Proton translocation by cytochrome oxidase in (antimycin+myxothiazol)-treated rat liver mitochondria using ferrocyanide or hexammineruthenium as electron donor

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1. When O_2 was injected into an anaerobic suspension of valinomycin-treated rat liver mitochondria inhibited with rotenone, antimycin, and myxothiazol, a small amount of O_2 (0.23–0.33 ng-atom of O/mg of protein) was reduced extremely rapidly (within the 2 s time-resolution of the oxygen electrode). The subsequent steady-state rate of flow of electrons to oxygen was very low [< 3 nequiv. $\cdot s^{-1} \cdot (g \text{ of mitochondrial protein})^{-1}$]. 2. In the presence of valinomycin there was a rapid ejection of protons synchronous with the rapid phase of O_2 consumption corresponding to 0.38–0.61 nequiv. of H^+ $\cdot (mg \text{ of mitochondrial protein})^{-1}$. When valinomycin was replaced by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) there was a rapid alkalification of the medium corresponding to 0.20–0.42 nequiv. of H^+ $\cdot (mg \text{ of mitochondrial protein})^{-1}$. 3. When 2 mM- $Fe(CN)_6^{4-}$ was present to re-reduce endogenous cytochrome *c*, O_2 consumption was still biphasic but the second phase of O_2 consumption was very much more rapid [600 nequiv. $\cdot s^{-1} \cdot (g \text{ of protein})^{-1}$], and resulted in the virtually complete consumption of the O_2 in the pulse within 4 s. With 60 μM - $Ru(NH_3)_6^{2+}$ as reductant, O_2 consumption was even faster [1200 nequiv. $\cdot s^{-1} \cdot (g \text{ of protein})^{-1}$]. 4. In a medium containing 150 mM-choline chloride with $Ru(NH_3)_6^{2+}$ as reductant, the proton per reducing equivalent stoichiometry ($\Delta H^+_{\text{O}}/e^-$) was +0.95 in the presence of valinomycin and –0.94 in the presence of FCCP. 5. In choline chloride medium containing $Ru(NH_3)_6^{2+}$ and valinomycin, there was an uptake of K^+ ions corresponding to 1.86 K^+/e^- . It is concluded that nearly 1 proton is translocated outwards through cytochrome oxidase per oxidizing equivalent injected in this medium. 6. In low ionic strength sucrose-based medium, with $Ru(NH_3)_6^{2+}$ as reductant, $\Delta H^+_{\text{O}}/e^-$ was 1.05 in the presence of valinomycin, and –0.71 in the presence of FCCP. It is concluded that the translocation of protons is accompanied by net acid production in this medium.

INTRODUCTION

It is widely agreed that electron flow through cytochrome *c* oxidase of rat liver mitochondria, from cytochrome *c* to oxygen, is so arranged that it causes a displacement of negative charge into the mitochondria (or the equivalent displacement of positive charge out of the mitochondria), and that the protons required for the formation of water are taken from the matrix, as originally proposed by Mitchell (1966). Wikström (1977) reported that, in addition to the inward translocation of one negative charge per electron transferred to oxygen, there is an outward translocation of one proton per electron, bringing the total inward charge translocation stoichiometry to two negative charges (or their equivalent) per electron transferred. According to this mechanism there would be two protons removed from the matrix, and one proton appearing in the extra-mitochondrial medium, per electron equivalent traversing the enzyme. Evidence for this mechanism has been provided in a number of papers both by Wikström and others (see

Wikström *et al.*, 1981a, for review). However, there have been a number of arguments raised against this proposed proton translocation, and some experimental results have been obtained that were interpreted as being incompatible with it (for reviews see Mitchell, 1980; Papa & Lorusso, 1984).

