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Review

Decoupling of oxidative phosphorylation and photophosphorylation

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Contents

I. Introduction

One of the unresolved disputes in bioenergetics is whether proton-dependent energy conversion is mediated exclusively by the bulk phase electrochemical potential difference, $\Delta\mu_{H^+}$ [1], or by a more direct pathway. An increasing number of studies of the mechanism of oxidative phosphorylation and photophosphorylation appear to be incompatible with the assumption that bulk $\Delta\mu_{H^+}$, as measured by conventional techniques, is the only intermediate that mediates energy transfer between the electron transport chain and the ATPase. These studies have been reviewed and critically evaluated frequently (cf. Refs. 2–9). One important aspect of these studies is the finding that the chemiosmotic explanation of uncoupling, as due entirely to reduction of the magnitude of $\Delta\mu_{H^+}$, is not always correct [2,5,6,9]. In particular, a growing number of unusual uncoupling agents have been shown to uncouple oxidative- and photo-phosphorylation with relatively little effect on the magnitude of $\Delta\mu_{H^+}$. The effects of these agents, which have been defined as decouplers [2], are the subject of this review.

I-A. Coupling and uncoupling: historical background

The pharmacological effects of 'classical uncouplers' (e.g., dinitrophenols) have been known for more than a century. In parallel with the elucidation of the major pathways of intermediate metabolism, it became evident

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that these agents uncouple energy-yielding processes (e.g., aerobic metabolism) from energy consuming-processes [10]. It was, therefore, suggested that uncouplers either allow oxidation without phosphorylation or catalyze the hydrolysis of a phosphorylated intermediate [11]. Later, it was shown that uncouplers inhibit ATP synthesis, in mitochondrial suspension, without inhibition of respiration [12,13]. In the period that followed the discovery of oxidative phosphorylation by isolated mitochondria, the intensive search for the mechanism of this process was closely linked to the investigation of the mechanism of uncoupling. The classical studies of mitochondrial metabolism in the 1950's culminated in the formulation of the chemical hypothesis. Accordingly, electron transport is coupled to ATP synthesis by the formation and utilization of a 'high energy intermediate' [14]. A central aspect of the hypothesis was the suggestion that this intermediate is susceptible to hydrolysis (or breakdown) by uncouplers. The discovery of photophosphorylation in chloroplasts [15], and the fact that this process is also uncoupled by classical uncouplers, suggested that the two processes share a common mechanism [16].

The concept of a 'high energy intermediate', which is central to the chemical hypothesis, could explain, elegantly, all the major phenomenological observations related to coupling and uncoupling in oxidative phosphorylation and photophosphorylation. The only drawback, of course, was the fact that no chemical intermediate could be identified, despite an intensive, often frantic, search by the best biochemists of the time. Peter Mitchell revolutionized bioenergetics with the formulation of the chemiosmotic hypothesis, according to which a proton current produced by electron transport and consumed by the ATPase serves to couple oxidative phosphorylation [17]. While a similar idea was proposed independently by R.J.P. Williams [18], the uniqueness, and the great advantage of Mitchell's hypothesis, particularly as developed later, was the identification of the proton electrochemical difference across the coupling membrane ($\Delta\mu_{H^+}$) as the 'high-energy intermediate' [1]. It is not fully appreciated by current researchers in bioenergetics that the chemiosmotic hypothesis, as formulated by Mitchell, fully retained the concept of a single high-energy intermediate as the only connection between the oxidation and phosphorylation processes. Consequently, the explanation of uncoupling in the chemiosmotic hypothesis is essentially the same as in the chemical hypothesis: uncouplers break down the 'intermediate,' i.e., dissipate $\Delta\mu_{H^+}$ [1].

This explanation of uncoupling appeared, at first, to be spectacularly successful. Not only, as postulated by Mitchell, did classical uncouplers turn out to be lipophilic weak acids that can facilitate proton transport and thus dissipate $\Delta\mu_{H^+}$ [19], but the uncoupling of oxidative phosphorylation and photophosphorylation by various other ions and ionophores could also be explained as due to the collapse of $\Delta\mu_{H^+}$ [20,21]. Alternative theories of coupling could not accommodate easily the uncoupling produced by ions and ionophores.

The acceptance of the chemiosmotic hypothesis has led to the widespread identification of uncouplers as agents that dissipate $\Delta\mu_{H^+}$ (cf. Ref. 22). It is, however, necessary to emphasize that the classical concept of uncouplers rests on their effects on oxidative and photophosphorylation, rather than their mechanism of action. Accordingly, uncouplers were defined as agents that: (i) stimulate basal (non-phosphorylating) electron transport; (ii) inhibit ATP synthesis without inhibition of electron transport; (iii) stimulate ATP hydrolysis; (iv) inhibit various exchange reactions catalyzed by the ATPase [23].

I-B. Proton pathways in oxidative phosphorylation and photophosphorylation

It is now abundantly clear that the basic features of oxidative phosphorylation and photophosphorylation are adequately described by Mitchell's chemiosmotic hypothesis. This subject has been reviewed extensively over the years and will be summarized here only briefly (cf. Ref. 22, 24–27). The central role of the reversible proton-ATPase of the F_0F_1 type in oxidative phosphorylation and photophosphorylation is established beyond doubt. The enzyme or its homolog has been isolated from many coupling membrane systems, reconstituted and shown to be able to catalyze $\Delta\mu_{H^+}$-driven ATP synthesis [28,29]. It has also been demonstrated, beyond doubt, that the inner membrane of mitochondria and the plasma membrane of most aerobic bacteria contain, in addition to the H^+-ATPase, a variety of redox proton pumps which generate $\Delta\mu_{H^+}$ [30]. Similarly, photosynthetic membranes contain, in addition to the H^+-ATPase, light-driven proton pumps that generate $\Delta\mu_{H^+}$ [31].

These and related findings led to the acceptance of Mitchell's hypothesis that a proton current serves as a coupling device in both processes. However, it is still very far from being clear what are the exact proton pathways that constitute the coupling current(s), both *within* the redox and ATPase pumps and *between* these pumps. The chemiosmotic hypothesis in its various versions included specific mechanisms based on proximal coupling between electron transport and proton transport ('ligand conductance') [30]. However, it is still not known with certainty whether any of these mechanisms is correct. Moreover, it is becoming increasingly clear that for some of these pumps (e.g., H^+-ATPase, cytochrome oxidase), the mechanism is quite different, more adequately described as a distal 'conformational coupling' [32,33]. In addition, there is mounting evidence which is incompatible with the chemiosmotic assump-

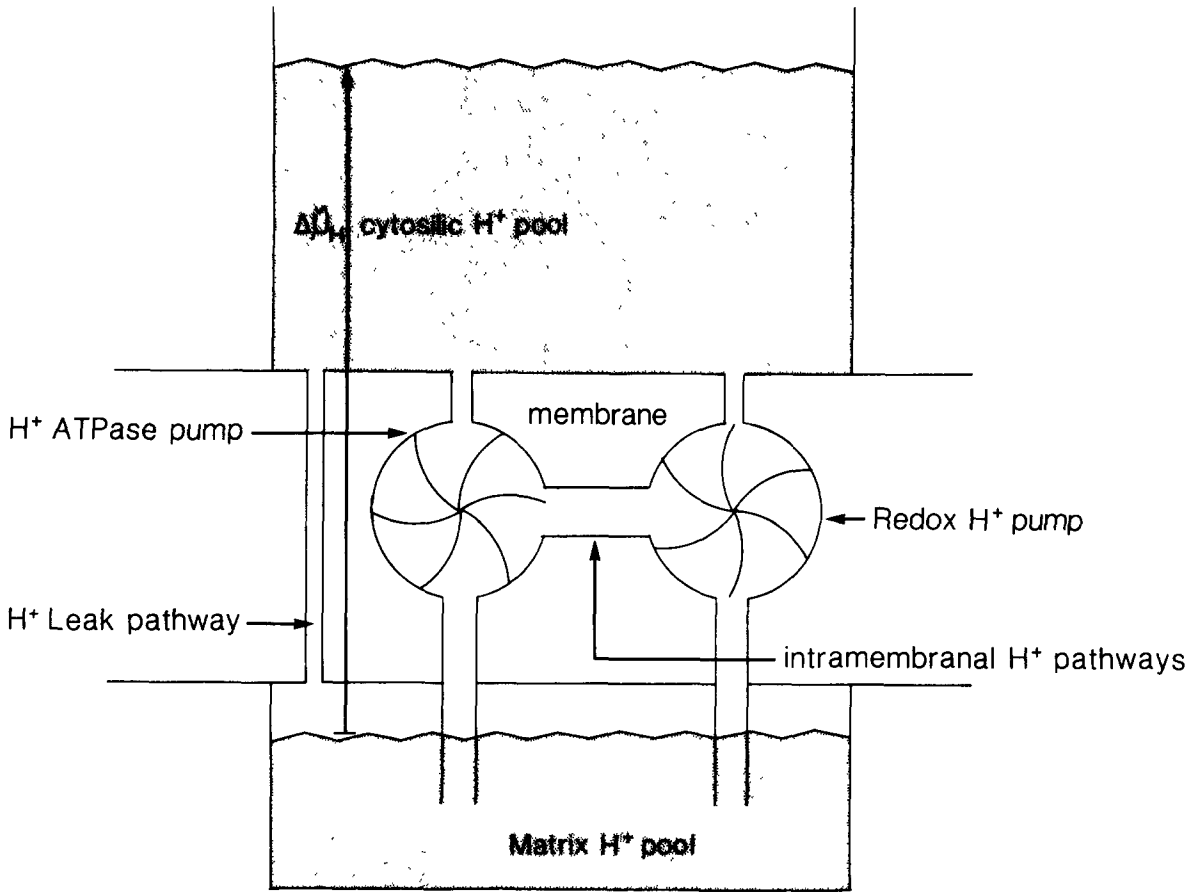

Fig. 1. An hydraulic representation of the parallel coupling model. In oxidative phosphorylation, energy from substrate oxidation is utilized to pump protons from the matrix to the cytosol. As the 'Head' pressure of the cytosolic pool is increased, fewer protons would be ejected into the cytosol and more protons would be transferred directly to the ATPase. The protons that flow through the ATPase drive ATP synthesis. For further details, see text.

