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Flux Ratios and Pump Stoichiometries at Sites II and III in Liver Mitochondria

EFFECT OF SLIPS AND LEAKS*

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Siro Luvisetto, Elena Conti, Maurizio Buso, and Giovanni Felice Azzone

From the Consiglio Nazionale delle Ricerche Unit for the Study of Physiology of Mitochondria and Institute of General Pathology, University of Padova, 35121 Padova, Italy

Addition of bovine serum albumin to state 4 mitochondria results in a depression of the proton leak and of the resting respiration of 70 and 25%, respectively. The conductance membrane potential diagram, both in the ohmic and in the non-ohmic region, shows that in the presence of bovine serum albumin the level of ohmic conductance is lowered while that of non-ohmic conductance is increased toward higher $\Delta\psi$ values. The same effect is observed during operation of the different proton pumps.

Addition of chloroform affects the conductance membrane potential diagram in the following manner: there is no effect in the ohmic region with all pumps, while there is an effect in the non-ohmic region either at site III or at sites II plus III but not at site II. This suggests a possible effect of chloroform at the level of the cytochrome oxidase proton pump.

During titration with oligomycin of the ATPase proton pump the conductance potential diagram shows a region of non-ohmicity only in the presence but not in the absence of an ATP-regenerating system.

Protonophoric uncouplers such as carbonyl cyanide *p*(trifluoromethoxy)phenylhydrazone and intrinsic uncouplers such as chloroform have different effects on the relationship between rates of charge translocation and of oxygen consumption, and thus on the pump stoichiometries, in that the slope of the diagram is modified by the latter but not by the former. The differential effects of protonophores and of intrinsic uncouplers on the stoichiometries have been analyzed by computer simulations and represent an additional criterion to distinguish between extrinsic and intrinsic mechanisms of uncoupling.

The chemiosmotic model of free energy transduction (1) assumes that the rate of the resting respiration is controlled by, and proportional to, the level of $\Delta\tilde{\mu}_H$.[1] Nicholls (2) reported that the decline of respiration during titration with respiratory inhibitors was accompanied by a decline of $\Delta\tilde{\mu}_H$ which was proportional at low, and non-proportional at high, values of $\Delta\tilde{\mu}_H$. Nicholls suggested that the non-ohmicity was a property of the lipid bilayer and that the proton conductance of the mitochondrial membrane be ohmic in the low $\Delta\tilde{\mu}_H$ range and non-ohmic in the high $\Delta\tilde{\mu}_H$ range. In subsequent studies Pietrobon *et al.* (3) argued that if the non-ohmicity were a property of the lipid bilayer it should have been independent of the type of proton pump under operation. Instead, the non-ohmic behavior was shifted in a different $\Delta\tilde{\mu}_H$ range according to the type of proton pump. Furthermore, the rate of the state 4 respiration was much higher than could be accounted for by the membrane proton conductance in the non-ohmic region (4–7). It was then proposed that the non-proportionality between respiration and $\Delta\tilde{\mu}_H$ was essentially due to intrinsic uncoupling (slip) of the proton pumps (4–6). It has been shown that both general anesthetics and gramicidin (in low salt media) induce a $\Delta\tilde{\mu}_H$-independent uncoupling which is accompanied by an increase of the apparent non-ohmicity (7–10). More recently, Brown (11) has performed titrations of reaction rates against $\Delta\tilde{\mu}_H$ and obtained, in contrast with earlier results, the same titrations with different pumps and inhibitors. He concluded that no slip need be invoked to explain these relationships.

The slip concept has implications on several controversial issues such as that of the stoichiometry of the pumps. Values for the H^+/O ratio between 6 and 8 have been reported during operation of the redox proton pumps at sites II plus III. Murphy and Brand (12, 13) have recently concluded that the stoichiometry of proton pumping changes with the variation of $\Delta\tilde{\mu}_H$ (output force of redox pumps), and thus that the mechanistic stoichiometry of the redox proton pumps is intrinsically variable.

What is referred to as measurement of the pump stoichiometry is usually an assay of the ratio between input and output flows, a ratio which is related to the phenomenological stoichiometry of the pumps by the equations of nonequilibrium thermodynamics:

$$J_H/J_O = (Zq)_{lf} \quad (1)$$

$$J_H/J_O = [Z(Z\Delta\tilde{\mu}_H/\Delta G_{ox} + q)]/(Zq\Delta\tilde{\mu}_H/\Delta G_{ox} + 1) \quad (2)$$

where J_H and J_O are the output and input flows, $\Delta\tilde{\mu}_H$ and ΔG_{ox} are the output and input forces, Z is the phenomenological stoichiometry, and q is the degree of coupling. Equation 1 indicates that the flux ratio corresponds to the phenomenological stoichiometry Z (multiplied by the degree of coupling q) only at level flow (lf). Equation 2 indicates that at any output force different from level flow the flux ratio is a complex function of several parameters.

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[1] The abbreviations used are: $\Delta\tilde{\mu}_H$, transmembrane proton electrochemical potential gradient; J_O, J_O^{sh}, rate of respiration in static head; J_e, rate of electron transfer; J_{Fe}, rate of ferrocyanide reduction; J_K, rate of K^+ efflux; J_{ATP}, rate of ATP hydrolysis; J_H^{ATP}, rate of ATPase-proton pumping; L_H^L, membrane proton-leak conductance; L_K, membrane K^+ conductance; $\Delta\psi$, transmembrane electrical potential gradient; n_e, H^+/e^- stoichiometry; BSA, bovine serum albumin; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; FCCP, carbonyl cyanide *p*(trifluoromethoxy)phenylhydrazone; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; $TPMP^+$, triphenylmethyl-phosphonium ion; RLM, rat liver mitochondria.

Recently, Beavis and Lehninger (14, 15), by applying the non-equilibrium thermodynamic analysis of van Dam *et al.* (16, 17), have compared the thermodynamic and the kinetic approaches to the determination of the stoichiometries. Given that an incomplete coupling between two metabolic fluxes is due to the fact that one of the intermediates takes part in side reactions, it follows that the ratio between the output and the input fluxes must be different from the mechanistic stoichiometry of the pump. Beavis (18, 19) has then proposed a very elegant and simple method to determine the upper and lower limit of the redox pump stoichiometries by selecting conditions where the side reactions of the intermediate are either minimized or maximized.

In the present study, we have analyzed (*a*) the effects of BSA and of chloroform, which reduce the passive proton leakage and act as a slip inducer, respectively, on the pattern of the so-called ohmic and non-ohmic behaviors of the various redox proton pumps, (*b*) the significance of the dependence of the flux ratio on the magnitude of $\Delta\psi$, (c) the effects of the variations of leaks and slips on the stoichiometries of the proton pumps at sites II and III of the respiratory chain, and (d) the slip in the ATPase proton pump. The evidence in favor and against the slip concept will be shortly reviewed.

