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Correlation of the turnover number of the ATP synthase in liposomes with the proton flux and the proton potential across the membrane

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The fluorescent indicator pyranine was used for recording the internal pH of liposomes. The proton permeability was deduced from the velocity of the internal pH increase which was caused by shifting the external pH from 7 to 9. From valinomycin titration of the proton permeability in the presence of internal and external KCl (0.1 M), the permeability coefficient of H^+ (P_H) was obtained as 10^{-4} cm/s at 22°C. The coefficient was twice this value with the ATP synthase isolated from *Wolinella succinogenes* present in the liposomal membrane (10 mg protein/g phospholipid). ADP and phosphate had no effect on the latter P_H. The protonophore TTFB (5 μmol/g phospholipid) increased the P_H by 3 orders of magnitude. The permeability coefficients of H^+ and K^+ were used for calculating the $\Delta\tilde{\mu}_H$ and the proton flux associated with the phosphorylation which was driven by gradients of H^+ and K^+. For the conditions of limiting permeability of K^+, the following conclusions were drawn. (1) In the steady state of rapid ion flux, the electrical potential across the liposomal membrane as calculated according to the Goldman equation, is directed opposite to the corresponding Nernst potential which is calculated from the K^+ gradient. (2) The maximum turnover numbers of phosphorylation require a $\Delta\tilde{\mu}_H$ of 200–220 mV across the liposomal membrane. These values of $\Delta\tilde{\mu}_H$ and the corresponding turnover numbers are close to those brought about by the bacterial electron transport and the coupled phosphorylation. (3) The velocity of phosphorylation is linearly related to the proton flux. The slope of the line can be explained on the basis of an H^+/ATP ratio of approx. 3.

Glossary

Symbol	Specification	Unit
B	buffering capacity	mol H^+/g phospholipid
C_H, C_K	concentration of H^+, K^+	mol/l
C^i, C^a	internal, external concentrations	mol/l
dpH^i/dt	velocity of internal pH change	s^{-1}

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Abbreviations: TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazol; Mes, 4-Morpholinoethanesulphonic acid; pyranine, 8-hydroxy-1,3,6-pyrenetrisulphonic acid.

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J_H	proton flux across liposomal membrane	mol $H^+ \cdot s^{-1} \cdot cm^{-2}$
k	F/RT	39 V^{-1}
O	specific surface of bilayer	$2.6 \cdot 10^6$ cm^2/g phospholipid
P_H	permeability coefficient of H^+	cm/s
P_H^{ap}	apparent permeability coefficient of H^+	cm/s
P_K	permeability coefficient of K^+	cm/s
V_i	internal volume of liposomes	ml/g phospholipid
$\Delta\psi$	Goldman potential (Eqn. 4)	mV
$\Delta\psi_N$	Nernst potential	mV
$\Delta\tilde{\mu}_H$	electrochemical proton potential across a membrane	mV.

Introduction

Liposomes containing the ATP synthase isolated from *Wolinella succinogenes* [1] were earlier found to catalyze ATP synthesis from ADP and phosphate [2]. The reaction was driven by gradients of H^+ and K^+ across the liposomal membrane in the presence of valinomycin. The turnover number was commensurable with that of the enzyme in the bacterial membrane, when the sum of the potentials, as calculated from the gradients according to the Nernst equation, was 280 mV. With the Nernst potential below 150 mV, the turnover number was more than an order of magnitude smaller. A similar relation of the turnover number to the potential across the membrane was observed with other ATP synthases both in liposomes or in their original membrane [3–7]. The reason for this relation has been investigated in the work presented.

The electron transport activity of cells and inverted vesicles of *W. succinogenes* was found to generate a $\Delta\tilde{\mu}_H$ of approx. 180 mV [8]. Thus the driving force of phosphorylation required with the liposomes seemed to exceed that necessary with the bacterial membrane by about 100 mV. To investigate this discrepancy, phosphorylation velocity and $\Delta\tilde{\mu}_H$ should be simultaneously measured with the liposomes. However, these measurements would be extremely difficult. The velocity of phosphorylation was found to decrease already after 5 s [2], while equilibration of the indicators of $\Delta\tilde{\mu}_H$ across the membrane would require distinctly greater time periods. Therefore, it was attempted to measure the permeability coefficients of H^+ and K^+. These coefficients were used to calculate the $\Delta\tilde{\mu}_H$ and the proton flux across the membrane.

