J. Membrane Biol. 49, 105–121 (1979)

Membrane Potential of Mitochondria Measured with an Electrode Sensitive to Tetraphenyl Phosphonium and Relationship between Proton Electrochemical Potential and Phosphorylation Potential in Steady State

Naoki Kamo*, Makoto Muratsugu, Ruji Hongoh, and Yonosuke Kobatake

Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan

Received 12 September 1979; revised 26 February 1979

Summary. The membrane potential of mitochondria was estimated from the accumulation of tetraphenyl phosphonium (TPP^+), which was determined with the TPP^+-selective electrode developed in the present study. The preparation and some operational parameters of the electrode were described. The kinetics for uptake by mitochondria of TPP^+ and DDA^+ (dibenzyldimethyl ammonium) were analyzed, and it was found that TPP^+ permeated the mitochondrial membrane about 15 times faster than DDA^+. The final amounts of accumulation of TPP^+ and DDA^+ by mitochondria were approximately equal. For the state-4 mitochondria, the membrane potential was about 180 mV (interior negative). Simultaneous measurements of TPP^+-uptake and oxygen consumption showed that the transition between states 3 and 4 was detectable by use of the TPP^+-electrode. After the TPP^+-electrode showed that state-4 was reached, the extra-mitochondrial phosphorylation potential was measured. The difference in pH across the membrane was measured from the distribution of permeant anion, acetate, so as to calculate the proton electrochemical potential. The ratio of extra-mitochondrial phosphorylation potential to proton electro-chemical potential, n was close to 3. This value of n was also found to be 3 when ATP was hydrolyzed under the condition that the respiratory chain was arrested. The implication that $n=3$ was discussed.

According to the chemiosmotic theory developed by Mitchell [25], the difference in proton electrochemical potential generated across the membrane is the sole energy-transducing intermediate between the respiratory chain and the proton-translocating ATP synthetase. The difference in the proton electrochemical potential, $\Delta\bar{\mu}_H$, is composed of electrical and concentration terms according to the relation:

$$\Delta\bar{\mu}_H = -2.3\,RT\Delta\mathrm{pH} + F\Delta\varphi$$

* *Address for reprint requests:* Department of Biophysics, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, 060 Japan.

0022-2631/79/0049-0105 $03.40

where $\Delta\varphi$ is the potential difference across the membrane, and ΔpH is the pH difference. These quantities are defined with respect to the outside of mitochondria. Here, R, T, and F have their usual thermodynamic significances. It is considered that the membrane potential mainly contributes to $\Delta\bar{\mu}_H$ in mitochondria [40].

The membrane potential of mitochondria, due to their smallness in size, cannot be measured directly by microelectrode. Skulachev and his coworkers [4] have pointed out that the lipid-soluble ions can diffuse passively across the membrane. When the membrane of cells or organelles is hyperpolarized, the lipid-soluble cation added to the medium is transferred electrophoretically into the cells or organelles, and hence the concentration of lipid-soluble cation in the medium decreases, and *vice versa*. At equilibrium, the lipid-soluble ions distribute between cells and medium in accordance with the Nernst equation, *viz.*

$$\Delta\varphi = \frac{RT}{zF} \ln \frac{C_e}{C_i}$$

where z stands for valency of the lipid-soluble ions ($z=1$ for tetraphenyl phosphonium cation, TPP^+), C_i, the concentration of the lipid-soluble ions in the cells, and C_e, that in the medium. This method of estimation of the membrane potentials with use of lipid-soluble ions has been applied to many organisms, organelles, membrane vesicles, and liposomes [12, 14, 16, 21, 37, 42].

In previous papers [31, 41], we developed an electrode sensitive to dibenzyldimethyl ammonium (DDA^+), one of lipid-soluble ions, and showed that the accumulation and efflux of DDA^+ was detected by the electrode developed. This method has advantages in that the electrode enables us to monitor the change in membrane potential of such small cells or vesicles easily and continuously. The membrane potential of mitochondria was estimated by use of the DDA^+-electrode [18]. However, DDA^+ required the presence of a lipid-soluble anion such as tetraphenyl boron (TPB^-) for permeation through the membrane, and the role of the anion was not made clear [14]. Contrary to DDA^+, tetraphenyl phosphonium (TPP^+) does not require the presence of the anion. Hence, TPP^+ is more suitable as a membrane potential indicator compared with DDA^+.

The present paper deals with the preparation of an electrode sensitive to TPP^+ and properties of the electrode. The membrane potential of mitochondria is measured with the electrode developed. The phosphorylation potential, ΔG_p, in state-4 condition is also measured and is

compared with $\Delta\bar{\mu}_H$, where the pH difference across the mitochondrial membrane is estimated from the distribution of a permeant acid, acetic acid.

Materials and Methods

Rat liver mitochondria were isolated in 250 mM sucrose and washed three times. The mitochondrial protein was determined by the method of Lowry [24], using bovine serum albumin as the standard.

The membrane potential of mitochondria was estimated from the accumulation of TPP^+ which was measured by use of an electrode sensitive to TPP^+. The selective electrode was constructed by use of a poly-vinylchloride-based membrane containing TPB^- as an ion-exchanger. After addition of 3 ml of 10^{-2} M sodium tetraphenylboron in tetrahydrofuran (THF) to 10 ml of THF solution containing 0.5 g poly-vinylchloride (PVC) and 1.5 ml of dioctylphthalate acted as plasticizer, the solvent was slowly evaporated at room temperature in a flat petri dish (60 cm^2 in area). The membrane thus obtained was transparent and 0.15–0.2 mm thick. A piece of the membrane was glued to a PVC tube with THF. A 10^{-2} M TPP^+ solution was placed inside the tube as an internal reference solution. This solution and a Ag−AgCl electrode were bridged with a small tube filled with a saturated KCl agar. Before use, it was necessary that the electrode was soaked overnight in a concentrated TPP^+ solution (ca. 10^{-2} M) for conditioning. The electromotive force between the TPP^+-electrode and calomel reference electrode in the sample solution was measured by an electrometer (Model TR-8651, Takeda Riken, Tokyo) connected to a pen-writing recorder. We have shown that for a case of *E. coli* intact cells, the amount of TPP^+ bound to the cell envelope must be corrected and can be estimated easily by use of the heat-treated cells (*manuscript in preparation*). We examined the binding of TPP^+ to the mitochondria heated at 60 °C for 30 min, and found that this amount was negligibly small compared with the uptake by intact mitochondria. Therefore, we calculated the membrane potential from the change in the TPP^+-electrode potential without any corrections. The membrane potential, $\Delta\varphi$, can be evaluated in accordance with the following equation.

