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PROTON ELECTROCHEMICAL GRADIENT AND PHOSPHATE POTENTIAL IN MITOCHONDRIA

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Summary

The paper reports an analysis of the relationship between $\Delta\tilde{\mu}_H$ the proton electrochemical potential difference, and ΔGp, the phosphate potential.

Depression of $\Delta\tilde{\mu}_H$ and ΔGp has been obtained by titration with: (a) carbonylcyanide trifluoromethoxyphenylhydrazone; (b) nigericin (+ valinomycin); (c) KCl (+ valinomycin); and (d) rotenone. The uncoupler depresses $\Delta\tilde{\mu}_H$ more than nigericin (+ valinomycin), KCl (+ valinomycin) and rotenone at equivalent ΔGp.

The ΔGp/$\Delta\tilde{\mu}_H$ ratio is about 3 at high values of $\Delta\tilde{\mu}_H$. When ΔGp and $\Delta\tilde{\mu}_H$ are depressed by nigericin (+ valinomycin) the ΔGp/$\Delta\tilde{\mu}_H$ ratio remains constant. When ΔGp and $\Delta\tilde{\mu}_H$ are depressed by uncouplers, the ΔGp/$\Delta\tilde{\mu}_H$ ratio increases hyperbolically tending to infinity while $\Delta\tilde{\mu}_H$ tends to zero. The absence of constant proportionality between ΔGp and $\Delta\tilde{\mu}_H$ indicates that the proton gradients driving ATP synthesis presumably operate within microscopic environments.

Introduction

Lardy [1] and Chance and Williams [2,3] considered respiratory control to be of kinetic origin. Klingenberg [4,5] considered respiratory control as due to an increased rate of reversed electron transfer. It is now generally accepted that oxidation reduction reactions and ADP phosphorylation are near equilibrium i.e. mitochondrial respiration is dependent on ΔGp, the phosphate potential [6—10]. If $\Delta\tilde{\mu}_H$ is the obligatory intermediate between electron transfer and ATP synthesis some sort of constant relationship should exist between $\Delta\tilde{\mu}_H$ and ΔGp.

Abbreviations: TPMP, triphenylmethylphosphonium; FCCP, carbonyl cyanide *p*-trifluoromethylphenylhydrazone.

In a previous paper [11] we showed that the relationship between rate of controlled respiration and $\Delta\tilde{\mu}_H$ is not that expected from a simple thermodynamic equilibration. In the present paper we investigate the relationship between $\Delta\tilde{\mu}_H$ and ΔGp in order to further ascertain the kinetic competence of $\Delta\tilde{\mu}_H$ as the sole and obligatory energy transducing intermediate. The approach is similar to that used in the preceding paper, namely determination of $\Delta\tilde{\mu}_H$ on the ion distribution under conditions of steady fluxes. Decline of ΔGp and depression of $\Delta\tilde{\mu}_H$ have been obtained by titrations against increasing concentrations of various ionophores. It appears that the effects of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), valinomycin, rotenone and nigericin + valinomycin are not equivalent. The capacity of uncouplers to cause depression of $\Delta\tilde{\mu}_H$, not proportional to the decline of ΔGp, results in an increase of the $\Delta G\text{p}/\Delta\tilde{\mu}_H$ ratio.

The observation is discussed in relation to the question of whether the proton circuits driving ATP synthesis involve microscopic events.

Experimental

Preparation of mitochondria and incubation medium and determination of $\Delta\tilde{\mu}_H$ were as described in the preceeding paper [11]. The matrix cation concentration $[\text{cation}]_m$ was calculated from Eqn. 1.

$$[\text{cation}]_m = \frac{C_p - C_s V_e}{V_i} \quad (1)$$

where C_p and C_s are the amounts of cations in the pellet and in the supernatant, V_e and V_i are the extramatrix and the matrix water in the pellet. Corrections for the amount of cation bound, in the case of Ca^{2+} were also as described in the preceding paper [11].

