was largely oxidized. To diminish the rate limitation imposed by the relatively low activity of D-3-hydroxybutyrate oxidation, L-malate with L-glutamate was used as the substrate. Recordings of this direct assay of quinone reduction have been shown in Fig. 1, and the further recordings shown in Fig. 9 illustrate an unusual but not consistent feature, namely, a biphasic reaction progress consisting of a fast initial phase followed by a slower phase. The extent of the initial phase is consistent with it being due to the reduction of quinone

by NAD(P)H present intramitochondrially at the onset of the assay. For instance, at a mitochondrial protein concentration of 1.2 mg/ml , the concentration of NAD(P)H could be as high as $6\mu\text{M}$, sufficient to support an extinction change corresponding to the reduction of $6\mu\text{M}$ -quinone, which is 0.075. This is entirely consistent with the extent of the fast initial phase shown in Fig. 9. However, the rate of the fast initial phase appears to be too slow to correspond to the rapid oxidation of intramitochondrial NAD(P)H (Figs. 2 and 3). The reason for this biphasic rate, therefore, remains unknown but the uncertainty does not affect the significance of these measurements of rotenone sensitivity. The percentage inhibition of the slow phase by rotenone is shown in Table 2; the values do not differ significantly from those obtained with the method, described in the next section, which measures the rate of oxidation of intramitochondrial NAD(P)H by quinones.

Rotenone sensitivity measured by fluorimetric assay of NAD(P)H oxidation with rapid mixing

Use of a rapid-mixing fluorimeter (see the Materials and Methods section) permitted observations to be made of NAD(P)H oxidation and its rotenone sensitivity under transient conditions that resembled those of proton-translocation experiments. Absolute rates of NAD(P)H oxidation were not measured and the rate of NAD(P)H oxidation was expressed either as the initial velocity in arbitrary units or as the half-time for the reaction (see Fig. 3). Rotenone sensitivity for any given quinone was the same whether calculated from the initial velocity or reaction half-time. The values given in Table 2 are those calculated from half-times, and are in good agreement with those calculated from the direct spectroscopic observations of quinone reduction.

Calculation of oxidation rates from cycles of NAD(P)H oxidation caused by small additions of quinones

When a suitable quinone is added to mitochondria in a small molar excess over the mitochondrial content of NAD(P)H, measurement of NAD(P)H fluorescence shows a cycle of NAD(P)H oxidation followed by reduction (Fig. 2). The initial rate of oxidation is too fast to measure unless rapid mixing is used but the duration of the oxidation of NAD(P)H could, hopefully, be used to calculate the average rate of reduction of the limited amount of added quinone. Fig. 10 shows recordings of NAD(P)H fluorescence of rat liver mitochondria and the effects of rotenone on the duration of cycles of oxidation caused by addition of quinones. There was clearly a marked prolongation of the cycle time but completion of the cycle was insufficiently sharp to allow quantitative time measurements. Because of the need for accurate

Fig. 10. Fluorimetric recordings of the effect of rotenone on the oxidation of rat liver mitochondrial NAD(P)H by duroquinone and ubiquinone₁

These experiments were performed under identical conditions to those outlined in the legend to Fig. 2 except that in both (b) and (d), rotenone (5 μg) was added to the mitochondrial suspension 5 min before the addition of quinone. The arrows indicate the addition of duroquinone (35 nmol) and ubiquinone₁ (55 nmol). (a), (b), (c) and (d) represent separate experiments. The vertical bar labelled '10%' corresponds to 10% of the total photo-multiplier signal at the beginning of the experiment. A downward deflexion corresponds to the oxidation of intra-mitochondrial NAD(P)H by added quinone.

Table 3. Succinate-quinone oxidoreductase activity in rat liver and ox heart mitochondria

The reduction of ubiquinone₁ and ubiquinone₂ by rat liver or ox heart mitochondria was determined by following decrease in extinction at 275 nm or, for duroquinone and ubiquinone₀, at 265 nm. Mitochondria (0.6–1.2 mg of protein) were suspended in 2.5 ml of 150 mM-KCl–20 mM-tris–HCl–1 mM-EDTA buffer, pH 7.4 at 25°C. Succinate (2.5 μmol), antimycin A (0.25 μg), KCN (2.5 μmol) and rotenone (0.5 μg) were added. The reaction was initiated by the addition of quinone (50–100 nmol). The rate of quinone reduction by endogenous substrate in the presence of rotenone was subtracted from the rate obtained in the presence of added succinate.