EXPERIMENTAL PROCEDURES

Materials—Rat liver mitochondria were prepared in 0.25 M sucrose, 10 mM Tris, 0.1 mM EGTA, according to standard procedures (20), and all the experiments were performed within 4 h of preparation. The mitochondrial protein was assayed with the biuret method using serum albumin as standard. The medium used for measurements referred to sites II + III, with succinate as substrate, contained 0.22 M sucrose, 10 mM succinate/Tris, 30 mM MOPS/Tris, 5 mM P_i/Tris, 0.1 mM EGTA/Tris, 5 μM rotenone, 1 μg/mg oligomycin, pH 7.4, *T* 25 °C. In the measurement referred to as site II, the medium was supplemented with 2 mM KCN and 1.5 mM $Fe(CN)_6(Tris)_3$, while in the measurements referred to as the site III succinate was omitted and the medium was supplemented with 2 mM ascorbate, 50 ng/mg antimycin, and variable amounts of $Fe(CN)_6(Tris)_4$ or TMPD. The ferrocyanide and ferricyanide-Tris salts were obtained from corresponding K^+ salts by replacement of K^+ with $Tris^+$ in a column containing the ion-exchanger resin Dowex 50W. Other media used are specified in the legends of figures. All reagents were of maximal purity commercial grade. BSA (fraction V), enzymes, inhibitors, and valinomycin were purchased from Sigma. The uncoupler FCCP was supplied by Dr. G. Heitler of Du Pont and chloroform by Riedel De Haen (99%).

Determinations of the Respiratory Rates and of K^+ Uptakes—The respiratory rates at sites II + III and at site III were estimated from the rates of oxygen consumption, whose concentration in the medium was measured polarographically with a Clark electrode (Yellow Spring) equipped with a Teflon membrane in a closed thermostated and stirred vessel. The zero oxygen point was determined with an excess of dithionite. The respiratory rate at site II was estimated from the rate of reduction of $Fe(CN)_6^{3-}$ measured spectrophotometrically on an AMINCO DW2a dual-wavelength spectrophotometer equipped with magnetic stirring and thermostatic control, following continuously the decrease in absorbance at 420 minus 480 nm.

The rate of K^+ uptake in valinomycin-treated mitochondria was measured with a Schott K^+ electrode (response time 1 s) and a glass combination electrode (Beckman) serving as reference bathed in a thermostated vessel, open to air. The electrodes were connected to a Radiometer 26 pH-meter, and the output was fed into a Perkin-Elmer Cetus Instruments model R100 A chart recorder (21, 22).

Determination of the ATPase Activity—The ATPase activity was evaluated by measuring the rate of ATP hydrolysis or the rate of proton flux through the ATPase. The rate of ATP hydrolysis, J_{ATP}, was measured spectrophotometrically on an AMINCO DW2a dual-wavelength spectrophotometer continuously following the decrease in absorbance at 340 minus 374 nm due to NADH oxidation in the presence of excess phosphoenolpiruvate, piruvate kinase, and lactate dehydrogenase. The experimental procedures were essentially the same as described in Pietrobon *et al.* (23). The rate of proton flux through the ATPase, J_H^{ATP}, was estimated from the pH change continuously measuring the absorbance change of phenol red (60 μM) at 576 − 620 nm. In this case the experimental procedure was essentially the same as described by Brown (11). Control experiments show identical results when the pH change was measured with a pH electrode.

Determination of $\Delta\psi$—The transmembrane electrical potential, $\Delta\psi$, was evaluated from the distribution of the lipophilic ion $TPMP^+$ (21). The concentration of TPMP in the incubation medium was followed continuously by using a TPMP-sensitive membrane electrode (response time about 10 s). The initial concentration of TPMP in the medium was 5 μM. The concentration of TPMP in the mitochondrial matrix was calculated from the amount of probe taken up by the mitochondria and the matrix volume which was taken as 1 μl/mg of protein under the prevailing experimental conditions (22). In the calculation of the membrane potential, TPMP binding was taken into account and corrected for. TPMP binding was evaluated by the amount of residual TPMP bound in mitochondria treated with excess of FCCP and antimycin (21, 33). No correction was used for the activity coefficient of TPMP in the matrix. Control experiments have shown that under the prevailing experimental conditions, namely the presence of 5 mM of P_i in the incubation medium, the transmembrane proton gradient, ΔpH, is negligible. The variations of $\Delta\psi$ can be considered to reflect to a good approximation those of $\Delta\tilde{\mu}_H$.

RESULTS

Effect of BSA on Leaks and Slips—The proportional relationship between respiration and proton electrochemical gradient has been attributed to membrane leaks, while the non-proportional relationship has been attributed either to non-ohmic behavior of the membrane (2, 24) or to slips in the pumps (3). The latter concept is supported by the fact that agents which increase the passive proton permeability cause an upward shift in the ohmic region of the $nJo/\Delta\psi$ relationship while agents which increase the intrinsic uncoupling cause a shift in the non-ohmic region (7–10). Furthermore, the quantitative assay of the passive proton permeability has been used as a tool to determine the relative contributions of leaks and slips during the basal respiratory rate (5). Since BSA is known to bind fatty acids which cause an increase of the proton leakage, we have analyzed the effect of BSA on the relationship between leaks and slips.

As discussed elsewhere (5) the passive proton permeability may be measured on the rate of K^+ efflux in respiratory inhibited mitochondria. The approach depends on the assumption that the rate of K^+ efflux depends on the rate at which protons diffuse into the matrix and collapse the K^+ diffusion potential. If BSA reduces the passive proton permeability, it should also reduce the rate of K^+ efflux. However BSA also binds valinomycin that leads to the requirement of increasing valinomycin concentrations in order to always render the rate of K^+ efflux non-limiting. Fig. 1 shows the effect of increasing BSA concentrations on the rate of K^+ efflux as measured at increasing valinomycin concentrations. It is seen that the higher the BSA concentrations the lower was the rate of K^+ efflux. The binding of valinomycin to BSA was indicated by the fact that, to reach the maximal rate of K^+ efflux, a higher valinomycin concentration was required parallel to the increase of the BSA concentration.