tion that protons are pumped exclusively into and out of the bulk water phases. This evidence has been discussed extensively in several recent reviews and will be only summarized here very briefly: (i) respiratory control (the stimulation of electron transport by ATP synthesis) is not exclusively mediated by $\Delta\mu_{H^+}$ [2–5]; (ii) inhibitors of electron transport inhibit ATP synthesis, initially, with little effect on $\Delta\mu_{H^+}$ [2–6]; (iii) uncoupling or inhibition of electron transport leads to increased $\Delta G_p/\Delta\mu_{H^+}$ ratios, suggesting that ΔG_p is not in equilibrium with $\Delta\mu_{H^+}$ [2–4]; (iv) in chloroplasts, protons generated by the photosystems appear to drive ATP synthesis without equilibration with the thylakoid lumen [7]; (v) ATP synthesis in some microorganisms (e.g., alkalophilic bacteria, *H. halobium*) is often associated with the generation of a very small $\Delta\mu_{H^+}$, which is insufficient to support the observed increase in ΔG_p [8]; (vi) there are uncoupler-resistant bacterial mutants in which the uncouplers effect on $\Delta\mu_{H^+}$ is not altered [9]; (vii) some agents uncouple energy conversion with little effect on $\Delta\mu_{H^+}$ [2,9]. It should be pointed out, without a detailed discussion of this evidence, that each issue on this list has been contested, on both experimental and theoretical grounds. Thus, there is certainly no consensus, at present, that this evidence is sufficient to reject the assumption that $\Delta\mu_{H^+}$ alone mediates energy conversion.

The modifications of the chemiosmotic hypothesis, which have been proposed to accommodate this evidence, are generally referred to as 'localized' coupling schemes. This description, however, is misleading, as the models differ substantially, particularly in regard to the degree of 'localization.' A frequently discussed model, which arises from the combined efforts of several investigators [3], postulates the existence of a coupling unit, a specific tight association between an ATPase and a redox pump in which protons are transferred directly within the coupling unit from the redox pump to the ATPase. The appearance of protons in the bulk phase is considered to be a leak from the coupling unit, but the significance and contribution of $\Delta\mu_{H^+}$ to energy transfer in this model are not sufficiently delineated [3]. This model is the most strongly 'localized' coupling scheme, and predicts 'localized' behavior in 'double-inhibitor titration' experiments. Some of these predictions have been confirmed; however, the naive interpretations of

these complicated experiments have been criticized [34]. Another model postulates that the coupling proton current flows on the membrane surface, which is not equilibrated with the adjacent bulk solution [35,36]. This model cannot be considered strongly localized, since each surface of the membrane constitutes a homogeneous compartment which is shared by many pumps. Other models of 'localized' coupling with various degrees of localization have been proposed (cf. Refs. 5,7,26).

We have advocated a 'parallel coupling' scheme in which both intramembranal and bulk proton pathways contribute (though not necessarily equally) to energy conversion [37]. In this model, intramembranal proton transport is suggested to occur by direct proton transfer between the pumps [38]. However, unlike the 'coupling unit' model, the coupling is not considered to be strongly localized, since the individual pumps diffuse independently in the membrane, while multiple collisions and aggregation facilitate proton transfer and allow partial equilibration of protons between the pumps [2]. Moreover, the parallel proton flow through the bulk is delocalized and thus leads to enhanced overall delocalization of protons. A schematic, hydraulic representation of some of the features of this model, which will be described in molecular detail later, is depicted in Fig. 1. An important feature of the 'parallel coupling' model, which is emphasized by the hydraulic representation, is the crucial importance of $\Delta\mu_{H^+}$ to the efficiency of coupling. When $\Delta\mu_{H^+}$ is low, the rate of proton pumping into the bulk would be high, compared to the rate of intramembranal proton transfer, and ATP synthesis would be driven largely by the bulk $\Delta\mu_{H^+}$ and thus strongly depend on its magnitude. However, as $\Delta\mu_{H^+}$ is increased, the 'head' pressure generated by $\Delta\mu_{H^+}$ would inhibit proton pumping into the bulk and increase the rate of intramembranal H^+-transfer. In this state, the magnitude of $\Delta\mu_{H^+}$ would not affect the rate of ATP synthesis strongly, and the rate of synthesis will depend strongly on the rate of electron transport. Unlike other 'localized' coupling schemes, this model retains an important role for $\Delta\mu_{H^+}$ in energy conversion and hence, the designation 'parallel coupling.' This important feature is emphasized to various degrees in other similar models (cf. Ref. 5). The assumption that the magnitude of $\Delta\mu_{H^+}$ controls the distribution of the proton current is a unique feature of this model. Recent supporting evidence for this model comes from a new study of the relationships between ΔG_p and $\Delta\mu_{H^+}$ in mitochondria. It was found that when $\Delta\mu_{H^+}$ was modulated by ionophores, such as valinomycin $+K^+$, the slope of the curve ΔG_p vs. $\Delta\mu_{H^+}$ was about 1 at high value of $\Delta\mu_{H^+}$ (suggesting a very weak dependence of ΔG_p on $\Delta\mu_{H^+}$), but increased to approx. 4 at low value of $\Delta\mu_{H^+}$, which is the slope expected for $\Delta\mu_{H^+}$-driven ATP synthesis [39].

The conventional approach to the evaluation of the various models of coupling is to derive various kinetic equations from the models and to compare the fit of the experimental data to these equations. Although the kinetic models have become more and more sophisticated over the years, I feel that it is still not possible to resolve the issue by this approach. Firstly, the many enzymes that participate in energy conversions are extremely complex, with several states of activation and a large number of ions and metabolites that modulate their activity. We are still far from an adequate kinetic description of even a single proton pump. Thus we cannot hope, at present, to construct an adequate kinetic model which combines a large number of enzymes and transport systems for which we do not have even approximate kinetic models. Even the 'passive' leak of protons through the membrane is so complex, kinetically, that we do not have an adequate kinetic description of this process. Secondly, the necessary simplifications required for present kinetics models include a number of adjustable parameters which may provide insights for model construction, but precludes rigorous model testing.

In our recent work, we have approached this issue from a different perspective. We argued that if indeed there is an intramembranal pathway of coupling, it should be possible to interfere specifically with this pathway. Ideally, one would hope to find an agent (or treatment) which specifically inhibits or uncouples the intramembranal pathway without significant side-effects, and, particularly, without any effect on the bulk to bulk $\Delta\mu_{H^+}$. This has not been fully accomplished as yet. However, scrutinizing the effects of a large variety of agents which interfere with coupling has yielded a spectrum of patterns of action, which suggests that there is more than one way to uncouple energy conversion and that there are distinct patterns of uncoupling which involve intramembranal processes rather than transmembrane transport of protons or other ions.

II. Uncoupling and $\Delta\mu_{H^+}$

If $\Delta\mu_{H^+}$ is the only intermediate between electron transport and ATP synthesis (as postulated by the chemiosmotic hypothesis), it is expected that uncoupling, as defined by the phenomenological criteria (listed above) would always be associated with reduction of the magnitude of $\Delta\mu_{H^+}$ [1]. Moreover, the titration of the rates of phosphorylation, State 4 respiration or the P/O by different uncouplers should produce a single curve, correlating these parameters with $\Delta\mu_{H^+}$.

Despite numerous qualitative demonstrations that uncouplers collapse $\Delta\mu_{H^+}$, only few studies provide a quantitative evaluation of the effects of uncouplers on the relationships between respiration, phosphorylation and $\Delta\mu_{H^+}$. Since these studies do not provide a unique

description of the dependence of the rate of ATP synthesis or electron transport on $\Delta\mu_{H^+}$, we do not know what is the 'true' relationship between these parameters. Moreover, because of the complex nature of these processes, and the existence of several activation states and direct effects on enzyme activity, it is impossible to predict these relationships from theoretical consideration and kinetic models. Thus, these relationships must be determined experimentally. The best approach to evaluate the effect of $\Delta\mu_{H^+}$ on the rate of phosphorylation is to use artificially generated $\Delta\mu_{H^+}$ of various magnitudes and to measure the induced rate of phosphorylation. Earlier data in chloroplasts [40], submitochondrial particles [41] and reconstituted TF_0F_1 [42] demonstrated the existence of a threshold $\Delta\mu_{H^+}$, a region of approximate linear dependence of the rate of phosphorylation on $\Delta\mu_{H^+}$ and a region of saturation at high $\Delta\mu_{H^+}$. More recently, Graber and his colleagues measured the rate of phosphorylation induced by artificially imposed $\Delta\mu_{H^+}$ in chloroplasts and reconstituted CF_0F_1-liposomes and provided a full description of these relationships for the various activation states of the CF_0F_1 enzyme [43–47]. It is now clear that previous measurements of the dependence of photophosphorylation on ΔpH by modulating light intensity largely reflect the light-dependent activation of the ATPase and not the dependence of the rate of the fully activated enzyme on $\Delta\mu_{H^+}$ [44]. Similar experiments were performed recently with the reconstituted F_0F_1 of *R. rubrum*. This bacterial enzyme does not require activation, which simplifies the analysis. The slopes of the curves of phosphorylation vs. ΔpH were very moderate [66]. An increase in ΔpH of 1 unit (i.e., 58 mV) resulted in only 2–5-fold stimulation of phosphorylation. This is in sharp contrast to photophosphorylation in chromatophores, in which very steep slopes were observed (cf. Ref. 67).

