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PROTON ELECTROCHEMICAL GRADIENT AND RATE OF CONTROLLED RESPIRATION IN MITOCHONDRIA

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Summary

The correlation between $\Delta\tilde{\mu}_H$, the proton electrochemical potential difference, and the rate of controlled respiration is analyzed. ΔpH (the proton concentration gradient) is measured on the distribution of [^{3}H]acetate, and $\Delta\psi$ (the membrane potential) on the distribution of $^{86}Rb^+$, $^{45}Ca^{2+}$ and [^{3}H]triphenylmethylphosphonium used either alone or simultaneously.

The effects of the addition of ADP + hexokinase (state-3 ADP) and of carbonylcyanide trifluoromethoxyphenylhydrazone (state-3 uncoupler) on respiration and $\Delta\tilde{\mu}_H$ are not equivalent: the uncoupler depresses $\Delta\tilde{\mu}_H$ more than ADP at equivalent respiratory rates.

The effects of the additions of nigericin-valinomycin and of ionophore A23187 (state-3 cation transport) and of carbonylcyanide trifluoromethoxyphenylhydrazone (state 3-uncoupler) on respiration and $\Delta\tilde{\mu}_H$ are also not equivalent: the uncoupler depresses $\Delta\tilde{\mu}_H$ more than A23187 and nigericin + valinomycin at equivalent respiratory rate. A23187 is very efficient in stimulating respiration with negligible $\Delta\tilde{\mu}_H$ changes.

Introduction

It is now generally accepted that energy conservation is accompanied by formation of a large proton electrochemical potential difference [1] ($\Delta\tilde{\mu}_H$), that $\Delta\mu_H$ can be utilized to drive ATP synthesis, and that abolition of $\Delta\tilde{\mu}_H$ is accompanied by uncoupling. Indeed, uncouplers are known to act as protonophores across artificial and natural membranes. All the above properties of $\Delta\tilde{\mu}_H$ do not

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

necessarily imply that it is the obligatory intermediate in energy transduction but they are consistent with the concept that $\Delta\tilde{\mu}_H$ is an energy store in thermodynamic equilibrium with the various energy transducing units. Whether $\Delta\tilde{\mu}_H$ possesses the kinetic competence to account for the total transfer of energy in energy transducing membranes is therefore an experimental problem attracting increasing interest.

Mitchell and Moyle [2] observed that, with respect to the state 4 level, $\Delta\tilde{\mu}_H$ is decreased 30 mV by ADP and 121 mV by uncouplers. Padan and Rottenberg [3] observed that, at similar respiratory rates, $\Delta\tilde{\mu}_H$ is decreased 5 mV by ADP and 22 mV by uncouplers. Nicholls [4] found that ADP decreases $\Delta\tilde{\mu}_H$ by 50 mV and that the rate of controlled respiration is an inverse linear function of $\Delta\tilde{\mu}_H$ over the range 220 to 166 mV [5]. Mitchell and Moyle [2] as well as Nicholls [4] conclude that their values are compatible with $\Delta\tilde{\mu}_H$ being the obligatory intermediate in energy transduction, while Padan and Rottenberg [3] suggest also a direct interaction between electron transport and ATP synthesis without $\Delta\tilde{\mu}_H$ as intermediate.

In all these investigations the kinetic competence of $\Delta\tilde{\mu}_H$ has been analyzed from the ion distributions and therefore under conditions of steady fluxes. This does not allow for the transient changes of $\Delta\tilde{\mu}_H$ during transitions of the metabolic state. However, the procedures suitable for following the kinetics of the $\Delta\tilde{\mu}_H$ changes have other limitations and are considered to provide less reliable values of $\Delta\tilde{\mu}_H$.

In the present investigation we have extended the analysis of the correlation between rate of controlled respiration and $\Delta\tilde{\mu}_H$ by using the ion distribution methods under steady flux conditions. To obtain information on the kinetic competence of $\Delta\tilde{\mu}_H$, the capacities of ADP and of a number of different ionophores which stimulate the rate of controlled respiration and depress $\Delta\tilde{\mu}_H$ have been determined. The experiments indicate that the effects of ADP, carbonylcyanide trifluoromethyl phenylhydrazone (FCCP), valinomycin + nigericin and A23187 [6,7] are not equivalent. It is conceivable that the rate of controlled respiration induced by the ionophores is affected by the kinetic parameters of cation transport. However a predominant role of localized proton circuits in controlling the rate of electron transfer is also feasible.