Fig. 2 shows the effect of BSA on the rate of proton leaks and on the basal respiratory rate. In accord with Fig. 1 addition of increasing BSA concentrations caused a reduction of the rate of K^+ efflux from about 60 to about 18 nmol × mg^{-1} × min^{-1} while causing a depression of the basal respiratory rate from 20 to 15 nmol × mg^{-1} × min^{-1}. In the *inset* of Fig. 2, it is also shown that the passive proton permeability accounts for about 40 and 20% of the basal respiratory rate, multiplied by the stoichiometry $H^+/O = 7$ as suggested by Beavis (18), in the absence and presence of 1% BSA, respectively.

Fig. 3 shows an experiment where the so-called conduct-

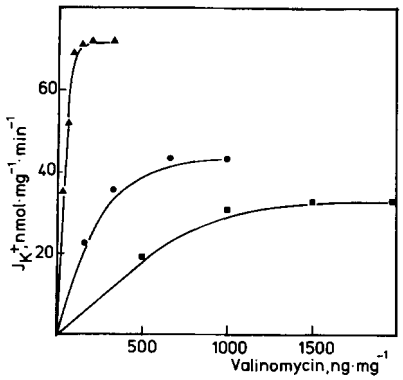

FIG. 1. **Rate of K^+ efflux as function of increasing amounts of valinomycin.** Different curves were obtained in the absence (▲) or in the presence of constant amount of BSA (●, 0.25%; ■, 0.5%). After incubation of RLM (1 mg/ml) for 2 min in the presence of succinate and BSA, antimycin (0.05 μg/mg) was added, followed after 5 s by valinomycin (variable concentrations), and the rate of initial K^+ efflux was measured.

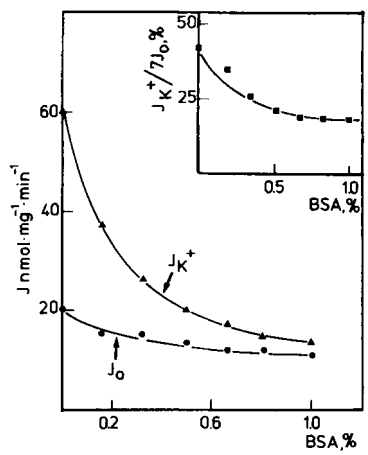

FIG. 2. **Comparison between the effect of increasing amount of BSA on the rate of initial K^+ efflux (▲) and the rate of respiration in state 4 (●).** *Inset,* ratio between JK^+/nJo ($n = 7$) as function of BSA concentration. Experimental procedure was similar as described in the legend of Fig. 1 except that the concentration of valinomycin was varied at each concentration of BSA on the basis of the results of Fig. 1.

ances ($nJe/\Delta\psi$) as a function of $\Delta\psi$ were measured in the absence or presence of BSA for both the pumps at sites II and III together (*A*), the pump at site II (*B*), and the pump at site III (*C*). Consider first the flow-force relationship in the absence of BSA. It is seen that the forms of these relationships were similar when the titrations were carried out either with the combined proton pumps at sites II and III or with the isolated proton pump at site II. On the other hand the form of the flow-force relationship differed in the case of the titration of the isolated proton pump at site III in two respects. First, the values of the conductance both in the ohmic and in non-ohmic regions were higher due to the higher rate of the resting respiration. Second, the transition from the ohmic to the non-ohmic region occurred at a higher $\Delta\psi$ due to the higher $\Delta\psi$ during operation of the cytochrome oxidase proton pump. This suggests that both the potential which initiates the non-ohmic relationship and the rate of the resting respiration are pump and not membrane properties. Brown (11) has reported rates of resting electron transfer of 50, 50, and 200 nmol of electron × mg^{-1} × min^{-1} during operation of the proton pumps at sites II plus III, of site III alone, and of site II alone, respectively. By multiplying these rates for the H^+/e^- ratios of the pumps assumed by Brown, namely 3, 2, and 1, one obtains rates of proton pumping (and therefore of proton conductance) of 150, 100, and 200 ng of ion $H^+ \times mg^{-1} \times min^{-1}$, respectively. The resting potentials measured by Brown were about 20 mV lower compared with those measured in the present study. It should be noted that Brown estimated such high rates of electron transfer in the bc_1 complex indirectly from the steady pH change due to the scalar proton release and not directly from the ferricyanide reduction. Furthermore, Brown's experiments were conducted in a KCl medium in the presence of nigericin, whereas our experiments were conducted in a sucrose medium in the presence of 5 mM P_i.

Now consider the effect of BSA. Addition of BSA caused, in the $nJe/\Delta\psi$ *versus* $\Delta\psi$ diagram, a level of ohmic conductance considerably lower with respect to that of native mitochondria, and an almost negligible change in the so-called non-ohmic conductance, *i.e.* the conductance was shifted only slightly toward lower values. Furthermore, and most important, the transition point is shifted by BSA toward higher $\Delta\psi$ values. This shift reflects the $\Delta\psi$ rise consequent to the leak inhibition caused by BSA. In conclusion, the experiment shows that the respiratory rate-proton motive force relationship depends in its ohmic region on the leak conductance of the membrane, and thus presumably reflects strictly the membrane properties which account for the passive proton permeability.

Chloroform as Slip Inducer—In previous work from this laboratory (7, 8), it was shown that chloroform induces a stimulation of the resting respiratory rate which is not accompanied by a decline of the membrane potential and by an appreciable increase of the proton conductance. The effect of chloroform is accompanied by a large shift of $nJe/\Delta\psi$ *versus* $\Delta\psi$ diagram in the non-ohmic and by a slight shift in the ohmic region. On the basis of these and other results, chloroform has been suggested to act as a slip inducer (8).

Fig. 4 shows the effect of chloroform on the relationship between conductance and $\Delta\psi$ as measured on the combined proton pumps at sites II and III (*A*), on the isolated proton pump at site II (*B*), and the isolated proton pump at site III (*C*). In Fig. 4*A* it appears that addition of chloroform, which had an almost negligible effect on the $\Delta\psi$ value at each antimycin concentration (7, 8), resulted in an upward shift of the conductance *versus* $\Delta\psi$ relationship both in the ohmic and in the non-ohmic region. Furthermore, also the initiation of the non-ohmic region was shifted toward lower membrane potential. These results are compatible with an enhancing action of chloroform on both the membrane conductance and the slips. The experiment of Fig. 4*B* with the isolated pump at site II indicates that while the slight effect on the membrane conductance was still present the effect on the slip was completely absent, suggesting that the induction of the slip occurred entirely at the level of the cytochrome oxidase proton pump. This suggestion is confirmed by the experiment of Fig. 4*C* which shows the effect of chloroform on the pump at site III. It is seen that the effect of chloroform was negligible in the ohmic and very marked in the non-ohmic region.

