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# Variable Proton Conductance of Submitochondrial Particles†

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ABSTRACT: The relationship between the rate of substrate oxidation and the protonmotive force (electrochemical proton gradient) generated by bovine heart submitochondrial particles has been examined. Unexpectedly, oxidation of succinate generated a higher protonmotive force than the oxidation of NADH, although the rate of proton translocation across the membrane was inferred to be considerably lower with succinate as substrate. The data suggest that the flow of electrons through site 1 of the respiratory chain may increase the conductance of the mitochondrial membrane for protons. Upon reduction of the rate of succinate oxidation by titration with malonate, the protonmotive force remained essentially constant until the extent of inhibition was greater than 75%. The general conclusion from this work is that a constant passive membrane conductance for protons cannot be assumed.

The conservation of energy, released via respiratory chain electron flow, as an electrochemical proton gradient (protonmotive force = $\Delta p$) is the major feature of the chemiosmotic mechanism in mitochondria as well as bacteria and chloroplasts (Mitchell, 1966). Many important contributions seem to have confirmed the link between respiration and protonmotive force (Boyer et al., 1977), but still there is little evidence on the factors that can modulate respiration, particularly if and how the protonmotive force itself can influence the rate of respiration and consequently the rate of proton translocation.

An important role between respiration rate and magnitude of the electrochemical proton gradient is played by the membrane; Mitchell's concept is that of a "passive" membrane (Mitchell, 1978) whose low effective proton conductance [0.11 nequiv of $H^+$ $min^{-1}$ (mg of protein)$^{-1}$ $mV^{-1}$] (Mitchell & Moyle, 1967a) should be protonmotive force independent and therefore limit the state 4 respiration in mitochondria (Mitchell, 1968). More recently, however, Nicholls (1974a, 1977) found that the dielectric characteristics of the membrane, thus the proton conductance, can change under different

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conditions such as different respiratory rates. He studied this phenomenon in mitochondria derived from two physiologically different tissues, namely, rat liver and cold-adapted guinea pig's or hamster's brown adipose tissue, which have different features, the most interesting being that exogenous purine nucleotides can physiologically modulate the conductance of the membrane in mitochondria from brown adipose tissues (Nicholls, 1974a,b, 1977) and consequently the maximal $\Delta p$.

As the knowledge of the electrical characteristics of the membrane (in terms of proton conductance) is essential for the identification of $\Delta p$ as the determinant of respiratory control (Boyer et al., 1977; Mitchell, 1968; Nicholls, 1974b), we have studied the relationship between the rate of proton translocation (proton current) and the protonmotive force in bovine heart submitochondrial particles (SMP) oxidizing flavoprotein- and $NAD^+$-linked substrates at different rates. As we found a nonlinear relation between proton current and protonmotive force, our data will be compared with those already reported by other authors and by us (Sorgato et al., 1978a,b) in order to try and explain some of our unexpected results in terms of a minimal form of the chemiosmotic mechanism.

## Materials and Methods

Mg–ATP bovine heart submitochondrial particles were prepared according to Ferguson et al. (1977). The membrane potential and pH gradient values were estimated from the extent of uptake of $S^{14}CN^-$ and [$^{14}$C]methylamine into the particles with the flow dialysis technique, with the procedures and assumptions detailed elsewhere (Sorgato et al., 1978a). It is noteworthy that the assumption that $SCN^-$ can reach a Nernst equilibrium distribution across biological membranes is supported by the work of Catterall et al. (1976), which has shown that both the distribution of this ion and direct measurements with an electrode gave similar estimates of the membrane potential for neuroblastoma cells. Oxygen consumption was measured with a Clark-type oxygen electrode in a chamber of 1-mL capacity. NADH absorption was followed at 340 nm in a Unicam SP1800 spectrophotometer. All experiments were carried out at room temperature. Protein was determined by the biuret method (Gornall et al., 1949). $KS^{14}CN$ and [$^{14}$C]methylamine hydrochloride were purchased from the Radiochemical Center (Amersham, England), and rotenone was from Sigma. Carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) was a gift of Dr. P. G. Heytler (E. I. du Pont de Nemours & Co., Inc., Wilmington, DE), and nigericin was a gift of Dr. R. L. Hammill (Lilly Research Laboratories, IN). All other reagents were of the highest grade commercially available. The basic medium used in all experiments was composed of 10 mM Na-Hepes, 200 mM sucrose, 2 mM $MgCl_2$, 50 mM KCl, and 5 mM $KH_2PO_4$, at pH 7.5. A value of 1 $\mu$L/mg of protein for the internal volume of the particles was always adopted (Sorgato et al., 1978a).

