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# Unique relationships between the rates of oxidation and phosphorylation and the protonmotive force in rat-liver mitochondria

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**The rate of ATP synthesis ($J_P$) in isolated rat-liver mitochondria was strongly dependent on the magnitude of the protonmotive force ($\Delta\tilde{\mu}_{H^+}$) across the mitochondrial inner membrane. Addition of different concentrations of various uncouplers or malonate to mitochondrial incubations in State 3 led to a depression of $\Delta\tilde{\mu}_{H^+}$ and a concomitant decrease in $J_P$. A unique relationship between $J_P$ and $\Delta\tilde{\mu}_{H^+}$ was obtained, which was independent of the way in which $\Delta\tilde{\mu}_{H^+}$ was varied. This unique relationship was observed when $K^+$ (in the presence of valinomycin) was used as a probe for $\Delta\psi$. Different relationships between $J_P$ and $\Delta\tilde{\mu}_{H^+}$ were observed when $K^+$ was used as a probe for $\Delta\psi$ and when $K^+$ was measured after separation of the mitochondria by centrifugation without silicone oil. This led to a serious underestimation of $\Delta\psi$, specifically when uncouplers were present, and non-unique flow-force relationships were thus obtained. Anomalous relationships between $J_P$ and $\Delta\tilde{\mu}_{H^+}$ were also found when $TPMP^+$ was used as a probe for $\Delta\psi$. However, in uncoupler incubations the presence of $TBP^-$ strongly affected the $TPMP^+$ accumulation ratio without any effect on the $K^+$ accumulation or on $J_P$ and in the presence of $TBP^-$ unique relationships between $J_P$ and $\Delta\tilde{\mu}_{H^+}$ were again obtained. This indicates that the accumulation of $TPMP^+$ inside the mitochondria is not a straightforward function of $\Delta\psi$ but also depends on conditions like the presence of $TBP^-$ or uncouplers. We conclude that there is a unique relationship between the rate of phosphorylation and the protonmotive force in mitochondria and that under some conditions the behaviour of $TPMP^+$ is anomalous.**

## Introduction

The chemiosmotic coupling theory of Mitchell [1] is supported by a great number of qualitative and quantitative observations. For instance, the synthesis of ATP can be driven by an artificial bulk-phase proton electrochemical gradient [2–7] (either in the form of $\Delta$pH or $\Delta\psi$) and it has been shown in submitochondrial particles [6] and in

Abbreviations: $TPMP^+$, triphenylmethylphosphonium cation; $DMDPP^+$, dimethyldiphenylphosphonium cation; $TPB^-$, tetraphenylboron anion; DMO, 5,5-dimethyloxazolidine-2,4-dione; DNP, 2,4-dinitrophenol; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; $AP_5A$, P1,P5-di(adenosine-5′)-pentaphosphate; Mops, 4-morpholinepropanesulfonic acid.

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chloroplasts [7] that a rate of phosphorylation can be achieved which is comparable with the rates that are obtained during photophosphorylation and during oxidative phosphorylation, respectively. Also, solute transport in bacteria can be driven by artificial proton gradients (for a review, see Ref. 8).

Furthermore, it has been found that the relationship between the rate of oxidation ($J_O$) and the rate of phosphorylation ($J_P$) in mitochondria is consistent with a quantitative description of mitochondrial oxidative phosphorylation based on the chemiosmotic coupling theory [9]. Also, in mitochondria in State 4 a proportional relationship was found between the phosphate potential ($\Delta G_P$) and the proton electrochemical potential gradient ($\Delta\tilde{\mu}_{H^+}$) with a constant ratio ($ATP/H^+$ stoichiometry) of 3.0 (Woelders et al. [10]). Recently, we reported that, also in agreement with the quantitative description of chemiosmotic coupling in mitochondria, the rates of oxidation and phosphorylation show a unique dependence on the magnitude of the $\Delta\tilde{\mu}_{H^+}$, which is independent of the way in which $\Delta\tilde{\mu}_{H^+}$ is varied [11]. Also, a unique relationship between the rate of phosphorylation and the protonmotive force has been observed in chloroplasts [12] and in submitochondrial particles [13].

However, also many results have been reported that are difficult to reconcile with the chemiosmotic coupling theory. For instance, a non-proportional relationship between $\Delta G_P$ and $\Delta\tilde{\mu}_{H^+}$ has been reported by our group [14–16] and others [17]. Furthermore, in a number of papers [18–22], non-unique relationships between the rates of mitochondrial oxidation and phosphorylation and the $\Delta\tilde{\mu}_{H^+}$ were reported. Also, in chromatophores from phototrophic bacteria, non-unique relationships between $J_P$ and $\Delta\tilde{\mu}_{H^+}$ were described [23]. Such non-unique relationships would indicate that the kinetic parameters of the enzymes involved in the oxidation and in the phosphorylation pathway, are different under different experimental conditions, or that the rates of oxidation and phosphorylation do not uniquely depend on the magnitude of $\Delta\tilde{\mu}_{H^+}$ as the sole energetic intermediary.

It is clear that, with respect to the force–force and the flow–force relationships in oxidative and photophosphorylation, there is no general agreement. Unfortunately, different laboratories obtained different results in comparable experiments. One of the reasons that different results are obtained by different authors may very well be that different methods are used to determine the magnitude of the membrane potential. For instance, in our present paper we show that, when $K^+$ is used as a probe for $\Delta\psi$, anomalous flow–force relationships may be obtained when mitochondria are separated from the medium by centrifugation without using silicone oil. This method was applied by Padan and Rottenberg [21] and Azzone et al. [18], who used $K^+$ or $Rb^+$ as a probe for $\Delta\psi$. We obtained unique $J_O/\Delta\psi$ and $J_P/\Delta\psi$ relationships when $K^+$ was measured after silicone oil centrifugation (this paper) or when $K^+$ was measured during the incubation using a $K^+$ electrode [11].

In most other investigations in which anomalous flow–force relationships were obtained, lipophilic cations such as triphenylmethylphosphonium ($TPMP^+$) were used as probes for $\Delta\psi$ (although in Ref. 23 anomalous flow–force relationships were described in chromatophores, in which $\Delta\psi$ was measured using the carotenoid band shift). In the experiments presented in this paper, we measured the distribution of the two probes $K^+$ and $TPMP^+$ in the same incubation. We found that either a unique or a non-unique relationship between the rate of phosphorylation and $\Delta\tilde{\mu}_{H^+}$ can be obtained depending on whether $\Delta\psi$ is calculated from the distribution of either $K^+$ or $TPMP^+$. The observed discrepancy between the two $\Delta\psi$ probes can well explain some of the discrepancies in the literature with respect to the flow–force relationships and is also of importance with respect to the force–force relationships. Possible mechanisms that lead to the observed discrepancy between the two $\Delta\psi$ probes will be discussed.