Methods

Preparation of liposomes. Liposomes were prepared essentially as described earlier [2]. Soybean phospholipid (0.1 g, Sigma, no. P 5638) was sonicated for 10 min at 0 °C in 2.5 ml of the incubation medium (0.1 M Mes/0.1 M glycylglycine/50 mM NaH_2PO_4/0.1 M KCl (pH 7.0)) containing 0.1 mM pyranine. Sonication was done using the Branson sonifier equipped with the microtip, at 40 W and 50% duty cycle. The suspension was frozen in liquid N_2 and then thawed at room temperature. This freeze-thawing procedure was repeated twice. For incorporation of the ATP synthase isolated from *W. succinogenes* [1], the enzyme (10 mg protein/g phospholipid) was added to the sonicated suspension and the mixture was freeze-thawed as described above.

The suspension was kept at 0 °C for at least 2 h, before the free external pyranine was separated by passing the liposomal suspension (0.2 ml) through a 1-ml column of Sephadex G-25 at room temperature. Equilibration and elution was done with the incubation medium.

Valinomycin and TTFB were applied in dimethylsulfoxide solution.

Recording of pyranine fluorescence. The liposomes were suspended (1 g phospholipid/l) at 22 °C in the incubation medium. The Eppendorf fluorometer was used with 1 cm cuvettes. The excitation wavelength was 436 nm. The fluorescent light was passed through a 470 nm cut-off glass filter.

The proportion of pyranine bound to the external liposomal surface (α) was evaluated from the very fast fluorescence signal which was caused by a shift of the external pH to 9 in the absence of ionophores. From this signal the fluorescence of the total external pyranine was calculated using the buffer equation with p$K = 7.2$. α is represented by the ratio of this fluorescence and $Fl_{\max}$ (see Fig. 1 and legend of Fig. 2).

Phospholipid. Phospholipid was determined as inorganic phosphate after ashing the liposomes [9]. The average molecular weight of the phospholipids was assumed as 800.

The specific surface of the liposomal membrane is $O = 2.6 \cdot 10^6$ cm^2/g phospholipid. This value corresponds to a surface requirement of a single phospholipid molecule of 0.7 nm^2.

Internal volume of the liposomes. The liposomes were prepared as described above. However, the external pyranine and phosphate was replaced by NaCl using a 1-ml column of Dowex 1-X8 (50–100 mesh). The internal volumes were calculated from the liposomal contents of inorganic phosphate or pyranine, using the corresponding concentrations in the liposomal preparation buffer (incubation medium). Inorganic phosphate [9] was determined after lysis of the liposomes with 1% dodecylsulfate. The pyranine content was calculated from the fluorescence of the liposomal suspension with the external pH > 10, using the fluorescence of a known amount of pyranine.

Buffering capacity of the liposomes at pH 7–9. The buffering capacity of the phospholipids was measured by pH-titration of a suspension of intact liposomes in 0.1 M KCl. The external pH was recorded as a function of the amount of KOH added using a glass electrode. The capacity of the internal buffer solutes was calculated in steps of 0.1 pH units according to the buffer equation using the concentrations and pK values of the individual buffer solutes in the liposomal preparation buffer (incubation medium) and the internal volume.

Results

Liposomes were prepared from soybean phospholipids using sonication. The suspension was freeze-thawed with or without ATP synthase (10 mg protein/g phospholipid) present. Freeze thawing caused enlargement of the liposomes and complete incorporation of the enzyme. For determining the internal buffering capacity of the liposomes their average internal volume had to be measured.

Internal volume

The liposomes were prepared in a buffer containing 50 mM phosphate. After the external phosphate was removed by means of an anion-exchange column, the average internal volume of the liposomes was determined from the phosphate content. From the average internal volume (1.63 ml/g phospholipid), the corresponding inner diameter was calculated as 38 nm. The calculation was done assuming globular shape and unilamellar structure of the liposomes. Electron microscopic inspection of the preparation after negative staining confirmed the unilamellar structure of the liposomes and indicated that their diameter ranged between 20 and 150 nm. Incorporation of the ATP synthase did not alter the size of the liposomes.

Buffering capacity of the liposomes

For evaluating proton permeability coefficients from internal pH changes, the internal buffering capacity of the liposomes had to be known. Buffering was caused by the phospholipids and the buffer solutes present within the liposomes. The buffering capacity of the phospholipids was determined by pH titration. The average slope of the titration curve between pH 7 and 9 was 55 μmol H$^+$/g phospholipid per pH unit. The buffering capacity so obtained referred to external surface of the phospholipid bilayer. The buffering capacity of the internal phospholipid was assumed to be the same. The capacity of the buffer solutes within the liposomes was calculated to be 105 μmol H$^+$/g phospholipid per pH unit. The total internal buffering capacity used with Eqn. 3 ($B =$ 160 μmol H$^+$/g phospholipid) represents the sum of the two values.