Experimental

Rat liver mitochondria were prepared as described previously [8]. The last washing was carried out with an EDTA-free medium. Mitochondria were incubated in 0.2 M sucrose, 10 mM succinate-Tris, 10 mM Tris · Cl, pH 7.4, 0.3 mM KCl (+ valinomycin), 1 μM rotenone and 3 mM P_i-Tris. P_i-Tris was replaced with 10 mM acetate-Tris when the permeant cation was Ca^{2+}. When three permeant cations were measured simultaneously, 30 mM acetate was used. Changes to this medium are described in the legends to the figures. The medium was bubbled thoroughly with oxygen (every 2 min) and the reaction started by the addition of mitochondria. The reaction was terminated by centrifugation at 15 000 rev./min in the rotor S-12 of the Sorvall RC2B centrifuge. The determination of $\Delta\psi$ and ΔpH in the presence of the various cations was carried out essentially as described in the preceding paper [9] with

the following variations. Each centrifuge tube in duplicate, was supplemented with either a labelled permeant cation $^{86}Rb^+$, $^{45}Ca^{2+}$ or [^{3}H]triphenylmethylphosphonium ($TPMP^+$), or [^{3}H]acetate. After centrifugation the supernatant and residual water from the walls were carefully removed and the weight of the pellet determined gravimetrically. The matrix water was calculated by correcting the total pellet water for the extra matrix water. The latter was determined in parallel experiments with [^{14}C]sucrose, and was found to be relatively constant under the prevailing experimental conditions (centrifugation time, incubation medium, etc.), i.e. about 1.8 $\mu l \cdot mg^{-1}$ protein. A variation of the extramatrix water was observed when Mg^{2+} was added to the medium, presumably due to a more extensive aggregation of the pellet. The mitochondrial pellet was dissolved in a Triton-containing liquid scintillation medium and counted in a Packard TriCarb 2455 liquid scintillation spectrometer. The distribution of radioactivity between pellet and supernatant was then determined. The $[cation]_0$ was calculated directly from the number of counts in the supernatant. The $[cation]_i$ was calculated by dividing the total amount of cation per mg protein by the matrix volume per mg protein. With K^+ and $TPMP^+$, the amount of cation in the pellet was corrected for the cation present in the extramitochondrial water and no further correction was attempted for the degree of binding. In the case of Ca^{2+} the amount of cation taken up was also corrected for a constant binding of 30 nmol $\cdot mg^{-1}$ protein. This figure was arrived at in dependent experiments where the increase of matrix volume was measured as a function of the amount of Ca^{2+} taken up. Whether the amount of Ca^{2+} uptake was varied by varying the amount of Ca^{2+} added or by adding variable amounts of uncoupler there was no increase of matrix volume below an uptake of 30 nmol $\cdot mg^{-1}$ protein. It is likely that this is the amount of divalent cation which remains bound to phospholipid binding sites at the matrix side of the inner membrane. The use of the total amount of ions, although corrected as described above, implies an activity coefficient of 1.

ΔpH was calculated on the basis of the distribution of acetate. As discussed previously [9] the counts of [^{3}H]acetate in the pellet were corrected for the number of counts in an uncoupler supplemented sample. The concentration of acetate in the matrix was then calculated by dividing the corrected counts of acetate for the matrix volume as measured gravimetrically.

The centrifugation procedure to measure the ion distribution has been questioned on the basis of the argument that anaerobiosis is reached rapidly in the pellet with subsequent alteration of the ion distribution between inner and outer spaces [10]. However, it has repeatedly been shown [11,12] that similar results are obtained whether or not separation of the subcellular structures from the medium is carried out. Secondly, although a shift from the matrix to the extramatrix space certainly occurs within the pellet after centrifugation, this does not affect the total ion content of the pellet. The ion distribution between the matrix space and the medium during incubation is reflected in the ion distribution between total pellet (matrix plus extramatrix space) and supernatant, and may be modified only to the extent that there is ion diffusion from the pellet to the supernatant. This is however too slow, due to the restricted surface, to affect significantly the ion content in the pellet. A similar argument also applies to the ratio between matrix and extramatrix volumes as measured from the [^{14}C]sucrose content of the pellet.

$[Mn^{2+}]_i$ was measured on the basis of the ESR sextet signal of free Mn^{2+} after chelation of external Mn^{2+} with EDTA as described in previous studies [10,11].

Respiration was measured with a Clark oxygen electrode in a thermo-equilibrated cuvette. Addition of the various ionophores was made sequentially to the same sample. The time interval between each addition was 60—120 s which was sufficient to obtain a constant respiratory rate. In parallel experiments ionophores were added in a single concentration to each sample. The respiratory rates obtained under the two conditions were identical. All data are the average of 6—8 independent experiments (standard deviation not greater than 5%). Chemicals were of the highest purity available. [^{3}H]Triphenylmethylphosphonium was kindly provided by Dr. R. Kabach.