## Materials and Methods

Rat-liver mitochondria were isolated as described in Ref. 24, but the isolation medium consisted of 275 mM mannitol and 2 mM Mops (pH 7.0) instead of 250 mM sucrose. The protein content was measured by the biuret method, using

bovine serum albumin (Sigma, essentially acid-free) as a standard. Oxygen concentrations were measured using a Clark oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH). The incubation medium contained 10 mM succinic acid, 1 mM L-malic acid, 10 mM $H_3PO_4$, 2 mM EGTA, 4 mM $MgCl_2$, 10 mM glucose, 0.1 mM KCl and 10 mM Mops. The medium was brought to pH 7.0 with Tris and contained mannitol to a final osmolality of 290 mosmol/kg. The medium was oxygenated prior to the incubations by bubbling pure oxygen through it. Mitochondria (1 mg of protein per ml) were incubated at 25 °C in a magnetically stirred oxygraph vessel with the lid removed. The oxygen concentration never dropped to values lower than 125 $\mu$M $O_2$ (= 50% saturation to air) before all the samples had been taken. Per mg protein 1 nmol rotenone was added. In all experiments, except the one shown in Fig. 3, the medium contained 0.1 mM KCl and 360 pmol valinomycin was added per mg protein. $TPMP^+$ or $DMDPP^+$, when used, was present in a concentration of 5 $\mu$M (iodide salts). DMO, when used, was present in a concentration of 2 $\mu$M. $\Delta\tilde{\mu}_{H^+}$ was varied using different concentrations of uncouplers (DNP (Tris salt), FCCP, gramicidin) or the competitive inhibitor for succinate dehydrogenase, malonate (Tris salt). In all experiments, except the one shown in Fig. 4, phosphorylation was maximally stimulated by the addition of 2 U hexokinase (Sigma Type 5) and 0.5 mM ADP (disodium salt). 0.5 mM $AP_5A$ (trilithium salt) was also included to inhibit adenylate kinase activity. Finally, 14 nCi [$^3$H]$TPMP^+$ or [$^3$H]$DMDPP^+$ were added per ml. When $\Delta\psi$ and $\Delta$pH were both to be measured, 0.7 $\mu$Ci [$^3$H]$TPMP^+$ and 0.1 $\mu$Ci [$^{14}$C]DMO were used per ml. All added aqueous solutions had a pH of 7.

Control experiments showed that a period of 2 min is ample time for the equilibration of the probes across the mitochondrial inner membrane. A time of 2–3 min after addition of the radioactive probes was allowed before the first sample was drawn.

Two 500 $\mu$l samples were drawn at a 2-min interval for determination of $J_P$ and were quenched immediately by vigorous mixing with 500 $\mu$l phenol/ chloroform/ isoamylalcohol, 38 : 24 : 1 (v/v/v) plus 50 $\mu$l 0.25 M EDTA during 1 min on a vortex mixer [25]. Then the samples were stored at −20 °C to be assayed later. In between taking the two $J_P$ samples a 600 $\mu$l sample was drawn (from the same oxygraph vessel) for determination of $\Delta\tilde{\mu}_{H^+}$ and was immediately centrifuged in an Eppendorf centrifuge at 9000 × $g$ during 1 min in a tube containing 0.1 ml silicone oil (AR200/AR20 = 3 : 1, Wacker GmbH, München, F.R.G.) unless specified otherwise. Directly after centrifugation, 0.2 ml was taken from the supernatant and transferred to another tube and the rest of the supernatant (and some of the oil) was aspirated. The tube was washed by completely filling it with Millipore-prepared water which was kept separate from the pellet by the remaining oil. After aspiration of the water and virtually all of the oil the pellets were stored at −20 °C to be processed later.

To prepare the $J_P$ samples for assay they were thawed, centrifuged and the aqueous phase was transferred to another tube. Then the samples were extracted with one volume of chloroform and one volume of *n*-hexane, respectively. Glucose 6-phosphate (and ATP) was assayed with standard enzymatic methods. The $\Delta\tilde{\mu}_{H^+}$ samples were processed as follows: the pellet was resuspended in 135 $\mu$l Millipore-prepared water. Then, the supernatant as well as the pellet fractions were deproteinised. A small volume of 2 M $HClO_4$ was added to reach a final concentration of 0.2 M and, after 10 min on ice, the tubes were centrifuged at 4 °C for 2 min at 9000 × $g$. Triplicate 10 $\mu$l aliquots were diluted 50 times with Millipore-prepared water for assay of the $K^+$ concentration. The $K^+$ concentration was determined using a Hitachi graphite furnace polarised Zeeman atomic absorption spectrophotometer (A.A.S. 180-80). In all the manipulations described above, care was taken to avoid contamination with $K^+$ of the water, solutions, tubes and pipette tips that were used. From the rest of the pellet and supernatant fractions one 100-$\mu$l or two 50-$\mu$l samples were counted in 4 ml Insta Gel (Packard). The counts were corrected for quenching and channel cross-talk. $\Delta\psi$ was calculated, using the Nernst equation, from the accumulation of $K^+$, $TPMP^+$ or $DMDPP^+$, without any correction for probe binding.

Thus, the distributions of $K^+$ and of $TPMP^+$ or $DMDPP^+$ were always measured in the same sam-

ple and this sample was always drawn from the same incubation as the one in which the rate of phosphorylation was measured.

In previous experiments it was checked that virtually all the matrix $K^+$ is mobile. We measured the $K^+$ content of mitochondria that had been energised with the medium and conditions described above (in State 4) and then received 80 $\mu$M DNP and 10 mM malonate. The $K^+$ content then dropped to $2.1 \pm 1.4$ nmol $K^+$/mg protein ($n = 3$). Without DNP and malonate the average $K^+$ content was 94 nmol/mg protein (in State 4).

