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HOMEOSTASIS OF THE PROTONMOTIVE FORCE IN PHOSPHORYLATING MITOCHONDRIA

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The relationship between the respiration rate and the magnitude of the electrochemical proton potential ($\Delta\mu_{H^+}$) in rat liver mitochondria was investigated. (1) Under the active-state conditions, the action of inhibitors of either phosphorylation (oligomycin) or respiration (rotenone, malonate) on the respiration and $\Delta\mu_{H^+}$ was measured. Both inhibitors diminished the respiration, whereas rotenone resulted in a decrease of $\Delta\mu_{H^+}$, and oligomycin produced an increase of this potential. The effect of the inhibitors was much more pronounced on the respiration rate than on $\Delta\mu_{H^+}$; for example, the excess of oligomycin produced a 90% inhibition of the respiration while $\Delta\mu_{H^+}$ was changed only by 9%. (2) Under the resting-state conditions, small concentrations of the uncoupler stimulated the respiration while changing $\Delta\mu_{H^+}$ to a relatively small extent. The uncoupler concentrations which doubled and tripled the respiration rate produced only 5 and 9% decrease of $\Delta\mu_{H^+}$, respectively. (3) The present results enabled us to propose a model describing the interrelationship between respiration and $\Delta\mu_{H^+}$.

Introduction

According to the chemiosmotic approach to mitochondrial energy transformations [1], functioning of the respiratory chain generates a protonmotive force ($\Delta\mu_{H^+}$) which, in turn, is the driving factor for ATP synthesis. Therefore, $\Delta\mu_{H^+}$ can be regarded formally as an intermediate in the system of oxidative phosphorylation. Thus, inhibition or activation of the respiratory chain should lead to a decrease or an increase, respectively, of the $\Delta\mu_{H^+}$ value in phosphorylating mitochondria. On the other hand, stimulation or inhibition of processes utilizing $\Delta\mu_{H^+}$ should result in a decrease or an increase of $\Delta\mu_{H^+}$, respectively. Indeed, it has been observed that alterations of the respiratory chain activity influence $\Delta\mu_{H^+}$. Its value can reach various levels depending on the respiratory substrate used [2], most probably due to differences in the flux through the respiratory chain under these conditions. Moreover, inhibitors of the respiratory chain can influence $\Delta\mu_{H^+}$. This has been observed under the resting [3–6], active [6] and uncoupled [5] states.

ATP synthesis is, potentially, a very potent $\Delta\mu_{H^+}$-utilizing process. Transition from the resting to the active state of respiring mitochondria, equivalent to a large increase of the flux through ATP synthase, is accompanied by a decrease of $\Delta\mu_{H^+}$. The magnitude of this decrease varies between 8 and 50 mV in various reports [2,3,7–9]. In some recent papers, it has been reported that the

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TPMP, triphenylmethylphosphonium bromide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

increase of the flux through ATP synthase is accompanied by even a smaller change of $\Delta\mu_{H^+}$ or no change at all [10,11]. The latter observation is not easy to accommodate with the concept of the bulk-to-bulk $\Delta\mu_{H^+}$ as a direct intermediate of oxidative phosphorylation.

In all these studies, the protonmotive force was calculated from the distribution of Rb^+ (plus valinomycin) or lipophilic cations ($\Delta\psi$) and of weak acids (ΔpH) between the mitochondrial matrix and the external medium. It could be, therefore, defined as corresponding to the electrical potential and the proton concentration gradient between the two bulk phases, and such a definition is usually ascribed to the symbol of $\Delta\mu_{H^+}$ (cf. Ref. 12). Parallel with the chemiosmotic hypothesis of Mitchell [1], a concept of localised proton gradient was proposed [13] according to which protons ejected by the respiratory chain are kept close to, or even within, the mitochondrial inner membrane and may not fully equilibrate with bulk phases on both of its sides. This concept has been recently invoked to accommodate apparent inconsistencies between the magnitude of $\Delta\mu_{H^+}$, or changes thereof, with the rate of ATP synthesis [7,9–11,14, 15].

