Fatty Acid Uncoupling of Oxidative Phosphorylation in Rat Liver Mitochondria†

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ABSTRACT: Free fatty acids (FFA) are known to uncouple oxidative phosphorylation in mitochondria. However, their mechanism of action has not been elucidated as yet. In this study we have investigated in detail the patterns of uncoupling by the FFA oleate and palmitate in rat liver mitochondria and submitochondrial particles. The patterns of uncoupling by FFA were compared to uncoupling induced by the ionophores valinomycin (in the presence of K^+) and gramicidin (in the presence of Na^+) and the proton translocator carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). The most striking difference in the pattern of uncoupling relates to the effect on the proton electrochemical potential gradient, $\Delta\tilde{\mu}_H$. Uncoupling by ionophores, particularly valinomycin, is associated with and most likely caused by a major reduction of $\Delta\tilde{\mu}_H$. In contrast, uncoupling by FFA is not associated with a significant reduction of $\Delta\tilde{\mu}_H$, indicating another mechanism of uncoupling. We suggest the use of the term decouplers for uncoupling agents such as FFA and general anesthetics that do not collapse the $\Delta\tilde{\mu}_H$ [Rottenberg, H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3313–3317]. The protonophore CCCP and to some extent the ionophore gramicidin indicate a mixed mode of uncoupling since their effect on $\Delta\tilde{\mu}_H$ is moderate when compared to that of valinomycin. Another distinguishing feature of uncouplers that collapse the $\Delta\tilde{\mu}_H$ is their ability to stimulate ADP-stimulated respiration (state 3) further. Decouplers such as FFA and general anesthetics do not stimulate state 3 respiration. The effects of FFA on energy conversion in submitochondrial particles are weaker than in intact mitochondria and depend on the free energy load of the driven process. Reverse electron transport (high load) is completely uncoupled by FFA. Oxidative phosphorylation (intermediate load) is partially uncoupled, and transhydrogenase (low load) is hardly affected. These data are compatible with the parallel coupling model in which both $\Delta\tilde{\mu}_H$ and an intramembranal proton transfer contribute to energy conversion. Accordingly, decouplers such as FFA and general anesthetics only uncouple the intramembrane pathway. FFA increases the oligomycin sensitivity of both ATP hydrolysis and ATP-generated $\Delta\tilde{\mu}_H$. Also, the $\Delta\tilde{\mu}_H$ generated by ATP is sensitive to FFA. These data suggest that the ATPase complex is the site for FFA action. A working hypothesis according to which the F_0 membrane-associated complex serves as a proton capacitor that is discharged by FFA is suggested.

Free fatty acids (FFA)[1] are potent uncouplers of oxidative phosphorylation in mitochondria (Pressman & Lardy, 1956; Wojtczak & Lehninger, 1961; Borst et al., 1962). However, the mechanism of uncoupling by free fatty acids has not been elucidated as yet. It is now widely believed that oxidative phosphorylation is mediated by proton transport as outlined in the chemiosmotic hypothesis (Mitchell, 1966, 1979). One of the most satisfying attributes of the chemiosmotic hypothesis is its explanation of uncoupling. Mitchell has suggested that uncouplers are proton translocators, i.e., weak acids that increase the membrane proton permeability and collapse the proton electrochemical gradient, $\Delta\tilde{\mu}_H$, by shuttling protons across the membrane. A similar explanation was offered for the uncoupling effect of electrogenic ionophores and electrogenic transport systems (e.g., Ca^{2+}) that collapse the membrane potential and thereby the proton electrochemical potential gradient (Mitchell, 1966). This explanation of uncoupling in mitochondria, chloroplasts, and bacterial cells has been verified in numerous cases for a diverse and large number of potent uncouplers and ionophores (Rottenberg, 1970; Mitchell, 1979; McLaughlin & Dilger, 1980; Nichols, 1982). However, it has been demonstrated recently that there are important exceptions to this general concept. We have shown that general anesthetics such as halothane and chloroform uncouple oxidative phosphorylation without significant reduction of $\Delta\tilde{\mu}_H$ (Rottenberg, 1983). The significance of this finding is that it suggests that there are alternative pathways for energy conversion in oxidative phosphorylation that bypass the solution bulk phases on either side of the membranes (Rottenberg, 1985). Recent studies on the kinetics and energetics of oxidative phosphorylation are compatible with the notion that the bulk to bulk proton electrochemical potential gradient is not the only driving force for ATP synthesis [for reviews, see Ferguson & Sorgato (1982), Westerhoff et al. (1984), Ferguson (1985), and Rottenberg (1985)]. We have screened diverse and large numbers of molecules that uncouple oxidative phosphorylation and have found many, including fatty acids, alcohols, detergents, and various drugs, that do not have significant effects on $\Delta\tilde{\mu}_H$ within the uncoupling concentration range (H. Rottenberg and K. Hashimoto, unpublished observation). We have concentrated in this study on the characteristics of the uncoupling by free fatty acid (FFA) of oxidative phosphorylation in rat liver mitochondria and submitochondrial particles since these are among the most potent of the above-mentioned group. In addition, in contrast to many of the other reagents that exhibit additional inhibitory

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[1] Abbreviations: $\Delta\tilde{\mu}_H$, the proton electrochemical gradient; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DMO, 5,5-dimethyl-2,4-oxazolidinedione; ANS, 1-anilinonaphthalene-8-sulfonate; FFA, free fatty acids; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

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effects on the mitochondrial enzymes, FFA at low concentrations show relatively few inhibitory effects. To further our understanding of the similarities and differences between FFA and more classical uncouplers, we have compared, under identical conditions, the various effects of palmitate, CCCP, valinomycin, and gramicidin on oxidative phosphorylation processes. In an effort to locate the site of action of FFA, we investigated the effect of palmitate on the proton-pumping efficiency of the ATPase and the redox pumps. On the basis of our results, we suggest the following working hypothesis: FFA uncouple oxidative phosphorylation by dissipation of an intramembranal proton capacitor, which is probably located on or close to the F_0 complex of the mitochondrial ATPase.

Materials and Methods

Rat liver mitochondria were prepared by conventional differential centrifugation, as described (Hashimoto & Rottenberg, 1983). Rat liver submitochondrial particles were prepared by sonication of mitoplasts isolated after digitonin treatment of rat liver mitochondria, as described (Thayer & Rubin, 1979). Respiration rates were determined by the use of an oxygen electrode. Phosphorylation rates were determined by the incorporation of $[^{32}P]P_i$ into ATP (Lindberg & Ernster, 1956). ATPase rates were determined by the release of $[^{32}P]P_i$ from [^{32}P]ATP. Reverse electron transport rates were determined from the fluorescence of the product NADH, essentially as described before (Rottenberg & Gutman, 1977). Transhydrogenase rates were determined from the fluorescence of the product NADPH, as described (Danielson & Ernster, 1963). The value of $\Delta\tilde{\mu}_H$ was calculated from the measurement of $\Delta\psi$ and ΔpH. $\Delta\psi$ was determined from the distribution of ^{86}Rb in the presence of valinomycin and ΔpH from the distribution of [^{14}C]DMO. ^3H_2O served as a marker for the water volume and [^{14}C]sucrose for the estimation of the matrix fraction of the pellet, as described in detail elsewhere (Rottenberg, 1979, 1984). Enzymes and biochemicals were from Sigma and radiolabeled compounds from Amersham. Fatty acids were prepared in dilute ethanol solution (1–3 mM) under argon and sealed under argon in small ampules. Ampules were opened on the day they were to be used.

