Modulation of the Kinetics and the Steady-State Level of Intermediates of Mitochondrial Coupled Reactions by Inhibitors and Uncouplers†

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ABSTRACT: (1) In oxidative phosphorylation and ATP-driven uphill electron transfer from succinate to NAD, double-reciprocal plots of rates vs. substrate concentrations of the energy-driven reactions are a family of parallel lines at several fixed subsaturating concentrations of the substrates or at several moderate concentrations of the inhibitors of the energy-yielding reactions. Thus, as shown elsewhere [Hatefi, Y., Yagi, T., Phelps, D. C., Wong, S.-Y., Vik, S. B., & Galante, Y. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1756–1760], partial uncoupling decreases the V_{max}^{app} and increases the K_m^{app} of the substrates of the energy-driven reactions, resulting in a decrease of V_{max}/K_m as a function of increased uncoupling. However, partial limitation of the flow rates of the energy-yielding reactions decreases both the V_{max}^{app} and the K_m^{app} of the substrates of the energy-driven reactions, resulting in no change in V_{max}/K_m. This is true as long as the rate limitation is moderate (e.g., <60%), under which conditions the steady-state membrane potential ($\Delta\psi$) remains essentially unchanged. At high inhibition of the energy-yielding reactions, or at moderate inhibition in the presence of low levels of an uncoupler to cause partial uncoupling, then the family of double-reciprocal plots is no longer parallel and tends to converge toward the left. Under these conditions, steady-state $\Delta\psi$ and V_{max}/K_m also decrease as inhibition is increased. (2) The relationship between the magnitude of steady-state $\Delta\psi$ and the rate of the energy-driven reaction was studied in oxidative phosphorylation, ATP-driven electron transfer from succinate to NAD, and respiration-driven uniport calcium transport by intact mitochondria. The rates of the driven reactions were modulated by partial uncoupling or partial inhibition of the energy-yielding reactions. Addition of increasing amounts of an uncoupler caused parallel decreases in the rates of the energy-driven reactions and the magnitude of $\Delta\psi$. When the rates were diminished by addition of increasing amounts of an inhibitor of the energy-yielding reactions, then the correlation between steady-state $\Delta\psi$ and the rate of the energy-driven reaction depended on the nature of the latter reaction. The correlation was (a) excellent for uniport calcium transport, (b) partial for oxidative phosphorylation, and (c) poor for ATP-driven uphill electron transfer. It was noted that in (a) the excellent correlation was between two bulk phase phenomena and that in (b) and (c) the more energy-demanding reaction (namely, uphill electron transfer) showed the poorer correlation. (3) In uncoupled submitochondrial particles the steady-state redox level of a known intermediate (cytochromes $c + c_1$) was studied as a function of partial inhibition of electron flow, once at the dehydrogenase and a second time at the oxidase ends of the respiratory chain. These experiments were also repeated in the presence of added cytochrome *c* to increase the pool size of the intermediate (i.e., cytochromes $c + c_1$) up to 33-fold. In all cases, there was an excellent correlation between the rate of electron flow and the steady-state redox level of cytochromes $c + c_1$. (4) The above results have been discussed with regard to the premise that $\Delta\tilde{\mu}_{H^+}$ (or $\Delta\psi$ in the case of submitochondrial particles) is the sole or the principal form of protonic energy transfer.

While it is generally accepted that the driving force for oxidative and photosynthetic phosphorylation is the gradient energy of protons, the premise that bulk phase $\Delta\tilde{\mu}_{H^+}$ is the obligate intermediate in these processes is widely questioned [see, for example, Kell (1979), Melandri et al. (1981), Rottenberg (1979, 1983), Slater (1980), Sorgato et al. (1980), Vinkler (1982), Williams (1978), and Zoratti et al. (1982)]. Among the approaches used to explore this issue has been the study of the effects of inhibitors, uncouplers, and ionophores on the rates of electron transfer and ATP synthesis, and the steady-state level of $\Delta\tilde{\mu}_{H^+}$. For example, Sorgato et al. (1980) showed that 60% inhibition of respiration by addition of malonate to bovine heart SMP[1] oxidizing succinate resulted in 70% inhibition of the rate of ATP synthesis without affecting the magnitude of $\Delta\tilde{\mu}_{H^+}$. In other studies, they have shown that 96% inhibition of ATP hydrolysis by addition of AMP-P(NH)P to SMP at constant ΔG_p resulted in only 6% decrease in the value of $\Delta\psi$ (Sorgato et al., 1981). Zoratti et al. (1982) have obtained similar results and have shown in addition that valinomycin + K^+ depressed $\Delta\tilde{\mu}_{H^+}$ as much as complete uncoupling but without diminishing the rate of ATP synthesis. They have concluded, therefore, that "uncoupling of oxidative phosphorylation is not due to depression of $\Delta\tilde{\mu}_{H^+}$ per se". Vinkler & Korenstein (1982) have shown that external electric field driven ATP synthesis by lettuce chloroplasts is uncoupler insensitive and slower than light-driven ATP synthesis. In other studies, Vinkler (1981) has shown that both partial uncoupling and limitation of electron transfer by lowering light intensity will decrease the rate of photophosphorylation. However, uncoupling increases K_m^{app} for ADP while the limitation of electron-transfer rate decreases it. Thus, she has concluded on the basis of these results that the rate of ATP synthesis is controlled by a mechanism "which is different than the formation of the electrochemical gradient". These and other observations and conclusions (e.g., Kayalar et al., 1976) indicated the need for a careful analysis of the effects of

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[1] Abbreviations: SMP, sonicated submitochondrial particles; $\Delta\tilde{\mu}_{H^+}$, transmembrane electrochemical potential of protons; AMP-P(NH)P, adenyl-5′-yl imidodiphosphate; ΔG_p, phosphate potential; $\Delta\psi$, membrane potential; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; $DSMP^+$, 2-[(dimethylamino)styryl]-1-methylpyridinium ion; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; ATPase, adenosinetriphosphatase.

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uncouplers and inhibitors on the kinetics and the steady-state level of intermediates of mitochondrial coupled processes.