Slip in the ATPase Proton Pump—Pietrobon *et al.* (23) reported a comparison between the titration of the ATPase proton pump with oligomycin and of the redox proton pump with antimycin. In both cases a non-ohmic behavior was observed although the region of non-ohmicity occurred at a considerable lower $\Delta\tilde{\mu}_H$ in the case of the ATPase proton pump. The observation was taken to support the view that the non-ohmic behavior is a pump and not a membrane property. However, Brown (11) has recently claimed that

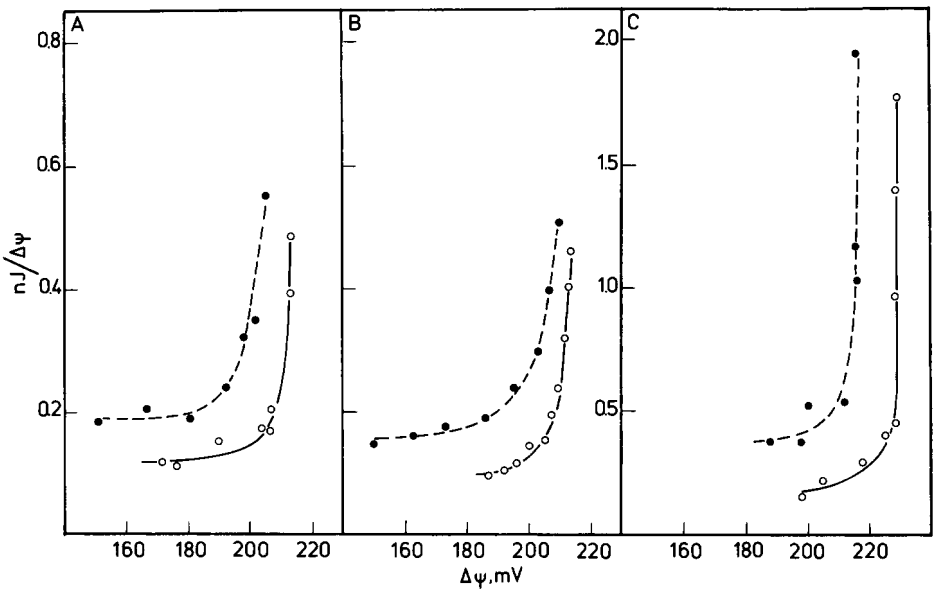

FIG. 3. **Relationship between *nJe*/Δψ (redox pump conductance) and Δψ in the presence (○) or in the absence (●) of albumin (0.6%).** *Panel A*, sites II + III, $n = 3.5$; *panel B*, site II, $n = 1$; *panel C*, site III, $n = 2.5$. RLM (1 mg/ml) were incubated with different media (see text) for 2 min. In *panels A* and *B*, succinate (5 mM) was then added followed, after 2 min, by increasing concentrations of malonate (0–5 mM) and the rate of oxygen consumption or rate of ferrocyanide reduction and Δψ were measured. In *panel C*, instead of succinate, increasing concentrations of ferricyanide were added, and after 2 min the rate of oxygen consumption and Δψ were measured.

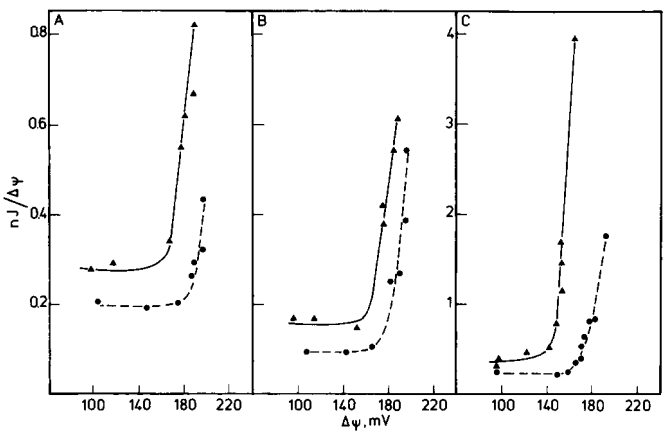

FIG. 4. **Relationship between *nJe*/Δψ and Δψ in the presence (▲) or in the absence (●) of chloroform (15 mM).** *Panel A*, sites II + III, $n = 3.5$; *panel B*, site II, $n = 1$; *panel C*, site III, $n = 2.5$. Experimental procedures were similar to those reported in the legend of Fig. 3 except for the use of antimycin (0–12.5 ng/mg) as redox inhibitor in *panel A*, *B*, and TMPD (0–400 μM) in the presence of ascorbate (2 mM) as redox substrate in *panel C*.

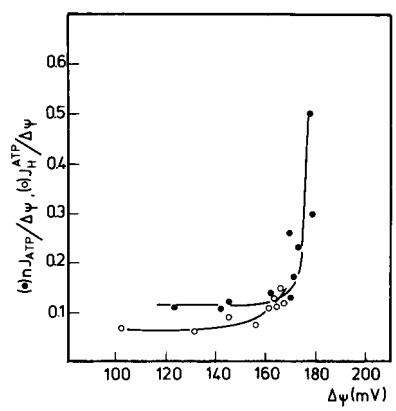

FIG. 5. **Ratio between the rate of ATP hydrolysis, multiplied by the H^+/ATP stoichiometry, (●) or rate of ATPase proton pumping (○) and Δψ in static head as a function of Δψ in titrations with oligomycin.** The dimension of $n_P J_{ATP}/\Delta\psi$ and $J_H^{ATP}/\Delta\psi$ are nmol × mg^{-1} × min^{-1} × mV^{-1}. n_P is taken as equal to 4. Medium composition was 0.2 M sucrose, 10 mM KCl, 1 mM P_i, 1 mM mannitol, 1 mM EDTA, 2 mM $MgCl_2$, 100 μM acetate, 5 μM rotenone, pH 7.4, *T* 25 °C. To measure J_{ATP}, RLM (1.5 mg/ml) was incubated for 3 min with increasing concentrations of oligomycin (0–0.2 μg/mg); then ATP (3 mM) was added, and after 2 min, the rate of ATP hydrolysis was measured. To measure J_H^{ATP}, RLM (1.5 mg/ml) were incubated in the presence of ATP (1.68 mM), ADP (0.17 mM), succinate (5 mM); after 5 min, antimycin (50 ng/mg) was added followed by titration of ATP hydrolysis with oligomycin. The transmembrane electrical potential Δψ were measured in parallel samples.

ATPase and redox proton pumps show identical flow force relationships.