$$\Delta\varphi = 2.3\frac{RT}{F}\log\left(\frac{v}{V}\right) - 2.3\frac{RT}{F}\log\left[10^{F\Delta E/2.3RT} - 1\right]$$

where v, V and ΔE stand for the mitochondrial volume, the volume of incubation medium, and the deflection of the TPP^+-electrode potential from the base line prior to injection of mitochondria. This equation has been derived using the conditions that TPP^+ distribution between mitochondria and medium follows the Nernst equation and that the mass-conservation law is applicable. Details should be consulted in the previous paper [31]. The temperature was kept constant at 25 °C. The medium was bubbled with $O_2 - CO_2$ (95:5) gas when necessary.

The oxygen consumption was followed with a Clark-type electrode, and the output was connected to a channel of pen-writing recorder, in which the change in the TPP^+-electrode potential was also recorded.

The ΔpH was determined from the distribution of [^{14}C]-acetic acid, which is a largely dissociated form in the present condition and distributes according to $\Delta\text{pH} = \log [Ac]_i/[Ac]_e$ [19]. Here, subscripts i and e signify the quantities of internal and of

external mitochondria. For starting the reaction, the mitochondria were suspended in a medium containing [^{14}C]-acetate and [^{3}H]-sucrose which was stirred magnetically and thermostated at 25 °C. The media used are described in the legends. After 3 min, 3 ml of the mitochondrial suspension were transferred to a centrifuge tube, followed by weighing quickly. The suspension was centrifugated at 13,000 × *g* for 1 min. The supernatant was decanted, and residual water attached to the wall was carefully removed with filter paper. The incubation medium without radio-isotopes was pipetted into the pellet until the weight became equal to that obtained prior to centrifugation, and the pellet was resuspended carefully. Two hundred µl of both the supernatant and this suspension was transferred to a scintillation vial containing 200 µl of 0.2% SDS (sodium dodecylsulfate) solution. After solubilization, Bray's solution (20 ml) was poured and the radio activity was counted by a Beckman LSC-200 liquid scintillation spectrometer. The window of the ^{3}H-channel was set so that the spillover from the ^{14}C-channel was 5%, and the ^{3}H-counts were corrected for this spillover. The amount of acetate in the mitochondria was corrected for the acetate in the extra-mitochondrial water estimated from the counts of [^{3}H]-sucrose in the pellet.

The matrix water was determined with [^{3}H]-water and [^{14}C]-sucrose, where [^{3}H]-water gives the total pellet water and [^{14}C]-sucrose, the extramatrix water, respectively. The procedure was the same as that employed for the determination of ΔpH. The matrix water in state-4 mitochondria was estimated to be 1.4 µl per mg of mitochondrial protein.

Phosphate was determined by the method employed by Fiske-Subbarow [11]. ATP was determined with hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49), ADP with pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) as described by Bergmeyer [5]. Production of NADPH or disappearance of NADH was monitored with a Gilford AL-200 spectrometer. After the electrode potential showed that state 4 had been reached (details are written later), a sample of about 3 ml of the suspension was removed and 0.2 ml of 3 N $HClO_4$ was added and agitated to stop the reaction. After neutralization with 0.2 ml of 3 N KOH, the sample was centrifugated at 13,000 × *g* and the concentrations of ATP, ADP and P_i in the supernatant were measured as described above. The terminologies for mitochondrial respiratory states proposed by Chance and Williams [8] are used.

Results and Discussion

The response of the electrode was linear with the logarithm of the TPP^+ concentration with a slope of 59 mV per decade concentration until the concentration decreased down to around 5×10^{-7} M. The electrode may also respond to certain substances other than TPP^+. The presence of such substances in a sample solution interfers the detection of the change in TPP^+ concentration. The effect of the interference is evaluated in terms of the selectivity coefficients, K_i, defined as

$$E = (\text{const}) + (RT/F) \ln \{[\text{TPP}^+] + K_i[i]^{1/z_i}\}$$

where E, $[\text{TPP}^+]$, $[i]$ and z_i are the emf observed, the concentration (strictly, activity) of TPP^+, that of the interfering substance i and the valence of i, respectively, and (const) is a constant depending only on the

concentration of the internal reference solution. At first, DDA^+ was chosen as the interfering substance and K_i obtained was 7×10^{-3}. The method for determination of K_i is written in the reference [22]. This means that the present electrode responds to TPP^+ about 100 times more sensitively than to DDA^+. According to theoretical and experimental studies on ion-selective electrode [e.g., 21], the following equation holds in the selectivity coefficient:

$$K_{si} = K_{sj} K_{ji}$$

where K_{si} represents the selectivity coefficient of i with respect to the primary ion, s. In the present discussion, s corresponds to TPP^+, i, various interfering ions, and j, DDA^+. The value of K_{ji} had been examined in the previous papers [31, 41] and found to be less than 10^{-5} for all interfering ions studied. Then, K_{si} (the selectivity coefficient with respect to TPP^+, i.e., K_i in the above equation) is expected to be less than 10^{-7}, since $K_{sj} = 7 \times 10^{-3}$. Actually, the k_i values were determined for various substances such as inorganic salts and oxidizable substrates used in the present experiments. We found that they were less than 10^{-6}. This implies that the error caused by the presence of such substance 10^4 times more concentrated than the TPP^+ concentration is smaller than 1%. In the following experiments, the substances used did not interfere with the measurements of change in TPP^+ concentration. The electrode system responded in 2–5 sec to a doubling of the TPP^+ concentration. The electrode was stored in 10^{-2} M TPP^+ solution when not in use. The relationship between the observed electromotive force and TPP^+ concentration was unchanged for at least half a year.

The effects of oxidative substrates, inhibitors and uncouplers on the uptake of TPP^+ were essentially the same as that obtained previously with use of a DDA^+-selective electrode [31, 41]; mitochondria accumulate TPP^+ when an oxidizable substrate or ATP was applied, and cessation of energy supply by inhibitors of uncouplers resulted in an efflux of TPP^+ accumulated previously. The presence of TPB^- up to 10% of TPP^+ scarcely affects the accumulation of TPP^+ (data not shown), meaning that TPP^+ does not require the presence of TPB^- for its electrophoretic permeation of the membranes as described above.