Another approach, which we have used recently, is to clamp $\Delta\mu_{H^+}$ at different values by ionophores and permeable ions and to measure the rates of phosphorylation and respiration. In general, ionophores produced titration curves that were not as steep as protonophores. That is, a larger reduction of $\Delta\mu_{H^+}$ must be induced by ionophores to produce the same effect on phosphorylation or respiration as a protonophore. As discussed below, in the case of ionophores and ions there is no reasonable alternative mechanism to influence these reaction rates but the collapse of $\Delta\mu_{H^+}$ and hence, we consider the titration with ionophores to be a reliable indicator of the true dependence of phosphorylation and respiration on $\Delta\mu_{H^+}$. Since protonophores produce steeper curves than ionophores, it appears that protonophores affect the reactions more directly in addition to collapsing $\Delta\mu_{H^+}$. Moreover, electron transport inhibitors produce even steeper curves (cf. Ref. 69), suggesting a direct pathway of coupling.

We can compare the titration curves of the rate of phosphorylation vs. $\Delta\mu_{H^+}$ produced by ionophores and protonophores with those arising from the studies of artifically induced $\Delta\mu_{H^+}$ [40–47]. This comparison (Fig. 2, see below) suggests that ionophores and ions uncouple solely by their effect on $\Delta\mu_{H^+}$ and thus produce titration curves that represent the 'true' effect of $\Delta\mu_{H^+}$ on phosphorylation and electron transport. Protonophores appear, by this criterion, to uncouple by another mechanism in addition to the reduction of $\Delta\mu_{H^+}$. Below, I review briefly the mechanism of uncoupling by ionophores and protonophores.

II-A. Uncoupling by ionophores and permeating ions

The most indisputable aspect of the chemiosmotic hypothesis is the explanation of various processes of ion transport in organelles and microorganisms as being driven by the components of the proton electrochemical potential, $\Delta\psi$ and ΔpH (cf. Ref. 22). It has been demonstrated that $\Delta\mu_{H^+}$ drives the transport of a large number of proton-coupled systems, both symporters (i.e., cotransport) or antiporters (i.e., exchange). In addition, $\Delta\psi$ can drive directly the electrogenic transport of permeating ions, while ΔpH can drive the transport of permeating weak acids or bases which diffuse across the membrane in their neutral form. Provided that the rates of transport of these ions are comparable to the rates of proton pumping by the redox pumps, and that the transport process can be sustained, these processes can lead to reduction of $\Delta\psi$, ΔpH or both and hence, to uncoupling. For instance, in mitochondria it has been known for a long time that Ca^{2+} is accumulated by respiring mitochondria while exhibiting all the phenomenological aspects of uncoupling (e.g., stimulation of respiration and ATPase and inhibition of phosphorylation and exchange reactions). The chemiosmotic explanation is simple: Ca^{2+} uptake is electrogenic, hence fast Ca^{2+} uptake will collapse $\Delta\psi$ and lead to uncoupling. These predictions of the chemiosmotic hypothesis were verified experimentally [20,48]. Similarly, the uncoupling effects of ionophores, such as valinomycin in the presence of high concentration of potassium [49], are explained as a result of the increased permeability of potassium, which at high concentration is taken up by the mitochondria at high rates, thus collapsing the membrane potential [20,21]. In mitochondria $\Delta\psi$ is the major component of $\Delta\mu_{H^+}$ and hence, collapsing of $\Delta\psi$ alone is sufficient to cause uncoupling. In contrast, in chloroplasts in which ΔpH is the major component of $\Delta\mu_{H^+}$, permeable ions and electrogenic ionophores are relatively without effect on photophosphorylation, but agents that collapse ΔpH are strong uncouplers. For instance, amines, which are accumulated by chloroplasts due to the permeability of their uncharged species [50], are very potent uncouplers in chloroplasts, but not

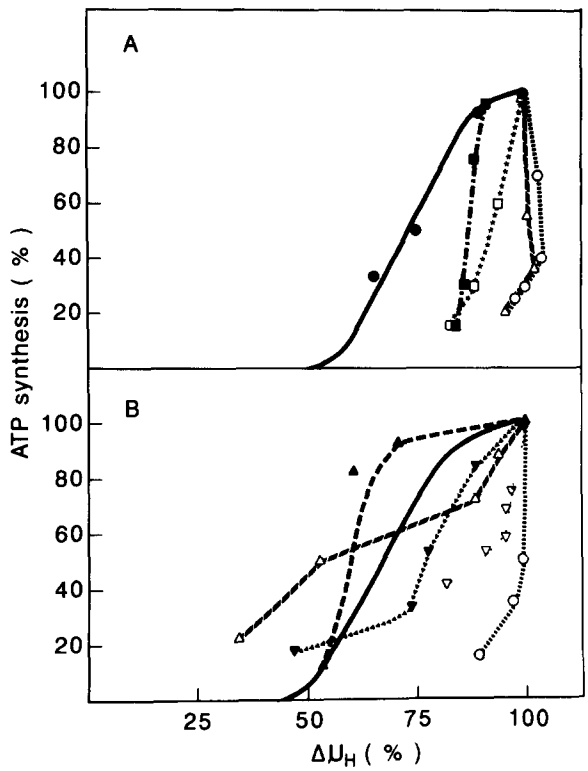

Fig. 2. The dependence of the rate of ATP synthesis on $\Delta\mu_{H^+}$ obtained with various uncouplers in chloroplasts (A) and mitochondria (B). (A) The results in chloroplasts are taken from the data of Pick et al. [65]. The solid line shows the dependence of the rate of ATP synthesis on artificially imposed $\Delta\mu_{H^+}$ in CF_0F_1 liposomes [44]. Nigericin (●); NH_4 (■), SF-6847 (□), gramicidin (△), palmitate (○). (B) The results in rat liver mitochondria are from Refs. 64, 107 and 108. The solid line shows the expected dependence of ATP synthesis on $\Delta\mu_{H^+}$ based on the results with CF_0F_1 [47] and submitochondrial particles [41]. Valinomycin $+K^+$ (▲) gramicidin, $+K^+$ (△), gramicidin, low salt (▽), FCCP (▼), palmitate (○).

in mitochondria. Their uncoupling of photophosphorylation is correlated with the collapse of ΔpH [52]. Similarly, nigericin, which catalyzes electroneutral exchange of H^+ for K^+ and collapses ΔpH, is a potent uncoupler of photophosphorylation in chloroplasts [52] but is without effect on oxidative phosphorylation in mitochondria. Since the mechanism of the effects of ionophores and permeating ions, in most cases, is well established, and because there is no evidence or feasible mechanism for a direct effect on either the H^+-ATPase or the redox H^+-pump, we may consider the characteristic of uncoupling by these agents to be the 'true' and unambiguous manifestation of the effect of $\Delta\mu_{H^+}$ on both phosphorylation and respiration. As Fig. 2 shows, nigericin collapses ΔpH and inhibits photophosphorylation in chloroplasts in a pattern which is almost identical to the dependence of ATP synthesis on artificial $\Delta\mu_{H^+}$ in CF_0F_1 liposomes [44]. Ammonium uncoupling deviates somewhat from this pattern, particularly at high concentration, most likely because of excessive swelling that ruptures the membrane. In mitochondria, inhibition of ATP synthesis by valinomycin ($+K^+$) exhibits a similar (though not identical) pattern, suggesting that this agent uncouples only by reduction of $\Delta\mu_{H^+}$.

II-B. Uncoupling by protonophores

Mitchell has postulated that uncouplers are lipophilic weak acids which increase the proton permeability of the membrane by shuttling protons between the surfaces of the membrane [1]. This has been verified for classical uncouplers (e.g., phenol derivatives) and a large number of non-classical uncouplers in studies of both artificial and natural membranes [19,53]. The term protonophores is now used to describe uncoupling agents that increase the proton permeability of membranes by the shuttling mechanism. However, it is much more difficult to prove that the shuttling of protons between the membrane surfaces, and the resulting decrease of $\Delta\mu_{H^+}$, is the only mechanism by which protonophores uncouple oxidative phosphorylation and photophosphorylation. Most of the attempts to verify (or disprove) this hypothesis have been based on correlations between the uncoupling potency in mitochondria and the protonophoric potency in phospholipid bilayers for a large and diverse group of uncouplers. The earliest attempt [54] resulted in a rather weak correlation and suggested that these two activities of protonophores were not strongly related. However, later studies [19] yielded much better correlation, and it was demonstrated that the earlier measurements of proton conductance in planar bilayers were unreliable because of the leakiness of these membranes [53]. Moreover, later studies [55] demonstrated the important effect of the pK on the pH profile of the uncoupling potency, which must be taken into account in these correlations for valid evaluation. Recent studies with liposomes, rather than planar bilayers, have demonstrated that within a group of similarly structured protonophores (e.g., substituted phenols) the correlation is indeed very good [56]. Moreover, the potency of these agents for increasing the proton permeability of phosphatidylcholine vesicles was highly correlated with their lipid/water partition coefficients and their acidity, as predicted from the shuttling mechanism [53]. Mitochondria uncoupling, as measured by the stimulation of respiration, was also correlated strongly with the partition coefficient and acidity and hence, also with the protonophoric activity. However, the dependence on acidity was much stronger for the mitochondrial uncoupling potency. Moreover, non-classical uncouplers showed even stronger dependence of uncoupling in mitochondria on their acidity in comparison with their protonophoric activity in liposomes [57]. Myoshi et al. argued that this may be related to the higher dielectric constant of mitochondria [58] which stabilizes the an-

ion. When the pH profile of the uncoupling of each protonophore was taken into account, both classical and non-classical uncouplers fitted closely the same curve [57]. Thus, it was concluded that these data strongly supported the chemiosmotic explanation of uncoupling. It must be pointed out, however, that as good as these correlations might be, they do not prove the hypothesis. The actual proton current carried by a protonophore across the mitochondrial or chloroplast membranes in partially uncoupled mitochondria cannot be evaluated from these studies with artificial membranes. Hence, the extent of the contribution of this current to the uncoupling cannot be evaluated.

