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Measurement of the proton-motive stoichiometry of the respiratory chain of rat liver mitochondria: the effect of *N*-ethylmaleimide

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The apparent proton-motive stoichiometry as measured by the oxygen-pulse technique in KCl medium is depressed by the rapid uptake of inorganic phosphate, unless endogenous phosphate is depleted or uptake is inhibited. In sucrose or choline chloride media, where the internal pH is more acid than in KCl media, uptake may be greatly diminished. In the absence of significant phosphate uptake, the observed stoichiometry of around 8, obtained with no added substrate or respiratory inhibitors, appears to be characteristic of NADH oxidation without significant participation of the proton-translocating NAD(P) transhydrogenase. A mechanistic stoichiometry of at least 8 is indicated.

Introduction

When the oxygen-pulse method is used to measure $\leftarrow H^+/O$ ratios in suspensions of rat liver mitochondria, it has long been recognised that the presence of inorganic phosphate in the suspending medium may lead to serious underestimation of $\leftarrow H^+$. Mitchell and Moyle [1] found that by slowing down the phosphate porter at low temperature it was possible to record and extrapolate a phosphate-induced decay of ΔpH. It was concluded that the $\leftarrow H^+/O$ values of near 6.0 obtained both at 25° and at 5°C in the absence of added P_i were likely to be correct, especially in view of the fact that additions of P_i approximately equivalent to the endogenous content depressed apparent $\leftarrow H^+/O$ only slightly.

Later when Scholes and Mitchell [2] found a stoichiometry of 8 using *Paracoccus denitrificans*, this was interpreted as indicating transfer of reducing equivalents from NADPH through the proton-translocating transhydrogenase. When, therefore, with rat liver mitochondria either in the presence of *N*-ethylmaleimide, or in low $[K^+]$ media (instead of the 150 nM KCl used in earlier experiments), it was found that the same value of 8 was obtained [3], and that the transhydrogenase stoichiometry indeed appeared to be 2 [3,4], it was reasonable to apply the same interpretation. It was suggested that, since *N*-ethylmaleimide was known to inhibit succinate dehydrogenase and NAD-linked enzymes strongly, but not the NADP-linked isocitrate dehydrogenase, operation of the complete respiratory chain fron NADPH was thereby promoted, with endogenous isocitrate re-reducing $NADP^+$.

This interpretation was rejected by Brand, Reynafarje and Lehninger [5,6] who found that re-

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Abbreviations: $\leftarrow H^+/O$, number of protons translocated outwards across the mitochondrial inner membrane per oxygen atom consumed; $\rightarrow K^+/O$ (or $\rightarrow Ca^{2+}/O$), number of potassium (or calcium) ions taken up by the mitochondria per oxygen atom consumed; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; P_i, inorganic phosphate.

moval of P_i by anaerobic washing resulted in an increase of the so-called $\leftarrow H^+$/site ratio from 2 to near 3 in O_2 pulse experiments, with no further stimulation by *N*-ethylmaleimide. Subsequent addition of 0.1 mM P_i depressed the observed ratio to 2. Both *N*-ethylmaleimide and mersalyl raised the stoichiometry over the same concentration range as was required to inhibit the P_i porter. Lowering the temperature to 5°C raised the apparent stoichiometry. Using a rate method in LiCl medium, they found that *N*-ethylmaleimide increased $\leftarrow H^+$/site without affecting $\rightarrow K^+$/site. They concluded that endogenous P_i levels were sufficient to cause serious underestimation of $\leftarrow H^+/O$ in the absence of *N*-ethylmaleimide, and that *N*-ethylmaleimide did not affect the actual $\leftarrow H^+/O$ stoichiometry.