The flux of TPP^+, denoted by J, is proportional to the difference of the electrochemical activity across the mitochondrial membrane [17].

$$J = P[C_e - C_i \exp(F\Delta\varphi/RT)] \quad (1)$$

where C_i, C_e and P stand for TPP^+-concentration in the mitochondria, TPP^+-concentration in medium, and permeability coefficient, respectively. In Eq. (1), the positive direction is defined as flow from the medium to mitochondria. The membrane potential, $\Delta\varphi$, is assumed to be constant and is not affected by the influx of TPP^+. Since the increase of intramitochondrial TPP^+-concentration is due to J, which is defined as moles per unit area and time, the following equation is obtained:

$$\frac{dC_i}{dt}=(A/v)\,J=\alpha J \qquad (\alpha=A/v) \tag{2}$$

where A and v are the surface area and volume of mitochondria, respectively. The mass conservation law requires the following equation:

$$vC_i+VC_e=VC_o. \tag{3}$$

Here, C_o stands for the initial concentration before injection of mitochondria, and V, the volume of medium.

Combining Eqs. (1), (2) and (3), we obtain the following differential equation:

$$\frac{dC_i}{dt}+\alpha P\left[\frac{v}{V}+\exp(F\Delta\varphi/RT)\right]C_i=\alpha PC_o. \tag{4}$$

The solution to this equation is given by Eq. (5) under the initial condition that $C_i=0$ when $t=0$.

$$C_i=\frac{C_o}{\dfrac{v}{V}+\exp(F\Delta\varphi/RT)}\left[1-\exp\left[-\alpha P\left\{\frac{v}{V}+\exp(F\Delta\varphi/RT)\right\}t\right]\right]. \tag{5}$$

Let us define U as the amount of TPP^+ accumulated in mitochondria, and then, we obtain that $U=vC_i$. Equation (5) is rewritten as follows:

$$\frac{U}{U_e}=1-\exp\left[-\alpha P\left\{\frac{v}{V}+\exp(F\Delta\varphi/RT)\right\}t\right] \tag{6}$$

where

$$U_e=vC_o/[v/V+\exp(F\Delta\varphi/RT)].$$

Letting t equal infinity in Eq. (6) leads to the relation $U=U_e$, meaning that U_e is the TPP^+-accumulation at equilibrium. Rearrangement of Eq. (6) gives

$$\log\frac{U_e-U}{U_e}=-\frac{\alpha P}{2.3}\left\{\frac{v}{V}+\exp(F\Delta\varphi/RT)\right\}t. \tag{7}$$

This equation implies that $\log(U_e - U)/U_e$ *vs.* t plot gives a straight line provided that $\Delta\varphi$ and v stay constant. The method for estimation of U with use of the deflection of electrode potential was described previously [41].

Equation (7) was applied to uptake of TPP^+ and DDA^+ by mitochondria, and the results were shown in Fig. 1. The uptake of DDA^+ was measured with the selective electrode for DDA^+ developed previously [41]. The equilibrium amounts of uptake of DDA^+ and TPP^+, U_e, were not substantially different from each other. Results shown in Fig. 1 indicate that TPP^+ can permeate the mitochondrial membrane about 15-fold faster than DDA^+. As described above, TPP^+ does not require a lipid-soluble anion such as TPB^-. These facts suggest that TPP^+ is more surpassing for the membrane potential indicator than DDA^+.

Figure 2*A* shows the results of simultaneous measurements of TPP^+-uptake and of oxygen consumption for the transition between state 3 and state 4, indicating that the change in the TPP^+ electrode potential

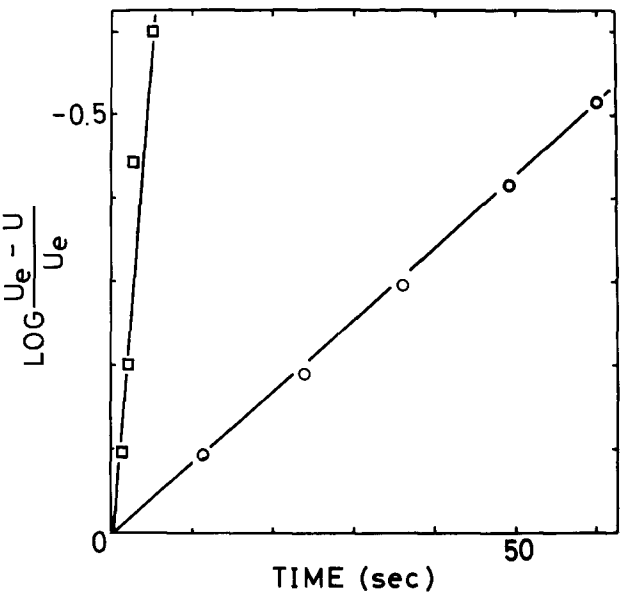

Fig. 1. Comparison of permeability coefficient between DDA^+ and TPP^+. Uptake of such lipid-soluble ions by mitochondria was analyzed according to Eq. (7). The medium used was 10 mM potassium phosphate (pH 7.4), 0.1 mM EDTA and 0.25 M sucrose. TPP^+-concentration was 10^{-4} M, and DDA^+-concentration was 10^{-4} M supplemented with 10^{-6} M TPB^-. When $t=0$, mitochondria were injected into the medium to initiate the uptake of the lipid-soluble ions. Mitochondrial concentration was 2.0 mg protein/ml. ○: DDA^+; □: TPP^+