More relevant experiments are those in which both the proton fluxes and the uncoupling activity induced by protonophores are compared in mitochondria under conditions which are as closely matched as possible. Such studies [59] show that, although the uncoupling and protonophoric activities of uncouplers in mitochondria are correlated, the correlation is not very strong. Thus, when the protonophoric activities at concentration of uncouplers, which produce 50% uncoupling were compared, large variations in the extent of the reversal of valinomycin-induced stimulation of H^+-pumping were noted. In a more recent study, the increased proton currents induced by different concentrations of a protonophore were compared directly with the increased rates of respiration and inhibition of phosphorylation. Based on the estimated rates of proton pumping, it was concluded that the protonophore-dependent increased rates of proton current could not fully account for the uncoupling [60]. Similarly, in chloroplasts, protonophore-induced increases in proton current were found to be insufficient to account for the inhibition of photophosphorylation, whereas increased proton currents by nigericin and NH_4^+ were sufficiently high to explain the inhibition [61]. It was also demonstrated recently that the deactivation of the ATPase in CF_0F_1-liposome by protonophores is not due to increased proton permeability [62]. Similarly, in beef heart submitochondrial particles the effect of protonophores on the kinetic parameters of oxidative phosphorylation was different from the effect of ionophores, suggesting a different mechanism of action [63].

We have used a different approach in which we evaluated the relationships between the effect of protonophores on the rates of respiration and phosphorylation and their effect on $\Delta\mu_{H^+}$ [64,65]. Protonophores produced curves that could not be fitted to the dependence of phosphorylation on $\Delta\mu_{H^+}$, as evaluated from studies with artificially generated $\Delta\mu_{H^+}$, or from the effect of ionophores (Figs. 2, 4). In all cases, both in mitochondria and chloroplasts, protonophores show stronger uncoupling activity than would be expected from their effect on $\Delta\mu_{H^+}$. Hence, we concluded that protonophores have a mixed mode of uncoupling, only part of which could be accounted for by the reduction of $\Delta\mu_{H^+}$.

It was shown recently by Krulwich and her colleagues that uncoupler resistant mutants of *Bacillus* species, in which protonophores do not inhibit ATP synthesis, are as sensitive as the wild type to the effect of protonophores on proton permeability [9]. It was concluded that the mutants enhanced their direct coupling to counteract the effect of protonophores on $\Delta\mu_{H^+}$. The mutations affect the fatty acid desaturase system, resulting in an increase of the saturated/unsaturated fatty acids ratio of the phospholipids [70].

The mechanism by which protonophores uncouple energy conversion in addition to their effect on $\Delta\mu_{H^+}$ is yet to be established. Earlier suggestions that uncoupling results from a covalent binding of the protonophores to the ATPase or other components of oxidative phosphorylation [71] are not very likely. Although some protonophores have a reactive moiety, their uncoupling activity does not appear to be related to this effect [72]. Moreover, very potent protonophores produce full uncoupling in concentrations which are well below the concentration of the enzymes of oxidative phosphorylation [61].

A simple mechanism which could explain most of the data relating to protonophore uncoupling, and which is based on their essential physicochemical properties, i.e., lipophilicity and acidity, is outlined below. The protonophore anion is suggested to be capable of interacting with the proton pumps removing protons from occluded sites and shuttling them to the surface. In principle, this mechanism is so similar to the transmembrane shuttling mechanism that all the evidence that is compatible with the latter mechanism is also compatible with this mechanism. The assumption that both of these mechanisms operate simultaneously can explain the discrepancy between the effect of protonophores on transmembrane proton currents and the uncoupling activity. It may likewise explain, in part, the data on uncoupler resistant mutants, in which the mutation can be assumed to protect occluded protons from access by the protonophore, possibly by a change of lipid-protein complexes [9]. It remains to be evaluated whether such effect leads to intrinsic uncoupling [73] or decoupling (see below). One aspect of protonophore action, which appears to be harder to accommodate with this mechanism, is the fact that some potent protonophores completely uncouple at concentrations significantly below the concentration of proton pumps [61]. It can be accommodated with more localized models by suggesting a very fast turnover of the protonophore shuttling cycle, such that each protonophore may interact with large numbers of pumps during a single turnover of the ATPase [74]. The potency of protonophores can be more easily accommodated with the more delocalized 'parallel coupling' model, which assumes rapid equi-

libration of intramembranal protons between larger numbers of pumps.

III. Decouplers: uncouplers of the intramembranal proton pathway

There are many lipophilic compounds which uncouple oxidative phosphorylation and photophosphorylation but do not belong in the two categories of uncouplers described above, ionophores and protonophores. Some of those agents (e.g., ionic detergents) disrupt the membrane structure, thereby inducing an increase in membrane conductance of various ions including protons, leading to the collapse of $\Delta\mu_{H^+}$. Although the mechanisms of the increased ion conductance is different from that of ionophores and protonophores, we may still consider these agents to be (chemiosmotic) uncouplers, since the uncoupling results largely from the collapse of $\Delta\mu_{H^+}$. However, while screening a large number of chemicals and drugs that affect oxidative phosphorylation, we tentatively identified a number of agents which appear to uncouple oxidative phosphorylation without significant reduction of $\Delta\mu_{H^+}$ (Rottenberg and Hashimoto, unpublished data). Many of these belong to distinct categories, such as anesthetics, free fatty acids and non-ionic detergents. We have named these agents 'decouplers' to distinguish them from the more familiar uncouplers which collapse $\Delta\mu_{H^+}$.

In studying the properties of some of these agents, as described below, it became clear that their mechanisms of action may be different from each other and that the profile of their uncoupling effects is not uniform. Often, their action can be attributed to a specific combination of effects which results in a specific profile of uncoupling. One possible mechanism of uncoupling which does not lead to large reductions of $\Delta\mu_{H^+}$ is 'intrinsic' uncoupling of the proton pumps, as discussed above [73,75]. An important test, which may distinguish between intrinsic uncouplers of the ATPase and decouplers, is the effect of these agents on ATP synthesis driven by artificially generated $\Delta\mu_{H^+}$. Decouplers of the intramembranal pathway should have no significant effect on this process, while intrinsic uncouplers should be as potent inhibitors of ATP synthesis in this process as in oxidative- and photo-phosphorylation. Based on studies of decoupling by anesthetics, free fatty acids and gramicidin both in mitochondria and chloroplasts (see below), we offer the following phenomenological description of the common features of decouplers: (i) a stimulation of basal ('State 4') respiration, but only up to the level of a phosphorylating system ('State 3'); (ii) inhibition of oxidative- or photo-phosphorylation (ATP synthesis); (iii) a relatively small effect on membrane ion permeabilities, including protons in the decoupling range; (iv) a relatively small effect on $\Delta\mu_{H^+}$ when generated by redox or light driven proton pump (in the 'decoupling' range); (v) stimulation of ATPase; (vi) no inhibition of ATP synthesis when driven by artificially generated $\Delta\mu_{H^+}$; (vii) a reduction of $\Delta\mu_{H^+}$ when generated by ATP hydrolysis; (viii) the decoupling activity is enhanced at high temperatures (37°C).

As a working hypothesis we suggest the following mechanism: decouplers release protons from the intramembranal proton transfer pathway ('pool'). This hypothesis predicts the effects of decouplers listed above, except for (vii) and (viii). We believe that these unpredicted effects provide clues to the mechanism of intramembranal coupling and we shall attempt to account for these in a molecular model which we present at the end of this review.

III-A. General anesthetics

Most anesthetics, both general anesthetics and local anesthetics, interfere with oxidative phosphorylation. However, this diverse group of membrane perturbing agents produces a large number of effects in addition to uncoupling. Almost all anesthetics strongly inhibit NADH dehydrogenase [76]. Local anesthetics also inhibit directly the ATPase and even the isolated F_1 [77,78]. Both local and general anesthetics affect ion transport in mitochondria and other membranes [79,80]. It is, therefore, somewhat difficult to study the uncoupling effects of anesthetics in isolation from these direct effects on various membrane enzymes. Nevertheless, it was shown that the combination of amines local anesthetics with lipophilic anions produced typical uncoupling patterns, presumably by shuttling protons across the membrane [81]. We have shown that the general anesthetics, halothane and chloroform, produce the characteristic effects of decouplers of oxidative phosphorylation, e.g., stimulation of State 4 respiration up to the level of State 3 (with succinate as substrate) and inhibition of ATP synthesis without effect on $\Delta\mu_{H^+}$ (provided that EGTA was included to inhibit Ca^{2+} cycling) [82]. These findings were largely confirmed by other investigators (cf. Refs. 60, 83, 84). Thus, by some of the criteria listed above, general anesthetics may qualify as decouplers. However, in at least one important aspect, general anesthetics differ from other decouplers. Both uncoupler-stimulated ATPase activity and basal ATPase were further stimulated by chloroform and halothane, suggesting specific (possibly intrinsic) uncoupling of the ATPase [82,73]. Pietrobon et al. [73] concluded that both the redox pumps and the ATPase are intrinsicly uncoupled by chloroform.