Acceptor	Oxidoreductase activity (nmol/min per mg of protein)	
	Rat liver mitochondria	Ox heart mitochondria
Duroquinone	0	0
Ubiquinone ₀	32.0	19.8
Ubiquinone ₁	29.7	101.5
Ubiquinone ₂	29.1	84.2

measurements of the relative contributions of rotenone-sensitive and -insensitive pathways of quinone reduction, this method was considered to be unsuitable.

Absence of proton translocation associated with reduction of quinones by succinate

The rates of quinone reduction by mitochondria in the presence of succinate, antimycin A and rotenone are shown in Table 3. Ubiquinone₀, ubiquinone₁ and ubiquinone₂ are reduced by succinate at rates comparable with those observed when L-malate+L-glutamate were used (Table 1). Reduction of duroquinone by succinate was not demonstrable. Measurement of pH changes during reduction of quinones by succinate showed that proton translocation did not occur when duroquinone, ubiquinone₀, ubiquinone₁ and ubiquinone₂ were used with either rat liver or ox heart mitochondria. Fig. 11 illustrates this with ox heart mitochondria and ubiquinone₁: the early part of the Figure shows proton translocation coupled to succinate oxidation by oxygen with a $\rightarrow H^+/2e^-$ value of 3.8.

Discussion

This study demonstrates that the rotenone-sensitive reduction of duroquinone, ubiquinone₀, ubiquinone₁ and ubiquinone₂ by intramitochondrial NAD(P)H is associated with proton translocation in mitochondria from both ox heart and, with the exception of ubiquinone₀, rat liver. According to Mitchell (1966), proton translocation and NAD(P)H oxidation should be identical in phase, since the latter is considered to drive the former directly. This prediction could not be tested for technical reasons; the half-time for

Fig. 11. pH recordings of proton translocation by ox heart mitochondria with succinate as substrate

Conditions for pH measurements are described in detail in the Materials and Methods section. An upward deflexion corresponds to an acidification of the suspending medium. Ox heart mitochondria (approx. 10 mg of protein) were suspended in a 150 mM-KCl–3.3 mM-glycylglycine–2 mM-succinate medium, pH 7.2 at 30°C, to which rotenone (0.5 µg) was added and the pH adjusted to 7.2 with 50 mM-KOH. After 15 min the following additions were made: air-saturated 150 mM-KCl (O, ng-atoms of oxygen added are indicated) and ubiquinone₁ (20 nmol). During the time-interval shown in the Figure, KCN (0.1 µmol) and antimycin A (AA, 0.2 µg) were added and the pH was adjusted with 50 mM-HCl.

NAD(P)H oxidation on mixing with quinone was 1.0 s or less, whereas the 10–90% rise time of the pH-measuring apparatus was at least 1.5 s. Within these experimental limitations the phases of proton translocation and NAD(P)H oxidation were identical in that they were complete within 3–4 s. That the acidification of the mitochondrial incubation medium observed on oxidizing intramitochondrial NAD(P)H with quinones corresponds to proton translocation across the mitochondrial membrane from interior to exterior is indicated by the effects of carbonyl cyanide *m*-chlorophenylhydrazone; acidification previously established by NAD(P)H oxidation was rapidly reversed, and subsequent additions of quinone failed to elicit acidification (Fig. 8).

A further prediction arising from Mitchell's (1966) chemiosmotic hypothesis is that the $\rightarrow H^+/2e^-$ ratio in the presence of valinomycin and K^+ ions should be constant over a range of values for $2e^-$. This prediction was fulfilled provided that the burst of NAD(P)H oxidation caused by added quinone was sufficiently fast to escape analytical interference from proton flow back across the membrane. This condition was met if the amount of intramitochondrial NAD(P)H was not exceeded by the amount of quinone added (e.g. see Fig. 6).

A considerable body of evidence, reviewed by Kröger & Klingenberg (1967), indicates that ubiquinone₁₀ is a respiratory carrier common to oxidation of both NADH and succinate, and that the synthesis of 1 mol of ATP from ADP and P_i is

coupled to the reduction of 1 mol of ubiquinone₁₀ by NADH but not by succinate. If Mitchell's (1966) proposals for chemiosmotic coupling of respiratory-chain oxidoreductions to ATP synthesis were in essence correct, then the predicted $\rightarrow H^+/2e^-$ value would be 2.0 for quinone reduction by NADH, and zero for reduction by succinate. This prediction was fulfilled in our experiments (Table 2, Figs. 5, 7, 8 and 11), provided that correction was made for rotenone-insensitive oxidation of NAD(P)H by quinone. Objections to our use of this correction factor can be largely discounted by our results with ox heart mitochondria and ubiquinone₁ and ubiquinone₂; the sensitivity to rotenone was about 80% or more and the correction factor was therefore quite close to unity (Table 2).