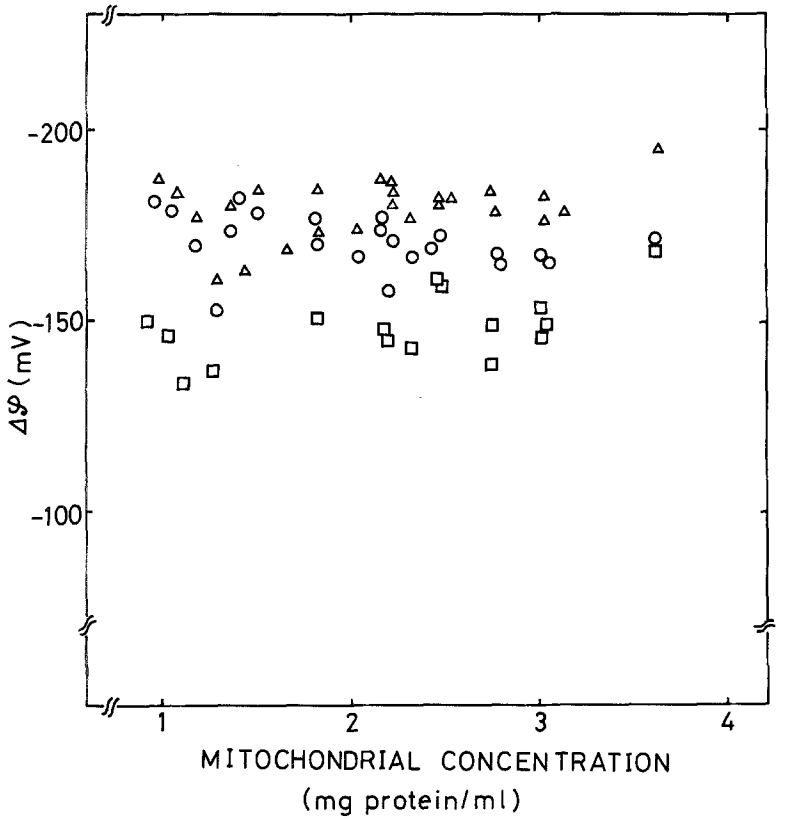

Fig. 3. Membrane potential of mitochondria in state 1 (respiration due to endogeneous oxidizable substrates), ○; state 3 (in the presence of 5 mM succinate, 5 μM rotenone, 0.1 mM ADP), □; and state 4 (in the presence of 5 mM succinate, 5 μM rotenone), △. The state-3 membrane potential was calculated from the bottom of the trace of TPP^+-electrode potential as shown in Fig. 2. In this calculation, the volume change in mitochondria between states 3 and 4 was ignored. Incubation medium contained (in mM): 40, sucrose; 40, KCl; 0.4, EDTA; 10, $MgCl_2$; 50, Tris-HCl (pH 7.5); 4.8, phosphate. TPP^+ concentration was 3×10^{-5} M

Under state-4 condition, the respiratory chain and extra-mitochondrial adenine nucleotide pool are close to thermodynamic equilibrium [30]. Then, the chemiosmotic theory requires that, under state-4 condition, $\Delta\bar{\mu}_H$ must also be in near equilibrium with phosphorylation potential, ΔG_p, which is defined as the free energy change of ATP synthesis. When the trace of TPP^+ electrode potential showed that state 4 was reached after addition of ADP into the reaction medium containing exogeneous ATP, a sample of the suspension was removed and the concentrations of ATP, ADP and P_i were analyzed. The phosphorylation potential in mV scale, ΔG_p, was calculated in accordance

again, the membrane potential returns approximately to the original level prior to addition of ADP. The reason for the time lag in the return of the membrane potential to the original state-4 level is not clear at present. Since as described above and shown in Fig. 1 the TPP^+-electrode responded quickly enough, the time lag is not explained by the response time of the electrode.

Comparison between TPP^+-electrode deflection and the oxygen consumption suggests that transition between states 3 and 4 can be detected with the membrane potential measurements by use of the TPP^+-electrode. This fact is further confirmed by use of "ADP-regenerating" system containing glucose and hexokinase. Figure 2*B* demonstrates that the presence of ADP results in the higher concentration of TPP^+ in the medium, and it is noted that the trace of TPP^+-electrode does not return to the original state-4 level (*see* the trace in the absence of atractyloside). This means that the membrane potential of state-3 mitochondria is smaller than that of state-4 mitochondria (interior negative). The change in TPP^+-concentration in the medium is partially attributed from the volume change of mitochondria [13].

To measure the concentration of ADP by means of spectroscopy, we must raise it in state-4 mitochondria where ratio of ATP/ADP is usually 100 or more. Therefore, ATP was added exogeneously into the incubation medium. As reported by Slater *et al.* [43], the transition from state 3 to state 4 becomes obscure in the presence of exogeneous ATP (several mM). An addition of Mg^{2+} made the transition clear, and K^+ also had a little effect [43]. Therefore, the medium used in the following experiments contains $MgCl_2$ and KCl.

Figure 3 shows the membrane potentials of mitochondria in state 1, state 3 and state 4 with varying mitochondrial concentrations. This figure indicates that the membrane potential of mitochondria estimated by the present method are relatively constant irrespective of mitochondrial concentration. The values of $\Delta\varphi$ shown in Fig. 3 are approximately equal to those obtained previously with the DDA^+-electrode [18]. In addition, the values obtained here are compatible with those obtained by previous investigators. Nicholls [32], Akerman and Wikstrom [1], and Laris *et al.* [23] have reported that the membrane potential of mitochondria in state-4 is 150 mV (with valinomycin plus ^{86}Rb), at least 170 mV (with safranine), and 150–180 mV (with cyanine dye). They also stated that the membrane potential of state-3 mitochondria is smaller than that of state-4 mitochondria. It is noted that the methods for measuring the membrane potential employed by these investigators and by us are different.