One experimental approach to this question involves the use of oxygen pulses to initiate electron flow from electron-donating reductants of cytochrome *c* in anaerobic suspensions of intact mitochondria inhibited with antimycin. In a previous publication from this laboratory (Moyle & Mitchell, 1978), it was reported that the results of such experiments were incompatible with the translocation of protons by cytochrome oxidase. Proton translocation was indeed observed in all media tested, corresponding to about 0.5 H^+/e^- , but it was ascribed to another segment of the respiratory chain. When the proton-conducting uncoupler FCCP was included in place of valinomycin, and EDTA (or EGTA) was present to prevent Ca^{2+} movements, H^+ ions were expected to be much more mobile across the mitochondrial membrane

Abbreviations used: val, valinomycin; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $\Delta H^+_{\text{O}}/e^-$ (val), change in number of H^+ ions in the medium per electron transferred to O_2 measured in the presence of valinomycin; $\Delta H^+_{\text{O}}/e^-$ (FCCP), as preceding but with FCCP replacing valinomycin; $\Delta K^+_{\text{O}}/e^-$ (val), change in number of K^+ ions in the medium per electron transferred to O_2 measured in the presence of valinomycin; $\leftarrow H^+/e^-$, number of protons translocated outwards across the mitochondrial membrane per electron transferred to O_2 ; $\rightarrow H^+/e^-$, as preceding but translocated inwards; TMPD, *NNN'*-tetramethyl-*p*-phenylenediamine; $TMPDH^+$, protonated TMPD; $TMPD^+$, the oxidized free radical form of TMPD; UHDBT, 5-(*n*-undecyl)-6-hydroxyl-4, 7-dioxobenzothiazole.

than any other ion, and were therefore expected to flow into the mitochondria in an amount closely corresponding to the amount of electrons transferred to oxygen. In all media examined, it was found that less than the expected number of protons disappeared from the outer medium in these FCCP experiments. It was further noted that the number of protons appearing outside in experiments using valinomycin, corresponded rather closely to twice the number of these missing protons. It was therefore suggested that some of the ferricyanide ions, produced in high concentration on the outer surface of the mitochondria, were re-reduced by a protonated reductant (perhaps ubiquinol or the protonated semiquinone radical) involved in a genuinely proton translocating segment of the respiratory chain above the antimycin block (Mitchell, 1980).

In experiments of this sort, allowance has to be made for the fact that antimycin does not completely block the flow of electrons from ubiquinol to cytochrome *c*. This antimycin-insensitive leak ($80 \text{ nequiv.} \cdot \text{s}^{-1} \cdot \text{g}^{-1}$) corresponds to a significant fraction of the total rate of O_2 consumption supported by ferrocyanide. In previous publications from this laboratory (Moyle & Mitchell, 1978; Mitchell & Moyle, 1979) data were corrected by subtracting from the observed quantity of protons that fraction presumed to be due to antimycin-insensitive respiration. With the introduction of myxothiazol (Trowitzsch *et al.*, 1980; von Jagow & Engel, 1981) which, when used in conjunction with antimycin A, slows electron flow through the cytochrome *c* reductase to $< 3 \text{ nequiv.} \cdot \text{s}^{-1} \cdot \text{g}^{-1}$, it became possible to do this type of experiment without the need to correct for the effects of residual inhibitor-insensitive respiration.

In the present paper we describe O_2 -pulse experiments similar to those done previously, but using rotenone + antimycin + myxothiazol to block the oxidation of reductants more completely than before, and employing ferrocyanide or hexammineruthenium (II) as electron donor in various suspension media. Our observations show that, under certain conditions, very nearly 1.0 H^+ ion is translocated from the matrix to the outer medium per electron travelling from extra-mitochondrial electron donors to oxygen, and that the equivalent of nearly 2.0 negative charges pass inwards per electron transferred to oxygen. These results are not explicable by the model previously suggested (Mitchell, 1980), but are compatible with the translocation of protons by cytochrome oxidase, as described by Wikström (1977).

EXPERIMENTAL

The methods of isolating rat liver mitochondria, of measuring and recording pH and making additions of anaerobic solutions of ionophores, inhibitors and standard acid or alkali (50 mM-HCl , KOH , or choline hydroxide in water) were as previously described (Mitchell *et al.*, 1979). (When freeing standard acid and standard K^+ solutions of O_2 by evacuating in Thunberg tubes, care was taken to evacuate for the minimum effective time, to minimize the loss of water vapour.)

