Studies on the Mechanism of Oxidative Phosphorylation
FLOW-FORCE RELATIONSHIPS IN MITOCHONDRIAL ENERGY-LINKED REACTIONS*

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The relationship between the steady-state level of membrane potential (Δψ) and the rates of energy production and consumption has been studied in mitochondria and submitochondrial particles. The energy-linked reactions investigated were oxidative phosphorylation (with NADH, succinate, and β-hydroxybutyrate as respiratory substrates) and nucleoside triphosphate-driven transhydrogenation from NADH to NADP and uphill electron transfer from succinate to NAD. Results have shown the following. 1) Attenuation of the rates of the energy-producing reactions results in a parallel change in the rates of the energy-consuming reactions with little or no change in the magnitude of steady-state Δψ. 2) At low rates of energy production and consumption, steady-state Δψ decreases. However, this is due largely to the energy leak of the system which lowers static-head Δψ when the rate of energy production is slow. 3) When the rate of energy production and static-head Δψ are held constant, and the rate of energy consumption is diminished by partial inhibition or the use of suboptimal conditions (e.g. subsaturating substrate concentrations), even a small decrease in the rate of energy consumption results in an upward adjustment of the level of steady-state Δψ. The lower the rate of energy input, the greater the upward adjustment of steady-state Δψ upon suppression of the rate of energy consumption. 4) The above results have been discussed with regard to the role of bulk-phase ΔμH+ or Δψ in the mitochondrial energy transfer reactions.

It is now well established that, in oxidative and photosynthetic phosphorylation, the driving force for the synthesis of ATP is protonic energy (1, 2). Discrete enzyme complexes transduce oxidative energy into a proton motive force, which is then transmitted to F0-F1 type enzyme complexes where ATP is made (2-4). The manner in which protonic energy is conveyed from source to sink is not known, however, and has been the subject of intensive investigation and vigorous debate in recent years. Several models for the mechanism of protonic energy transfer have been proposed by various investigators. At one extreme is the original chemiosmotic model of Mitchell (5), which holds that protonic energy is communicated from source to sink as the transmembrane electrochemical potential of protons (ΔμH+) via the aqueous phase that surrounds the energy transducing membranes (1, 5). Thus, according to this model, protonic energy would be delocalized over the entire membrane, which must be in the form of a closed vesicle to disallow energy dissipation. At the other extreme is the recently advanced mosaic model. This model proposes that protonic energy transfer is strictly localized and takes place between an energy-yielding and an energy-consuming enzyme complex, which together form a coupling unit (6, 7). There are also models in between these extremes, which suggest that protons are conducted within the membrane (8-10) or at the membrane lipid-water interfaces (11, 12). The reasons for the proposed alternatives to the chemiosmotic model of proton transfer have been discussed in excellent recent reviews (7-9, 11, 13-15) and cannot be detailed here. However, for the reader's appreciation of the problem, two examples will be cited.

1) In mitochondria, attenuation of the rate of respiration results in a parallel decrease in the rate of ATP synthesis. However, up to about 60% inhibition of respiration, ΔψH+ undergoes little or no change (16-19). More recent studies (20-22) have suggested that there is a steep linear relationship between the rate of ATP synthesis and the change in ΔψH+ or Δψ (see "Results").

2) The steady-state level of Δψ, which is the major component of ΔμH+ (especially in SMP*), can be considerably diminished by addition of valinomycin + K+ with little or no effect on the rate of ATP synthesis (17, 23).

In view of these and other observations (23-27), many workers in the field feel that ΔμH+ may not be the principal mode of energy transfer in mitochondria and bacteria. However, the alternative models proposed are not entirely adequate either. Moreover, none of these models provides a satisfactory explanation for the relationship between ΔμH+ and the kinetics of energy production and utilization. Yet, this is of crucial importance, because any chemical or physical assault that destroys ΔμH+ also uncouples the energy-linked processes of mitochondria, chloroplasts, chromatophores, and bacterial membranes.

The studies presented in this paper explore the relationship in mitochondria and SMP between steady-state Δψ and the rate changes of several energy-linked reactions. Results have indicated that the rate of energy utilization (e.g. rate of ATP

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† The abbreviations used are: SMP, submitochondrial particles; Δψ, membrane potential; ΔμH+, transmembrane electrochemical potential of protons; DCCD, N,N'-dicyclohexylcarbodimide; DSMP*, 2-[(dimethylamino)styryl]-1-methylpyridinium ion; ANS, 1-anilino-8-napthalene-8-sulfonate; S-13, 2,5-dichloro-3-t-butyl-4'-nitroanilidinylidene; CCCP, carbonyl cyanide m-chlorophenylhydrazone; NTP, nucleoside triphosphate; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

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synthesis) is related to the rate of energy production (e.g. rate of respiration), not to the magnitude of $\Delta \psi$. However, steady-state $\Delta \psi$ does respond to changes in the rates of energy input and outflow, and its magnitude appears to depend on the relative magnitudes of these two rates.

**MATERIALS AND METHODS**

Bovine SMP (28) and rat-liver mitochondria (29) were prepared as described previously. Protein concentration was determined by the procedure of Lowry et al. (30) or by the biuret method (31) in the presence of 0.1% sodium deoxycholate. All the assays were performed at 30 °C. ATPase activity was measured, using the ATP regenerating system as before (32, 33). GTPase activity was estimated from the release of Pi, which was determined colorimetrically as described (32, 34).

Assays of Energy-Linked Reactions—Oxidative phosphorylation activity of SMP was measured as before (28). The reaction mixtures contained, in a final volume of 0.6 ml, 0.25 mM succrose, 50 mM Tris acetate, pH 7.5, 0.6 mM EDTA, 25 mM glucose, 5 mM MgCl₂, 20 mM $[^3]$Hpotassium phosphate (1-5 x 10⁻⁹ cmol/ml), 42 g of hexokinase, and 30 g of SMP. ADP was added at the concentrations indicated in the figure legends. The respiratory substrate was 0.5 mM NADH, 5 mM potassium succinate, or 30 mM sodium DL-β-hydroxybutyrate plus 2 mM NADH as indicated. The rates of steady-state changes of oxygen/min/mg of SMP protein in the absence and presence of 1.2 mM ADP were in Figs. 1 and 2, 1060 and 1770 with NADH as the respiratory substrate, 490 and 800 with succinate as the respiratory substrate, and 169 and 177 with β-hydroxybutyrate plus NAD as the respiratory substrate, respectively; and, in Figs. 5-7, 750 and 115 with NADH as the respiratory substrate, 320 and 500 with succinate as substrate, and 115 and 118 with β-hydroxybutyrate plus NAD as substrate, respectively. Oxidative phosphorylation activity of rat-liver mitochondria was assayed in a 0.6 ml reaction mixture containing 0.25 g mannitol, 20 mM $[^3]$Hpotassium phosphate (1 x 10⁻⁹ cpmp/ mol), 10 mM KCl, 5 mM MgCl₂, 10 μM rotenone, 5 mM sucinate, 42 g of hexokinase, 25 mM glucose, 400 μM ADP, and 60 g of rat liver mitochondria.

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Assayed, and DSMP+ in the case of mitochondria.

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The low rates of ATP synthesis and the variable relationships between $J_{ATP}$ and $\Delta \psi$ or $\Delta \psi$ reported by others prompted us to examine the flow-force relationships in the mitochondrial energy-linked reactions, using our highly active SMP preparations which exhibit ATP synthesis rates as high as 3000 nmol (min - mg)$^{-1}$. Described below are, therefore, two types of experiments, one in which the rate of energy production was attenuated and its effect studied on $\Delta \psi$ and the rate of energy utilization and another in which the rate of energy production was held unchanged, but the rate of energy utilization was suppressed and its effect studied on the magnitude of $\Delta \psi$. The energy-coupled systems studied were oxidative phosphorylation (with NADH, succinate, and $\beta$-hydroxybutyrate + NAD as respiratory substrates) and NTP-driven uphill electron transfer from succinate to NAD and transhydrogenation from NAD to NADP.

**Effect of Attenuation of the Rate of Energy Production on $\Delta \psi$ and the Rate of Energy Utilization**

Fig. 1 shows the relationship between the rate of ATP synthesis and the magnitudes of static-head and steady-state $\Delta \psi$ as the rate of respiration was attenuated and the rate of ATP synthesis decreased in parallel. The respiratory substrates used were NADH, succinate, and $\beta$-hydroxybutyrate + NAD, which were oxidized by SMP at the rates given under "Materials and Methods." The oxidation rates of NADH and $\beta$-hydroxybutyrate were suppressed by addition to the assay of increasing amounts of Seconal, which inhibits electron transfer at the level of Complex I (NADH:ubiquinone oxidoreductase), and the oxidation rate of succinate was attenuated and its effect studied on the magnitude of $\Delta \psi$. The energy-coupled systems studied were oxidative phosphorylation (with NADH, succinate, and $\beta$-hydroxybutyrate + NAD as respiratory substrates) and NTP-driven uphill electron transfer from succinate to NAD and transhydrogenation from NAD to NADP.

**Effect of Attenuation of the Rate of Energy Utilization on Steady-state $\Delta \psi$**

Oxidative Phosphorylation—Fig. 2 shows the relationship between steady-state $\Delta \psi$ and varied rates of energy outflow during oxidative phosphorylation at fixed rates of energy input. The rate of respiration was set at high, intermediate, and low levels with the use, respectively, of NADH, succinate, and $\beta$-hydroxybutyrate + NAD as respiratory substrates. ATP synthesis there is little or no detectable change in steady-state $\Delta \psi$ as $J_{ATP}$ is lowered from about 2800 to about 1400 nmol (min - mg)$^{-1}$.

As seen in Fig. 2, essentially a similar relationship was observed between steady-state $\Delta \psi$ and the ATP-driven rates of uphill electron transfer from succinate to NAD, as the rate of ATP hydrolysis was progressively diminished by the use of subsaturating concentrations of ATP. Similar results were obtained when the rate of ATP hydrolysis was suppressed by addition of increasing amounts of oligomycin (data not shown).

The above experiments demonstrate the effects of three factors on steady-state $\Delta \psi$. These are (a) the rate of energy input (i.e. coupled respiration in Fig. 1 and ATP hydrolysis in Fig. 2), (b) the rate of energy utilization by the driven reactions (i.e. ATP synthesis in Fig. 1 and uphill electron transfer in Fig. 2), and (c) energy leak through the system (38-40). The latter is apparent from the drop in static-head $\Delta \psi$ when the rate of energy input was suppressed to very low levels. The results reveal two important points. (1) The major decrease in steady-state $\Delta \psi$, which occurs when the rate of energy input is attenuated, appears to be due mainly to suppression of the static-head $\Delta \psi$. (2) At high rates of energy input, where static-head $\Delta \psi$ is stable, changes in the rates of the energy-producing and the energy-consuming reactions have minimal or no detectable effect on steady-state $\Delta \psi$.

**Effect of Attenuation of the Rate of Energy Utilization on Steady-state $\Delta \psi$**

Oxidative Phosphorylation—Fig. 3 shows the relationship between steady-state $\Delta \psi$ and varied rates of energy outflow during oxidative phosphorylation at fixed rates of energy input. The rate of respiration was set at high, intermediate, and low levels with the use, respectively, of NADH, succinate, and $\beta$-hydroxybutyrate + NAD as respiratory substrates. ATP synthesis there is little or no detectable change in steady-state $\Delta \psi$ as $J_{ATP}$ is lowered from about 2800 to about 1400 nmol (min - mg)$^{-1}$.

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and β-hydroxybutyrate + NAD as oxidizable substrates. The rate of ATP synthesis was varied by employing various subsaturating concentrations of ADP. The changes in steady-state $\Delta \psi$ were monitored by the absorbance change of oxonol VI (filled symbols) as well as by the fluorescence change of ANS (open symbols), using two different preparations of SMP. As seen in Fig. 3, the two methods of monitoring $\Delta \psi$ changes gave qualitatively similar results. The reason for the difference in oxidative phosphorylation activities of the two experiments in which succinate was used as respiratory substrate was that the two preparations of SMP exhibited somewhat different succinoxidase activities.

In Fig. 3, the experimental point at the lowest $\Delta \psi$ on each curve represents $J_{\text{ATP}}$ at saturating [ADP]. As the ADP concentration was lowered, $J_{\text{ATP}}$ decreased, and this diminution in the rate of energy utilization was accompanied by an increase in steady-state $\Delta \psi$. It is also seen that the higher the rate of respiration (energy input), the sooner steady-state $\Delta \psi$ approached the static-head level as $J_{\text{ATP}}$ was diminished. Similar results are shown in Fig. 4. In these experiments, the rate of energy production was varied by employing succinate and β-hydroxybutyrate as respiratory substrates, as well as by using succinate in the presence of the competitive inhibitor malonate (compare circles and triangles). Furthermore, the rate of ATP synthesis was controlled not only by addition of subsaturating concentrations of ADP, but also by partial inhibition of ATP synthases, once with tributyltin chloride (filled circles) and another time with DCCD (filled squares). In Fig. 4, the data for the latter two sets of experiments (filled and open circles and squares) are marked with letters. The same letters indicate the same concentration of ADP in the absence (open symbols) and the presence (filled symbols) of the ATP synthase inhibitor. Therefore, at the same ADP concentration, $J_{\text{ATP}}$ was lower and steady-state $\Delta \psi$ was higher when the ATP synthases were fractionally inhibited by tributyltin chloride or DCCD. Otherwise, the data for succinate or β-hydroxybutyrate ± ATP synthase inhibitor fell on the same curve.

Thus, as seen in Figs. 3 and 4, regardless of the manner in which energy input or outflow was altered, there was a corresponding adjustment in the steady-state level of $\Delta \psi$. These results indicate that the magnitude of $\Delta \psi$ depends on the relative magnitudes of the rates of energy production and consumption.

Another important point is demonstrated in Fig. 5. The top curve shows, as before, the relationship between steady-state $\Delta \psi$ and the rate of ATP synthesis, supported by succinate oxidation, at different subsaturating concentrations of ADP. The middle and bottom curves show similar experiments car-
ried out in the presence of S-13 or two different concentrations of CCCP to achieve partial uncoupling. It is seen that the presence of uncouplers resulted in considerable lowering of $\Delta\psi$ and diminution of $\nu_{ATP}$ at each ADP concentration. Nevertheless, under conditions of constant energy drain by uncouplers, these suppressed levels of $\Delta\psi$ still responded to the rate of ATP synthesis as it was further diminished by lowering [ADP].

**Transhydrogenation and Uphill Electron Transfer**—The transhydrogenase experiments were performed, at first, with ATP as the energy source. However, it was found that ATP hydrolysis energizes the system too strongly to allow the formation of a steady-state $\Delta\psi$ below the static-head level. Even in the presence of moderate concentrations of ATPase inhibitors (see dashed line in Fig. 6) it was difficult to obtain sufficiently low levels of steady-state $\Delta\psi$ to permit the kind of manipulations reported in Figs. 3-5. Therefore, ATP was replaced with GTP as the energy source. Although at $V_{\text{max}}$ the rate of GTP hydrolysis by the bovine mitochondrial ATPase complex is comparable to that of ATP, the apparent $K_m$ for GTP is roughly 100-fold greater than that of ATP (41), and at 1-2 mM concentration, GTP is much more slowly hydrolyzed.

Therefore, with GTP as the energy source, the effects of energy input and outflow on steady-state $\Delta\psi$ were studied during transhydrogenation from NADPH to NADP (Fig. 6) and electron transfer from succinate to NAD (Fig. 7). In both experiments, the rate of energy input was suppressed by addition of oligomycin where indicated, and the rate of energy utilization was altered by using subsaturating levels of NADH in Fig. 6 and increasing amounts of malonate to lower the rate of succinate oxidation in Fig. 7. It is seen that in these situations also, the relationship between steady-state $\Delta\psi$ and the rates of energy production and consumption was essentially the same as that reported in Figs. 3 and 4 for oxidative phosphorylation. Thus, at saturating substrate concentrations, suppression of the rate of GTP hydrolysis by oligomycin lowered $\Delta\psi$, and at a fixed rate of energy input, the use of suboptimal conditions for the energy-requiring reactions (decrease of the rate of energy utilization) increased the steady-state level of $\Delta\psi$.

**DISCUSSION**

Results presented elsewhere (18) showed that the redox state of cytochrome c in SMP was precisely correlated with the rate of electron transfer from succinate to molecular oxygen, when respiration was attenuated up to 90% once by addition of increasing amounts of malonate to inhibit succinate oxidation and a second time by addition of increasing amounts of NaN3 to inhibit oxygen reduction. In these experiments, SMP were treated with an uncoupler in order to remove coupling constraints from the electron transport system, and cytochrome c was added up to 33 times the combined concentrations of cytochromes c1, c2 in SMP in order to expand the concentration of this intermediate in case the expanded pool should exhibit buffering. In addition, it should be pointed out that cytochrome c in uncoupled SMP represents a redox potential pool of about 250 ± 30 mV, which includes the Rieske iron-sulfur protein, cytochrome c1, cytochrome a, and Cu2+. Nevertheless, the redox state of this large potential pool was found to be precisely correlated with the rate of electron flow through the system.

However, as the data of Figs. 2 and 7 demonstrate, such a correlation does not exist between $\Delta\psi$ and the rates of energy-consuming reactions in SMP. Indeed, the data show that (a) steady-state $\Delta\psi$ remains essentially unchanged over a wide range of rate changes of the energy-consuming reactions and (b) when steady-state $\Delta\psi$ does drop, it is mainly because of the failure in static-head $\Delta\psi$ rather than because of the rate of energy utilization by the driven reactions.

On the other hand, the solid lines of Figs. 3-7 show that $\Delta\psi$ does respond to the rate changes of the energy-driven reactions when the latter are attenuated by the use of suboptimal substrate concentrations or by addition of specific inhibitors. In the first set of experiments (Figs. 1 and 2), the rate of energy consumption was altered by attenuating the rate of energy production. Under these conditions, steady-state $\Delta\psi$ remained essentially unchanged until the energy leak of the system at low rates of energy input became a significant factor and diminished the level of static-head $\Delta\psi$. In the second set of experiments (Figs. 3-7), the rate of energy input was held constant in each experiment, and static-head $\Delta\psi$ remained unchanged throughout that experiment. Under these conditions, steady-state $\Delta\psi$ responded to even very small changes in the rate of energy consumption. It might also be noted that when the rate of energy input was maintained at a fixed high level (e.g. by NADH oxidation in Fig. 3), the considerable suppression of the rate of energy utilization (ATP synthesis)

**FIG. 6.** Relationship between steady-state membrane potential and NTP-driven transhydrogenase activity at variable concentrations of NADH and variable rates of NTP hydrolysis. SMP at 10 mg/ml in 0.25 M sucrose and 50 mM Tris acetate, pH 7.5, were preincubated with 0 (a), 1 (, ), 2 (O) µg of oligomycin/ml for ≥30 min on ice. The NADH concentration range used was 0-50 µM. Conditions for the assay of NADH-NADP transhydrogenase activity and for monitoring oxonol VI absorbance changes are described under "Materials and Methods." The GTPase activities (µmol/min/mg of SMP) were 0.036, 0.032, and 0.026.

**FIG. 7.** Relationship between membrane potential and GTP-driven reverse electron transfer from succinate to NAD at two rates of GTP hydrolysis and the presence of variable concentrations of malonate. SMP were treated in A with 1 µg of oligomycin/ml as described in Fig. 6. The malonate concentration range used was 0.2-2 mM. The data points at the lowest $\Delta\psi$ represent experiments in the absence of malonate. GTPase activities and oxonol VI absorbance changes in the absence and presence of 1 µg of oligomycin/ml were the same as described in Fig. 6. Conditions for measurement of reverse electron transfer activity from succinate to NAD and for measurement of oxonol VI absorbance changes are described under "Materials and Methods."
resulted in a relatively small increase in steady-state $\Delta \psi$. By comparison, when the rate of energy input was held at a fixed low rate (e.g. by $\beta$-hydroxybutyrate oxidation in Fig. 3), then small decreases in the rate of energy utilization were accompanied by relatively large increases in steady-state $\Delta \psi$.

Thus, we seem to have two sets of discordant results. The data of Figs. 3–7, as seen in the preceding paragraphs, are fully consistent with bulk-phase $\Delta \psi$ being the intermediate which links the energy-yielding systems of mitochondria to the energy-consuming ones. However, the data of Figs. 1 and 2 are not consistent with this conclusion, because in these experiments steady-state $\Delta \psi$ did not respond to very large rate changes of the energy-consuming reactions, except at very low rates of energy input where static-head $\Delta \psi$ began to decline.

One possible interpretation of the results, which would agree with both sets of data, is as follows. As seen in Figs. 3, 4, 6, and 7, the magnitude of $\Delta \psi$ is exquisitely sensitive to the rate changes of the energy-producing and the energy-consuming reactions. To reconcile these data with those of Figs. 1 and 2, one could assume that by some mechanism not involving $\Delta \psi$ the rate of energy consumption is adjusted to the rate of energy production. If this were so, then steady-state $\Delta \psi$ would remain essentially constant when the rate of energy production (and consequently the rate of energy consumption) is altered. Under these conditions, bulk-phase $\Delta \psi$ or $\Delta \mu_M$ could still be the source of energy for the driven reactions, even though its magnitude would not influence their rates (see Fig. 8, scheme I). The mechanism by which the rates of the energy-consuming reactions are adjusted to the rates of the energy-producing ones could involve intramembranous or membrane interface protons (8–12) or physical contact of the energy-transducing complexes via lateral diffusion in the membrane (42, 43). That the mitochondrial energy-transducing complexes are capable of rapid translational motion has been demonstrated for Complexes III and IV (44).

It is also possible that, as has been suggested by others (6–11, 15), the bulk-phase $\Delta \psi$ or $\Delta \mu_M$ is not the principal mode of energy transfer in mitochondria. In that case, $\Delta \mu_M$ would still have to be in communication with the mitochondrial energy-transducing systems, but this communication might be relatively slow and differently affected by the experimental conditions employed (see Fig. 8, Scheme II). However, it should be added that this indirect involvement of $\Delta \mu_M$ may not be applicable to all the energy-linked reactions of mitochondria. For example, our previous results (18) have shown that there is an excellent correlation between the magnitude of $\Delta \psi$ and the rate of uniport calcium transport in bovine heart mitochondria.

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