We did not detect an effect on the oligomycin sensitivity of the ATPase in intact liver mitochondria [82] but observed a decrease in oligomycin sensitivity in beef heart submitochondrial particles (Rottenberg, Matsumo-Yagi and Hatefi, unpublished data). This suggests that at least part of the effect of these anesthetics is due

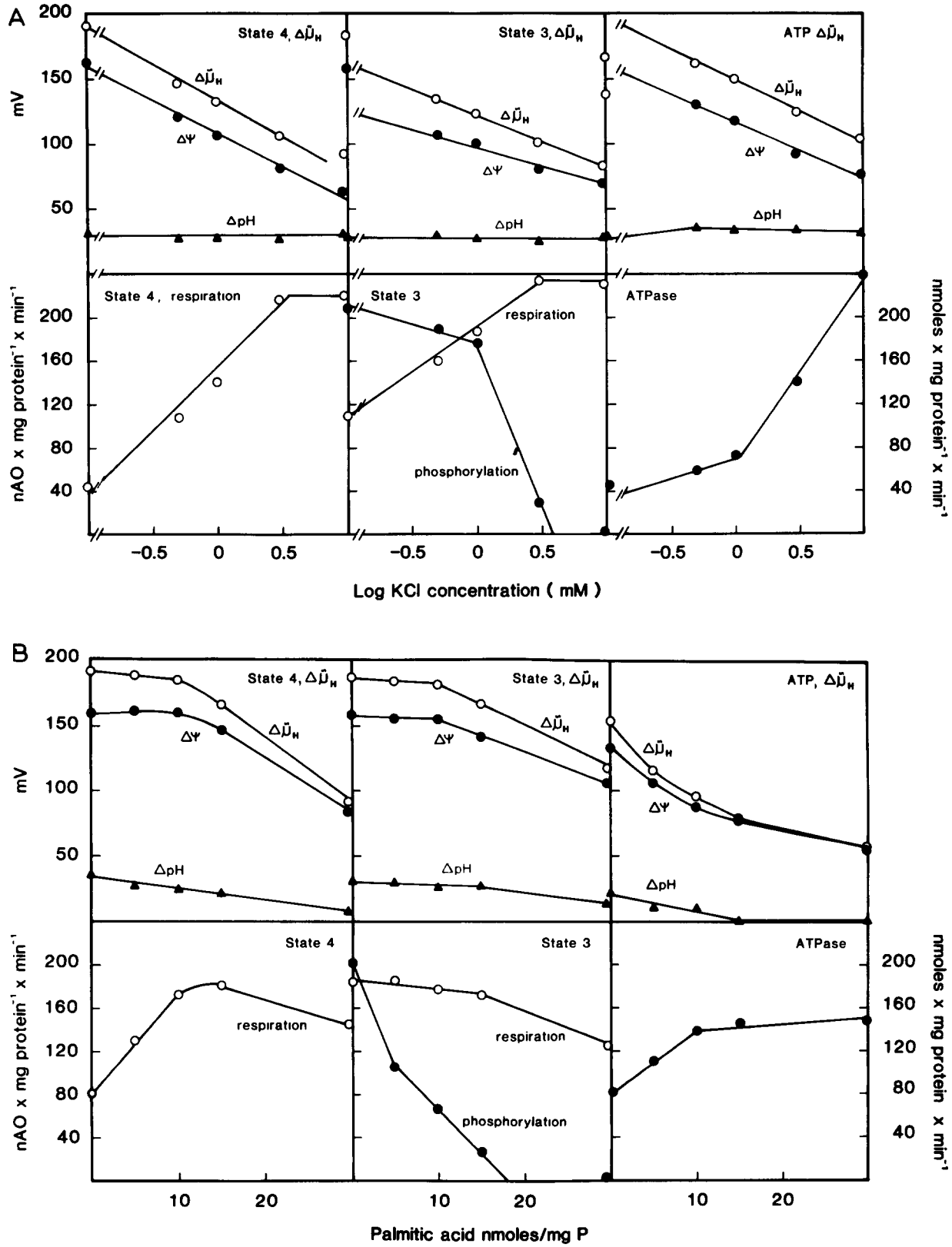

Fig. 3. Comparison of the effect of KCl (in the presence of valinomycin) and palmitate on energy conversion in rat liver mitochondria [64]. (A) In the left panels is shown the effect of increasing KCl concentrations on $\Delta\mu_{H^+}$ and the rate of respiration in State 4. The middle panels show the effect of increasing KCl concentration on $\Delta\mu_{H^+}$ and the rates of respiration and phosphorylation in State 3. The right panels show the effect of increasing KCl concentrations on $\Delta\mu_{H^+}$ generated by the ATPase and on the rate of the ATPase reaction. The bottom figure shows the corresponding effects of palmitic acid.

to direct effect on the ATPase that may be characterized as intrinsic uncoupling. It was recently shown that carnitine partially inhibits the uncoupling effects of general anesthetics, suggesting that part of their effects is the result of stimulation of a phospholipase A_2. The latter would lead to the release of free fatty acids and enhance the decoupling effect (see below) [83,84]. Thus, even under conditions that eliminate many of the inhibitory side-effects of these anesthetics, their uncoupling effects appear to be a mixture of intrinsic uncoupling, release of free fatty acids and direct decoupling. It is exceedingly difficult to separate these effects. Moreover, these anesthetics have the disadvantage of being both water-insoluble and extremely volatile. This makes the task of controlling their membrane concentration exceedingly difficult. While the potency of the anesthetics is increased at high temperature (37°C), their increased volatility at this temperature further complicates these studies. For these reasons, we have not pursued these studies and instead have concentrated our efforts on the search for more suitable decouplers.

III-B. Free fatty acids

Free fatty acids, at low concentrations, uncouple oxidative phosphorylation in mitochondria [85]. Mitochondrial preparations often contain sufficient amounts of free fatty acids to uncouple oxidative phosphorylation. Thus the use of albumin to remove the free fatty acids is necesary in order to obtain well-coupled mitochondria [86]. Although it was generally believed that free fatty acids uncouple by a reduction of $\Delta\mu_{H^+}$, this has never been established. High concentrations of free fatty acids indeed increase proton permeability of phospholipid membranes [87]. However, the basal proton permeability of mitochondrial and chloroplast membranes is much higher than that of phospholipid membranes, particularly at high values of $\Delta\mu_{H^+}$ [88]. Thus, a very high concentration of free fatty acids would be required to produce a significant increase in H^+ permeability in phosphorylating mitochondria or chloroplasts. We have shown that the uncoupling of oxidative phosphorylation in rat liver mitochondria by oleate and palmitate is not accompanied by significant reduction of $\Delta\mu_{H^+}$ [64]. Fig. 3 illustrates the difference in the uncoupling patterns between free fatty acids (palmitate) and ionophores (valinomycin $+K^+$) in rat liver mitochondria. In contrast to valinomycin, palmitate stimulates State 4 respiration and inhibits ATP synthesis without significant effect on $\Delta\mu_{H^+}$ (measured from the distribution of ^{86}Rb and [^{14}C]DMO). However, the stimulation of ATPase is associated with a reduction of $\Delta\mu_{H^+}$. Moreover, palmitate only stimulates State 4 to the level of State 3, while valinomycin stimulation of State 4 exceeds this level significantly. Similar decoupling effects of palmitate were also observed in chloroplasts [65]. Here, too, the decoupling was not associated with a reduction of $\Delta\mu_{H^+}$ or increased H^+ permeability in clear distinction from ionophores and permeable ions (e.g., nigericin and NH_4^+, see Figs. 2 and 4). In submitochondrial particles, palmitate uncoupled oxidative phosphorylation and reverse electron transport, but had no effect on ATP synthesis driven by an artifically imposed $\Delta\mu_{H^+}$ [75]. This latter observation clearly shows that palmitate does not collapse $\Delta\mu_{H^+}$ and does not cause intrinsic uncoupling of the ATPase. Studies with reconstituted cytochrome oxidase confirmed the conclusion that the stimulation of respiration by palmitate is not the result of increased proton permeability [89]. Also, recent studies of the effect of fatty acids on Ca^{2+} transport in mitochondria show that at the uncoupling range, palmitate did not affect membrane potential or electrogenic Ca^{2+} transport [90]. In contrast, Luvisetto et al. [60], while confirming our observation that oleate inhibition of ATP synthesis is not associated with a significant reduction of $\Delta\mu_{H^+}$, did observe significant reduction of membrane potential (measured by $TPMP^+$ electrode) associated with oleate stimulation of State 4 respiration. These investigators also concluded from the effect of oleate on valinomycin-induced K^+ efflux that oleate, in the uncoupling range, does increase the proton permeability of mitochondria. They explain the uncoupling effect of oleate in mitochondria as a combination of intrinsic uncoupling of the H^+ redox pump, inhibition of the ATPase and increased H^+ permeability. More recently, Schönfeld et al. [68] have studied the uncoupling of mitochondria by palmitate and observed even a larger reduction of $\Delta\psi$ associated with stimulation of State 4 respiration. They concluded that palmitate is a protonophore indistinguishable from classical uncouplers. We believe that a major source of the discrepancy between the results of Luvisetto et al. [60], Schönfeld et al. [68] and others [64,65,75,89,90] arises from different experimental procedures. Free fatty acids (FFA) are insoluble in physiological pH [91]. When a drop of concentrated ethanol solution of fatty acids is added to buffered solutions there will be instant precipitation of insoluble fatty acid salts. To insure incorporation into the membrane, it is necessary to add diluted solution to vigorously vortexed mitochondrial suspension. Using these precautions, one observes a very high potency of FFA uncoupling. This is in contrast to many published studies (cf. Ref. 92), including that of Luvisetto et al. [60], in which the uncoupling activity of oleate is observed only at concentrations which are at least 20-fold higher than those observed in our previous study [64]. When only a small fraction of the added FFA is incorporated into the membrane, a small difference in the mode of stirring and incubation conditions may result in large differences in actual membrane concentrations in parallel assays of $\Delta\psi$, respiration and phosphorylation. Moreover, Luvisetto et al. [60] ap-