Moyle and Mitchell pointed out [7] that under the strictly defined conditions when mitochondria were pulsed with O_2 in the presence of rotenone and antimycin, with diaminodurene or added ferrocytochrome *c* as reductants at the cytochrome oxidase level, no *N*-ethylmaleimide-sensitive loss of extramitochondrial protons occurred, and $\rightarrow K^+/O$ vaues were consistent with observed $\leftarrow H^+/O$. In a general survey of respiratory chain stoichiometries under a wide range of conditions [8], attention was again drawn to the fact that charge translocation (measured as $\rightarrow K^+$ in the presence of valinomycin) was always in excellent agreement with apparent $\leftarrow H^+/O$ in media containing only endogenous phosphate. Support for the view that transhydrogenase was operating to give $\leftarrow H^+/O > 6$ was provided by the observation that, in the presence of 10 mM Mg^{2+} (an inhibitor of transhydrogenase) or malate (NAD-linked), apparent $\leftarrow H^+/O$ fell from 8 to near 6, while with added citrate to increase the level of intramitochondrial isocitrate (NADP-linked), a value of 8 was maintained [8].

We here present the results of our continued investigations of this problem, which has been a serious hindrance to the formation of a consensus regarding the proton-motive stoichiometry of the respiratory chain. It is difficult to see how substantial progress can be made towards understanding the molecular mechanisms involved until this, still controversial, question of the $\leftarrow H^+/O$ ratios is resolved.

Materials and Methods

Isolation of rat liver mitochondria and measurement of $\leftarrow H^+/O$ by the O_2 pulse method were as previously described [9]. Mitochondria were depleted of P_i by anaerobic incubation for 30 min at 6 mg protein/ml in specially made glass centrifuge tubes with ground-in glass stoppers. After spinning down, the mitochondrial pellet was resuspended and transferred immediately to the electrode vessel. P_i was measured using a modification of the Fiske and Subbarow method with $SnCl_2$ as reductant [10]. Ca^{2+} uptake was measured using a custom-made 6 mm diameter electrode (Russell pH Ltd., Auchtermuchty, Fife, U.K.). NAD^+ was assayed by the method of Klingenberg [11], $NADP^+$ using the isocitric dehydrogenase method of Ciotti and Kaplan [12] and citrate according to Dagley [13]. These assays were performed on samples rapidly and anaerobically expelled by nitrogen pressure from a stirred flask into glass centrifuge tubes containing perchloric acid, also under nitrogen. The sampling tube from the flask was continuously flushed with nitrogen.

Reagents were used as commercially supplied, and were of analytical grade or of the highest purity available, except for the following. α-Methylisocitrate was prepared at the Plymouth Polytechnic by the method of Plaut et al. [14]. FCCP was a gift from Dr. P.G. Heytler of E.I. du Pont de Nemours and Co. Inc. (Wilmington, Delaware, U.S.A.)

Results

H^+/O measurements

Table I shows typical results of oxygen pulse experiments in KCl medium where, after an anaerobic wash to remove endogenous P_i, the P_i-depleted mitochondria were compared with untreated mitochondria from the same original preparation. Apparent $\leftarrow H^+/O$ was significantly increased after P_i depletion, becoming less susceptible to enhancement by *N*-ethylmaleimide. Restoration of phosphate restored both the original low apparent $\leftarrow H^+/O$ and the *N*-ethylmaleimide susceptibility. Much of the endogenous calcium was removed along with the phosphate, leaving a deficiency of mobile counter-ions. The addition of

TABLE I

EFFECT OF P_i DEPLETION ON $\leftarrow H^+/O$ (SEE METHODS)

The medium contained 150 mM KCl, 3.3 mM glycylglycine, 30 μg carbonic anhydrase per ml, mitochondria at a final concentration of 6.0 mg protein per ml and 40 μM $CaCl_2$ as indicated in a total volume of 3.3 ml at a final pH of 7.0–7.1. After 20 min anaerobic equilibration at 25°C, 50 μl pulses of air-saturated 150 mM KCl were injected. Subsequent pulses were at 10 min intervals. Additions: NEM (*N*-ethylmaleimide) at 33 μmol per g protein or 500 nmol potassium phosphate were added 5 min before the second and third pulses. The contents of the electrode vessel were assayed for inorganic phosphate at the end of each experiment.

