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Energy-storage capacity of the mitochondrial proton-motive force

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Resting state respiration of rat-liver mitochondria in the presence of oligomycin was rapidly blocked with cyanide and the dissipation of the membrane potential was followed with a tetraphenylphosphonium-sensitive electrode. From the rate of this dissipation and the electric capacitance of the mitochondrial membrane the energy stored in form of the membrane potential was calculated as about 7 μJ/mg protein. In the absence of oligomycin, dissipation of the membrane potential was slower, as it was partly compensated by proton ejection by mitochondrial ATPase hydrolyzing endogenous ATP. This allowed to calculate the total energy storage capacity of the proton-motive force. It amounted to the equivalence of 3.3 nmol ATP/mg protein or about 130 μJ/mg protein. The stoichiometry of proton-pumping ATPase utilizing endogenous ATP was estimated as three protons per molecule ATP.

Introduction

According to the chemiosmotic concept of oxidative phosphorylation [1], the proton electrochemical potential, also designated as the proton-motive force ($\Delta\tilde{\mu}_{H^+}$), is regarded as an intermediate in the energy transduction between the respiration and ATP synthesis. Although it represents a physical rather than chemical factor in energy coupling, old concepts of energy storage in form of the intermediate can applyl here as well. First attempts to evaluate the amount of energy accumulated in energized mitochondria were made by Eisenhardt and Rosenthal [2] and Azzi and Chance [3]. The former authors based their calculation on the initial rate of ATP synthesis after addition of ADP to energized mitochondria, the latter ones on measurements of Ca^{2+} retention by mitochondria after respiratory inhibition. Values equivalent to 0.3[3] –0.8[2] nmol ATP/mg mitochondrial protein were obtained. In contrast to these low values, in a more recent study Lemasters and Hackenbrock [4] reported on the synthesis of 2.25 nmol ATP/mg protein when $\Delta\psi$ was decreased by 35% after inhibition of mitochondrial respiration. This was extrapolated by the authors as being equivalent to 6.4 nmol ATP/mg protein for the whole span of $\Delta\psi$.

These discrepancies prompted us to reinvestigate the problem. We also made an attempt to differentiate between the energy storage capacity of the two components of $\Delta\tilde{\mu}_{H^+}$, namely the membrane potential ($\Delta\psi$) and the pH difference (ΔpH). In this investigation we adopted the procedure, first used by Lemasters and Hackenbrock [4], of measuring dissipation of the membrane potential when its restoration is prevented by a suitable inhibitor. Under such conditions the rate

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid; TPP^+, tetraphenylphosphonium cation.

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of the dissipation of the membrane potential could be a measure of the proton current due to proton leak through the inner mitochondrial membrane. This approach enabled us to calculate the energy of $\Delta\psi$ in physical units and not only in equivalents of ATP. On the other hand, comparing the rate the $\Delta\tilde{\mu}_{H^+}$ dissipation in the presence and absence of oligomycin, we were able to relate its energy to the amount of endogenous high energy phosphate bonds.

These results were published in a preliminary form in symposium proceedings [5].

Materials and Methods

Mitochondria from rat liver, isolated by a conventional procedure, were used throughout. The procedure of inhibition of mitochondrial respiration and measurinag dissipation of the membrane potential was as described elsewhere [5]. The incubation medium contained, unless otherwise indicated, 80 mM KCl, 3 mM $MgCl_2$, 5 mM phosphate (K salt), 1 mM EGTA (Tris salt), 10 mM succinate (Tris salt), 2 μM rotenone and 20 mM Hepes + 20 mM Mops adjusted with Tris to pH 7.2. It was placed in a chamber thermostated at 25°C and fitted with a Clark type oxygen electrode, a TPP^+-sensitive electrode [6] and a calomel reference electrode. To this medium TPP bromide was added in several small portions to make its final concentration 10–15 μM. This step served to calibrate the TPP electrode. Thereafter, mitochondrial suspension was introduced to a final concentration of 1–2 mg protein/ml and let to equilibrate for about 2 min. Most of TPP^+ was taken up by mitochondria, indicating their resting state membrane potential of 170–210 mV. The readings of the electrode were corrected for TPP^+ binding as described by Rottenberg [7]. To stop mitochondrial respiration KCN was then rapidly injected into the incubation chamber to make its final concentration 2 mM and readings of the TPP^+ electrode were recorded continuously. To calculate real changes of the membrane potential a correction for the response time of the measuring system (a 'deconvolution'), similar to that described by Krab et al. [8], was also introduced. The time constant of our measuring system was determined from the time-course of the electrode response after a rapid addition of KCN together with the protonophore carbonyl cyanide *m*-chlorophenylhydrazone, assuming that in that case the exit of TPP^+ from mitochondria was practically instantaneous.