In an earlier paper (Hatefi et al., 1982), we showed that partial (up to 90%) uncoupling of several energy-linked reactions catalyzed by SMP (i.e., respiration-driven ATP synthesis and transhydrogenation from NADH to NADP, NTP–P_i exchange, and ATP-driven NAD reduction by succinate) resulted in decrease of V_{max}^{app} and increase of K_m^{app} and that ln (V_{max}/K_m) decreased linearly as a function of uncoupler concentration to the extent examined. The significance of these results is that V_{max}/K_m is a measure of the apparent first-order rate constant for enzyme–substrate interaction and reflects the affinity between enzyme and substrate to form a complex. Therefore, the finding that V_{max}/K_m (hence, enzyme–substrate affinity) increases as a function of the degree of energization of SMP suggested that this increase might be a consequence of an energy-induced change in the conformation of the respective energy-linked enzymes. That the extent of conformation change of F_1-ATPase in SMP is a function of the degree of membrane energization has been ascertained (see Figure 3 below).

This paper presents an analysis of the effect of inhibitors of respiration and ATP hydrolysis on the kinetics of the reactions driven by these exergonic processes and a correlation of the rate changes effected by the inhibitors with the steady-state level of the membrane potential as measured by appropriate dyes in both intact mitochondria and SMP. In addition, similar inhibitor effects have been shown to operate at the level of uncoupled electron-transport system, and the rate changes have been correlated with the steady-state redox level of cytochrome *c* as an obligate respiratory chain intermediate. The results have shown that the steady-state redox level of cytochrome *c* registers the inhibition of electron flow through the respiratory chain. The steady-state level of the membrane potential responds partially to the inhibition of respiration in oxidative phosphorylation and minimally to inhibition of ATP hydrolysis in ATP-driven NAD reduction by succinate. In respiration-driven calcium transport, a better correlation seems to exist between inhibition of respiration and calcium transport and the decrease in the steady-state level of the membrane potential. However, under the conditions of uniport calcium transport, the static head membrane potential also diminished as the inhibition of respiration became severe.

Materials and Methods

Heavy beef heart mitochondria (Hatefi & Lester, 1958), phosphorylating SMP (Hansen & Smith, 1964), and nonphosphorylating SMP (Hanstein et al., 1974) were prepared according to the references given. Oxidative phosphorylation and ATP-driven NAD reduction by succinate were assayed as before (Hatefi et al., 1982). Unless otherwise indicated, in oxidative phosphorylation assays ADP was 1.1 mM, potassium phosphate, pH 7.5, was 20 mM, and SMP was added at 0.1 mg/mL. Calcium uptake by mitochondria was measured with an Orion Model 92-20 calcium electrode under the conditions described by Åkerman & Wikström (1976). Absorption spectra were recorded by Cary 118 and Aminco DW-2a spectrophotometers, oxygen consumption was measured by a Clark-type electrode, and fluorescence studies were carried out in an SLM photon-counting fluorescence spectrophotometer. Protein was estimated by biuret (Gornall et al., 1949) in the presence of 1 mg of deoxycholate/mL. The fluorescence response of $DSMP^+$ (Mewes & Rafael, 1981) was calibrated at pH 7.5 with KCl in the presence of heavy beef heart mitochondria, rotenone, and valinomycin. The fluorescence response of the dye was linear between 50 and 200 mV calculated $\Delta\psi$.

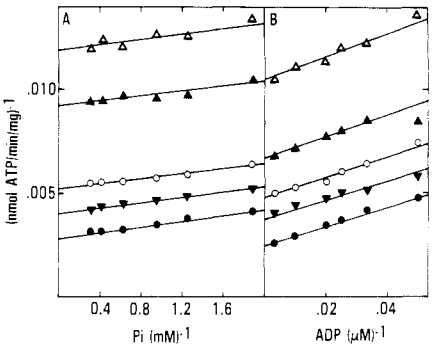

FIGURE 1: Double-reciprocal plots showing the effect of several fixed concentrations of malonate on the rate of ATP synthesis by SMP and on the K_m^{app} for P_i (panel A) and ADP (panel B). The substrate was 5 mM succinate, and the malonate concentrations were (●) zero, (▼) 40, (○) 80, (▲) 160, and (△) 300 μM. The assay temperature was 30 °C. The V_{max}^{app} in the absence of malonate was 397 nmol min^{-1} (mg of protein)$^{-1}$ when ADP was the variable substrate and 356 nmol min^{-1} mg^{-1} when P_i was the variable substrate.

ADP and NAD were obtained from P-L Biochemicals; ATP and oligomycin were from Boehringer; DL-β-hydroxybutyrate, HQNO, Tris, and cytochrome *c* were from Sigma; TMPD was from Eastman Kodak; rotenone was from S. B. Penicke; ascorbate, CCCP, and valinomycin were from Calbiochem; [^{32}P]P_i was from ICN; [^{33}P]P_i was from New England Nuclear; succinic and malonic acids were from Baker; $DSMP^+$ was from Gallard Schlesinger. All other chemicals were reagent grade. Aurovertin (Dr. B. Beechey, Shell Research Laboratories), efrapeptin and nigericin (Dr. R. L. Hamill, Eli Lilly), oxonol VI (Dr. W. G. Hanstein, University of Bochum), and UHDBT (Dr. B. L. Trumpower, Dartmouth Medical School) were the generous gifts of the colleagues named.

Results

Effects of Respiratory Chain and ATPase Inhibitors on the Kinetics of ATP Synthesis and NAD Reduction by Succinate. As was shown elsewhere (Hatefi et al., 1982), partial uncoupling of mitochondria results not only in decrease of the apparent V_{max} of various energized reactions but also in increase of the apparent K_m of their substrates. Both these changes are functions of the extent of uncoupling, and ln (V_{max}/K_m) increases linearly with the increase in the degree of membrane energization.

Inhibitors of the energy-yielding reactions, as mentioned earlier, also inhibit the rate of the coupled energy-requiring reactions [see, for example, Sorgato et al. (1980) and Zoratti et al. (1982)]. However, in contrast to uncouplers, this modulation has an opposite effect on the K_m^{app} of the substrates of the driven reactions. As seen in the double-reciprocal plots of Figure 1, when the rate of succinate oxidation by SMP was lowered by addition of increasing fixed concentrations of malonate, the decreases in V_{max}^{app} for ATP synthesis were accompanied by decreases in K_m^{app} for both ADP and P_i, giving an uncompetitive pattern of inhibition. Vinkler (1981) made somewhat similar observations with chloroplasts when she compared the effects of uncoupling and electron transfer limitation on the rate of ATP synthesis and K_m^{app} for ADP. Both uncoupling and electron-transfer limitation decreased the rate of photophosphorylation, but uncoupling increased while electron transfer limitation decreased K_m^{app} for ADP. She concluded from these results that electron-transfer controls ATP synthesis by a mechanism other than the formation of the electrochemical gradient. However, as will be seen below,

