MEASUREMENTS OF MITOCHONDRIAL $\leftarrow H^+/O$ QUOTIENTS: EFFECTS OF PHOSPHATE AND *N*-ETHYLMALEIMIDE

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1. Introduction

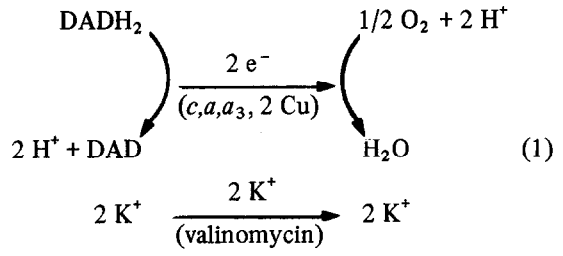
Some years ago [1] we discovered that the $\leftarrow H^+/O$ value of 6, measured by the respiratory-pulse technique [2] in suspensions of rat liver mitochondria in a 150 mM KCl medium without added substrate, could be increased to 8 by preincubating the mitochondria with NEM. The action of the NEM appeared to be explained by our observation that 'it largely inactivated succinate dehydrogenase and the NAD-linked enzymes but did not inactivate the NADP-linked isocitrate dehydrogenase (or the NADH oxidase and NAD(P) transhydrogenase). The conditions, after NEM treatment, thus favoured the involvement of the complete redox chain from NADPH to oxygen in the respiratory pulses' ([1] and see [3]). It is noteworthy that the maximum quantity of oxygen normally injected as air-saturated saline in the respiratory-pulse experiments (1 μg atom O per g protein) is equivalent to only about 12% of the total intramitochondrial NAD + NADP + isocitrate + citrate (2.6 μmol NAD, 3.5. μmol NADP and about 3 μmol isocitrate + citrate per g protein), the NAD and NADP being practically all reduced after the anaerobic preincubation that routinely precedes the oxygen injection, even in the absence of added reductant substrate. Thus, according to our interpretation, the $\leftarrow H^+/O$ quotient observed under various conditions may be raised towards a maximum of 8 by NEM because the NEM favours the use of NADPH or isocitrate as endogenous reductant in the mitochondria. This interpretation is consistent with a $\leftarrow H^+/2\ e^-$ value of 2 per 'site' or effective redox loop in the respiratory chain: 2 for loop 0, 2 for loop 1 and 4 for the Q cycle or loops 2 + 3 [4].

Brand et al. have recently put forward a quite different interpretation [5]. They have proposed that the $\leftarrow H^+/O$ quotients measured by the oxygen-pulse technique are underestimated by a third or by a half because the re-uptake via the phosphoric acid porter of mitochondrial P_i that leaks out during the anaerobic preincubation causes a previously unrecognised collapse of ΔpH that is too rapid to be corrected for by the usual extrapolation procedure. According to their view, NEM raises the observed extrapolated $\leftarrow H^+/O$ quotient to the 'true' value by inhibiting the phosphoric acid porter. This interpretation leads to a 'true' $\leftarrow H^+/2\ e^-$ value of 3 or 4 per 'site' or effective redox loop.

To decide between the alternative interpretations we have measured the effect of NEM on the apparent $\leftarrow H^+/O$ quotient given by the oxidation of $DADH_2$ via cytochrome *c* oxidase and cytochrome *c* in rat liver mitochondria treated with antimycin. In this system, previous work on cytochrome *c* oxidase [6], and measurements of $\rightarrow e^-/O$ quotients by means of

Abbreviations: pH_o, pH of suspension medium; pK_o, pK (i.e., $-\log_{10}(K^+$ ion activity)) of suspension medium; p*K*, acid ionisation constant; ΔpH, pH difference between the bulk aqueous phases on either side of the mitochondrial cristae membrane; $DADH_2$, 2,3,5,6-tetramethylphenylenediamine; c^{2+}, ferrocytochrome *c*; c^{3+}, ferricytochrome *c*; EGTA, ethyleneglycolbis(aminoethyl)-tetraacetic acid; $\leftarrow H^+$ or $\leftarrow K^+$, outward H^+ or K^+ translocation; $\rightarrow H^+$ or $\rightarrow K^+$, inward H^+ or K^+ translocation; $\rightarrow e^-$, inward electron translocation (inferred from $\rightarrow K^+$); val, valinomycin; FCCP, carbonylcyanide trifluoromethoxyphenylhydrazone; NEM, *N*-ethylmaleimide

$\rightarrow K^+/O$ quotients in the present paper, show that the only significant process producing ΔpH, apart from a very slow leak through the antimycin block, is as given in eq. (1):



The upper part of eq. (1) represents the oxidation of $DADH_2$ via (external) cytochrome *c* and cytochrome *c* oxidase, and the lower part represents the electrophoretic import of K^+, catalysed by valinomycin.