Status of the liposomal pyranine

The pyranine associated with the liposomes was present in two different states [10,11]. This was concluded from the fluorescence response of liposomal suspensions that was caused by a sudden increase of the external pH (addition of 60 mM NaOH in the experiment of Fig. 1: see curve II and III). The smaller proportion responded very fast even in the absence of the protonophore. The response of the residual part was slow, but was considerably accelerated by a protonophore, valinomycin (see Fig. 1) or Triton X-100. The

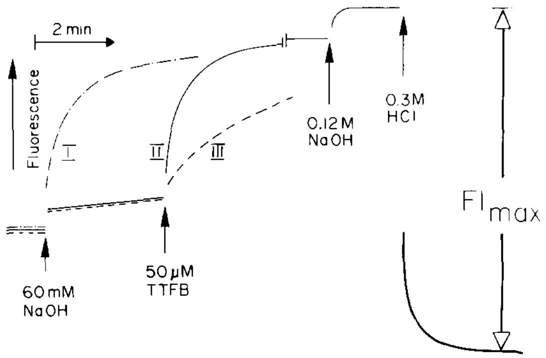

Fig. 1. Recording of the pyranine fluorescence of a liposomal suspension after increasing the external pH. Liposomes containing 25000 (curve I, ·–·–·), 50 (curve II, ———) or 5 nmol valinomycin/g phospholipid (curve III, — — —) were suspended (1 g phospholipid/l) in the incubation medium. The first (60 mM) and second addition of NaOH (0.12 M) brought the external pH to 8.7 and to more than 10 respectively. The subsequent addition of HCl caused the external pH to drop below pH 5. The corresponding minimum fluorescence signal was used as the basis for calculating the internal pH according to the equation given in the legend of Fig. 2.

proportion of the fast responding pyranine could not be decreased by repeated filtration of the liposomes through an anion exchanger or Sephadex gel which was found to bind free pyranine. It was constant for at least 24 h, provided that the filtration was done 2 h after freeze-thawing or later. These findings suggested that the fast responding pyranine was bound to the external surface of the liposomes, while the residual part was separated from the external medium by the liposomal membrane. From the fast responding fluorescence signal the amount of externally bound pyranine was determined as 7.5%. Titration of the bound species gave a p*K* which was close to that of the internal pyranine (7.2). The p*K* of free pyranine was measured as 7.3. The amount of pyranine which was bound to the membrane was estimated by comparing the contents of pyranine and phosphate of the liposomes after removal of the external anions. It was found that the pyranine content exceeded that of phosphate by approx. 4%. This results supported the view that less than 10% of the total pyranine is bound to the liposomal membrane.

Clement and Gould [11] suggested that the very fast responding part of the fluorescence was also caused by the internal aqueous pyranine. They discussed that the fast phase was due to unlimited proton flux, which was subsequently inhibited by a Goldman potential caused by counterflux limitation. This situation did not apply under the conditions used here. From the capacitance of the lipid bilayer (1 $\mu F/cm^2$) [12]) it was calculated that 1 pmol H^+/cm^2 had to pass through the membrane for establishing an inhibiting potential of 100 mV. As the internal buffering capacity ($B/O = 62$ pmol H^+/cm^2) was nearly 2 orders of magnitude greater, the corresponding fluorescence response of the internal pyranine would be negligible.

Permeability coefficient of protons

In the experiment illustrated in Fig. 1, the fluorescence of three liposomal suspensions that contained different amounts of valinomycin was recorded. The internal and external pH was 7.0. The addition of 60 mM NaOH caused the external pH to rise to 8.7. After the very fast phase of fluorescence increase which was due to the externally bound pyranine and was not resolved kinetically, the response of the internal pyranine was recorded. The fluorescence signal increased rapidly with the largest amount of valinomycin (25 μmol/g phospholipid) present. With the two smaller amounts of valinomycin, a slow rate of fluorescence increase was observed until the protonophore TTFB was added. The second addition of NaOH raised the pH above 10 and the subsequent addition of HCl caused the pH to drop below 5. The corresponding difference in fluorescence intensity (Fl_{max}) was used as a reference for evaluating the internal pH.