Results

Classical uncouplers are characterized as inhibitors of ATP synthesis that release the respiratory control, i.e., stimulate "static head" (state 4) respiration. These agents do not inhibit uncontrolled respiration, and they stimulate ATPase activity (Slater, 1967). Figure 1 shows the effect of oleate on oxidative phosphorylation processes in rat liver mitochondria. State 4 succinate oxidation (in the absence of phosphorylation) is stimulated by oleate to the level of state 3 respiration (during phosphorylation). The same concentrations slightly stimulate state 3 respiration but have no effect on CCCP-stimulated respiration. In the same concentration range ATP synthesis is completely inhibited (Figure 1A). The coupling parameters, the P/O ratio, and the respiratory control ratio (Figure 1B) are strongly reduced in this concentration range. At concentrations above 20 nmol/mg of protein, the respiration rate is gradually inhibited. In contrast to common uncouplers, oleate has little effect on the proton electrochemical potential, $\Delta\tilde{\mu}_H$ either at state 4 or state 3, in the uncoupling range. At higher concentrations (where respiration is inhibited), $\Delta\tilde{\mu}_H$ is gradually reduced. These data resemble the effects of general anesthetics (Rottenberg, 1983) on mitochondria but are in sharp contrast to classical uncouplers and to uncoupling by ionophores (Nichols, 1982). This characteristic of uncoupling by oleate is shared by other fatty acids and other membrane-perturbing agents. Table I shows the dependence of the uncoupling potency on the type of long-chain fatty acids. In shorter fatty acids the potency is determined largely by the partition coefficients (H. Rottenberg and K. Hashimoto, unpublished observations). Long-chain fatty acids have very high partition coefficients, and thus their membrane content is nearly identical with the total amount of added acid. Among the saturated fatty acids, palmitate (C_{16}) is the most potent. Unsaturation increases the potency of the uncoupling, as evidenced from the comparison of stearic and oleic acids. Also, it appears that the location of the double bond closer to the membrane surface increases the potency since oleate is more potent than *cis*-vaccinate. The configuration around the double bond appears to be unimportant since *trans*-vaccinate is almost as effective as *cis*-vaccinate. In subsequent experiments we use palmitate instead of oleate, since palmitate is a more stable species, less susceptible to peroxidation. The uncoupling effect of FFA is also observed with submitochondrial particles. Figure 2 shows the effect of palmitate on energy coupling in

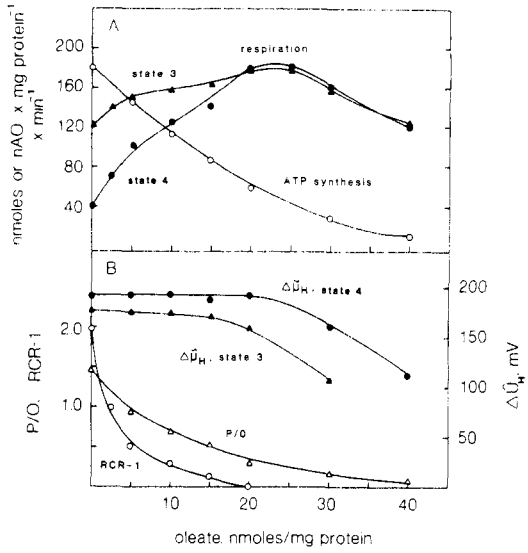

FIGURE 1: Effect of oleate on oxidative phosphorylation in rat liver mitochondria. Rat liver mitochondria were incubated (25 °C) in a basic medium containing 0.2 M sucrose, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM Na_2HPO_4, 5 mM HEPES (pH 7.4), 2 mM EGTA, 1 μM rotenone, 0.1 μM valinomycin, and 5 mM succinate. Oleic acid was added to the mitochondrial suspension from a 3 mM solution in ethanol. Mitochondrial protein concentration was 2 mg of protein/mL. For measurements of respiration rate in state 4 [A (●)] there were no further additions. For measurement of respiration rate in state 3 [A (▲))] 1 mM ADP was added. For measurement of ATP synthesis [A (○)] 1 mM ADP and ^{32}P (0.1 μCi/mL) were added. $\Delta\tilde{\mu}_H$ (B) was calculated from the measurement of $\Delta\psi$ and ΔpH as described under Materials and Methods. P/O and respiratory control ratio (B) were calculated from the results shown in (A).

Table I: Concentration of Fatty Acids Required for 50% Uncoupling of Oxidative Phosphorylation (P/O with Succinate as a Substrate)[a]

fatty acid	50% uncoupling (P/O) (nmol/mg of protein)
lauric acid, $CH_3(CH_2)_{10}COOH$	50
myristic acid, $CH_3(CH_2)_{12}COOH$	25
palmitic acid, $CH_3(CH_2)_{14}COOH$	10
stearic acid, $CH_3(CH_2)_{16}COOH$	40
oleic acid, $CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$	10
cis-vaccinic acid, $CH_3(CH_2)_5CH{=}CH(CH_2)_9COOH$	25
trans-vaccinic acid, $CH_3(CH_2)_5CH{=}CH(CH_2)_9COOH$	30

[a] Conditions are the same as in Figure 1. All acids were added as ethanol solution (1–3 mM).

