# Flow-force relationships in mitochondrial oxidative phosphorylation

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The rates of oxidation and phosphorylation in isolated rat-liver mitochondria have a steep dependence on the protonmotive force ($\Delta\tilde{\mu}_H$) across the membrane. These experimentally observed relationships proved to be independent of the way in which $\Delta\tilde{\mu}_H$ was varied. These results were obtained when the membrane potential ($\Delta\psi$) was calculated from the distribution of $K^+$ (in the presence of valinomycin). When triphenylmethylphosphonium ($TPMP^+$) was used as a probe for $\Delta\psi$, slightly different flow-force relationships were obtained. We conclude that unique relationships exist between $\Delta\tilde{\mu}_H$ and the rates of oxidation and phosphorylation, and that under some conditions the behaviour of the probe $TPMP^+$ is anomalous.

*Mitochondria* *Energy transduction* *Localized chemiosmotic coupling* *Protonmotive force* *Oxidation* *Phosphorylation*

## 1. INTRODUCTION

According to the chemiosmotic theory of Mitchell [1], electron transport in the respiratory (or photo-redox) chain is coupled to transmembrane proton transport. Thus, a difference of the electrochemical potential of protons is built up between the aqueous phases at both sides of the mitochondrial inner membrane ($\Delta\tilde{\mu}_{H+}$). At sufficient magnitude, the $\Delta\tilde{\mu}_{H+}$ may drive the endergonic phosphorylation of ADP to ATP. Stimulation of oxidation, which can be obtained by the initiation of phosphorylation or by adding uncouplers, is thus explained as a consequence of a lowered $\Delta\tilde{\mu}_{H+}$ when protons are allowed to flow back into the mitochondria. Both oxidation and phosphorylation are directly coupled to and dependent on $\Delta\tilde{\mu}_{H+}$ and are only indirectly coupled to each other. The rates of oxidation flux ($J_o$) and phosphorylation flux ($J_p$) should therefore be independent of the way in which $\Delta\tilde{\mu}_{H+}$ is varied, provided that the kinetic parameters of respiration or phosphorylation, respectively, are not affected. Indeed, a unique relationship between $J_p$ and $\Delta\tilde{\mu}_{H+}$ has been found in chloroplasts [2] and in submitochondrial particles [3]. However, anomalous flow-force relationships have been observed in other systems, for instance in rat-liver mitochondria [4]. These observations have been explained by assuming that the bulk-phase $\Delta\tilde{\mu}_{H+}$ is not the (only) high-energy intermediate in mitochondrial oxidative phosphorylation [5]. It was implied that there is a local force (e.g. local $\Delta\tilde{\mu}_{H+}$) close to the membrane which differs in magnitude from – but communicates with – the bulk $\Delta\tilde{\mu}_{H+}$. It may, however, be that the difference in reported flow-force relationships is partly due to differences in the methods that are used to determine $\Delta\tilde{\mu}_{H+}$. We therefore reinvestigated some flow-force relationships in rat-liver mitochondria and compared different methods for the determination of $\Delta\tilde{\mu}_{H+}$. We obtained unique relationships between $J_o$ and $\Delta\tilde{\mu}_{H+}$ and between $J_p$ and $\Delta\tilde{\mu}_{H+}$ when the measured $K^+$ distribution (in

*Abbreviations:* TPMP, triphenylmethylphosphonium; DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $AP_5A$, $P^1,P^5$-di-(adenosine-5$^1$-)pentaphosphate; DMO, 5,5-dimethyloxazolidine-2,4-dione

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the presence of valinomycin) was used to calculate the membrane potential ($\Delta\psi$). When $TPMP^+$ was used to measure $\Delta\psi$, a unique $J_o/\Delta\tilde{\mu}_{H+}$ relationship was also found, but sometimes the behaviour of the probe $TPMP^+$ was anomalous.