For determining the permeability coefficient of protons (P_H) according to Eqn. 1, proton fluxes, J_H, had to be calculated from the velocities

$$J_H = P_H(C_H^i - C_H^a) \qquad (1)$$

of the fluorescence changes. This was done using the velocity of the internal pH change (dpH^i/dt), the internal buffering capacity (B) and the specific surface of the phospholipid bilayer (O) according to Eqn. 2.

$$J_H = \frac{dpH^i}{dt}\frac{B}{O} \qquad (2)$$

Combination of Eqns. 1 and 2 gave Eqn. 3, where P_H was replaced by the apparent

$$P_H^{ap} = \frac{B\frac{dpH^i}{dt}}{O(C_H^i - C_H^a)} \tag{3}$$

permeability coefficient (P_H^{ap}). P_H^{ap} would apply, if the proton flux was not only a function of the concentration gradient of protons, but was also influenced by an electrical potential ($\Delta\psi$) across the membrane. The occurrence of a $\Delta\psi$ in experiments such as shown in Fig. 1 could not be excluded in the absence of further information.

Fig. 2A was obtained by conversion of the fluorescence signals of Fig. 1 to values of internal pH (pH^i) (see legend of Fig. 2). This conversion implied correction for the fluorescence signals caused by the external pyranine. From the time course of pH^i, P_H^{ap} was evaluated (Fig. 2B) using the points drawn in Fig. 2A. The various values of P_H^{ap} so obtained (Fig. 2B) were nearly invariant with time and were valid throughout the pH range used (pH 7–9). In the absence of TTFB, P_H^{ap} was nearly the same both with 5 and 50 nmol valinomycin/g phospholipid. This suggested that the proton flux was not limited by the counterflux of K^+ under these conditions. In a separate experiment (not shown) it was found that the proton flux was not limited by the concentration of K^+ used. (0.1 M).

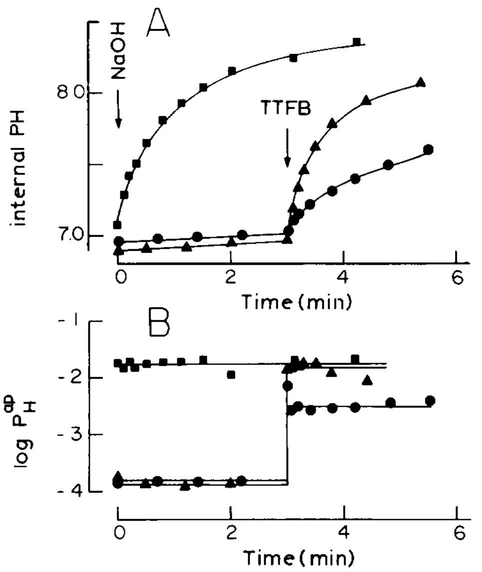

Fig. 2. (A). Time-course of the internal pH of the liposomal suspension after an increase of the external pH. Evaluation of the experiment of Fig. 1 (■, curve I; ▲, curve II; ●, curve III). The internal pH (pH^i) was calculated from the corresponding fluorescence signal (Fl) and Fl_{max} (see Fig. 1) according to the following equation:

$$pH^i = pK - \log\left\{\frac{(1-\alpha)Fl_{max}}{Fl - \frac{\alpha Fl_{max}}{1+10^{pK-pH^a}}} - 1\right\}$$

The pK (7.2) refers to the liposomal pyranine. α (0.075) designates the fraction of the externally bound pyranine (see Methods section). pH^a designates the external pH. (B) The apparent permeability coefficients of H^+ (P_H^{ap}) were calculated from the velocities of pH^i increase at the points indicates, according to Eqn. 3 with the $B = 160$ µmol H^+/g phospholipid.

Upon the addition of 5 µmol TTFB/g phospholipid, P_H^{ap} increased by 2 orders of magnitude with 50 and much less with 5 nmol valinomycin/g phospholipid present. This indicated that the proton flux was limited by the counterflux of K^+ under these conditions. Values of P_H^{ap} similar to those with TTFB present were measured in the absence of TTFB with a very large amount of valinomycin (25 µmol/g phospholipid) present.