Mitochondrial adenine nucleotides were determined fluorometrically by enzymatic assays [9].

Results

When the respiration of mitochondria under resting state (State 4) conditions was rapidly blocked by cyanide, the decline of the membrane potential ensued which could be followed with the TPP^+ electrode (Fig. 1A). If oligomycin was added before cyanide (closed symbols in Fig. 1A), generation of the proton-motive force by proton-pumping ATPase utilizing endogenous ATP was also blocked and therefore the decline of the membrane potential could be assumed to reflect solely the proton back-flow (proton leak, J_1) through the inner mitochondrial membrane (cf. Ref. 5). Thus, the slope of the corresponding curve of Fig. 1A (calculated by numerical differentiation) multiplied by membrane electrical capacitance (C) could be taken as a measure of the proton current according to the equation

$$J_1 = -\frac{d\Delta\psi}{dt} \cdot C \qquad (1)$$

When oligomycin was omitted, the decline of $\Delta\psi$ after blocking the respiration was slower and lasted longer (open symbols in Fig. 1A). This could be explained by functioning of proton-pumping ATPase, utilizing endogenous ATP, which partly compensated the back-flow of protons. Thus, the value of $(-d\Delta\psi/dt) \cdot C$ in the absence of oligomycin is a difference between the proton influx and the proton efflux driven by mitochondrial ATPase (J_{ATP}), utilizing endogenous ATP.

Our preparations of mitochondria incubated aerobically with succinate contained between 3.8 and 9.4 nmol ATP/mg protein and this value decreased to 0–0.6 nmol/mg protein after a few min incubation with cyanide in the absence of oligomycin. If phosphate in the incubation medium was substituted by 5 mM arsenate, the amount of mitochondrial ATP was below 1 nmol/mg protein

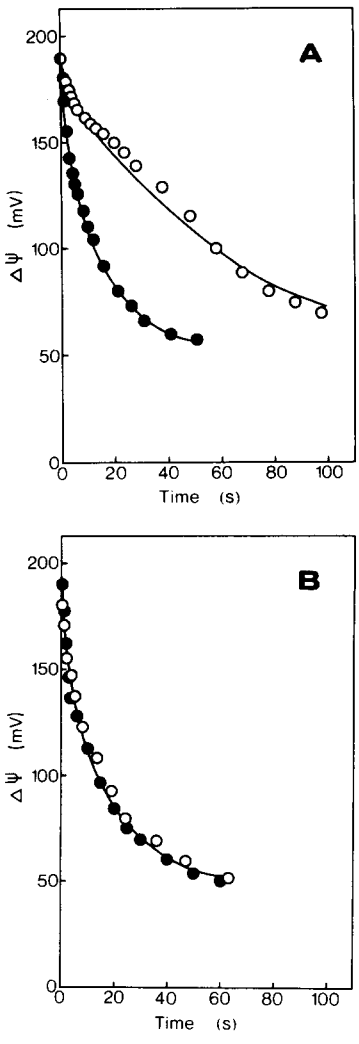

Fig. 1. Dissipation of the membrane potential after blcoking mitochondrial respiration with cyanide. Mitochondria (1.7 mg protein/ml) were incubated for about 2 min with 10 mM succinate prior to a rapid addition of KCN to 2 mM final concentration. The results are corrected for TPP^+ binding and 'deconvoluted' for the response time of the recording device. ○, no oligomycin; ●, oligomycin 2 μg/ml was added before cyanide. (A) The medium contained inorganic phosphate (see Materials and Methods); (B) Phosphate was substituted by 5 mM arsenate. Experiment No. 3 of Table I.