parently did not take any precaution to prevent the peroxidation of oleic acid. Peroxidized fatty acids are good detergents and collapse $\Delta\mu_{H^+}$ at high concentration. However, there is no uncoupling at low concentrations (Rottenberg, unpublished data). We have recently reinvestigated the effect of oleic acid on rat liver mitochondria with additional precaution to prevent peroxidation and have found further enhanced decoupling activity even compared to our original study. The concentration of oleic acid required for 50% uncoupling was 1.5 nmol/mg protein. This level is almost 100-fold more potent than that observed by Luvisetto et al. [60]. In this concentration range there was no significant effect on $\Delta\psi$ as measured by either ^{86}Rb distribution or by $[^3H]TPP^+$ distribution (Rottenberg, unpublished data). Thus, it appears that unsaturated fatty acids are much more potent decouplers than saturated fatty acid, but this effect is difficult to observe unless precautions are taken to prevent peroxidation. Another significant difference between our study and that of Luvisetto et al. and Schönfeld et al. is their use of lipophilic cation electrode for the estimation of $\Delta\psi$ which requires the use of high concentrations of lipophilic cations. We have observed synergistic uncoupling effects of lipophilic cations and fatty acids on mitochondria (Rottenberg, unpublished data). Apparently, lipophilic cations, at high concentration, enhance the permeability of the fatty acid anion, thus enhancing the protonophoric potency of fatty acids. This is very similar to the enhancement of the uncoupling potency of lipophilic amines by lipophilic anions [81].

Adreyev et al. [93] have suggested recently that the uncoupling effect of fatty acids is dependent on the activity of the ADP/ATP translocator. However, the modulation by carboxyatractyloside of FFA uncoupling, observed by Skulachev's group, is strongly dependent on the assay conditions and only results in relatively small attenuation of the FFA effect (cf. Ref. 68). Preliminary studies in our laboratory suggest that the translocator conformation has an effect on the binding of FFA to the ADP/ATP translocator on the matrix surface of the inner membrane possibly due to its positive surface charge [94]. This would affect the amount of fatty acid available for decoupling. However, this relatively minor effect on the potency of FFA uncoupling is not essential to FFA uncoupling, as is evident from uncoupling in cytochrome oxidase vesicles [90] and chloroplasts [65]. The results of our experiments also provide evidence that FFA inhibit ATP synthesis due to decoupling rather than a result of inhibition of the ATPase [60,73]. The synthesis of ATP in submitochondrial particles is inhibited when the reaction is driven by substrate oxidation but not when it is driven by artificially induced $\Delta\mu_{H^+}$ [75]. In chloroplasts, $\Delta\mu_{H^+}$ can be generated by adding a redox mediator such as pyocyanine. Pyocyanine produces an artificial coupling site by shuttling protons across the membrane [95]. Fatty acids do not inhibit ATP synthesis driven by pyocyanine-generated $\Delta\mu_{H^+}$ at the low concentration range which inhibits PS I- and PS II-driven phosphorylation [65]. This is not the result of high rates of proton pumping, since the degree of inhibition does not depend on the rate of pumping. Also, in chloroplasts, phosphorylation driven by PS I is more sensitive to FFA uncoupling than phosphorylation driven by PS II, suggesting a different decoupler sensitivity for different proton pathways [65].

At present, there is little information regarding the mechanism of action of FFA. However, experiments with fluorescent fatty acid suggest that in mitochondrial membranes, particularly at the uncoupling range, FFA are tightly associated with membrane proteins (Rottenberg, unpublished data). We have observed an enhancement of the oligomycin sensitivity of the ATPase by FFA and inhibition of $\Delta\mu_{H^+}$ when generated by ATP hydrolysis, suggesting direct effect on the ATPase [64]. Thus, these preliminary observations suggest that the main site of free fatty acid decoupling is the F_0F_1 complex. However, direct interaction with redox proton pumps may also contribute to decoupling [90].

III-C. Gramicidins

The linear gramicidins are short, hydrophobic, peptide antibiotics produced by *Bacillus brevis*. The major component of the extracted mixture is gramicidin A, a peptide of 15 alternating L- and D- uncharged hydrophobic amino-acid residues in which the amino-terminal headgroup, valine, is blocked by a formyl group and the carboxy-terminal tail group is blocked by ethanolamine [96,97]. The uncoupling activity of the gramicidins has been known for a long time [10]. In 1965 it was discovered that the uncoupling of oxidative phosphorylation in mitochondria by gramicidin is associated with massive monovalent salt uptake and swelling similar to uncoupling by valinomycin [98,99]. Soon afterwards, it was discovered that gramicidin forms a dimeric channel which increases the membrane permeability to monovalent cations [100,101]. It was logical to assume that the mechanism of uncoupling by gramicidin was due to this ionophoric activity because such a mechanism would lead to the collapse of $\Delta\psi$, ΔpH or both. Earlier observations appeared to confirm this suggestion [102–104] and therefore this was considered to be the established mechanism of uncoupling (cf. Ref. 105). In our study of the uncoupling activity of fatty acid in mitochondria [64], we included gramicidin for comparison as a typical ionophore. We expected the pattern of uncoupling to be exactly like valinomycin. To our surprise, gramicidin showed much greater potency as uncoupler in relation to its effect on $\Delta\mu_{H^+}$ (see Figs. 2B, 4B). These results led us to conclude that it acts

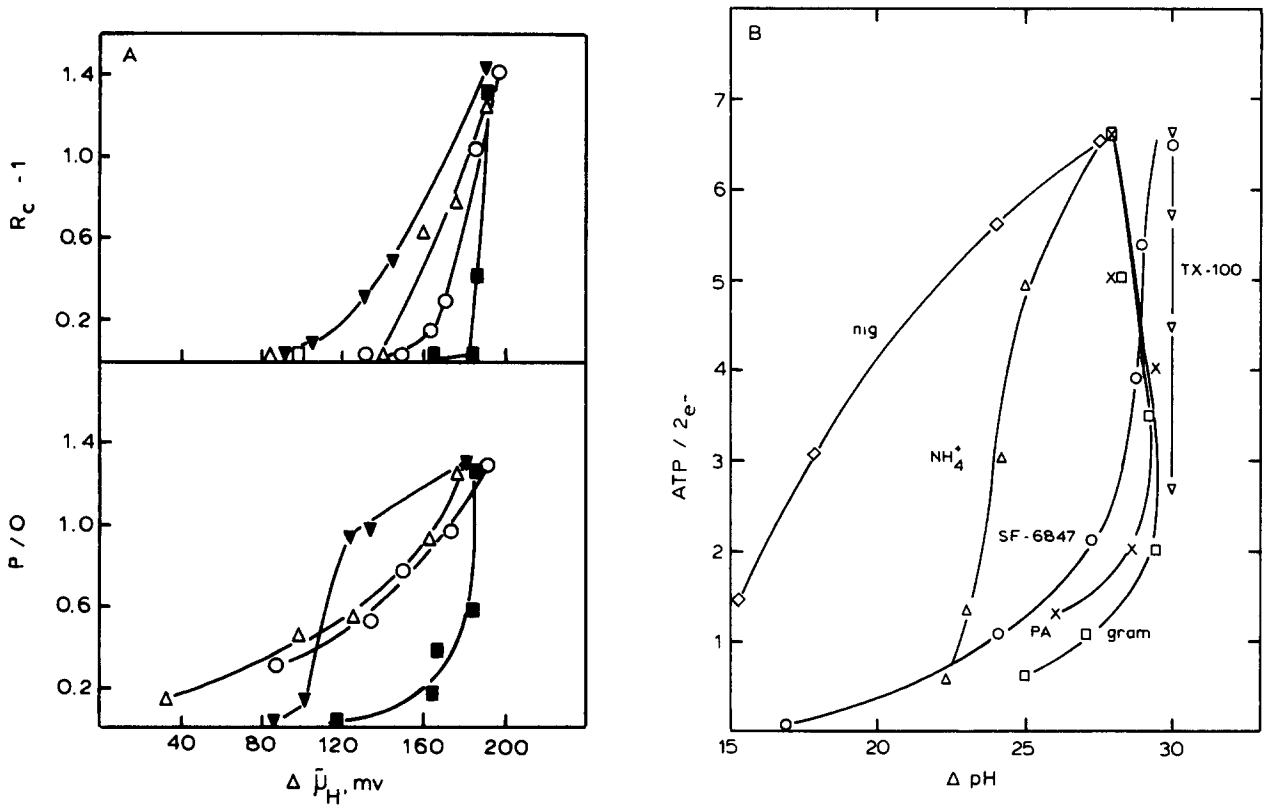

Fig. 4. Summary of the effect of various uncouplers in mitochondria and chloroplast on the relationships between the $\Delta\mu_{H^+}$ and the P/O and the respiratory control ratio. (A) is from Ref. 68 and shows the results in rat liver mitochondria with valinomycin $+K^+$ (▽), gramicidin $+Na^+$ (△), FCCP (○) and palmitate (■) (B) Shows the results of similar experiments with chloroplasts [65].

partially as an ionophore and partially as a decoupler. Also unexpectedly, gramicidin's effect on the $\Delta\mu_{H^+}$ generated by ATP was much greater than its effect on the $\Delta\mu_{H^+}$ generated by the redox pump. As discussed above, this is one of the characteristics of decouplers. Even more unexpected was the finding that, while gramicidin (like valinomycin) did not reduce the ΔpH generated by the redox pumps, it completely abolished, at very low concentrations, the ΔpH generated by the H^+-ATPase [64].