The O_2 content of water in equilibrium with air (saturated with water vapour) at 25.0°C and 760 mm barometric pressure was taken to be $516 \text{ ng-atom of O/ml}$ (Montgomery *et al.*, 1964). The O_2 contents of solutions of 150 mM-KCl , $150 \text{ mM-choline chloride}$ and $250 \text{ mM-sucrose/10 mM-choline chloride}$ were found to be 498 ,

502 and $474 \text{ ng-atom of O/ml}$ respectively, by comparing the O_2 content of these solutions with that of water, as previously described (Mitchell *et al.*, 1979). The O_2 values were corrected for barometric pressure at the time of the experiment.

All experiments were carried out at 25°C and $\text{pH } 7.0\text{--}7.1$. Anaerobic medium (3.3 ml) was introduced into the stirred electrode vessel under N_2 , and sufficient mitochondrial suspension was added to give 6.0 mg of mitochondrial protein/ml. Care was taken to allow residual oxygen to be consumed before adding valinomycin, so as to avoid extensive uptake of K^+ ions. An anaerobic solution of $132 \text{ mM-K}_4\text{Fe(CN)}_6$ or $20 \text{ mM-hexammineruthenium (III) chloride}$ was added approx. 5 min after the mitochondria. A further 10 min preincubation was allowed before the addition of rotenone, antimycin, myxothiazol and *N*-ethylmaleimide. This allowed stabilization of metabolite concentrations and ionic gradients and, when using the ruthenium compound, it allowed the slow reduction of the ruthenium by endogenous mitochondrial substrates. *N*-Ethylmaleimide was added 5 min before the first O_2 pulse. A second O_2 pulse was normally given 10 min after the first; but in this paper only first pulses are reported. It is important not to use excess antimycin as that increases the proton conductance. Rotenone, antimycin and myxothiazol were added as anaerobic solutions in ethanol as late as possible in the preincubation, because the very slow leakage of O_2 into our stirred electrode vessel was of the same order of magnitude as the very small residual electron flux obtained with this combination of inhibitors ($< 3 \text{ nequiv.} \cdot \text{s}^{-1} \cdot \text{g}^{-1}$), and could lead to a gradual oxidation of the reducing agents. Solutions of $\text{K}_4\text{Fe(CN)}_6$, hexammineruthenium chloride and *N*-ethylmaleimide were prepared on the day of use.

When measuring K^+ movements in media of low K^+ content, calibrating amounts of KCl were added immediately before and after the O_2 pulse, although the sensitivity of the K^+ electrode was found to be theoretical in the K^+ concentration range of the experiments ($0.6\text{--}0.8 \text{ mM}$). O_2 was added as $50 \mu\text{l}$ of air-saturated medium (choline chloride or sucrose/choline chloride as appropriate) containing virtually no K^+ , and a correction was made for the dilution of the K^+ in the suspension. Our K^+ electrodes (Electrical Instruments Ltd., Richmond, Surrey, U.K.) showed some sensitivity to H^+ changes ($1.0\text{--}1.5\%$ of the sensitivity of an ideal H^+ electrode), and the K^+ traces were therefore corrected (at the time points indicated in Fig. 3) for the pH changes measured simultaneously in the same electrode-vessel. Both H^+ and K^+ traces were corrected for baseline drift and plotted as $\Delta\text{H}_0^+/e^-$ or $\Delta\text{K}_0^+/e^-$ on the assumption that 2 electrons were transferred through the oxidase per O atom injected. The symbols on the continuous traces indicate the time-points for which these calculations were made. These corrected traces were extrapolated back to a time half-way through the apparent duration of the respiratory pulse. The H^+ calibration pulses were extrapolated similarly, so that the buffering power measured was the external buffering power.

Chemicals were obtained from commercial sources as follows: mannitol, sucrose, rotenone, KCl , choline chloride and $\text{K}_4\text{Fe(CN)}_6$ were from BDH, Poole, Dorset, U.K.; choline hydroxide, antimycin A, valinomycin and *N*-ethylmaleimide were from Sigma Chemical Co., Poole, Dorset, U.K.; myxothiazol and FCCP were from

Boehringer Corporation, Lewes, East Sussex, U.K.; hexammineruthenium chloride was from Aldrich, Gillingham, Dorset, U.K.. They were used as supplied with the exceptions of rotenone, which was recrystallized twice from ethanol, and choline hydroxide, which was decolourized with charcoal. Much of the hexammineruthenium chloride used was a kind gift from Dr. P. Hinkle (Cornell University, Ithaca, NY, U.S.A.), and much of the myxothiazol used was a kind gift from Dr. W. Trowitzsch (GBF, Mascheroder Weg 1, D-3300 Braunschweig, Germany).

