Uncoupling of Oxidative Phosphorylation. 2. Alternative Mechanisms: Intrinsic Uncoupling or Decoupling?†

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ABSTRACT: The mechanism of uncoupling of oxidative phosphorylation by carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), oleic acid, and chloroform is further investigated by measuring in the presence of a certain concentration of each type of uncoupler (i) the mitochondrial P/O and respiratory control ratios upon progressive inhibition of the redox pumps and (ii) $\Delta\tilde{\mu}_H$ and the rate of either electron transfer or adenosine 5′-triphosphate (ATP) hydrolysis in static head upon progressive inhibition of either the redox or the adenosine triphosphatase (ATPase) proton pumps. Chloroform exhibits in all the experiments a behavior very different from that of FCCP and oleic acid. For example, upon addition of antimycin to chloroform-supplemented mitochondria, the respiratory control ratio remains unchanged and the P/O ratio slightly increases (in a certain range of inhibition) instead of decreasing as expected for an increased membrane conductance (and as indeed measured in the presence of either FCCP or oleic acid). From the kinetic model of chemiosmotic free energy coupling described by Pietrobon and Caplan [Pietrobon, D., & Caplan, S. R. (1986) *Biochemistry 25*, 7690–7696] all the results can be simulated by making the assumptions that (i) chloroform acts specifically at the level of the proton pumps and intrinsically uncouples electron transfer and ATP hydrolysis/synthesis from proton translocation and (ii) FCCP and oleic acid have a mixed behavior and act both as protonophores and as intrinsic uncouplers of the redox pumps (but not of the ATPases). The consistency of the results with the alternative hypothesis that the three agents interfere either with localized energy coupling sites or with a direct interaction between proton pumps is discussed.

The overwhelming evidence that the typical lipophilic weak acid uncouplers of oxidative phosphorylation, such as FCCP,[1] increase the proton conductance of both natural and artificial phospholipid membranes [for review see McLaughlin and Dilger (1980); cf. also Luvisetto et al. (1987), preceding paper in this issue] has been considered a strong support for the chemiosmotic hypothesis of free energy coupling as formulated by Mitchell (1966). We have shown that another type of uncoupler of oxidative phosphorylation, the oleic fatty acid, also increases the proton conductance of the inner mitochondrial membrane (preceding paper). However, in the preceding paper it has also been shown that the increase in membrane proton conductance induced by either FCCP or oleic acid does not quantitatively account for the stimulation of the rate of electron transfer, while, on the other hand, it does account for most of the stimulation of the rate of ATP hydrolysis. Moreover, we have also shown that chloroform, another uncoupler of oxidative phosphorylation, only very slightly increases the proton conductance of the mitochondrial membrane. This very small increase does not account for the stimulation of either the rate of electron transfer or that of ATP hydrolysis.

All three agents, then, appear to cause the transfer of a certain number of electrons without translocation of a stoichiometric number of protons across the membrane from the mitochondrial matrix to the external medium. In addition, chloroform gives rise also to hydrolysis of ATP not accompanied by the translocation across the membrane of a stoichiometric number of protons. The interesting question that these results necessarily pose is, what is the mechanism that gives rise to such behavior?

To explain the finding that part of the increase in membrane conductance due to FCCP (not measured directly but calculated from the rate of electron transfer and $\Delta\tilde{\mu}_H$) was titrated down upon inhibition of the redox proton pumps, it has been proposed that FCCP in addition to its protonophoric effect intrinsically uncouples electron transfer from proton pumping through a specific interaction with the redox proton pumps (Pietrobon et al., 1981; Pietrobon & Zoratti, 1982; Walz, 1983). A similar type of mechanism has been proposed to explain the differential inhibition of electron transfer (or ATP hydrolysis) and of proton pumping of fluorescamine and its derivatives (Ramirez et al., 1980; Tu et al., 1981).

On the other hand, to explain the finding that the rate of ATP synthesis was inhibited at constant $\Delta\tilde{\mu}_H$ by anesthetics and fatty acids (cf., however, preceding paper), Rottenberg (Rottenberg, 1983; Rottenberg & Hashimoto, 1986) has

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[1] Abbreviations: J_e, rate of electron transfer; J_p, rate of ATP synthesis; J_{ATP}, rate of ATP hydrolysis; J_H^l, proton flux through leaks; L_H^l, membrane proton-leak conductance; RCR, respiratory control ratio; $\Delta\psi$, transmembrane electrical potential gradient; ΔpH, transmembrane pH gradient; $\Delta\tilde{\mu}_H$, transmembrane proton electrochemical potential gradient (in absolute value); n_e, H^+/e^- stoichiometry; n_p, H^+/ATP stoichiometry; f_e, fraction of active redox pumps; f_p, fraction of active ATPase pumps; P_i, inorganic phosphate; DMO, 5,5-dimethyloxazolidine-2,4-dione; $TPMP^+$, triphenylmethylphosphonium ion; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; ATP, adenosine 5′-triphosphate; ATPase, adenosinetriphosphatase; ADP, adenosine 5′-diphosphate.

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proposed that these agents uncouple by "interfering with intramembrane processes that mediate direct energy transfer between the electron transport complexes and the ATPase" and, more precisely, by interacting with a proton capacitor closely associated with the ATPases and capable of receiving protons directly from neighboring redox pumps. The comparison between the behavior of fatty acids, FCCP, and valinomycin led Rottenberg to conclude that FCCP (and also gramicidin) in addition to its protonophoric action also interacts with a local mechanism of energy coupling between redox and ATP synthase pumps (Rottenberg & Hashimoto, 1986). A mechanism of uncoupling essentially based on the interference with localized energy coupling sites or with a direct interaction between proton pumps has been proposed also as an explanation for the results of the so-called uncoupler–inhibitor titrations in the case of some typical lipophilic weak acid uncouplers and valinomycin [Hitchens & Kell, 1983; Herweijer et al., 1986; cf., however, Pietrobon and Caplan (1985a, 1986a,b)].

In the kinetic model of chemiosmotic free energy coupling described by Pietrobon and Caplan (1986b), it is possible to introduce intrinsic uncoupling in the proton pump models (Pietrobon & Caplan, 1985b), thus allowing the simulation of the effects of an increase of intrinsic uncoupling, at the level of either the redox or the ATPase pumps, on the overall process of oxidative phosphorylation. This modeling study has suggested to us new experiments to further investigate the mechanism of uncoupling by FCCP, oleic acid, and chloroform. Below it will be shown that the results of these experiments (as well as those of the preceding paper) can be simulated by assuming that the three agents act as intrinsic uncouplers of the redox pumps and that chloroform in addition acts also as an intrinsic uncoupler of the ATPases. Moreover, the consistency of the results with the alternative hypotheses that the three agents interfere either with localized energy coupling sites or with a direct interaction between proton pumps is discussed.

Materials and Methods

Rat liver mitochondria were prepared according to standard procedures (Massari et al., 1972), and all experiments were performed within 4 h of preparation. All reagents used were the same as described in the preceding paper. The procedures for the assay of the rates of respiration, the rates of ATP hydrolysis and synthesis, and the values of $\Delta\psi$ were as described in the preceding paper.

For the determination of the $n_e J_e/\Delta\psi$ parameter and of the $n_p J_{ATP}/\Delta\psi$ parameter, the rate of respiration and $\Delta\psi$ or the rate of ATP hydrolysis and $\Delta\psi$ were measured in parallel samples under identical conditions. Further details on the experimental conditions are included in the legends of the Figures 1 and 3.