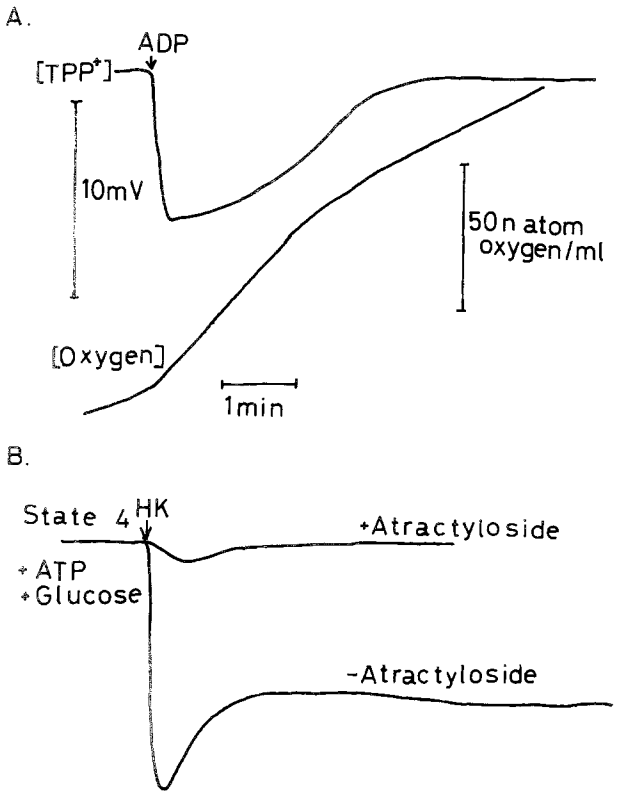

Fig. 2. (*A*): Simultaneous measurements of TPP^+ uptake and of oxygen uptake at the transition from state 4 to state 3. The downward deflection in the trace of TPP^+-electrode potential means the efflux of TPP^+ accumulated previously. The vertical bar indicates a change of 10 mV in TPP^+-electrode potential. The upward shift in the trace of oxygraph means a decrease in oxygen concentration in the medium. State 4 was attained in the presence of succinate (5 mM), and ADP (0.1 mM) was added at the time indicated by the arrow in the figure. Incubation medium contained 250 mM sucrose, 10 mM Tris-H_3PO_4 (pH 7.44), 0.1 mM EDTA, 2 mM $MgCl_2$, 5 μM rotenone, and 10^{-5} M TPP^+. The mitochondrial concentration was 0.87 mg protein/ml. (*B*): The change in TPP^+-electrode potential when ADP is regenerated by the combination of hexokinase and glucose. The steady state (state 4) was attained by adding succinate (4 mM) and ATP (100 μM) in the presence of 5 mM glucose. The arrow with *HK* indicates the time when 70 U hexokinase was applied to the suspension. The upper trace is the result in the presence of atractyloside (0.75 μM) and the lower, in the absence of atractyloside. Incubation medium was the same as in *A*, and mitochondrial concentration was 1.62 mg protein/ml

corresponds well to the oxygen uptake. In the figure, the downward deflection of the TPP^+-electrode potential means an increase of TPP^+ concentration in the medium. Then, an addition of ADP to state-4 mitochondria results in the efflux of TPP^+, which means the depolarization of mitochondria, and results in the increase of the rate of oxygen consumption. When the oxygraph trace shows that state 4 is attained

with the following equation:

$$\Delta G_p = \frac{1000}{F}\left(\Delta G_p^0 + 2.3\,RT \log \frac{[\mathrm{ATP}]}{[\mathrm{ADP}][\mathrm{P_i}]}\right)$$

where ΔG_p^0 stands for the standard free energy of synthesis of ATP at a given pH and Mg^{2+} concentration, and ΔG_p^0 has been determined by Rosing and Slater [38]. Note that the ΔG_p estimated in the present paper is identical with the extra-mitochondrial phosphorylation potential. Table 1 shows a comparison between ΔG_p and $\Delta\varphi$. In mitochondria, ΔpH is known to be small, and the membrane potential contributes mainly to $\Delta\bar{\mu}_H$ [27, 34, 36, 39, 40]. The right-most column shows the ratio of ΔG_p to $\Delta\varphi$, indicating that this value is approximately equal to 3. As reported by previous investigators, pH difference across the membrane was about 0.4 (24 mV in mV scale, interior alkaline) and at most, 0.6 (36 mV). Then,

Table 1. Comparison between ΔG_p and $\Delta\varphi$

	Expt. No.	ATP (mM)	ADP (μM)	ΔG_p (mV)	$\Delta\varphi$ (mV)	$\Delta G_p/\Delta\varphi$
A)	1	2.82	32	558	181	3.08
	2	2.86	28	562	183	3.07
	3	2.98	39	554	174	3.19
	4	2.92	30	560	161	3.48
B)	1	2.57	22.7	574	173	3.32
	2	2.69	36.1	564	174	3.24
	3	2.69	24.0	574	177	3.24
	4	2.69	42.5	558	175	3.19
C)	1	2.06	24.2	578	183	3.16
	2	1.79	21.0	578	192	3.01
	3	4.22	30.8	590	188	3.14
	4	0.82	37.4	542	187	2.90
	5	0.82	25.0	554	186	2.98
	6	0.94	56.4	536	181	2.96

Mitochondria (final concentration, ca. 1.5 mg protein/ml) were injected in the medium described below in the presence of 5 μM rotenone and 3×10^{-5} M TPP^+, followed by addition of ATP and 5 mM succinate. The medium was bubbled with O_2-CO_2 gas; then ADP was added. When TPP^+-electrode showed the return of state 4, the sample was removed and ΔG_p was determined. Medium *A* (in mM): 150, sucrose; 20, KCl; 30, Tris-HCl (pH 6.2); 0.25, EDTA; and 7, $MgCl_2$. Phosphate concentration is 5.44 mM. Medium *B* (in mM): 50, sucrose; 50, KCl; 0.4, EDTA; 50, Tris-HCl (pH 7.7); and 5, $MgCl_2$. Phosphate is 6.61 mM. Medium *C* (in mM): 40, sucrose; 40, KCl; 50, Tris-HCl (pH 7.5); 0.4, EDTA; and 10 $MgCl_2$. Phosphate is 4.76 mM.