RESULTS

No exogenous reductant

When an anaerobic suspension of mitochondria, inhibited with rotenone, antimycin and myxothiazol, was given a small pulse of O_2 , the time-course of O_2

Fig. 1. Changes in medium H^+ and O_2 concentrations following O_2 addition to anaerobic mitochondria inhibited with rotenone, antimycin and myxothiazol in the absence of exogenous reductant

The stirred, anaerobic, electrode vessel (volume 3.3 ml) contained: choline chloride, 150 mM; glycylglycine, 1 mM; EGTA, 1 mM; carbonic anhydrase, 25 $\mu\text{g}/\text{ml}$; mitochondrial protein, 6 mg/ml; rotenone, 0.4 μM ; antimycin, 36 $\mu\text{g}/\text{g}$ of protein; myxothiazol, 150 $\mu\text{g}/\text{g}$ of protein; *N*-ethylmaleimide, 0.2 mM. After anaerobic preincubation at 25 °C and pH 7.0–7.1, as described in the Experimental section, 50 μl of air-saturated medium containing 25.0 ng-atom of O was injected. (a) Changes in medium O_2 concentration were determined with a Clark-type electrode and are expressed as a percentage of added O_2 . The solid line represents the continuous readout from the O_2 electrode. ● indicates the time points for which the raw data were calculated in terms of ng-atom of O. The broken line indicates the presumed time course when allowance is made for the slow response of the O_2 electrode. (b) Changes in medium pH in the presence of either FCCP (1 μM) or valinomycin (200 $\mu\text{g}/\text{g}$ of mitochondrial protein) were determined with a glass electrode, corrected for baseline drift and expressed in terms of H^+ equiv. appearing in the outer medium (ΔH^+) per oxidizing equiv. injected. ●, valinomycin; ○ FCCP.

Fig. 2. $\Delta H^+_0/e^-$ in oxidant-pulse experiments using mitochondria inhibited with rotenone, antimycin and myxothiazol in the absence of exogenous reductant

(a) KCl medium; (b) choline chloride medium. Conditions were as for Fig. 1 except that in (a) choline chloride was replaced by KCl and valinomycin was at 10 $\mu\text{g}/\text{g}$ of mitochondrial protein. Data are expressed in terms of oxidizing equivalents consumed. ●, ▲, valinomycin; ○, △, FCCP; ●, ○, 0.25 ng-atom of O injected per mg of protein; ▲, △, 0.375 ng-atom of O injected per mg of protein.

consumption was markedly biphasic. A small amount of O_2 (0.23–0.33 ng-atom of O/mg of protein) was consumed very rapidly (Fig. 1a), and the remainder of the oxygen was reduced at a steady-state rate of < 1.5 ng-atom $\cdot$ s $^{-1}$ $\cdot$ (g of protein) $^{-1}$. When valinomycin was present to allow K^+ ions to move freely across the membrane, there was a rapid ejection of protons (0.38–0.61 nequiv./mg of protein) corresponding to the initial burst of respiration (Fig. 1b). When valinomycin was replaced by FCCP, so that the ionic conductance of the membrane was dominated by the H^+ conductance, protons were taken up by the mitochondria (0.20–0.42 nequiv./mg of protein). When the ΔH^+_0 was determined in terms of the oxidizing equivalents used, as in Fig. 2(a), either by injecting small amounts of O_2 that were completely reduced, or by injecting larger O_2 pulses and measuring the amount of O_2 remaining in the suspension, it was found that $\Delta H^+_0/e^-$ (val) extrapolated to 0.94 and $\Delta H^+_0/e^-$ (FCCP) to -0.64 . Similar results were obtained in both 150 mM-KCl (Fig. 2a) and 150 mM-choline chloride (Fig. 2b), and in sucrose-based medium (not shown).