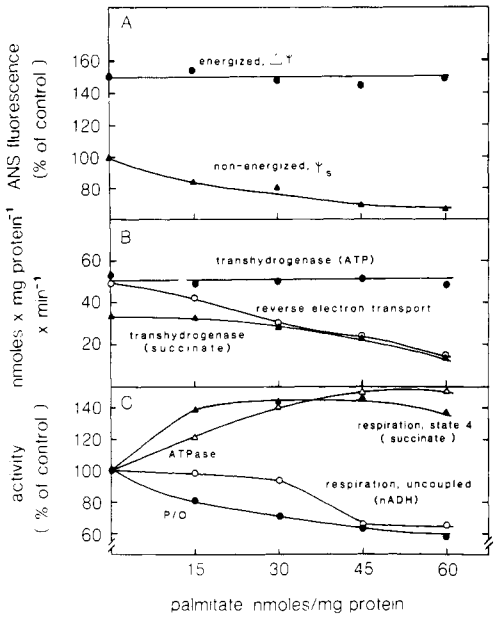

FIGURE 2: Effect of palmitate on energy transduction in rat liver submitochondrial particles. Submitochondrial particles were incubated in a basic medium containing 0.2 M sucrose, 5 mM Na_2HPO_4, and 5 mM $MgCl_2$ (pH 7.4). T = 25 °C. Protein concentration was 0.5 mg of protein/mL. For measurement of respiration at state 4 [panel C (▲)] succinate (5 mM) was added. For measurement of ATPase [panel C (△)] 3 mM ATP ([^{32}P]ATP) was added. For measurement of oxidative phosphorylation, ADP (1 mM) succinate (5 mM), and ^{32}P were added. Panel C (●) shows the measured P/O ratio. For measurement of NADH oxidase, 1 mM NADH and 5 μM CCCP were added. ANS fluorescence was measured in the presence of 10 μM ANS (ex = 400 nm; em = 470–500 nm). The nonenergized fluorescence [indicating ψ_s, panel A (▲)] was measured in the basic medium. The enhancement of fluorescence at state 3 (indicating $\Delta\psi$, panel A (●)] was calculated from the fluorescence level in the presence of succinate (5 mM) and ADP (1 mM) before and after the addition of CCCP (5 μM). The rate of reverse electron transport [panel B (○)] was measured from the fluoresence of NADH (ex = 340 nm; em = 430–470 nm) in a basic medium containing succinate (5 mM), KCN (1.5 mM), and NAD^+ (0.4 mM). The reaction was started by the addition of ATP (2 mM). The rate of transhydrogenase was measured in a basic medium supplemented with 2 μM rotenone, 100 mM ethanol, 750 μM NAD^+, 250 μM $NADP^+$, and alcohol dehydrogenase. The reaction was started by the addition of either 1.5 mM ATP (●) or 5 mM succinate (▲).

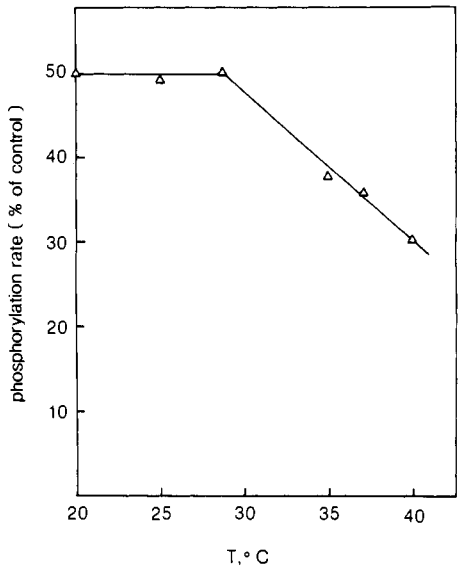

FIGURE 3: Temperature dependence of the inhibition of ATP synthesis by palmitate. Conditions are as in Figure 1, except for the temperature and the addition of palmitate (10 nmol/mg of protein).

rat liver submitochondrial particles. In general, uncoupling by FFA in submitochondrial particles requires higher concentrations of FFA. This is partly due to the fact that the mitochondrial inner membranes are concentrated approximately 2.5-fold in this preparation, as compared to intact mitochondria (when related to protein content). However, even when this factor is taken into account, it is evident that the uncoupling is less effective. Even at 60 nmol/mg of protein where inhibition of respiration is already observed, the P/O is still about 60% of control. Thus, it appears that uncoupling is not complete in this preparation (Figure 2C). There is only a mild stimulation of either state 4 respiration or ATPase (Figure 2C). This is in contrast to the effect of the classical uncoupler CCCP that greatly stimulates respiration and ATPase (3–4-fold) in these particles. Reverse electron transport is more strongly inhibited (Figure 2B), but this is partially due, at least at high concentration, to the inhibition of NADH oxidase activity (Figure 2C). Interestingly, transhydrogenase activity is not inhibited at all when energized by ATP and only slightly at high concentration when energized by succinate, probably due to inhibition of succinic oxidase in this range. Reverse electron transport, oxidative phosphorylation, and transhydrogenase differ by the "load" of free energy against which the coupled reaction must proceed under our assay conditions. The highest load is in reverse electron transport (Rottenberg & Gutman, 1977), and the lowest load is in the transhydrogenase. It thus appears that the effectiveness of uncoupling by palmitate depends on the free energy load. Indeed, if oxidative phosphorylation is measured at high phosphate potential (i.e., in the presence of ATP), the efficiency of uncoupling increases (not shown). These results suggest that FFA do not totally uncouple energy conversion in submitochondrial particles (see Discussion). Figure 2A shows the effect of palmitate on ANS fluorescence in submitochondrial particles. In nonenergized particles the extent of fluorescence is a function of the surface potential (Robertson & Rottenberg, 1983). Increasing the concentration of palmitate increases the negative surface charge of the membrane, thereby reducing the binding of the anion ANS^- and its resulting fluorescence. In coupled, respiring submitochondrial particles the formation of membrane potential (inside positive) induces the uptake and internal binding of ANS^-, resulting in enhanced fluorescence (Jasaitis et al., 1973; Robertson & Rottenberg, 1983). The extent of enhancement is a function of the membrane potential. Figure 2A shows that the enhancement of ANS^- fluorescence is not affected by palmitate, indicating that palmitate does not reduce the magnitude of $\Delta\psi$. In low salt medium in these particles $\Delta\psi$ is the major component of $\Delta\tilde{\mu}_H$. This is evident from the very weak response of 9-aminoacridine to substrate oxidation (not shown) and from the fact that 10 mM NH_4Cl which collapses ΔpH has relatively little effect on the rate of oxidative phosphorylation. In NH_4Cl-treated particles, phosphorylation is inhibited by palmitate while the stimulation of ANS fluorescence is unaffected. Thus, it appears that, in these particles also, FFA do not collapse the $\Delta\tilde{\mu}_H$ at the uncoupling concentration range. Similar results (Figure 2) were obtained with oleate (not shown). We have previously noticed that the uncoupling efficiency of anesthetics is more pronounced at higher temperatures. This may be related to the fact that mitochondria are less tightly coupled at high temperatures (Rottenberg, 1978; Rottenberg et al., 1985). The uncoupling by FFA is also more potent at higher temperatures. Figure 3 shows the inhibition of ATP synthesis by 10 nmol/mg of protein as a function of temperature. There is no effect of temperature between 20 and 30 °C. However, above 30 °C the potency

