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ATP synthase-mediated proton fluxes and phosphorylation in rat liver mitochondria: dependence on $\Delta\tilde{\mu}_H$

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The dependence of the proton flux through the ATP synthases of rat liver mitochondria on a driving force composed mainly of a potassium diffusion potential was determined and compared with the relationship between rate of phosphorylation and $\Delta\tilde{\mu}_H$ given by titrations with the respiratory inhibitor malonate. The two functions are in good agreement in the lower part of the $\Delta\tilde{\mu}_H$ range covered. However, the maximal proton fluxes through the ATP synthases are much lower than needed to account for the rate of State 3 phosphorylation sustained by the same mitochondria oxidizing succinate. Possible reasons for this behavior are discussed.

Introduction

According to the chemiosmotic hypothesis the driving force for ATP synthesis in all energy-conserving systems is the transmembrane electrochemical proton gradient, $\Delta\tilde{\mu}_H$. The rate of ATP synthesis, J_{ATP}, is therefore expected to be a function of $\Delta\tilde{\mu}_H$. Sigmoidal flow-force relationships are predicted from the results of modeling studies [1]. Indeed, application of artificial H^+ (electro) chemical gradients to chloroplasts (Refs. 2 and 3 and references therein), bacteria [4], submitochondrial particles [5,6] and reconstituted systems [7] has permitted the mapping of such sigmoids. A more complex picture is obtained during electron-transfer or light-induced phosphorylation. While the upper plateau level is rarely attained, the results are consistent with sigmoidal flow-force relationships in a number of cases (e.g., Refs. 2 and 3 and references therein and Refs. 8–11). However, at least in bacterial chromatophores [12] and mitochondria the form of the J_{ATP} vs. $\Delta\tilde{\mu}_H$ relationship seems to depend on the means chosen to modulate the relevant parameters (for reviews, cf. Refs. 13–15). In mitochondria, very steep J_{ATP}-vs.-$\Delta\tilde{\mu}_H$ relationships are obtained when respiratory inhibitors are used [11,16–20] while use of uncouplers or K^+ flows to dissipate $\Delta\tilde{\mu}_H$ gives rise to weaker (and different) dependencies of J_{ATP} on $\Delta\tilde{\mu}_H$ [11,19]. These discrepancies have been considered as evidence in favor of either some form of direct coupling between electron-transport chain and ATP synthases [11,12,18] or of localized energy-transducing domains [15]. Al-

Abbreviations: $\Delta\tilde{\mu}_H$, transmembrane electrochemical proton gradient; $\Delta\psi$, transmembrane electrical potential difference; ΔpH, transmembrane pH gradient; J_{ATP}, rate of ATP synthesis; J_H^{ATP}, rate of proton translocation through ATP synthases; J_H^{leak}, proton flux through leaks; J_o, rate of oxygen consumption; (np)TPMP, (null point)triphenylmethylphosphonium; DMO, 5,5-dimethyloxazolidine-2,4-dione; P_i, inorganic phosphate; As_i, inorganic arsenate; Mops, 4-morpholinepropanesulfonic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; J_K^{eff}, rate of K^+ efflux; TPB, tetraphenylborate; FCCP, carbonylcyanide-*p*-fluoromethoxyphenylhydrazone.

ternatively, it has been proposed that the physiological dependence of J_{ATP} on $\Delta\tilde{\mu}_H$ might indeed be very steep, as indicated by the titrations with respiratory inhibitors; results at variance with this would be due to experimental artefacts or unrecognized interference with regulatory mechanisms or with kinetic characteristics of the enzymes [13,17].

Recently we have shown that the difference between titrations with malonate and FCCP is not due to anaerobiosis of the mitochondria in the centrifugation step of the $\Delta\tilde{\mu}_H$ determination procedure [20]. In this work we aimed to determine the dependence of the proton flux through the mitochondrial ATP synthases on the magnitude of an applied $\Delta\tilde{\mu}_H$ (consisting mainly of a K^+ diffusion potential) and to compare it to the J_{ATP} vs. $\Delta\tilde{\mu}_H$ relationship obtained during titrations with malonate. If the latter indeed represents the physiological flow-force relationship, and a fully delocalized protonic coupling mechanism applies, the two relationships should coincide, once the H^+/ATP stoichiometry is taken into account.

Experimental limitations hamper the precise determination of the driving force associated with a given proton flux. Nonetheless, it can be concluded that the dependence of J_H^{ATP} on the applied $\Delta\tilde{\mu}_H$ is indeed steep, and, in the lower part of the $\Delta\tilde{\mu}_H$ range covered, in good agreement with the J_{ATP} vs. $\Delta\tilde{\mu}_H$ curves obtained titrating with malonate. However, the highest J_H^{ATP} values measured are considerably lower than necessary to account for the J_{ATP} sustained by the same mitochondrial preparation oxidizing succinate if H^+/ATP is taken as 3 (+1), and do not vary appreciably in response to $\Delta\tilde{\mu}_H$ increases. This behavior is tentatively explained in terms either of regulatory phenomena affecting ATP synthase activity or of a partial direct coupling between respiratory chain and ATP synthases.

Materials and Methods

Measurement of proton fluxes

The experimental procedure is similar to that described in Ref. 21. Mitochondria are incubated in an open thermostatted vessel containing a K^+ electrode (Schott) and a glass (Beckmann) electrode, used as reference. The incubation medium (cf. legend to Figs. 1 and 3) contains ADP and either an excess of hexokinase and glucose or 2 mM arsenate. Arsenate behaves as a phosphate analog: it is utilized by the ATP synthases to form an analog of ATP, ADP-As_i, which however is rapidly hydrolyzed mostly still in the matrix [22–25]. In the presence of arsenate, therefore, a futile cycle is set up which results in a permanent State-3-like situation. As_i alone does not stimulate respiration to full State-3 levels, but it does so if some ADP is also present in the system. This is presumably due to carrier-mediated ADP-As_i/ADP exchange [23]. Exogenous ADP-induced depletion of matrix ATP may also play a role. 2 mM As_i was used, since higher concentrations do not result in higher rates of respiration; at this concentration As_i has a slight uncoupling effect, visible as an increase of membrane proton leak conductance in the presence of oligomycin (see below).