This type of experiment has been discussed in detail by Wikström & Casey (1985) who (omitting myxothiazol) found approximately 0.7 nequiv. of H^+ /mg of protein.

Fig. 3. Changes in medium H^+ and K^+ concentrations in oxidant-pulse experiments using mitochondria inhibited with rotenone, antimycin and myxothiazol in the presence of added reductants

The conditions, media and reductants were as in Table 1, and as described in the Experimental section. (a) $Fe(CN)_6^{4-}$, KCl medium; (b) $Fe(CN)_6^{4-}$, choline chloride medium; (c) $Fe(CN)_6^{4-}$, sucrose medium; (d) $Ru(NH_3)_6^{2+}$, KCl medium; (e) $Ru(NH_3)_6^{2+}$, choline chloride medium; (f) $Ru(NH_3)_6^{2+}$, sucrose medium. $\bullet$, $\Delta H_0^+/e^-$ (val); $\circ$, $\Delta H_0^+/e^-$ (FCCP); $\blacktriangle$, $\Delta K_0^+/e^-$ (val).

Papa *et al.* (1983) observed a biphasic ejection of protons, using rapid-mixing techniques. About 0.7 nequiv. of H^+ /mg of protein was ejected in the fast phase when the mitochondria were inhibited with rotenone and antimycin, and 0.35 nequiv. of H^+ /mg of protein was ejected when UHDBT was also present. There is therefore substantial agreement on that point. These experiments have an important bearing on the question whether the proton ejection, seen in the absence of added reductant, also occurs in the presence of added reductant, and whether it should be subtracted from the proton ejection seen in the latter case, to determine the proton ejection due solely to electron flow from exogenous reductant to oxygen. The crucial observation is that the $\Delta H_0^+/e^-$ stoichiometry in these experiments without added reductant is not greater than 1.0 (see Fig. 2), and may be as low as 0.5 if allowance

is made for net acid production (see Wikström & Casey, 1985). It seems therefore, that, regardless of the origin of the protons ejected in the absence of added reductant, their inclusion will not cause overestimation, but could cause slight underestimation, of the $\leftarrow H^+/e^-$ stoichiometry of the oxidase. In any case, we think that Wikström & Casey (1985) are probably correct in ascribing the proton ejection associated with the oxidation of endogenous reductants to the action of the oxidase. We therefore conclude that no correction need be made for protons ejected as a result of the oxidation of endogenous electron carriers in the experiments described in this paper.

Ferrocyanide as reductant

When a small pulse of O_2 was injected into an anaerobic suspension of valinomycin-treated mitochondria

Table 1. $\Delta H_D^+/e^-$ and $\Delta K_D^+/e^-$ in oxidant pulse experiments using mitochondria inhibited with rotenone, antimycin and myxothiazol in the presence of added reductants

In all experiments the following additions were made to the final concentrations shown: mitochondrial protein, 6 mg/ml; carbonic anhydrase, 25 μ g/ml; rotenone, 0.4 μ M; antimycin, 36 μ g/g of protein; myxothiazol, 150 μ g/g of protein; *N*-ethylmaleimide, 0.2 mM. After anaerobic pre-incubation, as described in the Experimental section, 50 μ l of air-saturated medium was injected. Changes in medium pH and K^+ concentration were determined with glass electrodes in the presence of either 1 μ M-FCCP or 200 μ g of valinomycin/g of protein (10 μ g/g of protein in media containing 150 mM-KCl) and expressed in terms of H^+ or K^+ equiv. appearing in the outer medium per oxidizing equiv. injected. Data are given as mean, S.E.M. and number of experiments.