In a number of experiments, the total aqueous volumes and the extra-matrix aqueous volumes were measured using 7 $\mu$Ci [$^3$H]$H_2O$ and 0.6 $\mu$Ci [$^{14}$C]mannitol in incubations that were performed in parallel to the incubations in which $\Delta\tilde{\mu}_{H^+}$ (or $\Delta\psi$) and $J_P$ were measured. When different parameters were to be measured 'in parallel incubations', one incubation was started to which all reagents except the radioactive markers were added. The volume of this incubation was then divided over two identical oxygraph vessels and the respective radioactive probes were added. Samples from both vessels were taken and were centrifuged at the same time. Further procedures were identical, except that, when [$^3$H]$H_2O$ was to be measured, enough silicone oil was left to cover the mitochondrial pellets to prevent evaporation of [$^3$H]$H_2O$. In all experiments in which the mitochondrial volumes were measured we found that the matrix volumes in malonate and in uncoupler incubations were the same at the same value of $\Delta\psi$. Furthermore, the small decrease in $\Delta\tilde{\mu}_{H^+}$ relative to the State-3 value attained in the experiments shown in this paper resulted in only a very small decrease in the matrix volumes (three experiments; 36 incubations: the average volume was $0.93 \pm 0.13$ $\mu$l/mg protein when uncouplers were used or $0.95 \pm 0.11$ when malonate was present). Therefore, in some experiments where the matrix volumes were not measured, a constant matrix volume of 0.9 $\mu$l/mg protein and an extra-matrix aqueous volume of 2.5 $\mu$l/mg protein were assumed for the calculation of $\Delta\psi$.

[$^3$H]DMDPP$^+$ was synthesized by the reaction of [$^3$H]$CH_3I$ with excess methyldiphenylphosphine (from Aldrich) in ethylether and subsequent washing of the precipitate with ethylether. The final precipitate was dried and dissolved in ethanol. Radioactivity and chemical purity were assayed using thin-layer chromatography on Merck Kieselgel 60 F 254 in ethanol/$H_2O$, 2:1. Unlabeled DMDPP$^+$ was prepared using the same procedure. $^3H_2O$, [$^{14}$C]mannitol and [$^{14}$C]DMO (solid, dissolved in 10 mM Mops buffer (pH 7) to 0.1 mCi/ml and stored at $-20\,^\circ$C) were obtained from the Radioactive Centre, Amersham, England. [$^3$H]TPMP$^+$ and [$^3$H]$CH_3I$ were obtained from New England Nuclear. DNP was from Merck-Schuchard, Hohenbrunn, F.R.G. FCCP was a gift from Dr. P.G. Heytler.

## Results

In our present experiments we used $K^+$ (in the presence of valinomycin) and TPMP$^+$ as probes for the mitochondrial membrane potential. The mitochondria were separated from the medium by silicone oil centrifugation and $K^+$ and TPMP$^+$ were measured in the pellet and supernatant fraction.

TPMP$^+$ is moderately soluble in the silicone oil we used. Therefore we checked whether TPMP$^+$ would be lost to the silicone oil during centrifugation of the mitochondria through the oil and subsequent sample work-up. No [$^3$H]TPMP$^+$ could be recovered in the silicone oil after centrifugation of the mitochondria (not shown). Therefore it may be concluded that in the short time that passes between entrance of the mitochondria in the oil phase and separation of the silicon oil from the pellet (maximally 2 min in our experiments) no TPMP$^+$ is lost from the mitochondria.

A number of investigations have been reported in which mitochondria were separated from the medium by centrifugation of the samples in tubes without silicone oil (e.g., Refs. 18–21 and 26). We investigated whether different values of $\Delta\psi$ would be obtained when mitochondria were separated from the medium with and without silicone oil, when either $K^+$ or TPMP$^+$ was used as probes for $\Delta\psi$. Mitochondria were incubated in the presence of $K^+$ (and valinomycin) and [$^3$H]TPMP$^+$. $\Delta\psi$ was lowered by the addition of either malonate or DNP. After ample time for equilibration of the probes (3 min), two samples were drawn and centrifuged at the same time in tubes with and

TABLE I

$\Delta\psi$ CALCULATED FROM THE $K^+$ AND THE $TPMP^+$ DISTRIBUTION

From each incubation, two samples were drawn, one of which was centrifuged in a tube with (+) and the other in a tube without (−) silicone oil. $K^+$ and $TPMP^+$ were measured in the pellet and supernatant fractions. Phosphorylation was maximally stimulated by the addition of ADP and hexokinase. Medium composition and other conditions were as described in the Materials and Methods section. $\Delta\psi$ was lowered by addition of malonate or DNP. In the calculation of $\Delta\psi$ the mitochondrial matrix and extra-matrix aqueous volumes were assumed to be 0.9 and 2.5 μl/mg of protein, respectively (see Materials and Methods section). Averaged values are given ± standard deviation ($n = 3$). The standard deviation pertains to the variation in between incubations, which were all performed with the same mitochondrial preparation, and should not be considered as a measure of variation between different mitochondrial preparations.

| | $\Delta\psi$ calculated from the $K^+$ accumulation (kJ/mol) | | |
|---|---|---|---|
| | (+) | (−) | (+)−(−) |
| 0.6 mM Malonate | 12.95 ± 0.14 | 12.80 ± 0.37 | 0.15 ± 0.38 |
| 8 μM DNP | 13.98 ± 0.17 | 13.33 ± 0.30 | 0.65 ± 0.42 |
| | $\Delta\psi$ calculated from the $TPMP^+$ accumulation (kJ/mol) | | |
| | (+) | (−) | (+)−(−) |
| 0.6 mM Malonate | 14.70 ± 0.23 | 14.64 ± 0.18 | −0.06 ± 0.09 |
| 8 μM DNP | 14.87 ± 0.13 | 14.86 ± 0.13 | 0.01 ± 0.12 |

without silicone oil. We found no appreciable difference between the $TPMP^+$-based $\Delta\psi$ values obtained using the procedures with and without silicone oil. On the other hand, omission of the silicone oil in the centrifugation procedure led to an underestimation of the $K^+$ accumulation, especially in the uncoupler incubations. This underestimation was found to be up to 2 kJ/mol when we used our normal protocol, but the extent of the underestimation depended on the time that the pellet remained in contact with the supernatant. In the experiment shown in Table I we minimized the time used for taking a sample from the supernatant and for aspiration of the rest of the supernatant so that maximally 10 s elapsed between arresting the centrifuge and aspiration of the supernatant. As is shown in Table I, still a considerable underestimation of the $K^+$ accumulation of 0.65 kJ/mol was found when DNP was present. When malonate was used to depress $\Delta\psi$ instead of DNP, only a very small underestimation of the accumulation was found.