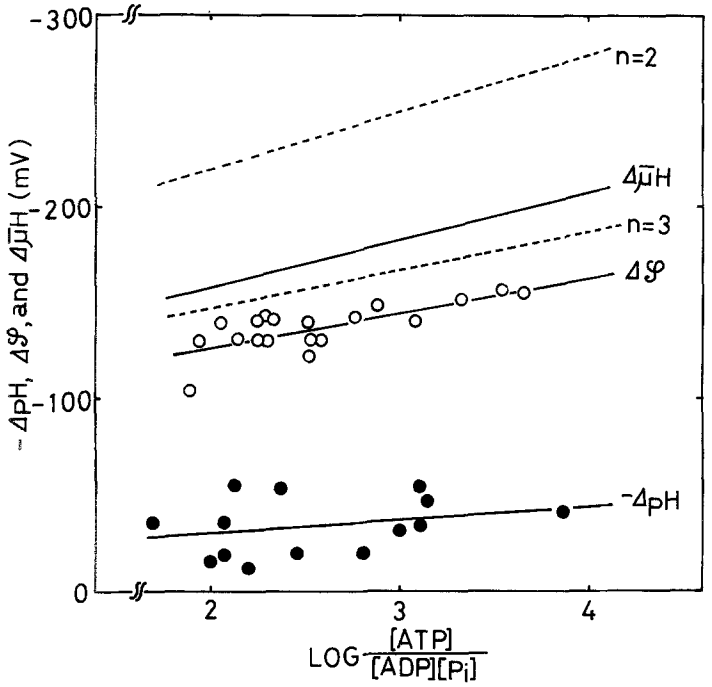

Fig. 4. The relationship of [ATP]/[ADP][P_i] to $\Delta\varphi$, ΔpH and $\Delta\bar{\mu}_H$ produced by hydrolysis of ATP under the condition that the respiratory chain is arrested. ○, $\Delta\varphi$; ●, $-\Delta$pH. The solid lines denoted by $\Delta\varphi$ and by ΔpH represent the line calculated with the least-squares method with those data, and are expressed as Eqs. (8) and (9). The solid line denoted by $\Delta\bar{\mu}_H$ is obtained by adding Eqs. (8) and (9). The dotted lines express the theoretical relationship between $\Delta\bar{\mu}_H$ and ΔG_p assuming that $n=2$ or $n=3$. Incubation medium contained: 55 mM sucrose, 40 mM KCl, 50 mM Tris-HCl (pH 7.5), 0.4 mM EDTA, 5 mM $MgCl_2$, 5 μM rotenone, and 1 μM antimycin A. TPP^+ concentration was 3×10^{-5} M

$\Delta\bar{\mu}_H$ can be calculated and the ratio of ΔG_p to $\Delta\bar{\mu}_H$, defined as n, is around 2.7 which is approximately equal to 3.

Next, we examine the ratio of ΔG_p to $\Delta\bar{\mu}_H$ produced by hydrolysis of ATP under the condition that the respiratory chain is arrested. In Fig. 4, the membrane potential (○) and ΔpH (●) are plotted against log[ATP]/[ADP][P_i]. The solid lines denoted by $\Delta\varphi$ and by ΔpH represent the line calculated by the least-squares method with these data, and are given by the following equations:

$$\Delta\varphi(\mathrm{mV}) = -92.5 - 17.4 \log \frac{[\mathrm{ATP}]}{[\mathrm{ADP}][\mathrm{P_i}]}, \tag{8}$$

$$\Delta\mathrm{pH}(\mathrm{mV}) = 16.4 + 7.2 \log \frac{[\mathrm{ATP}]}{[\mathrm{ADP}][\mathrm{P_i}]}. \tag{9}$$

Therefore, addition of Eqs. (8) and (9) yields the relation of $\Delta\bar{\mu}_H$ to [ATP]/[ADP][P_i] such as Eq. (10)

$$\Delta\bar{\mu}_H(\text{mV}) = -108.9 - 24.6 \log \frac{[\text{ATP}]}{[\text{ADP}][\text{P}_i]}. \tag{10}$$

Equation (10) is drawn by the solid line denoted by $\Delta\bar{\mu}_H$ in Fig. 4. The dotted lines express the theoretical relationship between $\Delta\bar{\mu}_H$ and ΔG_p assuming that $n=2$ or 3. The data shown in this figure indicate that the value of n is approximately 2.7 which is close to 3, as is obtained in the case of state 4 attained in the presence of oxidizable substrates. From the results obtained, it is concluded that the ratio of ΔG_p to $\Delta\bar{\mu}_H$, n, is about 3 for intact mitochondria energized by oxidation of substrates and by the hydrolysis of ATP. According to Mitchell's chemiosmotic theory, 2 protons are translocated per ATP synthesis and transported to the extramitochondrial space. The finding obtained in the present study is not consistent with this theory. A similar conclusion was obtained by several investigators [3, 33, 35, 40, 48].

It has been reported that ATP/ADP translocator is electrogenic: ADP is transported through the membrane with a different charge from ATP, e.g., ADP^{3-} against ATP^{4-} [19]. Then, the relationship of ATP/ADP between intra- and extramitochondrion can be expressed as Eq. (11)

$$2.3\,RT \log \left(\frac{[\text{ATP}]_e}{[\text{ADP}]_e} \Big/ \frac{[\text{ATP}]_i}{[\text{ADP}]_i} \right) = -F\Delta\varphi \tag{11}$$

where subscript e and i stand for the quantity of external and of internal mitochondrion. It is noted that Mg−ATP and Mg−ADP are not transported *via* the translocator [20], and then, Eq. (11) is valid under the assumption that Mg concentration is the same between intra- and extramitochondrion. The phosphate ($H_2PO_4^-$) is transported *via* phosphate/H^+ symport, and Eq. (12) holds

$$\frac{[\text{P}_i]_e}{[\text{P}_i]_i} = \frac{[\text{H}^+]_i}{[\text{H}^+]_e}. \tag{12}$$

Addition of Eqs. (11) and (12) leads to the relation

$$2.3\,RT \log \frac{[\text{ATP}]_e}{[\text{ADP}]_e[\text{P}_i]_e} = 2.3\,RT \log \frac{[\text{ATP}]_i}{[\text{ADP}]_i[\text{P}_i]_i} - \Delta\bar{\mu}_H. \tag{13}$$

Assuming that the standard free energy of phosphorylation, ΔG_p^0, in both intra- and extramitochondrion are the same, Eq. (13) describes that the difference between intra- and extramitochondrial phosphorylation potential is equal to the proton chemical potential difference across the membrane, namely,

$$\Delta G_p(\text{out}) = \Delta G_p(\text{in}) - \Delta \bar{\mu}_H. \tag{14}$$

It is noted that $\Delta \bar{\mu}_H$ is a negative value. Several authors [9, 10, 15, 43] have pointed out that extramitochondrial phosphorylation potentials (G_p(out)) are larger than the intramitochondrial phosphorylation potentials (G_p(in)). Since $\Delta \mu_H$ arisen between mitochondrial inner-membrane is found to be about 200 mV, Eq. (14) indicates the difference between ΔG_p(out) and ΔG_p(in) is about 200 mV in mV scale, which is equivalent to 4.6 kcal/mol. This value is in good agreement with those reported by Slater *et al.* [43]. Davis and Lumeng [9, 10] and Heldt *et al.* [15], however, reported a smaller value, i.e., 2.3–2.8 kcal/mol.