Medium	Reductant... $Fe(CN)_6^{4-}$		$Ru(NH_3)_6^{2+}$		$\Delta K^+/e^-$
	$\Delta H_D^+/e^-(val)$	$\Delta H_D^+/e^-(FCCP)$	$\Delta H_D^+/e^-(val)$	$\Delta H_D^+/e^-(FCCP)$	
150 mM-KCl/1 mM-glycylglycine/ 1 mM-EGTA	0.87 (± 0.02 , $n = 19$)	-0.87 (± 0.02 , $n = 6$)	0.86 (± 0.02 , $n = 13$)	-0.91 (± 0.01 , $n = 10$)	-
150 mM-Choline chloride/1 mM- glycylglycine/ 1 mM-EGTA	0.97* (± 0.01 , $n = 4$)	-0.98 (± 0.01 , $n = 5$)	0.95 (± 0.01 , $n = 9$)	-0.94 (± 0.01 , $n = 8$)	-1.86 (± 0.03 , $n = 9$)
240 mM-Sucrose/ 10 mM-choline chloride/1 mM- glycylglycine/ 1 mM-EGTA	0.99† (± 0.01 , $n = 3$)	-0.83 (± 0.02 , $n = 4$)	1.05 (± 0.01 , $n = 8$)	-0.71 (± 0.01 , $n = 7$)	-1.82 (± 0.04 , $n = 6$)

* The choline medium for ferrocyanide experiments contained EDTA in place of EGTA, and glycylglycine was omitted as the buffering power of EDTA was sufficient.

† The sucrose medium for ferrocyanide experiments contained 10 mM-MgCl₂ in place of 10 mM-choline chloride as the rate of O₂ consumption was otherwise too slow, and glycylglycine was omitted as the buffering power of Mg-EGTA was sufficient.

dria in medium containing 150 mM-KCl and 2 mM-K₄Fe(CN)₆, the oxygen was consumed within 4 s and protons appeared in the outer medium (Fig. 3a). In control experiments, in which valinomycin was replaced by uncoupler, so that the ionic conductance of the membrane was dominated by the H^+ conductance, H^+ ions were expected to enter the mitochondria with an $\rightarrow H^+/e^-$ stoichiometry of close to 1.0 ($\Delta H_D^+/e^- = -1.0$), and any translocated protons should have been lost back into the matrix. In practice it was found that less than the expected number of protons were removed from the outer phase (Fig. 3a), as previously reported (Moyle & Mitchell, 1978; Wrigglesworth & Nicholls, 1982). However, it will be seen in Table 1 that, in KCl-based medium, the mean value for $\Delta H_D^+/e^-(FCCP)$ was -0.87, somewhat closer to the expected value of -1.0 than observed in previous experiments done in the absence of myxothiazol (Moyle & Mitchell, 1978). This 'shortfall' might indicate that net acid is released in the outer medium during the pulse of respiration, though there are other possible explanations, as will be discussed below.

In a sucrose-based medium of low ionic strength, the value $\Delta H_D^+/e^-$ in valinomycin experiments was very close to 1.0, but the 'shortfall' in the FCCP experiments was somewhat larger than in KCl-based medium [$\Delta H_D^+/e^-(FCCP) = -0.83$; see Fig. 3c and Table 1]. In a medium of high ionic strength in which KCl was replaced by choline chloride, not only was the $\Delta H_D^+/e^-(val)$ very close to 1.0, but there was almost no 'shortfall' [$\Delta H_D^+/e^-(FCCP) = -0.98$; see Fig. 3b and Table 1].

It is concluded that, in the choline medium, practically all the added oxygen is reduced by electrons originating from electron-donating reductants of cytochrome oxidase [cytochromes *c* and *c*₁ and K₄Fe(CN)₆], and that

practically 1 proton is translocated from the inner to the outer phase per oxidizing equivalent added. In KCl- and sucrose-based media, there is a small deficit or 'shortfall' in the quantity $\Delta H_D^+/e^-(FCCP)$, which requires further discussion (see below).

Hexammineruthenium(II) as reductant

Experiments similar to those described above were performed in which the hexammineruthenium ion was the source of electrons for the reduction of endogenous cytochrome *c* or for the direct donation of electrons to cytochrome oxidase (Hochman *et al.*, 1981). This compound, when in the reduced Ru(II) form, is slowly oxidized by O₂, and it was found convenient to add it to the anaerobic mitochondrial suspension in the oxidized Ru(III) form, and allow it to become slowly reduced, by endogenous reductants in the mitochondria, during the preincubation before the respiratory inhibitors were added. Under our experimental conditions, 60 μ M-hexammineruthenium could be largely reduced in this way, as judged from the time course of net acid production and from the amount of O₂ that could be rapidly reduced in subsequent pulses. This concentration of hexammineruthenium(II) was found to be sufficient to sustain rapid reduction of the added O₂ pulse.