The experimental conditions required to demonstrate proton translocation coupled to the oxidation of NAD(P)H by quinone bear a close resemblance to those required for ATP synthesis or active transport of anions. For instance, ATP synthesis coupled to ubiquinone₁ reduction is inhibited by rotenone (Schatz & Racker, 1966) as is proton translocation (Fig. 7). Grinius *et al.* (1971) demonstrated that anion uptake by ox heart submitochondrial particles could be coupled to the reduction of ubiquinone₀ by NADH only if rotenone was not added. The reduction of ubiquinone₀ by succinate was not coupled to anion uptake. Reduction of naphthoquinone by NAD(P)H is coupled neither to ATP synthesis (Ernster, 1961) nor to proton translocation.

The mechanisms whereby proton translocation is coupled to respiratory-chain oxidoreduction are disputed. Mitchell (1966) favours a vectorial respiratory chain arranged in loops of alternating hydrogen carriers and electron carriers. Others (e.g. Chance, 1972) prefer models whereby proton translocation is not a primary and intrinsic property of the respiratory-chain carriers but is in some way driven secondarily by an as yet uncharacterized energy-conservation mechanism. Our experiments do not discriminate between such mechanisms for respiration-driven proton translocation but it is nevertheless impressive that Mitchell (1966) effectively predicted a stoichiometric $\rightarrow H^+/2e^-$ ratio for NAD(P)H oxidation by added quinone. That Mitchell's (1966) proposed scheme for oxidoreduction loops between NADH and ubiquinone₁₀ (Scheme 1a) may have taken some liberties with the sequence of respiratory carriers need not invalidate his more general proposals for the mechanism of respiration-driven proton translocation, and Scheme 1(b), supported by our observations, is entirely consistent with the chemiosmotic hypothesis.

The exact locations at which duroquinone, ubiquinone₀, ubiquinone, and ubiquinone₂ react with the respiratory chain are not known although they appear to be close to the functional position of ubiquinone₁₀ (Ruzicka & Crane, 1971a,b; Boveris *et al.*, 1971). Our finding that duroquinone is reduced by NADH but not succinate indicates that at least this quinone reacts with the respiratory chain at a point before the pathways of NADH and succinate oxidation converge. The simplest interpretation of our observations is that the quinones used acted analogously to ubiquinone₁₀ as acceptors for the iron-sulphur-flavoprotein complexes of the NADH dehydrogenase and succinate dehydrogenase, as shown in Scheme 1(b). This implies that, of the three vectorial hydrogen carriers required in the respiratory chain for Mitchell's view (1966) of NADH oxidation by oxygen, the first is flavoprotein, the second is ubiquinone₁₀, and the third is not currently identified (Skulachev, 1971).

Further experimental evidence about the possible looped structure of the NADH dehydrogenase could come from studies of the accessibility of the membrane-bound NADH dehydrogenase to permeant and non-permeant inhibitors, and from the effects of the membrane potential on the apparent redox potential of the iron-sulphur proteins that constitute the electron-carrying link of the loop (Mitchell, 1968). These latter experiments have effectively been done (Gutman *et al.*, 1970, 1971, 1972; Albracht & Slater, 1971), and it is clear that an iron-sulphur protein at the ubiquinone end of the sequence (centre 2 of Orme-Johnson *et al.*, 1971) can lower its oxidoreduction mid-potential by 200mV or more in response to conditions that should establish a membrane poten-

tial of similar magnitude. The required polarity of membrane potential would be outer-side positive and inner-side negative in Scheme 1, and this appears to be the case (Lieberman & Skulachev, 1970). Short-circuiting of the looped NADH dehydrogenase between FMN and ubiquinone₁₀ should, on this scheme, abolish ATP synthesis normally coupled to this segment of the respiratory chain. Such a short-circuited mechanism possibly explains the changes of phosphorylation efficiency occurring in iron- or sulphate-deficient *Candida utilis* mitochondria (Light *et al.*, 1968; Haddock & Garland, 1971) that lack some, if not all, of the iron-sulphur proteins of the electron-carrying limb of the NADH dehydrogenase (Clegg *et al.*, 1969; Ragan & Garland, 1971).

This work was generously supported by the Medical Research Council. H. G. L. is in receipt of a Postdoctoral Fellowship of the Medical Research Council of Canada. We thank Mr. T. A. Gray for preparing ox heart mitochondria, Mr. A. Tucker and Mr. D. Couttie for skilled technical assistance and Miss G. Barnes for her participation in the early part of this work. The rapid-mixing fluorimeter and storage oscilloscope were provided through a grant from the Royal Society.