For the determination of the P/e^- ratio, the rates of respiration and ATP synthesis were also measured in parallel samples under identical conditions.

The respiratory control ratio, RCR, was determined as described in the legend of Figure 5.

Results

We have measured the rate of electron transfer from succinate to oxygen and $\Delta\psi$ in rat liver mitochondria at increasing concentrations of antimycin (an inhibitor of the bc_1 complex) in static head without external agents and in the presence of a certain amount of FCCP, oleic acid, or chloroform. Control experiments have shown that under the prevailing experimental conditions ΔpH is very small (10–20 mV) and varies very little upon redox inhibition, so that, in our conditions, the variations of $\Delta\psi$ can be considered to reflect to a good approximation those of $\Delta\tilde{\mu}_H$. Figure 1 shows the ratio between the rate of electron transfer (multiplied by the H^+/e^- stoichiometry) and $\Delta\psi$ at different extents of redox inhibition, $n_e J_e/\Delta\psi$, as a function of $\Delta\psi$.

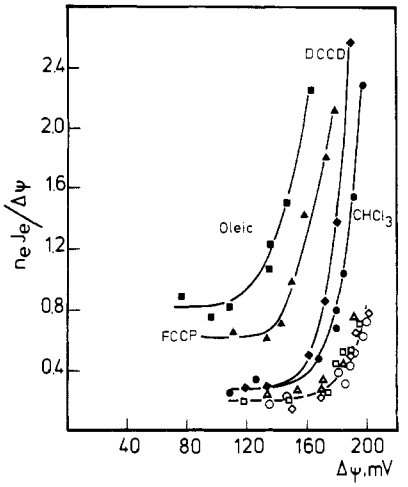

FIGURE 1: Ratio between the rate of electron transfer from succinate to oxygen (multiplied by n_e, the H^+/e^- stoichiometry) and $\Delta\psi$ in static head as a function of $\Delta\psi$ in titrations with antimycin in the presence of a constant amount of oleic acid (40 nmol/mg, ■), FCCP (20 pmol/mg, ▲), chloroform (15 mM, ●), and DCCD (120 nmol/mg, ◆) and in the absence of uncoupling agents (open symbols). The dimensions of $n_e J_e/\Delta\psi$ are nmol mg^{-1} min^{-1} mV^{-1}. n_e is taken as equal to 4. Medium composition: 0.2 M sucrose, 30 mM Tris/MOPS, 5 mM P_i/Tris, 1 mM EDTA, 5 μM rotenone, 1 μg/mg oligomycin; pH 7.4, $T = 25$ °C. Mitochondria (1 mg/mL) were incubated for 5 min in the presence of increasing concentrations of antimycin (0–12.5 ng/mg). Then succinate (10 mM) was added, and after 2 min, the given concentration of uncoupling agent was added and the rate of respiration and $\Delta\psi$ were measured.

The condition of stationary state requires that in static head the rate of proton efflux through the redox pumps is equal to the rate of passive proton influx. Thus, the rate of electron transfer multiplied by the H^+/e^- stoichiometry has been often considered to be equal to the rate of passive proton influx [e.g., Nicholls (1974) and Sorgato and Ferguson (1979)]. This equality implies the assumption of a stoichiometric relationship between electron transfer and proton efflux, i.e., the assumption of complete coupling of the redox proton pumps. Under this assumption the ratio between the rate of electron transfer multiplied by the H^+/e^- stoichiometry and $\Delta\tilde{\mu}_H$, $n_e J_e/\Delta\tilde{\mu}_H$, is equal to the membrane proton conductance.

However, it has been shown that the nonlinear relation found in this type of experiment in the absence of uncouplers (lower curve in Figure 1) does not reflect a nonohmic conductance of the membrane (Pietrobon et al., 1981, 1983; Zoratti et al., 1986). The more direct evidence is given by the fact that in static head the rate of electron transfer multiplied by the H^+/e^- stoichiometry is higher than the rate of passive proton influx at the same $\Delta\tilde{\mu}_H$ (Zoratti et al., 1986; see also in Figure 3 of the preceding paper the rates in the absence of uncoupling agent). The difference between $n_e J_e$ and the rate of passive proton influx has been considered to reflect the presence of a certain extent of intrinsic uncoupling (slip) in the redox pumps, whereby electrons can be transferred without proton translocation (Zoratti et al., 1986). The hypothesis of slipping proton pumps had been previously formulated to explain the nonlinear behavior of $n_e J_e/\Delta\tilde{\mu}_H$ and $n_p J_{ATP}/\Delta\tilde{\mu}_H$ (where n_p is the H^+/ATP stoichiometry and J_{ATP} is the rate of ATP hydrolysis) in titrations with inhibitors of the pumps (cf. Figures 1 and 3) (Pietrobon et al., 1981, 1983). By use of the proton

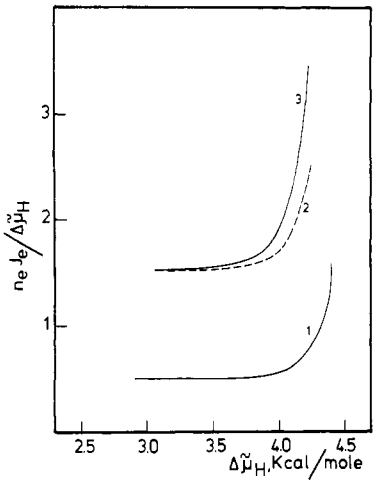

FIGURE 2: Simulations of $n_e J_e/\Delta\tilde{\mu}_H$ as a function of $\Delta\tilde{\mu}_H$. The simulations are performed by using the six-state proton pump model of Pietrobon and Caplan (1985b) and an ohmic parallel pathway for passive diffusion of protons (leak). Kinetic parameters are as in Pietrobon et al. (1986). The affinity (negative Gibbs free energy change) of the redox reaction is kept constant at 19.66 kcal/mol. ΔpH is kept constant at 0. Therefore $\Delta\tilde{\mu}_H \equiv \Delta\psi$. The different stationary states are obtained by decreasing the number of active redox pumps. Curve 1: Leak conductance $L_H^l = 0.5$ mol kcal^{-1} s^{-1}; rate constants of the slip transition $\alpha_{25} = 9.45 \times 10^{-13}$ s^{-1}, $\alpha_{52} = 1.4$ s^{-1}. Curve 2: $L_H^l = 1.5$; α_{25} and α_{52} as in curve 1. Curve 3: $L_H^l = 1.5$; $\alpha_{25} = 18.9 \times 10^{-13}$ s^{-1}, $\alpha_{52} = 2.8$ s^{-1}.

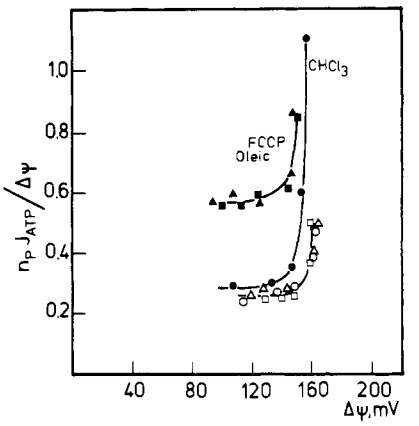

FIGURE 3: Ratio between the rate of ATP hydrolysis (multiplied by n_p, the H^+/ATP stoichiometry) and Δψ in static head as a function of Δψ in titrations with DCCD in the presence of a constant amount of oleic acid (20 nmol/mg, ■), FCCP (10 pmol/mg, ▲), and chloroform (10 mM, ●) and in the absence of uncoupling agents (open symbols). The dimensions of $n_p J_{ATP}/\Delta\psi$ are nmol mg^{-1} min^{-1} mV^{-1}. n_p is taken as equal to 4. Medium composition: 0.2 M sucrose, 30 mM Tris/MOPS, 0.2 mM EGTA, 2 mM $MgCl_2$, 5 mM P_i/Tris, 1 mM phosphoenolpyruvate, 0.1 mM NADH, excess pyruvate kinase, and lactate dehydrogenase; pH 7.4, $T = 25$ °C. Mitochondria (0.5 mg/mL) were incubated for 7 min in the presence of increasing concentrations of DCCD (0–10 nmol/mg). Then ATP (3 mM) was added, and after 3 min, the given concentration of uncoupling agent was added and the rate of ATP hydrolysis and Δψ were measured.

pump model studied by Pietrobon and Caplan (1985b) it has been shown that the nonlinear relation of Figure 1 can indeed be simulated when a certain degree of intrinsic uncoupling in the pumps[2] is assumed (Pietrobon et al., 1986).