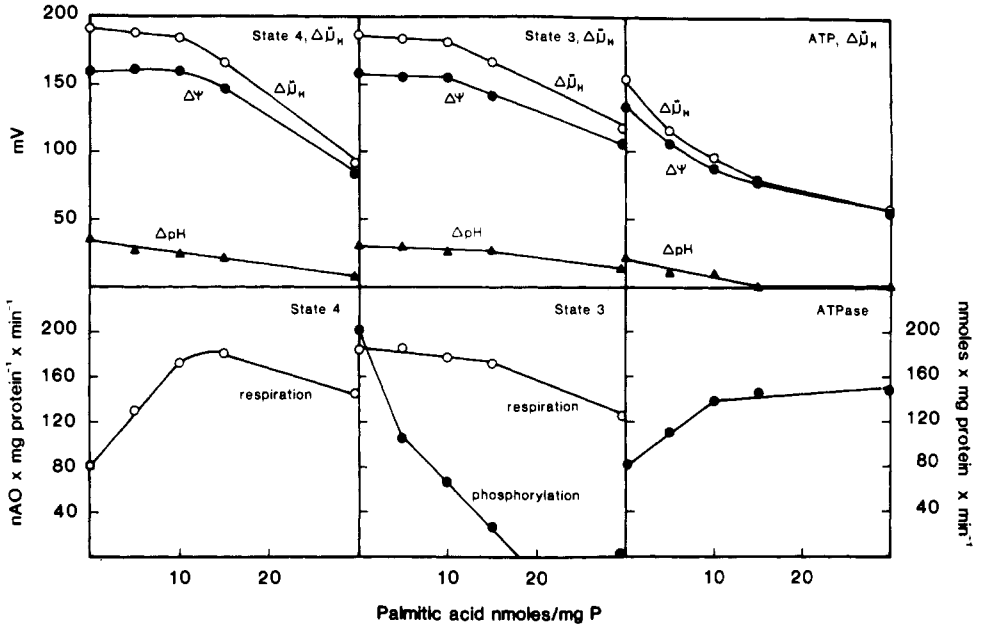

FIGURE 4: Effect of palmitate on mitochondrial function of 37 °C. Rat liver mitochondria were incubated in a medium containing 0.2 M sucrose, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM Na_2HPO_4 (pH 7.4), 2 mM EGTA, 2 μM rotenone, 0.1 μM valinomycin, and 5 mM succinate, except for the experiments on ATPase- and ATP-generated $\Delta\tilde{\mu}_H$ in which the medium was 0.2 M mannitol, 0.075 M sucrose, 2 mM EDTA, 20 mM HEPES (pH 7.4), 2 μM rotenone, 0.1 μM valinomycin, and 3 mM ATP. For state 3 measurements, 1 mM ADP was added to the basic medium. The rate of phosphorylation was measured from the incorporation of ^{32}P into ATP and the rate of ATPase from the hydrolysis of [^{32}P]ATP. ΔpH and $\Delta\psi$ measurements are detailed under Materials and Methods. Protein concentration was 2 mg of protein/mL. Palmitate was added as ethanolic solution (3 mM).

is greatly increased with temperature.

In order to elucidate more clearly the differences between the uncoupling pattern of FFA and more conventional uncouplers and ionophores, we embarked on a comparative study (Figures 4–8) of the uncoupling pattern of palmitate, valinomycin (+potassium), gramicidin, and CCCP. All the reagents are compared under the same incubation conditions and assayed by the same procedures, thus enhancing the validity of the comparison. Moreover, uncoupling is characterized at 37 °C, as against the conventional studies at 25 °C. This makes the study more relevant to physiological coupling conditions and also increases the differences between the various coupling modes. Figure 4 shows (left panel) the effect of palmitate on state 4 respiration in rat liver mitochondria (bottom) and in parallel the effect on ΔpH, $\Delta\psi$, and the calculated $\Delta\tilde{\mu}_H$ (top panel). It is observed (similar to oleate effect at 25 °C) that the stimulation of respiration is not associated with a signficant reduction of $\Delta\tilde{\mu}_H$. The inhibition of respiration at higher concentratons is associated with a reduction of $\Delta\tilde{\mu}_H$. In state 3 (center panel) phosphorylation is inhibited, at low concentration, without significant effect on the respiration. At higher concentrations, the respiration is also inhibited. $\Delta\tilde{\mu}_H$ (top panel) is not inhibited in the uncoupling concentration range but is reduced in the inhibitory range. The right hand panel shows the effect on the ATPase reaction. Under these conditions ATP hydrolysis is only slightly stimulated by palmitate and $\Delta\tilde{\mu}_H$ is moderately inhibited.

Figure 5 shows similar experiments with valinomycin and increasing concentrations of KCl. Full stimulation of state 4 respiration, obtained at about 3 mM, is associated with 50% reduction of $\Delta\tilde{\mu}_H$. Notice that valinomycin collapses only the membrane potential since it only affects potassium permeability and not proton permeability. These results, which are predicted by the chemiosmotic mechanism, are in sharp contrast to the results obtained with palmitate. Similarly at state 3 (center panel) the valinomycin + KCl inhibition of phosphorylation and further stimulation of respiration are associated with more than 50% reduction of $\Delta\tilde{\mu}_H$. This is in sharp contrast to palmitate, which hardly affected $\Delta\tilde{\mu}_H$ in the uncoupling range. Notice also that low concentrations of potassium, in the presence of valinomycin, while greatly reducing the magnitude of $\Delta\tilde{\mu}_H$, only slightly inhibit the rate of phosphorylation (Hofer & Pressman, 1966; Rottenberg, 1970). The right-hand panel shows the effect on ATPase. A large stimulation of ATPase is associated with a large reduction of $\Delta\tilde{\mu}_H$. It can be argued (see Discussion) that valinomycin ($+K^+$) uncoupling is the best example of classical, chemiosmotic, uncoupling since the only parameter that appears to be affected is the membrane potential, thus amounting to "voltage clamping" of $\Delta\tilde{\mu}_H$. Protonophores may also serve to "clamp" $\Delta\tilde{\mu}_H$ by increasing the membrane proton permeability (McLaughlin & Dilger 1980). However, in addition, these may also interact directly with the proton pumps, affecting active proton transport directly and not only through clamping of $\Delta\tilde{\mu}_H$. Comparing Figures 4 and 5 shows clearly the major difference between classical, chemiosmotic, uncoupling which is due to the collapse of $\Delta\tilde{\mu}_H$ and the nonclassical uncoupling which is not dependent on the collapse of $\Delta\tilde{\mu}_H$.

Another interesting difference is that classical uncouplers are capable of further stimulation of state 3 respiration where the nonclassical uncouplers, as exemplified by FFA or general anesthetics (Rottenberg, 1983), do not stimulate state 3 respiration (see Discussion). Figure 6 shows the uncoupling pattern of gramicidin. Gramicidin is a channel-forming ionophore that induces permeability to protons and other monovalent cations (Pressman, 1976). In our test medium the uncoupling depends on both protons and sodium (but not potassium, which is absent). In general, the pattern of uncoupling of oxidative phosphorylation is quite similar to valinomycin, although the collapse of $\Delta\tilde{\mu}_H$ is somewhat less pronounced. An unusual feature is the total collapse of $\Delta\tilde{\mu}_H$ by very low concentrations of gramicidin in ATP-dependent energization.