The mitochondria are allowed to reach the stationary state (as judged by constant values of the rate of respiration and $\Delta\tilde{\mu}_H$ in control experiments), and antimycin (50 ng/mg protein) and valinomycin (400 ng/mg protein) are then added to block the redox pumps and create a K^+ diffusion potential, which can be modulated by varying $[K^+]_o$. K^+ concentrations were determined taking into account medium contamination (measured by atomic absorption spectrometry) and the contribution by K^+ leaked from the mitochondria during storage in the stock suspension. Valinomycin was added either together or a few (4–10) seconds after antimycin. Control experiments showed the results not to differ when using the two procedures.

The K^+ diffusion potential causes protons to enter the matrix via both membrane leak pathways and the ATPases in the absence of oligomycin, but only via the former in its presence. The difference between the flows ± oligomycin (at the same diffusion potential) gives therefore a measure of J_H^{ATP}. For electroneutrality reasons, protons entering the matrix electrogenically will be compensated by efflux of K^+: monitoring K^+ movements is thus a convenient way to monitor H^+ fluxes, preferable to direct determinations of pH changes, which may be affected by electroneutral flows accompanying, e.g., P_i movements. K^+

efflux will also be caused by electrogenic anion efflux or influx of cations other than H^+. Migration of such species, probably negligible in our media and conditions, would cause an overestimation of the K^+ (H^+) fluxes, which would be automatically corrected when taking the difference between fluxes in the presence and absence of oligomycin. In control experiments, the J_H/J_K ratio was 0.65–0.8 in the absence and 0.8–0.9 in the presence of oligomycin. The value of J_K was routinely determined from the slope of the quasi-linear part of the trace, immediately following addition of valinomycin, and appropriate calibrations. The part of the trace used for K^+ flux determinations was recorded between approx. 3 and approx. 10 s after addition of valinomycin in the absence of oligomycin, and over a somewhat more extended time span in its presence.

Even at the highest measured K^+ fluxes, the apparatus used (electrodes, a Radiometer pHM 26 millivoltmeter and a Perkin-Elmer R 100 recorder) was not rate limiting. Low concentrations (0.1–0.2 mg/ml) of mitochondria were used under conditions of highest flows. Doubling the amount of protein, antimycin and valinomycin led to doubling of the measured J_K^{eff}. Potassium efflux rates some 3-times higher than the maximum values of the present experiments could be measured if an excess of the uncoupler FCCP was added together with valinomycin. The amount of valinomycin used can also catalyze the aerobic uptake of K^+ at a rate more than 3.5-times higher than the maximum J_K^{eff} measured in this work. This indicates that the amount of valinomycin added was not limiting. A valinomycin titration of J_K^{eff}, under conditions of maximal flows (low $[K^+]_o$, active ATP synthases) is presented as inset in Fig. 3. The increase of J_K^{eff} beyond approx. 0.3 μg valinomycin/mg is presumably due to the proton-conducting properties of this ionophore. This uncoupling effect causes an overestimation of both leak and ATPase-mediated H^+ flows, which disappears when taking the difference.

Null-point titrations of K^+ fluxes in energized, valinomycin-treated mitochondria

Mitochondria were incubated either in State 3 or in State 4 (in the presence of 1 μg/mg oligomycin). Upon addition of valinomycin (50–200 ng/mg) either K^+ efflux (at low K^+ concentrations) or K^+ influx ensued. Plots of the rate of K^+ influx or efflux vs. $[K^+]_o$ were linear within the K^+ concentration range used (20–400 μM). A typical experiment is shown in Fig. 1. Null-point K^+ concentrations were used for the calculation of the matrix K^+ concentration as outlined below.

Estimation of diffusion potentials

Calculation of K^+ diffusion potentials (59.2 $\log[K^+]_i/[K^+]_o$ mV; i for in, o for out) requires knowledge of the matrix K^+ activity, which may vary [26] and is not easy to determine accurately. A major purpose of this work was to compare the J_H^{ATP} vs. $\Delta\tilde{\mu}_H$ relationship obtained by varying $\Delta\psi_{diff}$ with the J_{ATP} vs. $\Delta\tilde{\mu}_H$ curves obtained from titrations with respiratory inhibitors. In the latter case $\Delta\tilde{\mu}_H$ is measured by probe distribution (see below): it is therefore important that the potentials measured by the two methods be comparable. To achieve this, the null-point for K^+ fluxes was determined as described above to estimate $[K^+]_i$. In these experiments $J_K = 0$ when $\Delta\psi$ (State 4 or 3) $= (RT/F)\ \ln([K^+]_i/[K^+]_o)$. $[K^+]_i$ can therefore be calculated from $[K^+]_o$ and $\Delta\psi$ (measured in parallel experiments by the TPMP distribution technique). In this manner the calculated K^+ dif-

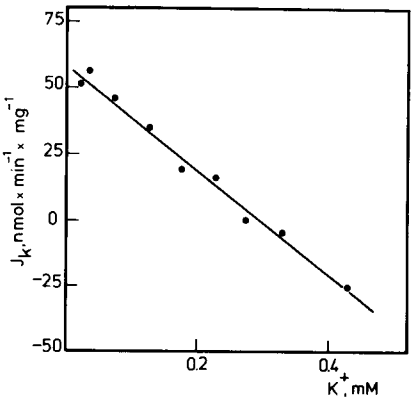

Fig. 1. A null-point titration of K^+ fluxes in State 3. Medium composition: 0.2 M sucrose, 30 mM Tris/Mops, 10 mM succinate/Tris, 5 mM P_i/Tris, 4 mM $MgCl_2$, 0.5 mM EGTA, 4 μM rotenone, 6 mM glucose, 0.3 mM ADP/Tris, 1.1 U/ml hexokinase, 3 μM TPMP, 1 μM TPB (pH 7.4); 1 mg mitochondrial protein/ml; 200 ng valinomycin/mg. *T*, 25°C. Null-point K^+ concentration in this experiment was taken as 293 μM.

fusion potentials are linked to the probe distribution-based $\Delta\psi$ values. If the measured State 4 (or 3) $\Delta\psi$ turned out to be wrong for any reason, the same correction would apply to the diffusion potentials.