Mitochondria	Additions	Observed $\leftarrow H^+/O$	Total P_i (nmol/ml)
Preparation A			
untreated	(i) –	5.9	
	(ii) NEM	7.2	227
P_i-depleted,	(i) –	7.0	
supplemented with Ca^{2+}	(ii) NEM	7.1	74
Preparation B			
untreated	(i) –	6.1	
	(ii) P_i	5.7	
	(iii) P_i + NEM	7.4	459
P_i-depleted,	(i) –	7.1	
supplemented	(ii) P_i	6.0	
with Ca^{2+}	(iii) P_i + NEM	7.3	264

calcium chloride restored it to about its original level. With untreated mitochondria, after about 40 min in the electrode vessel, endogenous inorganic phosphate varied in different preparations between 220 and 350 nmol/ml. Samples freshly taken from the mitochondrial stock suspension stored at 0°C contained significantly less. The value for preparation B includes 150 μM added phosphate. We conclude with Brand et al. [5] that P_i depletion or inhibition of the P_i porter with *N*-ethylmaleimide have similar effects on observed $\leftarrow H^+$.

In a similar type of experiment using Ca^{2+} as counter-ion (Fig. 1) we found that, while apparent $\leftarrow H^+$ was increased by *N*-ethylmaleimide, $\rightarrow Ca^{2+}$ was completely unchanged. Furthermore, only in the presence of *N*-ethylmaleimide was the $\leftarrow H^+/\rightarrow Ca^{2+}$ ratio close to the expected value of 2.0. Hence it is confirmed that the increase in apparent $\leftarrow H^+/O$ with *N*-ethylmaleimide involves an electroneutral process rather than an

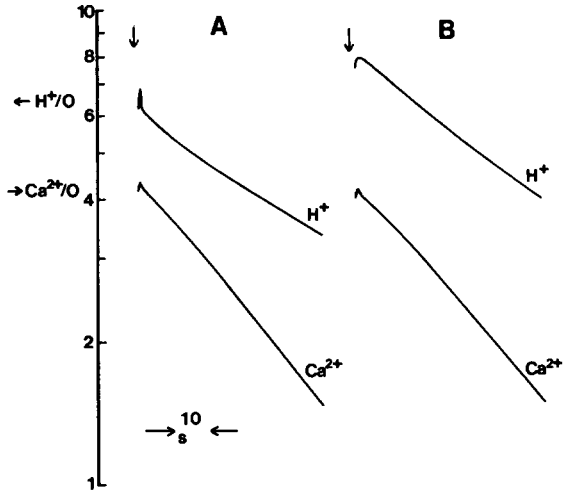

Fig. 1. Time-courses of $\leftarrow H^+/O$ and $\rightarrow Ca^{2+}/O$ plotted semi-logarithmically following pulses of O_2 added at the arrows. Conditions were as for Table I, but with oligomycin present at 1 mg per g protein and with 100 μM added $CaCl_2$ (A) Control; (B) *N*-ethylmaleimide present.

increase in the actual $\leftarrow H^+/O$ stoichiometry. The rapid initial H^+ spike frequently seen in the absence of *N*-ethylmaleimide may be the response of the recording system to the protons rapidly taken up with P_i, although the full extent is not seen. This latter point was further investigated by using lower concentrations of *N*-ethylmaleimide, so that any P_i-induced collapse would be slow enough to be monitored by the pH recording system. We would anticipate that if *N*-ethylmaleimide changed the effective respiratory chain length, intermediate concentrations might give intermediate $\leftarrow H^+/O$ values, but with normal pH decay characteristics, whereas an uptake of phosphate might be seen as a rapid initial decay. Our findings were in accordance with the latter expectation (Fig. 2). A similar effect of low *N*-ethylmaleimide concentrations was seen in the presence of rotenone with duroquinol as substrate.

Nicotinamide nucleotide assays.