Apparently, after the mitochondria were sedimented to a pellet and consequently deprived of oxygen and respiratory substrates, $K^+$ leaks out from the pellet to the supernatant when the pellet and the supernatant are not separated by a layer of silicone oil. The fact that this occurs to a much greater extent in the presence of an uncoupler may be explained by the fact that the efflux of $K^+$ will cause a diffusion potential that tends to keep the $K^+$ inside the mitochondria. The dissipation of this diffusion potential and thereby the efflux of $K^+$ will be accelerated when protons are allowed to enter the mitochondria via the protonophore. Thus, as is shown in Fig. 1, omission of silicone oil from the centrifugation procedure may lead to

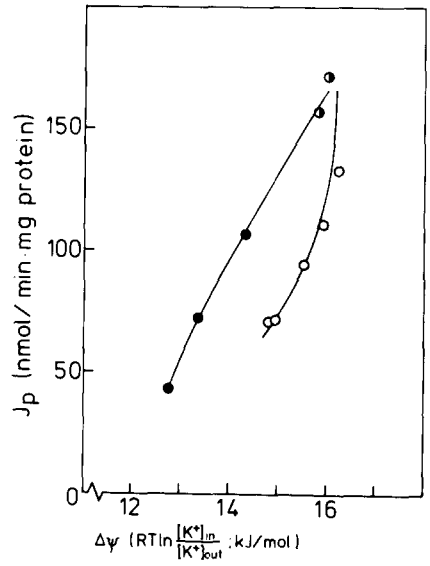


Fig. 1. Relationship between rate of phosphorylation ($J_P$) and $\Delta\psi$, when the mitochondria are separated from the medium by centrifugation without silicone oil. $\Delta\psi$ was calculated from the $K^+$ accumulation. $K^+$ was measured in the pellet and supernatant fraction after centrifugation. The mitochondrial matrix and extra-matrix aqueous volumes were determined in parallel incubations. Phosphorylation was maximally stimulated by the addition of ADP and hexokinase and $\Delta\psi$ was varied by adding 0–0.8 mM malonate (○) or 0–9 μM DNP (●). Medium composition and other conditions were as described in the Materials and Methods section.

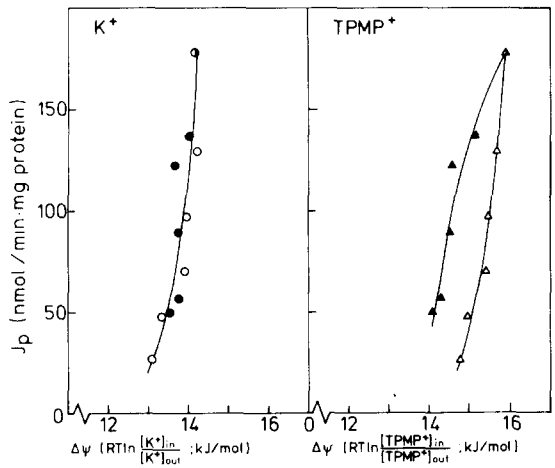


Fig. 2. Relationship between $J_P$ and $\Delta\psi$. $\Delta\psi$ was calculated from the accumulation of either $K^+$ (left panel, circles) or $TPMP^+$ (right panel, triangles). For each incubation, the accumulation ratios of $K^+$, $TPMP^+$ and DMO were measured in the same sample and samples for the measurement of $J_P$ were taken from that same incubation. Mitochondrial matrix and extra-matrix aqueous volumes were measured in parallel incubations. Phosphorylation was maximally stimulated by the addition of ADP and hexokinase and $\Delta\tilde{\mu}_{H^+}$ was varied by adding 0–1.6 mM malonate (open symbols) or 0–16 $\mu$M DNP (closed symbols). Medium composition and other conditions were as described in the Materials and Methods section. $\Delta$pH values (DMO) are not shown but they did not vary significantly. The average $\Delta$pH value in the malonate incubations was $1.61 \pm 0.16$ ($n = 5$) and in the DNP incubations was $1.73 \pm 0.32$ ($n = 5$).

anomalous flow-force relationship, in which underestimated values of $\Delta\psi$ are present.

In the experiment shown in Fig. 2, the mitochondrial membrane potential was calculated from the distribution of both $K^+$ (in the presence of valinomycin, Fig. 2A) and $TPMP^+$ (Fig. 2B). The mitochondria were separated from the medium by silicone oil centrifugation. Respective measurements of $K^+$ and $TPMP^+$ were performed each time in the pellet and supernatant fraction from one and the same sample. In the incubation medium 10 mM glucose was present and ADP and excess hexokinase were added to maintain a steady, maximally stimulated rate of phosphorylation (State 3). Addition of either a respiratory inhibitor or an uncoupler to mitochondrial incubations in State 3 led to a decrease in the membrane potential and a concomitant decrease in the rate of phosphorylation. When $\Delta\psi$ was calculated from the distribution of $K^+$, a unique relationship was found between $J_P$ and $\Delta\psi$, that is independent of the way in which $\Delta\psi$ was varied. These results confirm the data from the experiments reported in our previous paper, in which $\Delta\psi$ was measured using a $K^+$-sensitive glass electrode. On the other hand, when $\Delta\psi$ was calculated from the $TPMP^+$ distribution, there was no unique relationship between $J_P$ and $\Delta\psi$; at a certain rate of phosphorylation a lower value of $\Delta\psi$ was found when $\Delta\psi$ was depressed by adding DNP than when malonate was used to lower $\Delta\psi$. The same results were observed when another protonophore, e.g. FCCP, or a pore-former, e.g. gramicidin, was used instead of DNP (not shown).