In experiments similar to those given in Fig. 1 and 2, P_H^{ap} was determined as a function of the amount of valinomycin, in the absence of TTFB

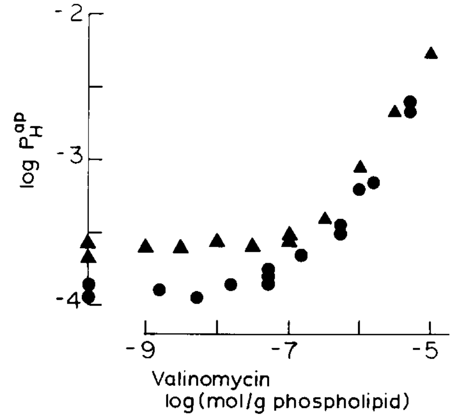

Fig. 3. P_H^{ap} as a function of valinomycin concentration in the absence of TTFB. P_H^{ap} was calculated according to Eqn. 3. dpH^i/dt was determined as described in Fig. 2. The dots (●) were obtained with liposomes lacking protein and the triangles (▲) with liposomes containing the ATP synthase isolated from *W. succinogenes* (10 mg protein/g phospholipid). The data given on the ordinate were measured without valinomycin.

(Fig. 3). P_H^{ap} was 10^{-4} cm/s without valinomycin and did not significantly increase with amounts of valinomycin up to 0.1 μmol/g phospholipid. This indiated that the proton flux was apparently not limited by the counterflux of K^+. Therefore P_H^{ap} was equal to the true P_H of the liposomal membrane under these conditions. With larger amounts of valinomycin, P_H^{ap} was found to increase. This was probably due to a direct unspecific effect of valinomycin on P_H. Similar results were obtained with liposomes containing the ATP synthase isolated from *W. succinogenes*. However, the P_H was $2 \cdot 10^{-4}$ cm/s in this case. The presence of ADP and phosphate had no effect on this P_H.

The P_H of the phospholipid bilayer determined in this work (10^{-4} cm/s) is in good agreement with the 'proton-hydroxide permeability coefficient' published by the groups of Nichols [12] and Deamer [13]. It should be mentioned that proton permeability coefficients ranging between 10^{-9} cm/s and 3 cm/s have been reported by others [14–16].

In the experiment of Fig. 4, the P_H^{ap} in the presence of TTFB was titrated with valinomycin. The P_H^{ap} increased with increasing amounts of valinomycin until a slight saturation was observed with about 1 μmol valinomycin/g phospholipid where P_H^{ap} was 0.1 cm/s. This value was regarded to be the true P_H in the presence of TTFB. Greater amounts of valinomycin caused the P_H^{ap} to increase further, probably by unspecifically increasing P_H.

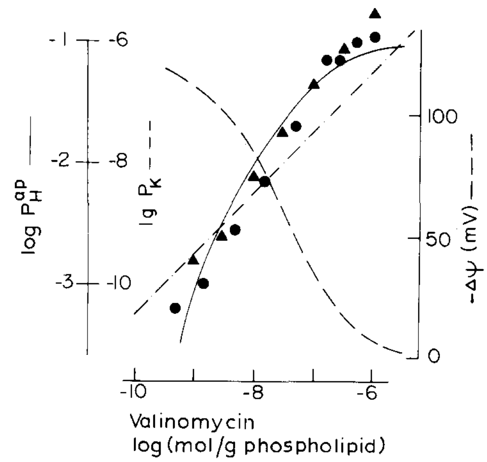

Fig. 4. P_H^{ap} as a function of valinomycin concentration in the presence to TTFB (5 μmol/g phospholipid). P_H^{ap} (● and ▲) was determined as shown in Fig. 2. The dots (●) were obtained with liposomes lacking protein and the triangles (▲) with liposomes containing the ATP synthase isolated from *W. succinogenes* (10 mg protein/g phospholipid). The solid line (P_H^{ap}) was calculated according to Eqn. 6 with $P_H = 0.1$ cm/s. The dashed line ($\Delta\psi$, - - - - - -) was calculated according to Eqn. 4 with the values of P_K given by the dotted line (·–·–·–·) which was calculated from the titration curve given in Ref. 17.

The increase of P_H^{ap} with the amount of valinomycin suggests that proton flux is limited by the counterflux of K^+ in the presence of TTFB. This limitation should create a $\Delta\psi$ which is given by the Goldman equation (Eqn. 4).

$$\Delta\psi = \frac{1}{k} \ln \frac{C_H^a P_H + C_K^a P_K}{C_H^i P_H + C_K^i P_K} \tag{4}$$