Table I: SMP-Catalyzed Reactions Showing Uncompetitive Inhibition as a Function of Rate Modulator Concentration[a]

reaction	variable substrate	energy source	rate modulator	$\Delta\mu_{H^+}$ effector
ATP synthesis	ADP or P_i	succ oxidation	malonate	
ATP synthesis	ADP or P_i	succ oxidation	malonate	valinomycin
ATP synthesis	ADP or P_i	succ oxidation	malonate	nigericin
ATP synthesis	ADP or P_i	succ oxidation	[succ]	
ATP synthesis	ADP[b] or P_i	BOH (NAD) oxid	[NAD]	
ATP synthesis	ADP or P_i	asc/TMPD oxid	[TMPD]	
succ → NAD	succ	ATP hydrolysis	oligomycin	
succ → NAD	NAD[c]	ATP hydrolysis	oligomycin	
succ → NAD	NAD[c]	ATP hydrolysis	efrapeptin	
succ → NAD	NAD	ATP hydrolysis	malonate	
NADH → O_2	NADH		KCN	CCCP
NADH → O_2	NADH		UHDBT	CCCP
succ → O_2	succ[b]		KCN	CCCP
succ → O_2	succ[b]		HQNO	CCCP

[a] Oxidative phosphorylation was assayed as described elsewhere (Hatefi et al., 1982) with the following changes. In the first three experiments succinate and malonate concentrations were the same as in Figure 1, valinomycin and nigericin concentrations were respectively 1.27 and 0.5 μg/mg of protein, and K^+ concentration was 36 mM. In the next three experiments, there was no respiratory inhibitor, and the rate of respiration was controlled by addition of subsaturating to saturating levels of substrates, which were 0.1–5.0 mM succinate; 0.025–2.0 mM NAD plus 30 mM DL-β-hydroxybutyrate (BOH), and 0.66–2.5 mM TMPD plus 11.7 mM sodium ascorbate (higher TMPD concentrations were inhibitory). The reaction times with each substrate were respectively, 3, 5, and 2 min. β-Hydroxybutyrate oxidation was initiated by the addition of NAD and ascorbate oxidation by the addition of TMPD. Assay temperature was 30 °C. ATP-driven electron transfer from succinate to NAD was also assayed as before (Hatefi et al., 1982). The variable substrates were at the following concentrations: sodium succinate, 0.1–2.0 mM; NAD, 0.01–0.5 mM. The rate modulator concentrations were the following: malonate, 0.2–0.8 mM; oligomycin, 0.125–0.27 μg/mg of protein; efrapeptin, 0.38–1.025 μg/mg of protein. The latter two inhibitors were incubated with SMP for 30 min at 0 °C and then added to the reaction mixture. Assay temperature was 38 °C. NADH and succinate oxidation were assayed at 30 °C and pH 7.4 in a reaction mixture containing 0.25 M sucrose, 50 mM potassium phosphate, 1 μM CCCP, and 7.5–150 μM NADH or 0.12–2.2 mM succinate. NADH oxidation was monitored spectrophotometrically at 340 nm in the presence of 20 μg of SMP/mL, and succinate oxidation was monitored polarographically in the presence of 1.1 mg of SMP/mL. The final concentrations of rate modulators were as follows: KCN, 0.01–0.1 mM; UHDBT, 25–100 nM; HQNO, 2–13.5 μM. All inhibitors and CCCP were preincubated with SMP as above, and in the case of UHDBT the preincubation and assay pH was 7.0. [b] Double-reciprocal plots converged slightly toward the right. [c] Double-reciprocal plots converged slightly toward the left.

such results as shown in Figure 1 have a simple kinetic interpretation.

As stated above (Figure 1), uncompetitive inhibition was observed in ATP synthesis with respect to either ADP or P_i as the variable substrate when in the presence of succinate as the respiratory substrate the rate of electron transfer was limited by addition of several fixed concentrations of malonate as inhibitor. The inhibition pattern was the same when the experiments were conducted in the presence of valinomycin + K^+ or nigericin + K^+ to convert $\Delta\tilde{\mu}_{H^+}$ respectively to ΔpH or $\Delta\psi$ (Table I). Similar results were obtained when respiration was limited by addition of several fixed, subsaturating concentrations of succinate or NAD (in the presence of β-hydroxybutyrate) as the oxidizable substrate (Table I).[2] An uncompetitive pattern of inhibition was also observed in ATP-driven NAD reduction by succinate with either NAD or succinate as the variable substrate when the rate of ATP hydrolysis was diminished by addition of several fixed concentrations of either oligomycin or efrapeptin (Table I). In addition, uncompetitive inhibition was obtained in substrate oxidation by completely uncoupled SMP when NADH or succinate was the variable substrate and the rate of electron transfer was limited by addition of several fixed concentrations of KCN, UHDBT, or HQNO (Table I).[3] What these results

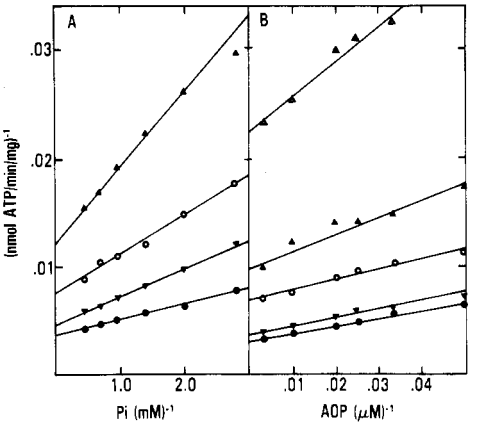

FIGURE 2: Double-reciprocal plots showing the effect of fixed concentrations of malonate in the presence of 50 nM CCCP on the rate of ATP synthesis and the K_m^{app} of P_i (panel A) and ADP (panel B). Substrate and malonate concentrations were the same as in Figure 1.

indicate is that the overall kinetics of oxidative phosphorylation, ATP-driven NAD reduction by succinate, and even uncoupled electron transfer from NADH and succinate to oxygen conform to the kinetics of enzymes possessing separate and non-overlapping catalytic sites which are linked by a mobile intermediate or transport chain (Renosto et al., 1981). This kind of kinetic interplay between separate catalytic sites is referred to as a hybrid or nonclassical ping-pong, or rapid equilibrium random ping-pong, mechanism. Other examples of enzymes exhibiting this kind of kinetics are methylmalonyl-CoA:pyruvate carboxytransferase (Northrop, 1969), pyruvate carboxylase (Barden et al., 1972), pyruvate dehydrogenase (Tsai et al., 1973), sulfite oxidase (Kessler & Rajagopalan, 1974), α-ketoglutarate dehydrogenase (Hamada et al., 1975), glutamate synthase (Rendina & Orme-Johnson, 1978), xanthine oxidase (Coughlan & Rajagopalan, 1981),

[2] Decreasing the rate of NADH or succinate oxidation by lowering the assay temperature from 30 to 20 °C decreased the V_{max}^{app} for ATP synthesis by >50% but had no effect on K_m^{app} for NADH, succinate, or ADP.