## Results

In a previous paper (Sorgato et al., 1978a) concerning the generation and magnitude of the protonmotive force in bovine heart submitochondrial particles, we reported that the protonmotive force developed in submitochondrial particles, suspended in a $P_i$–Tris medium, had approximately the same value (140–145 mV) with either NADH or succinate as substrate, each of which supported a different rate of oxygen consumption and proton translocation.

This rather surprising result could be tentatively explained by assuming that particles cannot maintain protonmotive forces of higher magnitudes without damaging their membrane through dielectric breakdown (Nicholls, 1977). In the same paper we also reported that submitochondrial particles are in fact capable of maintaining a higher $\Delta p$ (185 mV) when incubated in a sucrose–Hepes–KCl medium and oxidizing NADH. Thus, a protonmotive force of 150 mV cannot always be considered to be the upper limit, but as in $P_i$–Tris buffer where the protonmotive force comprised solely a membrane potential, it may be that the protonmotive force is limited by the capacity of the membrane to tolerate an extreme value of the membrane potential.

These findings led us to investigate, in more detail, the apparent capacity of the mitochondrial membrane to modulate its electric characteristics in response to the generation of proton currents of different intensities, as can happen when submitochondrial particles are respiring on substrates that activate a different number of $H^+$ translocating sites [the intensity of the proton current being calculated by multiplying the $H^+/O$ ratio times the respiration rate (Mitchell & Moyle, 1967a,b)]. The $H^+/O$ ratio has been taken equal to 6 for succinate oxidation and 9 for NADH oxidation (Brand, 1977).

The relationship between proton currents and protonmotive forces in particles suspended in a sucrose–Hepes–KCl medium was rather intriguing, as shown in Table I. The data indeed confirm the value of $\Delta p$ (185–195 mV) maintained by submitochondrial particles when NADH is substrate but indicate that succinate oxidation is capable of generating a higher protonmotive force up to $\sim$230 mV. This result was very surprising because the respiration rate in the presence of succinate is usually lower than that with NADH, and consequently the proton currents are inferred to be markedly different (see Table I).

As mentioned earlier, the fact that different substrates can give rise to the same protonmotive force, although their respiration rates and associated proton currents are different, can be explained by the assumption that the proton conductance of the membrane is not always a constant and that the inner membrane of mitochondria can behave as an ohmic conductor only up to a certain value of $\Delta p$ and proton current (Nicholls, 1974a; Kell et al., 1978a). Therefore, above these values, the membrane would behave nonohmically; as a consequence, since the linear relationship between the proton current and the protonmotive force no longer exits, the former would increase without a concomitant increase of the latter. This disproportionate increase between the proton current and the $\Delta p$ can be attributed to a variation of the proton conductance only if the stoichiometry of $H^+$ translocating per energy conserving site, measured under non-steady-state conditions (Mitchell & Moyle, 1967b; Nicholls, 1974b), remains invariant under steady-state conditions (Mitchell, 1966, 1968). Although it has already been demonstrated that such behavior is found in mitochondria from several sources (Nicholls, 1974a, 1977), the same explanation cannot be applied to our results as the higher value of the protonmotive force is found with the substrate, succinate, that generates the lower rate of proton translocation (Table I).

For exclusion of the idea that the lower value of the protonmotive force observed with NADH as substrate was caused by the exhaustion of NADH during the experiment, which lasts $\sim$10 min before the addition of an uncoupler, ethanol and alcohol dehydrogenase were used as a NADH regenerating system. The exhaustion of ethanol itself was ruled out by making subsequent additions of extra ethanol during the experiment, which did not alter the magnitude of $\Delta p$. Moreover, possible damage of the membrane by ethanol was