and decreased to as little as 0.1 nmol/mg protein after inhibition of respiration by cyanide. This was due to the well known effect of arsenate promoting ATP hydrolysis (cf. Ref. 10). In spite of this pronounced decrease in the contnet of endogenous ATP, mitochondria pipetted into the arsenate-containing medium developed practically the same membrane potential as in the phosphate-containing medium. It was observed that in the arsenate medium oligomycin had no effect on the rate of $\Delta\psi$ dissipation (Fig. 1B). This confirms that endogenous ATP was responsible for the difference in $\Delta\psi$ dissipation in the presence and absence of oligomycin and that oligomycin allows to differentiate between the net proton leak (J_1) and that partly compensated by proton pumping ATPase.

The rates of $\Delta\psi$ decline ($d\Delta\psi/dt$) in the presence and absence of oligomycin plotted vs. the actual value of $\Delta\psi$ are shown in Fig. 2. As already discussed in a previous publication [5], the plot in the presence of oligomycin reveals a nonlinear character, reflecting non-ohmic characteristics of the inner mitochondrial membrane with respect to protons (cf. Ref. 11). Since $d\Delta\psi/dt$ in the absence of oligomycin represents a difference between the dissipation rate of $\Delta\psi$ due to proton leak and the rate of its restoration by proton-pumping ATPase,

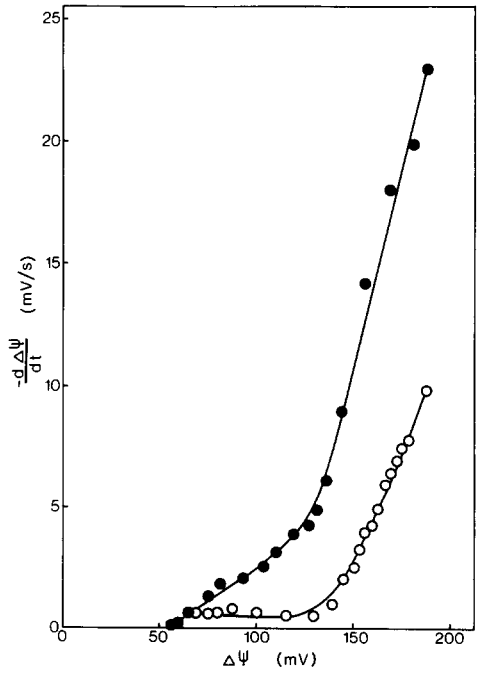

Fig. 2. Effect of oligomycin on the dependence of the dissipation rate of the membrane potential ($d\Delta\psi/dt$) on the actual value of the membrane potential ($\Delta\psi$). Conditions, experiment No. and indications same as in Fig. 1A.

the difference between the two plots of Fig. 2 corresponds to the net proton flux through the ATPase utilizing endogenous ATP (J_{ATP}). The time dependence of the two fluxes is shown in Fig. 3. Integration of these plots with respect to time from zero to infinity allows to calculate the amount of electric charges (Q) transferred by the two fluxes, according to the formula

$$Q = C\int_0^\infty \left|\frac{d\Delta\psi}{dt}\right| \cdot dt \qquad (2)$$

Since $d\Delta\psi/dt$ can be regarded as a measure of the proton current, its value multiplied by the actual $\Delta\psi$ is proportional to the power (P) of corresponding proton fluxes. This can be calculated for J_1 simply from data of Fig. 2 (closed symbols)

$$P_1(t) = \left|\frac{d\Delta\psi}{dt}\right| \cdot \Delta\psi(t) \cdot C \qquad (3)$$