The phosphate potential was determined essentially as described by Slater et al. [6]. Time of incubation was 7 min except in the case of rotenone where it was prolonged to 15 min. The length of the incubation period was selected after ascertaining that the ATP/ADP ratio had become independent of the time of incubation. The reaction was terminated with perchloric acid. After cooling and neutralization with triethanolamine/KOH, the samples were centrifuged and the supernatants were assayed for nucleotides. ATP was determined in the presence of hexokinase, glucose-6-phosphate dehydrogenase and NADP. The formation of NADPH was followed with an Eppendorf fluorometer. The amount of net ATP synthesis was obtained by correcting for the amount of ATP found in a sample, incubated under identical conditions, supplemented with $2 \cdot 10^{-6}$ M FCCP and antimycin A. This latter ATP is presumably due to residual adenylate kinase activity. ADP was determined, also fluorimetrically, in the presence of pyruvate kinase, phosphoenolpyruvate, lactic dehydrogenase and NADH. The phosphate potential was calculated by employing a value of −7.9 kcal/mol for the standard free energy of hydrolysis of ATP [6,12,13]. The data reported in the figures are the averages of 12—16 independent experiments.

Chemicals were of the highest purity commercially available. [^{3}H]Triphenylmethylphosphonium was kindly provided by Dr. R. Kabach.

Results and Discussion

The effects of FCCP, nigericin and valinomycin on ΔGp and $\Delta\tilde{\mu}_H$

Fig. 1 shows the effect of FCCP, rotenone and nigericin (+ valinomycin) on ΔGp. The values of ΔGp with FCCP and rotenone were determined either in the presence or in the absence of valinomycin. The difference in ΔGp values due to treatment with valinomycin was negligible. FCCP, rotenone and nigericin caused a decline in ΔGp which was proportional to the amount of the inhibition. ΔGp fell from values of 530 mV, in the absence of inhibitors, to values of 420 mV with 80 pmol nigericin · mg^{-1} protein of 450 mV with 100 pmol rotenone · mg^{-1} protein, and of 419 mV with 200 pmol FCCP · mg^{-1} protein. The decline of ΔGp from values around 530—550 mV. in the absence of ionophores, to values of 420 mV at the high ionophore concentrations implies a decrease of the ATP concentration in the medium from about 190 to 15 μM. This latter ATP concentration can be determined enzymatically with sufficient accuracy to obtain reproducible results. ΔGp values below 410 mV were determined only occasionally since they correspond to ATP concentrations in the medium lower than 10 μM.

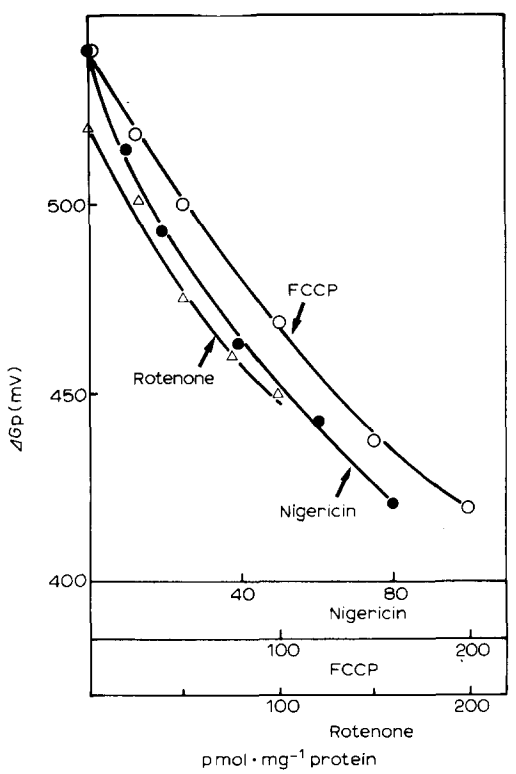

Fig. 1. Effect of FCCP and nigericin (+ valinomycin) and rotenone on ΔGp. The medium contained 0.2 M sucrose, 10 mM succinate-Tris, 3 mM P_i-Tris, 10 mM Tris-Cl, pH 7.4, 1 mM EDTA, 200 μM ADP, 1 μM rotenone and 4 mg mitochondrial protein. In the samples titrated with nigericin 0.5 mM KCl and 0.2 μg valinomycin/ml were also added. In the samples titrated with rotenone, succinate was replaced with 1 mM β-hydroxybutyrate. Time of incubation 7 min, with rotenone 15 min.