Table I: Relation between Rate of Proton Translocation and Magnitude of the Protonmotive Force in Bovine Heart Submitochondrial Particles Respiring on Succinate, NADH, and Succinate Plus NADH (in Presence and Absence of Rotenone)[a]

| substrate | respiratory rate [μg of O $min^{-1}$ (mg of protein)$^{-1}$] | $H^+$ current [μg of $H^+$ $min^{-1}$ (mg of protein)$^{-1}$][b] | Δψ (mV) | 59(ΔpH) (mV) | Δp (mV) |
|---|---|---|---|---|---|
| expt 1 | | | | | |
| succinate (10 mM) | 0.155 | 0.930 | 132 | 90 | 222 |
| $NAD^+$ (1 mM) | 0.235 | 2.155 | 93 | 95 | 188 |
| succinate (10 mM) + $NAD^+$ (1 mM) | 0.241 | 0.420 (succinate) 1.539 (NADH) | 110 | 83 | 193 |
| expt 2 | | | | | |
| succinate (20 mM) | 0.200 | 1.2 | 135 | 78 | 213 |
| $NAD^+$ (1 mM) | 0.310 | 2.790 | 100 | 95 | 195 |
| succinate (20 mM) + $NAD^+$ (1 mM) | 0.380 | 0.420 (succinate) 2.790 (NADH) | 115 | 70 | 185 |
| succinate (20 mM) + $NAD^+$ (1 mM) + 0.4 μg of rotenone | nd[c] | nd | 138 | 83 | 221 |
| succinate (20 mM) + $NAD^+$ (1 mM) + 5 μg of rotenone | 0.200 | 1.2 | 133 | 78 | 211 |
| expt 3 | | | | | |
| succinate (20 mM) | 0.240 | 1.44 | 150 | 88 | 238 |
| $NAD^+$ (1 mM) | 0.220 | 1.98 | 103 | 100 | 203 |
| succinate (20 mM) + $NAD^+$ (1 mM) | 0.230 | nd | 130 | 80 | 210 |
| expt 4 | | | | | |
| succinate (20 mM) | 0.150 | 0.900 | 137 | 90 | 227 |
| $NAD^+$ (1 mM) | 0.230 | 2.070 | 97 | 100 | 197 |
| succinate (20 mM) + $NAD^+$ (1 mM) | nd | nd | 115 | 85 | 200 |
| succinate (20 mM) + $NAD^+$ (1 mM) + 5 μg of rotenone | 0.150 | 0.900 | 120 | 93 | 213 |

[a] Respiration rates and protonmotive force determinations were usually carried out by using the same amount of particles. In the upper chamber of the flow dialysis apparatus, 1 mL of the basic medium, over which a water-saturated $O_2$ stream was continuously blown, contained 7.95 mg of protein (expt 1), 7.5 mg of protein (expt 2), 4.375 mg of protein (for the determination of the membrane potential in expt 3), 5.25 mg of protein (for the determination of the pH gradient in expt 3), and 8.25 mg of protein (expt 4). For determination of the membrane potential and pH gradient, 20 μM $KS^{14}CN$ (60 μCi/μmol) and 20 μM [$^{14}$C]$MeNH_2$·HCl (55.5 μCi/μmol) were added, respectively. When NADH was a substrate, 1% (v/v) ethanol and 0.05 mg of alcohol dehydrogenase were present. The reaction was started by the addition of 1 mM $NAD^+$. When 0.4 μg of rotenone, dissolved in ethanol, was added to have 50% inhibition of NADH dehydrogenase activity or 5 μg to get 100% inhibition, the amount of absolute ethanol added was diminished so to always have the same final percentage of alcohol present at the beginning of the experiment. Rotenone was routinely allowed to incubate with the particles from 5 to 10 min before the initiation of the experiment in the flow dialysis cell and at least 3 min in the $O_2$ electrode cell. When succinate was a substrate, 10 or 20 mM sodium succinate was allowed to incubate for at least 3 min in order to activate succinate dehydrogenase before starting the experiment by adding radioactive label. 5 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) was added after ~10 min from the addition of the radioactive label. All experiments were done at room temperature. [b] The intensity of the proton current was calculated by multiplying the respiration rate times the $H^+/O$ ratio of the substrate used. The $H^+/O$ ratio of 6 and 9 was taken when succinate and NADH were the substrate (Brand, 1977). When succinate and NADH were oxidized together in the absence of rotenone, the relative proton current contribution of succinate was calculated by multiplying the $H^+/O$ ratio for succinate times the respiratory rate of succinate in the presence of NADH. This was calculated by substracting from the total oxygen consumption due to the oxidation of both substrates together, as measured with a Clark-type oxygen electrode, the relative contribution of NADH oxidation, which was followed spectrophotometrically at 340 nm in the presence of succinate. [c] nd = not determined.

excluded since the size of the succinate-supported protonmotive force was not diminished by the presence of ethanol alone (data not shown) or when the regenerating system was present together with rotenone to block NADH oxidation (see Table I).