In Fig. 3 the data from the experiment shown in Fig. 2 are represented in such a way that the $K^+$- and $TPMP^+$-based $\Delta\psi$ values from one set of incubations are plotted in the same graph. When no uncoupler or respiratory inhibitor was added, a higher value of $\Delta\psi$ was calculated on the basis of the $TPMP^+$ distribution than on the basis of the $K^+$ distribution. This may be caused by an overestimation of the $TPMP^+$ accumulation due to binding of the lipophilic probe to the membrane [27]. This difference between the $K^+$ and $TPMP^+$ accumulation values can be seen to remain constant when various concentrations of malonate

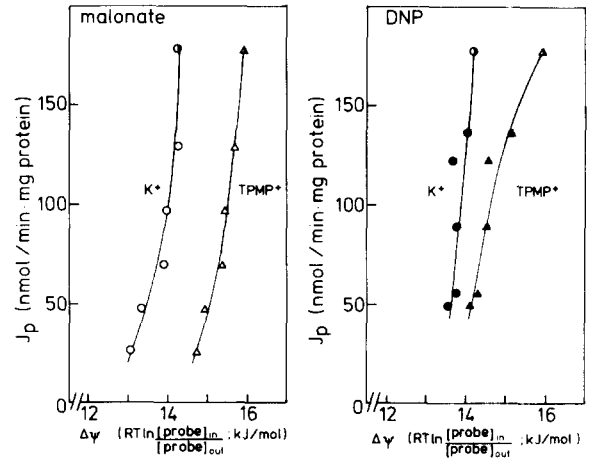


Fig. 3. Data from the experiment shown in Fig. 2 represented in such a way that the $K^+$- and $TPMP^+$-based $\Delta\psi$ values from one set of incubations are shown in the same graph. Left panel: malonate incubations; right panel: DNP incubations. The symbols have the same meaning as in Fig. 2.

were added (Fig. 3A). However, the difference between the $K^+$ and $TPMP^+$ values decreased when increasing concentrations of uncoupler were added (Fig. 3B). This suggests that the binding of $TPMP^+$ to the membrane is decreased when energised mitochondria are uncoupled. Thus, in other words, the discrepancy between the two probes would be caused by the fact that the binding parameters of the $TPMP^+$ binding to the membrane are not constant and may be altered when energised mitochondria are uncoupled. In de-energised mitochondria (in the presence of 100 mM KCl and excess valinomycin, no exogenous substrates and excess antimycin A) we measured the partitioning coefficient for the partitioning of $TPMP^+$ between the aqueous phase and the mitochondrial inner plus outer membranes (cf. Ref. 27) and observed no difference when either 12 $\mu$M DNP or 1 mM malonate was present (results not shown). We have tried to measure $TPMP^+$ binding in energised mitochondria, using phosphorus NMR, but the $TPMP^+$ signal was too weak, even at high concentrations of $TPMP^+$.

Because we know of no suitable method to discriminate in energised mitochondria between accumulated $TPMP^+$ that is present free in the matrix aqueous phase and $TPMP^+$ that is bound to the membrane, we cannot test this hypothesis. Therefore we used another probe for $\Delta\psi$ to see whether the results obtained with either $K^+$ or $TPMP^+$ could be confirmed.

We decided to use the dimethyldiphenylphosphonium cation ($DMDPP^+$). The ability of $TPMP^+$ to permeate through the mitochondrial membrane is believed to be due to the presence of the phenyl groups, which tend to spread out evenly the single positive charge over a relatively large area. $DMDPP^+$ is closely related to $TPMP^+$. The difference with $TPMP^+$ is that $DMDPP^+$ has one phenyl group less, and a methyl group instead. Therefore the compound can be expected to have a lower affinity for binding to the mitochondrial membranes.

Another expected consequence is, of course, that permeation of $DMDPP^+$ through the mitochondrial inner membrane is slower than that of $TPMP^+$. As is shown in Fig. 4, the uptake of $DMDPP^+$ was, indeed, rather slow. In another experiment (not shown) uptake still proceeded

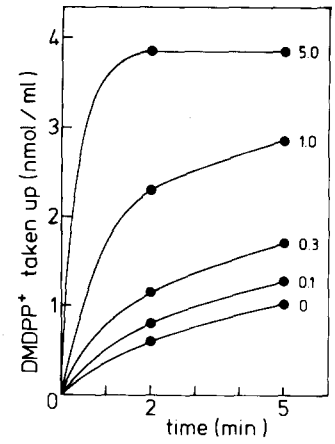


Fig. 4. Time-courses of uptake of $DMDPP^+$ by mitochondria, in the presence of 0–5 $\mu$M $TPB^-$. The concentrations of $TPB^-$ that were used are indicated in the graph (in $\mu$M). No ADP, malonate or uncoupler was added. Medium composition and other conditions were as described in the Materials and Methods section.

after 20 min of incubation and the gradient of $DMDPP^+$ that had been reached was still lower than that of $K^+$, suggesting that equilibrium of the $DMDPP^+$ accumulation with $\Delta\psi$ was not reached. The rate of entry of $DMDPP^+$ into the mitochondria could be increased by adding tetraphenyl boron anion ($TPB^-$). As is shown in Fig. 4, the uptake of $DMDPP^+$ strongly increased and equilibrium was reached within 2 min when 5 $\mu$M $TPB^-$ was present in the incubation medium.

In the experiment shown in Fig. 5, $DMDPP^+$ was used as a probe for $\Delta\psi$ in the presence of 5 $\mu$M $TPB^-$ and a comparison was made between the three probes $K^+$, $TPMP^+$ and $DMDPP^+$. $TPMP^+$ and $DMDPP^+$ (with 5 $\mu$M $TPB^-$) were used in parallel incubations together with $K^+$ and valinomycin. From each incubation the rate of phosphorylation and the probe distribution (both $TPMP^+$ and $K^+$ or both $DMDPP^+$ and $K^+$) were determined. In this way $K^+$ may serve as a common reference for the comparison of the two probes $DMDPP^+$ and $TPMP^+$. ADP and hexokinase were present to maintain a maximal steady rate of phosphorylation. $\Delta\tilde{\mu}_{H^+}$ was varied using different concentrations of either malonate or DNP. The conditions and methods were the same as in the other experiments and were as described in the Methods section.