In the presence of atractyloside, inhibitor of ATP/ADP translocator, the membrane potential of mitochondria does not change even when the ATP level in the medium is reduced by adding glucose and hexokinase (*see* Fig. 2*B*). This finding suggests that the membrane potential and pH difference are equilibrated with the intramitochondrial phosphorylation potentials rather than the extramitochondrial phosphorylation potentials, as is quite reasonable from the morphology of ATPase of mitochondria. The stoichiometry of H^+ translocated per ATP hydrolyzed has been investigated for rat liver mitochondria [26], bovine heart submitochondria [46], and reconstituted vesicles incorporated purified ATPase [44]. For all systems, the value of 2 has been reported; therefore, we obtain that

$$\Delta G_p(\text{in}) = -2\Delta \bar{\mu}_H \tag{15}$$

although Alexandre *et al.* [2] reported that H^+/ATP is 3 for rat liver mitochondria. Berry and Hinkle [6] reported that the ratio of $\Delta \bar{\mu}_H$ to ΔG_p is 2 for submitochondrial particles. It is noted that the phosphorylation potential measured for submitochondrial particles corresponds to ΔG_p(in) for mitochondria. But, this value is controvertible: Sorgato *et al.* estimated 3 of $\Delta G_p/\bar{\mu}_H$ for submitochondria [45]. Introduction of Eq. (15) into Eq. (14) yields the relationship between ΔG(out) and $\Delta \bar{\mu}_H$, i.e.

$$G_p(\text{out}) = -3\Delta \bar{\mu}_H. \tag{16}$$

Equation (16) is consistent with the present experimental results.

Davis *et al.* [10] have suggested that the slow respiration at state 4 is required to maintain ΔG_p(out) higher than ΔG_p(in). The efflux of ATP from mitochondria caused by addition of hexokinase and glucose in the presence of oligomycin to inhibit the ATPase evoked the slight increase of respiration (N. Kamo and M. Murastugu, *unpublished data*). These facts indicate that the expulsion of ATP from mitochondria requires the energy.

Lehninger *et al.* [e.g., 7] re-examined the stoichiometry of H^+ ejection during mitochondrial electron transport (the H^+/site ratio) and have concluded that the previous value obtained by Moyle and Mitchell [28] is underestimated. Based on this finding and the measurement of H^+/ATP ratio, Lehninger and his coworkers [47] have proposed a modified chemiosmotic theory in which $4\,H^+$ are ejected per pair of electrons traversing each conserving site of the respiratory chain. Direct tests of the $H^+/2e^-$ ratio for sites 2 and 3 were carried out by Alexandre, Reynafarje and Lehninger [2], who confirmed the modified chemiosmotic theory. According to this theory, the ratio of ΔG_p(out) to $\Delta\bar{\mu}_H$ should be near 4. Moyle and Mitchell [29], however, have opposed this interpretation and insisted that $2\,H^+$ are ejected per energy-conserving site of the respiratory chain. Further study should be necessary.

References

1. Akerman, K.E.O., Wikctrom, M.K.F. 1976. Safranine as a probe of the mitochondrial membrane potential. *FEBS Lett.* **68**:191
2. Alexandre, A., Reynafarje, B., Lehninger, A.L. 1978. Stoichiometry of vectrial H^+ movements coupled to electron transport and to ATP synthesis in mitochondria. *Proc. Nat. Acad. Sci. USA* **75**:5296
3. Azzone, C.F., Pozzan, T., Massari, S. 1978. Proton electrochemical gradient and phosphate potential in mitochondria. *Biochim. Biophys. Acta* **501**:307
4. Bakeeva, L.E., Grinius, L.L., Jasaitis, A.A., Kuliene, V.V., Levitsky, D.O., Liberman, E.A., Severina, I.I., Skulachev, V.P. 1970. Conversion of bio-membrane produced energy into electric form. II. Intact mitochondria. *Biochim. Biophys. Acta* **216**:13
5. Bergmeyer, J.U., 1971. Methods of Enzyme Analysis. Academic Press, New York
6. Berry, E.A., Hinkle, P.C. 1978. Estimation of electrochemical proton gradient in submitochondrial particles. *Fed. Proc.* **37**:1753
7. Brand, M.D., Lehninger, A.L. 1977. H^+/ATP ratio during ATP hydrolysis by mitochondria: Modification of the chemiosmotic theory. *Proc. Nat. Acad. Sci. USA* **74**:1955
8. Chance, B., Williams, G.R. 1955. A method for the localization of sites for oxidative phosphorylation. *Nature (London)* **176**:250
9. Davis, E.J., Lumeng, L.L. 1975. Relationships between the phosphorylation potentials generated by liver mitochondria and respiratory state under conditions of adenosine diphosphate control. *J. Biol. Chem.* **250**:2275