In view of these controversies, we wish to propose that the concept of the bulk-to-bulk protonmotive force can still hold if one assumes that its value is highly homeostated. In the present paper, experiments will be presented which show that, within certain very narrow limits of $\Delta\mu_{H^+}$, small changes thereof produce large alterations of the rates of electron flow and ATP synthesis. Similar conclusions have also been reached in studies on brown adipose tissue mitochondria [16,17]. The homeostasis of $\Delta\mu_{H^+}$ may be an important factor in cellular metabolism, since the distribution of various metabolic effectors (e.g., Ca^{2+}, ATP/ADP ratio, respiratory substrates) between the cytosol and the mitochondria depends strongly on the magnitude of $\Delta\mu_{H^+}$.

Materials and Methods

Rat liver mitochondria were isolated by the standard differential centrifugation procedure [18] using a medium containing 225 mM mannitol, 75 mM sucrose, 50 μM EGTA and 1 mM Tris-HCl (pH 7.0). Only mitochondrial preparations exhibiting the respiratory control above 5 with glutamate plus malate were used. Protein was determined by the biuret method using bovine serum albumin as standard. Mitochondrial respiration was measured at 25°C with a Clark type electrode. The incubation medium contained 100 mM KCl, 20 mM Tris-HCl, 5 mM KH_2PO_4/K_2HPO_4, 5 mM $MgCl_2$ and 1 mM EGTA (pH 7.2). It was assumed that the medium contained 470 ng atom O/ml when saturated with air [19]. The transmembrane electric potential ($\Delta\psi$) and the pH gradient (ΔpH) between the matrix and the external medium were calculated from the distribution of [^{3}H]TPMP [20] and [^{14}C]acetate [3], respectively. The matrix space was calculated from the difference between the ^3H_2O space and the [^{14}C]sucrose space.

[*methyl*-^{3}H]TPMP was obtained from New England Nuclear (Boston, MA, U.S.A.), other radiochemicals were from Amersham International, U.K. and the Institute of Nuclear Research (Świerk, Poland). Chemicals and biochemicals were of the highest purity commercially available.

Results

Mitochondria were titrated with inhibitors of either the respiration (rotenone or malonate, depending on the substrate) or ATP synthesis (oligomycin) and the transmembrane electric potential ($\Delta\psi$) was measured. With glutamate plus malate as respiratory substrates (Fig. 1), the inhibition of the respiration by rotenone in the resting state was accompanied by a sharp decrease of $\Delta\psi$. In the active state (in presence of ADP-regenerating system), both rotenone and oligomycin produced a diminution of the respiration. However, inhibition by rotenone resulted in a decrease of $\Delta\psi$, whereas inhibition by oligomycin increased this potential. With either inhibitor, the effect on the respiration rate was much more pronounced than that on the $\Delta\psi$ value. In fact, the change of $\Delta\psi$ upon titration with oligomycin, maximally corresponding to the difference between $\Delta\psi$ values in the resting and the active states, amounted to 15 mV, which is only 9% of its initial, i.e., active state, value, whereas the respiration was inhibited by 90%.

The $\Delta\psi$ value for the resting state, identical

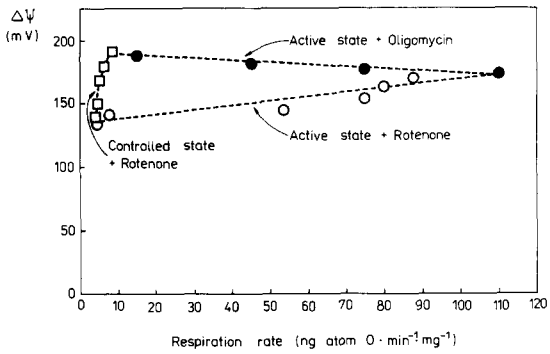

Fig. 1. The effect of rotenone and oligomycin on the respiration rate and $\Delta\psi$ in mitochondria respiring in the active and the resting states. Mitochondria (4.2 mg protein) were incubated in 1.8 ml of the standard medium supplemented with 10 mM glutamate, 2 mM malate, 0.1 nM [^{3}H]TPMP and either 10 μM carboxyatractyloside (resting state) or 2 mM ADP (active state).