The $\Delta\psi$ is calculated from the valinomycin titration curve of P_K [17] using $P_H = 0.1$ cm/s. Both the curves for P_K and $\Delta\psi$ are given in Fig. 4. With values of $P_K < 10^{-10}$ cm/s, $\Delta\psi$ (-120 mV) is determined by the internal and external H^+ concentrations, and J_H vanishes because the concentration gradient of H^+ is balanced by $\Delta\psi$. With values of $P_K > 10^{-6}$ cm/s, $\Delta\psi$ vanishes and J_H is no longer limited by the counterflux of K^+. The effect of $\Delta\psi$ on J_H should be determined by the Nernst-Planck equation (Eqn. 5 [18]). To test this hypothesis

$$J_H = \frac{P_H k\, \Delta\psi \left(C_H^i\, e^{k\Delta\psi} - C_H^a\right)}{e^{k\Delta\psi} - 1} \tag{5}$$

the theoretical titration curve of P_H^{ap} was calculated from the titration curve of $\Delta\psi$ using Eqn. 6 which was obtained from Eqns. 5 and 1 after

$$P_H^{ap} = \frac{P_H k\, \Delta\psi \left(C_H^i\, e^{k\Delta\psi} - C_H^a\right)}{\left(e^{k\Delta\psi} - 1\right)\left(C_H^i - C_H^a\right)} \tag{6}$$

replacing P_H in Eqn. 1 by P_H^{ap}. The theoretical titration curve of P_H^{ap} (Fig. 4) is not linear in contrast to that of P_K. As the measured values of P_H^{ap} are close to the theoretical curve, it is concluded that even the greater proton fluxes through the liposomal membrane in the presence of the protonophore TTFB are well described by Eqns. 4 and 5. In the presence of 10^{-9} to 10^{-7} mol valinomycin/g phospholipid, only the counterflux of K^+ has to be taken into account, while those of other ions are negligible.

Discussion

Correlation of proton flux and velocity of ATP synthesis

In an earlier publication the turnover number of phosphorylation was measured with liposomes containing the ATP synthase [2]. Phosphorylation was driven by gradients of H^+ and K^+. The liposomal preparation was much the same as that used here for determining P_H. However, values of the internal pH between 5 and 6 had to be applied with the external pH at 9, in order to obtain high turnover numbers.

It is desirable to know J_H in the steady state of phosphorylation. Unfortunately, J_H cannot be measured directly with the indicator used ($pK = 7.3$), and suitable indicators are not available. However, J_H can be calculated according to Eqns. 4 and 5 from the experimental conditions on the basis of the assumption that the P_H determined here (Fig. 3) is valid in the steady state of phosphorylation. The P_H of the lipid bilayer is nearly independent of pH and surface charge [16] and should, therefore be valid also at the lower internal pH. The question is, whether the P_H of the ATP synthase is the same at various velocities of phosphorylation or not. As a test on this question, the velocity of phosphorylation is plotted as a function of the corresponding J_H (Fig. 5). The values are calculated using the P_H obtained with liposomes containing the ATP synthase (Fig. 3) and the concentrations of H^+ and K^+ that are valid at the start of the phosphorylation reaction (closed symbols in Fig. 5). The relation of J_H to the velocity of phosphorylation can be approximated by a straight line which passes through the origin on extrapolation. From the slope of the line and the average surface density of the ATP synthase on the liposomal membrane (10^{14} cm^2/mol ATP synthase), the H^+/ATP ratio is obtained as 6. This ratio supports the view that the P_H used for calculating J_H is valid in the various steady states of phosphorylation. The ratio is twice the widely accepted value of ATP synthesis, because only half of J_H is mediated by the ATP synthase. This is a consequence of the finding that the P_H of liposomes is doubled by the incorporation of the enzyme (Fig. 3). According to Eqn. 5 J_H increases proportionally with P_H, provided that $\Delta\psi$ is not greatly affected by the alteration of P_H (Eqn. 4).

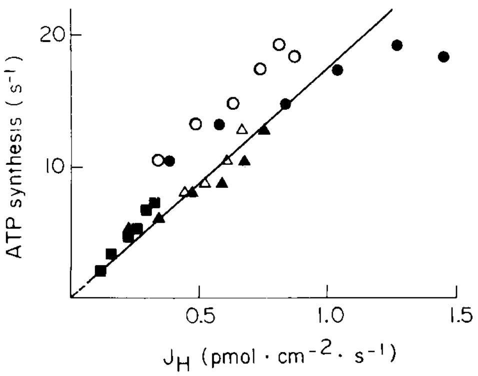

Fig. 5. Correlation of the turnover number of the ATP synthase and the calculated proton flux across the liposomal membrane. The turnover numbers were taken from Ref. 2. The corresponding values of J_H were calculated from the initial experimental conditions (closed symbols) using Egns. 4 and 5 with $P_H = 2 \cdot 10^{-4}$ cm/s and $P_K = 10^{-8}$ cm/s. The external pH was 9.0 and the internal concentration of K^+ was 20 mM in all the experiments. The internal pH was 5.0 (●, ○), 5.5 (▲ △) or 6.0 (■). In each set of experiments the external concentration of K^+ was varied between 15 and 120 mM. Valinomycin (45 nmol/g phospholipid) was present. The values of J_H corresponding to the open symbols were calculated using the internal and external concentrations of H^+ and K^+ which result as a consequence of the initial proton flux for 5 s.