## 2. MATERIALS AND METHODS

Rat-liver mitochondria were isolated as in [6], but 275 mM mannitol, 2 mM Mops (pH 7.0) was used instead of 250 mM sucrose as the isolation medium. Protein was determined by the biuret method, using bovine serum albumin as a standard. Oxygen consumption was measured using a Clark oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH). Mitochondria were incubated at 25°C in magnetically stirred oxygraph vessels in a medium containing 10 mM succinic acid, 1 mM malic acid, 10 or 5 mM $H_3PO_4$, 2 mM EGTA, 4 mM $MgCl_2$, 10 mM glucose and 10 mM Mops. The medium was brought to pH 7.0 with Tris and contained mannitol to a final osmolality of 290 mosmolal; 0.4 $\mu$g rotenone was added per mg protein.

$TPMP^+$ and $K^+$ were used as probes for $\Delta\psi$, either separately or combined in one experiment in parallel incubations. When different parameters were to be measured in 'parallel incubations', one incubation was started, to which all reagents except the radioactive markers were added. From this incubation a 700 $\mu$l aliquot was transferred to another oxygraph vessel, which then received the radioactive probes. The $K^+$ measurements were always carried out in the same vessel in which $J_o$ or $J_p$ was determined. [$^3$H]$TPMP^+$ and [$^{14}$C]DMO distributions were initially measured in parallel incubations ([$^{14}$C]DMO is used as a probe of $\Delta$pH). These experiments are not shown but are discussed here. In our later experiments, presented in this paper, [$^3$H]TPMP and [$^{14}$C]DMO distributions and $J_o$ were measured in the same incubations.

When the $K^+$ distribution was measured, the medium contained 50 $\mu$M KCl and 0.5 $\mu$g valinomycin was added per mg protein. The extramitochondrial $K^+$ concentration was measured using a $K^+$-sensitive glass electrode (Ingold, Urdorf, Switzerland). The intramitochondrial $K^+$ content was calculated by subtraction of the extramitochondrial $K^+$ content from the total $K^+$ content of the incubation. The total $K^+$ content was measured after anaerobiosis was reached, in the presence of excess uncoupler. Previous experiments showed that under these conditions only very little of the $K^+$ is retained in the mitochondria. In the experiments presented in figs 1 and 2, the mitochondrial matrix volumes were calculated from the $K^+$ content relative to fully de-energised mitochondria, assuming a matrix volume of 0.4 $\mu$g/mg protein in fully de-energised mitochondria and assuming osmotic equilibrium with a univalent $K^+$ salt. This method gave results that were in good agreement with the method in which $^3H_2O$ and [$^{14}$C]mannitol were used (see below).

[$^3$H]$TPMP^+$ and DMO distributions were measured as follows: the medium contained 5 $\mu$M (50 $\mu$M in some experiments) $TPMP^+$. 2 min before sampling 7 $\mu$Ci [$^3$H]$TPMP^+$ and 0.2–0.3 $\mu$Ci [$^{14}$C]DMO were added per ml incubation volume. A control experiment showed that a period of 2 min was ample for equilibration of the probes across the mitochondrial membrane. A 600 $\mu$l sample was drawn from the oxygraph vessel and was layered on top of 500 $\mu$l silicone oil (AR200/AR20, 3:1, Wacker, Munich) in Eppendorf centrifuge tubes. Directly after centrifugation for 1 min at 9000 × $g$, a sample was drawn from the supernatant and the rest of the supernatant and most of the oil were aspirated. The tube was washed with water which was kept separate from the pellet by a layer of oil. After aspiration of the water, the pellet was resuspended in water and deproteinised with 0.1 M $HClO_4$. Samples originating from the supernatant and the pellet were counted. The $^3$H and $^{14}$C counts were corrected for quenching and channel cross-talk. Mitochondrial matrix volumes and adhering medium volumes were determined using exactly the same silicone oil centrifugation procedure with 10 $\mu$Ci $^3H_2O$ and 0.8 $\mu$Ci [$^{14}$C]mannitol per ml incubation volume in parallel incubations (see [7]). To obtain a correct measurement of the adhering medium volume and of $\Delta$pH, it is essential that oil is used to separate the mitochondria from the medium and that the centrifugation tubes are washed with water after aspiration of the supernatant and before the pellet is resuspended. If only $TPMP^+$ (and not DMO) is measured, the oil (and the washing of the tube) can be omitted, provided that centrifugation and separation of the pellet from the supernatant are achieved within minutes

(cf. [4]). $TPMP^+$ is slightly soluble in silicone oil. However, practically identical values of the apparent $TPMP^+$ distribution were obtained when samples from one mitochondrial incubation were centrifuged in tubes with or without silicone oil.