Figure 7 shows the uncoupling pattern by the weak acid protonophore CCCP, a traditional classical uncoupler. It is

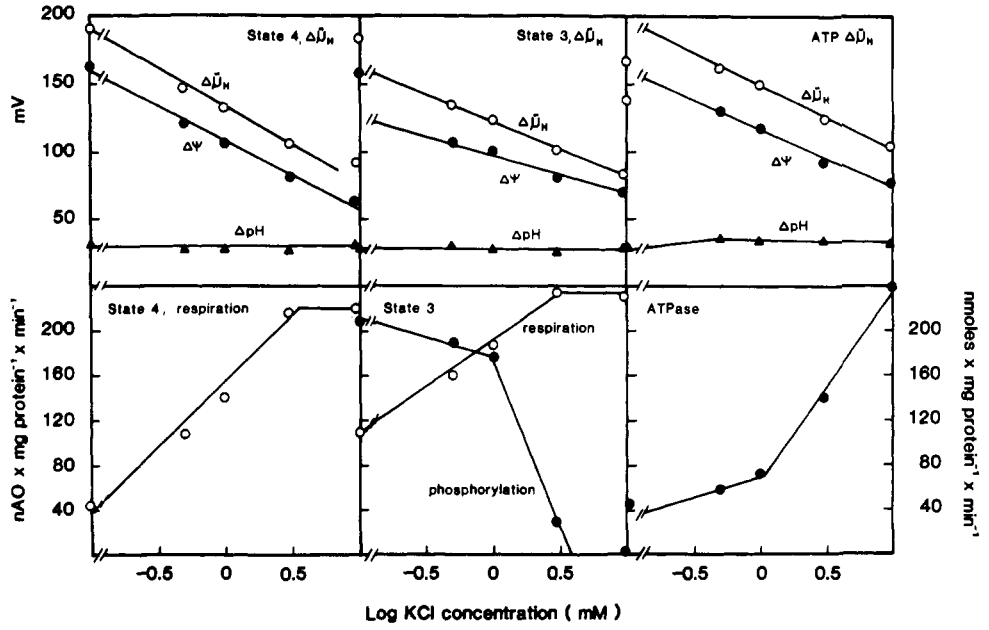

FIGURE 5: Effect of KCl (in the presence of valinomycin) on mitochondrial function. Conditions are as in Figure 4, except for the addition of KCl, which replaces NaCl.

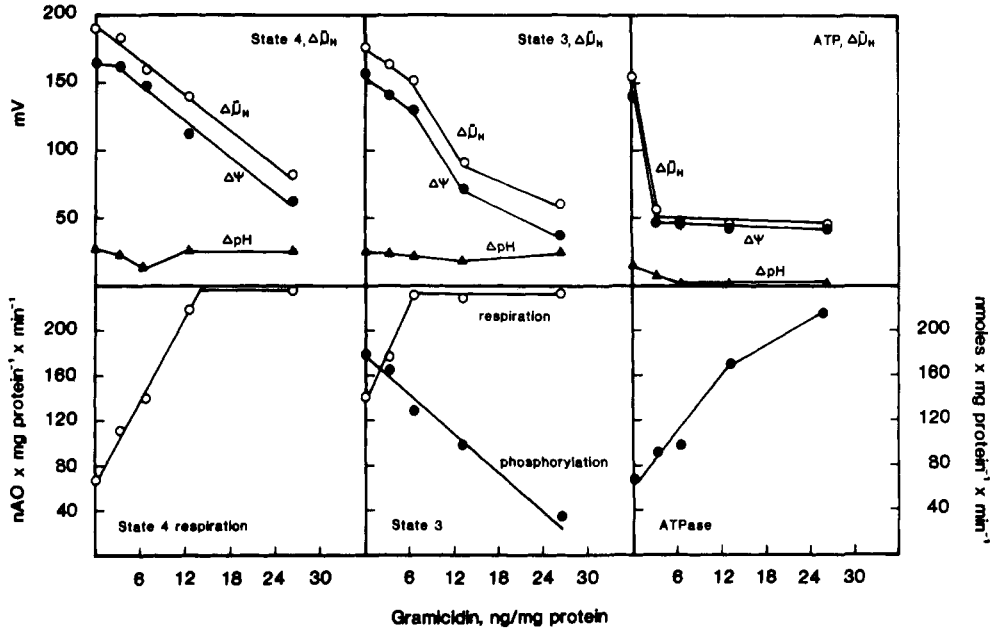

FIGURE 6: Effect of gramicidin on mitochondrial function. Conditions are as in Figure 4 except for the addition of gramicidin.

observed that although stimulation of state 4 respiration and inhibition of phosphorylation are associated with the reduction of $\Delta\tilde{\mu}_H$, the reduction is not as large as is the case with valinomycin (+K). In other words, a small reduction of $\Delta\tilde{\mu}_H$ is associated with a large stimulation of state 4 respiration and a large inhibition of phosphorylation. Also, the stimulation of state 3 respiration is smaller than that of valinomycin (+K). The relationship between the magnitude of $\Delta\tilde{\mu}_H$ and the respiratory control ratio, and $\Delta\tilde{\mu}_H$, and the P/O ratio for the uncoupling reagents is found in Figure 8. If all the uncouplers work by collapsing $\Delta\tilde{\mu}_H$, we expect all the data fall on a single curve. This is clearly not the case. Since we are fairly sure that valinomycin (+K) uncoupling works through the clamping of $\Delta\tilde{\mu}_H$, its pattern probably represents the "true" $\Delta\tilde{\mu}_H$-dependent uncoupling. In this case uncoupling requires a large reduction of $\Delta\tilde{\mu}_H$. At the other extreme, palmitate shows little effect on $\Delta\tilde{\mu}_H$ in the uncoupling range, and its pattern probably represents intramembranal uncoupling not associated with a direct effect on $\Delta\tilde{\mu}_H$. By these criteria the uncoupling pattern of CCCP and gramicidin is of a mixed type showing both classical patterns, which are no doubt due to the known effects of these agents on the passive proton permeability together with more direct effects where the protonophores are able to release stored intramembranal protons. Not shown in Figure 8, but also fitting the same pattern, is the stimulation of state 3 respiration.

If the effect of the rate of phosphorylation on the rate of respiration is not dependent on $\Delta\tilde{\mu}_H$ (Padan & Rottenberg, 1973), we expect phosphorylation and intramembrane uncoupling to bring the rate of respiration of the same level (state 3), while the collapse of $\Delta\tilde{\mu}_H$ should further stimulate the respiration. These predictions are compatible with the effect of the different uncouplers on state 3 respiration. The results of our studies suggest to us that fatty acids interact directly with the proton pumps, dissipating an intramembranal proton capacitor.