While the values of J_H apply at the start of the phosphorylation reaction (closed symbols in Fig. 5), the corresponding velocities of phosphorylation are calculated from the amounts of ATP formed after 5 s. After this time period the ion gradients and the corresponding values of J_H are smaller than the initial ones. To test the effect of this discrepancy on the relation between J_H and the velocity of phosphorylation, the J_H after 5 s (open symbols in Fig. 5) are calculated from the corresponding concentrations of H^+ and K^+ which result from a constant initial J_H for 5 s. A significant difference between the two values is seen only with the liposomes containing maleinate buffer ($pK = 6.2$) at an initial internal pH of 5.0 (dots in Fig. 5). In this case the turnover number should be correlated with a corresponding average J_H. This correction would not markedly affect the fit of the data to the straight line. At the highest initial J_H, the internal pH rises to 5.4 within the 5 s. However, the $\Delta\tilde{\mu}_H$ drops by only 6 mV (not shown).

In summary, Fig. 5 suggests that the turnover number of the ATP synthase within the liposomes is linearly related to J_H between 0.1 and 1 pmol/s per cm^2. Both the H$^+$/ATP ratio and the P_H appear to be nearly constant in this range. The P_H determined in the experiment of Fig. 3 seems to apply also in the steady state of rapid phosphorylation. The breakdown of $\Delta\tilde{\mu}_H$ during the time period (5 s) required for measuring phosphorylation is negligible under the experimental conditions used. It is to be mentioned that the free energy of the ion gradients ($\Delta\tilde{\mu}_H$, considering P_K and P_H according to Eqn. 4) at the smallest value of J_H still exceeds the corresponding phosphorylation potential by a factor of 2.

Correlation of proton flux and $\Delta\tilde{\mu}_H$

In Fig. 6 the initial values of J_H (Fig. 5) are plotted against the corresponding $\Delta\tilde{\mu}_H$ which are given by Eqn. 7. As a consequence of Eqn. 5, the

$$\Delta\tilde{\mu}_H = \Delta\psi + \frac{\Delta \mathrm{pH}}{k} \qquad (7)$$

relation of the two parameters is exponential. Furthermore, three different curves are obtained which correspond to the three different sets of experimental conditions (closed symbols in Fig. 6). The experimental data within a set refer to a common internal pH (5, 5.5 or 6), but to different external concentrations of K$^+$. Depending on the internal pH, similar values of $\Delta\tilde{\mu}_H$ may correspond to largely different values of J_H.

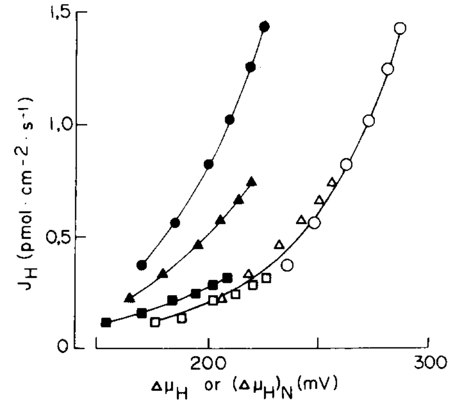

Fig. 6. Theoretical correlation of J_H to $\Delta\tilde{\mu}_H$. The data on the ordinate are the initial J_H given on the abscissa of Fig. 5. $\Delta\tilde{\mu}_H$ (closed symbols) was calculated according to Eqn. 7 with $\Delta\psi$ given by Eqn. 4. $(\Delta\tilde{\mu}_H)_N$ (open symbols) was calculated from Eqn. 8 with $\Delta\psi_N$ obtained from the internal and external concentration of K$^+$ according to the Nernst equation.