In comparing the experimental conditions used by Pietrobon *et al.* (23) and by Brown (11), we note that the formers used an ATP-regenerating system while the latter did not. Fig. 5 shows an experiment where the ATPase proton pump has been titrated with oligomycin. The experiment was carried out both in the absence and in the presence of an ATP-regenerating system. It is seen that the two titrations differed drastically due to the fact that in the absence of an ATP-regenerating system the ATPase activity was markedly lower. As a consequence, the region of non-proportionality between proton motive force and pump activity (non-ohmic region) was practically abolished in the absence of the ATP-regenerating system.

Measurement of the Flux Ratios on the Basis of the H^+ Leak—Zoratti *et al.* (5) have used the rate of K^+ efflux at various Δψ as a measure of the effect of Δψ on the rate of H^+ influx and thus as an assay of the H^+ leaks. Murphy and Brand (12, 13) have used the rate of K^+ efflux as the basis for a steady-state assay of charge/O ratios in the following manner. In steady state, where the net charge displacement is zero, *i.e.* charge influx is equal to charge efflux, the following holds:

$$nJ_O^{sh} = J_H^{pump} \quad (3)$$

$$J_H^{pump} = J_H^{leak} \quad (4)$$

Hence:

$$n = J_H^{leak}/J_O^{sh} \quad (5)$$

where J_H^{pump} and J_H^{leak} are the proton fluxes due to redox pumping and to membrane leakage, respectively. From Equation 5 it follows that it should be possible to calculate the value of *n*, the charge/O ratio, by measuring J_O^{sh} and J_H^{leak} in respiring and anaerobic mitochondria, respectively.

In the experimental approach of Murphy and Brand, valinomycin-treated mitochondria were left respiring in a low K^+ medium supplemented with an increasing concentration of a respiratory inhibitor and, after reaching a steady state, given a pulse of excess antimycin or myxothiazol. Rates of K^+ efflux and of respiration were then measured. On the basis of this approach, Murphy and Brand have concluded that the stoichiometry of proton pumping is membrane potential invariant at the level of the cytochrome-bc_1 complex while it decreases from 4 at low to 2 at high membrane potential at the level of the cytochrome oxidase. The approach of Murphy and Brand is original in two respects, namely, in that it allows measurement of: (*a*) the flux ratio in the vicinity of static head, and (*b*) the effect of the leak on the flux ratio.

Fig. 6 shows an experiment similar to those of Murphy and Brand where valinomycin-supplemented mitochondria incubated in a low K^+ medium and oxidizing succinate with ferricyanide as electron acceptor were titrated with malonate, and rates of electron transport and of K^+ efflux (after antimycin) were measured in the absence and presence of 0.6% BSA. It is seen that (*a*) in the bc_1 complex there was a clear effect of the membrane potential on the flux ratio; (*b*) the flux ratio was markedly affected by the presence of BSA due to the effect of BSA on the rate of K^+ efflux but not on the rate of ferricyanide reduction; (*c*) when the flux ratio was plotted as a function of the rate of K^+ efflux an anomalous result was obtained; namely, that the flux ratio was decreased with the decrease of the leak.

Following the Murphy and Brand procedure for the assay of the flux ratio, it appears that the BSA-induced leak decrease leads to a contradictory phenomenology. On one hand the plots of the flux ratios *versus* membrane potential indicate that, as expected, at all potentials the flux ratios are shifted to a higher $\Delta\psi$ range when $\Delta\psi$ is increased by the addition of BSA, *cf.* Fig. 6. On the other hand the plots of the flux ratios *versus* the leak rate indicate that the flux ratio decreases with the decrease of the leak rate, *cf. inset* of Fig. 6. This is contrary to the expectation since a decrease of the leak should increase the flux ratio. These ambiguities are due essentially to the fact that the resting respiration cannot be accounted for by the rate of the leaks. Thus, the Murphy and Brand procedure while it suggests an effect of the membrane potential on the flux ratio, it does not appear to yield consistent results as to the effects of both membrane potential and leaks on the flux ratios.

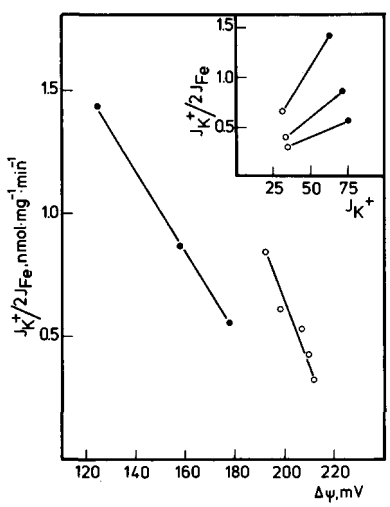

FIG. 6. **Ratio between the rate of K^+ efflux after addition of valinomycin to respiration-inhibited mitochondria and rate of ferricyanide reduction as a function of the transmembrane electrical potential, either in the presence (○) or in the absence (●) of albumin (0.6%).** *Inset,* flux ratio as function of the rate of K^+ efflux. RLM (1 mg/ml) were incubated in the presence of ferricyanide (1.5 mM) and increasing concentrations of malonate for 2 min. Succinate (5 mM) was then added, and the rate of ferricyanide reduction was measured. After 2 min antimycin (50 ng/mg), followed after 2 s by valinomycin (150 ng/mg), was added and the rate of K^+ efflux was measured.

Upper and Lower Limits for Pump Stoichiometries—For the assay of the mechanistic stoichiometries of the pumps at sites II and III in the absence and in the presence of uncoupling agents, Beavis suggested an approach (18, 19) based on the measurement of the slope of the relationship between the rates of charge translocation and of oxygen consumption. In this approach, titration with a respiratory inhibitor gives rise to a transport-respiration diagram with a slope corresponding to the lower limits, and titration with valinomycin to a slope corresponding to the upper limits of the mechanistic charge/O stoichiometry of the redox pump. By applying the same approach one may obtain phosphorylation-respiration diagrams and then the upper and the lower limits for the mechanistic P/O ratio of oxidative phosphorylation. Beavis has also shown that addition of protonophoric agents has no effect on the slope of the relationship in the phosphorylation-respiration diagram.

Fig. 7 shows the effect of FCCP on the slope of the charge translocation-respiration diagram. It is seen that, as in the case of the phosphorylation-respiration diagram (19), the addition of FCCP resulted in a shift to the right of the charge translocation-respiration relationship. However, addition of FCCP did not modify the slope of the relationship. Results similar to those reported in Fig. 7 were obtained when the titrations were carried out under conditions of lower limits with respiratory inhibitors.