Estimating the error affecting the calculated $\Delta\psi_{diff}$

Diffusion potentials calculated as described will be completely correct only if the membrane conductance for ions other than K^+ is zero. This is obviously not the case.

If antimycin and valinomycin are added to State 3 mitochondria across whose membrane TPMP is distributed at equilibrium, a release or a (transient) uptake of TPMP will result, depending on whether the actual (as opposed to calculated from Nernst's law) diffusion potential is lower or higher than the State 3 $\Delta\psi$. If the changes in medium TPMP concentration are monitored with a fast-responding specific electrode it is therefore possible to carry out null-point titrations as a function of $[K^+]_o$, determining at which $[K^+]_o$ ($[K^+]_o^{npTPMP}$), TPMP uptake no longer occurs upon adding antimycin and valinomycin. Traces taken from such a titration are shown in Fig. 2. TPB was added to the medium to accelerate TPMP equilibration and thus provide a faster response. Since the electrode responds also to TPB, and TPB is extensively bound by the mitochondria, such experiments are not well suited for the measurement of $\Delta\psi$ values. TPB did not affect the $\Delta\psi^{St3}$ values, since the $[K^+]_o$ at null point for K^+ fluxes was the same ± TPB. Antimycin, although added together with valinomycin, appears to act slightly before the ionophore, inducing a depolarization which is then transiently reversed.

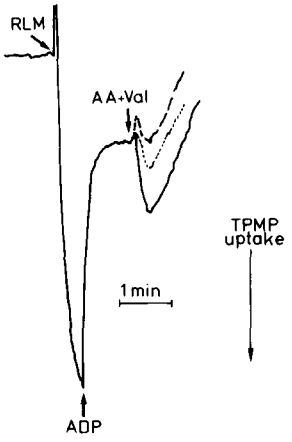

Fig. 2. TPMP electrode traces from a null-point titration of TPMP movement. Medium composition and conditions as in Fig. 1, except that ADP was added where shown. $[K^+]_o$, 21 μM (———); 61 μM (- - - - -); 71 μM (— — —). Where indicated: 50 ng antimycin/mg, 400 ng valinomycin/mg. Null-point K^+ concentration in this experiment was taken as 71 μM. AA, antimycin; RLM, rat liver mitochondria.

At the null point (for TPMP movement) K^+ concentration ($[K^+]_o = [K^+]_o^{npTPMP}$):

$$\Delta\psi_{diff}^{npTPMP} = \Delta\psi^{St3}$$

Applying Nernst's law one would, however, calculate:

$$\Delta\psi_{diff}^{npTPMP}\,(\text{calculated}) = \frac{RT}{F}\ln\frac{[K^+]_i}{[K^+]_o^{npTPMP}}$$

Since:

$$\Delta\psi^{St3} = \frac{RT}{F}\ln\frac{[K^+]_i}{[K^+]_o^{npK}}$$

(where $[K^+]_o^{npK}$ is the medium K^+ concentration at null point for K^+ movements in respiring, valinomycin-treated mitochondria), the error committed on $\Delta\psi_{diff}^{npTPMP}$ calculations by applying Nernst's law is given (at 25°C) by:

$$\text{error} = 59.2\log\frac{[K^+]_o^{npK}}{[K^+]_o^{npTPMP}}\,(\text{mV})$$

It becomes possible therefore to evaluate the overestimation affecting Nernst's law diffusion potential values at $[K^+]_o^{npTPMP}$. Because the relatively sluggish TPMP electrode response causes an underestimation of the K^+ concentration at the null-point, error estimates are upper limits.

Evaluation of matrix acidification

When measuring proton fluxes, the ensuing K^+/H^+ exchange is expected to lead to matrix acidification, i.e., ΔpH will decrease and become inverted ($pH_{in} < pH_{out}$). Quantitative assessment of inverted ΔpH in mitochondria is complicated by lysosomal contamination and probe binding [27]. 9-Aminoacridine uptake and fluorescence

quenching was used to monitor the process. The calibration procedure described by Casadio and Melandri [28], involving measuring fluorescence quenching induced by pH jumps and the relaxation of the signal, was used to quantify the dye response.

Comparison of experiments in iso- and iperosmolar media

In view of the uncertainties affecting the $\Delta\tilde{\mu}_H$ values it was felt desirable to ascertain the effect of a clear-cut appreciable increase of $\Delta\tilde{\mu}_H$ (in the low $[K^+]_o$ region) on J_H^{ATP} in order to establish whether a plateau had been attained in the J_H^{ATP}-vs.-$\Delta\tilde{\mu}_H$ plots.

State 3 and State 4 rates of respiration and $\Delta\psi$, J_H^{ATP} values and null-point (for K^+ and TPMP fluxes) K^+ concentrations were determined in approximately isoosmolar media and in media of higher osmolarity (cf. legend to Table I). The osmolarity increase induces a decrease of the rate of State 3 respiration. If this decrease were due to inactivation of electron transfer it would not be relevant for the present investigation. If on the other hand the decrease is due to ATPase inactivation it must be taken in due consideration in quantitating the effect of the osmolarity increase on J_H^{ATP}. Control experiments (not shown) indicate that the osmolarity increases used cause a decrease (max. 33%) in the rate of respiration of mitochondria treated with excess FCCP. The corresponding inhibition of State 3 respiration is, however, consistently higher (max. 69%). Thus, at high osmolarity the ATP synthases are indeed inhibited and constitute the major respiration-limiting factor. This conclusion is supported by the higher State 3 $\Delta\psi$ values found at higher osmolarities: opposite variations would be expected if respiratory inhibition were due to limitation of respiration chain activity.

The extent of inactivation depends on the solute used to increase the osmolarity, sucrose being more effective than Tris-HCl. Because of ATP synthase inhibition, the rates of H^+ influx are lower at higher osmolarity; at the same time, the K^+ gradient responsible for creating the diffusion potential increases because of the osmotic behavior of the mitochondria [29–32]. Thus, at a given $[K^+]_o$, a shift from a lower to a higher osmolarity medium must result in higher values of both $\Delta\psi$ and ΔpH.