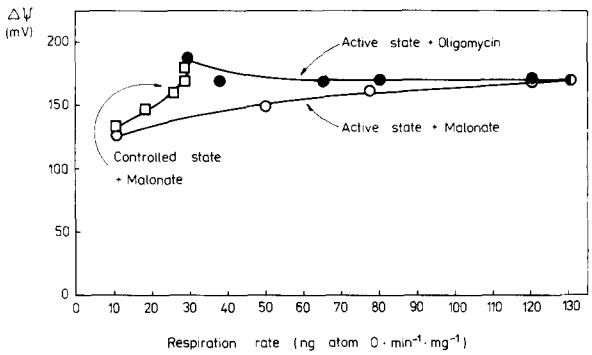

Fig. 2. The effect of malonate and oligomycin on the respiration rate and $\Delta\psi$. Mitochondria (3.8 mg protein) were incubated in 1.8 ml of the standard medium supplemented with 20 mM succinate, 2 μM rotenone and 0.1 nM [^{3}H]TPMP. Other conditions as in Fig. 1.

with that for the active state maximally inhibited with oligomycin, marks the upper limit of $\Delta\psi$ which can be attained by mitochondria. The plot of the active state plus rotenone intercepts with the plot of the resting state plus rotenone at the point which indicates another critical value of $\Delta\psi$, further designated as the lower limit, below which ADP no longer stimulates the respiration. The values for both the upper and the lower limit differ somewhat depending on mitochondrial preparation, but the difference between these limits remains fairly constant and amounts to about 50 mV (Table I).

TABLE I

LIMITS OF $\Delta\psi$ IN PHOSPHORYLATING MITOCHONDRIA

The value of $\Delta\psi$ in the resting state is designated as the upper limit, whereas the value at which ADP no longer produces an increase of the respiration rate is defined as the lower limit. Experimental conditions were as in Fig. 1.

Expt. No.	Upper limit (mV)	Lower limit (mV)	Difference between the limits (mV)
1	155	105	50
2	173	130	43
3	159	108	51
4	198	134	64
5	192	135	57
Mean ± S.D.	175 ± 19	122 ± 15	53 ± 8

Similar results as those shown in Fig. 1 were obtained using succinate (plus rotenone) as the respiratory substrate. The inhibition of the respiration by oligomycin or malonate was accompanied by a relatively small increase or decrease, respectively, of $\Delta\psi$ (Fig. 2). Similarly as with glutamate plus malate, the difference between the upper and the lower limits of $\Delta\psi$ amounted to about 50 mV.

In experiments shown in Figs. 1 and 2 and Table I, only the electric component of $\Delta\mu_{H^+}$ was measured. It can be, however, presumed that conclusions drawn from these experiments can be extended over the behaviour of the total $\Delta\mu_{H^+}$, since, as shown in Table II, $\Delta\psi$ constituted the major and approximately constant part of $\Delta\mu_{H^+}$ under our experimental conditions.

It has been shown [3,21] that the rate of the resting-state respiration depends on the magnitude of the proton leak and the 'slip' of the proton pump. To elucidate the importance of these factors in controlling the respiration, the proton leak was increased by titrating mitochondria respiring with various substrates with the protonophore CCCP. As shown in Fig. 3, low concentrations of CCCP produced the same increase of respiration with either glutamate plus malate or β-hydroxybutyrate. Only at higher concentrations of the protonophore, the respiration rate with various substrates attained different levels, most likely depending on different activities of the respective dehydrogenases.

The evaluation of the contribution of a particu-

TABLE II

THE RELATION BETWEEN THE MAGNITUDE OF $\Delta\psi$ and ΔpH AND THE RESPIRATION RATE AS CONTROLLED BY THE AVAILABILITY OF ADP OR BY THE UNCOUPLER

The standard incubation medium contained 10 mM glutamate, 2 mM malate, 15 mM glucose, 0.2 mM ATP and various amounts of hexokinase or FCCP. The medium was saturated with oxygen.