Results and Discussion

Comparison between ADP and uncouplers

In Fig. 1 stimulation of respiration and depression of $\Delta\tilde{\mu}_H$ were obtained by adding increasing amounts either of carbonylcyanide trifluorimethoxyphenylhydrazone (FCCP) or of hexokinase/glucose/Mg^{2+} and ADP. The combination of herokinase/glucose/Mg^{2+} and ADP was used in order to maintain a constant state-3 respiratory rate over the 5 min incubation period. $\Delta\psi$ was measured on the distribution of K^+. The effects of FCCP and of ADP were not equivalent. ADP stimulated respiration more effectively than FCCP, while FCCP depressed $\Delta\tilde{\mu}_H$ more effectively than ADP. At identical respiratory rates, $\Delta\tilde{\mu}_H$ was smaller with FCCP. The slope of the plot, respiratory rate vs. $\Delta\tilde{\mu}_H$, was steeper with ADP than with FCCP.

The use of parallel instead of simultaneous determinations of $\Delta\tilde{\mu}_H$ and respiration may give rise to two sources of error, the first and more trivial being

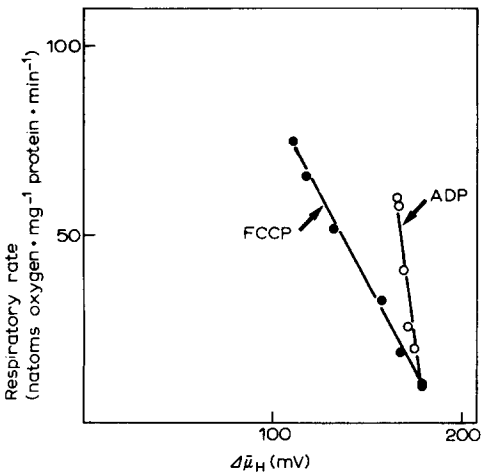

Fig. 1. Effect of ADP and uncouplers on respiration and $\Delta\tilde{\mu}_H$. The medium contained 0.2 M sucrose, 10 mM succinate-Tris, 10 mM Tris-Cl, 3 mM P_i-Tris pH 7.4, 1 μM rotenone, 2 mM $MgCl_2$, 0.3 mM KCl, 0.2 μg valinomycin/ml and 4 mg mitochondrial protein. Stimulation of respiration was obtained by increasing amounts of either FCCP or hexokinase + ADP. Time of incubation, 5 min.

the variability of the mitochondrial preparations and the other the possibility that $\Delta\tilde{\mu}_H$ may not remain constant in the course of the incubation.

The procedure of stimulating the respiratory rate and depressing $\Delta\tilde{\mu}_H$ by adding increasing concentrations of uncouplers and ADP/hexokinase/glucose/Mg^{2+}, permits a continuous variation of the two parameters. The sources of error arising from determining respiration and $\Delta\tilde{\mu}_H$ in parallel are then minimized since the effect of uncouplers and of ADP/hexokinase/glucose/Mg^{2+} are compared on the basis of the slopes rather than on single values.

The results of Fig. 1 agree with the conclusion of Nicholls [4,5] that a close correlation exists between rate of controlled respiration and $\Delta\tilde{\mu}_H$ and that the rate of controlled respiration is an inverse linear function of $\Delta\tilde{\mu}_H$ in a range of high $\Delta\tilde{\mu}_H$ values. At higher concentrations of uncouplers, when the possibility of increasing the respiratory rate is exhausted, a small increase of respiratory rate is accompanied by a large decline of $\Delta\tilde{\mu}_H$ (not shown and Nicholls [5]). On the other band the conclusion that $\Delta\tilde{\mu}_H$ is the sole determinant of the rate of controlled respiration is not in accord with the difference in slopes induced by ADP and uncouplers in the experiment of Fig. 1. Padan and Rottenberg [3] attributed the more marked depression of $\Delta\tilde{\mu}_H$ by uncoupler to a direct interaction between electron transport and ATP synthesis. To test this hypothesis the relation between respiratory rate and $\Delta\tilde{\mu}_H$ is analyzed below in the presence of various ionophores.