The lower curve (1) in Figure 2 is a simulation of the behavior of the $n_e J_e/\Delta\tilde{\mu}_H$ ratio as a function of $\Delta\tilde{\mu}_H$ obtained with the same model when the number of active redox pumps is decreased in the presence of a constant proton leak conductance of 0.5 mol s^{-1} kcal^{-1} and a small extent of intrinsic uncoupling in the proton pump model [as in Pietrobon et al. (1986)]. The upper dashed curve (2) is obtained with the same intrinsic uncoupling but with a proton conductance 3 times higher. The upper continuous curve (3) is obtained by doubling the rate constants of the "slip" transition in the model and again with a proton conductance 3 times higher (1.5 mol s^{-1} kcal^{-1}). Figure 2 shows that the $n_e J_e/\Delta\tilde{\mu}_H$ ratio represents the actual conductance of the membrane only at low values of $\Delta\tilde{\mu}_H$, i.e., at high concentrations of inhibitor, where most of the uncoupled electron transfer has been inhibited. The part of the "apparent proton conductance" (as we will call from now on the $n_e J_e/\Delta\tilde{\mu}_H$ ratio) that is titrated down upon inhibition with little change of $\Delta\tilde{\mu}_H$ may be taken to reflect the intrinsic uncoupling of the pump. In fact, this portion increases as the degree of intrinsic uncoupling is increased (cf. curves 2 and 3). Note that if one compares curve 2 with curve 1, the portion which is titrated down is slightly lower in the presence of a higher membrane conductance (and therefore a lower $\Delta\tilde{\mu}_H$ in static head) because in the model the slip is $\Delta\tilde{\mu}_H$ dependent and decreases as $\Delta\tilde{\mu}_H$ decreases (Pietrobon et al., 1986).

Figure 1 shows that the increase of the apparent conductance induced by chloroform is progressively titrated down by antimycin until, at low Δψ; $n_e J_e/\Delta\psi$ reaches a constant value only slightly higher than that in the absence of chloroform. Since this value represents more closely the actual membrane conductance, Figure 1 shows, in complete agreement with the results of the preceding paper, that chloroform increases very little the actual conductance of the membrane. Moreover, the fact that what we have defined as the apparent conductance is first increased by chloroform and then progressively titrated down by antimycin supports the view that this apparent conductance reflects a property of the pumps rather than a property of the membrane. Figure 1 also shows, again in complete agreement with the results of the preceding paper, that FCCP and oleic acid have a mixed behavior. Both agents increase the conductance of the membrane (at low Δψ the value of $n_e J_e/\Delta\psi$ is 3–4 times higher than that in the absence of external agents), but in addition, they also interact directly with the pumps, as shown by the fact that part of the increase in the apparent conductance is titrated down with antimycin. A comparison between the experimental results in Figure 1 and the simulations in Figure 2 shows that the behavior of the three agents in this type of experiment can be simulated by assuming that they interact directly with the redox pumps, producing intrinsic uncoupling of electron transfer from proton translocation.

It has been reported that DCCD, at concentrations higher than those which inhibit the ATPases, inhibits differentially proton pumping and electron-transfer activities of the cytochrome oxidase and the bc_1 complex [see, for a review, Azzi et al. (1984)]. We have then performed a titration with antimycin in the presence of a certain amount of DCCD. Figure 1 shows that the behavior of DCCD is very similar to that of chloroform in this type of experiment. In another experiment we have measured the ionic dissipative current and the rate of electron transfer in static head as a function of DCCD, and the results are again very similar to those obtained in the preceding paper for chloroform (not shown). Therefore, DCCD, as chloroform, interacts directly with the redox pumps, possibly leading to intrinsic uncoupling of electron transfer from proton translocation.

[2] The cyclic six-state kinetic model of a proton pump of Pietrobon and Caplan (1985b) has a "slip" transition that allows uncoupled cycles to occur (e.g., in the case of the ATPase, cycles that hydrolyze ATP without proton pumping or, in the reverse direction, cycles that translocate protons without making ATP), thus introducing the possibility of intrinsic uncoupling of the pumps.

To test the effect of the three agents on the ATPase proton pump, we have measured the rate of ATP hydrolysis and $\Delta\psi$ at increasing concentrations of DCCD (in the concentration range where it essentially acts as an inhibitor of the ATPases) in static head without external agents and in the presence of a certain amount of FCCP, oleic acid, or chloroform. Figure 3 shows the behavior of the ratio between the rate of ATP hydrolysis (multiplied by the H^+/ATP stoichiometry) and $\Delta\psi$, $n_p J_{ATP}/\Delta\psi$, at different extents of ATPase inhibition, as a function of $\Delta\psi$. Under the assumption of complete coupling of the ATPase pumps, the ratio $n_p J_{ATP}/\Delta\psi$ is equal to the passive proton conductance of the membrane. However, the behavior shown in Figure 3 in the absence of external agents together with the difference between rate of passive influx of protons and the rate of ATP hydrolysis in static head reported in the preceding paper indicates that the ATPases are not completely coupled protons pumps (Pietrobon et al., 1983). Therefore $n_p J_{ATP}/\Delta\tilde{\mu}_H$ is also only an apparent conductance that more closely represents the actual proton conductance of the membrane only at low values of $\Delta\tilde{\mu}_H$. The portion of the apparent conductance that is titrated down upon inhibition of the ATPases reflects the intrinsic uncoupling of the ATPase proton pumps.

Figure 3 shows that chloroform gives rise in this experiment to a pattern similar to that shown in Figure 1 for the same agent. The increase of the apparent conductance induced by chloroform is almost completely titrated down by DCCD with little change of $\Delta\tilde{\mu}_H$ until it reaches a constant value, which is only slightly higher than that in the absence of chloroform. However, at variance from Figure 1, in the presence of FCCP and oleic acid the portion of the apparent conductance being titrated down does not significantly increase with respect to that measured in static head without external agents. The comparison between simulations and experimental results in Figure 3 leads to the conclusion that FCCP and oleic acid do not interact significantly with the ATPases, while chloroform does. This conclusion is in agreement with that reached in the preceding paper on the basis of the comparison between the rates of proton influx and ATP hydrolysis. The behavior of chloroform in Figure 3 can be simulated by assuming that it intrinsically uncouples ATP hydrolysis from proton translocation.