The power of proton ejection due to hydrolysis of endogenous ATP can be calculated by multiplying by $\Delta\psi$ and C the difference between $d\Delta\psi/dt$ values in the presence and absence of oligomycin (taken for the same value of $\Delta\psi$)

$$P_{ATP} = \left|\left(\frac{d\Delta\psi}{dt}\right)_{+oligo} - \left(\frac{d\Delta\psi}{dt}\right)_{-oligo}\right| \cdot \Delta\psi(t) \cdot C \qquad (4)$$

The plots of $|d\Delta\psi/dt| \cdot \Delta\psi$ vs. time for the proton leak and the proton flux through ATPase (equivalent to P_1/C and P_{ATP}/C, respectively) are presented in Fig. 4. The areas below these plots multiplied by membrane capacitance are therefore the measure of the energy (E) of respective fluxes. This can be calculated by integration of the functions presented in Fig. 4.

$$E = C\int_0^\infty \left|\frac{d\Delta\psi}{dt}\right| \cdot \Delta\psi(t) \cdot dt \qquad (5)$$

The results of integrations presented in Eqns. 2 and 5, i.e., values of Q/C and E/C, designated as 'charge-transfer factor' and 'energy factor', respectively, for four typical experiments are shown in Table I. It can be seen that these 'factors' for the proton leak (J_1) are similar in all experiments, whereas the same values for the flux through ATPase (J_{ATP}) differ markedly but, as expected, are proportional to the amount of high-energy

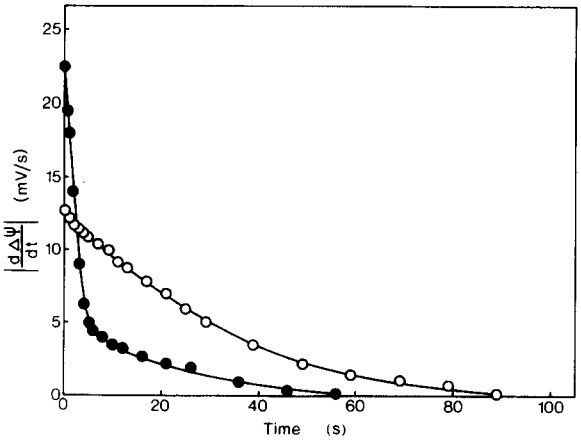

Fig. 3. Time dependence of the dissipation of the membrane potential due to proton leak and of the proton flux through ATPase utilizing endogenous ATP. ●, Proton leak; ○, flux through ATPase (difference between the rate of dissipation of the membrane potentia in the absence and presence of oligomycin). Because the two proton fluxes proceed in opposite directions, for sake of simplicity absolute values of $d\Delta\psi/dt$ are presented in this Figure. Same experiment as in Figs. 1 and 2.

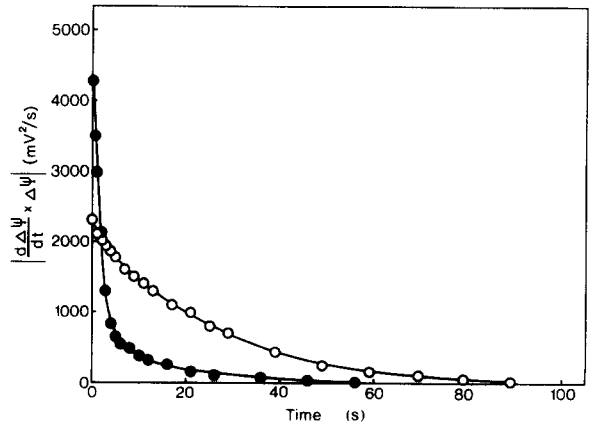

Fig. 4. Time dependence of the power of proton fluxes after blocking mitochondrial respiration with cyanide. The ordinate shows absolute values of the rate of membrane potential change multiplied by the actual value of the membrane potential. When multiplied by the membrane capacitance (Eqn. 3) it equals to the power of respective proton fluxes. ●, Proton leak; ○, proton flux through ATPase (values obtained as described in Fig. 3). Same experiment as in preceding figures.