Conditions of incubation	Respiration rate (ng atom $O \cdot min^{-1} \cdot mg^{-1}$)	$\Delta\psi$ (mV)	ΔpH (mV)	$\Delta\mu_{H^+}$ (mV)	$\Delta\mu_{H^+}/\Delta\psi$
Controlled state	15	169	44	213	1.26
Intermediate states (limited hexokinase)					
40% active state	27	163	38	201	1.23
62% active state	42	156	42	198	1.27
93% active state	63	151	25	176	1.17
Active state (excess of hexokinase)	68	149	33	182	1.22
Partial uncoupling (titration with FCCP)					
46% active state	31	166	37	203	1.22
63% active state	43	156	38	194	1.24
93% active state	63	127	14	141	1.11

lar reaction in the control of the overall process can be based on the concept of the control strength [22,23] which is a measure of the extent to which the flux through the overall process is modified by a small change of the rate of a particular reaction.

The control strength (c_i) of the proton leak in the resting state respiration was determined in the present investigation by adding small amounts of the protonophore, as described by Groen et al. [21]. With all respiratory substrates used, this value

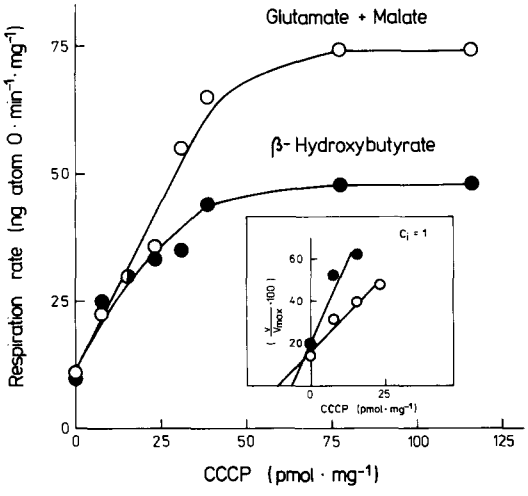

Fig. 3. Stimulation by uncoupler of mitochondrial respiration with various respiratory substrates in the resting state. Mitochondria (2.6 mg protein) were incubated in 1.7 ml of the standard medium supplemented with 10 mM glutamate plus 2 mM malate or 20 mM β-hydroxybutyrate.

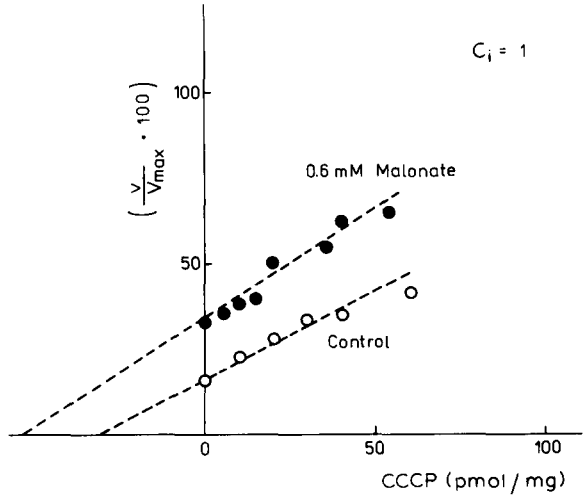

Fig. 4. Stimulation by uncoupler of mitochondrial respiration in the resting state. Mitochondria (3.8 mg protein) were incubated in 1.7 ml of the standard medium supplemented with 20 mM succinate and 2 μM rotenone. Where indicated, 0.6 mM malonate was also present.

was close to 1. In these calculations, it was assumed that the resting-state respiration was entirely related to the proton leak. Although some authors [5,24,25] postulate that slips of proton pumps may also be responsible for resting-state respiration, it can be concluded with some approximation that the proton leak is an important, if not the only, controlling factor of the resting-state respiration. This was confirmed by the observation that in mitochondria respiring with succinate, addition of low concentrations of malonate did not alter the value of the control strength of the proton leak (Fig. 4). In fact, malonate at the concentration used did not change the rate of the resting-state respiration but decreased by about 60% the active-state respiration (not shown).