The rate of phosphorylation was determined as follows. The medium contained 1 mM $AP_5A$ to inhibit adenylate kinase activity and was saturated with oxygen. 2 mg mitochondrial protein per ml and 0.8 U hexokinase per mg protein were added. The reactions were started by the addition of ATP (0.5 mM). At 3 and 5 min after the addition of ATP, 500 $\mu$l samples were drawn and quenched by mixing with 0.5 ml organic quench mixture + 50 $\mu$l 0.25 M EDTA [8]. Glucose 6-phosphate was assayed with standard enzymatic methods.

$^3H_2O$ and [$^{14}$C]mannitol were obtained from the Radiochemical Centre, Amersham, England. [$^3$H]$TPMP^+$ and [$^{14}$C]DMO were obtained from New England Nuclear. DNP was from Merck-Schuchard, Hohenbrunn, FRG. FCCP was a gift from Dr P.G. Heytler. Hexokinase (lyophilised powder) was from Sigma, St. Louis, MO.

## 3. RESULTS AND DISCUSSION

As shown in fig.1, there is a steep dependence of the rate of oxidation by rat-liver mitochondria on the magnitude of $\Delta\psi$. Moreover, identical relations are obtained when $\Delta\tilde{\mu}_{H+}$ ($\Delta\psi$) is lowered by adding the protonophore DNP (or FCCP, not shown) or by stimulating ATP synthase activity by the addition of hexokinase. Likewise, as shown in fig.2, a unique relationship between the rate of phosphorylation and $\Delta\psi$ was obtained when $\Delta\psi$ was lowered by adding FCCP or by inhibiting respiration with malonate.

In all our experiments we obtained unique relationships between $J_o$ and $\Delta\tilde{\mu}_{H+}$ (or $\Delta\psi$) and between $J_p$ and $\Delta\tilde{\mu}_{H+}$ (or $\Delta\psi$), when $\Delta\psi$ was calculated from the $K^+$ distribution (in the presence of valinomycin). In these experiments the $K^+$ distribution was measured – using a $K^+$-sensitive glass electrode – in the same incubation as that in which the rates of oxidation or phosphorylation were determined. However, when $\Delta\psi$ was calculated from the distribution of radioactively labelled $TPMP^+$ which was measured in parallel incubations (cf. [4]), anomalous flow-force relationships were obtained regardless of

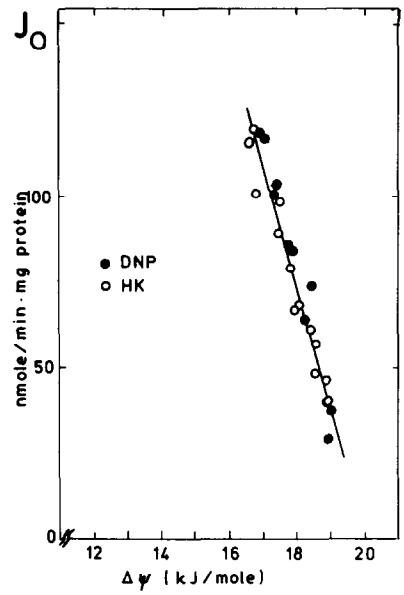


Fig.1. Dependence of the rate of mitochondrial oxidation on $\Delta\psi$. $\Delta\psi$ was varied using 0–16 $\mu$M DNP (●) or 0–0.3 U hexokinase (○). $\Delta\psi$ was calculated from the $K^+$ distribution between the inside and outside of the mitochondria.