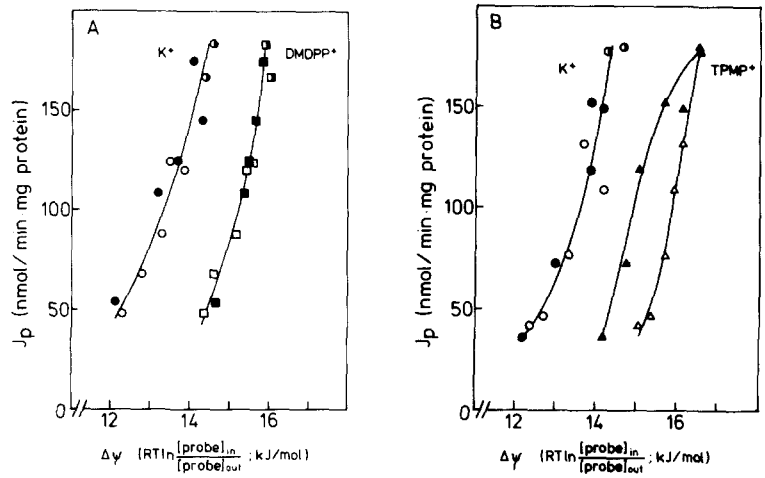


Fig. 5. Relation between $J_P$ and $\Delta\psi$. Phosphorylation was maximally stimulated by the addition of ADP and hexokinase. ($\Delta\tilde{\mu}_{H^+}$) was varied by adding 0–1.6 mM malonate (open symbols) or 0–16 $\mu$M DNP (closed symbols). Medium composition and other conditions were as described in the Materials and Methods section. $\Delta\psi$ was calculated from the accumulation of $K^+$ (circles), $DMDPP^+$ (squares) and $TPMP^+$ (triangles). $TPMP^+$ and $DMDPP^+$ were used in parallel incubations, which means that an incubation was started to which all reagents except the radioactive markers were added; this volume was then divided over two identical oxygraph vessels and [$^3$H]$TPMP^+$ was added to the one; and [$^3$H]$DMDPP^+$ (5 $\mu$M) and $TPB^-$ (5 $\mu$M) were added to the other vessel. $J_P$ and the $K^+$ accumulation were measured both in the $TPMP^+$ and the $DMDPP^+$ incubations. The results from the $DMDPP^+$ incubations are shown in Fig. 5A and those of the $TPMP^+$ incubations in Fig. 5B. For each incubation, the accumulation ratios of $K^+$ and the respective phosphonium probe were measured in the same sample and samples for the measurement of $J_P$ were taken from that same incubation. In the calculation of $\Delta\psi$, the mitochondrial matrix and extra-matrix aqueous volumes were assumed to be 0.9 and 2.5 $\mu$l/mg of protein, respectively (see the Materials and Methods section).

When $J_P$ was plotted as a function of the $\Delta\psi$ values obtained with the different probes, it can be seen that, for all the DNP and malonate concentrations used, both the $K^+$ accumulation values and the $J_P$ values obtained in the $DMDPP^+$ incubations are very well comparable with the values obtained in the $TPMP^+$ incubations. This means that there was no inhibition of phosphory-

TABLE II

EFFECT OF $TPB^-$ ON THE $\Delta\psi$ CALCULATED FROM THE ACCUMULATION OF $TPMP^+$ AND $K^+$

Phosphorylation was maximally stimulated by the addition of ADP and hexokinase and concentrations of DNP and malonate were chosen to give equal rates of phosphorylation and equal $K^+$ distribution across the mitochondrial matrix membrane. Medium composition and other conditions were as described in the Materials and Methods section. Incubations with and without 5 $\mu$M $TPB^-$ were performed in parallel, which means that one incubation was started, to which all reagents except the $TPMP^+$ and $TPB^-$ were added. This volume was then divided over two identical oxygraph vessels and $TPB^-$ (5 $\mu$M) was added to one, and [$^3$H]$TPMP^+$ (5 $\mu$M) was added to both vessels. In the calculation of $\Delta\psi$, the mitochondrial matrix and extra-matrix aqueous volumes were assumed to be 0.9 and 2.5 $\mu$l/mg protein, respectively (see the Materials and Methods section). Averaged values are given ± standard deviation ($n = 3$). The standard deviation pertains to the variation in between incubations which were all performed with the same mitochondrial preparation.

| | $\Delta\psi$ $TPMP^+$ (kJ/mol) | | $\Delta\psi$ $K^+$ (kJ/mol) | | $\Delta\psi$ $TPMP^+ - \Delta\psi$ $K^+$ (kJ/mol) | |
|---|---|---|---|---|---|---|
| | $-TPB^-$ | $+TPB^-$ | $-TPB^-$ | $+TPB^-$ | $-TPB^-$ | $+TPB^-$ |
| 0.56 mM Malonate | 15.80 ± 0.11 | 15.92 ± 0.07 | 13.75 ± 0.27 | 13.64 ± 0.27 | 2.05 ± 0.33 | 2.29 ± 0.33 |
| 12 $\mu$M DNP | 14.65 ± 0.10 | 15.84 ± 0.10 | 13.02 ± 0.19 | 13.28 ± 0.25 | 1.63 ± 0.32 | 2.56 ± 0.29 |

lation and there was no change in the permeability of the mitochondrial inner membrane for protons when $DMDPP^+$ and $TPB^-$ were present.

A unique relationship between $J_P$ and $\Delta\psi$ was obtained both with $K^+$ and $DMDPP^+$. The $DMDPP^+$-based $\Delta\psi$ values are higher than those obtained with $K^+$ but the difference between the values obtained with these two probes is constant. In contrast, an anomalous $J_P/\Delta\psi$ relationship was again found when $TPMP^+$ was used as a probe for $\Delta\psi$.

The difference between the results obtained with $TPMP^+$ and $DMDPP^+$ (with $TPB^-$) may be attributed to differences in molecular structure and characteristics of the two probes. Another reason may, of course, be the presence of $TPB^-$ which was added to facilitate permeation of $DMDPP^+$ through the membrane.

To test this, another comparison was made between $TPMP^+$ and $K^+$ (in the presence of valinomycin). This time $TPMP^+$ was used with and without 5 $\mu$M $TPB^-$ in parallel incubations. The experimental conditions were the same as in the experiment shown in Figs. 2, 3 and 5. Phosphorylation was maximally stimulated by the addition of ADP and hexokinase and concentrations of DNP and malonate were chosen to give approximately equal rates of phosphorylation and equal $K^+$ distribution across the mitochondrial matrix membrane.

As shown in Table II, in the absence of $TPB^-$ the same result was obtained as has already been shown in Fig. 3, i.e., that the difference between the $K^+$- and the $TPMP^+$-based $\Delta\psi$ values was smaller when DNP was present. Strikingly, this was not found when 5 $\mu$M $TPB^-$ was present in the incubation. Instead, the difference between the $TPMP^+$- and the $K^+$-based $\Delta\psi$ values was the same (or even somewhat bigger) in the DNP incubations as in the malonate incubations. Thus, the effect of DNP on the $TPMP^+$ $\Delta\psi$ value relative to the $K^+$ value is somehow prevented or counterpoised by $TPB^-$.