TABLE I

DISSIPATION OF THE MEMBRANE POTENTIAL AND HYDROLYSIS OF ENDOGENOUS ADENINE NUCLEOTIDES AFTER INHIBITION OF MITOCHONDRIAL RESPIRATION WITH CYANIDE

Experimental conditions as described under Materials and Methods and in Fig. 1. 'Charge transfer factors' (columns 3 and 4) and 'energy factors' (columns 5 and 6) represent the areas below the curves in Figs. 3 and 4, respectively (see Eqns. 2 and 5). The decrease in the content of high-energy phosphate bonds of endogenous adenine nucleotides (Column 13) was calculated as follows: $\Delta \sim P = 2\Delta ATP - \Delta ADP$.

Expt. No.	$\Delta\psi$ (mV)	Charge transfer factor (mV)		Energy factor (mV^2)		Adenine nucleotides (nmol/mg protein)						
						initial		after KCN		difference		
s		J_1	J_{ATP}	J_1	J_{ATP}	ATP	ADP	ATP	ADP	ΔATP	ΔADP	$\Delta \sim P$
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
1	206.4	148.9	557.9	19800	83700	6.2	4.2	0.2	4.4	−6.0	0.2	−11.8
2	198.7	142.5	743.0	18200	112600	9.4	4.5	0.6	2.6	−8.8	−1.9	−19.5
3	188.8	136.4	371.0	16700	51500	6.4	2.5	0.5	4.4	−5.9	1.9	−9.9
4	178.3	135.0	216.5	15100	29500	3.8	2.8	0.0	4.4	−3.8	1.6	−6.0
Mean	193.1	140.7		17500								
±S.D.	±12.2	±6.4		±2000								

phosphate bonds disappearing, i.e., the net amount of ATP hydrolyzed, after blockage of the respiratory chain with cyanide (column 13).

Calculations and Discussion

Pauly et al. [12] determined the electric capacitance of the inner mitochondrial membrane as 0.5–0.6 $\mu F/cm^2$ by dielectric measurements of mitochondrial suspensions. Mitchell [1,13] assumed the value of 1 $\mu F/cm^2$ as mor realistic and recalculated it for liver mitochondria as 400 μF/mg protein. Substituting this value for C in Eqns. 2 and 5 and the 'charge-transfer factor' and 'energy factor' from Table I for the resultants of relevant integrations (see Figs. 3 and 4 for graphic presentation), one obtains mean values for the amount of charges transferred and for the energy of the proton flux expressed in physical units:

$140.7\ mV \cdot 400\ \mu F/mg\ protein = 5.6 \cdot 10^{-5}$ C per mg protein

$17500\ (mV)^2 \cdot 400\ \mu F/mg\ protein = 7 \cdot 10^{-6}$ J per mg protein. Because of the uncertainty of the value for membrane capacitance, these figures represent only a rough approximation and point to the order of magnitude rather that to precise values of energy and charge capacities of the transmembrane potential.

To recalculate these values into equivalents of ATP hydrolyzed (or synthesized), the way of expressing mitochondrial energy storage by other authors [1–4], we assumed average Gibbs free energy of ATP hydrolysis (ΔG) at our experimental conditions as close to 40 kJ/mol. The standard free energy of ATP hydrolysis under conditions close to those in the matrix of respiring mitochondria (pH 8.0, ionic strength $I = 0.2$ and Mg^{2+} concentration of about 1 mM) was calculated by Rosing and Slater [14] as 32.7 kJ/mol. Assuming further the intramitochondrial concentration of inorganic phosphate as 10 mM, one can calculate that the initial and final 'instantaneous' free energy changes of ATP hydrolysis in experiments presented in Table I (except for expt. No. 4 where the final amount of ATP was below 0.1 nmol/mg protein) were 45–46 and 37–40 kJ/mol, respectively. Therefore, the average value of 40 kJ/mol, adopted here, seems to be a reasonable and safe approximation. Thus, the amount of energy of the proton leak calculated in this way comes out to be equivalent to about 0.175 nmol ATP/mg protein. As will be discussed in more detail later on, this value relates, however, to the energy associated with one component of the proton-motive force only, namely the membrane potential ($\Delta\psi$).