The extent of inhibition of the ATP synthases in high-osmolarity media has been determined from respiration data in order to normalize and compare J_H^{ATP} values. The State 4 to State 3 transition is accompanied by a $\Delta\tilde{\mu}_H$ decrease of some 20 mV (e.g., Refs. 33–35) which may cause a decrease of the respiratory activity due to leaks (Ref. 21 and references therein) and intrinsic uncoupling of the pumps [36]. In high-osmolarity media, the lower ATP synthase activity results in higher State 3 $\Delta\psi$ values (cf., e.g., Ref. 11). Because the dependence of intrinsic uncoupling of the mitochondrial pumps on $\Delta\tilde{\mu}_H$ has not yet been determined, it is impossible to ascertain exactly to what extent the decrease of the State 3 respiratory rate reflects ATP synthesis inhibition. However, limiting values can readily be found.

The ratio of the ATP synthase activities in high- and low-osmolarity media may be expressed as:

$$\text{activity ratio} = \frac{K\left[{}^{h}J_o^{St3} - \left({}^{h}J_o^{St4} - {}^{h}\Delta J_o\right)\right]}{K\left[{}^{l}J_o^{St3} - \left({}^{l}J_o^{St4} - {}^{l}\Delta J_o\right)\right]}$$

where K is a proportionality constant, the superscripts h and l indicate high- and low-osmolarity media and ΔJ_o is the extent of respiration, due to leaks and intrinsic uncoupling, abolished because of the $\Delta\tilde{\mu}_H$ variation. Being:

$${}^{h}J_o^{St3} < {}^{l}J_o^{St3},\ {}^{h}J_o^{St4} \approx {}^{l}J_o^{St4},$$

and, since

$${}^{h}\left(\Delta\tilde{\mu}_H^{St4} - \Delta\tilde{\mu}_H^{St3}\right) < {}^{l}\left(\Delta\tilde{\mu}_H^{St4} - \Delta\tilde{\mu}_H^{St3}\right)$$

being:

$${}^{h}\Delta J_o \leqslant {}^{l}\Delta J_o$$

it can be readily seen that the minimal and maximal values of the activity ratio are given by:

$$\frac{{}^{h}J_o^{St3} - {}^{h}J_o^{St4}}{{}^{l}J_o^{St3}} \leqslant \text{ratio} \leqslant \frac{{}^{h}J_o^{St3}}{{}^{l}J_o^{St3}}$$

We arbitrarily chose the average of the extreme values as an estimate of the ATP synthase activity

in the high-osmolarity media. Qualitatively, results and conclusions do not change if either the minimal or maximal values are used instead.

Determination of $\Delta\tilde{\mu}_H$ in steady states

$\Delta\psi$ and ΔpH measurements concerning the malonate titrations were carried out using the probe distribution method, as described in Ref. 37. Experiments were conducted in parallel to and under the same conditions as J_{ATP} determinations. ΔpH was found to be close to zero, in stationary states, under all the conditions used. The data are therefore presented in terms of $\Delta\psi$ only. Other $\Delta\psi$ determinations were carried out utilizing the TPMP-sensitive electrode described below.

Determinations of J_{ATP}

Mitochondria (normally 2 mg/ml) were incubated in an open, thermostatted vessel, in the presence of the chosen concentration of respiratory inhibitor, and phosphorylation was started by the addition of ADP after 2–4 min of incubation. Four 1-ml samples were withdrawn from the suspension within 1 min after addition of ADP and quenched in $HClO_4$. The ATP content of the samples was determined by standard enzymatic techniques after centrifugation of the denatured protein, neutralization with TRA/KOH and separation of the precipitated $KClO_4$. The rate of ATP synthesis was calculated from the linear regression analysis of [ATP] vs. time plots. Medium compositions are specified in the legend to Fig. 1. Conditions were the same as in parallel determinations of $\Delta\tilde{\mu}_H$ and of J_H^{ATP} vs. $\Delta\psi_{diff}$ relationships.

Materials

Rat liver mitochondria were prepared by standard procedures in a sucrose-based medium [38]. Protein concentrations were determined by the biuret method using bovine serum albumin as standard. Enzymes, nucleotides, inhibitors and ionophores were purchased from Sigma, labeled probes from Amersham. Arsenate was prepared by reacting As_2O_5 (Aldrich) with water. The TPMP electrode was built using the body of a Radiometer F2112 Selectrode; a Beckman combination glass electrode was used as reference. Outputs from this apparatus and from the K^+ electrode were fed to Radiometer pHM26 millivoltmeters and to Perkin-Elmer R100 recorders. Fluorescence measurements were carried out using a Perkin-Elmer 650-40 fluorimeter equipped with temperature control and stirring devices, using 400 and 460 nm as excitation and emission wavelengths, respectively.

Results

Fig. 3 shows typical J_K^{eff} data plotted vs. the diffusion potentials calculated from Nernst's law. The lower curve, in the presence of oligomycin, reflects the membrane proton leak (J_H^{leak}) under the experimental conditions. Note that the basal membrane leak conductance was higher than normal (cf. Ref. 21) due to the presence of arsenate and of relatively high amounts of valinomycin.