The regenerating system produces acetaldehyde, which, at concentrations of 12 mM and above, can substantially inhibit oxidative phosphorylation by rat liver mitochondria (Cederbaum et al., 1974). Therefore, the question arises as to whether the presence of acetaldehyde might be responsible for the lower values of Δp observed in experiments with NADH as substrate. Several lines of evidence militate against such an explanation. (1) Addition of 20 mM acetaldehyde to submitochondrial particles oxidizing either NADH (in the absence of the regenerating system) or succinate had no effect on the value of the membrane potential observed for either substrate. The implication is that acetaldehyde does not cause general damage to the membrane. (2) As shown in Table I (and see below), the value of Δp observed with both NADH and succinate present as substrates was lower than that found with succinate alone. The failure of succinate oxidation in this experiment to generate the value of Δp seen with succinate as the sole substrate cannot be attributed to an effect of acetaldehyde (see 1 above) or to a decreased rate of succinate oxidation (see Figure 1). (3) It would be expected than any inhibitory effect of acetaldehyde on Δp would be more apparent with high particle concentrations at which a greater accumulation of acetaldehyde might occur. Yet, the same value of Δp was observed over a wide range of particle concentrations [Table I and Sorgato et al. (1978a)]. Although acetaldehyde must be produced in the flow dialysis cell, the steady-state concentration is presumably rather low owing to constant loss of this volatile compound (bp 21 °C) by boiling and dialysis. It should be pointed out that the regenerating system was used to supply NADH because two difficulties arise when NADH is itself used as substrate. First, the net consumption of a proton by NADH oxidation causes substantial changes in the medium pH under our reaction conditions, and, second, for reasons that we do not understand, [$^{14}$C]methylamine in the diffusate from the flow dialysis cell does not reach a stable steady state, thus making estimation of the pH gradient difficult.

The above considerations also draw attention to features of the flow dialysis procedure that may require consideration. The experiments are done with relatively high concentrations of membrane particles or vesicles and over relatively long periods of time so that considerable changes in the concentrations of substrates and products can occur during an experiment. The use of a regenerating system obviates this

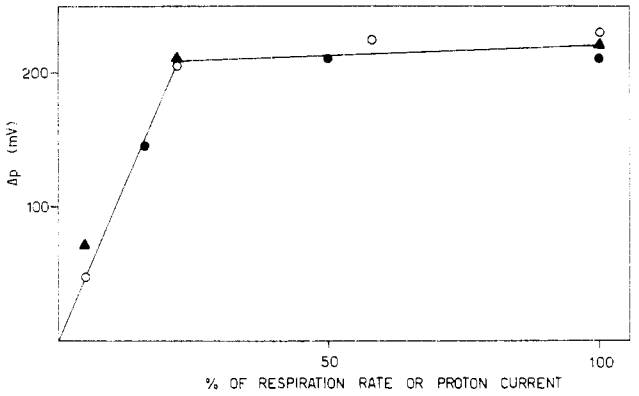


FIGURE 1: Effect on $\Delta p$ of reducing the rate of succinate oxidation by titration with malonate. Both in the flow dialysis and in the $O_2$ electrode cells the protein concentration was 5.5 mg/mL in the experiments indicated with ● and 7.9 mg/mL in the experiments indicated with ○ and ▲. For inhibition of succinate oxidation, increasing amounts of malonate were added (from 0 to 25 mM). Malonate was usually allowed to preincubate, in the presence of succinate and the particles, from 5 to 10 min in order to allow the respiration to become linear. When malonate was inhibiting succinate oxidation by ~95%, only the membrane potential was determined, as at that inhibition the pH gradient would have been insignificant. All other conditions were as in Table I.

problem to the extent that the concentration of respiratory chain substrate remains relatively constant although the concentrations of the other substrates of the system will change.