The effects of FCCP and nigericin on $\Delta\tilde{\mu}_H$ as calculated on the K^+ distribution are shown in Fig. 2. 200 pmol FCCP · mg^{-1} protein depressed $\Delta\tilde{\mu}_H$ to 45 mV, while 80 pmol nigericin · mg^{-1} protein depressed $\Delta\tilde{\mu}_H$ to 118 mV. In Fig. 2B the values of ΔGp are plotted against the values of $\Delta\tilde{\mu}_H$. Since the decline of ΔGp induced by nigericin was accompanied by a smaller depression of $\Delta\tilde{\mu}_H$, with respect to the depression induced by FCCP, the slope of the plot was much steeper in the case of nigericin than in the case of FCCP.

In the preceding paper [11] it was found that the rates of respiration induced by ADP and ionophores were not accompanied by similar levels of $\Delta\tilde{\mu}_H$. In the experiment of Fig. 2 a similar discrepancy is observed. Nigericin + valinomycin cause a decline of ΔGp with minor variation of $\Delta\mu\tilde{H}$: levels of ΔGp around 420 mV are obtained while $\Delta\tilde{\mu}_H$ is still above 100 mV. However with FCCP, levels of ΔGp around 420 mV are reached when $\Delta\tilde{\mu}_H$ falls below 50 mV.

As discussed in the preceding paper [11] the calculation of $\Delta\psi$ on the K^+ distribution in the presence of nigericin is incorrect, since nigericin induces a K^+ efflux and therefore leads K^+ out of electrochemical equilibrium. This means, as discussed previously, that the values of $\Delta\psi$ calculated on the K^+ distribution in the presence of nigericin are lower than the real $\Delta\psi$. Therefore the discrepancy between the effects of FCCP and of nigericin + valinomycin is larger than apparent from the data of Fig. 2. To test this conclusion, decline of ΔGp and depression of $\Delta\tilde{\mu}_H$ have been obtained in the experiments of Figs. 3 and 4, in the absence of nigericin.

In Fig. 3 the depression of $\Delta\tilde{\mu}_H$ and ΔGp was obtained by increasing the amount of valinomycin at three KCl concentrations, 0.3—0.1 and 1.5 mM. It is seen that the increase of valinomycin caused no decline of ΔGp at 0.3 mM KCl, except at the highest valinomycin concentration. On the other hand the decline was marked at 1.0 and 1.5 mM KCl. The decline of ΔGp was accompanied only by minor depression of $\Delta\tilde{\mu}_H$. As a consequence the slope of the