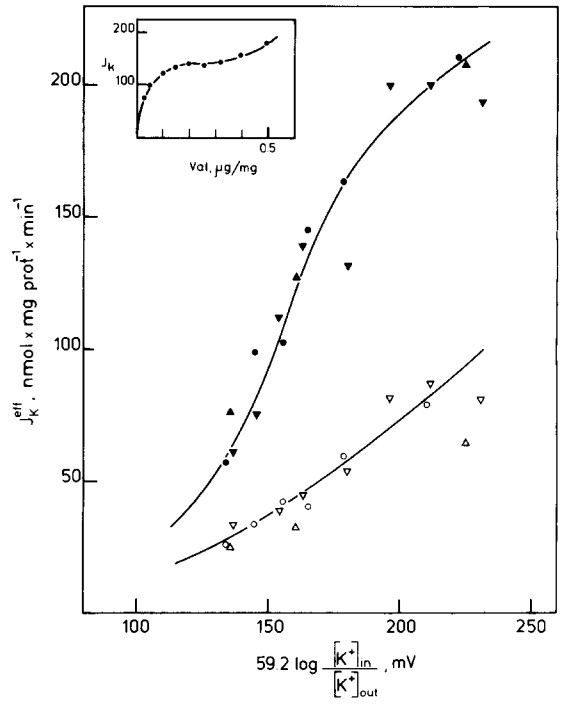

Fig. 3. Relationship between rate of K^+ efflux and 59.2 log$[K^+]_{in}/[K^+]_{out}$ mV. Three experiments are shown. Medium composition: 0.20 M sucrose, 30 mM Tris/Mops, 10 mM Acetate/Tris, 0.5 mM EDTA/Tris, 5 mM glycerol, 5 µM Rotenone, 2 mM Asi, 0.3 mM ADP/Tris (pH 7.4). Open symbols: +1 µg oligomycin/mg. 0.2–4.0 mg. RLM/ml, 14–800 µM $[K^+]_o$. Inset: J_K^{eff} as a function of valinomycin concentration; no oligomycin; 1 mg mitochondrial protein/ml. 76 µM $[K^+]_o$.

The upper curve (-oligomycin) reflects also the contribution of proton flux through the ATP synthases. The difference between the two curves is therefore a measure of J_H^{ATP} as a function of $\Delta\psi_{diff}$. At the highest diffusion potentials (lowest $[K^+]_o$'s) the rapid rise of the upper curve is replaced by a gentler increase, similar to that of the lower curve. This behavior was observed in all the 13 experiments performed.

Fig. 4 shows plots of J_H^{ATP} (defined as $J_K^{eff}(-\text{oligomycin}) - J_K^{eff}(+\text{oligomycin})$), divided by the H^+/ATP stoichiometry, vs. 59.2 $\log[K^+]_i/[K^+]_o$ ('Nernst diffusion potential') and of J_{ATP} vs. $\Delta\psi$. The former relationship was obtained using both arsenate- and phosphate/hexokinase-containing media, and adopting stoichiometry values of 3 and 4 H^+/ATP, respectively. The latter relationship was determined in titrations with malonate. The results of five experiments are shown. In three of them both the J_H^{ATP}/n vs. Nernst diffusion potentials and the J_{ATP} vs. $\Delta\psi$ relationships were determined on the same mitochondrial preparation and diffusion potential and State 3 $\Delta\psi$ values were linked as described in Materials and Methods. The two curves coincide in the lower $\Delta\psi$ range, while at higher values of the driving force, J_H^{ATP}/n, but not J_{ATP}, appears to reach a plateau. Furthermore, the maximal J_H^{ATP}/n values are much lower than expected on the basis of the rate of aerobic phosphorylation by the same mitochondrial preparations.

In experiments of the type shown in Fig. 2 (cf. Materials and Methods) the null-point for TPMP movement was reached at 65–80 μM $[K^+]_o$; at these K^+ concentrations J_K^{eff} $(= J_H^{ATP} + J_H^{leak})$ was 130–170 nmol · mg^{-1} · min^{-1}. The null-point for K^+ movement in State 3 mitochondria (cf. Fig. 1, Materials and Methods) was obtained instead at 280–310 μM $[K^+]_o$. The corresponding calculated (maximal) overestimation of the diffusion potential ranged from 32 to 38 mV. This error estimate applies to calculated diffusion potentials 30–40 mV higher than the State 3 $\Delta\psi$. The overestimation is expected to be somewhat larger at higher diffusion potentials, and to become progressively smaller as potentials and fluxes decrease. The error committed applying Nernst's law should also be smaller, at equal $[K^+]_i/[K^+]_o$ ratios, in oligomycin-treated mitochondria, because membrane conductance and proton fluxes are lower. In this case an experiment analogous to those described for State 3 mitochondria yielded a similar error estimate at a calculated (Nernst) diffusion potentials of some 254 mV and J_K^{eff} (J_H^{leak}) value of 81 nmol · mg^{-1} · min^{-1}.

Acidification of the mitochondrial matrix after addition of valinomycin and antimycin was visible as a variation of 9-aminacridine fluorescence in the lower $[K^+]_o$ range. In our media, relatively rich in weak acids, the size of the inverted ΔpH

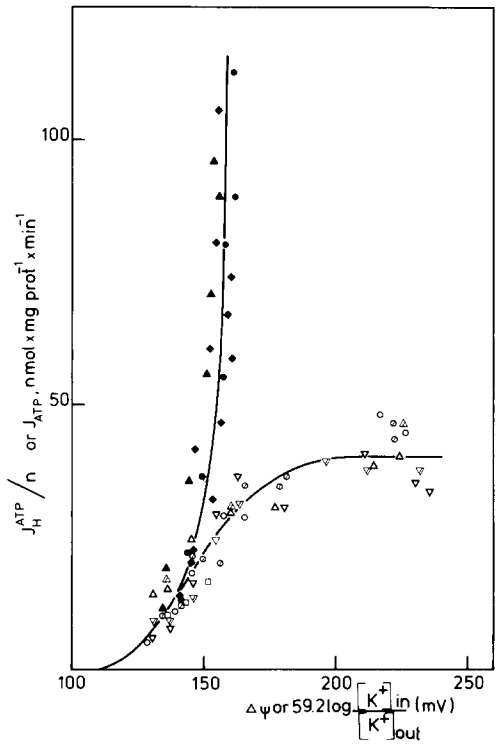

Fig. 4. Relationships between J_{ATP} and $\Delta\psi_{St3}$ (titrations with malonate, closed symbols) and between J_H^{ATP}/n and 59.2 $\log[K^+]_{in}/[K^+]_{out}$ (open symbols). Symbols having the same shape and orientation refer to experiments performed with the same mitochondrial preparation, with TPMP-based and calculated (Nernst) $\Delta\psi$ values linked as described in the text. Barred symbols (∅, △, ▽, ⊠) refer to experiments in As_i-containing media (cf. legend to Fig. 3), the others to phosphate/hexokinase media (cf. Fig. 1; +5 mM glycerol, no TPB). J_H^{ATP} was calculated as the difference between J_K^{eff} ± oligomycin at the same $[K^+]_o$; n, the H^+/ATP stoichiometry, was taken as 3 and 4 for As_i- and P_i-experiments, respectively. In the experiments involving modulation of K^+ diffusion potentials, $[K^+]_o$ varied between 13 and 875 μM and mitochondrial concentration between 0.2 and 4 mg protein/ml.

formed at the end of K^+ efflux varied from 0.5–0.7 pH units at 10–20 μM $[K^+]_o$ to below detection threshold (approx. 0.3 units) at about 0.5 mM $[K^+]_o$. The rate of 9-aminacridine fluorescence quenching also decreased as $[K^+]_o$ increased: at the lowest potassium concentrations quenching required only 20–30 seconds for completion, with about half of the quenching taking place in the first 10 seconds.