Regarding the inner mitochondrial membrane

as a capacitor, the energy accumulated in form of the membrane potential can also be calculated from the formula

$$E = 0.5\, C(\Delta\psi)^2 \qquad (6)$$

Substituting 193.1 mV for resting state $\Delta\psi$ (Table I), the value of 7.5, μJ/mg protein is obtained, which is very close to the value calculated above. Good agreement of these two values points to the reliability of the integration procedure (Eqn. 5) and indicates that the discharge of a capacitor is a good model for dissipation of the mitochondrial membrane potential.

Multiplying the 'energy factor' of J_{ATP} (Table I) by 400 μF/mg protein and recalculating the product into equivalents of ATP, one obtains values between 0.3 (expt. No. 4) and 1.1 (expt. No. 2) nmol/mg protein, whereas the real net amount of ATP hydrolyzed in these experiments was between 6.0 and 19.5 nmol/mg protein, i.e., almost 20-times higher.

At this point it may be appropriate to stress again the degree of approximation of such calculations, resulting in the first line from an uncertainty of the value for membrane capacitance and from taking an average value for the free energy of ATP hydrolysis, as discussed earlier. However, even if the resulting figures were underestimated by a factor of 2 or 3, there still remains a large discrepancy between the calculated values for the ATP equivalent of the proton flux through ATPase (J_{ATP}) and the real amounts of ATP which was hydrolyzed. The obvious explanation of these differences is the fact that, taking membrane capacitance as 400 μF/mg protein, we only calculated the energy of dissipation of the electric component ($\Delta\psi$) of the proton-motive force. Meanwhile, the concentration component (ΔpH) was dissipated parallelly and also in form of the proton flux. This component should therefore be regarded as an additional current generator. Its capacity, which is apparently much higher than that of $\Delta\psi$, depends on the buffering capacity of the matrix compartment (assuming the buffering capacity of the external medium as approaching infinity). Therefore, in order to calculate the total energy stored in form of $\Delta\tilde{\mu}_{H^+}$, the total capacity of the system, and not only the electric capacitance of the inner membrane, should be taken for calculations depicted by Eqns. 1–5. This total capacity can be evaluated from Eqn. 5 by substituting the energy of ATP hydrolysis (values of column 13 in Table I multiplied by $\Delta G = 40$ kJ/mol) for E and the 'energy factor' for J_{ATP} for the resultant of the integration:

$$C = \frac{\Delta \sim P\Delta G}{\text{'energy factor' for } J_{ATP}} \qquad (7)$$

The outcome of such calculations is shown in column 2 of Table II as the total (formal) capacitance of the system. Although its value is expressed here for formal reason in capacitance units, it comprises both the electric capacitance and the capacity of the concentration component of $\Delta\tilde{\mu}_{H^+}$. The degree of approximation of this value mainly derives from the approximation of free energy of ATP hydrolysis and therfore may be slightly underestimated. Multiplying by this value the 'energy factor' of the proton leak (J_1 in Table I), the energy accumulated in form of the proton-motive force can be calculated as 122 μJ/mg protein (Table II, column 3).

A different calculation procedure but leading of course to the same result is the following. The value of the 'energy factor' corresponding to one nmol of hydrolyzed ATP is obtained by dividing data of column 6 by those of column 13 of Table I. The result is shown in column 5 of Table II. Now, dividing the 'energy factor' for J_1 (column 4 of Table I) by this value, the energy of the proton leak expressed in equivalents of ATP is obtained (column 4, Table II) as 3.0 nmol/mg protein on the average. It is worthy to note that this figure is a resultant of direct comparison of the energy of proton fluxes with the amount of concomitantly hydrolyzed ATP. It is therefore not affected by either of the two arbitrarily assumed facors. Multiplying the latter value by free energy of ATP hydrolysis (40 kJ/mol), the energy of $\Delta\tilde{\mu}_{H^+}$ expressed in physical units can be obtained (column 3, Table II).