Moreover, in chloroplasts, uncoupling by gramicidin appears to be completely independent of its effect on $\Delta\mu_{H^+}$ [65]. At the uncoupling range of photophosphorylation, there is no significant effect on the magnitude of either $\Delta\psi$ or ΔpH (Figs. 2A, 4A). Thus, in this system gramicidin behaves as a pure decoupler. In addition, gramicidin did not inhibit ATP synthesis driven by the artificial coupling site induced by pyocyanine at the concentration range that inhibits PS II- or PS I-driven photophosphorylation. Much higher concentrations of gramicidin were needed to inhibit pyocyanine-driven ATP synthesis. This inhibition did correlate with reduction of ΔpH. Like other decouplers, gramicidin also decreases ΔpH in chloroplasts when generated by the ATPase [65]. The difference between the decoupling profile of gramicidin in chloroplasts and mitochondria arises from the fact that in chloroplasts, at steady state, the major component of $\Delta\mu_{H^+}$ is ΔpH, whereas in mitochondria it is $\Delta\psi$ [22]. Therefore, to collapse $\Delta\mu_{H^+}$ in chloroplasts it is necessary to induce proton efflux. For collapsing $\Delta\mu_{H^+}$ in mitochondria, it is sufficient to induce monovalent cation uptake. Even though gramicidin is selective for protons over other monovalent cations, the proton concentration, even at the very low intrathylakoid pH, is still only in the micromolar range (10 μM) and hence, relatively high concentrations of gramicidin are required to collapse ΔpH when the latter is generated by electron transport.

In contrast in mitochondria, where $\Delta\psi$ is the major component of $\Delta\mu_{H^+}$, millimolar concentrations of monovalent cations (Na^+, K^+ or NH_4^+) easily collapse $\Delta\psi$ and account for the higher potency of gramicidin as ionophore in the presence of these cations. For the same reason, gramicidin is quite potent in collapsing flash-induced $\Delta\psi$ in chloroplasts suspensions provided the medium contains monovalent cations [102]. However, in the absence of monovalent cations, gramicidin behaves very much like a pure decoupler, uncoupling oxidative phosphorylation without effect on $\Delta\mu_{H^+}$ [106–110]. At high monovalent cations concentration, gramicidin un-

coupling is a mixture of decoupling and the classical behavior of ionophores as exemplified by valinomycin (Fig. 2B). There is, however, a very clear difference between these two ionophores. Valinomycin, even at high concentrations, will not uncouple oxidative phosphorylation unless high concentrations of potassium salts are added [47], while gramicidin is a potent decoupler, even in the absence of monovalent salts.

III-D. The mechanism of gramicidin decoupling

One of the unexpected features of the action of all decouplers is the manifestation of specific effects on the H^+-ATPase. As noted above, all the decouplers studied to date reduced the magnitude of $\Delta\mu_{H^+}$ when generated by the H^+-ATPase. This finding suggests that the principal site of action of decouplers is associated with this pump, and by implication that this pump plays a central role in intramembranal coupling. The results of our studies of gramicidin decoupling in mitochondria add an additional important detail to this general observation. As already discussed above, in mitochondria, where proton concentration is of the order of 10^{-7} to 10^{-8} M, the ionophoric activity of gramicidin should be due entirely to monovalent cation transport, because monovalent cations are present at much higher concentrations (10^{-4} M to 10^{-1} M). Thus, gramicidin (like valinomycin) should collapse $\Delta\psi$, but not ΔpH. ΔpH should actually increase (to compensate for the reduction of $\Delta\psi$). Indeed, this pattern is observed when $\Delta\mu_{H^+}$ is generated by the redox pumps [64,107]. However, when $\Delta\mu_{H^+}$ is generated by H^+-ATPase, ΔpH collapsed at extremely low gramicidin concentration [107], even in the presence of a high concentration of monovalent cations [64]. This finding strongly suggests that gramicidin has access to protons generated by the ATPase H^+-pump before they equilibrate with the bulk phase. The apparent ability of gramicidin to accept protons from the H^+-ATPase directly led us to question whether the dimeric channel, which is responsible for the transport of ions across the membrane, was necessary for the decoupling activity of gramicidin. Therefore, we investigated the decoupling activity of gramicidin derivatives which cannot form dimeric head-to-head channels [111]. We have identified two such derivatives, desformyl gramicidin and des(formylvalyl) gramicidin, which decouple oxidative phosphorylation almost as well as gramicidin itself [107]. These derivatives, in which the formyl headgroup has been removed, cannot form the conductive head-to-head dimer channel. Under low salt conditions, these derivatives show very little ionophoric activity in mitochondria [107]. Yet, their decoupling activity is almost as strong as that of gramicidin. These results suggest that a transmembrane channel is not necessary for decoupling oxidative phosphorylation. It is possible that a monomer may form the same helical structure that produces the head-to-head channel [112]. Such a half-channel could not extend from surface to surface, but only from the surface to the membrane core. It is interesting that the derivatives, unlike gramicidin itself, were not capable of collapsing ΔpH when the latter was generated by the H^+-ATPase. This suggests that the site of proton extraction which collapses ATPase-induced ΔpH is near the surface, while the site of proton extraction in decoupling of oxidative phosphorylation is in the membrane core. We discovered later [108] that at high concentration of Rb^+ or K^+ salts these gramicidin derivatives do form a transmembrane channel, most probably of the anti-parallel double-helix ('pore') type. However, at the low salt concentrations in which we conducted our experiments on decoupling, there was little channel activity. Therefore, the decoupling activities of the derivatives cannot be attributed to a transmembrane channel [107].

Additional evidence for the ability of gramicidin to release occluded intramembranal protons comes from studies in chloroplasts [113]. Theg and Junge found that protons that are generated by PS II are deposited first into a 'membrane domain' and that these occluded protons can be released by gramicidin [114]. These protons were found to be delocalized [115]. Moreover, Junge and his colleagues showed that removal of F_1 also allows these protons to escape into the bulk [116]. It is also known that photophosphorylation driven by PS II is much more sensitive to gramicidin than phosphorylation driven by either PS I or cyclic electron transfer [65,117]. Thus, it appears that an intramembranal proton pool shared by PS II and the ATPase can be drained by gramicidin.

III-E. Molecular mechanism of decoupling: a working hypothesis

Because the molecular mechanisms of the proton pumps are still very poorly understood, it cannot be expected that a precise molecular mechanism for decoupling can be established at present. However, we believe that the information obtained from the experiments with ionophores, protonophores and decouplers provide important clues to the molecular mechanism of proton pumping. It is, therefore, not premature to attempt to combine this information with our present knowledge of the molecular aspects of proton pumping.

The most advanced understanding of a proton pump mechanism comes from the studies of bacteriorodopsin [118]. This simple pump consists of a single peptide and a retinal chromophore. Many of the intermediate steps in the H^+-pumping cycle have been characterized by spectroscopic techniques. The generation of a number of random and site-directed mutations (cf. Refs. 118, 119, 120) enabled the evaluation of the participation of

several amino acid residues in the proton pumping cycle. Discussion of these studies is outside the scope of this review; however, the picture that emerges may be relevant to other proton pump mechanisms. The chromophore retinal is believed to serve as a light-driven gate of the proton 'pore' through the protein. The photochemical driving reaction, the isomerization of the retinal chromopore, serves to move a proton from one acidic residue (Asp-96) to another (Asp-85) over a strong permeability barrier a short distance within the protein (5 Å). The proton pathways, within the pump, from the membrane surfaces to and from these groups appear to be less well defined. It is possible that pumped protons could escape the protein within the membrane before reaching the surface. Such a mechanism would allow intramembranal proton pathways between the bacteriorodopsin molecules and other proton pumps.

The proton ATPase of the F_0F_1 type is a much more complex enzyme. Even the simplest known species, the *E. coli* F_0F_1, consists of at least 22 subunits consisting of 8 different peptides [28]. The ATPase of higher organisms is even more complex. Nevertheless, recent studies, mostly with the *E. coli* enzyme, have resulted in an emerging consensus for the proton pathway within this enzyme. The F_1 complex is located outside the membrane and catalyzes the reversible reaction, ADP + $P_i \leftrightarrows$ ATP, as well as the energy-dependent binding of ADP and release of ATP. This complex is not considered to participate directly in proton pumping. The emerging consensus is that energy from the proton flow through F_0 is transferred to the F_1 complex by conformational interactions between peptides of F_0 and F_1. Within F_0, which in *E. coli* consists of one copy of subunit *a*, two copies of *b* and approximately 10 copies of *c*, only *a* and *c* are believed to be directly involved in proton transport. Subunit *c* has one polar group (Asp-61), which is buried in the hydrophobic core of the membrane, that appears to play a crucial role in H^+ translocation and energy conversion (cf. Refs. 121, 122). This group can be specifically reacted with the lipophilic carboxylic reagent DCCD. The covalent binding of one molecule of DCCD per F_0 inhibits proton pumping, oxidative phosphorylation and ATPase activity. Studies of several F_0 mutations have led to the identification of three other polar residues on subunit *a* (Arg-210, Glu-219, His-245), which are believed to participate in proton transport (cf. Refs. 123, 124). Although the exact structure of this subunit is not known, models based on hydropathy patterns suggest that these polar groups are buried in the membrane but are positioned close to membrane surfaces. Thus, a model has emerged [125,28], according to which the DCCD binding group (Asp-61) on subunit *c* serves to shuttle protons between groups of subunit a on two sides of the membrane (Fig. 5). This model bears a certain resemblance to the model of the bacteriorodopsin pump in that