The odd behavior of the membrane toward the two substrates, NADH and succinate, prompted us to investigate the behavior of the SMP in the presence of both reductants. When they are oxidized simultaneously, the oxygen consumption is higher (see respiration rates of expt 1 and 2 in Table I) than that due to a single substrate. The $H^+$ current due to the individual substrates, when oxidized together, can be calculated from the total oxygen consumption by both substrates together and from the rate of NADH oxidation, followed spectrophotometrically, in the presence of succinate (see Table I footnotes). The proton current due to the simultaneous oxidation of succinate and NADH is lower than the sum of the proton currents of the single substrates but still higher than the proton current with succinate alone. Indeed, the succinate oxidation rate seems to be inhibited when NADH is present.

The protonmotive force resulting from the oxidation of both succinate and NADH is very close to the one obtained when NADH is the only substrate, suggesting that the oxidation of NADH per se does not allow the membrane to support a proton gradient as high as when succinate is the sole substrate. If this were indeed the case, the inhibition of NADH dehydrogenase by rotenone in the presence of succinate oxidation should restore the protonmotive force to its higher value. In fact, when rotenone is inhibiting NADH oxidation by either 50 or 100%, $\Delta p$ rises to a value very close to that with succinate alone (expt 2 and 4 of Table I).

The relationship between the magnitude of $\Delta p$ and the size of the proton current was examined further in experiments in which the rate of respiration was reduced by the addition of inhibitors. Figure 1 shows that when the rate of succinate oxidation was reduced by titration with malonate, the magnitude of $\Delta p$ did not markedly decrease until the extent of inhbition exceeded 75%. This result indicates that in submitochondrial particles the membrane conductance toward protons does not behave ohmically so that a constant $\Delta p$ is maintained over a wide range of proton translocation rates. This contrasts with an earlier experiment in which titration of the rate of NADH oxidation with rotenone showed that the membrane potential declined almost in parallel with the respiration rate, behavior closer to an ohmic conductance of the membrane for protons (Kell et al., 1978a). In more recent experiments we have attempted to study the effect of rotenone on $\Delta p$ measured by flow dialysis using the same reaction medium, but with NADH as substrate, as used in the experiments of Figure 1. These experiments have been hampered by the difficulty of accurately measuring the extent of inhibition by rotenone at the relatively high concentrations of particles required for the flow dialysis experiments. However, despite this difficulty it does appear that $\Delta p$ decreases by only 30 mV when the rate of NADH oxidation is inhibited ~50% by rotenone. Thus, in this case also a nonohmic membrane conductance for protons is indicated although the relationship between the proton current generated by NADH oxidation and the magnitude of $\Delta p$ requires further investigation.

A detailed study on a titration similar to that of Figure 1 was carried out by Nicholls (1974a) on rat liver mitochondria. In such a system there was good linear relationship between the proton current and the protonmotive force except at high respiratory rates: the $\Delta p$ value was decreased 26% when succinate oxidation was inhibited 50% by the presence of malonate and ~37% when malonate concentration was such to lower respiration to 40% of its initial value. In the case of submitochondrial particles, even when succinate oxidation is limited to 20% of its original value, the resulting protonmotive force is almost unchanged (Figure 1).

A possible criticism of our findings is that the technique we use to estimate the magnitude of the components of the protonmotive force is not sufficiently sensitive to detect high values, particularly of the membrane potential (the membrane potential being the major contributor to $\Delta p$), so that the maximum rate of succinate oxidation could maintain a membrane potential that is higher than the upper limit of detection by our experimental methods. For exclusion of this possibility, an estimation of the membrane potential was carried out in the presence of succinate and nigericin. Nigericin exchanges protons with potassium ions, so that if one adds nigericin after the protonmotive force has built up, potassium ions present in the medium should exchange for those hydrogen ions pumped into the lumen of the particles, resulting in an increase in the membrane potential at the expense of the pH gradient (Ashton & Steinrauf, 1970; Montal et al., 1970; Pressman et al., 1967). Several controls performed under these conditions revealed that the membrane potential, as detected during succinate oxidation alone, could be increased up to 15–20 mV upon addition of nigericin (0.5 $\mu$g/mg of protein), thus confirming that the values normally estimated were not at an artifactual low level.

In Table I the relative contributions of the electrical and chemical components to the protonmotive force are given. It appears that, comparing succinate and NADH as substrate, the most variable is the membrane potential contribution, which was consistently lower when NADH dehydrogenase was operating. On the other hand, the pH gradient was almost always slightly lower when succinate is present.