10. Davis, E.J., Lumeng, L., Boffoms, D. 1974. On the relationships between the stoichiometry of oxidative phosphorylation and the phosphorylation potential of rat liver mitochondria as functions of respiratory state. *FEBS Lett.* **39**:9
11. Fiske, C.H., Subbarow, Y. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**:375
12. Griniuviene, B., Chmieliauskaite, V., Grinius, L. 1974. Energy-linked transport of permeant ions in *Escherichia coli* cells: Evidence for membrane potential generation by proton-pump. *Biochem. Biophys. Res. Commun.* **56**:206
13. Hackenbrock, C.R. 1966. Ultrastructual basis for metabolically linked mechanical activity in mitochondria. I. Reversible ultrastructual changes with change in metabolic steady state in isolated liver mitochondria. *J. cell Biol.* **30**:269
14. Harold, F.M., Papineau, D. 1972. Cation transport and electrogenesis by *Streptococcus faecalis*. I. The membrane potential. *J. Membrane Biol.* **8**:27
15. Heldt, H.W., Klingenberg, M., Milovancev, M. 1972. Differences between the ATP/ADP ratios in the mitochondrial matrix and in the extramitochondrial space. *Eur. J. Biochem.* **30**:434
16. Hirata, H., Altendorf, K., Harold, F.M. 1973. Role of an electrical potential in the coupling of metabolic energy to active transport by membrane vesicles of *Escherichia coli*. *Proc. Nat. Acad. Sci. USA* **70**:1804
17. Johnson, F.M., Eyring, H., Polissar, M.J. 1954. The Kinetic Basis of Molecular Biology. p. 545. John Wiley & Sons, New York
18. Kamo, N., Muratsugu, M., Kurihara, K., Kobatake, Y. 1976. Change in surface charge density and membrane potential of intact mitochondria during energization. *FEBS Lett.* **72**:247
19. Klingenberg, M., Rottenberg, H. 1977. Relation between the gradient of the ATP/ADP ratio and the membrane potential across the mitochondrial membrane. *Eur. J. Biochem.* **73**:125
20. Klingerberg, M. 1976. The ADP-ATP carrier in mitochondrial membranes. *In:* The Enzymes of Biological Membrane. Vol. 3. Membrane Transport. A. Martonosi, editor. Plenum, New York
21. Komor, E., Tanner, W. 1976. The determination of the membrane potential of *Chlorella vulgaris*. *Eur. J. Biochem.* **70**:197
22. Lakshminarayanaiah, N. 1976. Membrane Electrode. p. 86. Academic, New York
23. Laris, P., Bahr, D.P., Chaffee, R.R.J. 1975. Membrane potentials in mitochondrial preparations as measured by means of a cyanine dye. *Biochim. Biophys. Acta* **376**:415
24. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265
25. Mitchell, P. 1966. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. *Biol. Rev.* **41**:445
26. Mitchell, P., Moyle, J. 1968. Proton translocation coupled to ATP hydrolysis in rat liver mitochondria. *Eur. J. Biochem.* **4**:530
27. Mitchell, P., Moyle, J. 1969. Estimation of membrane potential and pH difference across the criste membrane of rat liver mitochondria. *Eur. J. Biochem.* **7**:471
28. Mitchell, P., Moyle, J. 1967. Respiration-driven proton translocation in rat liver mitochondria. *Biochem. J.* **105**:1147
29. Moyle, J., Mitchell, P. 1978. Measurements of mitochondrial H^+/O quotients: Effects of phosphate and N-ethylmaleimide. *FEBS Lett.* **90**:361
30. Muraoka, S., Slater, E.C. 1969. The redox states of respiratory-chain components in rat-liver mitochondria. *Biochim. Biophys. Acta* **180**:221
31. Muratsugu, M., Kamo, N., Kurihara, K., Kobatake, Y. 1977. Selective electrode for dibenzyldimethyl ammonium cation as indicator of the membrane potential in biological system. *Biochim. Biophys. Acta* **464**:613

32. Nicholls, D.G. 1974. The influence of respiration and ATP hydrolysis on the proton-electrochemical gradient across the inner membrane of rat-liver mitochondria as determined by ion distribution. *Eur. J. Biochem.* **50**:305
33. Nicholls, D.G., Berson, V.S.M. 1977. Inter-relationships between proton electrochemical gradient, adenine-nucleotide phosphorylation potential and respiration, during substrate-level and oxidative phosphorylation by mitochondria from brown adipose tissue of cold-adapted guinea-pigs. *Eur. J. Biochem.* **75**:601
34. Padan, E., Rottenberg, H. 1973. Respiratory control and proton electro-chemical gradient in mitochondria. *Eur. J. Biochem.* **40**:413
35. Papa, S. 1976. Proton translocation reactions in the respiratory chains. *Biochim. Biophys. Acta* **456**:39
36. Quagliariello, E., Genchi, G., Falmieri, F. 1971. Respiration-dependent anion uptake by rat liver mitochondria. *FEBS Lett.* **13**:253
37. Ramos, S., Schuldiner, S., Kaback, H.R. 1976. The electrochemical gradient of protons and its relationship to active transport in *Escherichia coli* membrane vesicles. *Proc. Nat. Acad. Sci. USA* **73**:1892
38. Rosing, J., Slater, E.C. 1972. The value of ΔG^o for hydrolysis of ATP. *Biochim. Biophys. Acta* **267**:257
39. Rottenberg, H. 1973. The mechanism of energy-dependent ion transport in mitochondria. *J. Membrane Biol.* **11**:117
40. Rottenberg, H. 1975. The measurement of transmembrane electrochemical proton gradients. *J. Bioenerg.* **7**:61
41. Shinbo, T., Kamo, N., Kurihara, K., Kobatake, Y. 1978. A PVC-based electrode sensitive to DDA^+ as a device for monitoring the membrane potential in biological systems. *Archiv. Biochem. Biophys.* **187**:414
42. Skulachev, V.P. 1975. Energy coupling in biological membranes: Current state and perspectives. *In:* Energy Tranducing Mechanisms. Biochemistry Series One. Vol. 3, pp. 31–73. E. Racker, editor. Butterworths, London
43. Slater, E.C., Rosing, J., Mol, A. 1973. The phosphorylation potential generated by respiring mitochondria. *Biochim. Biophys. Acta* **292**:534
44. Sone, N., Yoshida, M., Hirata, H., Okamoto, H., and Kagawa, Y. 1976. Electrochemical potential of protons in vesicles reconstituted from purified, proton-translocation adenosine triphosphatase. *J. Membrane Biol.* **30**:12
45. Sorgato, M.C., Ferguson, S.J., Kell, D.B., John, P. 1978. The protonmotine force in bovine heart submitochondrial particles. *Biochem. J.* **174**:237
46. Thayer, W., Hinkle, P.C. 1973. Stoichiometry of adenosine triphosphate-driven proton translocation in bovine heart submitochondrial particles. *J. Biol. Chem.* **248**:5395
47. Vercesi, A., Reynafarje, B., Lehninger, A.L. 1978. Stoichiometry of H^+ ejection and Ca^{2+} uptake coupled to electron transport in rat heart mitochondria. *J. Biol. Chem.* **253**:6379
48. Wiechmann, A.H.C.A., Beem, E.P., Dam, K. van 1975. The relation between H^+ translocation and ATP synthesis in mitochondria. *In:* Electron Transfer Chains and Oxidative Phosphorylation. E. Equadliariello, S. Papa, F. Palmieri, E.C. Slater and N. Siliprandi, editors. pp. 335–342. North-Holland, Amsterdam