The presence of NEM does not change the comparatively well-defined electron-translocation process by which ΔpH is produced in this system, as judged by NEM-independent $\rightarrow K^+/O$ quotients close to 2.0. We have therefore been able to use this system, for which the $\leftarrow H^+/O$ quotient should be 2.0, to test the proposition [5] that the apparent $\leftarrow H^+/2\ e^-$ quotient per effective redox loop in the respiratory chain is a spurious underestimate by a third or by a half unless P_i translocation is blocked (e.g., by NEM).

Our observations show that the apparent $\leftarrow H^+/2\ e^-$ value is close to 2.0 and that it is NEM independent.

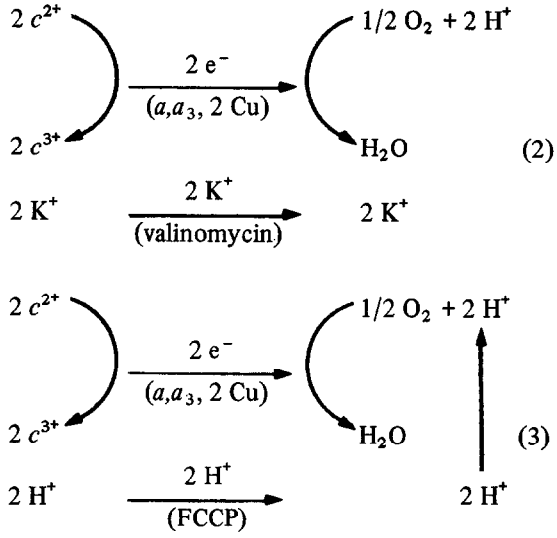
2. Materials and methods

The method of isolating rat liver mitochondria, and the methods of measuring and recording pH_0 and pK_0 and oxygen concentration were as described [2,7,8]. Cytochrome *c* was from horse heart (Type III, Sigma, London). It was reduced by treatment with ascorbate, followed by dialysis (see [9]), and made up as a stock 2% solution in 10 mM Tris/HCl buffer at pH 7.0. The stock $DADH_2$ solution (10 mM in water) was made up freshly for each day's experiments.

The general experimental procedures for the O_2-pulse experiments with the antimycin- and rotenone-treated mitochondria were as before [6]. To allow for the small protonmotive and electrogenic effect of the antimycin-insensitive respiration during the O_2 pulses with ferrocytochrome *c* or $DADH_2$ as reductant, we estimated the rate of oxygen consumption in the absence of added reductant and expressed it as a fraction of that in the presence of each reductant. This fractional antimycin-insensitive respiratory rate was multiplied by the observed $\leftarrow H^+/O$ or $\rightarrow K^+/O$ quotient in the absence of added reductant to obtain the amount of $\leftarrow H^+/O$ or $\rightarrow K^+/O$ to be subtracted from the estimated quotients in the presence of each reductant. This correction of the quotients never exceeded 17% and 8% of the estimated values in the O_2-pulse experiments with ferrocytochrome *c* and $DADH_2$ as reductant, respectively.

3. Results and discussion

O_2-pulse experiments with suspensions of rat liver mitochondria treated with antimycin and rotenone, using ferrocytochrome *c* as reductant, showed that the $\rightarrow K^+/O$ quotient in the presence of valinomycin, and the corresponding $\rightarrow H^+/O$ quotient for secondary inward electrophoretic proton translocation in the presence of FCCP were close to 2, as shown in fig.1 A and B, and as represented in eq. (2) and eq. (3):



The small pulse of primary outward proton translocation in fig.1A, and the equivalent slight shortfall of net alkalinisation in fig.1B, are accounted for by the normally protonmotive antimycin-insensitive respira-

tion, which proceeds at about 10% of the rate of ferrocytochrome *c* oxidation in this type of experiment (and see [6]). Correcting for this effect (see section 2) the mean secondary electrophoretic $\rightarrow K^+/O$ and $\rightarrow H^+/O$ quotients, corresponding to the primary $\rightarrow e^-/O$ quotient for the cytochrome *c* oxidase reaction, were found to be 1.98 ± 0.06 (8 values) and 1.96 ± 0.07 (10 values), respectively.