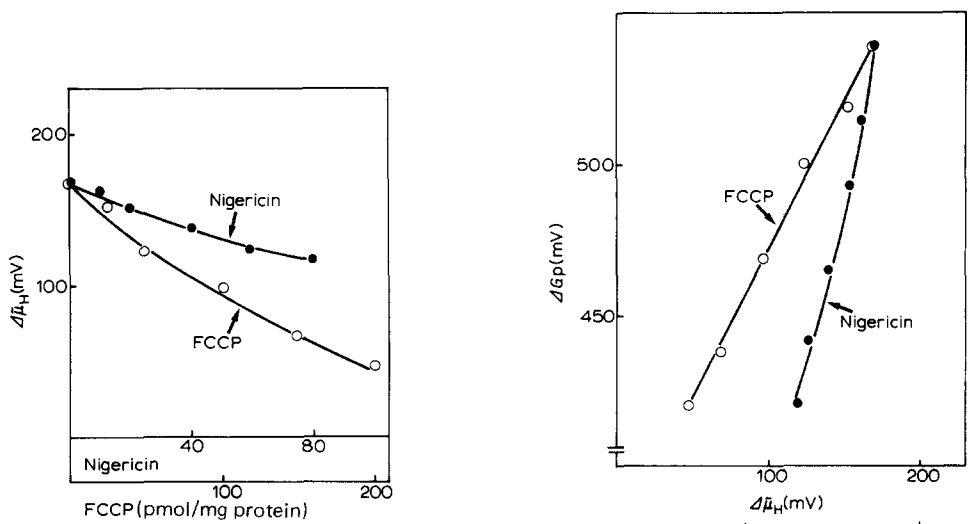

Fig. 2. Effect of FCCP and nigericin (+ valinomycin) on $\Delta\tilde{\mu}_H$ and ΔGp. $\Delta\tilde{\mu}_H$ measured on K^+. Experimental conditions as in Fig. 1. $\Delta\tilde{\mu}_H$ was measured as described in Experimental.

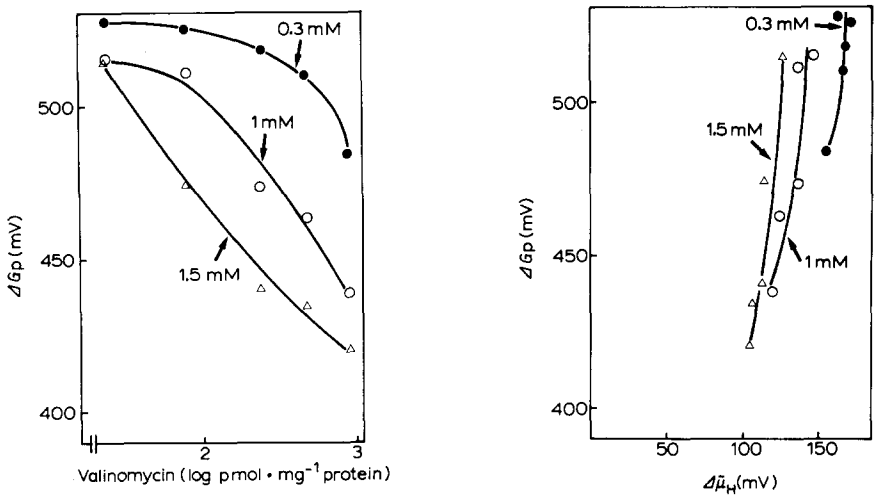

Fig. 3. Effect of valinomycin and KCl on ΔGp and $\Delta\tilde{\mu}_H$. $\Delta\tilde{\mu}_H$ measured on K^+. Experimental conditions as in Fig. 1 except that the concentrations of KCl and of valinomycin were as indicated in the figure.

plot ΔGp vs. $\Delta\tilde{\mu}_H$ was very steep, low values of ΔGp being reached at still high values of $\Delta\tilde{\mu}_H$. The experiments of Fig. 3 thus show that a marked decline of ΔGp in the presence of a minor change of $\Delta\tilde{\mu}_H$ can also be obtained in the absence of nigericin.

In Fig. 4 the depression of ΔGp and $\Delta\tilde{\mu}_H$ in valinomycin treated mitochondria was obtained by increasing KCl between 0.3 and 20 mM. It is seen that ΔGp fell from 525 to 400 mV. This is due to the fact that increase of $[K^+]_0$ leads to considerable swelling and membrane stretching. The plot of ΔGp vs. $\Delta\tilde{\mu}_H$ shows