Fig. 8 shows the effect of chloroform on the slope of the charge translocation-respiration diagram. It is seen that the presence of chloroform resulted in a marked modification of the diagram causing a marked increase of the slope of the plot. Furthermore, in the presence of chloroform the relationship between charge translocation and respiration tended to

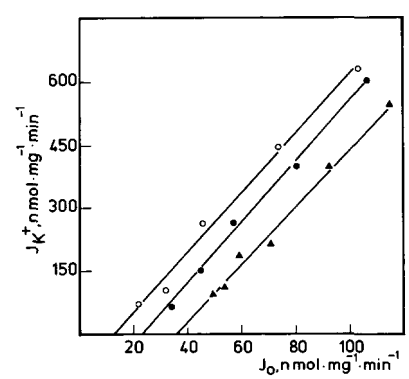

FIG. 7. **Determination of the upper limit of the mechanistic K^+/O ratio in the absence (○) and in the presence of FCCP (●, 15 pmol/mg; ▲, 30 pmol/mg).** Medium composition: 0.24 M sucrose, 5 mM succinate, 10 mM cholinchloride, 10 mM Tris, 0.1 mM EGTA/Tris, 2 mM P_i/Tris, 4 mM KCl, 2 μM rotenone, 1 μg/mg oligomycin, pH 7.4, *T* 25 °C. The initial rate of K^+ uptake following the addition of various concentrations of valinomycin is plotted as function of the corresponding rate of oxygen consumption. Each set of data was obtained with succinate as substrate and with valinomycin variable from 0 to 400 ng/mg. The slope of the lines fitting the experimental points represent the upper limit. The fitting lines are described by the equations: $J_K = [7.7(J_O) - 95]$ in the absence of FCCP, $J_K = [7.6(J_O) - 173]$ in the presence of 15 pmol/mg of FCCP, and $J_K = [7.4(J_O) - 262]$ in the presence of 30 pmol/mg of FCCP, respectively.

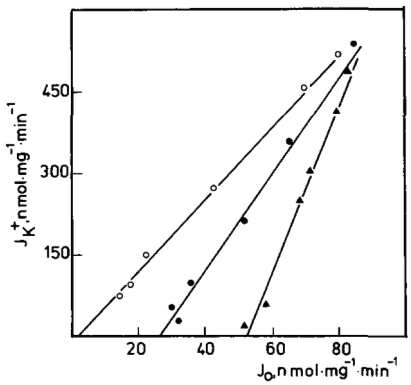

FIG. 8. **Determination of the upper limit of the mechanistic K^+/O ratio in the absence (●) and in the presence of chloroform (●, 10 mM; ▲, 17.5 mM).** The medium composition and experimental procedure are essentially the same as described in Fig. 7. In the absence of chloroform the line fitting the experimental data is represented by the equation $J_K = [7.25(J_O) - 22]$. In the presence of chloroform the lines are described by the equations $J_K = [8.1(J_O) - 208]$ and $J_K = [15.5(J_O) - 813]$, respectively.

become steeper particularly at lower transport and respiration rates, and this leads to exceedingly high and practically unacceptable values of stoichiometries of the pumps. Again, as in the case of the experiment of Fig. 7, similar results were obtained when the titrations were carried out under conditions of lower limits with respiratory inhibitors.

DISCUSSION

The Effect of BSA on Leaks—The establishment of the extent to which the basal respiratory rate is due to leaks or slips, respectively, is a relevant problem in bioenergetics. We have previously discussed an approach to this problem based on Equation 4. If the basal respiratory rate is due to leaks ($nJ_o = L_H^L \Delta\tilde{\mu}_H$), the magnitude of the unequivalence $nJ_o \neq L_H^L \Delta\tilde{\mu}_H$ provides also a quantitative measure of the slip. By comparing the basal respiratory rate with the rate of the proton leaks, Zoratti *et al.* (5) have concluded that, with mitochondria oxidizing succinate, the resting respiration is accounted for about ⅓ by leaks and about ⅔ by slips, respectively. In the present experiments, we find that the extent of leaks is slightly higher, say about 40%. Addition of BSA, however, modifies significantly the ratio between leaks and slips. The relationship between $nJ_o/\Delta\psi$ *versus* $\Delta\psi$ is altered both in the ohmic and in the non-ohmic regions as a reflection of the diminished and enhanced roles of leaks and slips, respectively. Due to the depression of passive proton leakage, at 1% BSA the rate of the leaks accounts for no more than 20% of the resting respiratory rate. This implies that the depression of the leak results in a proportional enhancement of the slips as predicted in the proton pump model of Pietrobon and Caplan (25) where the rate of the slip is markedly dependent on $\Delta\tilde{\mu}_H$. The increase of $\Delta\tilde{\mu}_H$ induced by BSA may be considered as the main reason for an increase of the rate of the slip.

Slip in ATPase, Cytochrome Oxidase, and bc_1 Complex—The experiment of Fig. 5 indicates that, under conditions of thermodynamic control, the rate of the ATPase activity is considerably lower in the absence than in presence of an ATP-regenerating system. At the lower rate of ATPase activity, the flow-force relationship is strictly proportional and the phenomenon of non-ohmicity is abolished. This explains why the non-ohmicity cannot be observed in the absence of an ATP-regenerating system. In fact, the non-proportionality occurs in the static head region, where the output force is very close to the input force. Note also that the static head $\Delta\tilde{\mu}_H$ for the redox proton pump is considerably higher than that of the ATPase proton pump, *i.e.* the output force of the redox is higher than that of the ATPase proton pump. This explains the different range of non-ohmicity for the redox and ATPase proton pumps.

Pietrobon *et al.* (3) reported a non-proportionality between inhibition of the respiration and of $\Delta\tilde{\mu}_H$ during operation both of the proton pump in the bc_1 complex and of the combined proton pumps at sites II and III. Brown (11) has concluded that during titrations with respiratory inhibitors the non-ohmic behaviors were identical whether in the case of the combined pumps at sites II and III or of the individual pumps at the same sites. From the data of Brown (*cf.* Fig. 2 of Ref. 11), it appears that the proton pumping (and hence the proton conductance) is 100 and 200 ng of ion $H^+ \times mg^{-1} \times min^{-1}$ for the proton pump at sites III and II, respectively. An almost equal difference was taken by Pietrobon *et al.* (3) as an evidence for the operation of slips in the proton pumps and to calculate the percentage of resting respiration due to leak and slip. Both in our and in Brown's experiments, the extent of non-ohmicity depends on the rate of resting respiration; however, in our hands the resting respiration is higher for cytochrome oxidase and lower for the bc_1 complex, while the opposite is true in Brown's experiments.