While it may be agreed that the $\leftarrow H^+/O$ values of 6 obtained in KCl medium in the absence of *N*-ethylmaleimide are incorrect and the actual stoichiometry is nearer 8, it might still be argued that a figure of 6 is nevertheless correct for NADH oxidation, but that in the absence of rotenone

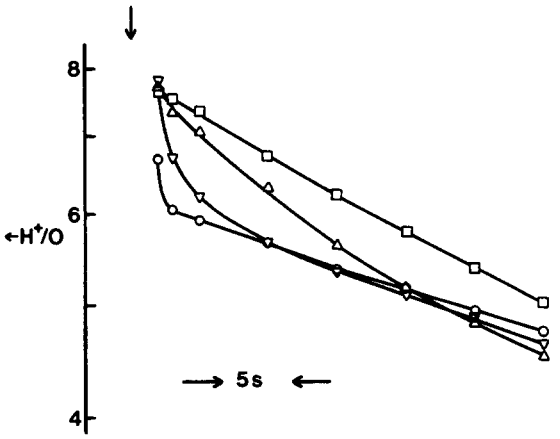

Fig. 2. Effect of low concentrations of *N*-ethylmaleimide on ← H^+/O and initial pH_o decay. Conditions were as for Table 1, except that the concentration of glycylglycine was reduced to 1 mM, and 100 μM added $CaCl_2$ was present. Oxygen injections were at intervals of 10 min. *N*-ethylmaleimide additions were as follows: ○, none; ▽, 10 μmol per g protein; △, 13 μmol per g protein; □, 16 μmol per g protein.

what is measured is the stoichiometry for NADPH oxidation. Mitchell and Moyle investigated the redox changes of the respiratory carriers following an oxygen pulse [15], but did not differentiate between NADP and NAD. We have therefore extracted and assayed the individual nicotinamide nucleotides.

Fig. 3 shows the time-course of oxidation of nicotinamide nucleotides following a pulse of oxygen in KCl medium. Sampling took about 3 s. The first sample following the pulse, plotted at 3 s, was taken between 1.5 and 4.5 s. The primary reductant in every case appears to be NADH (open circles). The re-reduction of NAD^+ in A was quantitatively accounted for by a fall in endogenous citrate. Addition of α-methylisocitrate, a specific inhibitor of the NADP-linked isocitrate dehydrogenase [14] slowed the overall nicotinamide nucleotide re-reduction and revealed oxidation of NADPH (B). In the prescence of *N*-ethylmaleimide, while nicotinamide nucleotide re-reduction was slowed to about the same extent as with α-methylisocitrate, no NADPH oxidation could be seen, suggesting that the *N*-ethylmaleimide inhibition is predominantly at the level of transhydrogenase (cf. Ref. 16). This inhibition is evident in a comparison of the FCCP-containing