The experiments of fig.1, C and D, which were the same as those of fig.1, A and B, except that the mitochondria were preincubated with 0.2 mM NEM, show that NEM has no detectable effect on the electron-translocating cytochrome *c* oxidase reaction.

When $DADH_2$ was used as reductant for cytochrome *c* in mitochondrial suspensions treated with antimycin and rotenone, the O_2-pulse experiments shown in fig.1, E and F, gave the expected electrophoretic $\rightarrow K^+/O$ quotient close to 2, (2.03 ± 0.10, 8 values), confirming that the $DADH_2$ was reacting exclusively with cytochrome *c*, as indicated in eq. (1). In this type of experiment, the normally proton-motive antimycin-insensitive respiration proceeded at only 5% of the rate of $DADH_2$ oxidation, and the $\rightarrow K^+/O$ and $\leftarrow H^+/O$ quotients were corrected accordingly (see section 2). The proton pulse produced by $DADH_2$ oxidation (fig.1E) gave an apparent $\leftarrow H^+/O$ quotient close to 2 (1.98 ± 0.08, 8 values). The rather rapid decay of this proton pulse may probably be explained by the fact that the medium contained 10 mM sulphate, and that the cristae membrane contains a proton-linked sulphate porter system. The control with FCCP present (fig.1F) showed that, unlike cytochrome *c* oxidation, which produced an equivalent of net alkali (fig.1B), $DADH_2$ oxidation, via cytochrome *c* and cytochrome *c* oxidase, caused no net pH_0 change when FCCP allowed the H^+ ions