Though one would be tempted to conclude that flavin-linked substrates can develop higher membrane potentials and, in turn, site 1 linked substrates higher pH gradients, or that the relative contribution of the components of the protonmotive force somehow depends on the origin of the proton current, there is as yet no explanation available for the variation in the relative contributions of the membrane potential and pH gradient shown in Table I.

The reaction medium used in the present work was the same as that employed by Bashford & Thayer (1977) in an investigation of the protonmotive force generated by submitochondrial particles. On the basis of the extent of quenching of 9-aminoacridine fluorescence, they concluded that NADH oxidation supported a pH gradient of ~165 mV. Although they used a slightly different preparation of submitochondrial particles, comparison of their result with the pH gradient of ~90 mV obtained from the extent of methylamine uptake (Table I) shows that a larger pH gradient is indicated by the 9-aminoacridine method. Similar discrepancies have been observed previously with both submitochondrial particles (Sorgato et al., 1978a) and thylakoids (Benedetti & Garlaschi, 1977). Bashford & Thayer (1977) also indirectly estimated that the response of the spectroscopic probe oxonol VI corresponded to a membrane potential of ~65 mV in the presence of NADH. Thus, whereas in the present work the uptake of $S^{14}CN^-$ and [$^{14}$C]methylamine has shown that the membrane potential and pH gradient are almost equal in magnitude (Table I) when NADH is the substrate, the use of 9-aminoacridine fluorescence and oxonol VI suggests that the pH gradient is the dominant term. We are inclined to the view that 9-aminoacridine fluorescence quenching overestimates the pH gradient and thus that the indirect determination (Bashford & Thayer, 1977) of the membrane potential from the changes in absorbance of oxonol VI was an underestimate.

A further difference from the results of Bashford & Thayer (1977) is that they observed an identical response of oxonol VI, and thus, by implication, an identical membrane potential, induced by both NADH and succinate oxidation. In contrast, our data (Table I) consistently indicated that respiration with succinate generated the larger membrane potential.

## Discussion

The data presented in this paper indicate that there is a complex relationship between the proton current (rate of proton translocation) and the protonmotive force in the membrane of submitochondrial particles suspended in a sucrose–Hepes–KCl medium and that there is an upper limit on the value of the protonmotive force attainable. Nicholls (1974a, 1977) suggested that the ability of the membrane to limit its maximal protonmotive force is the effective way in which the mitochondrial membrane prevents its own dielectric breakdown. A mechanism of this kind predicts that the same value of the protonmotive force should be generated with either NADH or succinate as substrate. Yet, it is both evident (Table I) and puzzling that in the sucrose–Hepes–KCl medium flavoprotein-linked succinate oxidation generates a higher protonmotive force than NADH oxidation, although in a $P_i$–Tris medium the two substrates generated a similar protonmotive force which comprised solely a membrane potential (Sorgato et al., 1978a).

Consideration must be given to several explanations for the failure of the higher NADH respiration rate to generate the maximal value of the protonmotive force. First, if sufficient NADH respiration rate was not coupled to the generation of the protonmotive force, then the effective proton current linked to NADH oxidation could really be below the proton current linked to succinate oxidation. This situation would be realized if, for example, the preparation of submitochondrial particles contained substantial amounts of particles which exhibited an uncoupled NADH oxidase activity and no succinate dehydrogenase activity. An explanation along these lines is inherently unlikely and can almost certainly be excluded by the observation that addition of the uncoupler carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) stimulated the NADH respiration rate by approximately 3-fold while succinate respiration was increased by less than 1.5-fold. Other explanations for the lower protonmotive force sustained by NADH oxidation on the basis of experimental artifacts, e.g., exhaustion of or damage by ethanol, have been ruled out by the appropriate controls detailed under Results.

The magnitude of the protonmotive force is expected to be limited by the rate at which the membrane can conduct protons down their electrochemical gradient. If during electron flow through the first proton-translocating region of the respiratory chain the membrane conductance is increased above the level found when only the second and third regions are operating, then the consequence could be a lower protonmotive force during NADH oxidation. Such a mechanism would also explain why the simultaneous oxidation of NADH and succinate generated the protonmotive force characteristic of NADH alone and also account for the increase in the protonmotive force when NADH oxidation was blocked by rotenone (Table I).