The corrections to be applied to $\Delta\psi_{diff}$ values and the decrease of pH_{in} during K^+ efflux may justify the suspicion that the imposed $\Delta\tilde{\mu}_H$ might not have exceeded the State 3 $\Delta\tilde{\mu}_H$. The plateau exhibited by J_H^{ATP} (cf. Fig. 4) would then be an artefact. To clarify this point we performed osmolarity-shift experiments as described in the Materials and Methods section. Table I presents some representative results.

The inhibition of ATPase activity was accompanied by higher $\Delta\psi^{St3}$ values, the difference between high- and low-osmolarity media ranging from 3 to 12 mV. Nonetheless, the null-point for TPMP movement (see above) was always reached at higher potassium concentrations in the higher-osmolarity media. This indicates that at the same $[K^+]_o$ the actual diffusion potential at high osmolarity always exceeded that generated at low osmolarity by more than the difference between the two State 3 $\Delta\psi$'s. An estimate of the variation in $\Delta\psi_{diff}$ can be obtained on the basis of Nernst's law, following the procedure outlined in the Materials and Methods section to calculate $[K^+]_i$. Since the overall membrane permeability to protons is lower at high osmolarity, the error committed applying Nernst's law is lower in this case, and the difference ${}^h\Delta\psi_{diff} - {}^l\Delta\psi_{diff}$ (see Table I), estimated as 59.2 $\log {}^h[K^+]_{in}/{}^l[K^+]_{in}$, thus represents an underestimate of the $\Delta\psi_{diff}$ increase. $\Delta\tilde{\mu}_H$ increases are even larger, because of the lower rate of matrix acidification at higher osmolarities. The $\Delta\tilde{\mu}_H$ increase due to the osmolarity shift is expected to cause a sharp increase of the corrected J_H^{ATP} if applied in a $\Delta\tilde{\mu}_H$ region where the flow depends steeply on the force. This behavior is indeed observed if the media contain relatively high K^+ concentrations (Table I), thus validating the method. Viceversa, at lower K^+ concentrations, corresponding to the plateau region of the curve in Fig. 4, the osmolarity increase had negligible effects on the corrected J_H^{ATP}. This indicates that the plateau of Fig. 4 is not an artefact. A range of driving force thus exists where the proton

TABLE I

THE EFFECT OF OSMOLARITY ON J_H^{ATP}

J_H^{ATP} was calculated as the difference between J_K^{eff} ± oligomycin; the ATPase activity ratio was calculated as $\frac{1}{2}[{}^hJ_o^{St3}/{}^lJ_o^{St3} + ({}^hJ_o^{St3} - {}^hJ_o^{St4})/{}^lJ_o^{St3}]$; $[K^+]_i$ was measured as described in the text; $\Delta\psi^{St3}$ was measured using the TPMP-sensitive electrode, without binding corrections. The assays were carried out as in Fig. 4 in phosphate/hexokinase media containing different amounts of sucrose, Tris/Mops and Tris-HCl^- in order to change the osmolarity: (a) low-osmolarity medium: 0.13 M sucrose, 25 mM Tris/Mops, 30 mM Tris/Cl^-; high-osmolarity medium: idem, +0.15 M Tris/Cl^-; (b) and (d) low-osmolarity medium: 0.13 M Tris/Cl^-; high-osmolarity medium + 0.3 M sucrose: (c) low-osmolarity medium: 0.13 M sucrose, 25 mM Tris/Mops; high-osmolarity medium: idem, + 0.1 M Tris/Cl^-. Letters (a)–(d) identify experiments performed with different mitochondrial preparations. Medium K^+ concentrations given are the averages of the concentrations in the two media, which differed by no more than 5 μM. n.d., not determined.

	$[K^+]_o$ (μM)	J_H^{ATP} high osmolarity (A)	J_H^{ATP} low osmolarity (B)	ATPase activity ratio (C)	${}^hJ_H^{ATP}/{}^lJ_H^{ATP}$ (corrected) $\left(\frac{A}{B\times C}\right)$	${}^h\Delta\psi^{St3} - {}^l\Delta\psi^{St3}$ (mV)	${}^h\Delta\psi_{diff} - {}^l\Delta\psi_{diff}$ (mV)
a	20	63.2	99.5	0.6	1.06	3	6
b	31	37.5	126.8	0.24	1.23	12	n.d.
b	56	29.7	130.8	0.24	0.95	12	n.d.
c	158	101.4	117.0	0.58	1.49	4	13
c	258	79.3	66.8	0.58	2.05	4	13
d	372	58.5	33.1	0.48	3.68	11	22

flux through the ATP synthases does not respond to $\Delta\tilde{\mu}_H$ increases, despite the fact that its magnitude is much lower than nJ_{ATP} at equivalent $\Delta\tilde{\mu}_H$.

Discussion

The purpose of this work was to determine the correlation between proton flux through the ATP synthases and the driving force, $\Delta\tilde{\mu}_H$, and to compare it with the J_{ATP} vs. $\Delta\tilde{\mu}_H$ relationship given by titrations with malonate. If the latter yields the true, physiological dependence of J_{ATP} on $\Delta\tilde{\mu}_H$, and if phosphorylation proceeds only via bulk-to-bulk $\Delta\tilde{\mu}_H$, the two curves should coincide.