The kinetic model of chemiosmotic free energy coupling described by Pietrobon and Caplan (1986b) gives rise to nonlinear relations between the rate of electron transfer and the rate of ATP synthesis, similar to those measured in many energy coupling membranes (Pietrobon, 1986). This model allows us to simulate the effects of intrinsic uncoupling not only on the operation of the single proton pump but also on the coupling between a primary and a secondary proton pump. Classical variables measuring the degree of coupling are the P/O and the respiratory control ratio (RCR). It has been shown that the simulated behavior of these variables upon inhibition of the redox pumps as a function of the fraction of inhibited pumps is remarkably different depending on whether the uncoupling (resulting in certain values of P/O ratio and RCR) is assumed to be at the level of the membrane (leak) or at the level of the pumps (slip, i.e., intrinsic uncoupling) (Pietrobon, unpublished results). This has led us to measure the P/O ratio and RCR in mitochondria at different degrees of inhibition of the redox pumps in the presence of a certain amount of one or the other of the three uncoupling agents.

Figures 4 and 5 show the P/e^- and the respiratory control ratio, respectively, as a function of the concentration of antimycin in the presence of a given concentration of FCCP,

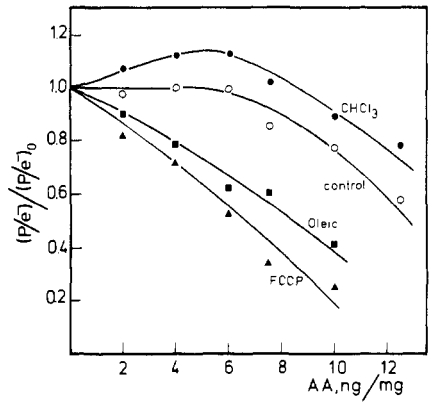

FIGURE 4: Normalized P/e^- ratio as a function of antimycin in the presence of a constant amount of chloroform (15 mM, ●), oleic acid (40 nmol/mg, ■), and FCCP (20 pmol/mg, ▲) and in the absence of uncoupler (○). Medium as in Figure 1, but without oligomycin. Rat liver mitochondria (1 mg/mL) were incubated for 5 min in the presence of increasing amounts of antimycin (0–12.5 ng/mg); succinate (10 mM) was then added followed after 2 min by the addition of the uncoupling agent. After 2 min, ADP (1 mM) was added and the rate of electron transfer, J_e, and the rate of ATP synthesis, J_p, were measured. $(P/e^-)_0$ is the value of J_p/J_e in the absence of antimycin. Control: $(P/e^-)_0 = 0.87$. FCCP: $(P/e^-)_0 = 0.47$. Oleic acid: $(P/e^-)_0 = 0.43$. Chloroform: $(P/e^-)_0 = 0.53$.

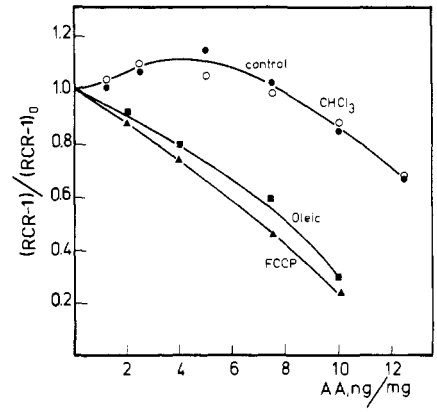

FIGURE 5: Normalized respiratory control ratio (RCR) as a function of antimycin in the presence of a constant amount of chloroform (15 mM, ●), oleic acid (40 nmol/mg, ■), and FCCP (20 pmol/mg, ▲) and in the absence of uncoupler (○). Medium as in Figure 1. Procedure as in Figure 4 but FCCP (0.2 μM) was added instead of ADP. The RCR is calculated as the ratio between the maximal rate of respiration in the presence of FCCP and the rate of respiration in its absence. $(RCR\text{-}1)_0$ refers to the value in the absence of antimycin. Control: $(RCR)_0 = 8.42$. FCCP: $(RCR)_0 = 2.7$. Oleic acid: $(RCR)_0 = 2.03$. Chloroform: $(RCR)_0 = 2.82$.

chloroform, or oleic acid and, as a control, in its absence. The concentrations of each agent are such as to give similar P/e^- ratios and RCRs in the absence of antimycin. The P/e^- ratio in Figure 4 is the ratio between the initial rates of ATP synthesis, J_p, and of electron transfer, J_e, upon addition of ADP to mitochondria in state 4. The RCR in Figure 5 is the ratio between the maximal rate of respiration in the presence of excess FCCP and the rate of respiration in state 4.

Figure 4 shows that in the absence of external agents the P/e^- ratio is initially rather insensitive to the inhibition of respiration; i.e., addition of antimycin causes an almost proportional decrease of the rates of respiration and phosphorylation in an extended range of inhibition. Thus, the P/e^- ratio is only 20% lower under conditions where the ADP stimulated rate of respiration is almost 80% inhibited. The condition of stationary state requires that in state 3 the rate of proton efflux through the redox pumps be equal to the sum of the rates of proton influxes through the leaks and through the ATP

synthetases. Under the assumption that the proton pumps are completely coupled, from the condition of zero net proton flux, a relation between J_p and J_e can be immediately derived:

$$J_p/J_e = n_e/n_p - J_H^l/n_pJ_e \quad (1)$$

J_H^l is the rate of passive proton influx, and n_e and n_p are the H^+/e^- and H^+/ATP stoichiometries, respectively. The P/e^- ratio, J_p/J_e, is exactly equal to the mechanistic stoichiometry ATP/e^- in the absence of dissipative proton flux ($J_H^l = 0$) and approximately equal to it as long as the dissipative proton flux is small with respect to the rate of electron transfer in state 3 (multiplied by the H^+/ATP stoichiometry). Thus in a redox titration as in Figure 4 the lower is the membrane proton conductance, the more extended is the range of respiration inhibition within which the P/e^- ratio is expected to remain relatively constant.

The presence of intrinsic uncoupling in the proton pumps complicates the analysis of the predicted behavior of J_p/J_e in a redox titration, since eq 1 does not hold anymore. It has been shown that in the presence of intrinsic uncoupling of the redox pumps there is a range of inhibition within which the ratio J_p/J_e (which is lower than n_e/n_p) even increases (Pietrobon, 1986; cf. also Discussion and Figure 8), since the decrease of the rate of electron transfer in that range is mainly due to inhibition of the uncoupled electron flow. Moreover, it can be shown that in the presence of intrinsic uncoupling of the ATPases the ratio J_p/J_e (which is again lower than n_e/n_p) remains approximately constant in a very extended range of redox inhibition. This is due to the fact that the dissipative proton flow through the ATPases is steeply dependent on $\Delta\tilde{\mu}_H$, and therefore it rapidly decreases as inhibition proceeds as a consequence of the lowered $\Delta\tilde{\mu}_H$ (Pietrobon, unpublished results). The relative insensitivity of the P/e^- ratio to inhibition with antimycin shown in Figure 4 in the absence of external agents is then in agreement with the presence of a small membrane proton conductance and a small intrinsic uncoupling of the proton pumps in native mitochondria.

Figure 4 shows that in the presence of FCCP and oleic acid increasing concentrations of antimycin result in a more marked depression of the P/e^- ratio. The relative inhibition of the P/e^- ratio follows closely that of electron transfer in state 3 in the presence of oleic acid, and it is slightly higher than the inhibition of electron transfer in the presence of FCCP. The more marked depression of the P/e^- ratio upon redox pump inhibition in the presence of FCCP and oleic acid is in agreement with the increase in membrane proton conductance induced by these agents (cf. eq 1 and its discussion).