These calculations are not affected by the fact that the free energy of the membrane potential decreases when the latter collapses, because the 'energy factor' is always related to the amount of ATP which is simultaneously hydrolyzed (whose free energy also decreases with decreasing the

TABLE II

CALCULATION OF THE ENERGY OF PROTON LEAK UNTIL COMPLETE DISSIPATION OF THE PROTON-MOTIVE FORCE (EQUIVALENT TO THE ENERGY STORED IN FORM OF THE PROTON-MOTIVE FORCE)

Values of column 5 are obtained by dividing values of column 6 of Table I by those of column 13 of Table I. Data of column 4 can be obtained either by dividing values of column 3 by 40 kJ/mol or by dividing values of column 5 of Table I by those of column 5 of the present Table. For other explanations see text.

Expt. No.	Total (formal) capacitance (mF/mg protein)	Energy of J_1		Energy factor per nmol ATP ((mV)^2/nmol)
		in physical units (μJ/mg protein)	in equivalents of ATP (nmol/mg protein)	
(1)	(2)	(3)	(4)	(5)
1	5.64	112	2.79	7100
2	6.93	126	3.14	5800
3	7.69	128	3.21	5200
4	8.14	120	3.01	4900
Mean $\pm$ S.D.	7.10 $\pm$ 1.10	122 $\pm$ 7	3.04 $\pm$ 0.19	5800 $\pm$ 1000

phosphorylation potential). The value of 122 μJ/mg protein represents, however, a slight underestimation resulting from the fact that, in our experimental conditions, $\Delta\psi$ (and presumably also $\Delta\tilde{\mu}_{H^+}$) did not collapse completely. Because of drastically decreased membrane conductance at low membrane potential [11], when the membrane potential attained the value of about 50 mV its further decrease became so slow (Figs. 1 and 2) that the experiment was terminated at that point. However, the remaining energy amount is rather small and amounts to about 9 μJ/mg protein, as can be calculated using Eqn. 6 and substituting 7.1 mF/mg protein for C and 50 mV for $\Delta\psi$. Hence, the total amount of energy stored in form of $\Delta\tilde{\mu}_{H^+}$ in resting state liver mitochondria can be estimated as close to 131 μJ or the equivalence of 3.3 nmol ATP/mg protein assuming formally ΔG as 40 kJ/mol. The latter value does not mean, of course, that that much ATP could be synthesized due to energy accumulated in the proton-motive force, since there is a threshold potential below which no ATP formation occurs. Assuming this threshold as 100 mV [15,16], it can be calculated using Eqn. 6 that about 36 μJ/mg protein (i.e., equivalence of 0.9 nmol ATP, using the same recalculation factor) from the total energy stored in $\Delta\tilde{\mu}_{H^+}$ cannot be utilized to make ATP.

Our value for the energy storage capacity of $\Delta\tilde{\mu}_{H^+}$ is abotu half of that estimated by Lemasters and Hackenbrock [4]. However, their value of 6.4 nmol ATP/mg protein is considerably overestimated due to a linear extrapolation of the energy associated with the collapse of the memrbane potential down to zero. In contrast, our procedure is not affected by such error, as discussed in the preceding paragraph. Therefore, though our calculation of the energy in physical units may be somewhat uncertain due to a rather arbitrary assumption of an average ΔG, its expression in equivalents of ATP is rather safe from this uncertainty.

Comparing the values for energy storage in form of the entire $\Delta\tilde{\mu}_{H^+}$ (Table II) with that calculated for the electric component only (7.0 μJ/mg protein), one has to recall that a much higher energy storage capacity of ΔpH as compared to $\Delta\psi$ was already calculated by Mitchell [1] 20 years ago on the basis of his original formulation of the chemiosmotic theory. According to his estimation, the storage capacity of the pH difference is nearly a 100 times that associated with the membrane potential for the same span of $\Delta\tilde{\mu}_{H^+}$. Taking into account that ΔpH constitutes about 15%–20% of the entire $\Delta\tilde{\mu}_{H^+}$ [4,16], this is in close agreement with the present finding where the energy stored in $\Delta\tilde{\mu}_{H^+}$ appeared to be 17-times higher than that accumulated in form of $\Delta\psi$. It may be interesting to note that Mitchell's values for energy storage capacity of $\Delta\psi$ and ΔpH,

calculated on the basis of electric capacitance and the buffering capacity of mitochondria, when related to the real participation of either of these components in the total proton-motive force, amount to 0.2 and 3 nmol ATP/mg protein for $\Delta\psi$ and ΔpH, respectively, which is very close to the present results based on a direct experimental approach.