[3] In contrast to the effects of KCN, UHDBT, and HQNO on the K_m^{app} of NADH and succinate, Demerol decreased the rate of NADH oxidation without affecting the K_m^{app} for NADH, and 2-thenoyltrifluoroacetone lowered the rate of succinate oxidation but decreased the K_m^{app} for succinate only slightly. For example, a 4.6-fold decrease in V_{max}^{app} of succinate oxidation was accompanied by a change of K_m^{app} for succinate from about 0.4 to 0.25 mM. It is, therefore, interesting that when the site of inhibition is close to that of substrate binding, then rate limitation does not result in an uncompetitive pattern of inhibition.

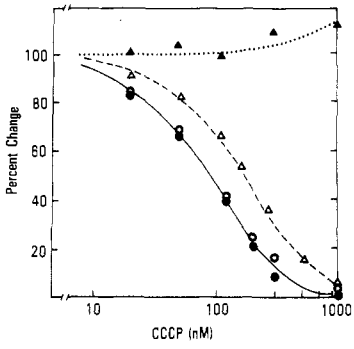

FIGURE 3: Effect of CCCP on V_{max}^{app} for ATP synthesis (●), aurovertin fluorescence enhancement (○), oxonol VI absorbance change (△), and the rate of succinate oxidation (▲). The experiments were carried out essentially as described before (Hatefi et al., 1982) in the presence of 5 mM succinate, 1.1 mM ADP, 20 mM potassium phosphate, pH 7.5, and 0.1 mg of SMP/mL. The rates of succinate oxidation measured polarographically with a Clark-type oxygen electrode and of ATP synthesis at 30 °C and absence of CCCP were respectively 500 and 625 nmol min^{-1} (mg of protein)$^{-1}$. Oxonol VI was added at 1.7 μM, and its absorbance change was monitored at 630 minus 603 nm on a DW-2a Aminco dual wavelength spectrophotometer. Aurovertin was added at 0.66 μM in the presence of 0.45 mg of SMP/mL and absence of ADP. The excitation and emission wavelengths for monitoring the fluorescence enhancement of aurovertin were respectively 366 and 470 nm (Chang & Penefsky, 1973). In this and subsequent figures, reaction rates and the extent of aurovertin fluorescence enhancement or oxonol VI absorbance change in the absence of added uncouplers or inhibitors were the control values for calculation of the percent changes shown.

and the *Penicillium* nitrate reductase (Renosto et al., 1981).

Figure 2 shows what happens to the parallel plots of Figure 1 when the experiments are conducted in the presence of low levels of CCCP (or valinomycin plus nigericin and K^+; results not shown) to cause partial uncoupling. The concentration of CCCP added was 50 nM, and the degree of uncoupling, as judged from the V_{max}^{app} of ATP synthesis, was about 30%. It is seen that the double-reciprocal plots at several fixed concentrations of malonate are no longer parallel and tend to converge toward the left. This is understandable since limitation of electron flow by addition of malonate decreases the K_m^{app} and uncouplers, as shown elsewhere (Hatefi et al., 1982), increase it. Severe inhibition of electron flow (i.e., greater than about 50%) by addition of high levels of malonate, HQNO, or KCN (data not shown) also resulted in nonparallel double-reciprocal plots similar to Figure 2. This deviation from parallel double-reciprocal plots appears to be related to the steady-state level of the membrane potential. The plots at increasing fixed concentrations of respiratory inhibitors remain parallel so long as the membrane potential remains essentially unchanged, and they tend to converge toward the left when the steady-state level of the membrane potential is diminished by addition of uncouplers or high concentrations of respiratory chain inhibitors.

Effects of Uncouplers and Respiratory Chain and ATPase Inhibitors on the Rates of Energy-Linked Reactions and the Steady-State Level of the Membrane Potential. Figure 3 shows that addition of increasing amounts of the uncoupler CCCP to SMP oxidizing succinate resulted, in parallel experiments, in comparable declines in the rate of ATP synthesis, the magnitude of respiration-induced aurovertin fluorescence, and the steady-state level of the membrane potential as monitored by the absorbance change of oxonol VI (the change in the fluorescence response of 8-anilinonaphthalene-1-sulfonic acid was exactly the same as that shown for oxonol VI). By contrast, extensive inhibition of respiration and phosphorylation (Figure 4, filled and open triangles, respectively) by addition

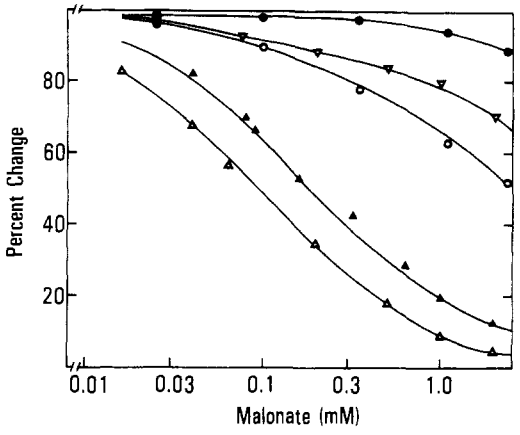

FIGURE 4: Effect of malonate on the rates of ATP synthesis (△) and succinate oxidation (▲), on oxonol VI absorbance change in the presence (○) (steady state) and absence (●) (static head) of ADP and hexokinase, and on aurovertin fluorescence enhancement (▽). Conditions were the same as in Figure 3, except that in the latter experiment aurovertin and SMP concentrations were 0.2 μM and 1.05 mg/mL, respectively.