The pH changes in the suspension, on injecting air-saturated medium, were very similar to those observed with ferrocyanide as reductant, except that the O₂ was consumed more rapidly (see Figs. 3d-3f, and Table 1). In low ionic strength sucrose-based medium $\Delta H_D^+/e^-(val)$ was 1.05, significantly above 1.00 at the 95% confidence level. However, the 'shortfall' in uncoupler experiments was again considerable [$\Delta H_D^+/e^-(FCCP) = -0.71$]. It is concluded that net acid is generated during

the pulse in this medium in both valinomycin and FCCP experiments (see below). A low ionic strength medium in which mannitol replaced sucrose gave the same results as the sucrose medium.

In the high ionic strength medium, in which choline replaced K^+ , there was almost no 'shortfall' [$\Delta H^+_{\text{O}}/e^-(\text{FCCP}) = -0.94$], as in the ferrocyanide experiments. In this medium, as in the sucrose medium, it was possible to monitor K^+ ion movements during the respiratory burst. It was found (Fig. 3e and Table 1) that 1.86 K^+ ions passed into the mitochondria for every electron passing to oxygen, indicating that the H^+ appearing during the pulse is ejected electrogenically from the mitochondrial matrix, and therefore represents true proton translocation.

DISCUSSION

The failure of the $\Delta H^+_{\text{O}}/e^-(\text{FCCP})$ traces to extrapolate to -1.0 could result from several different causes. Three types of cause will be considered: (a) the H^+ conductance with 1 μM -FCCP might not sufficiently dominate all other ionic conductances, and hence some other ions (e.g. K^+ , Ca^{2+} , Cl^-) may move during the respiratory burst; (b) a fraction of the added O_2 might react with hydrogenated reductants (either directly or through the cytochrome chain) generating net acid; (c) net acid might be generated by ionic interactions that do not involve redox reactions.

The first suggested explanation of the 'shortfall' was tested in two ways. The FCCP concentration was varied over the range 0.2–5.0 μM , and the extrapolated $\Delta H^+_{\text{O}}/e^-(\text{FCCP})$ values were plotted against the reciprocal of uncoupler concentration. It was found that $\Delta H^+_{\text{O}}/e^-(\text{FCCP})$ was sensitive to uncoupler concentration, but that 1 μM -FCCP was apparently sufficient to achieve maximum uncoupling. Control experiments were also done in which valinomycin and FCCP were simultaneously present. In this case the $\Delta H^+_{\text{O}}/e^-(\text{val} + \text{FCCP})$ traces were expected to approach the total net pH change in the combined inner and outer phases, at the K^+ ion concentrations present. (The acid calibrations in this case measure the total buffering power, as is appropriate.) In such experiments the 'shortfalls' were diminished somewhat (barely significant at the 95% confidence level), suggesting a slight inadequacy of uncoupler, but the still substantial 'shortfall' showed that net acid was indeed generated during the pulse.

The observation that 'shortfall' was larger in media of low than of high ionic strength suggests that ionic interactions between the hexacyanoferrate anions or the hexammineruthenium cations and the mitochondria may be responsible; for example, the transformation of the reduced ion to the oxidized ion in each case might flush a proton off the mitochondrial protein or phospholipid. In support of this type of suggestion is the observation that when anaerobic hexammineruthenium(III) chloride was added to the anaerobic suspension, a considerable acidification was observed immediately, which was followed by a slower acidification, as the ruthenium was reduced by mitochondrial reductants. This was investigated more quantitatively by repeating the experiments of Fig. 3 but, instead of injecting O_2 , injecting an equivalent amount of the oxidized ion [50 nequiv. of ferricyanide or hexammineruthenium(III)]. Ferricyanide caused no acid step in either high or low ionic strength media. Hexammineruthenium(III), on the other hand, gave an

acid step in low ionic strength medium equivalent to 0.2–0.4 of the oxidizing equivalents injected. This acid step was negligibly small in high ionic strength medium. Thus, ionic interactions involving $Ru(NH_3)_6^{3+}$ can explain all, or a large part, of the shortfall in $\Delta H^+_{\text{O}}/e^-(\text{FCCP})$ in low ionic strength media when hexammineruthenium(II) was the reductant, but not that in KCl medium or when the reductant was $Fe(CN)_6^{4-}$.