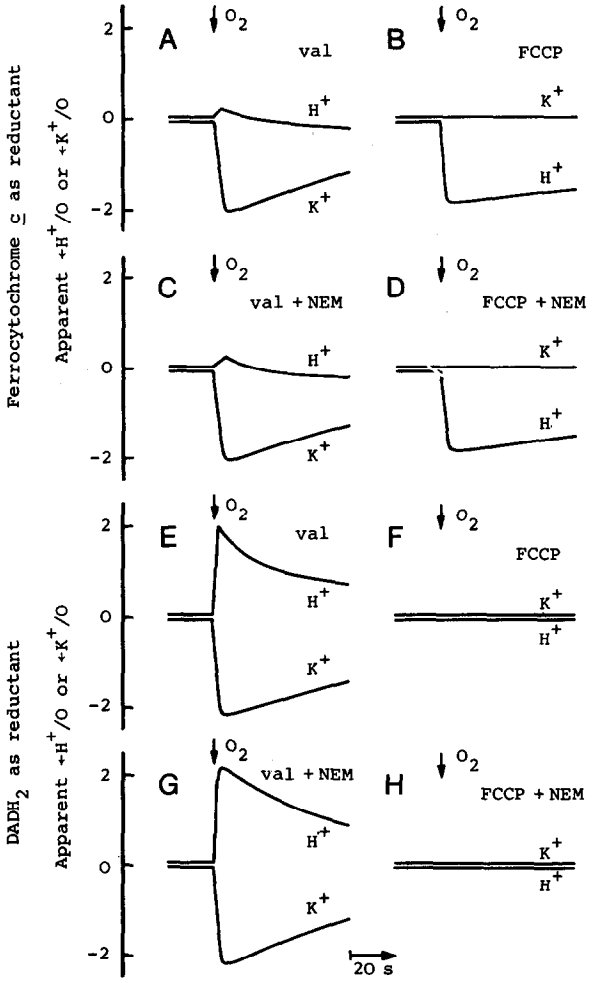


Fig.1. Changes of pH_0 and pK_0, plotted as apparent $\leftarrow H^+/O$ and $\leftarrow K^+/O$ quotients, following respiratory pulses using ferrocytochrome *c* (A–D) and $DADH_2$ (E–H) as reductant in mitochondria treated with rotenone and antimycin. Rat liver mitochondria (about 6 mg protein/ml) were preincubated anaerobically for 20 min at pH_0 7.0–7.1 at 25°C in 3.3 ml medium containing 240 mM sucrose, 3.3 mM glycylglycine, 10 mM $MgSO_4$, 1 mM EGTA (choline salt), carbonic anhydrase (30 μg/ml), 0.4 μM rotenone and antimycin (36 μg/g mitochondrial protein). As indicated, valinomycin (200 μg/g protein) or 1 μM FCCP was present. In experiments A–D, 0.025 mM ferrocytochrome *c* was present from the beginning of the anaerobic preincubation (see [6]), and in experiments E–H, 0.6 mM $DADH_2$ was present. Where indicated, 0.2 mM NEM was added 5 min before the injection of air-saturated sucrose solutions (the quantity of O_2 injected was 23.8 ng atoms O in A–D, and 47.6 ng atoms O in E–H). In all experiments the O_2 was reduced in 3–4 s. The time-course of pH_0 and pK_0, plotted as apparent $\leftarrow H^+/O$ and $\leftarrow K^+/O$, respectively, has not been corrected for the antimycin-insensitive respiration, but the pK_0 time-course has been corrected for a baseline shift caused by injecting the air-saturated (K^+-free) sucrose solution. The pK_0 was 3.1–3.2 when the O_2 pulses were injected after the 20 min preincubation period. The traces before the O_2 pulses have been drawn as two separate lines for purposes of clarity only. The actual traces correspond to the zero on the vertical axis. The outward H^+ or K^+ translocation represented by $\leftarrow H^+$ or $\leftarrow K^+$ corresponds to inward H^+ or K^+ translocation when given with a minus sign.

produced by the external oxidation of $DADH_2$ to be imported electrophoretically and internally neutralised by O^{2-} (see eq. (3)). This is in accordance with the known relatively low pK, and consequent deprotonation, of the $=NH_2^+$ groups produced by oxidation of the aromatic $-NH_2$ groups of $DADH_2$. As shown in fig.1, G and H, the only significant effect of preincubating the mitochondria with NEM on the O_2-pulse experiments, using $DADH_2$ as reductant, was that the decay of ΔpH_0 after the pulse was slower than before (fig.1E). This was presumably accounted for by an inhibition of the sulphate porter system by NEM. The values of the $\rightarrow K^+/O$ quotient and apparent $\leftarrow H^+/O$ quotient after NEM treatment were 2.01 ± 0.07 (8 values) and 1.98 ± 0.05 (8 values), respectively.

Our results show that, using the usual extrapolation procedure, the estimated $\leftarrow H^+/O$ and $\rightarrow K^+/O$ quotients are the same, within experimental error, as required by eq. (1), and the estimated $\leftarrow H^+/O$ quotient is not significantly changed after the phosphoric acid porter has been inhibited by treating the mitochondria with NEM. It follows, contrary to the proposition of Brand et al. [5,10], that the $\leftarrow H^+/O$ quotients estimated by our O_2-pulse technique are not depressed significantly by the re-entry of endogenous inorganic phosphate, which is normally present in the external medium at a concentration of about 0.1 mM at the end of the anaerobic preincubation period. This conclusion was previously indicated by our observation that, over a range of added phosphate concentrations, up to 0.5 mM at 5°C or up to 0.1 mM at 25°C, the extrapolated $\leftarrow H^+/O$ quotient was virtually unaffected, and corresponded to a $\leftarrow H^+/2\ e^-$ quotient close to 2 per effective redox loop [2]. Indeed, observations by Brand et al. ([10], fig.6) confirmed that the addition of 0.1 mM phosphate has little if any significant effect on the estimated $\leftarrow H^+/O$ quotient at 28°C.

4. Conclusion

The fact that NEM treatment does not significantly increase the estimated $\leftarrow H^+/O$ quotient, and that the estimated $\leftarrow H^+/O$ quotient corresponds to the expected value of 2 predicted by eq. (1) in the well-controlled experimental system described here leads to the primary conclusion that $\leftarrow H^+/O$ quotients are not normally underestimated because of the re-entry of mitochondrial inorganic phosphate via the phosphoric acid porter. It follows that, under similar conditions with respect to phosphate concentration and phosphoric acid translocation, but such that NEM does increase the $\leftarrow H^+/O$ quotient, this effect of NEM cannot be attributed to inhibition of the phosphoric acid porter. Thus, the only extant explanation of the $\leftarrow H^+/O$ enhancing effect of NEM in the usual O_2-pulse experiments is that NEM favours the involvement of the complete redox chain from NADPH to oxygen in the respiratory pulses. In another paper, we shall describe experiments that help to explain how NEM produces this remarkable effect, even when (as in experiments by Brand et al. [5,10]) rotenone has been added with the object of suppressing NADH oxidation.

We conclude, from the present work, and also from work on the stoicheiometry of calcium translocation reactions [11–13], that the $\leftarrow H^+/2\ e^-$ quotient per effective redox loop in the respiratory chain of rat liver mitochondria is 2, and not 3 or 4 as claimed by Brand et al. [5,10].

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