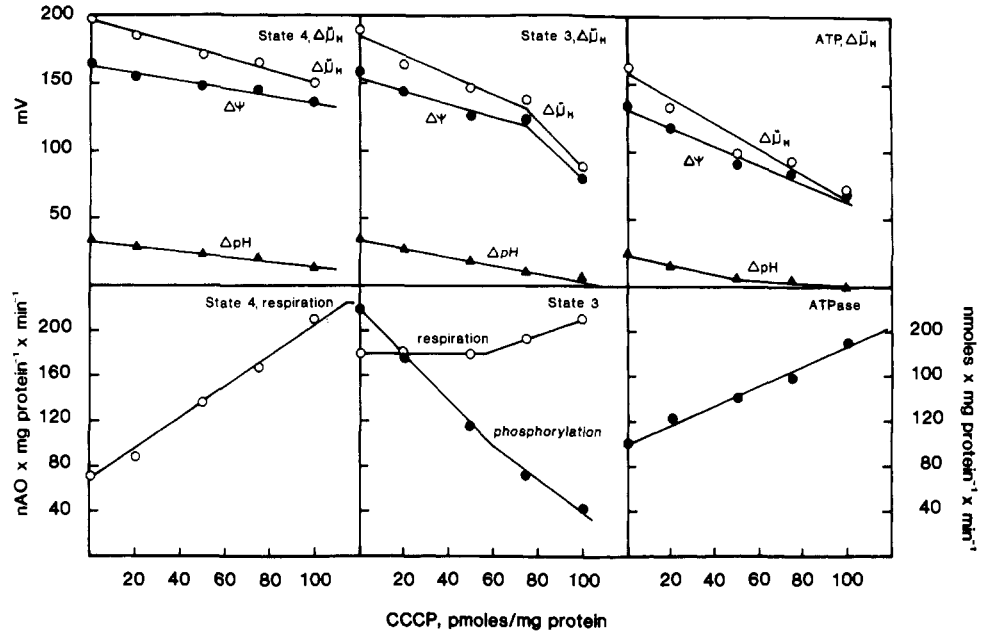

FIGURE 7: Effect of CCCP on mitochondrial function. Conditions are as in Figure 4 except for the addition of CCCP.

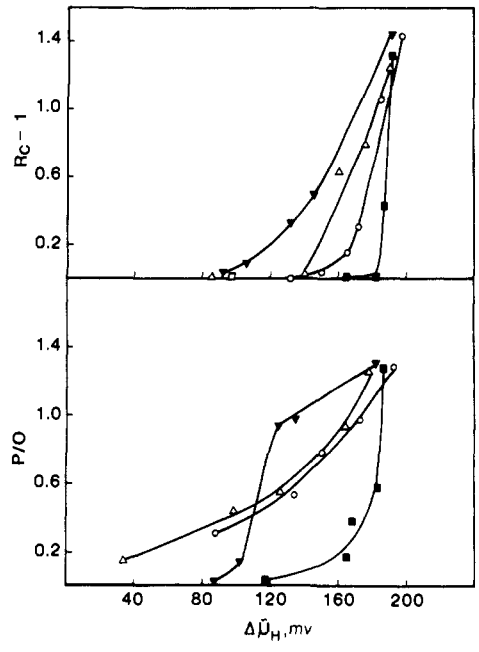

FIGURE 8: Relationships between respiratory control ratio (RCR-1) and $\Delta\tilde{\mu}_H$ and between the P/O ratio and $\Delta\tilde{\mu}_H$. Data are taken from Figures 4–7. Palmitate (■), gramicidin (△), CCCP (○), and KCl (▼).

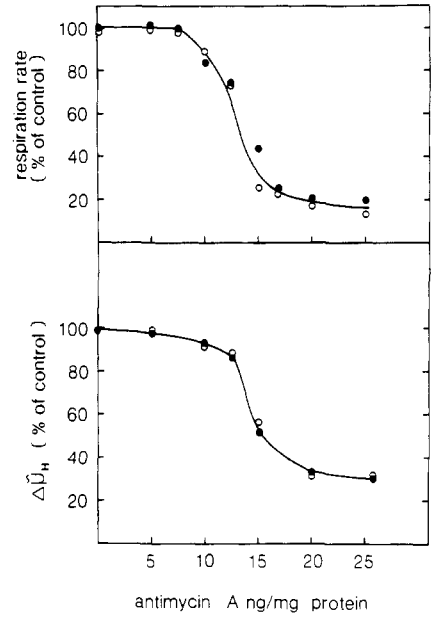

FIGURE 9: Effect of palmitate on the inhibition of state 4 respiration and $\Delta\tilde{\mu}_H$ by antimycin A. Medium was composed of 0.2 M sucrose, 5 mM Na_2HPO_4 (pH 7.4), 5 mM $MgCl_2$, 5 mM succinate, 2 μM rotenone, and 0.1 μM valinomycin, T = 37 °C. Palmitate when added was 10 nmol/mg of protein. Protein concentration was 2 mg/mL. $\Delta\tilde{\mu}_H$ was measured as described under Materials and Methods. Full circles, no palmitate; empty circles, with palmitate. Uninhibited respiration rate was 61 nmol mg^{-1} min^{-1} without palmitate and 89 with palmitate.

In an effort to find whether the interaction is more specific to the ATPase pump or to the redox pump, we studied the pattern of inhibition of these pumps by specific inhibitors in the presence and absence of palmitate. Figure 9 shows the effect of antimycin A on electron transport (top) and $\Delta\tilde{\mu}_H$ (bottom) in the absence and presence of palmitate. The concentration dependence shows the typical sigmoidal curve observed with this inhibitor. The inhibition of $\Delta\tilde{\mu}_H$ is somewhat delayed, compared to the inhibition of the respiration. However, when normalized to the state without antimycin, there is no significant difference in the pattern of inhibition of either electron transport or $\Delta\tilde{\mu}_H$ between the systems in the presence or absence of palmitate. In contrast, a similar titration of the ATPase activity with oligomycin (Figure 10) shows clear differences in the pattern of inhibition of ATPase activity (top) and $\Delta\tilde{\mu}_H$ (bottom). It appears that palmitate renders the ATPase more sensitive to oligomycin inhibition. While the meaning of this observation is not entirely clear, it suggests that there is a direct interaction between the ATPase and FFA which affects the function and coupling of the ATPase.