References

- Albracht, S. J. & Slater, E. C. (1971) *Biochim. Biophys. Acta* **245**, 508–511
- Boveris, A., Oshino, R., Erecinska, M. & Chance, B. (1971) *Biochim. Biophys. Acta* **245**, 1–16
- Brown, C. B., Russel, J. R. & Howland, J. L. (1965) *Biochim. Biophys. Acta* **110**, 640–642
- Chance, B. (1961) *Quinones Electron Transp., Ciba Found. Symp.* 327–345
- Chance, B. (1972) *FEBS Lett.* **23**, 3–20
- Chappell, J. B. (1964) *Biochem. J.* **90**, 225–237
- Clegg, R. A., Ragan, C. I., Haddock, B. A., Light, P. A., Garland, P. B., Swann, J. C. & Bray, R. C. (1969) *FEBS Lett.* **5**, 207–210
- Conover, T. E. & Ernster, L. (1962) *Biochim. Biophys. Acta* **58**, 189–200
- Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1959) *Data for Biochemical Research*, pp. 30, 36, 172, Oxford University Press, London
- Ernster, L. (1961) *Biol. Struct. Funct. Proc. IUB/IUBS Int. Symp. Ist.* **2**, 139–168
- Ernster, L., Dallner, G. & Azzone, G. F. (1963) *J. Biol. Chem.* **238**, 1124–1131
- Garland, P. B., Shepherd, D. & Yates, D. W. (1965) *Biochem. J.* **97**, 587–594
- Grinius, L. L., Guds, T. I. & Skulachev, V. P. (1971) *J. Bioenerg.* **2**, 101–113
- Gutman, M., Mayr, M. & Singer, T. P. (1970) *Biochem. Biophys. Res. Commun.* **41**, 40–44
- Gutman, M., Singer, T. P. & Beinert, H. (1971) *Biochem. Biophys. Res. Commun.* **44**, 1572–1578

- Gutman, M., Singer, T. P. & Beinert, H. (1972) *Biochemistry* **11**, 556–562
- Haddock, B. A. & Garland, P. B. (1971) *Biochem. J.* **124**, 155–170
- Heytler, P. G. (1963) *Biochemistry* **2**, 357–361
- Klingenberg, M. & Slenczka, W. (1959) *Biochem. Z.* **331**, 486–517
- Kröger, H. & Klingenberg, M. (1967) *Curr. Top. Bioenerg.* **2**, 151–193
- Lawford, H. G. & Garland, P. B. (1972) *Biochem. J.* **129**, 47P–48P
- Liberman, E. A. & Skulachev, V. P. (1970) *Biochim. Biophys. Acta* **216**, 30–42
- Light, P. A., Ragan, C. I., Clegg, R. A. & Garland, P. B. (1968) *FEBS Lett.* **1**, 4–8
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mitchell, P. (1966) *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation*, Glynn Research Ltd., Bodmin
- Mitchell, P. (1968) *Chemiosmotic Coupling and Energy Transduction*, Glynn Research Ltd., Bodmin
- Mitchell, P. & Moyle, J. (1967a) *Biochem. J.* **104**, 588–600
- Mitchell, P. & Moyle, J. (1967b) *Biochem. J.* **105**, 1147–1162
- Mitchell, P. & Moyle, J. (1967c) in *Biochemistry of Mitochondria* (Slater, E. C., Kaniuga, Z. & Wojtczak, L., eds.) pp. 53–62, Academic Press, New York and London
- Orme-Johnson, N. R., Orme-Johnson, W. H., Hansen, R. E. & Beinert, H. (1971) *Biochem. Biophys. Res. Commun.* **44**, 446–450
- Ragan, C. I. & Garland, P. B. (1971) *Biochem. J.* **124**, 171–187
- Ruzicka, F. J. & Crane, F. L. (1971a) *Biochim. Biophys. Acta* **223**, 71–85
- Ruzicka, F. J. & Crane, F. L. (1971b) *Biochim. Biophys. Acta* **223**, 221–223
- Schatz, G. & Racker, E. (1966) *J. Biol. Chem.* **241**, 1429–1438
- Skulachev, V. P. (1971) *Curr. Top. Bioenerg.* **4**, 127–190
- Slater, E. C. (1966) *Flavins Flavoproteins, Proc. Symp.* **487–510**
- Smith, A. L. (1967) *Methods Enzymol.* **10**, 81–86
- Strittmatter, P. (1964) in *Rapid Mixing Techniques in Biochemistry* (Chance, B., Eisenhardt, R. H., Gibson, Q. H. & Longberg-Holm, K., eds.), pp. 71–84, Academic Press, New York and London
- Ziegler, D. M. (1961) *Biol. Struct. Funct. Proc. IUB/IUBS Int. Symp. Ist.* **2**, 253–264