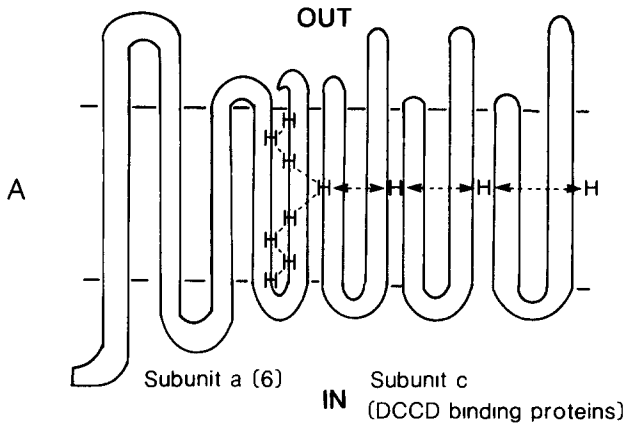

Fig. 5. A model for proton pathways in F_0F_1-ATPases. The vertical pathway from surface to surface is in accordance with Refs. 28, 125. Additional parallel pathway in the core of the membrane is provided by the multiple of subunits *c*.

the crucial coupling step is the transfer of protons between two groups on the same peptide over a relatively short distance. In the 'moving arm' model of bacteriorodpsin, light-driven proton pumping appears to be due to isomerization of the retinal in which the Schiff base serves to transfer the protons from one amino acid residue to another. The corresponding process in the F_0 could be a conformational change in subunit *c* driven by ATP dissociation from F_1 which is coupled to moving a proton by Asp-61 between two acidic groups on the inner and outer sections of subunit *a*. One aspect of the F_0 structure, which is not addressed by this model is the function of the large number of additional copies of subunit *c*. It has been proposed [125] that these subunits surround the other subunits in a circle. Rotation of this complex of subunits *c* in relation to the core of F_0 is suggested to be linked to the alternative site coupling and rotation of F_1 [126]. However, others have pointed out that a separate oligomeric complex of subunit *c* appears to exist and that the two complexes may rotate relative to each other [127–129]. Here I present a similar model (Figs. 5, 6) which postulates an entirely new function to the complex of multiple subunits *c*. I suggest that, in addition to moving protons between the two acidic groups of subunit *a* in a direction perpendicular to the membrane surface, the Asp-61 groups of subunit *c* serve to move protons laterally, parallel to the membrane surface, between proton pumps. This lateral movement is acomplished by the rotation of the complex. Assuming a fast exchange of protons with similar groups on other pumps (both other ATPases and redox pumps, see below) this 'network' of DCCD-binding groups in the core of the membrane would form an intramembranal pool of occluded protons. Other membrane proteins may also contribute to this pool. Dilley [132] has suggested that in chloroplasts, plastocyanin and PS II-associated

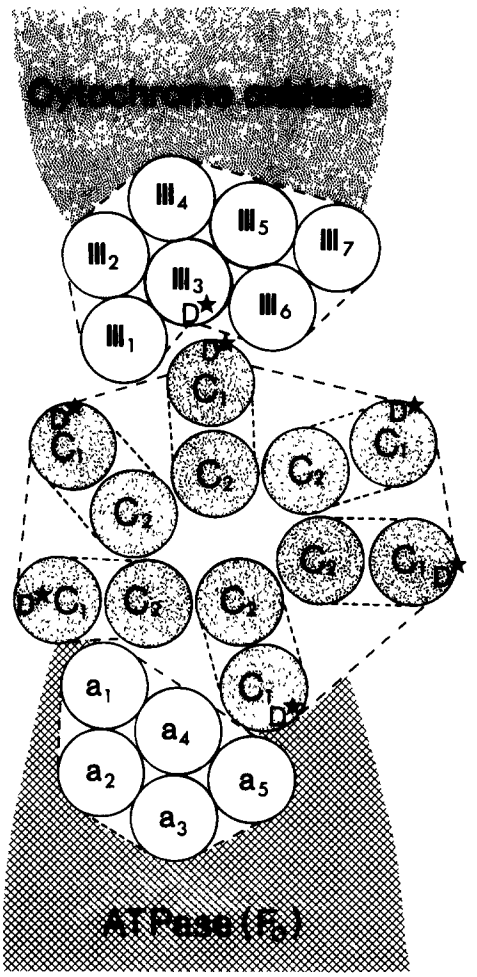

Fig. 6. A sectional view, at the core of the membrane of the model shown in Fig. 5. This view demonstrates the postulated role of rotation of the complex of subunits *c* in intramembranal coupling and the asymmetry of the model in regard to decoupler sensitivity which results from rotation in one direction; see text for further details.

peptides contribute to the localized proton domain. I further postulate that decouplers can release protons from the occluded proton 'pool.' This can be done either by providing a direct escape route for protons from the core of the membrane (e.g., gramicidin or gramicidin derivatives) or by loosening of the structure of the complex and thus allowing protons to diffuse out of the complex.

One important feature of decoupling that can be accounted for by the model is the fact that $\Delta\mu_{H^+}$-driven ATP synthesis is not inhibited by decouplers, while the generation of $\Delta\mu_{H^+}$ by ATP is inhibited. For that purpose I postulate that the complex always rotates in the same direction regardless of the direction of the reactions. For example, let us assume that the subunits *c* complex always rotates clockwise as in Fig. 6. When protons move from the outer surface to the inner surface, as is the case in $\Delta\mu_{H^+}$-driven ATP synthesis, the proton which is picked from the acidic group on outer the segment of subunit *a* is immediately deposited to the acidic group on the inner segment of subunit *a* after a very short clockwise rotation moving only a few Ångstroms. Thus, in this mode, the proton is always within the protein complex, protected from agents in the lipid phase and, therefore, cannot be removed by decouplers. However, when the enzyme is pumping protons, moving protons from the inner surface to the outer surface the complex of subunits *c* must make a full rotation before the protons can be deposited in the acidic group on outer the segment, since the complex always rotates clockwise. This has the advantage that the protons, before reaching the outer surface of F_0, can be donated to another pump and thus, produce intramembrane coupling (e.g., reverse electron transport). In this rotation the protons are exposed to the lipid phase and can be removed by decouplers. During oxidation- or photo-phosphorylation, this rotation allows the complex of subunits *c* to pick up protons directly from the redox pumps, resulting in intramembranal coupling. These protons, however, are susceptible to dissipation by decouplers. Only endogenous redox pumps and proteins which have the appropriate H^+-binding groups can participate in such processes. Thus, the artificial H^+-pumping site produced by redox mediators, such as pyocyanine or PMS in chloroplasts, cannot produce intramembranal coupling because both protons and electrons must be delivered together at the membrane surface. Hence, these processes are insensitive to decouplers. The model of proton pumping depicted in Fig. 6 results in asymmetry of the ATPase in regard to decouplers and in relation to ATP synthesis and hydrolysis in general. Evidence for such asymmetry also arises from kinetic studies (cf. Ref. 126).

Fig. 6 shows, schematically, proton transfer to the complex of subunits *c* from cytochrome oxidase. This transfer could occur directly when these complexes collide, or indirectly in transient aggregates through the network of the DCCD-binding groups and other participating proteins. The function of the DCCD-binding group of subunit III of cytochrome oxidase has not been fully elucidated as yet [130]. It is possible that one important aspect of its function is to mediate intramembranal proton transfer to the complex of subunits *c*. Since it appears that all redox pumps which participate in oxidative phosphorylation, have DCCD-binding groups, which are important for coupling [130,131], this may be the principal route of intramembranal proton transfer. The temperature dependence of decoupling is also explained by this model, since protein aggregation at low temperature [38] would hinder access of decouplers to these sites.

III-F. Conclusions and outlook

Current studies of the mechanism of uncoupling by various agents in mitochondria, chloroplasts and

bacteria suggest that in addition to the collapse of $\Delta\mu_{H^+}$, uncoupling could be the result of the release of occluded protons from the coupling membrane. The release of protons from pools of occluded protons which are shared by the pumps would lead to a particular profile of uncoupling effects which was defined as decoupling. The release of protons from specific sites within the pumps may lead to another profile which was defined as intrinsic uncoupling. The data obtained so far suggest that uncoupling by free fatty acids and gramicidin (in the absence of alkali cations) is mostly due to decoupling. General anesthetics appear to produce a mixture of decoupling and intrinsic uncoupling. Uncoupling by protonophores could be a mixture of intrinsic uncoupling and the collapse of $\Delta\mu_{H^+}$. Uncoupling by ionophores and permeable ions appear to be due to the collapse of $\Delta\mu_{H^+}$. These different classes of uncouplers should provide a useful tool in elucidating the proton pathways within and between the proton pumps. The challenge for investigators in this field is to find even more specific agents for each class of uncouplers so that a specific mechanism can be studied without interference from side-reactions. In addition, specific assays could be developed for the investigation of each type of uncoupling.

The information obtained from studies with decouplers and intrinsic uncouplers could be combined with information from molecular and genetic studies for the construction of models for proton transport. I have suggested a role for intramembranal DCCD binding groups in mediating proton exchange between the redox and ATPase H^+-pumps and suggested an assymetric model of the proton pathway in the F_0 complex of the H^+-ATPase which could explain these results. This model is open to experimental tests.

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