Chapter 7

THE MEMBRANES INVOLVED IN PROTON-MEDIATED FREE-ENERGY TRANSDUCTION: THERMODYNAMIC IMPLICATIONS OF THEIR PHYSICAL STRUCTURE

Hans V. Westerhoff, Douglas B. Kell, Frits Kamp, and Karel van Dam

TABLE OF CONTENTS

I. INTRODUCTION

The history of membrane-linked bioenergetics parallels the history of the biochemistry of intermediary metabolism. At first, oxidative phosphorylation was seen as a single process. Then, with the chemical and the chemiosmotic coupling hypotheses, the overall process was split up into clearly distinguishable parts. This reductionism has, in both fields, led to an enormous increase in our understanding. Thus, it is now rather well established that protons play the central role in oxidative and photophosphorylation.[1] Electron transfer chains, bacteriorhodopsin, and the ATP synthase (in this chapter we shall use this term to exclusively denote the F_0F_1 H^+-ATPase) can all function as proton pumps. If they are reconstituted into the same phospholipid vesicle, oxidative- or photophosphorylation is catalyzed, although it remains unclear whether proteinaceous devices additional to the minimal proton pumps are required.[2] Artificial electrochemical potential differences for protons in these and other more physiological systems can drive ATP synthesis. