rapid method for the isolation of 7S NGF in which the yields are much greater (70–80 mg from 100 mice) than for either of these two other procedures.

Registry No. Nerve growth factor, 9061-61-4.

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Intrinsic Uncoupling of Mitochondrial Proton Pumps. 1. Non-Ohmic Conductance Cannot Account for the Nonlinear Dependence of Static Head Respiration on $\Delta\tilde{\mu}_H$

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ABSTRACT: The passive membrane conductance L_H^l of rat liver mitochondria has been measured and compared with the quantity $nJ_e^{sh}/\Delta\tilde{\mu}_H^{sh}$ (n = H^+/e stoichiometry; J_e^{sh} = rate of electron transfer in static head) over a $\Delta\tilde{\mu}_H$ range. The two curves approach each other only in the lower part of the range, while they sharply diverge at large values of $\Delta\tilde{\mu}_H$. Thus $nJ_e^{sh}/\Delta\tilde{\mu}_H^{sh}$ cannot be considered to be a measure of L_H^l in the upper $\Delta\tilde{\mu}_H$ region. Only a fraction of the static head electron flow is accounted for by futile proton cycling via leaks. Contaminating open membrane fragments or completely leaky mitochondria can be responsible for only a small part of the residual rate of oxygen consumption. We conclude that a large part of static head respiration must have yet another cause and propose it to be intrinsic uncoupling of the respiratory chain enzymes.

The relationship between the electron-transfer rate J_e^{sh} and the output force for the redox H^+ pumps $\Delta\tilde{\mu}_H^{sh}$[1] in static head (state 4) mitochondria has been the object of considerable interest in the past ten years (Nicholls, 1974, 1977; Sorgato

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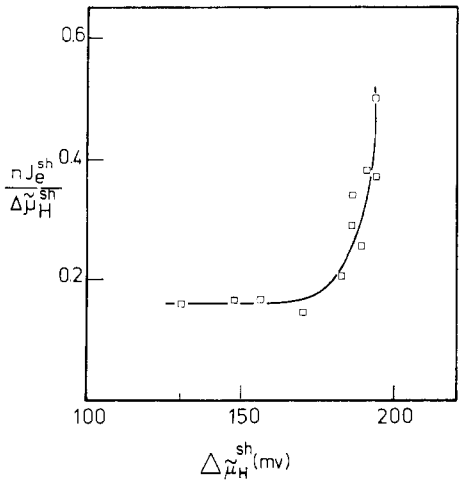

FIGURE 1: $nJ_e^{sh}/\Delta\tilde{\mu}_H^{sh}$ vs. $\Delta\tilde{\mu}_H^{sh}$ by titration with antimycin (n = 4). The dimensions of the ordinate axis are nmol of e mg^{-1} min^{-1} mV^{-1}. The medium composition was 0.24 M sucrose, 10 mM Tris/Mops, 5 mM glycerol, 2 mM P_i/Tris, 0.5 mM EGTA, 2 μM rotenone, and 1 μg of oligomycin/mg of protein, pH 7.4; T 25 °C. Mitochondria (2 mg of protein/mL) were incubated for 2 min in the presence of 0–27.5 ng of antimycin A/mg; 2 mM succinate was then added and J_e measured after 2.5 min. $\Delta\psi$ and ΔpH were determined in parallel experiments from the distribution of TPMP and DMO, respectively.

et al., 1979; Pietrobon et al., 1981, 1983; Krishnamoorthy & Hinkle, 1984). Under the assumptions that the mitochondrial proton pumps are completely coupled and that the membrane leak conductance L_H^l is constant, a given inhibition of the rate of respiration should result in a proportional reduction of $\Delta\tilde{\mu}_H$ (Nicholls, 1974; Pietrobon et al., 1981). L_H^l could be calculated at any J_e^{sh} (and corresponding $\Delta\tilde{\mu}_H^{sh}$) value as

$$L_H^l = nJ_e^{sh}/\Delta\tilde{\mu}_H^{sh} \quad (1)$$

where n is the stoichiometry of proton pumping, i.e., the H^+/e ratio. However, Nicholls (1974, 1977), followed by others (Sorgato et al., 1979; Pietrobon et al., 1981, 1983; Krishnamoorthy & Hinkle, 1984), found that during titrations with respiratory inhibitors there is no proportionality between depression of the rate of electron flow and decrease of $\Delta\tilde{\mu}_H$. $nJ_e^{sh}/\Delta\tilde{\mu}_H^{sh}$ is not constant; rather, curves such as the one shown in Figure 1 are obtained. The hypothesis was therefore put forward (Nicholls 1974) that the membrane leak conductance (L_H^l) is a function of $\Delta\tilde{\mu}_H$, increasing steeply above a threshold value of the latter parameter ("non-ohmic conductance" hypothesis).

Non-ohmic proton ("leak") current–voltage relationships have been observed with both artificial and natural membranes when the driving force was mainly an electric field (Krishnamoorthy & Hinkle, 1984; Gutknecht, 1984; O'Shea et al., 1984), but ohmic relationships have also been reported (Schlodder et al., 1982; Graeber, 1982; Cafiso & Hubbell, 1983). If the protons move in response to a pH gradient, ohmic relationships are obtained (Mitchell & Moyle, 1967; Kell & Morris, 1980; Arents et al., 1981; Schlodder et al., 1982; Graeber, 1982; Maloney & Hansen, 1982; Krishnamoorthy & Hinkle, 1984; O'Shea et al., 1984).

According to the non-ohmic conductance hypothesis, the form of titration curves such as that in Figure 1 is determined by the properties of the membrane. Hence, for a given organelle preparation only one relationship between the quantity $nJ_e^{sh}/\Delta\tilde{\mu}_H^{sh}$ ($=L_H^l$) and $\Delta\tilde{\mu}_H^{sh}$ should exist. Recently, Pietrobon et al. (1981, 1983) observed that different relationships could be obtained, depending on the final electron acceptor used or on the identity of the primary proton pump [redox chain or adenosinetriphosphatase (ATPase)]. The relationship between rate of oxygen consumption or ATP hydrolysis and $\Delta\tilde{\mu}_H$ at static head seemed to reflect, at least in part, the properties of the enzymes involved rather than those of the lipid bilayer. An alternative model was therefore presented: the observations of Nicholls and others would mainly be explained by incomplete coupling of the proton pumps ("molecular slip"). The passive proton conductance of the membrane, which might or might not be a function of $\Delta\tilde{\mu}_H$, would not account for all of the respiration by static head mitochondria, most of which would be due to the pumps themselves exhibiting a certain amount of failure. Some electrons would be transferred from substrate to oxygen without an associated proton translocation across the mitochondrial membrane ("redox" or "reaction" slip), while some protons would reenter the mitochondrial matrix in an enzyme-catalyzed process without a concomitant reversed electron flow ("proton slip"). The ATPase would behave similarly.

The purpose of this work has been to investigate further whether non-ohmic conductance through the lipid bilayer may by itself account for the observed behavior of respiration and $\Delta\tilde{\mu}_H$ (Figure 1) in static head mitochondria. We compared L_H^l, determined directly from measurements of proton passive flow in response to an electrochemical potential difference, and $nJ_e^{sh}/\Delta\tilde{\mu}_H^{sh}$ over a range of $\Delta\tilde{\mu}_H$ values. The two parameters should coincide if there is no contribution of intrinsic uncoupling to respiration. After the initiation of this work, two reports appeared, addressing the same problem from the same point of view and utilizing the same techniques (Krishnamoorthy & Hinkle, 1984; O'Shea et al., 1984). The authors observed a certain degree of non-ohmicity and concluded from this that the intrinsic uncoupling hypothesis is to be rejected. However, respiration and proton-flux data were not compared to check whether a quantitative agreement exists between L_H^l and $nJ_e^{sh}/\Delta\tilde{\mu}_H^{sh}$. We also present data that weaken an alternative explanation, namely, that most of state 4 respiration might be due to uncoupled respiration by broken or damaged mitochondria (Duszynski & Wojtczak, 1984).

Materials and Methods

Materials. Rat liver mitochondria were prepared according to standard procedures (Massari et al., 1972a). Antimycin, oligomycin, and rotenone were purchased from Sigma. [^{14}C]TPMP, [^{14}C]DMO, and [^{3}H]glycerol were obtained from Amersham.

Determination of J_H^l and L_H^l. The membrane proton-leak conductance, L_H^l, at any value of the driving force, $\Delta\tilde{\mu}_H$, can be determined as the ratio $J_H^l/\Delta\tilde{\mu}_H$. To measure passive proton flow through the bilayer, the mitochondrial proton pumps are blocked, and a transmembrane electrochemical proton gradient is created by establishing a valinomycin-induced K^+ diffusion potential. If the permeability coefficient for K^+ is sufficiently higher than that of other ions, the size of the diffusion potential can be calculated from Nernst's law. It can be modulated either by varying the potassium concentration in the medium or by varying the osmolarity of the medium itself, since mitochondria behave as osmometers

[1] Abbreviations: J, reaction rate or flow; L_H^l, membrane proton-leak conductance; $\Delta\psi$, transmembrane electrical potential gradient; ΔpH, transmembrane pH gradient; $\Delta\tilde{\mu}_H$, transmembrane electrochemical proton gradient; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; TPMP, triphenylmethylphosphonium; DMO, 5,5-dimethyl-2,4-oxazolidinedione; Tris, tris(hydroxymethyl)aminomethane; Mops, 4-morpholinepropanesulfonic acid; RLM, rat liver mitochondria; NADH, reduced nicotinamide adenine dinucleotide.

(Tedeschi & Harris, 1955, 1958; Rossi & Azzone, 1969; Rottenberg & Salomon, 1969; Massari et al., 1972a,b). When valinomycin is added to antimycin- and oligomycin-treated mitochondria, the rate of potassium efflux must, for electroneutrality reasons, be equal to the sum of the rates of anion efflux and influx of cations other than K^+. In the medium we used, essentially the only permeable ion beside K^+ is H^+ (OH^-). Chloride permeability is low (Azzi & Azzone, 1966). Thus H^+ influx in response to $\Delta\tilde{\mu}_H$ was followed by monitoring K^+ efflux in the presence of an excess of valinomycin and taking J_K^{eff} as a measure of J_H^l. Any unrecognized movement of other ions, such as Tris–H^+ or Cl^-, would result in an overestimation of J_H^l. This method offers advantages over a direct measurement of pH variations, as done in other investigations (Krishnamoorthy & Hinkle, 1984; O'Shea et al., 1984), because it avoids complications due to the operation of proton-linked transport systems of mitochondria, especially the phosphate and carboxylate carriers, which are hard to inhibit completely (Kaplan & Pedersen, 1983; LaNoue & Schoolwerth, 1979). Control experiments gave J_H/J_K ratios of 0.80–0.95.

For each determination of J_K^{eff} and K^+ diffusion potential, mitochondria (1–3 mg of protein/mL) were suspended in 5 mL of buffered medium of defined composition and osmolarity (cf. legend to Figure 2) in a thermostated vessel, open to the air. The suspension bathed a Schott K^+ electrode (response time < 1 s) and a glass combination electrode (Beckman) that served as reference, connected to a Radiometer 26 pH meter/mVolmeter. The output was fed to a Perkin-Elmer Model R100 A chart recorder. The medium contained a known concentration of K^+ ions. The contributions of mitochondrial leaked K^+ and the medium K^+ contamination (measured by atomic absorption spectroscopy, 6–25 μM) were taken into account.

We considered the possibility of a large, acid-inside ΔpH in respiration-inhibited mitochondria. Such a ΔpH might form following K^+ efflux from the mitochondria in the stock (anaerobic) suspension. It would be difficult to evaluate, and it would introduce an additional uncertainty in the magnitude of the driving force for H^+ uptake. To avoid this complication, a respiratory substrate was included in the medium, so that the mitochondria reach a well-defined and reproducible initial state (state 4) in each determination. Under our conditions mitochondria maintain a small (6–20 mV: phosphate was present) ΔpH, which was measured by the DMO distribution technique.

After a 1-min incubation, 0.05 μg of antimycin A/mg of protein (0.1–0.3 μM) was added. This amount of antimycin inhibits respiration essentially immediately (not shown). A residual antimycin-insensitive rate of O_2 consumption remained, not exceeding 1 nmol of oxygen atoms mg^{-1} min^{-1}. If this respiration is coupled to proton pumping, it will lead to a small underestimation of the rate of H^+ leak. Higher amounts of antimycin do not suppress respiration further and have instead an uncoupling effect, clearly visible as an increase in the rate of K^+ efflux. Antimycin-supplemented mitochondria were allowed to stand for 10 s, a time sufficient to inhibit electron flow and to collapse $\Delta\psi$ below the diffusion potential value (Figure 5 and analogous experiments not shown), while the amount of K^+ leakage, H^+ influx, and therefore ΔpH variation is negligible. Valinomycin (0.1 μg/mg; 0.1–0.3 μM) was then added, and K^+ efflux ensued. Control experiments indicated that this amount of valinomycin is sufficient to render K^+ diffusion not limiting [cf. also Krishnamoorthy & Hinkle (1984), inset of Figure 3]. Higher amounts were avoided because of the well-known uncoupling (proton-carrying) effect of this ionophore at high concentrations (Azzone et al., 1984). Rates of K^+ efflux were estimated from the slope of the initial, practically rectilinear part of the recording after valinomycin addition. A typical trace is shown in Figure 2. Calculations were based on suitable calibrations for each set of determinations. Determinations were carried out in triplicate or duplicate for each potassium concentration. L_H^l was calculated as $J_K^{eff}/\Delta\tilde{\mu}_H$. $\Delta\tilde{\mu}_H$ was taken as the sum of the potassium diffusion potential and state 4 ΔpH. The K^+ diffusion potential was calculated from the inner (cf. below) and outer K^+ concentrations by using Nernst's law.

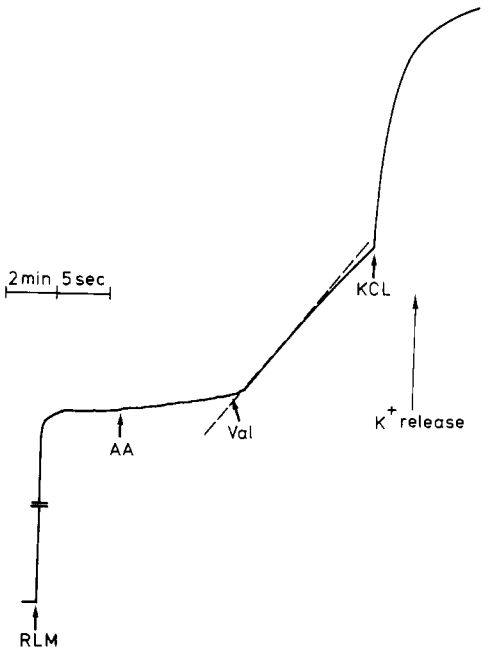

FIGURE 2: Typical K^+ electrode recording. The medium composition was as in Figure 1, except that glycerol was omitted and 2 mM succinate/Tris was included. Contaminating K^+ and K^+ contributed by added RLM = 29.4 μM. J_K^{eff} = 56.0 nmol mg^{-1} min^{-1}; RLM = 1 mg of protein/mL; T = 25 °C. See text for other details. KCl (20 μM) was added after valinomycin to show that the response of the electrode is not rate-limiting.

Determination of $[K^+]_{in}$. Addition of valinomycin to energized mitochondria will result in neither uptake nor release of potassium only if $\Delta\mu_K = \Delta\psi$, i.e., if

$$(RT/F) \ln ([K^+]_{in}/[K^+]_{out}) = \Delta\psi \qquad (2)$$

We performed null-point titrations of K^+ movement by determining the direction and the initial rate (J_K) of K^+ fluxes induced by addition of valinomycin to state 4 mitochondria suspended in media of accurately known and variable (15–200 μM) K^+ concentrations. Plots of J_K vs. $[K^+]_{out}$ were linear and intercepted the abscissa at different values of $[K^+]_{out}$ depending on medium osmolarity. These intercepts, together with static head $\Delta\psi$ values measured in parallel TPMP uptake experiments, were used to calculate $[K^+]_{in}$ according to eq 2 above.

Internal potassium concentrations determined in this manner depend on the measured static head $\Delta\psi$, which varies slightly (a few millivolts) from one mitochondrial preparation to the other. The procedure has however the advantage of establishing a link between TPMP-based $\Delta\psi$ measurements and calculated potassium diffusion potentials: if the former are affected by systematic error of any kind, the latter will also be, to the same extent. Therefore, the potentials will be strictly comparable, a feature of importance in this work.

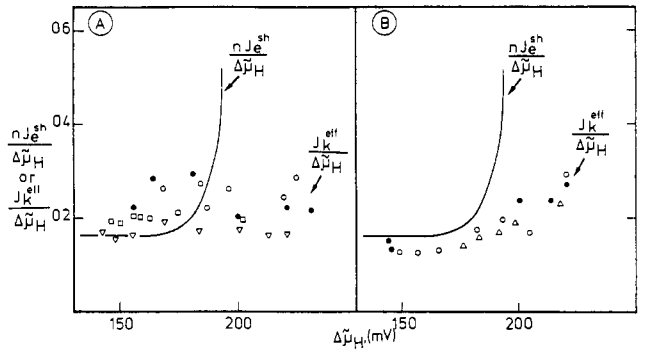

FIGURE 3: $J_K^{eff}/\Delta\tilde{\mu}_H$ ($=L_H^I$) vs. $\Delta\tilde{\mu}_H$ (lower curve, experimental points). The upper trace (solid line) is the curve of Figure 1, included to facilitate comparison. The dimensions of the ordinate axis are nmol mg^{-1} min^{-1} mV^{-1}. L_H^I data from three representative experiments are shown, consisting of determinations of the type shown in Figure 2 (different symbols indicate different experiments). For details see text.

Determination of $nJ_e^{sh}/\Delta\tilde{\mu}_H^{sh}$. Rates of respiration were determined from the rate of oxygen consumption by rat liver mitochondria incubated (2 mg/mL) in a closed, thermostated, and stirred vessel, measured with a Clark electrode (Yellow Springs). Mitochondria were incubated in the presence of varying amounts of inhibitor for 2–4 min. The respiratory substrate was then added and the rate of respiration determined from the slope of the trace. Determination of medium oxygen content and calibration of the electrode response were carried out by allowing submitochondrial particles to oxidize known (spectrophotometrically determined) amounts of NADH. $\Delta\tilde{\mu}_H$ was determined in parallel experiments as described by Zoratti et al. (1984), following the same order of additions and times of incubation. TPMP and DMO distributions were used to calculate $\Delta\psi$ and ΔpH. $\Delta\psi$ values were corrected for binding as described by Pietrobon et al. (1982).

Rate of NADH Oxidation by RLM and Calculation of the Contribution by Membrane Fragments to Static Head Respiration. "Broken" mitochondria were prepared for each experiment by osmotic shock. Mitochondria from the normal preparation were suspended for 5–10 min in cold H_2O, centrifuged, washed once with 0.25 M sucrose and 10 mM Tris/Mops (pH 7.4), and resuspended in the same medium. These preparations consumed little or no oxygen in the absence of added substrates.

The rate of mersalyl-insensitive NADH oxidation by "normal" (J^N_{NADH}) and broken (J^B_{NADH}) mitochondria was measured by following the rate of absorbance decrease at 340–374 nm with a double-beam spectrophotometer (Aminco DW2) and from determinations of oxygen consumption rates. These data were used together with the respiratory rates of static head normal (J^N_{succ}) and of broken mitochondria (J^B_{succ}) to calculate the contribution by open membranes to static head respiration as

$$\% = J^N_{NADH}J^B_{succ}/J^B_{NADH}J^N_{succ} \times 100 \qquad (3)$$

The rate of mersalyl-insensitive NADH oxidation by normal mitochondria was thus taken as a measure of the amount of contaminating open membrane fragments. Use of eq 3 depends on the assumption that these fragments behave like osmotically shocked mitochondria.

RESULTS

Comparison of Relationships Linking L_H^I and $nJ_e^{sh}/\Delta\tilde{\mu}_H$ to $\Delta\tilde{\mu}_H$. Figure 3B shows a plot of the quantity $J_{K^+}^{eff}/\Delta\tilde{\mu}_H$ ($=L_H^I$) vs. $\Delta\tilde{\mu}_H$ (lower curve, data from three representative experiments). The curve shown in Figure 1 is replotted here to facilitate comparison (solid line). The increase in L_H^I shown in Figure 3B was observed only in about half of the experiments performed while in the others the relationship was best described as an horizontal line; this behavior is shown in Figure 3A (data from four representative experiments). Since the rise of L_H^I is in accordance with the non-ohmic conductance hypothesis, this result will be given the most consideration in the discussion below.

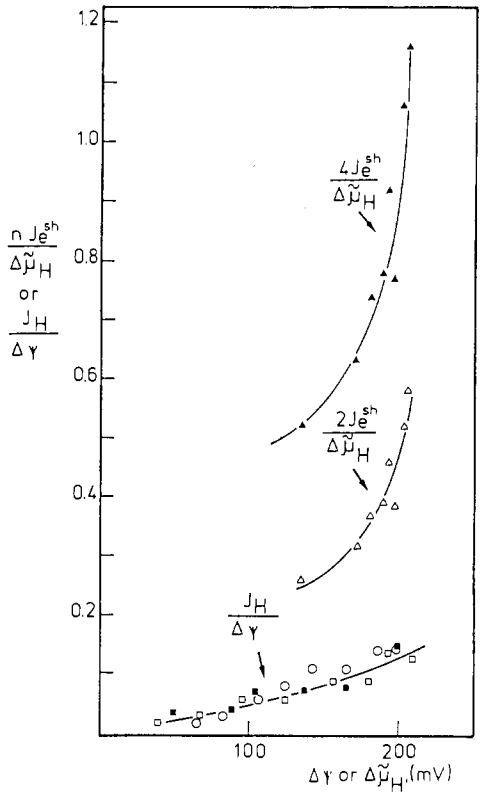

FIGURE 4: Data replotted from Krishnamoorthy & Hinkle (1984) [cf. their Figures 1 (succinate respiration) and 3]. The dimensions of the ordinate axis are as in Figure 3. Symbols used for the experimental points on the lower curve are the same as those in Krishnamoorthy & Hinkle (1984). In plotting $nJ_e^{sh}/\Delta\tilde{\mu}_H^{sh}$ vs. $\Delta\tilde{\mu}_H^{sh}$, n was taken as either 4 or 2 H^+/e.

Our values of J_K^{eff} are higher than the J_H^I values reported by Krishnamoorty & Hinkle (1984) over a comparable range of $\Delta\psi$ (cf. their Figure 3). Potassium efflux rates are however very similar since Krishnamoorthy and Hinkle report J_H/J_K ratios of 0.5–0.7. Our values are also higher than those reported by O'Shea et al. (1984) (cf. their Figure 3). The curve defined by their few points seems to rise more sharply and at somewhat lower $\Delta\psi$ values than ours or that of Krishnamoorty and Hinkle. We have replotted the data presented by the latter authors as $J_H/\Delta\psi$ vs. $\Delta\psi$ or $nJ_e^{sh}/\Delta\tilde{\mu}_H^{sh}$ vs. $\Delta\tilde{\mu}_H^{sh}$ in Figure 4, in order to facilitate comparisons. At variance from Krishnamoorthy and Hinkle, we could not detect any clear effect of the medium pH on the L_H^I vs. $\Delta\tilde{\mu}_H$ curves between pH 6.2 and 7.5.

The data of Figure 3 as well as those of Krishnamoorthy and Hinkle (Figure 4) indicate that the plots of L_H^I ($J_K^{eff}/\Delta\tilde{\mu}_H$ or $J_H/\Delta\psi$) and $nJ_e^{sh}/\Delta\tilde{\mu}_H^{sh}$ vs. the driving force define two different curves: they approach each other only in the lower $\Delta\tilde{\mu}_H$ range. The proton conductance of the mitochondrial membrane may well increase somewhat as $\Delta\tilde{\mu}_H$ increases, but this variation can account for only a part of the increase of the quantity $nJ_e^{sh}/\Delta\tilde{\mu}_H^{sh}$, which is sharper and steeper and occurs at lower $\Delta\tilde{\mu}_H$ values. From the present data it appears that in state 4, if $n = 4$, only about 30% of the electron flow

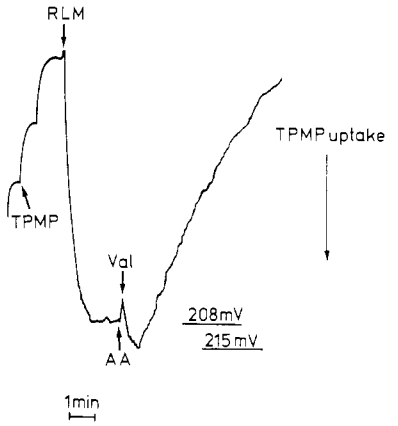

FIGURE 5: TPMP electrode trace recorded during an experiment. The medium composition was 0.4 M sucrose, 10 mM Tris/Mops, 2 mM P_i/Tris, 0.5 mM EGTA, 2 mM succinate/Tris, 2 μM rotenone, 1 μg of oligomycin/mg of protein, pH 7.4; T 25 °C; contaminating K^+ = 6.2 μM; K^+ contributed by added RLM (1 mg of protein/mL) = 15.3 μM. The K^+ diffusion potential was calculated as 226 mV. Total TPMP in the assay was 10 μM. Only the last two calibrating additions of TPMP (2 μM each) are shown.

is due to proton cycling, while the remaining ca. 70% is due to some other process. The value assigned to the stoichiometric coefficient n is of course important in such a comparison. We have taken n = 4 H^+/e (succinate to O_2). The results would be quantitatively different but the conclusions would not change if n were equal to 3.

A further point of importance is the correctness of the $\Delta\tilde{\mu}_H$ scale. In some 20 separate experiments we covered $\Delta\psi$ ranges extending on both sides of the usually measured static head $\Delta\psi$ values. The minimum and maximum K^+ diffusion potentials calculated during this work were 140 and 230 mV, respectively. It might be objected that these values are not reliable, since they depend on the exact values of $[K^+]_{in}$. While their correctness is important, what chiefly matters here is that they be comparable with TPMP-based measurements. The K^+ diffusion potentials used to construct Figure 3 were calculated by assuming a matrix K^+ concentration equal to 33% of the osmolarity of the media. In turn, this value of $[K^+]_{in}$ was calculated from null-point titrations of K^+ fluxes (cf. Materials and Methods) on the basis of a static head of 187 mV, which was the value measured in the experiment reported in Figure 1. The values for the two curves are thus self-consistent: were the $nJ_e^{sh}/\Delta\tilde{\mu}_H^{sh}$ vs. $\Delta\tilde{\mu}_H^{sh}$ curve to be shifted due to more precise measurements, the $J_K^{eff}/\Delta\tilde{\mu}_H$ vs. $\Delta\tilde{\mu}_H$ curve would also shift accordingly. Furthermore, we ascertained that the highest diffusion potentials we established are indeed larger than the static head $\Delta\psi$, using the following approach. We monitored the distribution of the lipophilic cationic $\Delta\psi$ probe TPMP during typical experiments, using a specific TPMP electrode. A typical trace is shown in Figure 5. Addition of antimycin to static head mitochondria caused a rapid depolarization, reversed upon addition of valinomycin. The concentration of probe in the medium was transiently lowered below the static head level, indicating that the K^+ diffusion potential exceeds the static head $\Delta\psi$. $\Delta\psi$ values shown in Figure 5 were calculated on the basis of a volume of 0.8 μL/mg and without any binding correction. Notice that since (a) the response of the electrode is relatively slow (cf. titration pulses), (b) the diffusion potential declines as K^+ is lost, and (c) K^+ loss is accompanied by shrinkage, the diffusion potential must initially have exceeded the static head by more than the 7 mV indicated. The K^+ diffusion potential calculated for this experiment was 226 mV. In a series of experiments of this type the $\Delta\psi$ calculated from the maximum TPMP trace deflection after addition of valinomycin was lower than the $\Delta\psi$ calculated from Nernst's law up to about 250 μM $[K^+]_{out}$ and was higher above. Clearly such TPMP uptake measurements cannot provide reliable values for the K^+ diffusion potentials. However, they do show that the range covered indeed extended on both sides of the static head. A further observation leading to the same conclusion is that state 4 mitochondria lose potassium upon addition of valinomycin unless the medium K^+ concentration is higher than 40–90 μM (depending on medium osmolarity). This means that the K^+ chemical gradient, and thus presumably the potential created by K^+ diffusion in experiments such as the one in Figure 2, exceeds the static head $\Delta\psi$ at low $[K^+]_{out}$ values.

Although H^+ uptake is driven by both $\Delta\psi$ and ΔpH, these two components of $\Delta\tilde{\mu}_H$ are not kinetically equivalent: non-ohmic or ohmic current–voltage relationships are obtained, depending on whether the driving force is respectively $\Delta\psi$ or ΔpH (Krishnamoorthy & Hinkel, 1984; O'Shea et al., 1984). Thus driving forces expressed as $\Delta\tilde{\mu}_H$ can strictly be compared only if they are made up in the same proportion of $\Delta\psi$ and ΔpH. This is the case in the present experiments, since both proton-flux measurements and determinations of the rate of respiration at various inhibitor concentrations were carried out with mitochondria (initially) in static head, for which $\Delta\tilde{\mu}_H$ was composed essentially of $\Delta\psi$, with ΔpH limited to a few millivolts. The contribution of such a ΔpH to proton influx is negligible (Krishnamoorthy & Hinkle, 1984; O'Shea et al., 1984).

We have thus satisfied ourselves that at the lowest K^+ concentrations used the driving force for H^+ uptake in the time span during which the initial J_K^{eff} was recorded was higher than in static head mitochondria, while J_K^{eff} was much lower than nJ_e^{sh} (with n = either 4 or 3 H^+/e). Thus it cannot be argued that the two curves, $nJ_e^{sh}/\Delta\tilde{\mu}_H^{sh}$ vs. $\Delta\tilde{\mu}_H^{sh}$ and $J_K^{eff}/\Delta\tilde{\mu}_H$ vs. $\Delta\tilde{\mu}_H$, actually coincide.

The Broken Mitochondria Hypothesis. Another possible explanation for the behavior of the rate of electron flow and $\Delta\tilde{\mu}_H$ during titrations with inhibitors has recently been advanced by Duszynski & Wojtczack (1984). If a fraction of a typical mitochondrial preparation is composed of open membrane fragments or damaged organelles, respiring at maximal rates without sustaining a $\Delta\tilde{\mu}_H$, and if this uncoupled respiration is preferentially titrated at low inhibitor concentrations, curves such as the one in Figure 1 will result. This hypothesis has been tested by using three different approaches.

NADH Oxidation by Mitochondrial Suspensions. Intact mitochondria oxidize NADH only via the mersalyl-sensitive, rotenone- and antimycin-insensitive cytochrome c shuttle mechanism (Bernardi & Azzone, 1982), and not via the respiratory chain. Thus we took the rate of mersalyl-insensitive NADH oxidation to be a measure of the contamination by open membranes. The procedures followed are outlined under Materials and Methods. In seven experiments, the rate of mersalyl-insensitive NADH oxidation varied from below the detection threshold (estimated at 0.2 nmol mg^{-1}) to 2.8 nmol mg^{-1} min^{-1}. This shows that at least in some preparations a small amount of open fragments was present. A quantitative estimate of their contribution to static head respiration is however made difficult by the high variability, from one preparation to the other, of the rates of NADH and succinate oxidation (as well as of their ratio) by osmotically shocked mitochondria. Part of the reason for this variability may be that only open membrane structures oxidize NADH, while succinate may be oxidized at high rates also by mitochondria

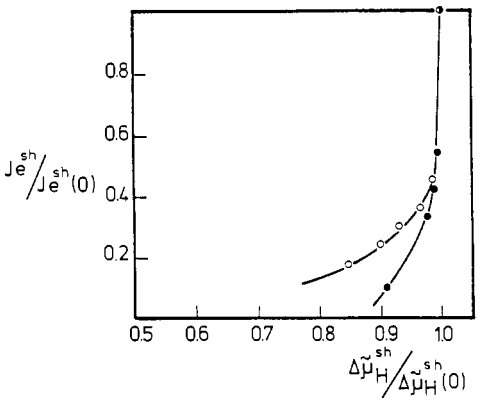

FIGURE 6: Titrations with antimycin (●) and malonate (○) of the rate of respiration and apparent $\Delta\tilde{\mu}_H$ of a mixture of osmotically shocked and normal static head mitochondria. The medium composition was 0.20 M sucrose, 20 mM Tris/Mops, 2 mM P_i/Tris, 0.5 mM EGTA, 5 μM rotenone, 5 mM glycerol, 4 μM TPMP, and 2 μg/mL oligomycin, pH 7.4; T = 25 °C. Normal and shocked mitochondria were mixed in a ratio of about 2.6 to 1 based on protein. Shocked mitochondria accounted for ca. 60% of the initial rate of respiration (40.7 nmol of oxygen atoms mg^{-1}). Antimycin = 0–11.5 ng/mg; malonate = 0–15 mM. Mitochondria (2.8 mg of protein/mL) were incubated for 3 min in the presence of inhibitor; then 20 mM succinate, 50 μM fumarate, and 215 μM malate were added, and respiration or probe uptake was measured after 1 min.

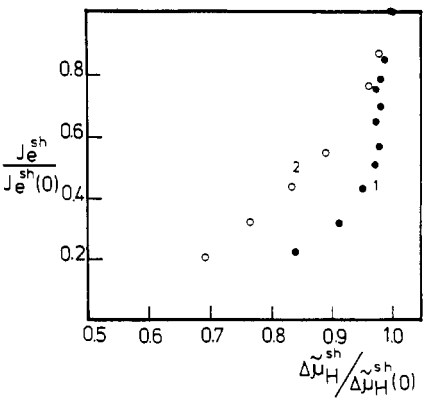

FIGURE 7: Relative rates of electron transfer and $\Delta\tilde{\mu}_H$ during titrations of static head mitochondria with antimycin (1, ●) and malonate (2, ○). Rates of electron transfer and $\Delta\tilde{\mu}_H$ have been divided by their values in the absence of inhibitors. The medium composition and procedure were as in Figure 6. Averages of four experiments are shown.

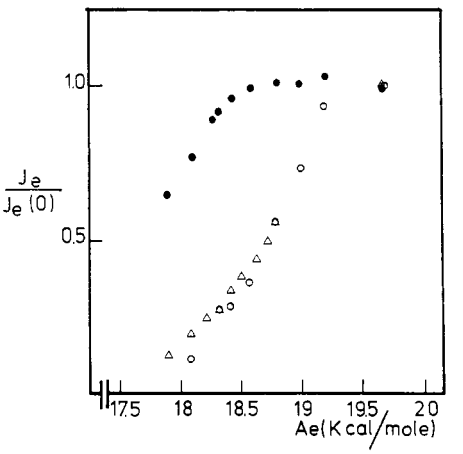

FIGURE 8: Titration by succinate limitation of the rate of electron transfer of static head (●), FCCP-treated (○), or osmotically shocked (△) mitochondria. Rates of respiration (relative to respiration with excess substrate) are plotted vs. the calculated affinity for succinate oxidation. The medium as in Figure 6, except that TPMP was omitted. FCCP = 100 pmol/mg of protein. Mitochondria were incubated for 3 min in the oxygraph cell; then 50 μM fumarate, 215 μM malate, and succinate varying between 50 μM and 20 mM were added, and the initial rate of respiration was measured. The concentration of malate added is that in equilibrium with 50 μM fumarate, assuming an equilibrium constant of 4.3 for the fumarate hydratase reaction. The affinity A_e is calculated as $A_e = RT \ln K(c_{succ}/c_{fum})^{1/2}$, with $K = 1.31 \times 10^{13}$ (at pH = 7.4 and for P_{O_2} = 0.2 atm).

that have been rendered leaky by the osmotic shock, while remaining impermeable to NADH. If the rates of oxidation of NADH and succinate by a preparation of submitochondrial particles, rather than by shocked mitochondria, are used in eq 3 (cf. Materials and Methods), the contribution by open membrane fragments to static head respiration can be calculated as varying from below 1% to about 13%.

Titrations with Malonate and Antimycin. A previous paper (Pietrobon et al., 1981) has already reported that different J_e^{sh} vs. $\Delta\tilde{\mu}_H^{sh}$ curves are obtained in titrations with malonate or antimycin. That different relationships are obtained depending on the inhibitor used constitutes by itself strong evidence against non-ohmic conductance as the sole determinant of the behavior of static head respiration and $\Delta\tilde{\mu}_H$ during titrations. It also constitutes evidence against the presence of a significant amount of broken or completly uncoupled (leaky) vesicles. Respiration by such vesicles would be expected to be preferentially titrated at low inhibitor concentrations with little effect on the measured $\Delta\tilde{\mu}_H$. Thus, as long as this part of the respiration is titrated, the two inhibitors should give rise to the same J_e^{sh} vs. $\Delta\tilde{\mu}_H^{sh}$ relationship. This behavior is verified in the experiment of Figure 6, which shows such a titration for a mixture of osmotically shocked and normal mitochondria. The two titration curves coincide as long as the electron flow due to the shocked mitochondria is being inhibited; when respiration by the undamaged mitochondria begins to be titrated, the two curves diverge. This behavior can be compared to that of Figure 7, which shows the same experiment for a normal mitochondrial suspension. While it is hard to decide whether or not the two curves coincide in their first part, it is clear that broken or leaky vesicles cannot account for a large fraction of the rate of respiration.

Titrations by Substrate Limitation. In Figure 8 rates of respiration, relative to those with excess substrate, are plotted vs. the affinity of the redox reaction in static head, FCCP-treated, and osmotically shocked mitochondria. Respiration is reduced to a given fraction of maximum at much higher succinate concentrations (affinities) in the case of broken or uncoupled mitochondria than in the case of untreated mitochondria. This behavior is inconsistent with the hypothesis that a large percentage of static head respiration be due to membrane fragments or leaky vesicles.

The conclusion may thus be drawn from the various approaches and results presented above that while some broken membranes and/or leaky vesicles are probably present in our preparations, they do not account for more than a small and variable fraction of the "excess" state 4 respiration.

Discussion

Mitochondrial state 4 respiration has long been attributed to an "incomplete coupling" of the energy conversion process. This situation has been elegantly described by using the formalism of nonequilibrium thermodynamics (Kedem & Caplan, 1965): a degree of coupling lower than 1 necessarily leads to attainment of a steady state (static head) rather than equilibrium and implies energy expenditure for the maintenance of an output force ($\Delta\tilde{\mu}_H$). On a mechanistic level, which is what we are concerned with in this paper, incomplete coupling

has been traditionally explained with the known basal permeability of the mitochondrial membrane to protons: flow of protons down their electrochemical gradient through leaks sets up a futile cycle with consequent energy dissipation. According to this picture, static head respiration results from the need to pump out of the matrix as many protons as reenter it via nonproductive pathways; electron transfer is always coupled to H^+ ejection through a fixed stoichiometry. The nonlinear relationship between rate of respiration and output force ($\Delta\tilde{\mu}_H$) obtained by titrating with respiratory inhibitors (see the introduction) has been considered to simply reflect the underlying nonlinear relationship between the membrane leak conductance and $\Delta\tilde{\mu}_H$.

In this work we have shown that the picture outlined above is inadequate. Protons do leak through the mitochondrial membrane, but this current explains only part of static head respiration (approximately 30% according to our measurements). Still another part may well have a trivial origin: it may arise from the presence of completely uncoupled or broken mitochondria; i.e., it would be an artifact arising in the organelle isolation procedures. Again, our data indicate that this cannot account for most of the remaining 70% or so of the rate of oxygen consumption in the resting state. The contribution by leaky or broken mitochondria in our preparations to state 4 respiration is variable and difficult to quantify, but it can confidently be stated not to exceed about 20% even in the worst cases.

This still leaves a substantial fraction of J_e^{sh} unexplained. We propose that this "excess" electron flow may be due to intrinsic uncoupling of the respiratory chain enzymes. Evidence that it may be so, based on model simulations and comparison with experimental data, is presented in the following paper (Pietrobon et al., 1985).

We have determined the dependance of the membrane leak conductance on $\Delta\tilde{\mu}_H$. In some of our experiments a non-ohmic behavior was observed. However, the deviation from a constant relationship (L_H^l vs. $\Delta\tilde{\mu}_H$) is slight, so much so that it may have been simply missed because of experimental noise in that part of our determinations that suggests instead an ohmic behavior. Our data are in reasonably good agreement with those recently presented by Krishnamoorthy & Hinkle (1984) and by O'Shea et al. (1984). The analysis presented by the former authors in terms of a trapezoidal energy barrier may thus be considered to satisfactorily explain our results as well.

Krishnamoorthy & Hinkel (1984) and O'Shea et al. (1984) have proposed that the observed current–voltage relationships explain the relation between rate of electron flow and output force, with no need to invoke intrinsic uncoupling or other explanations for an excess electron flow that, according to them, should not exist. A reexamination of the data presented by Krisnamoorthy and Hinkle shows that this conclusion was not warranted: a necessary requisite for it is that plots of $nJ_e^{sh}/\Delta\tilde{\mu}_H^{sh}$ vs. $\Delta\tilde{\mu}_H^{sh}$ and J_H^l vs. $\Delta\tilde{\mu}_H$ be superimposable. This is evidently not the case, as shown in Figure 4. Krishnamoorthy and Hinkle's data actually show an even greater discrepancy between the two curves than our own data. The data presented by O'Shea et al. (1984) do not allow a similar conclusion, since respiration measurements are not presented. The J_H^l vs. $\Delta\psi$ curves these authors present in their Figure 3 do seem to exhibit a sharper curvature, occurring at lower $\Delta\psi$ than in our case. The maximal value for J_H^l they present for mitochondria, approximately 47 nmol mg^{-1} min^{-1}, is close to the maximal values we observed. Any discrepancy between their data and ours thus would seem to be due mostly to the $\Delta\psi$ ($\Delta\tilde{\mu}_H$) scale. We reiterate (see Results) that the highest diffusion potentials we reached certainly exceeded state 4 $\Delta\psi$ values.

The group led by Jackson has argued against the intrinsic uncoupling idea on the basis of the coincidence of the values of cyclic electron flow multiplied by the H^+/e stoichiometry, nJ_e ($n = 2$), and dissipatory ion currents, J_{diss}, and on the basis of the independence of their ratio from light intensity in chromatophores (Cotton et al., 1984). However, as in other papers by the same group, the relevant experiments were performed in the absence of a suitable blocking agent for the F_0F_1 ATPase. The same group has clarified that under these circumstances practically all of the H^+ backflow through the membrane occurs via F_0F_1 (Clark et al., 1983; Cotton & Jackson, 1984). Because of the conductance properties of the ATPase, $\Delta\psi$ is held at values considerably lower than in the presence of venturicidin (Cotton & Jackson, 1984). Under these conditions the extent of slippage would be expected to be low [cf. following paper in this issue (Pietrobon et al., 1985)], especially in comparison with the high electron and ion fluxes involved, and it could easily have been missed in view of the experimental uncertainties mentioned by the authors. When the ATPases are blocked, the curve relating J_{diss} to $\Delta\psi$ exhibits a gentler rise, at higher $\Delta\psi$ values (Cotton & Jackson, 1984), and it may then reflect the membrane conductance properties. A comparison of J_e and J_{diss} under those conditions would be more useful for the issue we are contending with. (In all our experiments the ATPase was blocked with oligomycin.) Comparison of parts A and B of Figure 3 in Cotton & Jackson (1984) however shows that different J_{diss} vs. $\Delta\psi$ curves may be obtained (in the presence of venturicidin), apparently depending on the length of the illumination period to which the chromatophores are subjected. What the curves really reflect is therefore doubtful.

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Intrinsic Uncoupling of Mitochondrial Proton Pumps. 2. Modeling Studies†

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ABSTRACT: The thermodynamic and kinetic properties associated with intrinsic uncoupling in a six-state model of a redox proton pump have been studied by computing the flow–force relations for different degrees of coupling. Analysis of these relations shows the regulatory influence of the thermodynamic forces on the extent and relative contributions of redox slip and proton slip. Inhibition has been introduced into the model in two different ways, corresponding to possible modes of action of experimental inhibitors. Experiments relating the rate of electron transfer to $\Delta\tilde{\mu}_H$ at static head upon progressive inhibition of the pumps have been simulated considering (1) the limiting case that the nonzero rate of electron transfer at static head is only due to intrinsic uncoupling (no leaks) and (2) the experimentally observed case that about 30% of the nonzero rate of electron transfer at static head is due to a constant proton leakage conductance in parallel with the pumps, the rest being due to intrinsic uncoupling. The same simulations have been performed for experiments in which the rate of electron transfer is varied by varying the substrate concentration rather than by using an inhibitor. The corresponding experimental results obtained by measuring $\Delta\tilde{\mu}_H$ and the rate of electron transfer at different succinate concentrations in rat liver mitochondria are presented. Comparison between simulated behavior and experimental results leads to the general conclusion that the typical relationship between rate of electron transfer and $\Delta\tilde{\mu}_H$ found in mitochondria at static head could certainly be a manifestation of some degree of intrinsic uncoupling in the redox proton pumps. The physiological significance of this conclusion and its implications with regard to the interpretation of measurements of the stoichiometry of coupled processes are discussed.

An ion pump promotes the coupling between a scalar reaction and a transmembrane vectorial ion flow. Physiological imperfections in the coupling may be caused by two factors: (1) parallel pathways of ion flow (leaks) and (2) intrinsic uncoupling within the pumps. Many workers assume that ion pumps are completely coupled and that all uncoupling may be attributed to external leaks. Actually there is no thermodynamic or kinetic justification for such an assumption. Recently, a number of reports have appeared showing that a certain degree of intrinsic uncoupling is indeed present in ion pumps and transport systems.

Passive pump-mediated (ouabain-sensitive) Rb^+ fluxes in the absence of ATP and phosphate have been measured in phospholipid vesicles reconstituted with kidney (Na,K)ATPase (Karlish & Stein, 1982) and in gastric vesicles enriched in (H,K)ATPase (Soumarmon et al., 1984). It has been also reported that a (Na,K)ATPase-mediated Rb^+ uptake decoupled from Na^+ efflux can be induced in erythrocytes by applying an alternating current (ac) field in the range of 20 V/cm (Serpersu & Tsong, 1984). The anion transporter of the human red cell membrane, whose physiological function is a one-to-one exchange transport of extracellular with intracellular anions, also mediates net anion efflux (Knauf et al., 1977). This efflux is the result of intrinsic uncoupling of the transporter (Frölich et al., 1983). A DCCD[1]-sensitive ATPase

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