Comparison between various ionophores

Fig. 2 shows the effect of FCCP and nigericin + valinomycin on $\Delta\tilde{\mu}_H$ as measured on the distribution of Ca^{2+} and $TPMP^+$, rather than K^+. The values of $\Delta\psi$ at low K_0^+ are higher than those obtained with other permeant cations [2,9,12]. There is no way yet of deciding which values are correct. Secondly,

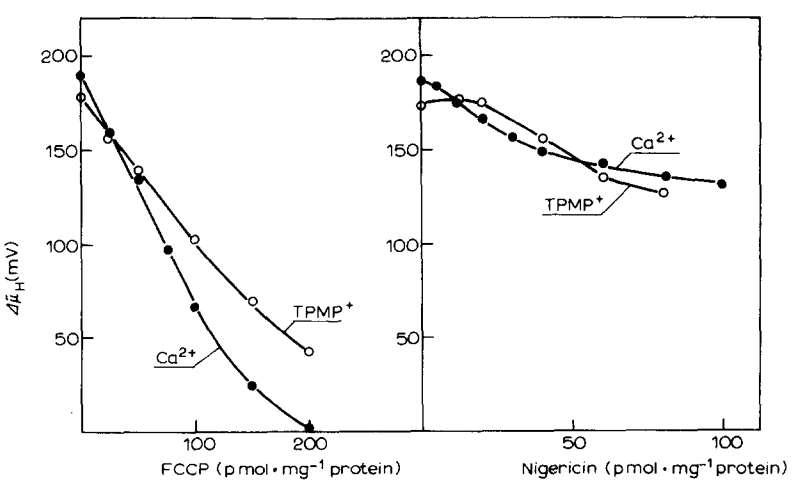

Fig. 2. Effect of uncouplers and valinomycin + nigericin on $\Delta\tilde{\mu}_H$. Experimental conditions as in Fig. 1 except that $MgCl_2$ was omitted and P_i was replaced with acetate in the presence of Ca^{2+}. Amount of permeant cations was 200 μM Ca^{2+}, 0.3—0.5 mM KCl (+0.2 μg valinomycin/ml) and 25 μM $TPMP^+$. As indicated in the abscissa depression of $\Delta\tilde{\mu}_H$ was obtained by increasing amounts of FCCP and nigericin (+ valinomycin). 4 mg mitochondrial protein. Time of incubation, 4 min.

addition of uncouplers to valinomycin-treated mitochondria leads to depletion of matrix K^+ and then to respiratory inhibition. This implies a metabolic shift from state 3 to state 5 which may be relevant for the correlation between $\Delta\tilde{\mu}_H$ and respiratory rate.

The use of the divalent cations has other limitations. $\Delta\tilde{\mu}_H$ must be measured in the presence of acetate and not of P_i. Secondly, there is a discrepancy between the values of $[cat^{2+}]_i$ based on Ca^{2+} on Mn^{2+} and Sr^{2+} distributions [9]. Thirdly, the values of $\Delta\psi$ based on matrix volume or total uptake are presumably overestimated, as found for Mn^{2+} by comparing ESR and total uptake data [9,11]. Fourthly, a disequilibrium exists between steady state distribution of divalent cations and $\Delta\psi$ calculated on the K^+ distribution. The disequilibrium is large at high $\Delta\psi$ values and disappears below values of $\Delta\psi$ of 100 mV [9]. Finally the uptake of 200 μM Ca^{2+} implies a $\Delta\bar{p}H$ of about 1 unit while that of 25 μM $TPMP^+$ implies a negligible ΔpH.

Other criticisms apply to $TPMP^+$, an organic cation which owes its lipophilicity to charge screening. First, it must be used in low concentrations because it damages the membrane; under these conditions, the changes of the matrix volume are negligible. Secondly, it is impossible to assess the extent to which binding in the matrix reduces the ion activity; such a reduction may become more significant under conditions of limited uptake.

In Fig. 2 the decline of $\Delta\tilde{\mu}_H$ induced by FCCP was more marked when determined in the presence of Ca^{2+} than in the presence of $TPMP^+$; at 200 pmol FCCP · mg^{-1} protein, $\Delta\tilde{\mu}_H$ was about zero on Ca^{2+} and about 40 mV on $TPMP^+$. In contrast the decline of $\Delta\tilde{\mu}_H$ induced by nigericin + valinomycin was more marked in the presence of $TPMP^+$ than in the presence of Ca^{2+}. At 60 pmol nigericin · mg^{-1} protein, $\Delta\tilde{\mu}_H$ was about 140 mV with Ca^{2+} and 135 mV with $TPMP^+$.

In Fig. 3 the values of $\Delta\tilde{\mu}_H$ obtained with the various amounts of FCCP and