## Discussion

To investigate the validity of the chemiosmotic coupling theory, it can be tested whether relationships between various energetic parameters are consistent with some basic predictions based on this theory. For instance, when mitochondria have reached equilibrium for the ATP-synthase and ATP/ADP and $P_i$ translocation reactions and the ATP/proton stoicheiometry is assumed to be constant, a proportional relationship is expected between the extramitochondrial phosphate potential ($\Delta G_P^{ex}$) and $\Delta\tilde{\mu}_{H^+}$. In several reports a non-proportional relationship has been shown (e.g., Refs. 14–17). However, as was shown by Woelders et al. [10], at least part of these observations may be explained as a consequence of a series of systematic deviations due to the methodology that was used for the measurement of $\Delta G_P^{ex}$ and $\Delta\tilde{\mu}_{H^+}$. More recently, Petronilli et al. [26] claimed to have excluded systematic errors and found a significant rate of net ATP synthesis and a rise of the energy charge in the presence of rather high concentrations up to 100 nmol FCCP per mg of protein. At the values of $\Delta G_P^{ex}$ that were eventually reached, low values of $\Delta\tilde{\mu}_{H^+}$ were found that were not compatible with a stoicheiometry of three or four protons per ATP. A serious drawback of these experiments, however, is that $\Delta\tilde{\mu}_{H^+}$ and $\Delta G_P$ were not measured in the same incubation and were not even determined under exactly the same conditions of incubation. This could lead to differences in the $\Delta\tilde{\mu}_{H^+}$ values between the '$\Delta G_P$' incubations and the '$\Delta\tilde{\mu}_{H^+}$' incubations. A number of mechanisms that could lead to such differences have been found and discussed by Woelders et al. [11].

Another discrepancy in the literature concerns the relationships between the magnitude of $\Delta\tilde{\mu}_{H^+}$ and the steady-state rate of $\Delta\tilde{\mu}_{H^+}$-dependent reactions. When $\Delta\tilde{\mu}_{H^+}$ is varied and all other kinetic and thermodynamic parameters of the particular reaction of interest are kept constant, a unique dependence of the rate of that reaction on the magnitude of $\Delta\tilde{\mu}_{H^+}$ is expected that is independent of the way in which $\Delta\tilde{\mu}_{H^+}$ is varied. Such unique relationships have indeed been shown in a number of publications [11–13]. However, in contrast also anomalous relationships have often been reported [18–22].

In our present paper, we have shown that, when $K^+$ (with valinomycin) was used as a probe for $\Delta\psi$ and the mitochondria were separated from the medium without using silicone oil, $\Delta\psi$ might be seriously underestimated in the presence of an

uncoupler. We conclude from our experiments that $K^+$ is lost from the pellet when no silicone oil is present to separate the pellet from the supernatant. The efflux of $K^+$ from the mitochondria in the pellet will generate a diffusion potential that tends to keep the $K^+$ inside the mitochondria. Therefore, any mechanism that causes dissipation of this diffusion potential or that will allow the efflux of $K^+$ without net-charge translocation will increase the $K^+$ efflux and thereby the underestimation of $\Delta\psi$. As in our experiments, such a mechanism could for instance be a protonophore, provided that there is a certain pH buffer capacity inside and outside the mitochondria. Also, underestimation of $\Delta\psi$ may be expected when nigericin is present, or when the mitochondrial $H^+/K^+$ antiporter is activated by extensive $K^+$ uptake and matrix swelling, which is reported to be the mechanism of uncoupling by high $[K^+]$ media in the presence of valinomycin [28,26]. In fact, with every cation that is used as a probe for $\Delta\psi$, it may be expected that whenever another cation is allowed to enter the mitochondria after sedimentation of the mitochondria, underestimation of the $\Delta\psi$ values will occur. The extent of the underestimation will depend on the abundance of the other cation, the buffer capacity for these ions inside and outside the mitochondria, and the mobility of these ions and the probe cation within the mitochondrial pellet. Such a mechanism may explain the anomalous $J_O/\Delta\tilde{\mu}_{H^+}$ relationships that were shown in Refs. 18 and 21 in which $\Delta\tilde{\mu}_{H^+}$ was varied using ADP, FCCP, nigericin and the $Ca^{2+}$ ionophore A23187, respectively, and $K^+$, $Rb^+$ or $Ca^{2+}$ were used as probes for $\Delta\psi$. However, it cannot explain the anomalous flow-force relationships that were obtained when $TPMP^+$ was used as a probe for $\Delta\psi$, since no important underestimation of $TPMP^+$ accumulation was found when silicone oil was omitted from the centrifugation procedure. This may be due to the lipophilic character of $TPMP^+$, that probably leads to a slow diffusion of the probe in the mitochondrial pellet. Furthermore, Zoratti and Pertronilli [20] showed that an anomalous $J_P/\Delta\psi$ relationship was also obtained when $TPMP^+$ was measured during the incubation, using a $TPMP^+$-sensitive electrode, instead of measuring it in the pellet and supernatant fraction after centrifugation.

Our present results agree with the results presented by Zoratti and Petronilli [20], as we also found anomalous $J_P/\Delta\tilde{\mu}_{H^+}$ relationships when $\Delta\psi$ was calculated from the distribution of $TPMP^+$ which was measured after separation of the mitochondria from the pellet using silicone oil centrifugation. However, when $\Delta\psi$ was calculated from the $K^+$ distribution, which was measured in the same incubations and actually in the same samples as the $TPMP^+$, unique relationships between $J_P$ and $\Delta\tilde{\mu}_{H^+}$ were obtained. This discrepancy between the two $\Delta\psi$ probes was found in a great number of experiments of which only few examples are shown.