A change of H^+/O ratio has been observed in cytochrome oxidase under several conditions. Removal of subunit III decreases the stoichiometry of proton pumping and so does treatment with dicyclohexylcarbodiimide or heating to 43–45 °C (26, 27). It has been suggested that the decrease of stoichiometry is due to prevention of dimerization of cytochrome oxidase. Murphy and Brand (13) have concluded that there is slip in the cytochrome oxidase but not in the bc_1 complex proton pump. We have observed that at identical membrane potentials the rate of the resting respiration depends on the nature and concentration of substrate used; for example, the resting respiration is higher with TMPD than with ferrocyanide. We have also observed that the concentrations of TMPD or of ferrocyanide can be varied so to obtain a rate of the resting respiration either low or high. This is accompanied by a parallel variation of the extent of non-ohmicity.

Does the Leak Concept Account for the Resting Respiration?—The slip concept, now accepted in the current literature (see Ref. 30 for critical review), is supported by the following three lines of evidence.

1) The resting respiratory rate of intact mitochondria is, on the average, about 18 nmols of oxygen $\times mg^{-1} \times min^{-1}$ corresponding to a rate of H^+ ion pumping of 108–126 ng of ions $\times mg^{-1} \times min^{-1}$ (for a H^+/O stoichiometry of 6 and 7, respectively). However the rate of K^+ efflux, corresponding to the passive proton leakage, in anerobic mitochondria, under conditions of equivalent $\Delta\tilde{\mu}_H$, is about 60 nmol $\times mg^{-1} \times min^{-1}$.

2) Addition of BSA lowers the rate of passive proton leakage to about 20 nmol $\times mg^{-1} \times min^{-1}$ causing a negligible change of the rate of the resting respiration.

3) The rate of pump operation as a function of $\Delta\tilde{\mu}_H$ when modulated by respiratory inhibitors reflects the thermodynamic and kinetic properties of the various pumps rather than the electrical properties of the membrane. For example the flow-force relationship becomes non-ohmic in a different $\Delta\psi$ range for the ATPase and the various redox proton pumps.

A new class of uncoupling agents has been discovered which causes uncoupling without increasing the proton conductance; these agents have been denoted as intrinsic uncouplers or slip

inducers. Intrinsic uncouplers cause a stimulation of the respiration without affecting the level of $\Delta\tilde{\mu}_H$. Intrinsic uncouplers modify the flow-force relationship by causing an increased non-ohmicity. The distinction between protonophores and intrinsic uncouplers is further supported by two other groups of experiments, namely (*a*) the titrations of the respiratory control ratios and of the P/O ratios with respiratory inhibitors reported in preceding papers (8–10), and (*b*) the kinetic assays aiming to establish the upper and lower limits of the pump stoichiometries reported here.

The experiments reported in Fig. 4 demonstrate that the chloroform-induced increase of non-ohmicity, observed when the pump operates at sites II + III, reflects an effect occurring exclusively at the level of site III. This favors the view that the non-ohmicity phenomenon is independent from the changes of the leak conductance. In fact, whatever the nature of the native non-ohmicity, the chloroform-induced increase of non-ohmicity is not accompanied by an increased leak and becomes apparent only during the assay of the flow-force relationship of the cytochrome oxidase proton pump.

The Warsaw group (31, 32) has suggested that the non-proportionality between respiration and $\Delta\tilde{\mu}_H$ is due to the presence of a population of damaged mitochondria (open membranes) which provide a parallel pathway for electron transfer (31). The concept of the parallel pathway differs basically from that of the slip in that the former is a mechanism of electron transfer completely without thermodynamic control. The present study provides additional evidence against the view of damaged mitochondria showing that the slip inducers increase the slopes of the transport-respiration diagrams. A higher degree of coupling parallel to the decrease of $\Delta\tilde{\mu}_H$ can be accounted for by a steep flow-force relationship in a non-ohmic slip or leak but not by a population of open membranes where the respiration is without thermodynamic control.

The slip has been defined originally as a failure of the pump, *i.e.* turnover of pumps which transfer electrons without moving H^+ ions across the membrane, a concept subsequently incorporated in the six-state proton pump model of Pietrobon and Caplan (25). However, this is not the only mechanism by which a slip can be visualized. In fact the evidence that the non-ohmicity is a pump and not a lipid bilayer property could also be visualized as the occurrence of leakage of charges at the protein-lipid interface of the redox and ATPase proton pumps. At all events the extent of charge leakage is linked to the operation of the pump and is not a passive property of the protein-lipid constituents of the membrane. This explains why inhibitions of pump activity and of charge leaks go in parallel.

Garlid *et al.* (24) have suggested that all charge movements across biological membranes, whether through leak or slip pathways, should be intrinsically non-ohmic and that these pathways occur most likely at the protein-lipid interfaces. The slip concept emphasizes that, whatever the molecular mechanism, the proton pump activities play a major role in determining the turnover of these charge translocation pathways.

The Effect of Leaks and Slips on the Stoichiometries—In 1955 Chance and Williams (28) raised the question as to whether the rate of phosphorylation should be divided by the total or the Δ rate of oxygen uptake and decided for the former solution. Most studies have followed this choice since 1955 without, however, discussing the theoretical implication of one or another solution. A correct choice would require the knowledge of the mechanism of the resting respiration and therefore of whether this resting respiration is, or not, transformed into coupled respiration when the latter is activated in the presence of, say, ATP synthesis or ion transport. For example, initiation of ATP synthesis following addition of ADP results in a $\Delta\tilde{\mu}_H$ depression of about 20 mV, but it is not known to what extent this depression interferes with the rate of either leaks or slips.

The rationale presented by Beavis and Lehninger (14, 15) exploits the ability to predict the direction of change of the leak-slip processes as the fluxes, ion transport ATP synthesis

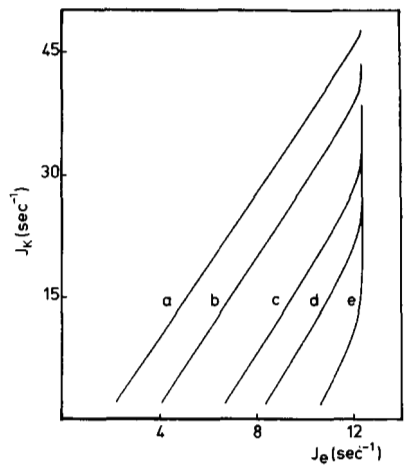

FIG. 9. **Computer simulations of the effect of protonophoric agents on the upper limit titrations.** The simulations are obtained with a chemiosmotic protonic circuit model constituted by three elements in parallel: a proton pump model as described in Ref. 25, with the kinetic parameters of pump B and affinity $A = 19.66$ kcal × mol^{-1} to simulate the proton efflux through a redox pump, an ohmic proton leak with different values of conductance L_H^L, and a pathway for potential-driven K^+ transport with conductance L_K. Each curve is obtained by increasing L_K from 0.5 to 20 mol × kcal^{-1} s^{-1} and with the following different values of proton leak conductance: *curve a*, $L_H^L = 0.5$ mol × kcal^{-1} × s^{-1}; *curve b*, $L_H^L = 2.5$; *curve c*, $L_H^L = 5.5$; *curve d*, $L_H^L = 7.5$; *curve e*, $L_H^L = 10.5$. In this simulation, slip transition in the redox proton pump model is present with rate constants equal to $\alpha_{25} = 9.45 \times 10^{-13}$ s^{-1}, $\alpha_{52} = 1.4$ s^{-1} corresponding to physiological value (see Refs. 6 and 25 for details).