of increasing amounts of malonate had only a slight effect on respiration-induced, static head membrane potential (Figure 4, filled circles) and partial effects on steady-state $\Delta\psi$ (open circles)[4] and aurovertin fluorescence enhancement (upside-down triangles). For example, at the malonate concentration where respiration and phosphorylation were inhibited by 52% and 65%, respectively, static head $\Delta\psi$ was essentially unchanged, and steady-state $\Delta\psi$ and aurovertin fluorescence enhancement were diminished, respectively, by only 16% and 12%. Sorgato et al. (1980) and Zoratti et al. (1982) have obtained results similar to the data of Figure 4 on the effect of respiration limitation on the rate of ATP synthesis and the steady-state magnitude of $\Delta\psi$ or $\Delta\tilde{\mu}_{H^+}$, and because the rate inhibitions were not reflected in the steady-state level of $\Delta\tilde{\mu}_{H^+}$, they have questioned the validity of the premise that bulk phase $\Delta\tilde{\mu}_{H^+}$ is the obligate intermediate in oxidative phosphorylation. While this concern has been expressed also by others (see introduction), there is no theoretical basis by which to predict the behavior of $\Delta\tilde{\mu}_{H^+}$ under these conditions. Nor have we been able to find literature data for the steady-state fluctuations of an obligatory intermediate upon modulation of flow rates in cases of enzyme systems whose overall kinetics conform to the rapid equilibrium random ping-pong mechanism (see above and Table I). Consequently, we felt that perhaps a better understanding of the relationship between steady-state $\Delta\tilde{\mu}_{H^+}$ and the rates of respiration and ATP synthesis might be gained by (a) examining other mitochondrial energy-linked reactions and (b) studying the effect of rate modulations of the respiratory chain in uncoupled SMP on the steady-state redox level of an intermediate as an example of a closely related system. These studies are described below.

Figure 5 shows the effect of inhibition of ATP hydrolysis by addition of graded amounts of oligomycin to SMP on the rate of ATP-driven NAD reduction by succinate and on the magnitude of steady-state $\Delta\psi$. Data for static head $\Delta\psi$ are also shown. It is seen that static head and steady-state $\Delta\psi$

[4] We appreciate that the absorbance change of oxonol VI may not be a linear function of $\Delta\psi$. However, as shown in Figure 3, incremental additions of CCCP, which is known to diminish $\Delta\psi$, resulted in parallel decreases in the rate of ATP synthesis and the oxonol VI response. Therefore, we feel that a comparison of the effects of inhibitors (Figures 4, 5, and 7) relative to the effect of CCCP on the oxonol VI response as a monitor of $\Delta\psi$ changes is valid, regardless of the absolute $\Delta\psi$ values involved.

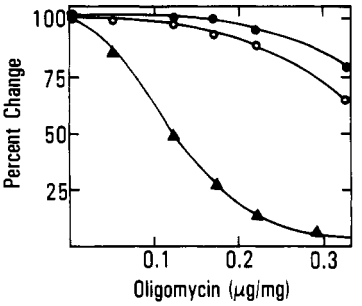

FIGURE 5: Effect of oligomycin on the rate of ATP-driven NAD reduction by succinate (▲) and on oxonol VI absorbance change in the presence (○) (steady state) and absence (●) (static head) of NAD. SMP (10 mg/mL) was incubated for 30 min at 0 °C with oligomycin and then added to the assay at 0.2 mg/mL. In the absence of oligomycin, the rates of NAD reduction and ATP hydrolysis were respectively 385 (at 38 °C) and 970 (at 30 °C) nmol min^{-1} (mg of protein)$^{-1}$.

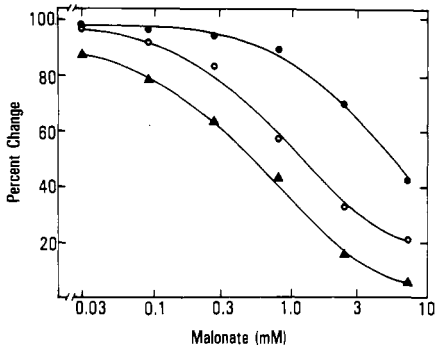

FIGURE 6: Effect of malonate on the rate of uniport calcium uptake (▲) driven by succinate oxidation by intact mitochondrdia and on membrane potential in the absence (●) (static head) and presence (○) (steady state) of calcium. The assay medium contained, at pH 7.5 and 30 °C, 0.25 M sucrose, 20 mM Tris, 10 mM Hepes chloride, 7 μM rotenone, 5 mM potassium succinate, and 2 mg of heavy beef heart mitochondria/mL. Calcium uptake was initiated by addition of 200 μM $CaCl_2$. Membrane potential was monitored by the fluorescence response of $DSMP^+$ (1 μM). The excitation and emission wevelengths were 479 and 589 nm, respectively. In the absence of malonate (potassium salt), the rate of calcium uptake was 210 ng of ion min^{-1} (mg of protein)$^{-1}$; the static head $\Delta\psi$, as estimated by calibration of the $DSMP^+$ fluorescence response (see Matierals and Methods), was 174 mV, and the steady-state $\Delta\psi$ was 115 mV. In the absence of malonate and presence of calcium, the rate of succinate oxidation was 104 nmol min^{-1} (mg of protein)$^{-1}$. Upon addition of malonate, this rate decreased in parallel with the rate of calcium uptake (data not shown).

were essentially unaffected when NAD reduction was inhibited by 50%; even at 90% inhibition of NAD reduction the $\Delta\psi$ values were lowered by only about 5 and 10%, respectively. Replacement of oligomycin with CCCP (data not shown) resulted in inhibition of NAD reduction and lowering of the initial absorbance change of oxonol VI essentially as shown for the forward reaction in Figure 3.

In another experiment, the relationship between the rate of respiration-driven uniport calcium transport and $\Delta\psi$ was studied in whole mitochondria. The rate of respiration was modulated as before by partial inhibition of succinate oxidation by malonate, and membrane potential was monitored by the fluorescence response of $DSMP^+$ (Mewes & Rafael, 1981). Figure 6 shows the data for calcium transport and $\Delta\psi$ under the conditions used for uniport calcium transport (i.e., absence of P_i), and Figure 7 is a control showing the effect of malonate on $\Delta\psi$ and state 3 rate of respiration in whole mitochondria. As seen in Figure 7, the effect of malonate in whole mitochondria on static head and steady-state $\Delta\psi$ as monitored by $DSMP^+$, and on the ADP-induced rate of respiration (proportional to the rate of ATP synthesis), was very similar to the effect obtained with SMP and a different dye (oxonol VI) for monitoring $\Delta\psi$ (Figure 4). Returning to Figure 6, it is seen that in contrast to the data of Figures 4, 5, and 7, the rate of calcium transport and the steady-state level of $\Delta\psi$ were diminished in parallel in response to inhibition of respiration by malonate. However, static head $\Delta\psi$ was also affected under these conditions at malonate concentrations ≥1.0 mM. The apparent similarity of the parallel curves of Figure 6 to those of Figure 3 prompted us to check the effect of CCCP on the rate of calcium transport and the associated $\Delta\psi$ as well. As seen in Figure 8, increasing amounts of CCCP had comparable effects in decreasing the rate of calcium uptake and lowering the magnitude of the static head and steady-state $\Delta\psi$.

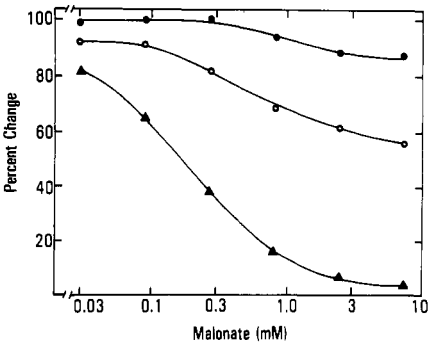

FIGURE 7: Effect of malonate on the rate of ADP-induced respiration (▲) and on membrane potential in the absence (●) (static head) and presence (○) (steady state) of ADP in intact mitochondria. Conditions were the same as in Figure 6, except that the reaction mixtures also contained 10 mM potassium phosphate and 5 mM $MgCl_2$ and the concentration of heavy beef heart mitochondria was 1 mg/mL. Each addition of ADP was 118 μM. In the absence of malonate, the state 3 rate of succinate oxidation was 316 nmol min^{-1} (mg of protein)$^{-1}$, static head $\Delta\psi$ was 199 mV, and steady-state $\Delta\psi$ was 170 mV.

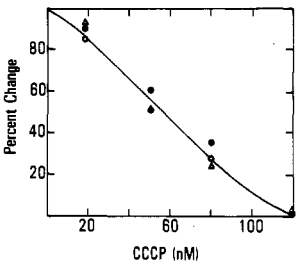

FIGURE 8: Effect of CCCP on the rate of respiration-driven calcium transport (△) by intact mitochondria and on the static head (●) and steady-state (○) $\Delta\psi$. Conditions were the same as in Figure 6, except that the concentration of heavy beef heart mitochondria was 1 mg/mL and each addition of $CaCl_2$ was 24 μM. In the absence of CCCP, the rate of calcium uptake was 152 ng of ion min^{-1} (mg of protein)$^{-1}$.

Effects of Malonate and Azide on the Rate of Succinate Oxidation and the Steady-State Redox Level of Cytochrome c. As was shown in Table I and discussed above, the overall kinetics of the oxidative phosphorylation system and the respiratory chain in uncoupled SMP conform to those of enzymes with independent catalytic sites connected by a mobile intermediate or a transport chain. Since the steady-state membrane potential as a possible obligatory intermediate in oxidative phosphorylation and uphill electron transfer appeared to be relatively insensitive to rate fluctuations of the energy-yielding and energy-requiring reactions, it was of interest to see how the steady-state redox level of a proven obligatory intermediate of the respiratory chain would respond to rate limitations at the substrate and oxygen ends of the chain. The experiments were carried out with SMP in the presence of high levels of CCCP to ensure complete disengagement of the respiratory chain from the energy-coupled reactions. The in-

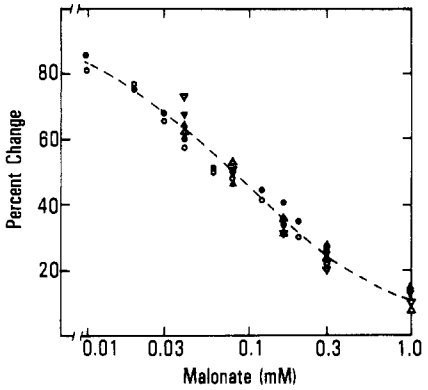

FIGURE 9: Effect of malonate on the rate of succinate oxidation by nonphosphorylating SMP (○, △, ▽) and the steady-state reduction level of cytochromes $c + c_1$ (●, ▲, ▼) in the absence (●, ○) and the presence of 12 (▲, △) and 33.3 μM (▼, ▽) added cytochrome c. The reaction mixtures at 30 °C contained, in a total volume of 3.0 mL, 0.25 M sucrose, 50 mM Tris–acetate, pH 7.5, 1 μM CCCP, 1.87 mg of SMP/mL, and malonate as shown. Where indicated, cytochrome c was added at the concentrations given above. The reaction was started by addition of 4.5 mM sodium succinate, and the reduction of cytochromes $c + c_1$ was followed by a DW-2a Aminco dual wavelength spectrophotometer at 550 minus 540 nm to steady state and then to maximal reduction upon anaerobiosis. The rate of oxygen consumption at each concentration of added malonate was estimated from the duration of the aerobic phase (from the time succinate was added until full reduction of cytochrome c due to anaerobiosis) and was also checked by polarography under the same conditions. From the extent of cytochrome c reduction at 550 minus 540 nm, it was calculated that at 12 and 33.3 μM added cytochrome c the cytochrome $c + c_1$ concentration was increased 11.9- and 33-fold, respectively. For other details see the text.

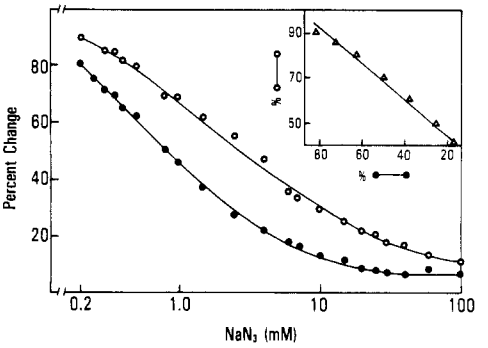

FIGURE 10: Effect of NaN_3 on the rate of succinate oxidation by nonphosphorylating SMP (○) and the steady-state oxidation level of cytochromes $c + c_1$ (●). Assay conditions were the same as in Figure 9, except that the SMP concentration was 2.0 mg/mL and NaN_3 was added at the concentrations shown instead of malonate. The reactions were started by addition of 5 mM succinate. For other details, see Figure 9 and the text.