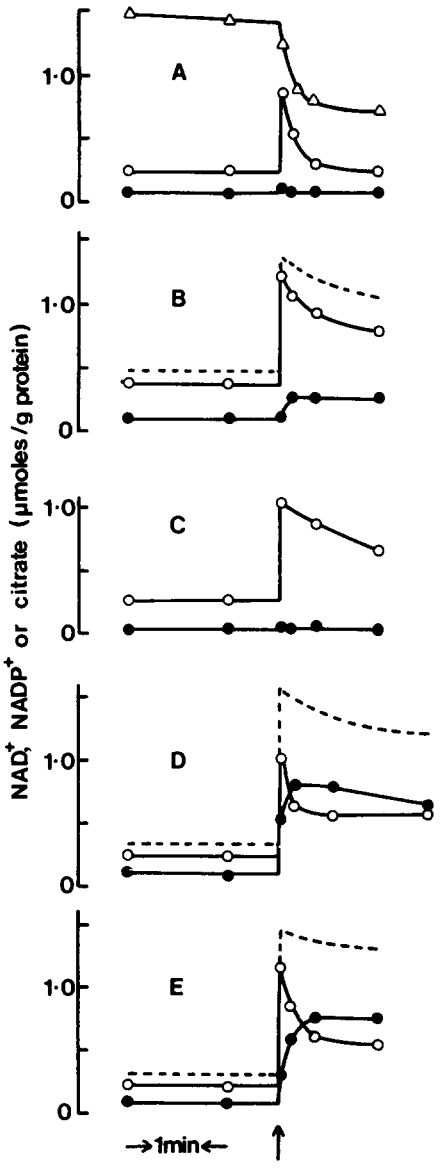

Fig. 3. Time-courses of nicotinamide nucleotide oxidation on pulsing with oxygen. The medium contained 150 mM KCl, 3.3 mM glycylglycine and 1 mM EDTA as the potassium salts, mitochondria to a final concentration of 6 mg protein per ml at pH 7.0–7.1, and either 10 μg valinomycin per g protein (A–C) or 1 μM FCCP (D,E). Total volume: 24 ml. After 20 min anaerobic incubation, oxygen (1.5 μg atom per g protein) was injected as air-saturated 150 mM KCl at the arrow, 3 ml samples (see Methods) were taken at the time indicated. ○, $NADH^+$; ●, $NADP^+$; — — — total oxidised nicotinamide nucleotides; △, endogenous citrate. A, control; B, +0.5 mM α-methylisocitrate; C, +33 μmol (*N*-ethylmaleimide) per g protein; D, +FCCP; E, +FCCP+ *N*-ethylmaleimide.

TABLE II

DISTRIBUTION OF CITRATE IN ANAEROBIC MITOCHONDRIAL SUSPENSIONS

Media, additions and experimental procedures were as described for Fig. 3 and Fig. 4, except that, after 18 min anaerobic incubation, KCN to 1 mM was added anaerobically. After 20 min, samples were taken and transferred into $HClO_4$, either directly, or after first centrifuging out the mitochondria. Concentrations are given as μmol per g protein ± S.D. NEM, *N*-ethylmaleimide.

Medium	Additions	Total citrate	Extramitochondrial citrate	% Released
KCl		1.21 ± 0.29 (8)	0.20 ± 0.10 (8)	17
	NEM	0.95 ± 0.22 (4)	0.28 ± 0.16 (4)	29
	FCCP	1.18, 1.42	1.22, 1.22	94
Sucrose		1.69 ± 0.12 (4)	1.72 ± 0.10 (4)	102

experiments D and E. Here presumably there is a more complete collapse of the protonic electrochemical potential than in the presence of valinomycin, allowing full redox equilibration of the nicotinamide nucleotides. The reason for the slow overall rate of reduction here is evident from Table II. In the presence of FCCP, or when the mitochondria were suspended in sucrose/choline chloride, the endogenous citrate was released into the outer medium. Fig. 4 illustrates the slow reduction in sucrose/choline chloride medium and the effect of added citrate. *N*-ethylmaleimide was without significant effect, and identical results were obtained in a 150 mM choline chloride medium (not shown). Further confirmation that the final, slow redox equilibration between NAD and NADP occurred through the transhydrogenase, rather than by a non-proton-translocating route, was obtained by adding FCCP or ATP 4 min after the oxygen pulse in a sucrose/choline chloride medium. FCCP induced a rapid equilibration, while with ATP, NAD became more oxidised and NADP became correspondingly more reduced by reversed electron transfer.

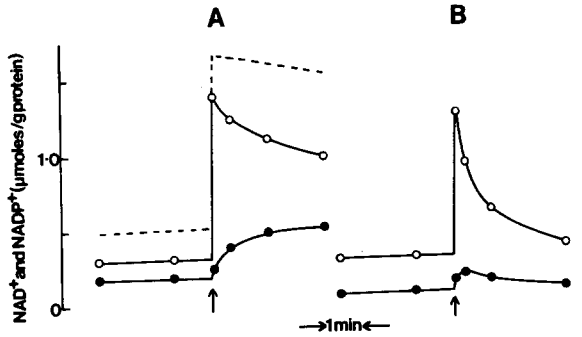

Fig. 4. Time-courses of nicotinamide nucleotide oxidation on pulsing with oxygen in low [K^+] medium. The medium contained 240 mM sucrose, 10 mM choline chloride, 3.3 mM choline glycylglycine, 1 mM choline EDTA, mitochondria to a final concentration of 6.0 mg protein per ml and 100 μg valinomycin per g protein. Total volume: 24 ml (pH 7.0–7.1). Oxygen (1.5 mg atom O per g protein) was injected as air-saturated 240 mM sucrose/10 mM choline chloride. ○, NAD^+, ●, $NADP^+$; — — — total oxidised nicotinamide nucleotide. A, no additions; B, plus 10 mM choline citrate.