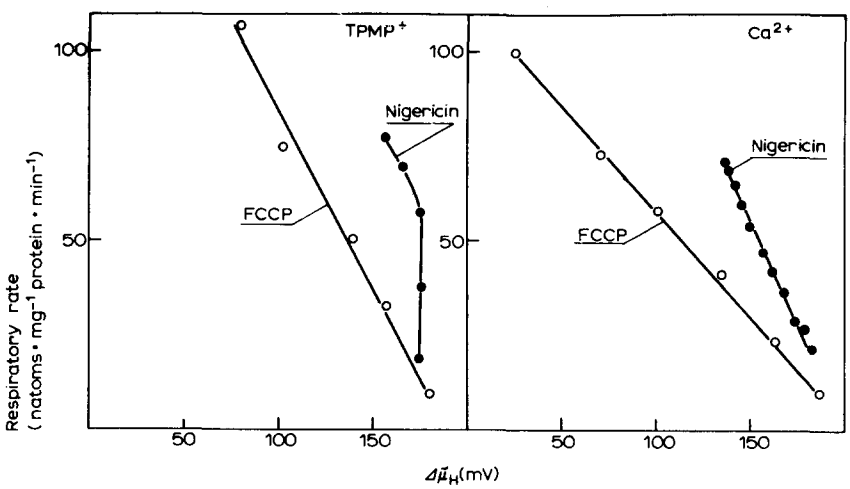

Fig. 3. Effect of ionophores on respiration and $\Delta\tilde{\mu}_H$. The values for $\Delta\tilde{\mu}_H$, as induced by the various concentrations of FCCP or nigericin (+ valinomycin) are taken from the experiments shown in Fig. 2. The values for respiration are taken from experiments carried out under identical conditions as explained in Experimental.

nigericin + valinomycin are plotted against the rates of controlled respiration. The effects were not equivalent. The stimulation of respiration, in respect to the depression of $\Delta\tilde{\mu}_H$, was more pronounced with nigericin + valinomycin than with FCCP. As in Fig. 1, the slope of the plot was steeper with nigericin + valinomycin than with FCCP. The patter was similar whether based on the values of $\Delta\tilde{\mu}_H$ obtained in the presence of Ca^{2+} or of $TPMP^+$ except that the slopes were always steeper in the latter case.

The comparison between effects of FCCP and nigericin + valinomycin may be criticized on the basis of the argument that in the former case there is a constant matrix volume due to the impermeability of the inner membrane to K^+ while in the latter case the matrix swells due to K^+ influx. The activity of matrix Ca^{2+} and $TPMP^+$ may not be identical when the membrane is rendered freely permeable to K^+. This question is analyzed in the experiment of Fig. 4. The concentration of free Mn^{2+} in the matrix was measured on the basis of the ESR sextet signal of Mn^{2+} $(H_2O)_6$, in mitochondria treated or not with valinomycin. Using this technique the bound Mn^{2+} is not measured and Mn^{2+} concentration becomes equal to Mn^{2+} activity. The plot of Fig. 4 provides the following information: (a) the concentration of free Mn^{2+} in the presence of the various amounts of FCCP is lower than the concentration of matrix Ca^{2+} calculated with the correction of 30 nmol · mg^{-1} protein Ca^{2+} bound (cf. also ref. 9); (b) in the presence of valinomycin the concentration of free Mn^{2+} was about 30% decreased; since in the presence of valinomycin the matrix volume undergoes a corresponding increase due to K^+ uptake, it appears that the activity of divalent cations is not altered by the occurrence of a simultaneous K^+ transport.

Further information about the validity of the $\Delta\tilde{\mu}_H$ measurements is provided by the experiments of Figs. 5—7. In these experiments three permeant cations

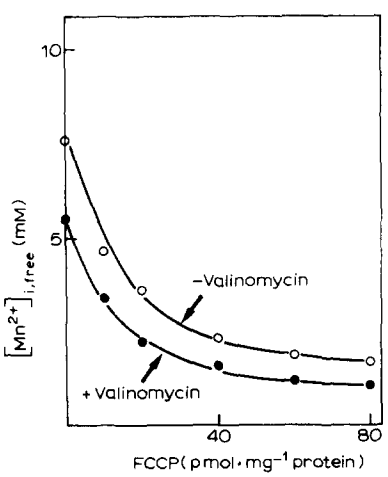

Fig. 4. Effect of uncouplers on $[Mn^{2+}]_i$ in the absence and presence of valinomycin. The medium contained 0.2 M sucrose, 10 mM succinate-Tris, 10 mM Tris-Cl, pH 7.4, 1 μM rotenone, 30 mM acetate-Tris, 300 μM $MnCl_2$, 0.3 mM KCl, 5 mM $MgCl_2$ and 3 mg protein/ml. When indicated valinomycin was 0.2 μg/ml. The determination of $[Mn^{2+}]_i$ was carried out as described in refs. 9—11.