termediate whose redox changes were followed was cytochrome c (actually cytochromes $c + c_1$), the substrate was succinate, and the rate modulators were malonate at the dehydrogenase end and azide at the oxidase end. As seen in Figure 9, the steady-state redox level of cytochromes $c + c_1$ reflected the changes in the rate of electron flow as succinate oxidation was inhibited up to 90% by addition of increasing amounts of malonate. This was quite unlike the behavior of $\Delta\psi$ as a possible intermediate of oxidative phosphorylation. We considered the possibility that perhaps the pool size of cytochrome c is too small in SMP to provide a buffer, even though on the basis of E_m the pool size at the level of cytochrome c includes cytochromes c_1 and a, Cu_a, and the iron-sulfur protein of complex III. Nevertheless, we repeated the experiment of Figure 9 in the presence of added cytochrome c to increase the $c + c_1$ pool size, once 12-fold and another time 33-fold. The results, which are also given in Figure 9, showed that increasing the cytochrome c concentration did not dampen its response to changes in the rate of succinate oxidation. Figure 10 shows a similar experiment, except that in this case succinate oxidation was inhibited at the oxidase end of the chain by addition of increasing amounts of sodium azide. Once again, the results, though somewhat different from those of Figure 9, indicated that the redox level of cytochrome c is influenced by the rate of electron flow through the system. The data shown in Figure 10 are for SMP in the absence of added cytochrome c. Essentially similar results were obtained when the cytochrome $c + c_1$ pool size was increased 12-fold by addition of cytochrome c.

There are two points with regard to the experiments of Figures 9 and 10 that need further comment. First, it is seen in Figure 9 that at all stages of inhibition of succinate dehydrogenation the percent reduction level of cytochrome c is numerically the same as the percent uninhibited rate of oxygen uptake, whereas in Figure 10 the percent oxidation level of cytochrome c does not coincide numerically with the percentage of the uninhibited rate of oxygen consumption. This is probably in part because of the way the percentages of reduction and oxidation of cytochrome c were calculated. In Figure 9, the steady-state reduction level of cytochrome c in the absence of malonate was taken as 100, and lower reduction levels in the presence of malonate were calculated as percentages of this value. In Figure 10, the fractional oxidation levels of cytochrome c in the absence and presence of azide were calculated relative to "complete" reduction after anaerobiosis, and then all oxidation levels of cytochrome c in the presence of azide were normalized as percentages of the oxidation level in the absence of azide. The point is that the importance of the data of Figures 9 and 10 is not the coincidence of the percentages of flow rates and the steady-state redox level of cytochrome c. These are, respectively, kinetic and thermodynamic parameters, and their coincidence in Figure 9 is probably fortuitous. Even at the same inhibition level of the flow rates in Figures 9 and 10, the corresponding redox levels of cytochrome c cannot be compared, because in the uninhibited system succinate dehydrogenation is the rate-determining step to the extent that in the absence of inhibitors and added cytochrome c the steady-state reduction level of endogenous cytochrome c in the experiment of Figure 9 was only about 12.7%. However, the point of these experiments is that regardless of the locus of inhibition, the rate changes were reflected in the steady-state redox level of cytochrome c (see also the inset of Figure 10) even when its concentration was increased by more than 30-fold.

The second point to be discussed is concerned with the experiments in which cytochrome c was added to SMP to increase its concentration. Since the topological position of cytochrome c in mitochondria is on the cytosolic surface of the inner membrane, it is generally assumed that sonic particles (i.e., SMP) do not reduce and oxidize added cytochrome c. This is incorrect, and the reason that added cytochrome c is reduced by SMP and succinate, as in the experiments of Figure 9, is not because of the presence of right side out particles in the SMP preparation. Not only are the added and endogenous cytochromes c in these experiments reduced rapidly and apparently monotonically but also even NADH as substrate reduces the same total amount of cytochrome c after anaerobiosis as does succinate. With NADH as the substrate, one would expect the right side out particles to be eliminated from consideration, because they should not react with NADH on the cytosolic side. Consequently, added cytochrome c should not be rapidly reduced by SMP in the presence of NADH,

because right side out particles should not admit NADH and the inside out particles should not react with cytochrome *c*. However, regardless of these considerations, the fact is that under the conditions of our experiments both succinate and NADH reduced endogenous or added cytochrome *c* rapidly and resulted in exactly the same level of cytochrome *c* reduction upon anaerobiosis.

Discussion

In mitochondria, uncouplers and inhibitors of the energy-yielding reactions have different effects on the membrane potential and the kinetics of the energy-driven reactions. Uncouplers diminish $\Delta\psi$ and the rate of the driven reactions in parallel and increase the K_m^{app} of the substrates of the driven reactions. As was shown elsewhere (Hatefi et al., 1982), the decrease in ln (V_{max}/K_m) is a linear function of increasing uncoupler concentration (i.e., decreasing free energy) in respiration-driven ATP synthesis and nicotinamide nucleotide transhydrogenation and in ATP-driven uphill electron transfer from succinate to NAD. Inhibitors of the energy-yielding reactions also diminish the rates of the energy-consuming reactions but have a different effect on $\Delta\psi$ and K_m^{app} of the substrates of the driven reactions.

The effect of these inhibitors on the latter parameter is opposite to the effect of uncouplers. Uncouplers *increase* the K_m^{app} of the substrates of the energy-driven reactions, whereas inhibitors of the energy-yielding reactions *decrease* these K_m values. The result is an uncompetitive pattern of inhibition of the driven reactions, as shown in Figure 1 for oxidative phosphorylation and in Table I for ATP-driven uphill electron transfer. This behavior, as discussed in the preceding section, is displayed by a number of complex enzymes whose kinetics conform to a rapid equilibrium random ping-pong mechanism. We have also shown that the mitochondrial respiratory chain in completely uncoupled SMP behaves in the same manner (Table I). Thus, the similar effect of electron-transfer limitation on the K_m^{app} of ADP observed by Vinkler (1981) in chloroplast photophosphorylation is not a peculiarity of energy-transducing systems and has a much simpler explanation unrelated to the nature of the intermediate in oxidative or photosynthetic phosphorylation.