Our results may be interpreted as follows. When the $\Delta\psi$ values calculated from the $K^+$ distribution are tentatively assumed to be correct or anyhow are taken as a reference parameter, it can be seen that the use of $TPMP^+$ as a probe for $\Delta\psi$ leads to overestimated values of $\Delta\psi$. This is attributed to probe binding, especially to the inner side of the mitochondrial inner membrane [27]. This overestimation is constant when increasing concentrations of malonate are used, but is strongly decreased when the mitochondria are uncoupled (see, e.g., Fig. 3). This, and not the overestimation per se, is the actual cause for the discrepancy between the two probes. Therefore, correction for probe binding, as has been proposed by Rottenberg [27] or as has been used by others, would not abolish the discrepancy between the two $\Delta\psi$ probes, since a constant $TPMP^+$ binding affinity is assumed in such correction models. If the presence of uncouplers indeed affects the $TPMP^+$ binding coefficient, this cannot be a specific effect of a certain uncoupler or type of uncoupler, since the same results are obtained with such different uncouplers as DNP and gramicidin. This, and also the fact that no difference of the binding coefficient was observed in the de-energised mitochondria, indicates that the effect would somewhat be related to the uncoupled state of mitochondria, e.g., the increased turnover of protons through the system, although we do not understand the mechanism. In the presence of $TPB^-$, no discrepancy between the two $\Delta\psi$ probes was observed and the overestimation of the $TPMP^+$ (or $DMDPP^+$) $\Delta\psi$ values relative to the $K^+$ values was the same in the presence of DNP and in its absence. Ap-

parently, $TPB^-$ prevents the effect of DNP or has the opposite effect, thus counterpoising the DNP effect.

As alternative to the above interpretation, it could be assumed that $TPMP^+$ binding affinity is constant and that, apart from a constant overestimation, the $TPMP^+$ $\Delta\psi$ values are correct. The fact that the $TPMP^+$- and $K^+$-based $\Delta\psi$ values approached one another when increasing concentrations of an uncoupler were added (e.g., see Fig. 3), should then be explained by assuming that the uncoupler somehow causes a relative increase in the $K^+$ accumulation. The only two mechanisms that may be proposed to have this effect are an increase in $K^+$ binding or a decrease in $K^+$ efflux through a $K^+/H^+$ antiporter mechanism. A saturable binding of $K^+$ has been observed [29] but is shown to be of no significance considering the relatively high $K^+$ concentration that is normally found in mitochondria. Even at lowered values of $\Delta\psi$, e.g., in the experiment shown in Fig. 3, the intramitochondrial $K^+$ concentration still ranged from 41 to 65 nmol/mg protein. Therefore it is very unlikely that the presence of an uncoupler would induce extra $K^+$ binding to such an extent that important deviations of the $K^+$-based $\Delta\psi$ values would be obtained. The $K^+/H^+$ antiporter mechanism that was reported by Bernardi and Azzone [28] is activated when the intramitochondrial $K^+$ concentration becomes too high and matrix swelling is induced. This is observed only when mitochondria are incubated in high $[K^+]$ media. At the moderate intramitochondrial $K^+$ concentrations observed in our experiments, as given above, a substantial underestimation of $\Delta\psi$ values caused by this $K^+/H^+$ antiporter mechanism, that then also must be assumed to be directly affected by the presence of an uncoupler, is not very likely. Also, that the above mechanisms would have led accidentally to unique $J_O/\Delta\tilde{\mu}_{H^+}$ and $J_p/\Delta\tilde{\mu}_{H^+}$ relationships in all our experiments would have been quite a coincidence.

That the overestimation of the $TPMP^+$-based $\Delta\psi$ value is not necessarily constant is more conclusively shown by the experiments with and without $TPB^-$. In these experiments, it is clearly shown that under identical conditions in parallel incubations a much higher $TPMP^+$ accumulation was obtained in the presence of $TPB^-$ than in its absence. The increase in the chemical potential difference of $TPMP^+$ may well exceed 1 kJ/mol. This was observed only when DNP was present. No influence on the $TPMP^+$ accumulation was found when malonate was used. At the same time no influence on the $K^+$ accumulation was found when either malonate or DNP was used. The observed higher $TPMP^+$ accumulation cannot be explained by assuming that $TPB^-$ somehow specifically leads to an increase in the membrane potential in partly uncoupled mitochondria (and not when a respiratory inhibitor is used). This explanation must be discarded because, whatever relationship between $J_P$ and the true $\Delta\psi$ is accepted, a rise in $\Delta\psi$ of about 1 kJ/mol or more would certainly cause a substantial rise in $J_P$, which was not found (see Fig. 5). Also, whatever relationship between the $K^+$ accumulation and the true $\Delta\psi$ is accepted, an increase in the $K^+$ accumulation would then also be expected which was also not found (see Fig. 5 and Table II). An increase in $TPMP^+$ binding could, of course, be considered as an obvious consequence of the partitioning of a lipophilic uncharged $TPMP^+$-$TPB^-$ complex into the membrane phase. However, this is clearly no satisfying explanation, since no significant increase in the $TPMP^+$ accumulation was induced by $TPB^-$ in the malonate incubations. Therefore the most plausible explanation for the inconsistent $TPMP^+$ $\Delta\psi$ values is that the binding affinity is not constant. Other mechanisms, however, e.g., charge-neutral leaking out of the $TPMP^+$, thus causing violation of the equilibrium condition, cannot be ruled out, although any evidence favouring them is lacking.

The binding of $TPMP^+$ is quantitatively very important as it may constitute 50% or more of the total accumulated $TPMP^+$. Naturally, changes in the binding parameters will seriously affect the observed $TPMP^+$ accumulation ratio. However, no independent estimation of the magnitude of $TPMP^+$ binding in energised mitochondria can be obtained. Therefore binding parameters have been determined in de-energised mitochondria and the obtained values have been applied to energised mitochondria. More seriously, these binding parameters have explicitly or implicitly been assumed to be constant, although no evidence exists that this assumption is justified. In the present

paper we have shown that the way in which the $TPMP^+$ accumulation relates to the true $\Delta\psi$ value may seriously change. This is most probably due to changes in the binding affinity. Whatever the reason for the aberration, inconsistent results may be obtained when $TPMP^+$ is relied on as a probe for $\Delta\psi$. Therefore, the anomalous flow-force relationships that are found when $TPMP^+$ is used as a probe for $\Delta\psi$ cannot be considered as a falsification of the chemiosmotic coupling theory. When $K^+$ was used as a probe for $\Delta\psi$ in mitochondria we obtained unique relationships between the steady-state rates of phosphorylation and oxidation and the magnitude of $\Delta\tilde{\mu}_{H^+}$ (this paper and Ref. 11) and a proportional relationship between $\Delta G_P^{ex}$ and $\Delta\tilde{\mu}_{H^+}$ in State 4 [10]. These results are fully in agreement with the chemiosmotic coupling theory.

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