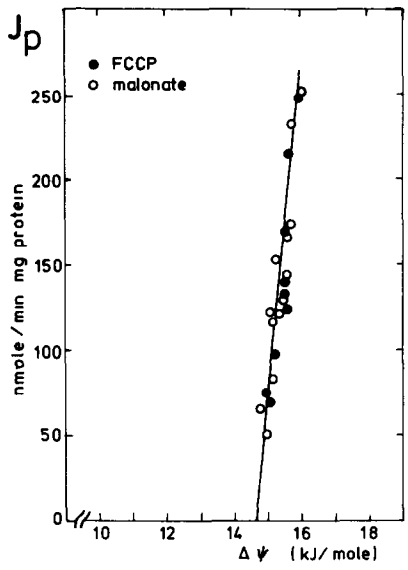


Fig.2. Dependence of the rate of mitochondrial phosphorylation on $\Delta\psi$. $\Delta\psi$ was varied using 0–47 pmol FCCP/mg protein (●) or 0–1 mM malonate (○). $\Delta\psi$ was calculated from the $K^+$ distribution between the inside and outside of the mitochondria.

whether valinomycin was present (not shown). In some of our experiments, this could be explained by the effect of ethanol and ethyl acetate which were added as solvents of the radioactive markers [$^3$H]TPMP$^+$ and [$^{14}$C]DMO. As shown in fig.3, the addition of 7 $\mu$l ethanol and 3 $\mu$l ethyl acetate per ml medium strongly increased the rate of oxidation at submaximal FCCP concentrations. Ethyl acetate alone had less effect and ethanol alone no appreciable effect. Also, the initial slope of the FCCP titration became steeper by the addition of ethyl acetate and ethanol, which indicates that the apparent proton-carrying activity of FCCP in the mitochondrial membrane was increased by the addition of the two solvents. Therefore, the mitochondria in the vessel in which the radioactive probes were added were more strongly uncoupled by FCCP than the mitochondria in the vessel in which the rate of phosphorylation was measured.

Another phenomenon that may cause anomalies is that the rate of oxidation of mitochondria that were submaximally uncoupled by FCCP slowly decreased to about 60% of the initial value in 5 min, whereas the oxidation of maximally uncoupled mitochondria was not affected. The decrease in the rate of oxidation was observed both in glass and in perspex oxygraph vessels. After preincubation of the vessel with submaximal concentrations of FCCP for 5 min before the addition of mitochondria, the decreased rate of oxidation

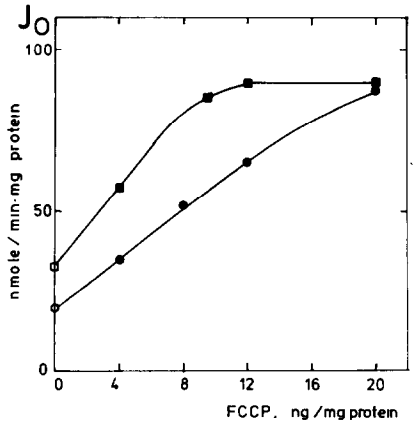


Fig.3. Mitochondrial oxidation as a function of the FCCP concentration with (■) and without (●) 3 $\mu$l ethyl acetate and 7 $\mu$l ethanol per ml.

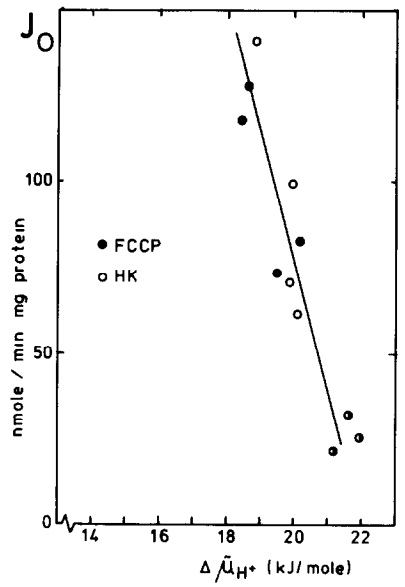


Fig.4. Dependence of the rate of mitochondrial oxidation on $\Delta\tilde{\mu}_{H+}$. $\Delta\tilde{\mu}_{H+}$ was varied using 0–44 pmol FCCP per mg protein (●) or 0–0.4 U hexokinase per mg protein (○). $\Delta\psi$ and $\Delta$pH were calculated from the distributions of [$^3$H]TPMP and [$^{14}$C]DMO, respectively.

was directly obtained and no further decline was observed. Apparently, FCCP is slowly exchanged between the mitochondria and the walls of the vessel.