The behavior of the chloroform-supplemented mitochondria in Figure 4 is markedly different from that of oleic acid supplemented or FCCP-supplemented mitochondria. Addition of antimycin in this case causes less than a proportional inhibition of the phosphorylation rate with respect to the electron transfer, with the consequence that, up to a 30% inhibition of the electron transfer, the P/e^- ratio increases instead of decreasing. As already mentioned, this behavior, although surprising, is what is predicted for an increased intrinsic uncoupling of the redox pumps (Pietrobon, 1986; cf. Discussion and simulations in Figure 8).

Figure 5 shows the effect of antimycin on the respiratory control ratio, RCR, as measured in the absence and in the presence of FCCP, oleic acid, or chloroform. In the absence of any agent there is a slight increase of the RCR at low antimycin concentrations and a decrease at high antimycin concentrations. This behavior is due to the fact that in antimycin titrations the inhibition pattern shows a higher sigmoidicity in the titration of the uncoupled (maximal) respiration rate than in the titration of the static head respiration rate. The higher sigmoidicity has been interpreted as an expression of a $\Delta\tilde{\mu}_H$ dependence of the control exerted by the bc_1 complex on the rate of electron transfer, the control by the bc_1 complex increasing as $\Delta\tilde{\mu}_H$ increases (Stoner, 1985). If there were no such $\Delta\tilde{\mu}_H$ dependence, one would expect a depression of the respiratory control ratio as a function of the redox inhibitor concentration since the maximal uncoupled rate, which is controlled only by the redox enzymes, is expected to be more sensitive to the addition of respiratory inhibitors than the static head rate, where the electron transfer is controlled by both the redox pumps and the leak. The higher the leak, the larger should be the depression of the RCR upon inhibition of the redox pumps.

Figure 5 shows that in the presence of FCCP and oleic acid the gradual inhibition of the respiration rate by antimycin is accompanied by a marked depression of RCR, in agreement with the increase in membrane proton conductance induced by these agents. In contrast, in the presence of chloroform the respiratory control ratio as a function of antimycin shows the same pattern as the control mitochondria in the absence of external agents.

Discussion

The results of the experiments presented in this paper are in complete agreement with, and further support, the conclusions reached in the preceding paper. The conclusion that oleic acid as well as FCCP increases the passive membrane conductance is further supported by the fact that the values of the parameters $n_eJ_e/\Delta\tilde{\mu}_H$ and $n_pJ_{ATP}/\Delta\tilde{\mu}_H$ at high concentration of pump inhibitor (low $\Delta\tilde{\mu}_H$) are higher in oleic acid (or FCCP) supplemented mitochondria than in native mitochondria (Figures 1 and 3). As extensively discussed under Results, with incompletely coupled ("slipping") proton pumps, the value of the apparent membrane proton conductance, given either by $n_eJ_e/\Delta\tilde{\mu}_H$ or $n_p/J_{ATP}/\Delta\tilde{\mu}_H$, closely represents the actual value of the membrane proton conductance at high concentrations of inhibitor (of the redox or the ATPase pumps, respectively), where the uncoupled reaction rate (electron transfer or ATP hydrolysis) is almost completely inhibited (Pietrobon et al., 1986; cf. simulations in Figure 2). Moreover, the conclusion that oleic acid (as FCCP) increases the membrane conductance is also supported by the observation that upon addition of antimycin the depression of both the P/O ratio and RCR is more marked in oleic acid (or FCCP) supplemented mitochondria than in native mitochondria (Figures 4 and 5).

On the other hand, the fact that chloroform only very slightly increases the membrane conductance is confirmed by the very small increase in the values of $n_eJ_e/\Delta\tilde{\mu}_H$ and $n_pJ_{ATP}/\Delta\tilde{\mu}_H$ at low $\Delta\tilde{\mu}_H$ in chloroform-supplemented mitochondria (Figures 1 and 3) and by the completely different behavior in the experiments of Figures 4 and 5 exhibited by chloroform with respect to that of FCCP and oleic acid. Particularly striking is the result that in chloroform-supplemented mitochondria (up to a certain inhibition of the respiratory rate) the P/O ratio increases upon inhibition of the redox pumps instead of decreasing as expected for an increased membrane conductance.

According to the classical chemiosmotic explanation of the uncoupling of oxidative phosphorylation, uncouplers act by increasing the proton (or in general the ionic) conductance of the membrane with a consequent depression of $\Delta\tilde{\mu}_H$ and inhibition of the $\Delta\tilde{\mu}_H$-driven pumps, on one hand, and stimulation of the $\Delta\tilde{\mu}_H$-generating pumps on the other. It is clear from

the results of this as well as of the preceding paper that chloroform uncouples oxidative phosphorylation through a different mechanism. Moreover, we have shown in the preceding paper (cf. also Figure 1) that also FCCP and oleic acid do not uncouple simply by increasing the membrane conductance since the measured increase of the proton dissipative flux does not quantitatively account for the stimulation of the rate of respiration.

The most obvious question arising from these results concerns the alternative (or additional) mechanism through which these agents exert their uncoupling action. Another question related to the first is whether the failure of the classical chemiosmotic explanation of uncoupling to account for these results necessarily implies a rejection of the delocalized chemiosmotic hypothesis, i.e., in other words, whether these results can be considered as additional independent evidence in favor of a localized mechanism of energy coupling [cf. Rottenberg (1983)]. With respect to this second question it is obvious that what we have denoted the classical chemiosmotic mechanism of uncoupling is not the only mechanism of uncoupling compatible with the delocalized chemiosmotic model. The hypothesis, originally proposed by Walz and colleagues (Pietrobon et al., 1981; Walz, 1983), that certain uncoupling agents might act at the level of the pumps, through a specific interaction, by intrinsically uncoupling the proton pumps is compatible with delocalized chemiosmosis at least in its broader meaning.

Intrinsic uncoupling, for example, of an ATPase pump, implies that, when the pump is operating in the hydrolytic direction, some molecules of ATP are hydrolyzed without coupled proton translocation and, when the pump is operating in the synthetic mode, some protons are translocated without coupled synthesis of ATP (Pietrobon & Caplan, 1985b). Thus, the behavior of an intrinsic uncoupler of the proton pumps in the experiments presented in this as well as the preceding paper is expected to be different from that of an extrinsic leak-inducer uncoupler. The discrepancy between the rate of passive proton influx and the rate of electron transfer in static head (multiplied by n_e, the H^+/e^- stoichiometry), which increases in the presence of all three agents tested (see preceding paper), can be readily explained by assuming that FCCP, oleic acid, and chloroform act as intrinsic uncouplers of the redox proton pumps. In fact, in this case the rate of proton efflux through the redox pumps is lower than the rate of electron transfer multiplied by n_e since some electrons (the more, the higher the concentration of intrinsic uncoupler) are transferred without stoichiometric translocation of protons. Similarly, the chloroform-induced increasing discrepancy between the rate of passive influx of protons and the rate of ATP hydrolysis (multiplied by the H^+/ATP stoichiometry) in static head can be explained by assuming that chloroform acts also as an intrinsic uncoupler of the ATPases.