Multiplying the 'charge transfer factors' for J_{ATP} (Table I) by the total capacity of the system as calculated above (7.1 mF/mg protein), the values of the charges transferred due to hydrolysis of endogenous ATP are obtained (Table III). Dividing these values by the amount of ATP hydrolyzed in a particular experiment, the H^+/ATP ratio close to 3 could be calculated (Table III). The degree of approximation of this evaluation is mainly determined by the approximation of the 'total capacity' (Eqn. 7) and this, in turn, depends on correctness of the value of ΔG for ATP hydrolysis. Since the latter value may be slightly underestimated in our calculations, the value of H^+/ATP may also be subject to some underestimation. It may be needless to add that the rather uncertain value of the electric capacitance is not involved in this calculation. The stoichiometry of 2 was obtained by Moyle and Mitchell [17] for proton-pumping ATPase in submitochondrial particles. The present value of 3 for intact mitochondria hydrolyzing endogenous ATP is, most likely, the resultant of the production of inorganic phosphate in the matrix compartment which, in turn, is exchanged for external HO^-, thus additionally increasing ΔpH.

The initial rate of the dissipation of the membrane potential in our experiments was close to 20 mV/s (Fig. 2). Substituting this value for $-d\Delta\psi/dt$ in Eqn. 1 and taking 7.1 mF/mg protein for the total capacitance of mitochondria, the magnitude of the initial proton current due to discharge of the entire $\Delta\tilde{\mu}_{H^+}$ comes out to be 142 μA/mg protein. This corresponds to the transfer of 88 nmol protons/min per mg protein. Assuming H^+/O stoichiometry for succinate oxidation as 6, the oxidation rate of about 15 ng atom oxygen/min is obtained. This is close to the rate of resting state respiration observed with our mitochondrial preparations (see, e.g., Refs. 5 and 16), indicating again that these calculations represent a reasonable approximation.

The present results allow better to understand the role of the two components of the proton-motive force. They clearly indicate that the membrane potential has a negligible part in mitochondrial energy storage, the major part being played by the proton concentration gradient (ΔpH). $\Delta\psi$ has, however, a number of other important functions. Since in mitochondria it constitutes the main driving force for proton re-entry through the ATPase complex, it is one of its rate-controlling

TABLE III

CHARGE TRANSFER DUE TO DISSIPATION OF THE PROTON-MOTIVE FORCE AND HYDROLYSIS OF ENDOGENOUS ADENINE NUCLEOTIDES

Values of columns 2 and 4 are products of multiplication of data of columns 3 and 4 of Table I by the total capacitance of the system (see column 2 of Table II). Figures of columns 3 and 5 are obtained by dividing the former data by the Faraday constant. H^+/ATP ratio (column 6) is calculated by dividing values of column 5 of this Table by those of column 13 of Table I.

Expt. No.	Charge transfer for J_1		Charge transfer for J_{ATP}		H^+/ATP
	(μC/mg protein)	(nmol H^+/mg protein)	(μC/mg protein)	(nmol H^+/mg protein)	
(1)	(2)	(3)	(4)	(5)	(6)
1	1057	11.0	3960	41.0	3.5
2	1012	10.5	5280	54.7	2.8
3	968	10.0	2630	27.3	2.8
4	959	9.9	1540	15.9	2.7
Mean ± S.D.	999 ± 45	10.4 ± 0.5			2.9 ± 0.4

factors. Moreover, the membrane potential maintains the high phosphorylation potential in the cytoplasmic compartment [18], plays a role in Ca^{2+} homeostasis [19,20] and probably regulates the association of the natural protein inhibitor with the ATPase complex [21], thus controlling the rate of ATP synthesis.

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