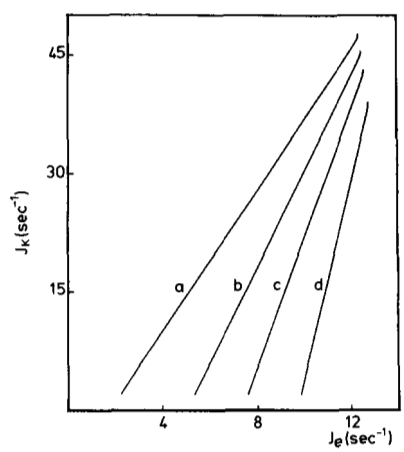

FIG. 10. **Computer simulations of the effect of slip inducers on the upper limit titrations.** Each different curve is obtained as described in Fig. 9 by using different rate constants of redox slip transition: *curve a*, $\alpha_{25} = 9.45 \times 10^{-13}$ s^{-1}, $\alpha_{52} = 1.4$ s^{-1}; *curve b*, $\alpha_{25} = 4.72 \times 10^{-12}$ s^{-1}, $\alpha_{52} = 7$ s^{-1}; *curve c*, $\alpha_{25} = 9.45 \times 10^{-12}$ s^{-1}, $\alpha_{52} = 14$ s^{-1}; *curve d*, $\alpha_{25} = 1.89 \times 10^{-11}$ s^{-1}, $\alpha_{52} = 28$ s^{-1}. In this simulation the proton leak conductance is constant and equal to $L_H^L = 0.5$ mol × kcal^{-1} × s^{-1}.

or respiration, are modulated by specific means. In this treatment it is assumed that

$$J_K = nJ_O - J_H^{leak} \quad (6)$$

where J_H^{leak} is the sum of the rates of H^+ leakage and pump slippage without distinguishing between the two mechanisms of uncoupling or, rather, by implicitly assuming that it makes no difference whether J_H^{leak} is determined by leaks or slips. Beavis and Lehninger predicted what should have been the effect of uncouplers under conditions of either upper or lower limit titrations. For the lower limit titrations the slope of the plots should decrease as the concentrations of FCCP are raised due to a larger decrease in leak as respiration is inhibited. For the upper limit titrations the slope of the plot should increase as the concentration of FCCP is increased due to an increase in leak. However, both effects were not observed, *i.e.* there was negligible or no change in slope at increasing FCCP concentrations. No explanation was offered for the discrepancy between predictions and observations. However, a closer look at the equations (14, 15) indicates that the effect of FCCP depends essentially on the values of the leak and coupling coefficients in the equations.

In the present study, the results obtained in Figs. 6 and 7 have been simulated by using the kinetic model of chemiosmotic free energy transduction of Pietrobon (29) which accounts for the coupling of two proton pumps in the presence of physiological rates of operation of the pumps and of physiological leak. The same model has already been used to simulate the effect of classical protonophoric agents and of slip inducers on (*a*) respiratory rates and respiratory control ratio and (*b*) P/O ratios (7, 8). To perform the simulations of the titrations performed according to Beavis, we have introduced in the model of Pietrobon, parallel to the pathway for the proton leakage, an element accounting for the valinomycin-catalyzed, potential-driven transport of K^+. The effect of the different valinomycin concentration was accounted for by varying the conductance of this pathway.

In Fig. 9 it is seen that, at increasing extents of protonophoric leaks, the slopes of the transport-respiration diagram were unchanged and only shifted to the right, proportionally to the protonophore concentration until saturation of the respiration was obtained. On the other hand as shown in Fig. 10, at increasing concentrations of slip inducers there was a non-proportional shift to the right with an increase in slope parallel to the increasing concentration of the slip inducer. The effect of redox slip was simulated by increasing the value of the constants α_{52} and α_{25} corresponding to the transition between the state 5 and 2 of the six-state proton pump model of Pietrobon and Caplan (25). The ranges of effects of protonophores and slip inducers used in the simulations were selected in order to be as close as possible to those presumably occurring under the experimental conditions described in Figs. 7 and 8 at the concentrations used of uncouplers.

The comparison between the simulations of Figs. 9 and 10 and the experiments of Figs. 7 and 8 indicate that it is not immaterial for the calculation of the stoichiometries whether the level of the resting respiration is determined by ohmic or non-ohmic leaks or slips. In fact, independently of whether one is measuring the lower or the upper limit of the stoichiometries, addition of classical protonophores, such as FCCP which cause an ohmic leak, or of intrinsic uncouplers, such as chloroform which cause an non-ohmic slip, results in different effects in that the former do not and the latter do modify the slope of the transport-respiration diagram and thus the upper limit of the mechanistic stoichiometry which can be determined under these conditions. This is presumably due to the very steep dependence of the slip on $\Delta\tilde{\mu}_H$, and thus on the fact that the depression of $\Delta\tilde{\mu}_H$ due to K^+ transport results in a large inhibition of the rate of the slip. The same effect is predicted in case the resting respiration would be due to a highly non-ohmic leak.

The differential effect of protonophores and of intrinsic uncouplers has two implications. First, it becomes an additional criterion to distinguish between the two mechanisms of uncoupling. Second, it leads to different calculations of the P/O and of the H^+/O ratios. In fact if the resting respiration would be due to an ohmic proton leakage, similar to that due to protonophores, the stoichiometry of the pump during K^+ transport should be calculated by taking into account only the K^+ transport stimulated respiration (the Δ oxygen uptake) but not the rate of the resting respiration. On the other hand if the resting respiration would be due to either highly non-ohmic leaks or slips, the stoichiometry of the pump should be calculated by taking into account both the K^+ stimulated and the rate of the resting respiration (and thus the total oxygen uptake). A steep flow-force dependence of the slip process is a convenient mechanism to optimize energy coupling during the transition from the resting to the activated respiration (whether for ATP synthesis or ion transport).

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