Discussion

FFA have been recognized as uncouplers of oxidative phosphorylation in mitochondria for many years. The earlier work on the effects of FFA on oxidative phosphorylation resulted from the realization that mitochondrial preparations, particularly "aged" mitochondria, as well as other aged membrane fractions, contained endogenous uncoupling activity (Pressman & Lardy, 1956; Wojtack & Lehninger, 1961; Borst

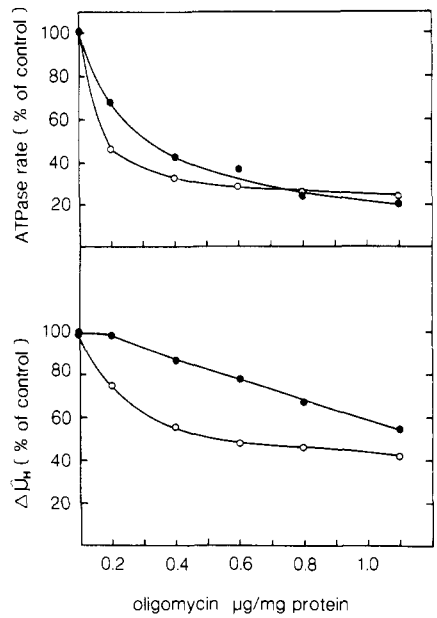

FIGURE 10: Effect of palmitate on the inhibition of ATPase and $\Delta\tilde{\mu}_H$ by oligomycin. Medium was composed of 0.2 M mannitol, 0.075 M sucrose, 0.2 mM EDTA, 20 mM HEPES (pH 7.4), 2 μM rotenone, and 0.1 μM valinomycin. Palmitate when present was 10 nmol/mg of protein. Protein was 2 mg/mL, T = 37 °C. Assays, as described under Materials and Methods. Suspensions were incubated for 3 min with the indicated oligomycin concentration prior to initiation of the assay by the addition of 3 mM ATP. Full circles, without palmitate; empty circles, with palmitate.

et al., 1962). This activity was identified as due to free fatty acids, a breakdown product of membrane "aging". These earlier studies established that FFA stimulate state 4 respiration, inhibit ATP synthesis, and stimulate ATPase activity. However, there are very few recent studies on the effect of FFA on liver or heart mitochondria (Piper et al., 1983; Matsuoka & Nakamura, 1979), and there were practically no studies aimed at elucidating the mechanism of the uncoupling in these systems. A special case is Brown adipose tissue mitochondria that are uncoupled by FFA through a tissue specific system (Locke et al., 1982).

Our results on the effect of oleate and palmitate on oxidative phosphorylation processes in rat liver mitochondria confirm and greatly extend the earlier observations and provide new clues to the mechanism of FFA action. We confirmed the stimulation of state 4 respiration and the parallel inhibition of ATP synthesis. The data on stimulation of ATP hydrolysis are more equivocal. Under our incubation conditions, the stimulation of ATPase activity is not as large as reported by others. This is partially due to the much reduced control of the ATPase at higher temperatures (Rottenberg, 1978) and to the absence of Mg^{2+} in our medium (Borst et al., 1962). Mg^{2+} was omitted because it reduces the magnitude of $\Delta\tilde{\mu}_H$ under these conditions. This study also differs somewhat from previous studies with respect to the concentration dependence of the uncoupling by various fatty acids (Borst et al., 1962). This probably results from the fact that FFA are sparingly soluble in aqueous suspensions. The addition of a concentrated solution (even in ethanol) to the mitochondrial suspension appears to result in the formation of metastable micelles that do not dissolve easily even when the concentration is below the critical micellar concentration. We have noticed that if we add an equal amount of palmitate (or oleate) from a concentrated ethanol solution (5 mM or more), the effect is considerably smaller than when added as a dilute solution (1–3 mM). The contrast is particularly noticeable in comparison to the study of Borst et al. (1962) where the uncoupling concentration appears to be at least 1 order of magnitude higher than reported here.

In a more recent study, FFA was applied as a human serum albumin complex. Effective uncoupling was observed at a free concentration of 5×10^{-7} M for stearate in heart mitochondria (Piper et al., 1983). If we take the partition coefficient of stearate to be 10^5 (Pjora et al., 1984), we can calculate an effective membrane concentration of 5×10^{-2} M, which is approximately equivalent to 50 nmol/mg of protein [assuming 1 μL/mg of protein as membrane volume (Rottenberg et al., 1981)]. This value compares favorably with our value of 40 nmol/mg of protein in liver mitochondria.

The amount of membrane-bound FFA that is required for maximal uncoupling far exceeds the effective concentrations of potent classical uncouplers and ionophores. It was previously argued that the fact that the amount of membrane-bound uncouplers required for uncoupling is much less than the amount of oxidative phosphorylation components suggests a delocalized action that is compatible with the chemiosmotic mechanism of uncoupling (Terada & VanDam, 1975). If we apply the same consideration to FFA, we find that the amount of membrane-bound FFA required for full uncoupling greatly exceeds the content of any specific enzyme or even the total content of proton pumps [1 nmol/mg of protein (Hatefi, 1985)]. If we assume that FFA act through interaction with membrane enzymes, then full uncoupling may require more than one molecule of FFA for each affected protein. However, the exact number would depend on the partition coefficient of the FFA within the membrane, between the bulk lipid phase and the affected enzyme, as well as the binding to other proteins not involved directly in the coupling. Nevertheless, the important conclusion is that the membrane concentration is sufficiently high to be compatible with direct interaction between FFA and the enzymes of oxidative phosphorylation.

The most important finding of this study is the observation that FFA uncouple mitochondria without significant reduction of $\Delta\tilde{\mu}_H$. In this they differ from ionophores and classical uncouplers and are similar to general anesthetics (see below). On the basis of their structure and interactions with phospholipid membranes, it is not expected that long-chain FFA would have pronounced protonophoric activity. First, the pK of FFA is much lower than required for effective uncoupling by proton transmembrane shuttling (McLaughlin & Dilger, 1980). Moreover, the maximal uncoupling is at high pH, in which the acids are totally ionized, contrary to the prediction of proton-shuttling mechanism (Borst et al, 1962). Finally, the rate of "flip-flop" of FFA could not be sufficient to produce significant proton current. While we have not measured the effect of FFA on proton permeability directly, the fact that they do not affect the magnitude of $\Delta\tilde{\mu}_H$ at state 4 or 3, in the uncoupling concentration range, would exclude transmembrane proton shuttling as a mechanism for their uncoupling activity. The reduction of $\Delta\tilde{\mu}_H$ associated with high concentration of FFA is most likely due to their inhibition of electron transport and, in any case, is not relevant to the uncoupling effect. Another novel finding of this study is the relatively weak uncoupling of oxidative phosphorylation in submitochondrial particles. There is an inherent difference in the energetics of oxidative phosphorylation between mitochondria and submitochondrial particles. In submitochondrial particles the phosphorylation reaction takes place in the medium where the ATPase free energy (phosphate potential) is initially very low (20–25 kJ/mol in our assay conditions). In intact mitochondria, the phosphorylation takes place in the

matrix where few turnovers of the ATPase are sufficient to raise the ATPase free energy above 35–40 kJ/mol. The effectiveness of FFA inhibition in submitochondrial particles is greater for reverse electron transport which proceeds against high redox free energy (Rottenberg & Gutman, 1977). In contrast, transhydrogenase, which proceeds against very low free energy load, is hardly inhibited by FFA. These relationships between the effectiveness of uncoupling and the free energy load suggest that FFA could not uncouple processes that proceed against lower load, because, in the latter cases, $\Delta\tilde{\mu}_H$ alone is a competent driving force.