To our knowledge, this is the first attempt at such a comparison. Rates of phosphorylation as high as those obtained by 'natural' means of energization have been reported to result from the application of both a ΔpH and a diffusion potential to submitochondrial particles [5] and chloroplasts (e.g., Ref. 39) in jump experiments, but a direct comparison of flow-force correlations has proved difficult. The properties of the system and the available techniques unfortunately pose limits to the assessment of the driving force values. In our experiments, a true stationary state cannot be established during K^+ efflux due to the continuous decrease of the K^+ gradient, the increase of $[K^+]_o$ playing a major role in this respect. On the other hand the time response of the TPMP electrode is not fast enough to provide an accurate measurements of the diffusion potential during the transients following valinomycin addition. Calculations based on Nernst's law result in a variable overestimation which is smaller when the ATPases are blocked by oligomycin and the permeability to protons correspondingly decreased. Thus, it is not strictly correct to calculate J_H^{ATP} as the difference of the J_K^{eff} values measured at the same $[K^+]_o$ in the absence and presence of oligomycin. However, the dependence of J_H^{leak} on $\Delta\tilde{\mu}_H$ is relatively weak (Fig. 3, cf. also Ref. 21), so that the error is relatively small.

The plots in Figs. 3 and 4 thus present a distorted view of the relationship between the various proton fluxes and the driving force: the potential (abscissa) scale becomes progressively overextended when moving towards higher values. Points in the 190–200 mV range may be overestimated by 30–40 mV, with smaller or larger corrections applying at lower or higher potentials, respectively. Further distortion is caused by matrix acidification which occurs at variable rates depending on $[K^+]_o$ and membrane proton conductance.

The question arises as to whether the two flow-force relationships of Fig. 4 might actually coincide, and the plateau exhibited by the plot of J_H^{ATP} values be an artefact. The comparison of results in media at different osmolarities (cf. Results), however, suggests that this is not the case; a certain range of $\Delta\tilde{\mu}_H$ values exists within which J_H^{ATP} appears to be limited by some factor other than the size of the driving force. This conclusion depends on the assumption of equal inhibition of the ATP synthases during determinations of rates of respiration and of proton fluxes in high-osmolarity media.

Since the discrepancy between State 3 J_{ATP} and the maximal J_H^{ATP}/n values appears to be real, it requires an explanation. A lower H^+/ATP stoichiometry would facilitate the agreement between the two flow-force relationships. However, n should be at most 2 overall to explain our data. The evidence is overwhelmingly in favor of higher stoichiometries in mitochondria.

Regulatory phenomena leading to deactivation of the ATP synthases deserve consideration. The prime candidate for such an inhibitory mechanism is the ATPase inhibitor protein (for a review, cf. Ref. 40), which is thought to interact with F_1 in a reversible, energization-dependent manner (e.g., Ref. 41), blocking ATP hydrolysis and possibly also ATP synthesis (Refs. 42–44, but cf., e.g., Ref. 45, 46). The binding of the inhibitor protein seems to be a decreasing function of $\Delta\psi$. In heart submitochondrial particles full activation of the ATP synthases upon energization requires many seconds or minutes (e.g., Ref. 42), but no lag is usually detectable when initiating phosphorylation by energized rat liver mitochondria. In our experiments the mitochondria were incubated in full State 3 conditions for 1 min, so that the ATP synthases were certainly activated (at State 3 levels) at the time of the addition of antimycin and valinomycin. In the lower $[K^+]_o$ range valinomycin addition led to a $\Delta\psi$ increase above State 3 levels (cf. Fig. 2), which would not be expected to

cause inhibition. The TPMP electrode (Fig. 2) indicates that at least in some cases a brief depolarization preceeds the K^+ diffusion-induced hyperpolarization. Reported rate constants for inhibitor protein dissociation under energized conditions in SMP's are in the order of 0.1–0.2 s^{-1} [42,44]. Sánchez-Bustamante et al. [47] have estimated at 20–30% and approx. 50% the fraction of free (activated) ATP synthases in State 3 and State 4 mitochondria, respectively. Since these fractions reflect equilibria, it follows that ATPase inhibition and activation processes must proceed at similar rates. Thus, any depolarization-induced inhibition would (1) be slight in view of the time involved and (2) revert on the same time scale following repolarization. An alternative view involves the modulation of the ATPase activity either by means of an effect of pH_i on the binding of the inhibitor protein [48] or by the operation of the respiratory chain (e.g., Refs. 49 and 50). Konings and co-workers have recently shown that the activity of transport proteins in some bacteria may be controlled by the redox state of components of the respiratory chain [51–53]. Such complex kinetic interactions, if they exist in mitochondria as well, may provide a clue to explain the observed multiplicity of flow-force relationships [11,19,20]. Notice that in our K^+ diffusion experiments the respiratory chain was completely blocked, and that its activity was progressively reduced in malonate titrations.

The idea of a (partial) direct coupling between the respiratory chain and the ATP synthases has received considerable attention recently (for reviews, cf. Refs. 13–15 and 54–55). In the 'coupling unit' hypothesis [15] few primary and secondary proton pumps utilize the same proton domain. In these domains the transmembrane proton electrochemical potential difference may be not only fluctuating, but also higher than that between the bulk water phases, thus justifying the higher rates of phosphorylation. In the 'collisional hypothesis' model recently put forward by Slater and co-workers [55], the energy required for ATP synthesis may be obtained also via random encounters between 'activated' respiratory chain components and ATP synthases. The mechanism involving bulk phase $\Delta\tilde{\mu}_H$ would only allow submaximal rates of phosphorylation, due to the slow diffusion of protons from the bulk phase to their binding sites on the enzyme. No such limitation would apply to energy (proton?) transfer between enzyme complexes. Respiration, which would provide the activated redox chain components, would therefore allow the attainment of higher rates of phosphorylation than an artificially created electrochemical proton gradient. Other schemes envisioning a direct transfer of protons from the redox pumps to the ATP synthases, such as Williams' "protons-in-membrane" model [56] or Kell's "proton-conducting network" [54] are also compatible with our results.