There may be a number of other less obvious reasons that could cause some incompatibility of the measurements performed in parallel incubations. We therefore conclude that flow-force relationships can be considered fully reliable only when all parameters, i.e. the flux of interest, $\Delta\psi$, $\Delta$pH and the mitochondrial matrix volume are measured in the same incubation. Because only two radioactive markers can conveniently be accommodated in one measurement, the closest approximation of this ideal set-up is to measure all parameters except the mitochondrial volume in one incubation. The matrix volume then has to be determined in a parallel incubation. In a number of experiments performed in this fashion, in which [$^3$H]TPMP$^+$ and [$^{14}$C]DMO were used as probes, identical relationships between $J_o$ and $\Delta\tilde{\mu}_{H+}$ were obtained when $\Delta\tilde{\mu}_{H+}$ was varied by the addition of either hexokinase or uncoupler (FCCP or DNP) in submaximal concentrations. A typical experiment is shown in fig.4. These results confirm the results

obtained earlier with $K^+$ as a probe for $\Delta\psi$. Nevertheless, in a quantitative sense the two probes $TPMP^+$ and $K^+$ need not give exactly the same results. Due to its lipophilic nature, part of the $TPMP^+$ is present in or adsorbed to the mitochondrial membrane. Therefore, $TPMP^+$ may respond to surface potential or to charges inside the membrane [9]. Apart from their dependence on $\Delta\psi$ these parameters may depend on the 'State' of the mitochondria (e.g. State 4, State 3 or uncoupled). In order to estimate the effect of probe binding we compared the values of $\Delta\tilde{\mu}_{H+}$ which were obtained when $\Delta\psi$ was measured with either 5 or 50 $\mu$M $TPMP^+$. Exactly the same values of $\Delta\tilde{\mu}_{H+}$ were obtained. At 50 $\mu$M $TPMP^+$ $\Delta$pH was slightly higher which was compensated for by a small decrease in $\Delta\psi$. Apparently, surface charges do not play an important role. The measurement of $\Delta\psi$ with $K^+$ (in the presence of valinomycin) may also have disadvantages. At the higher concentrations of $K^+$, the mitochondrial matrix volume is influenced by changes in $\Delta\psi$. Also, at very low values of $\Delta\tilde{\mu}_{H+}$ part of the $K^+$ may be held inside the mitochondria by a Donnan potential which must be compensated for by a negative $\Delta$pH (inside acid). In the experiments presented here the decrease in $\Delta\tilde{\mu}_{H+}$ was only small and most of the $K^+$ was retained in the mitochondria.

Zoratti and Petronilli [4] used $TPMP^+$ as a probe for $\Delta\psi$. They obtained $J_p/\Delta\psi$ relationships that were steeper when $\Delta\psi$ was lowered using malonate than when FCCP was added to decrease $\Delta\psi$. The parameters involved, i.e. $J_p$ and $\Delta\psi$, were measured in separate incubations using the same mitochondrial preparation. The distribution of DMO was also measured in separate incubations, but $\Delta$pH data were omitted as the values did not vary within experimental scatter. Mitochondrial matrix volumes were not measured. We observed that systematic deviations could easily be obtained when the different parameters were measured in separate incubations.

The unique flow-force relationships reported in this paper, together with our observation that the phosphate potential maintained by mitochondria in State 4 is proportional to the $\Delta\tilde{\mu}_{H+}$, reported earlier [10], are fully in agreement with the chemiosmotic coupling theory in its simplest form.

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