were present simultaneously 200 μM Ca^{2+}, 25 μM TPMP and 0.3 mM K^+ (+ valinomycin). A concentration of 30 mM acetate was used. This concentration was selected in order to increase the uptake of the univalent cations although it resulted in a smaller respiratory stimulation. Fig. 5 shows the effect of ionophore A23187, nigericin + valinomycin and FCCP on $\Delta\tilde{\mu}_H$ measured on Ca^{2+}. 250 pmol A23187 · mg^{-1} protein induced a decline of $\Delta\tilde{\mu}_H$ of less than 30 mV while 40 pmol nigericin · mg^{-1} protein induced a decline of about 50 mV. Larger declines of $\Delta\tilde{\mu}_H$ were obtained with higher amounts of A23187 and nigericin (not shown). 200 nmol FCCP · mg^{-1} protein reduced $\Delta\tilde{\mu}_H$ to zero. There is an important difference in the effect of FCCP as measured only in the presence of Ca^{2+} (Fig. 2) or in the presence of other permeant cations (Fig. 5), in that in the latter case low FCCP concentrations modified $\Delta\tilde{\mu}_H$ on Ca^{2+} only slightly.

Fig. 5B shows the plot of the rate of controlled respiration vs. $\Delta\tilde{\mu}_H$. The slope of the plot, which was not linear as in Figs. 1 and 3, decreased in the order A23187 > nigericin > FCCP.

Fig. 6 shows the effect of A23187, nigericin + valinomycin and FCCP on $\Delta\tilde{\mu}_H$ as measured on K^+. There was good agreement with the data of Fig. 5. 250 pmol A23187 · mg^{-1} protein caused a decline of $\Delta\tilde{\mu}_H$ of 60 mV (compare 30 mV on Ca^{2+}, Fig. 5). The effect of low nigericin concentrations on the K^+ distribution, was very marked. At the higher nigericin concentrations the effect was comparable to that of A23187. FCCP caused an almost linear decline of $\Delta\tilde{\mu}_H$ in the range 0—100 pmol · mg^{-1} protein. In the plots of Fig. 6B, again the slope was much steeper for A23187 than for FCCP. As for nigericin a break was observed between the low concentration range where the ionophore caused

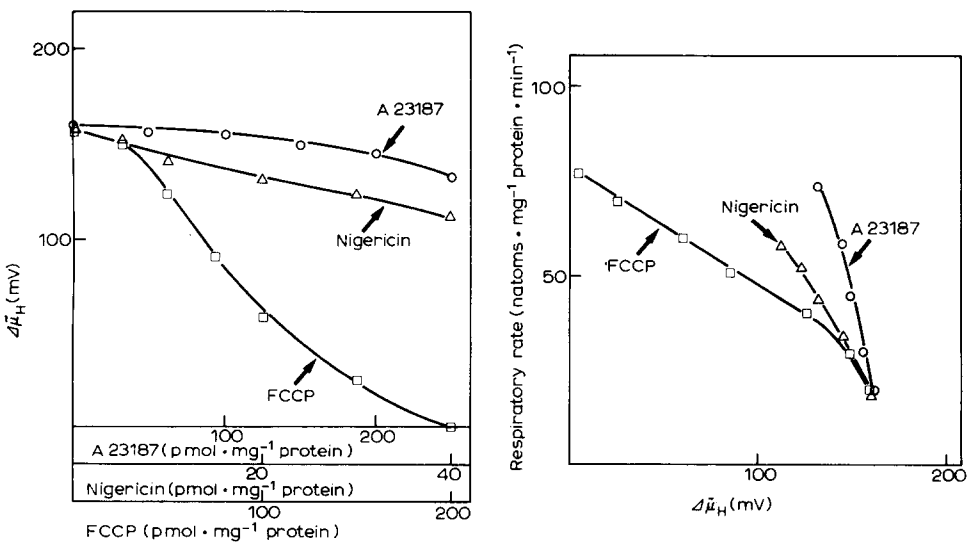

Fig. 5. Effect of A23187, nigericin (+ valinomycin) and FCCP on $\Delta\tilde{\mu}_H$ measured on Ca^{2+} distribution. Correlation with respiration. The medium contained 0.2 M sucrose, 10 mM succinate-Tris, 30 mM acetate-Tris, 10 mM Tris-Cl, pH 7.4, 1 μM rotenone, 0.3 mM KCl, 25 μM $TPMP^+$, 200 μM $CaCl_2$ and 4 mg mitochondrial protein. Valinomycin was 1 μg/ml except in the presence of nigericin when it was 2 μg/ml. Time of incubation 5 min.