As already mentioned, in the kinetic model of chemiosmotic free energy coupling described by Pietrobon and Caplan (1986b) it is possible to introduce intrinsic uncoupling in the proton pump models (Pietrobon & Caplan, 1985b). The model gives rise to nonlinear relations between the rate of electron transfer and the rate of ATP synthesis similar to those measured in many energy-coupling membranes (Pietrobon, 1986). Moreover, the introduction of intrinsic uncoupling in the proton pump models has allowed us to obtain simulated behaviors of inhibitor titrations in static head similar to those experimentally observed in mitochondria (Pietrobon et al., 1986). We can further use this model to simulate the effects of an increased intrinsic uncoupling at the level of either the redox or the

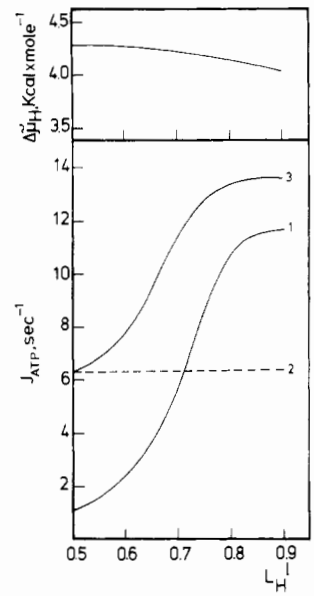

FIGURE 6: Simulations of the rate of ATP hydrolysis and of $\Delta\tilde{\mu}_H$ in static head as a function of the leak conductance, L_H^I, and of the extent of intrinsic uncoupling of the ATPase proton pumps. The simulations are obtained with the same model as in Figure 2 but with kinetic parameters as in pump A of Pietrobon and Caplan (1985b). The affinity of the ATP hydrolysis reaction is kept constant at 13 kcal/mol. Curve 1: Together with L_H^I the rate constants of the slip transition are varied from $\alpha_{52} = 2\ s^{-1}$, $\alpha_{25} = 1.17 \times 10^{-12}\ s^{-1}$ at $L_H^I = 0.5$ to $\alpha_{52} = 92\ s^{-1}$, $\alpha_{25} = 5.38 \times 10^{-11}\ s^{-1}$ at $L_H^I = 0.9$. Curve 2: Maximal rate of ATP hydrolysis when (i) the extent of intrinsic uncoupling is varied with L_H^I as in curve 1 and (ii) the fraction of active ATPases, f_p, is 0.3 (i.e., f_p is equal to the fraction of active ATPases at the maximum of the curve simulating the rate of ATP hydrolysis as a function of $\Delta\tilde{\mu}_H$ in the presence of a $\Delta\tilde{\mu}_H$-dependent inhibition of the pumps). Curve 3: As curve 2 but assuming that, together with the extent of intrinsic uncoupling, f_p varies from 0.3 at $L_H^I = 0.5$ to 0.61 at $L_H^I = 0.9$.

ATPase pumps on the concerted operation of the two proton pumps, i.e., on the overall process of oxidative phosphorylation. We can thus predict the consequences of inducing (with the addition of certain agents) an increased extent of intrinsic uncoupling in either (or both) pump on the behavior of $\Delta\tilde{\mu}_H$ and of the rates of ATP synthesis and electron transfer in the different stationary states and at the different degrees of redox inhibition in which they have been measured. Through a comparison between simulated behavior and experimental results we can then test whether the working hypothesis that chloroform acts as an intrinsic uncoupler of both redox and ATPase pumps, and that FCCP and oleic acid act both as protonophores and as intrinsic uncouplers of the redox pumps, can account for all the results reported in this as well as in the preceding paper.

We have already shown that the experimental behavior of the apparent conductance $n_e J_e/\Delta\tilde{\mu}_H$ (and by analogy also that of $n_p J_{ATP}/\Delta\tilde{\mu}_H$) can indeed be simulated within this working hypothesis (cf. simulations in Figure 2 and results in Figures 1 and 3).

Figure 6 shows the simulated behavior of the rate of ATP hydrolysis and $\Delta\tilde{\mu}_H$ in static head when it is assumed that the agent causes an increase of the intrinsic uncoupling of the ATPases together with the very slight increase in membrane proton conductance. The assumption of a progressively increasing extent of intrinsic uncoupling of the ATPases allows us to simulate (curve 1) the extensive stimulation of the rate of ATP hydrolysis and the relatively small changes of $\Delta\tilde{\mu}_H$ that were measured at increasing concentrations of chloroform (cf. Figure 2c of the preceding paper).

In order to simulate (curve 3) the further stimulation by chloroform of the rate of ATP hydrolysis in the presence of a concentration of FCCP that would otherwise maximally stimulate the rate of hydrolysis (cf. Figure 2a,c of the preceding paper) it was also necessary to assume, together with an increasing intrinsic uncoupling, a progressive release of the $\Delta\tilde{\mu}_H$-dependent inhibition of the ATPases. This inhibition was introduced in the simulations to mimic the $\Delta\tilde{\mu}_H$-dependent inhibition caused by the natural inhibitor protein and/or by tightly bound ADP (Chernyak & Kozlov, 1986), and it allowed us to simulate the characteristic bell-shaped dependence of the rate of ATP hydrolysis on FCCP concentration. When this inhibition is kept constant, the dashed curve labeled 2 in Figure 6 is obtained for the simulation. We have been able to simulate the experimental behavior of chloroform (curve labeled 3 in Figure 6) only by making the additional assumption of a chloroform-induced release of the ($\Delta\tilde{\mu}_H$-dependent) mechanisms that regulate the ATPase activity. An equivalent assumption is that the uncoupled rate of ATP hydrolysis induced by chloroform is not regulated by the usual (inhibitor protein and/or tightly bound ADP) regulatory mechanisms.

Both the assumed chloroform-induced effects, namely, increase of intrinsic uncoupling and release of the ATPase regulatory mechanisms, require a specific interaction of chloroform with the ATPases (and/or their specific lipid environment) leading to significant conformational changes of the pump. That this is likely to happen is suggested by the drastic (more than 1 order of magnitude) decrease in oligomycin sensitivity of the chloroform-stimulated ATP hydrolysis (preceding paper) and also by the fact that at much higher concentrations chloroform is able to detach F_1 from F_0 (Linnett et al., 1979).

Figure 7 shows a set of simulations corresponding to the experiments of Figure 1 of the preceding paper. In these simulations, as L_H^l is increased, the intrinsic uncoupling of the redox pumps is also increased (Figure 7a,b) or both the intrinsic uncoupling of the redox and ATPase pumps are increased (Figure 7c). The extent of intrinsic uncoupling of the ATPases at each L_H^l in Figure 7c is equal to that adopted in Figure 6 in the simulations of the rate of ATP hydrolysis. Moreover, to account for the additional inhibitory effects of oleic acid and chloroform (see preceding paper), there is a progressive decrease of the number of active redox pumps (Figure 7a,b) and also of the number of active ATPase pumps (Figure 7b) parallel to the increase of L_H^l. The simulated behavior in Figure 7 is reasonably close to the results shown in Figure 1 of the preceding paper at increasing concentrations of FCCP (a), oleic acid (b), and chloroform (c), respectively. In particular, by assuming that chloroform intrinsically uncouples the redox pumps, we have been able to simulate the chloroform-induced stimulation of the static head respiration at little change of $\Delta\tilde{\mu}_H$.