Discussion

Considerations presented below contain two kinds of approximation. Firstly, the determination of $\Delta\psi$ based on the distribution of a lipophilic cation is overestimated by the binding of the probe to mitochondrial membranes [26]. With TPMP used in the present investigation, this overestimation is assumed to amount to about 50 mV [26]. However, this error becomes relatively smaller when changes of $\Delta\psi$ rather than absolute values thereof are compared. Secondly, in most experiments presented here only the electric component of $\Delta\mu_{H^+}$, i.e., $\Delta\psi$, was measured. It was shown, however, that under experimental conditions applied, $\Delta\mu_{H^+}$ varied proportionally to $\Delta\psi$ and therefore the former value could be deduced by multiplying the latter by the proportionality factor of 1.22 (Table II).

Taking into account these corrections, the difference in $\Delta\mu_{H^+}$ between the resting and the active states in the present investigation was 14–18 mV. This figure is within the range of values reported in the literature [3,7–9]. It can be assumed that ATP is not appreciably synthesized at $\Delta\mu_{H^+}$ smaller than the 'lower limit' (Table I), below which ADP produces no increase of the respiration rate (Figs. 1 and 2). By multiplying the mean value of this 'lower limit' of $\Delta\psi$ by the proportionality factor and subtracting 50 mV for the binding of the probe, one obtains the value of 99 mV for the 'lower limit' of $\Delta\mu_{H^+}$. This agrees fairly well with observations of Zoratti et al. [27], Padan and Rottenberg [7] and Holian and Wilson [26] who found that ATP could be synthesized in mitochondria even at $\Delta\mu_{H^+}$ values as low as 100 mV.

The present results enable us to propose a model describing the interrelationship between respiration and $\Delta\mu_{H^+}$. The following observations provide the basis for these considerations.

Oxidative phosphorylation proceeds at a significant rate only when the magnitude of $\Delta\mu_{H^+}$ exceeds a certain threshold level

As discussed earlier, this threshold is probably equivalent to the value of about 100 mV at which ADP starts to stimulate mitochondrial respiration.

When phosphorylation proceeds, the activity of ATP synthase is very sensitive to changes of $\Delta\mu_{H^+}$

When phosphorylating mitochondria were titrated with respiratory inhibitors, the rate of respiration was always altered much more than the magnitude of $\Delta\mu_{H^+}$ (Figs. 1 and 2). Since the rate of respiration reflects the rate of phosphorylation, a small alteration of the level of $\Delta\mu_{H^+}$ produces a significant change in the rate of phosphorylation. This statement can be expressed in a more quantitative way by applying the concept of elasticity [22,23]. The elasticity ($\epsilon_R^{v_i}$) is a measure of the extent to which the flux (v_i) through a particular reaction of the pathway is influenced by a change of the concentration (dR) of one of the reactants (R) of this reaction:

$$\epsilon_R^{v_i} = \frac{dv_i}{dR} \cdot \frac{R}{v_i} \tag{1}$$

In our case, $\Delta\mu_{H^+}$ will be regarded as the reactant and therefore elasticity of ATP synthase with respect to $\Delta\mu_{H^+}$ can be calculated from the following expression:

$$\epsilon_{\Delta\mu_{H^+}}^{\text{ATP synth.}} = \frac{\partial \text{Phosph.}}{\partial \Delta\mu_{H^+}} \cdot \frac{\Delta\mu_{H^+}}{\text{Phosph.}} \tag{2}$$

Since the rate of ATP synthesis can be assumed to be proportional, within certain limits, to the rate of respiration, Eqn. 2 can be transformed as fol-

lows:

$$\epsilon_{\Delta\mu_{H^+}}^{\text{ATP synth.}} = \frac{\partial \text{Resp.}}{\partial \Delta\mu_{H^+}} \cdot \frac{\Delta\mu_{H^+}}{\text{Resp.}} \quad (3)$$

where Resp. denotes the rate of active-state respiration, $\Delta\mu_{H^+}$ is the active-state $\Delta\mu_{H^+}$ and $\partial\text{Resp.}/\partial\Delta\mu_{H^+}$ is the ratio of changes of the respiration rate and $\Delta\mu_{H^+}$ produced by a small concentration of the inhibitor. The value of $\epsilon_{\Delta\mu_{H^+}}^{\text{ATPsynth.}}$ for the active-state conditions can be calculated from the titration of the active state with respiratory inhibitors (Figs. 1 and 2). The titration curves can be replotted as changes of $\Delta\mu_{H^+}$ versus changes of the respiration rate. The slope of such plots in the region close to the active state conditions gives the value of $\partial\text{Resp.}/\partial\Delta\mu_{H^+}$. The elasticity calculated in this way amounted to 5–10.