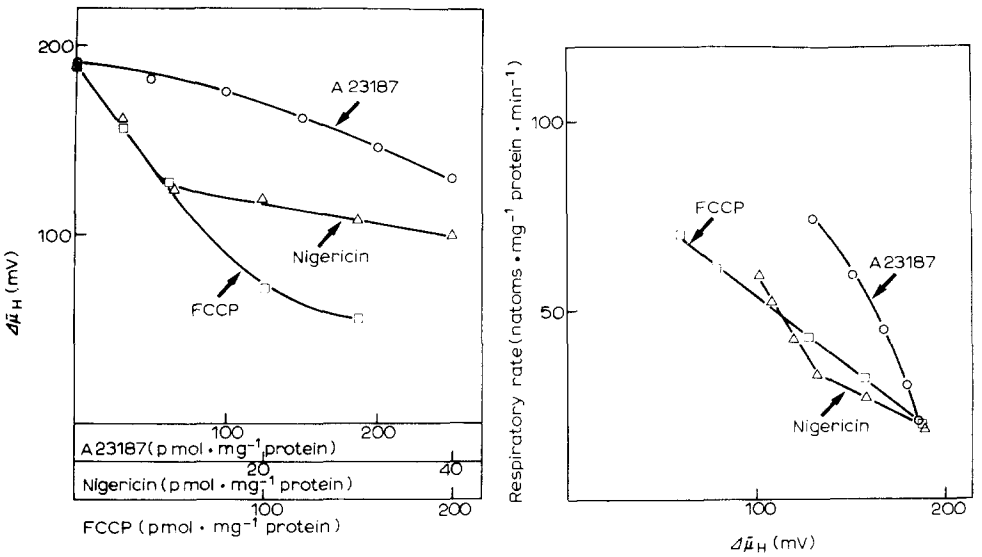

Fig. 6. Effect of A23187, nigericin (+ valinomycin) and FCCP on $\Delta\tilde{\mu}_H$ measured on K^+ distribution. Correlation with respiration. Experimental conditions as in Fig. 5.

a major decline of $\Delta\tilde{\mu}_H$, and the high concentration range where the effect on $\Delta\tilde{\mu}_H$ was more reduced.

Fig. 7 shows the effect of A23187, nigericin + valinomycin and FCCP, on $\Delta\tilde{\mu}_H$ as measured on $TPMP^+$. There was significant agreement between the decline of $\Delta\tilde{\mu}_H$ induced by A23187 and nigericin as measured on Ca^{2+} and $TPMP^+$ (Figs. 5 and 7). Concerning the effect of FCCP, at low uncoupler concentrations there was also on the $TPMP^+$ distribution a smaller decline similar to that observed with Ca^{2+} and in contrast with that observed with K^+. On the other hand the decline of $\Delta\tilde{\mu}_H$ at high FCCP was less marked on $TPMP^+$ than on Ca^{2+}. This is presumably due to the difficulty of accounting for the extent of binding under conditions of low $TPMP^+$ uptake. The plot of Fig. 7B, rate of controlled respiration vs. $\Delta\tilde{\mu}_H$, revealed the usual pattern. The relation was not linear. The slope decreased in the order A23187 > nigericin > FCCP.

A comparison of the data of Fig. 3 with those of Figs. 5—7 indicates that the respiratory stimulation was larger in the former than in the latters. This is presumably due to the presence of 30 mM acetate and 25 μM $TPMP^+$ in Figs. 5—7 that leads to a slight increase of the basal respiratory rate.

The calculation of $\Delta\psi$, on the Ca^{2+} distribution in the presence of A23187, and on the K^+ distribution in the presence of nigericin, is, in the experiments of Figs. 5 and 6, incorrect. Indeed application of the Nernst equation is allowed only when the permeant cations are at electrochemical equilibrium. This may be the case when the rate of electrical cation entry is much faster than that of electroneutral cation efflux. However when the rate of electroneutral cation efflux becomes appreciable as at the higher A23187 and nigericin concentrations, the cation distribution depends on the difference between rates of influx and of efflux. Addition of A23187 and of nigericin therefore results in a disequilibrium between Ca^{2+} and K^+ distribution, respectively, and real $\Delta\psi$. This