The simulations of Figure 7 have been obtained by progressively changing a number of parameters such as L_H^l, intrinsic uncoupling of the redox and/or ATPase pumps, and, when needed, number of active pumps. The arrows in Figure 7 indicate the position at which a given set of values of all the above parameters have been selected in Figures 8 and 9 to simulate the behavior of the P/O ratio and RCR upon inhibition of the redox pumps in the three different cases a, b, and c. The dashed curve in Figures 8 and 9 represents the simulated relative (with respect to zero inhibition) P/e^- ratio and RCR as a function of the inhibition factor f_e in the presence of a small extent of both proton leakage and intrinsic uncoupling of the proton pumps as required to simulate the

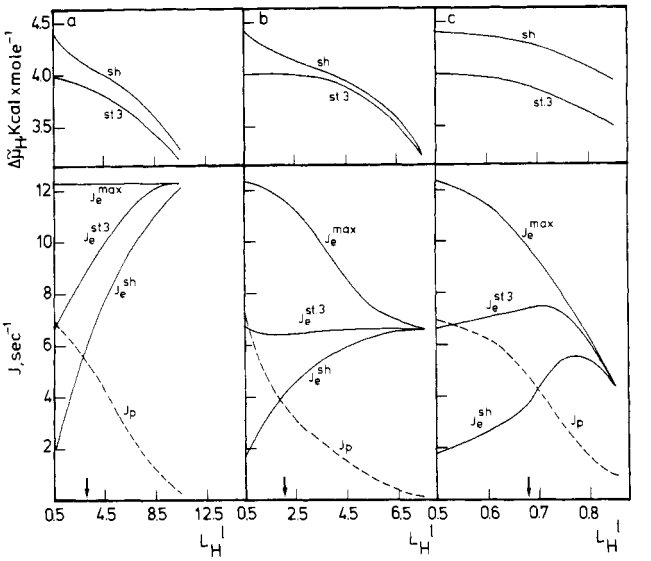

FIGURE 7: Simulations of the rates of ATP synthesis (dashed lines) and of electron transfer and of $\Delta\tilde{\mu}_H$ in static head and state 3 as a function of the leak conductance and of the extent of intrinsic uncoupling of the redox pumps (a, b) and of both the redox and the ATPase pumps (c). The simulations are obtained with the kinetic model of chemiosmotic energy coupling constituted by a redox proton pump model and an ATPase pump model as those used in Figures 2 and 6 with the same kinetic parameters and with constant affinities of 19.66 and 9 kcal/mol, respectively [see Pietrobon and Caplan (1986b) and Pietrobon (1986) for more details]. The third element of the model is an ohmic leak with conductance L_H^l. L_H^l and the rate constants of the slip transition of the redox pumps are varied to different extent in the simulations of panels a, b, and c, starting in every case from $L_H^l = 0.5$ mol kcal^{-1} s^{-1} and $\alpha_{52} = 1.4$ s^{-1}, $\alpha_{25} = 9.45 \times 10^{-13}$ s^{-1}, which are the values used in Pietrobon et al. (1986) to simulate the behavior of mitochondria in static head. In addition, in panel c the degree of intrinsic uncoupling of the ATPases is increased with L_H^l as in Figure 6. Panel a: The rate constants of the slip transition in the redox pump model are increased from $\alpha_{52} = 1.4$ s^{-1}, $\alpha_{25} = 9.45 \times 10^{-13}$ s^{-1} at $L_H^l = 0.5$ to $\alpha_{52} = 28$ s^{-1}, $\alpha_{25} = 1.89 \times 10^{-11}$ s^{-1} at $L_H^l = 10.5$. Panel b: The rate constants of the slip transition in the redox pump model are increased from the same values as in panel a at $L_H^l = 0.5$ to $\alpha_{52} = 9.8$ s^{-1}, $\alpha_{25} = 6.61 \times 10^{-12}$ s^{-1} at $L_H^l = 7.5$. The fraction of active redox pumps and the fraction of active ATPase pumps are varied from 1 at $L_H^l = 0.5$ to 0.53 and 0.29, respectively, at $L_H^l = 7.5$. Panel c: The rate constants of the slip transition in the redox pump model are increased from the same values as in (a) and (b) at $L_H^l = 0.5$ to $\alpha_{52} = 45$ s^{-1}, $\alpha_{25} = 3.04 \times 10^{-11}$ s^{-1} at $L_H^l = 0.85$. The rate constants of the slip transition in the ATPase model are varied with L_H^l as in Figure 6. The fraction of active redox pumps is varied from 1 at $L_H^l = 0.5$ to 0.35 at $L_H^l = 0.85$.

behavior of native mitochondria in static head (Pietrobon et al., 1986; cf. Figure 2). As found experimentally, the P/e^- ratio is almost constant in an extensive range of inhibition (it actually tends to slightly increase in the simulations because of the presence of a small intrinsic uncoupling in the redox pumps). The simulated RCR ratio in Figure 9 does not increase with inhibition as found experimentally (cf. control in Figure 5) because in the model there is a single redox proton pump and not, as in mitochondria, a chain of redox enzymes among which the control on the electron flux is distributed (Groen et al., 1982). As already mentioned, a $\Delta\tilde{\mu}_H$ dependence of this flux-control distribution may explain the observed increase of RCR upon addition of antimycin (Stoner, 1985). Note also that our simple chemiosmotic model is constituted only by three elements (the two proton pumps and the leak), and therefore the maximal theoretical value of P/e^-, n_e/n_p, is 1.333 (with $n_e = 4$ and $n_p = 3$ as assumed in the simulations). In mitochondria, because of the presence of the electrogenic adenine nucleotide translocator, the maximal theoretical value of P/e^- is 1 (assuming the same stoichiometry

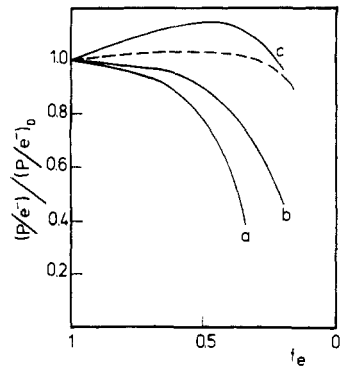

FIGURE 8: Simulations of the normalized P/e^- ratio as a function of the fraction of active redox pumps, f_e. Dashed curve: $L_H^1 = 0.5$; slip rate constants of the redox pump, $\alpha_{52} = 1.4\ s^{-1}$, $\alpha_{25} = 9.45 \times 10^{-13}\ s^{-1}$; slip rate constants of the ATPase, $\alpha_{52} = 2\ s^{-1}$, $\alpha_{25} = 11.7 \times 10^{-13}\ s^{-1}$. $(P/e^-)_0 = 1.04$. Curve a: $L_H^1 = 3.0$; slip rate constants of the redox pump, $\alpha_{52} = 5\ s^{-1}$, $\alpha_{25} = 3.37 \times 10^{-12}\ s^{-1}$; slip rate constants of the ATPase as in the dashed curve. $(P/e^-)_0 = 0.56$. Curve b: $L_H^1 = 2.0$; slip rate constants of the redox pump, $\alpha_{52} = 3.15\ s^{-1}$, $\alpha_{25} = 2.125 \times 10^{-12}\ s^{-1}$; slip rate constants of the ATPase as in the dashed curve; $f_e = 0.9$ (i.e., each abscissa value is multiplied by 0.9); $f_p = 0.45$. $(P/e^-)_0 = 0.48$. Curve c: $L_H^1 = 0.68$; slip rate constants of the redox pump, $\alpha_{52} = 6\ s^{-1}$, $\alpha_{25} = 4.05 \times 10^{-12}\ s^{-1}$; slip rate constants of the ATPase, $\alpha_{52} = 21\ s^{-1}$, $\alpha_{25} = 1.23 \times 10^{-11}\ s^{-1}$; $f_e = 0.77$. $(P/e^-)_0 = 0.65$.