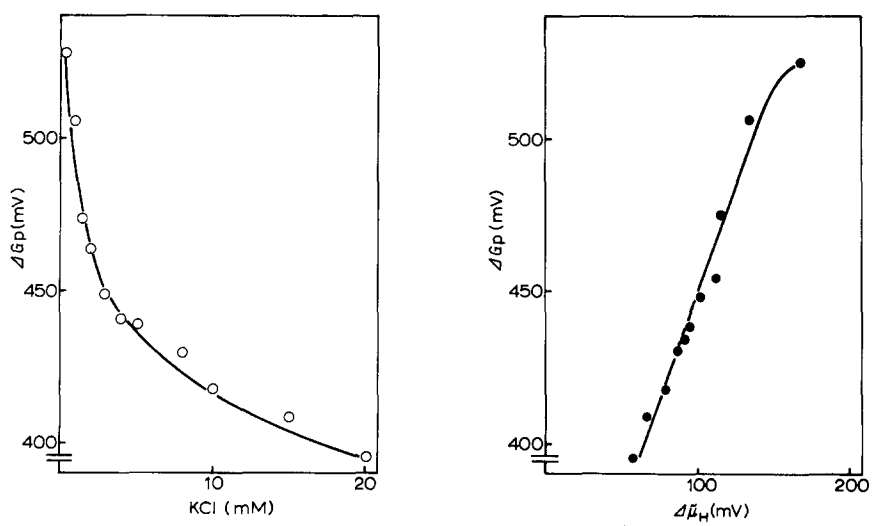

Fig. 4. Effect of KCl on ΔGp and $\Delta\tilde{\mu}_H$. $\Delta\tilde{\mu}_H$ measured on K^+. The medium contained 0.2 M sucrose, 10 mM succinate-Tris, 1 mM β-hydroxybutyrate, 3 mM P_i-Tris, pH 7.4, 1 mM EDTA, 200 μM ADP, 0.5 μg valinomycin/ml and 4 mg protein. The osmolarity was kept constant by decreasing the sucrose concentration parallel to the increase of the KCl concentration between 2 and 20 mM. Time of incubation 7 min

that the decline of ΔGp occurred parallel to a depression of $\Delta\tilde{\mu}_H$ between 120 and 50 mV.

A comment is needed as to the mechanism involved in the depression of $\Delta\tilde{\mu}_H$ in the experiments shown in Figs. 1—4. This is clear in the case of FCCP and of nigericin + valinomycin: (a) electrical entry of H^+ via FCCP and (b) electrical entry by K^+ via valinomycin followed by electroneutral efflux of K^+ via nigericin. In the case of the depression induced by high KCl concentrations, it is known that increase of KCl in the medium above 0.5 mM leads to extensive K^+ uptake, matrix osmotic swelling and membrane stretching. Increased H^+ influx and K^+ efflux then occur through the leaky membrane with a marked stimulation of the basal respiratory rate. In the case of the $\Delta\tilde{\mu}_H$ depression induced by high valinomycin concentrations, membrane damage is also likely although the molecular mechanism for this effect is not clear. In conclusion it appears that the slope of the plot ΔGp vs. $\Delta\tilde{\mu}_H$ tends always to be steeper under conditions where the effect of the ionophores is that of inducing a cycling of both H^+ and cations through the membrane.

In a preceding paper [11] we compared the values of $\Delta\tilde{\mu}_H$ calculated on the K^+ distribution with those calculated on the $TPMP^+$ and Ca^{2+} distribution. Fig. 5 shows a plot of ΔGp vs. $\Delta\tilde{\mu}_H$ as measured on either $TPMP^+$ or Ca^{2+} distribution. The values of $\Delta\tilde{\mu}_H$, based on Ca^{2+}, were obtained by titrating the effect of FCCP, of nigericin + valinomycin (cf. Fig. 2 ref. 11) and of rotenone in the presence of 200 μM Ca^{2+} and 10 mM acetate. These conditions are different from those used to measure ΔGp (involving no Ca^{2+} and 3 mM P_i). The implicit assumption is that the extent of depression of $\Delta\tilde{\mu}_H$ induced by the