Discussion

These results, while fully supporting the view that *N*-ethylmaleimide increases ← H^+/O by preventing rapid uptake of phosphate, leave several questions unanswered. (1) Why did Mitchell and Moyle [8] invariably see a close correlation between ← H^+/O and → K^+/O? (2) Why was NEM found to be ineffective in the presence of antimycin [7]? (3) Why does ← H^+/O appear to be lower with Mg^{2+} present? (4) Why does ← H^+/O appear to be lower with malate present?

We shall attempt to answer these questions in the following discussion. However, it will first be necessary to draw attention to a characteristic of pH equilibration by permeant acids which may not at first be obvious. In general the pH difference between two solutions of an acid HA and its anion A^-,

$$pH_I - pH_O = \log \frac{\{HA\}_O \{A^-\}_I}{\{HA\}_I \{A^-\}_O}$$

where braces denote activities and the subscripts I and O indicate the phases which we may call inner and outer, respectively. If these phases are separated by a membrane that is selectively permeable by HA so that at equilibrium $\{HA\}_O = \{HA\}_I$, then,

$$pH_I - pH_O = \log \frac{\{A^-\}_I}{\{A^-\}_O}$$

or

$$\log\{A^-\}_I = \Delta pH + \log\{A^-\}_O \text{ (see Fig. 5)}$$

In the case of an anaerobic mitochondrial suspension in a buffered medium subjected to a small pulse of oxygen, the final term may be regarded as constant, owing to the large ratio of external to internal volumes, and may be eliminated by an appropriate choice of units of activity as in Fig. 5. Since in this case $\{HA\}_O$ and thus $\{HA\}_I$ are also relatively unchanged, it is evident that changes in $\{A^-\}_I$ brought about by changing ΔpH must be accompanied by corresponding uptake or release of the protonated acid. Obviously this is not intended to give a precise indication of the extent of phosphate uptake which in any case is effectively accompanied by more than one proton at pH 7.0. A more detailed and quantitative treatment can readily be developed from the rationale outlined by Mitchell [17]; see also West [18].

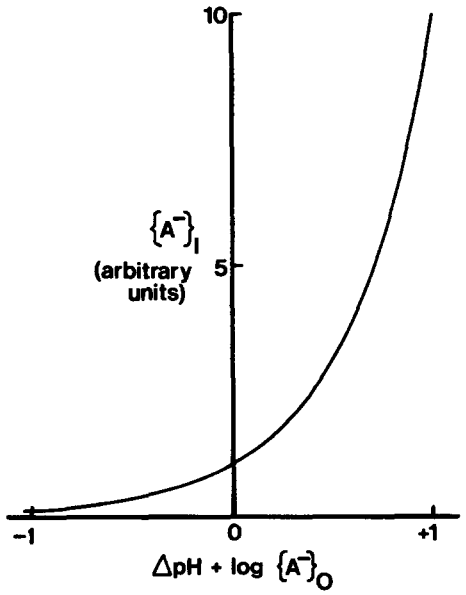

Fig. 5. General relationship of trans-membrane ΔpH and distribution of the anion of a permeant acid HA.

Returning to the question concerning $\rightarrow K^+/O$ measurements, it is clear from Fig. 5 that with an oxygen pulse giving an increase in ΔpH of 0.5 to 1 unit, the extent of proton uptake will be powerfully influenced by the starting ΔpH, i.e., the ΔpH in State 5. In media containing little or no added potassium, especially when valinomycin and a calcium chelator are present, there is an inevitable tendency for the internal pH to fall substantially as protons are taken up in exchange for endogenous potassium and calcium ions. For example, in 150 mM choline chloride + 1 mM EGTA at $pH_O = 7.0$, an approximate calculation, based on the observed proton uptake and the internal buffering power, suggests that after 20 min anaerobic incubation pH_I would have fallen by almost one unit. Since it was necessarily under these conditions that the comparisons of $\leftarrow H^+/O$ and $\rightarrow K^+/O$ were carried out by Mitchell and Moyle [8], it is not surprising that, after allowing the potassium to equilibrate, very little phosphate uptake occurred on pulsing with oxygen. In the LiCl medium used by Reynnafarje and Lehninger [6], however, uptake of Li^+ in exchange for H^+, presumably on the sodium/proton antiporter [19], counteracts the valinomycin-induced H^+ uptake. We have recently confirmed, using the O_2 pulse method, that in LiCl medium apparent $\leftarrow H^+/O$ was increased by *N*-ethylmaleimide while $\rightarrow K^+/O$ remained constant.