Some other characteristics of the J_H^{ATP}/n curve in Fig. 4 deserve to be mentioned. Data with either As_i/ADP or HK/ADP to maintain State 3 fall along the same curve. This is not a trivial result, since the position of the flow-force curve is influenced by the value of the output force (1), i.e., ΔGp_i, and As_i might be expected to change this parameter.

The position of the flow-controlling force range is comparable to that found for CF_0F_1 in experiments involving the sudden application of a ΔpH and/or a diffusion potential to chloroplasts [2,3,39]. It is remarkable that the sigmoids obtained by Graeber and co-workers differ considerably from the sixth-power dependence determined by, e.g., Jackson and co-workers [57] by varying the light intensity. The diffusion potentials applied in Graeber's experiments were estimated from the Goldmann equation, taking into account the permeabilities of several ions. Furthermore, most of the driving force consisted of a pH gradient, so that the calculated $\Delta\tilde{\mu}_H$ values should be fairly reliable. It is thus difficult to ascribe the discrepancy exclusively to erroneous driving force determinations.

The evidence available for both mitochondria and chloroplasts points therefore to the involvement of as yet poorly understood factors affecting the properties of the ATP synthases when electron-transport chains operate.

Appendix. Electrode response and rate under- or overestimation

The considerations presented below refer to the experimental system used in this work (electrode

monitoring K^+ concentration), but they have more general validity. Definitions: y: actual system-response parameter (recorder pen position on y-axis); x: variable monitored (medium K^+ concentration); S: conversion factor relating y to x, so that $y = Sx$ for an infinitely fast response or at equilibrium (taken as constant).

As determined in K^+ jump experiments, the system response follows the first-order law:

$$\frac{dy(t)}{dt} = k(y_\infty - y) = k(Sx_\infty - y) \qquad \text{(A-1)}$$

with $k \approx 0.69\ s^{-1}$. In experiments involving continuous variation of x in time Eqn. A-1 becomes:

$$\frac{dy(t)}{dt} = k(Sx(t) - y) \qquad \text{(A-2)}$$

$x(t)$ is not known a priori. However, a plausible form is Eqn. A-4 obtained by integrating Eqn. A-3:

$$\frac{dx(t)}{dt} = K\,e^{-K't} \qquad \text{(A-3)}$$

$$x(t) = x_0 + \frac{K}{K'}\left(1 - e^{-K't}\right) \qquad \text{(A-4)}$$

Eqn. A-3 describes an exponential decay, determined by the magnitude of K', of the rate of

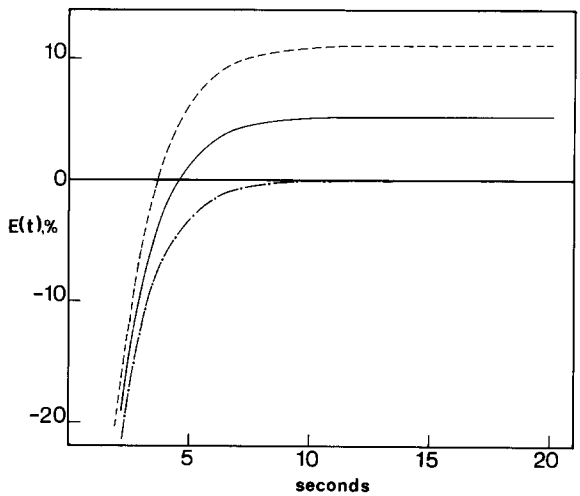

Fig. A-1. Plot of $E(t)$ vs. time. $E(t)$ is defined by Eqn. A-7 above. The curves are drawn for $n = 10$ (— — —), $n = 20$ (———) and $n = \infty$ (·–·–·).

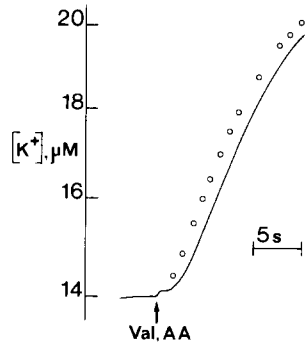

Fig. A-2. K^+ fluxes during a diffusion experiment. Recorded (lower curve) and corrected (dots) traces are shown. See text for the correction procedure.

K^+ efflux from the mitochondria in one of our experiments, i.e., of the rate of K^+ increase in the medium. K determines the initial efflux rate. Substitution of Eqn. A-4 into Eqn. A-2 and integration with the boundary condition $y(0) = Sx_0$ yields:

$$y(t) = Sx_0 + \frac{SK}{K'} + \frac{SK}{k - K'}e^{-kt} - \frac{kKS}{K'(k - K')}e^{-K't} \qquad \text{(A-5)}$$

Substituting A-4 and A-5 into A-2 and conveniently setting $n = k/K'$:

$$\frac{dy(t)}{dt} = SKe^{-kt/n}\frac{n}{n-1}\left(1 - e^{-(n-1)kt/n}\right) \qquad \text{(A-6)}$$

Comparison of A-3 with A-6 shows that the error committed by taking $dy(t)/dt$ as a measure of $dx(t)/dt$ is given by:

$$E(t) = 1 - \frac{n}{n-1}\left(1 - e^{-(n-1)kt/n}\right) \qquad \text{(A-7)}$$

Fig. A-1 shows a plot of this function for $k = 0.69\ s^{-1}$ and reasonable values of n.

In an alternative approach, recently presented by Wojtczak et al. [58], Eqn. A-2 is rearranged to:

$$Sx(t) = \frac{1}{k}\frac{dy(t)}{dt} + y \qquad \text{(A-8)}$$

and the point-by-point determination of the tangents to the recorded curve and of the y values are used to calculate $Sx(t)$. The plot thus obtained is the one that would have been recorded by a system with infinitely fast response. This

operation has been carried out on a few of our traces, and Fig. A-2 exemplifies the results. The first part of the calculated $Sx(t)$ curve shows much scatter due to the addition-induced disturbance, and has therefore been omitted. The initial lag evident in the recorded trace is due mostly to a delay in the action of valinomycin.

Figs. A-1 and A-2 indicate that the error affecting our flux measurements because of response-time limitations cannot lead to serious distortion of the results.

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