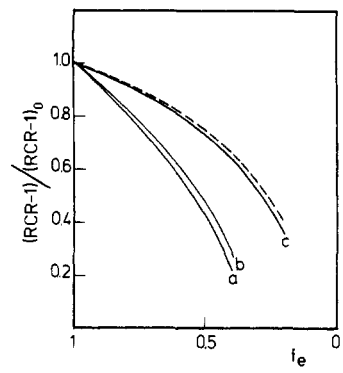

FIGURE 9: Simulations of the normalized RCR ratio as a function of the fraction of active redox pumps, f_e. For each curve parameters are as in Figure 8. Dashed curve: $(RCR)_0 = 7.06$. Curve a: $(RCR)_0 = 2.8$. Curve b: $(RCR)_0 = 2.44$. Curve c: $(RCR)_0 = 2.56$.

for the proton pumps and one negative charge translocated for each ADP exchanged with ATP).

The simulations a, b, and c in Figures 8 and 9 have been obtained in the presence of increased values of membrane proton conductance and intrinsic uncoupling of the redox pumps in cases a and b and of intrinsic uncoupling of both redox and ATPase pumps in case c (cf. arrows in Figure 7), resulting in values of the P/e^- ratio and RCR (in the absence of redox inhibitor) comparable (taking into account the above-mentioned difference) to those measured in the presence of FCCP, oleic acid, and chloroform, respectively. Indeed, the simulations of the P/e^- ratio and RCR as a function of the inhibition factor f_e are close to the experimental behavior exhibited in the presence of FCCP and oleic acid in cases a and b and to the experimental behavior exhibited in the presence of chloroform in case c. Of particular interest is the fact that we have been able to simulate the increase of the P/O ratio and the unchanged (with respect to the control) pattern of the RCR upon addition of antimycin exhibited by chloroform-supplemented mitochondria by assuming that this agent acts mainly as an intrinsic uncoupler of both redox and ATPase pumps. Actually the behavior of both the P/O ratio and RCR is such that it can be mainly attributed to the increased intrinsic uncoupling of the redox pumps, whereby an increased number of electrons is transferred at the maximal rate without proton pumping. In fact, in this case the decrease in the number of active redox pumps causes a higher relative inhibition of the uncoupled electron flow with respect to the coupled electron flow, thus explaining the pattern of both the P/O ratio and RCR.

The comparison between simulations and experiments leads to the conclusions that (i) the results presented in this as well as the preceding paper are consistent with a mixed action of FCCP and oleic acid as protonophores and intrinsic uncouplers of the redox proton pumps and with an action of chloroform as intrinsic uncoupler of both redox and ATPase pumps and (ii) the results do not necessarily require the rejection of the delocalized chemiosmotic model of free energy coupling unless other experiments are presented which are incompatible with the view that these agents act as intrinsic uncouplers.

On the other hand, there are other types of experimental observations [for reviews see Westerhoff et al. (1984) and Ferguson (1985)] that appear to be inconsistent with a delocalized model of free energy coupling and have led to the proposal of several mechanisms of localized protonic coupling in which pathways of proton flow are situated within or adjacent to the membrane and/or a direct interaction takes place between redox and ATPase pumps (Williams, 1961; Rottenberg, 1978, 1983; Kell, 1979; Westerhoff et al., 1984; Boyer, 1984; Slater et al., 1985).

In the "parallel coupling" mechanism proposed by Rottenberg (1978) [see also Slater et al. (1985)] ATP can be synthesized both through a delocalized chemiosmotic mechanism and through a direct interaction between redox and ATPase pumps, whereby protons can be transferred intramembranously between the two proton pumps. Rottenberg has proposed that chloroform and oleic acid specifically uncouple the intramembranal pathway by interfering with a proton capacitor closely associated with the ATPases and capable of receiving protons directly from neighboring redox pumps and has called them "decouplers" (Rottenberg, 1983; Rottenberg & Hashimoto, 1986). Kell and colleagues, in favoring the localized "coupling units" model of energy transduction (Westerhoff et al., 1984), have proposed that uncoupling agents act essentially by uncoupling the coupling units. The uncoupling mechanism should involve rapid shuttling of the uncoupler from one to the other coupling unit (Hitchens & Kell, 1983). Slater and colleagues have proposed in their "collision hypothesis" a similar mechanism for the uncoupling of the complexes of redox and ATP synthase pumps (Slater et al., 1985; Herweijer et al., 1986). To explain the results of the uncoupler–inhibitor titrations, where the effectiveness of a given concentration of uncoupler in inhibiting the rate of ATP synthesis is proportional to the fraction of inhibited ATPases, it has been assumed that the uncoupling process itself, not the movement of the uncoupler from one site to another, is the rate-limiting step.

Clearly, the above-mentioned and any other localized models of free energy coupling have to provide an explanation for the uncoupling of oxidative phosphorylation by FCCP, oleic acid, and chloroform consistent with their properties and with the behavior exhibited by these three agents in the experiments presented in this as well as in the preceding paper. The fact that our results can be explained by assuming that these agents act as intrinsic uncouplers of the pumps, without the need of invoking a localized protonic energy-coupling mechanism, does not exclude that the same results can be explained by a localized model of energy coupling, without the need of invoking intrinsic uncoupling. Furthermore, the two different

explanations may not be in opposition with respect to one another.

It is not difficult to conceive how in static head the decoupling action could give rise to the transfer of electrons not accompanied by proton translocation from the matrix to the external medium. This would be phenomenologically indistinguishable from an increased intrinsic uncoupling of the redox pumps and may certainly explain the increasing difference between rate of passive influx of protons and the rate of electron transfer (multiplied by H^+/e^- stoichiometry) in static head (preceding paper) and also the behavior of $n_e J_e/\Delta\tilde{\mu}_H$ (and RCR) upon inhibition of the redox pumps shown in Figure 1 (and Figure 5) in the presence of the uncoupling agents. What appears intuitively (and also on the basis of the published models of local coupling) more difficult to explain with a decoupling action is the increase of the P/O ratio observed up to a certain inhibition of the electron transfer in the presence of chloroform (Figure 4). Also rather difficult to explain is the fact that FCCP and oleic acid, in contrast with chloroform, do not appear to significantly act as decouplers when the ATPases (functioning in the hydrolytic mode) are the primary pumps (Figure 3 and cf. also Figure 4 of the preceding paper). Most likely these difficulties can be overcome by a more quantitative description of the parallel coupling model (or another localized model) of free energy coupling, especially if the possibility of slips is included. At the moment, in the absence of such model, the present results do not appear to be able to unequivocally discriminate between different energy-coupling mechanisms, but they might help to do so in the future.

We are currently working on a kinetic model of localized protonic coupling that incorporates the kinetic pump models proposed by Pietrobon and Caplan (1985b). In such localized protonic coupling, a common transition leads to direct transfer of protons between the two pumps. In this model, the event of decoupling (i.e., electron transfer without ATP synthesis) is generated through the same slip transitions [cf. Pietrobon and Caplan (1985b)] that give rise to intrinsic uncoupling of the pumps when the two proton pumps operate independently. This suggests the interesting possibility that the same specific interaction of the uncoupler with the pump leads to intrinsic uncoupling of the pumps when the pumps operate under conditions where the protonic circuits are delocalized and to decoupling when the pumps operate under conditions where the protonic circuits are localized.

ACKNOWLEDGMENTS

The simulations performed in the present study are based on a model elaborated by D. Pietrobon and S. R. Caplan at the Department of Membrane Research of the Weizmann Institute, Rehovot, Israel. We are therefore extremely grateful to Prof. S. R. Caplan for his fundamental contribution to the present approach. We thank Dr. M. Zoratti for useful discussion and M. Santato and L. Pregnolato for technical assistance.

Registry No. FCCP, 370-86-5; DCCD, 538-75-0; ATPase, 9000-83-3; $CHCl_3$, 67-66-3; H^+, 12408-02-5; oleic acid, 112-80-1.

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