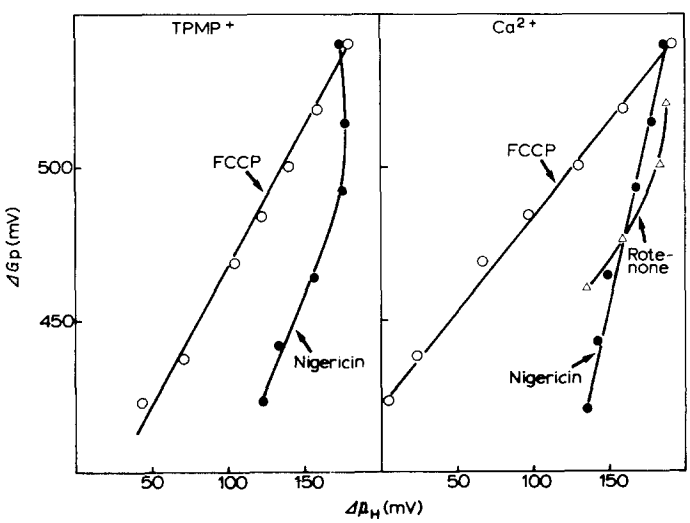

Fig. 5. Effect of FCCP, nigericin (+ valinomycin) and rotenone on ΔGp and $\Delta\tilde{\mu}_H$. $\Delta\tilde{\mu}_H$ measured on $TPMP^+$ and Ca^{2+}. The medium contained 0.2 M sucrose, 10 mM succinate-Tris, 3 mM P_i-Tris, 10 mM Tris-Cl, pH 7.4, 1 μM rotenone, 1 mM EDTA, 200 μM ADP and 4 mg mitochondrial protein. In the samples titrated with nigericin 0.5 mM KCl and 0.2 μg valinomycin/ml were also added. In the samples titrated with rotenone, succinate was replaced with β-hydroxybutyrate. $TPMP^+$ distribution was measured in the presence of 25 μM $TPMP^+$. Ca^{2+} distribution, in the presence of various uncoupler and nigericin concentrations, was measured in a medium were EDTA, P_i and ADP were omitted and 200 μM Ca^{2+} + 10 mM acetate were added.

various inhibitors is similar under the two experimental conditions. Fig. 5 shows, in accordance with the data of Fig. 2, a marked difference in the relationship ΔGp vs. $\Delta\tilde{\mu}_H$ between FCCP on one side and nigericin and rotenone on the other. The slope of the plot was much steeper in the case of nigericin (+ valinomycin) than in the case of FCCP. With $TPMP^+$ as permeant cation the initial decline of ΔGp occurred in the presence of an apparent slight increase of $\Delta\tilde{\mu}_H$ at the low nigericin concentrations. This is presumably due to the further uptake of $TPMP^+$ following K^+ release induced by nigericin. The fact that the values of $\Delta\tilde{\mu}_H$ during the FCCP titration are lower with Ca^{2+} than with $TPMP^+$ (cf. Fig. 2 of ref. 11) is presumably due to absence of correction for internal binding in the case of $TPMP^+$. The binding plays a major role at low $TPMP^+$ uptake. The lower values of $\Delta\tilde{\mu}_H$, during FCCP titration, with Ca^{2+} in respect to $TPMP^+$, lead to a more marked difference between effects of FCCP and nigericin when based on Ca^{2+} in respect to $TPMP^+$. The effect of rotenone was more like that of nigericin (+ valinomycin) than that of FCCP, i.e. it affected ΔGp more than $\Delta\tilde{\mu}_H$.

The $\Delta Gp/\Delta\tilde{\mu}_H$ ratio

From the plots of Figs. 2 and 5 it is possible to calculate the $\Delta Gp/\Delta\tilde{\mu}_H$ ratios as a function of $\Delta\tilde{\mu}_H$. In the case of the effect of nigericin the $\Delta Gp/\Delta\tilde{\mu}_H$ ratios are constant in the range of 3 [13—17]. However in the presence of FCCP the $\Delta Gp/\Delta\tilde{\mu}_H$ ratio tended to increase with the depression of $\Delta\tilde{\mu}_H$. In Fig. 6 are reported the various $\Delta Gp/\Delta\tilde{\mu}_H$ ratios obtained at the various FCCP concentrations (cf. Figs. 2 and 5). Fig. 6 shows that the plot $\Delta Gp/\Delta\tilde{\mu}_H$ is hyperbolical and that all ratios obtained with either K^+, or $TPMP^+$ or Ca^{2+} as permeant cation are lying on the same curve.