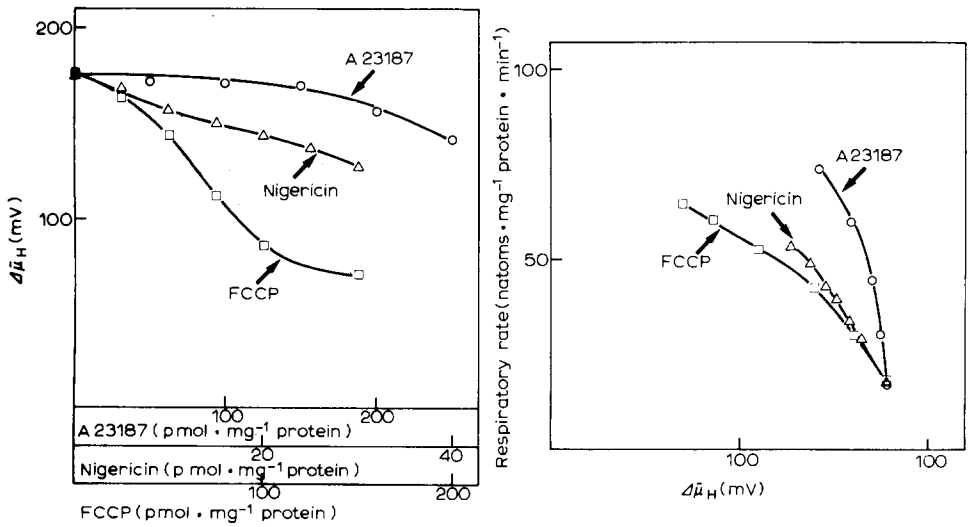

Fig. 7. Effect of A23187, nigericin (+ valinomycin) and FCCP on $\Delta\tilde{\mu}_H$ measured on $TPMP^+$ distribution. Correlation with respiration. Experimental conditions as in Fig. 5.

means that in the presence of A23187 and nigericin, the values of $\Delta\tilde{\mu}_H$ calculated on Ca^{2+} and K^+ are lower than the real $\Delta\tilde{\mu}_H$ values, and the slopes of the plots of respiratory rate vs. $\Delta\tilde{\mu}_H$ values should be even steeper than indicated in Fig. 5 and 6. The comparison with the values of $\Delta\psi$ based on the $TPMP^+$ distribution (cf. Fig. 7) furthermore indicates that the lowering of the apparent $\Delta\psi$ due to the additions of nigericin and A23187 is not large. FCCP at high concentrations caused a greater drop of $\Delta\tilde{\mu}_H$ when calculated on Ca^{2+} (or on Mn^{2+} see Fig. 4) than on $TPMP^+$ or K^+; this is presumably due to the uncertainty in the calculation of the matrix cation concentration at low $TPMP^+$ or K^+; the matrix cation concentration was calculated in the case of Ca^{2+} after a correction for a constant binding of 30 nmol · mg^{-1} protein and in the case of Mn^{2+} with the ESR technique. FCCP at low concentrations caused a greater drop of $\Delta\tilde{\mu}_H$ on K^+ than on Ca^{2+} or $TPMP^{2+}$; this is observed only when more than one permeant cation is present and will be discussed elsewhere.

From the experiments of Figs. 3 and 5—7 it appears that the plot, respiratory rate vs. $\Delta\tilde{\mu}_H$, has: (i) a steeper slope with nigericin + valinomycin than with FCCP in the presence of a single permeant species, and (ii) a decreasing slope for A23187 > nigericin + valinomycin > FCCP in the presence of three permeant species. A23187 is very efficient in stimulating respiration without depressing $\Delta\tilde{\mu}_H$. This capacity of A23187 is apparent also in Fig. 3 of Pfeiffer et al. [7] where 200 pmol · mg^{-1} protein ionophore causes an increase of the respiratory rate from 15 to 90 natoms oxygen · mg^{-1} protein · min^{-1}, with an increase of $[Ca^{2+}]_0$ amounting to a few % of the total Ca^{2+} uptake. Furthermore Hutson et al. [16] have shown that in the presence of A23187 half respiratory stimulation is obtained at 3.1 μM $[Ca^{2+}]_0$. Under the conditions of Fig. 5 an increase of $[Ca^{2+}]_0$ from 2 to about 10 μM, corresponding to a depression of $\Delta\psi$ of 20 mV, is accompanied by a stimulation of the rate of controlled respiration from 16 to 70 natoms · mg^{-1} protein · min^{-1}.

Conclusion

Large variations in the relationship between respiratory rate and $\Delta\tilde{\mu}_H$ are observed not only with the use of ADP and uncouplers but also with a number of different ionophores. There are two alternatives. One, $\Delta\tilde{\mu}_H$ is not the sole determinant of the rate of controlled respiration. For example in the case of the respiratory stimulation by Ca^{2+} + A23187 it may be that a predominant role is played by the kinetic parameters determining the interaction of Ca^{2+} with its carrier. A low K_m in the absence of Mg^{2+} leads to large respiratory stimulations at minimal variations of the Ca^{2+} concentrations. This is not the case of nigericin which exerts a more sluggish effect. It is known that in the case of the valinomycin induced K^+ transport, large variations of $[K^+]_0$ are required to affect significantly the respiratory rate. This points to a kinetic control of the respiratory stimulation induced by ionophores. Two, the determination of $\Delta\tilde{\mu}_H$ in the bulk phases does not provide a reliable estimate of the thermodynamic potential of the protons in the electron transfer unit, and thus of the force regulating the respiratory rate. This does not exclude proton gradients as obligatory intermediates in energy transduction, but necessitates discrimination between macroscopic and microscopic events especially with regard to the role the localized proton circuits.

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