Regarding the latter, however, we have observed a relationship between the membrane potential and respiration-modulated K_m^{app} of ADP and P_i in oxidative phosphorylation, which is of interest. It appears that as long as steady-state $\Delta\psi$ remains essentially unchanged as respiration is progressively inhibited (up to about 50–60%), then the K_m^{app} for ADP and P_i continue to decrease together with V_{max}^{app} for ATP synthesis. This results in parallel double-reciprocal plots, as shown in Figure 1, and an essentially constant value of V_{max}/K_m. When steady-state $\Delta\psi$ diminishes at high inhibition of respiration or by partial uncoupling, then the double-reciprocal plots tend to converge toward the left, and V_{max}/K_m decreases as a function of increased uncoupling or inhibition of respiration. These considerations suggest that V_{max}/K_m for ATP synthesis is coupled to the membrane potential. However, this conclusion does not mean that the membrane potential is the sole or the principal driving force for ATP synthesis. It may simply mean that whatever factors that affect V_{max}/K_m also influence the steady-state level of the membrane potential.

Regarding the relationship between $\Delta\psi$ and the rates of energy-driven reactions, the following observations were made. In oxidative phosphorylation catalyzed by SMP or intact mitochondria, the steady-state level of $\Delta\psi$ was minimally affected when inhibition of respiration caused up to about 50% inhibition of the rate of ATP synthesis. When electron transfer was further inhibited, then steady-state $\Delta\psi$ began to drop somewhat, and at about 90% inhibition of the rate of ATP synthesis the steady-state level of $\Delta\psi$ was diminished by about 40%. In ATP-driven NAD reduction by succinate, there was even less correspondence between the decrease in the rate of electron transfer and the magnitude of steady-state $\Delta\psi$ as ATP hydrolysis was inhibited by addition of increasing amounts of oligomycin. Thus, up to about 70% inhibition of the rate of NAD reduction, steady-state $\Delta\psi$ was essentially unchanged, and at about 90% inhibition of the former, $\Delta\psi$ was lowered by only about 10%. By contrast to oxidative phosphorylation and uphill electron transfer, there appeared to be a good correlation between the rate of respiration-driven uniport calcium transport and the magnitude of steady-state $\Delta\psi$. These points are demonstrated also in Figure 11, in which the percent rate changes for calcium transport, oxidative phosphorylation by mitochondria and SMP, and uphill electron transfer are plotted against the percent steady-state $\Delta\psi$ changes in each case. The correlation between the decrease in the rate of calcium transport and the decrease in the magnitude of $\Delta\psi$ agrees with the findings of Åkerman (1978), who concluded that the rate of uniport calcium transport in rat liver mitochondria is a function of the magnitude of the membrane potential. It is, therefore, interesting that there is good correspondence between one bulk phase phenomenon and another, namely, the magnitude of $\Delta\psi$ and the rate of calcium transport, but not between the former and the rates of ATP synthesis or uphill electron transfer (Figure 11). It is also interesting, and perhaps contrary to expectation, that in the case of ATP synthesis and uphill electron transfer, it is the more energy-demanding reaction, namely, uphill electron transfer, which shows a poorer correlation to the steady-state level of $\Delta\psi$.

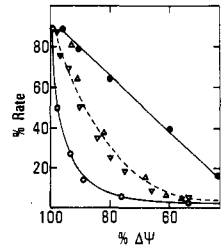

FIGURE 11: Secondary plots of the data of Figures 4–7, showing the relationships between steady-state $\Delta\psi$ and the percent rate changes of calcium uptake (●), ATP synthesis by SMP (▽), state 3 respiration rate by intact mitochondria (△), and uphill electron transfer from succinate to NAD (○).

In spite of these considerations, we appreciate that in principle one cannot predict the steady-state behavior of $\Delta\psi$ as a possible obligatory intermediate in relation to the flow rate of the reactions studied and that the effect of flow rate on the steady-state level of an intermediate may vary considerably depending on the rate constants of the partial reactions involved. However, in order to gain some understanding of the relationship between the flow rate and the steady-state level of a *proven* intermediate in a closely related system, we studied the effect of electron flow rate in uncoupled SMP on the steady-state redox level of cytochromes $c + c_1$. The electron flow rate was regulated by addition of increasing amounts once of malonate to inhibit succinate dehydrogenation and another time of sodium azide to inhibit cytochrome oxidase. In both cases, there was excellent correlation between the rate of respiration and the redox state of cytochromes $c + c_1$. A similar correlation was observed in Figure 3 for $\Delta\psi$ and the rate of ATP synthesis as affected by CCCP and in Figure 6 for $\Delta\psi$ and the rate of calcium transport but not in

Figures 4–6 for $\Delta\psi$ and the rates of ATP synthesis or uphill electron transfer (see also Figure 11). Strictly speaking, the results of Figures 9 and 10 cannot be compared to those of Figures 4–6. Nevertheless, the data of Figures 9 and 10 show that in the respiratory chain the steady-state level of a *known* intermediate is extensively influenced by the flow rate, whereas in oxidative phosphorylation, and particularly in ATP-driven uphill electron transfer, such a correspondence between the rate of the driven reaction and the steady-state level of $\Delta\psi$ does not exist. Whether these data are indeed inconsistent with the premise that the membrane potential is the sole or the principal intermediate in oxidative phosphorylation and ATP-driven uphill electron transfer in SMP remains to be seen. In this regard, the following point may be of interest.

As mentioned above, in both mitochondria and chloroplasts, partial uncoupling and partial inhibition of electron flow diminish V_{max}^{app} for ATP synthesis and alter the K_m^{app} of the substrates. However, in a photophosphorylation system reconstituted from bacteriorhodopsin and a chloroplast ATPase complex preparation, lowering of ΔpH from 3.32 to 2.78 decreased the V_{max} for ATP synthesis by about 10-fold without affecting the K_m values for ADP and P_i (Takabe & Hammes, 1981). By comparison, a 10-fold lowering of the rate of ATP synthesis by addition of nigericin to chloroplasts was accompanied by a 2.5-fold increase in the K_m^{app} for ADP (Vinkler, 1981). In the reconstituted system, protonic energy transfer occurs most likely through the bulk phase. The question is whether in the native chloroplast system there are other modes of energy communication, which in addition to the rate of ATP synthesis also influence the K_m of the substrates.

Acknowledgments

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Registry No. ATP, 56-65-5; NAD, 53-84-9; TMPD, 100-22-1; KCN, 151-50-8; UHDBT, 43152-58-5; HQNO, 341-88-8; CCCP, 555-60-2; Succ, 110-15-6; Ca, 7440-70-2; malonate, 141-82-2; oligomycin, 1404-19-9; efrapeptin, 56645-91-1; valinomycin, 2001-95-8; nigericin, 28380-24-7.

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