Energy for ATP synthesis can be provided in the various transducing systems either by metabolic reactions (respiration or illumination) or by the difference of proton electrochemical potential. If $\Delta\tilde{\mu}_H$ is the obligatory intermediate between metabolism and ATP synthesis, aerobic or illumination driven ATP

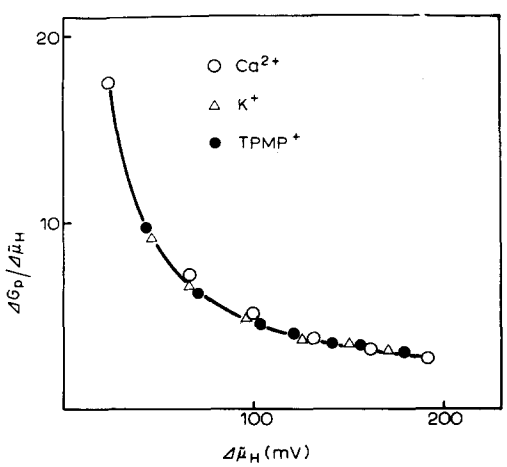

Fig. 6. Relationship between $\Delta Gp/\Delta\tilde{\mu}_H$ ratio and $\Delta\tilde{\mu}_H$. The values were taken from Figs. 2 and 5.

synthesis is equivalent to ion driven ATP synthesis. If ATP synthesis is driven by $\Delta\tilde{\mu}_H$ the following relation holds: $\Delta Gp/\Delta\tilde{\mu}_H = n$, where n is the number of H^+ translocated per ATP. However if n is a finite number, whether 2 or 4, an osmotic threshold might be predicted, i.e. a value of $\Delta\tilde{\mu}_H$ below which ATP synthesis is abolished [17]. The lower the n value, the higher is the osmotic threshold in mV. For ΔGp = 560 mV and n = 2 the osmotic threshold is 280 mV; for ΔGp = 560 and n = 4, the threshold is at 140 mV. Furthermore the lower ΔGp, the lower is the level of the osmotic threshold. The osmotic threshold concept predicts that ATP synthesis should be abolished when the $\Delta Gp/\Delta\tilde{\mu}_H$ ratio is higher than 2 or 4. An osmotic threshold during ion driven ATP synthesis has been reported in intact mitochondria [18], submitochondrial particles [19], chloroplasts [20,21] and bacterial chromatophores [22]. Junge et al. found a minimal $\Delta\psi$ for ATP synthesis in chloroplasts [23]. In the case of metabolism-driven ATP synthesis the results reported vary [24]. Graber and Witt [25] found a threshold of 50 mV in chloroplasts at low ΔpH, and no threshold at high ΔpH. Melandri et al. [26] found in chromatophores a $\Delta\tilde{\mu}_H$ threshold at 120 mV when energy supply was limited by uncoupler; however when energy supply was limited by electron transfer the $\Delta\tilde{\mu}_H$ threshold was at 300 mV. Jackson et al. [27] found that following short flash excitation in chromatophores the extent of ADP + P_i induced decay of the carotenoid shift was a constant fraction, 10% of the total decay. This is not in accord with an osmotic threshold if a large part of $\Delta\tilde{\mu}_H$ exists as $\Delta\psi$. Massari and Azzone [28] found lack of ADP induced stimulation of respiration below $\Delta\tilde{\mu}_H$ of 120 mV.

In the present study [29,30] the decline of ΔGp induced by either valinomycin alone or nigericin (+ valinomycin) is accompanied by negligible changes of the $\Delta Gp/\Delta\tilde{\mu}_H$ ratio. A constant $\Delta Gp/\Delta\tilde{\mu}_H$ ratio of about 3 is observed at the various concentrations of valinomycin and of nigericin (+ valinomycin). On the other hand the decline of ΔGp induced by uncoupler is accompanied by a major increase in the $\Delta Gp/\Delta\tilde{\mu}_H$ ratio. The occurrence of relatively high values of ΔGp in the presence of low values of $\Delta\tilde{\mu}_H$ gives rise to the hyperbolic relationship between $\Delta Gp/\Delta\tilde{\mu}_H$ and $\Delta\tilde{\mu}_H$. The ratio tends to infinity while $\Delta\tilde{\mu}_H$ tends to zero. It may be argued that the $\Delta Gp/\Delta\tilde{\mu}_H$ ratio should have been calculated on the endogenous ΔGp (taking into account the concentration of nucleotides and P_i in the matrix) rather than on the exogenous ΔGp. However the effect of $\Delta\tilde{\mu}_H$ is that of increasing the exogenous in respect to the endogenous ΔGp. Therefore the decrease of $\Delta\tilde{\mu}_H$ should decrease rather than increase the $\Delta Gp/\Delta\tilde{\mu}_H$ ratio.