The three sets of data fit to a common relation, when J_H is plotted as a function of $(\Delta\tilde{\mu}_H)_N$ (Eqn. 8), where the $\Delta\psi_N$ term is calculated from the K$^+$ gradient according to the Nernst equation (open symbols in Fig. 6).

$$(\Delta\tilde{\mu}_H)_N = \Delta\psi_N + \frac{\Delta \mathrm{pH}}{k} \qquad (8)$$

Using the Nernst equation instead of Eqn. 4 implies that J_H is not limited by the counterflux of K$^+$. However, the P_K which corresponds to the amount of valinomycin used in the experiment indicates limitation of J_H by the counterflux of K$^+$. As a consequence, the $\Delta\psi$ calculated according to Eqn. 4 is directed opposited to $\Delta\psi_N$ at the greater values of J_H. Therefore, $\Delta\tilde{\mu}_H$ is distinctly smaller than $(\Delta\tilde{\mu}_H)_N$ (Fig. 6). The difference is most pronounced with the data corresponding to an internal pH of 5, where it amounts to 62 mV. Thus the actual $\Delta\tilde{\mu}_H$ across the liposomal membrane which is valid in the steady state of rapid phosphorylation (220 mV) is apparently close to the $\Delta\tilde{\mu}_H$ (180 mV) generated by the electron transport across the membrane of inverted bacterial vesicles [8]. The maximum turnover number of the ATP synthase in liposomes (Fig. 6) is about half that measured with the bacterial vesicles, where phosphorylation is driven by electron transport [2]. This suggest that the isolated ATP synthase has retained nearly its original capacity of phosphorylation after incorporation into liposomes. Furthermore, the enzyme can operate with its original activity at values of $\Delta\tilde{\mu}_H$ commensurable with those brought about by electron transport. This is consistent with the view that the energy of the electron-transport reaction is exclusively transduced by means of the $\Delta\tilde{\mu}_H$ [19].

The exponential relation of the turnover number of ATP synthesis to $(\Delta\tilde{\mu}_H)_N$ was observed with many ATP synthases [3–7], and was interpreted as a threshold response of the enzymes. As shown in Fig. 6, this exponential relation is apparently a consequence of the relation of J_H to $(\Delta\tilde{\mu}_H)_N$, while the turnover number is linearly related to J_H (Fig. 5). This argues against a threshold response of the enzyme at free energies of the $\Delta\tilde{\mu}_H$ exceeding the phosphorylation potential. It rather appears that the velocity of phosphorylation and J_H are related to their common driving forces as expressed by Eqn. 5.

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References

1 Bokranz, M., Mörschel, E. and Kröger, A. (1985) Biochim. Biophys. Acta 810, 84–93

2 Bokranz, M., Mörschel, E. and Kröger, A. (1985) Biochim. Biophys. Acta 810, 332–339

3 Thayer, W.S. and Hinkle, P.C. (1975) J. Biol. Chem. 250, 5336–5342

4 Gräber, P., Junesch, U. and Schatz, G.H. (1984) Ber. Bunsenges. Phys. Chem. 88, 599–608

5 Sone, N., Yoshida, M., Hirata, H. and Kagawa, Y. (1977) J. Biol. Chem. 252, 2956–2960

6 Schmidt, G. and Gräber, P. (1985) Biochim. Biophys. Acta 808, 46–51

7 Zoratti, M., Pietrobon, D. and Azzone, G.F. (1982) Eur. J. Biochem. 126, 443–451

8 Mell, H., Wellnitz, C. and Kröger, A. (1986) Biochim. Biophys. Acta 852, 212–221

9 Chen, P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756–1758

10 Kano, K. and Fendler, J.H. (1978) Biochim. Biophys. Acta 509, 289–299

11 Clement, N.R. and Gould, J.M. (1981) Biochemistry 20, 1534–1538

12 Nichols, W.J., Hill, M.W., Bangham, A.D. Deamer, D.W. (1980) Biochim. Biophys. Acta 596, 393–403

13 Deamer, D.W. and Nichols, J.W. (1983) Proc. Natl. Acad. Sci. USA 80, 165–168

14 Nozaki, Y. and Tanford, C. (1981) Proc. Natl. Acad. Sci. USA 78, 4324–4328

15 Gutknecht, J. and Walter, A. (1981) Biochim. Biophys. Acta 641, 183–188

16 Gutknecht, J. (1984) J. Membrane Biol. 82, 105–112

17 Stark, G. and Benz, R. (1971) J. Membrane Biol. 5, 133–153

18 Adam, G., Läuger, P. and Stark, G. (1977) Physikalische Chemie und Biophysik, Springer-Verlag, Berlin

19 Mitchell, P. (1966) Biol. Rev. 41, 445–502