The comparison of uncoupling by the electrogenic ionophore valinomycin (+K) and FFA in mitochondria is most instructive. We were careful to keep all assay conditions identical so that quantitative relationship can be evaluated. The differences are striking. To obtain complete stimulation of state 4 respiration or inhibition of phosphorylation by valinomycin, $\Delta\tilde{\mu}_H$ must be reduced by more than 50%; the same uncoupling is obtained by FFA with almost no effect on $\Delta\tilde{\mu}_H$. Another distinctive feature is the effect on state 3 respiration. Valinomycin stimulates state 4 respiration to a much greater extent than either phosphorylation or FFA, which stimulate state 4 respiration to the same extent. This suggests that while valinomycin stimulation of respiration depends on reduction of $\Delta\tilde{\mu}_H$, the stimulation of respiration by FFA and phosphorylation does not depend on the reduction of $\Delta\tilde{\mu}_H$ (Padan & Rottenberg, 1973). It is very likely that the latter share the same locus for stimulation, possibly an intramembranal proton capacitor.

Because of this distinct pattern of uncoupling by FFA, which is similar to that of general anesthetics (Rottenberg 1983) and other membrane perturbing reagents (H. Rottenberg and K. Hashimoto, unpublished observation), we suggest the use of the term "decouplers" for these agents to distinguish them from classical uncouplers, which are by now synonomous with protonophores (Nichols, 1982; McLaughlin & Dilger, 1980). Since valinomycin does not affect proton permeability per se but reduces $\Delta\tilde{\mu}_H$ through its effect on $\Delta\psi$, we argue that its effects should be considered as representing the true effect of $\Delta\tilde{\mu}_H$ on state 4 respiration and phosphorylation. If protonophores were acting only through their effect on $\Delta\tilde{\mu}_H$, we would expect the same dependence on $\Delta\tilde{\mu}_H$ for protonophoric and ionophoric uncoupling. But, as Figure 8 clearly demonstrates, this is not the case. The fact that protonophores fall midway between valinomycin and FFA suggests a mixed mode of action for these agents. In part, their effect is due to the collapse of $\Delta\tilde{\mu}_H$ just as observed for valinomycin, but in addition they may be capable of releasing the intramembranal protons as was suggested for FFA. Indeed, a molecule capable of shuttling protons across the membrane should also be capable of shuttling protons from intramembrane sites to the surface.

It is still far from being clear where the site of action of FFA is; presumably it is the site of the intramembrane proton capacitors. The experiments on the effect of antimycin inhibition of electron transport and $\Delta\tilde{\mu}_H$ in the presence and absence of palmitate (Figure 9) did not indicate any effect on the proton-pumping efficiency of the redox pump by palmitate. Incidently, these experiments do not show the great discrepancy between inhibition of electron transport and $\Delta\tilde{\mu}_H$ reported by others (Nichols, 1974; Pietrobon et al., 1983). This appears to be due to the effect of temperature. At 37 °C, the respiratory control is much lower (Rottenberg, 1978), and the pattern of the relative inhibition of electron transport and $\Delta\tilde{\mu}_H$ is modified. In contrast, the oligomycin titration retained the discrepancy between the effect on ATPase and $\Delta\tilde{\mu}_H$ (Pietrobon et al., 1983) and showed increased oligomycin sensitivity in the presence of palmitate. While the exact meaning of this observation is not clear, it seems to suggest a more direct interaction with the ATPase. This is also compatible with the more pronounced effect of palmitate on $\Delta\tilde{\mu}_H$ when generated by ATP.

Recently, it was reported that external field induced ATP synthesis in beef heart submitochondrial particles yields up to 12 ATP for each short (60 μs) pulse and requires the presence of bovine serum albumin (Knox & Tsong, 1984). We have found that the requirement for serum albumin in this system is due to the extreme sensitivity of this high-yield process to the presence of FFA (Rottenberg, Winkler, and Korenstein, unpublished observation). Preliminary results with fluorescence energy transfer probes indicate close association between FFA and inner membrane proteins (Rottenberg and Contardi, unpublished observation) and also a close proximity between a fluorescent-labeled FFA and a fluorescent-labeled ATPase (Silverstein and Rottenberg, unpublished observation). We therefore suggest, as a working hypothesis, that FFA uncouple by an interaction with a proton capacitor which is closely associated with the ATPase, possibly subunits of F_0. This capacitor is postulated to be capable of receiving protons directly from neighboring redox pumps. The ATPase is, in turn, capable of utilizing the stored protons for ATP synthesis. The capacitor is also accessible to the external medium where the overflow of protons is discharged.

Recent experiments on the kinetics and energetics of oxidative phosphorylation and photophosphorylation raise serious doubts regarding the assumption that $\Delta\tilde{\mu}_H$ is the only intermediate between oxidation and phosphorylation [for reviews, see Ferguson & Sorgato (1982), Ferguson (1985), Westerhoff et al. (1984), and Rottenberg (1985)]. In particular, the observation that inhibition of electron transport is associated with inhibition of ATP synthesis without significant effect on $\Delta\tilde{\mu}_H$ is relevant to our own findings (Wilson & Forman, 1982; Mandolino et al., 1983). However, in their case it is possible that the activity of the ATPase is directly regulated by the redox reaction, hence leading to inhibition of ATP synthesis. Our results make this explanation unlikely. We have advocated a "parallel coupling" scheme in which both chemiosmotic (i.e., bulk-mediated) and intramembranal proton transfer contribute to energy transfer. In this scheme the decouplers such as free fatty acid or general anesthetics specifically uncouple the intramembranal pathway. The detailed mechanism of proton transfer between proton pumps is yet to be established. However, if the pumps, and particularly the ATPase pump, have the capacity to store protons in numbers that are sufficient for several turnovers of the chemical reaction, it is not hard to imagine that other colliding and/or aggregated pumps can accept or deliver protons directly to this proton capacitor.

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Registry No. NADH oxidase, 9032-21-7; ATP, 56-65-5; CCCP, 555-60-2; ATPase, 9000-83-3; H^+, 12408-02-5; K, 7440-09-7; gramicidin, 9072-60-0; transhydrogenase, 9072-60-0; valinomycin, 2001-95-8; lauric acid, 143-07-7; myristic acid, 544-63-8; palmitic acid, 57-10-3; stearic acid, 57-11-4; oleic acid, 112-80-1; *cis*-vaccinic acid, 506-17-2; *trans*-vaccinic acid, 693-72-1.

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