Conclusion

Whether $\Delta\tilde{\mu}_H$ is the obligatory high energy intermediate in energy transductions is controversial [17,31—35]. In the present study the relationship between ΔGp and $\Delta\tilde{\mu}_H$ is not constant but depends on the pathway for energy drain. While inophores inducing H^+-cation cycling maintain a fixed $\Delta Gp/\Delta\tilde{\mu}_H$ ratio, classical uncouplers inducing only electrical H^+ entry, cause an increase of the $\Delta Gp/\Delta\tilde{\mu}_H$ ratio at low $\Delta\tilde{\mu}_H$ values. This is in accord with the view of a discrete interaction between the two energy transducing units catalyzing electron transfer and ATP synthesis. This is shown in the scheme of

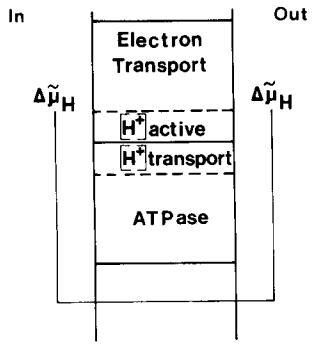

Fig. 7. Relationship between energy transducing units.

Fig. 7 where active transport is seen an intrinsic property of the energy transducing units. If energy is drained for ATP synthesis or for active transport at a rate faster than for equilibration with $\Delta\tilde{\mu}_H$, the stimulation of the respiration or the decline of ΔGp are more pronounced than the decline of $\Delta\tilde{\mu}_H$. This may be the case with ADP or nigericin (+ valinomycin) or A23187 or valinomycin which drain energy, the former at the level of the ATP synthesis and the latter at the level of active transport, respectively. The opposite occurs with FCCP which causes primarily a decline of $\Delta\tilde{\mu}_H$. It is likely that as the primary event leading to charge separation in each energy transducing unit is molecular in nature, so also the proton circuits connecting electron transfer and ATP synthesis transducing units are operating over microscopic environments. If this is so, then the determination of $\Delta\tilde{\mu}_H$ in the bulk phases may have little relation to the driving force at the catalytic site of the ATP synthetase.

The titrations with FCCP provide information about the dimension of the microscopic event for energy conservation. $\Delta\tilde{\mu}_H$ was depressed below 20 mV and ΔGp below 420 mV at FCCP concentrations of 200 pmol · mg^{-1} protein, i.e. $12.4 \cdot 10^{13}$ molecules FCCP · mg^{-1} protein. It has been calculated that 1 mg protein of rat liver mitochondria contains $7.2 \cdot 10^9$ mitochondria and each mitochondrion contains 20 000 respiratory chains [36]. The concentrations for full uncoupling then correspond to about 20 000 FCCP molecules per mitochondrion and 1 FCCP molecule per respiratory chain. The argument used here to assess the dimension of the energy conserving unit in rat liver mitochondria is similar to that used for chloroplasts and chromatophores. In this case it has been calculated that uncoupling requires 1 molecule of gramicidin per thylakoid (approx. 10^5 chlorophyll molecules) and 1 molecule of valinomycin per vesicle (approx. $5 \cdot 10^3$ bacteriochlorophyll molecules) for chloroplasts and bacterial chromatophores, respectively [37]. The limitations of this argument have already been discussed [38].

Note added in proof (Received November 7th, 1977)

Mitchell has recently proposed [39] a miniaturized version of the chemiosmotic hypothesis where the proton circulation is comparatively localised. In our view the critical question is not the extent of localisation of the proton cir-

culation but rather whether energy transducing membranes operate as fuel cells or as molecular energy machines (see ref. 17). The number of cycles of electron transfer required to increase the thermodynamic potential of a single mitochondrion at a level compatible with ATP synthesis is $2 \cdot 10^4$ in the case of a fuel cell and 1 in the case of a molecular energy machine.

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