A further obvious implication of Fig. 5 is that, in the presence of permeant acids, decreasing the extent of proton ejection will lead to a more than proportionate decrease in acid uptake. Thus, for a given pulse size, it is more difficult to detect the phosphate effect with the respiratory chain partly inhibited with rotenone or antimycin than when using the complete chain. Moyle and Mitchell's measurements done in the presence of antimycin [7] were complicated by the need to correct for a significant antimycin-insensitive respiratory leak rate, and by the fact that the outer mitochondrial membrane is impervious to cytochrome *c*, so that only the small fraction of the mitochondrial population with damaged outer membranes was supplied with reductant (see Wikström and Casey [20]). We recently repeated these measurements in

KCl medium, with myxothiazol present as well as antimycin, greatly decreasing the respiratory leak rate, and using ferrocyanide or hexammineruthenium (II) as substrate. We found an apparent $\leftarrow H^+/O$ of 1.74 ± 0.14 S.D. ($n = 19$) [21]. In the absence of *N*-ethylmaleimide, apparent $\leftarrow H^+/O$ was 1.48 ± 0.15 S.D. ($n = 17$) (West, I.C., Moody, A.J. and Mitchell, R., unpublished results).

In magnesium-containing media with low $[K^+]$, the low values of both $\leftarrow H^+/O$ and $\rightarrow K^+/O$ may result from the competitive inhibition by Mg^{2+} of valinomycin-mediated K^+ transport [22]. In fact, in this laboratory (Mitchell, R., unpublished results) $\leftarrow H^+/O$ and $\rightarrow K^+/O$ were increased by at least 0.5 by increasing $[K^+]$ from 0.7 to 2.0 mM in a medium containing 240 mM sucrose + 10 mM $MgSO_4$. Little or no NADPH oxidation was seen unless FCCP was present.

In experiments similar to that shown in Fig. 4A but with 10 mM choline L-malate present, it was found that both NAD and NADP became oxidised to the extent of about 1.5 μmol per g protein during the 20 min preincubation period, and the oxidation step on injecting oxygen was slightly smaller. It seems probable that the anaerobic oxidation of the nucleotides occurred by a reversal of the fumarase and succinate dehydrogenase reactions, and that on pulsing with oxygen, some of the reducing equivalents were supplied from succinate formed during the preincubation, thus lowering $\leftarrow H^+/O$ [8].

It seems clear that our published values of $\leftarrow H^+/O$ obtained in KCl medium in the absence of *N*-ethylmaleimide [1–4,7–9] have been underestimated owing to rapid turnover of the phosphate porter, and that these measurements should be performed under conditions where the phosphate effect is minimised. At the point where acidification of the outer medium is at its peak, normally about two seconds after the oxygen pulse, experiments of the type shown in Fig. 4A, where re-reduction of $NADP^+$ is slow, indicate that very little turnover of the transhydrogenase has occurred. It is true that the observed decay will be influenced by the slowly continuing transfer of reducing equivalents from NADPH, changing the extrapolation slightly, but a correction of the extrapolated $\leftarrow H^+/O$ on the basis of a revised decay rate (assuming $\leftarrow H^+/2e = 2.0$ for the transhydrogenase) proved to be insignificant.

We conclude that our previous estimation of the proton stoichiometry for NADH oxidation of 6.0 was incorrect, and that the actual value is at least 8.0.

Acknowledgements

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