It still seems possible that, as previously proposed (Moyle & Mitchell, 1978; Lorusso *et al.*, 1979; Mitchell, 1980), some net acid might be produced during the fast reduction of the O_2 pulse as a result of re-reduction of $Fe(CN)_6^{3-}$ by an endogenous reductant such as ubiquinol or the protonated semiquinone radical. Indeed, with antimycin-treated mitochondria prepared from young rats, it seems that electrons can flow rapidly from a hydrogenated reductant to ferricyanide or oxidized cytochrome *c* and, in doing so, generate extramitochondrial protons with a stoichiometry of 1 H^+/e^- (Wrigglesworth & Nicholls, 1982). It is not known if this process occurs in the combined presence of myxothiazol and antimycin, though similar experiments of Wikström & Penttilä (1982) suggest that it does not.

In spite of the above reservations, it is evident, however, that inhibitor-insensitive oxidation of an endogenous reductant by ferricyanide is inadequate to explain the observed proton ejection (Wrigglesworth & Nicholls, 1982; Wikström & Penttilä, 1982). In our experiments using hexammineruthenium as reductant in low ionic strength medium, if the 'shortfall' is attributed to net acid and subtracted from the $\Delta H^+_{\text{O}}/e^-(\text{val})$ data, there still remains $0.76 \leftarrow H^+/e^-$. In choline chloride medium the appropriate correction leaves us with $0.89 \leftarrow H^+/e^-$. This corresponds quite well with the extra K^+ ions that disappear during the pulse; 0.82 in sucrose and 0.86 in choline media. If the redox process that generated the net acid were responsible for these translocated protons (Moyle & Mitchell, 1978; Lorusso *et al.*, 1979), the $\leftarrow H^+/e^-$ stoichiometry of that redox process would have to be 2.6 in the sucrose medium and 14.8 in the choline chloride medium; which is not credible.

Shortfall in $\Delta H^+_{\text{O}}/e^-(\text{FCCP})$ can be investigated by correlating the ferricyanide generated with the oxygen consumed. Wikström & Krab (1978), in experiments somewhat similar to those described here, found that there was no 'shortfall' in the amount of ferricyanide produced. There was therefore no re-reduction of ferricyanide, though there was a 12–18% 'shortfall' of $\Delta H^+_{\text{O}}/e^-(\text{FCCP})$. Papa *et al.* (1983), in analogous experiments using TMPD/TMPDH⁺ as exogenous reductant, found no shortfall in $\Delta H^+_{\text{O}}/e^-(\text{FCCP})$ other than that due to protons released from the protonated form of their reductant; but they did observe a rapid re-reduction of the oxidized TMPD⁺, so that there was, in their experiments, a shortfall of TMPD⁺. However, Wikström & Penttilä (1982) found that myxothiazol slowed the re-reduction of TMPD⁺ without affecting the rapid proton ejection or the rapid TMPD oxidation. Proton ejection must therefore be related to TMPD oxidation, not to its re-reduction.

Two research groups have concluded that $\leftarrow H^+/e^-$ is 2 for cytochrome oxidase in antimycin-inhibited rat liver mitochondria, on the basis of a comparison of rates of change of H^+_{O} and K^+_{O} concentrations with the rate of ferrocyanide oxidation (Alexandre *et al.*, 1978; Azzone *et*

al., 1979). This high stoichiometry has been shown to be thermodynamically impossible under state-4 conditions (Wikström *et al.*, 1981*b*; Murphy *et al.*, 1985). It has not been confirmed by other experimental approaches. The experiments described in this paper are equally incompatible with $\leftarrow H^+/e^-$ of 0 and 2, but lead us to accept the proposal of Wikström (1977) that when electrons flow through cytochrome oxidase nearly $1.0 H^+/e^-$ is translocated from the matrix to the outer medium.

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