Data somewhat similar to those of the present paper have been reported about brown adipose tissue mitochondria from cold-adapted guinea pigs oxidizing glycerol 3-phosphate or palmitoyl-L-carnitine (plus malate) in the presence of exogenous nucleotides, such as GDP, which is believed to inhibit specifically the high proton permeability of the inner membrane of these mitochondria (Nicholls, 1977). When the rate of glycerol 3-phosphate oxidation, which is flavin linked, was increased by raising the concentration of glycerol 3-phosphate, the protonmotive force increased in parallel until a threshold upper limit was reached at ~220 mV (Nicholls, 1977). Further increases in the oxidation rate did not increase the magnitude of the protonmotive force, analogous to the results with succinate found in the present work. However, when the adipose tissue mitochondria were supplied with palmitoyl-L-carnitine (plus malate), whose oxidation sequentially involves both a flavoprotein and $NAD^+$, the maximum value of the protonmotive force attained was lower than the threshold value with glycerol 3-phosphate as substrate. More crucially, the rate of oxidation of palmitoyl-L-carnitine (plus malate) was such that the resulting proton current was at least equal to the minimum current due to glycerol 3-phosphate oxidation required to establish the threshold value of the protonmotive force. These results led to the conclusion that the threshold-limiting protonmotive force attainable by glycerol 3-phosphate oxidation was larger than that by NAD-linked respiration, but this raises the problem, also encountered in the present work, as to why the upper threshold value of the protonmotive force is not reached, despite the apparently adequate proton current, with NAD-linked respiration. In the light of presently available data, the conclusion is that electron flow through the first proton translocating region of the respiratory chain increases the membrane conductance toward protons, at least under some conditions. It is not known whether this site 1 region specific regulation of membrane conductance has any in vivo role, but it might be involved in the regulation of reverse and forward flow of electrons through the site 1, as explored by Nicholls (1977).

The distinctly nonohmic relationship between the protonmotive force in submitochondrial particles and the rate of proton translocation reported in the present paper (Figure 1) parallels the behavior that has now been observed with several types of mitochondria (Nicholls, 1974a,b, 1977), thylakoids (Schönfeld & Neumann, 1977), and membrane vesicles from *Paracoccus denitrificans* (Kell et al., 1978b). All of these observations raise the possibility that there exists in energy-

transducing membranes a component which is sensitive to the rate of proton translocation and the magnitude of the protonmotive force. This variability of the membrane conductance for protons, which we have also reported for submitochondrial particles suspended in a $P_i$–Tris reaction medium (Sorgato et al., 1978b), now needs to be taken into account in analyses of oxidative phosphorylation and respiratory control that are based on irreversible thermodynamics. Until now these studies have made the reasonable assumption of a constant proton leak or conductance (Hinkle et al., 1975; van Dam & Westerhoff, 1977). The decay of a pH change after giving an acid or respiratory pulse to mitochondria is also usually analyzed on the basis of a constant membrane conductance for protons (Mitchell & Moyle, 1967a,b). Presumably in this type of experiment the protonmotive force does not reach a sufficiently high value to induce changes in proton conductance, but it could be that under some experimental conditions this assumption will not always be justified.

Some comment is required as to why in previous work the same value of the protonmotive force was found in the $P_i$–Tris medium for both NADH and succinate oxidation and why the value of the protonmotive force was lower than in Hepes–KCl–sucrose with either substrate. We have redetermined the protonmotive force in $P_i$–Tris and have confirmed the earlier results, but we have, on occasion in recent experiments, noted that succinate generated a slightly higher protonmotive force than NADH. Further investigation of this point is required. Nevertheless, the proton conductance of the membrane seems to show the same behavior in $P_i$–Tris when succinate or NADH oxidation is progressively inhibited (Kell et al., 1978a; Sorgato et al., 1978b). It could be argued that the low values of the protonmotive force in the $P_i$–Tris medium are artifacts caused either by the failure to detect the pH gradient under these conditions or by damage to the particles by the components of this reaction medium. The reasons for rejecting these arguments are detailed elsewhere (Sorgato et al., 1978a).

If it is accepted that the protonmotive force is low in the low osmolarity and low ionic strength $P_i$–Tris medium, then one might tentatively suggest that the ionic strength and osmolarity of the medium directly affect the proton conductance. These variables and not thermodynamic equilibrium with the respiratory chain could then be determinants of the magnitude of the protonmotive force.

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