The activity of the respiratory chain is very sensitive to a change of $\Delta\mu_{H^+}$ in phosphorylating and resting-state mitochondria.

A support for this observation can be drawn from two types of experiments. (a) The uncoupler at low concentration added to mitochondria in the resting state markedly influences the rate of respiration, whereas $\Delta\mu_{H^+}$ is subject to a relatively small change only (Figs. 3 and 4, Table II). Thus, even a small decrease of $\Delta\mu_{H^+}$ causes a significant rise in the activity of the respiratory chain. (b) The inhibitor of ATP synthase, oligomycin, affects much more the rate of respiration than the $\Delta\mu_{H^+}$ value (Figs. 1 and 2). Therefore, a small change of $\Delta\mu_{H^+}$ significantly influences the flux through the respiratory chain. This again can be expressed in a quantitative way. The sensitivity of the respiratory chain to a change of $\Delta\mu_{H^+}$ ($\epsilon_{\Delta\mu_{H^+}}^{\text{Resp. chain}}$), calculated in a similar way as described previously from plots of titration of phosphorylating mitochondria with oligomycin, amounted to values close to 10.

The sensitivity of the respiratory chain to $\Delta\mu_{H^+}$ is lost at low values of $\Delta\mu_{H^+}$

It is known [28] that the active-state respiration is only slightly, if at all, stimulated by uncouplers. The rationale for this is the fact that the respiratory rate in this state is limited mostly by the efficiency of the respiratory chain and that rate-controlling steps no longer include the proton leaks [28]. However, uncouplers still produce a drastic decrease of $\Delta\mu_{H^+}$.

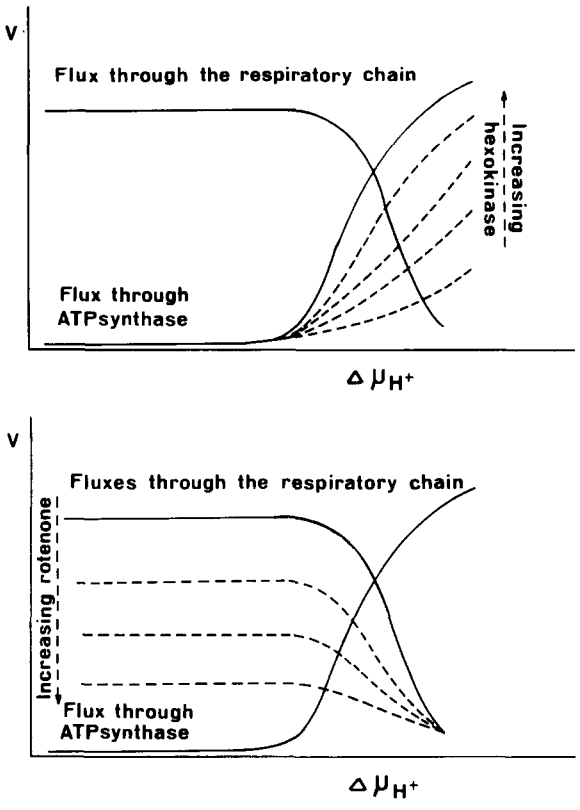

Fig. 5. A model of the dependence of the fluxes through the respiratory chain and ATP synthase on the magnitude of $\Delta\mu_{H^+}$. (A) The flux through ATP synthase is modified by availability of ADP. (B) The flux through the respiratory chain is modified by respiratory inhibitors.

All these observations allow us to propose the following general scheme (Fig. 5A). The flux through ATP synthase becomes significant when $\Delta\mu_{H^+}$ attains the threshold value and then increases rapidly with the increase of $\Delta\mu_{H^+}$. The steepness of the rising part of the curve depends on the availability of ADP (depicted as the dependence on the amount of hexokinase). The flux through the respiratory chain is maximal as long as $\Delta\mu_{H^+}$ is low but it decreases sharply when $\Delta\mu_{H^+}$ becomes high enough. Since the rate of both ATP synthesis and respiration can be expressed as the amount of protons transported through the mitochondrial membrane per unit of time, the interception of these two curves indicates the

situation in which respiration-driven proton extrusion is counterbalanced by proton inflow through ATP synthase. A steady state is then attained. All interception points are situated on the steep part of the respiration curve, illustrating why a large change of the respiration rate is accompanied by a small change of $\Delta\mu_{H^+}$. Thus, $\Delta\mu_{H^+}$ is homeostated between the resting and the active states.

This model describes changes of $\Delta\mu_{H^+}$ in mitochondria undergoing transition from the resting to the active state. When the flux through the respiratory chain is modified by specific inhibitors (assuming that ADP and inorganic phosphate are in excess) another picture emerges (Fig. 5B). Here, the interception points are situated on the steep part of the curve depicting the flux through ATP synthase. For a certain rate of respiration, these points are shifted towards lower values of $\Delta\mu_{H^+}$ as compared to the situation shown in Fig. 5A. This shift is observed in experiments shown in Figs. 1 and 2 (compare $\Delta\psi$ for the active state + oligomycin with $\Delta\psi$ for the active state + respiratory inhibitor). Nevertheless, also under these conditions, $\Delta\mu_{H^+}$ is homeostated. This seems to be a general property of phosphorylating mitochondria.

Taking this into account, one can understand better why large changes of fluxes through the respiratory chain and ATP synthase are accompanied by only minor alterations of $\Delta\mu_{H^+}$. In our opinion, this property of the system allows to explain observed relationships still in terms of the proton-motive force as the formal intermediate of energy coupling, without the necessity of introducing the concept of the localised proton gradient. It also opens a field for further speculations. For example, it may be expected that with a decreased degree of coupling, i.e., under conditions of an increased proton leak, the difference of $\Delta\mu_{H^+}$ between the controlled and the active states will diminish. This assumption needs experimental verification. If it appears to be true, it may explain why some authors [11] have claimed that $\Delta\mu_{H^+}$ does not change between the controlled and the active states in submitochondrial particles the degree of coupling of which is lower than that of intact mitochondria.

The present considerations may also shed new light on the discrepancies between $\Delta\mu_{H^+}$ values in mitochondria stimulated to the same rate of respiration by either hexokinase or an uncoupler [7,9,29]. In contrast to other authors [7,9], we have found [29] that these differences occur only at higher respiratory rates (see also Table II, 93% of active-state respiration). It is well known that uncouplers stimulate mitochondrial respiration, but it has also been observed [30,31] that, at higher concentrations, they can inhibit the respiration. Although the mechanism of this inhibition is not quite clear, from the point of view of our model it can be regarded as analogous to the effect of respiratory inhibitors on $\Delta\mu_{H^+}$. Thus, the action of uncouplers might have a mixed character, i.e., increasing the proton leak but decreasing the flux through the respiratory chain (Fig. 5B), while that of hexokinase would increase the flux through ATP synthase only (Fig. 5A).

Considerations presented so far concern mammalian mitochondria. However, it has to be stressed that similar relationship between $\Delta\mu_{H^+}$ and proton fluxes have also been described for other energy-coupling systems, as chloroplasts and bacterial membrane fragments. For example, a definite threshold value of $\Delta\mu_{H^+}$ below which no ATP synthesis occurs was also found in chloroplasts [32], bacterial chromatophores [33,34] (cf. however, Ref. 35) and intact photosynthetic bacteria [36]. In analogy to mitochondria, chromatophores and respiring bacterial membrane vesicles respond to conditions enabling ATP synthesis with only a small change of $\Delta\mu_{H^+}$ [33,35,37]. Furthermore the rate of coupled electron transport in thylakoids [38] and bacterial fragments [39] can be substantially inhibited without a significant lowering of $\Delta\mu_{H^+}$. These results allow to extend the concept of homeostasis of $\Delta\mu_{H^+}$ over a wide range of energy-coupling membranes.

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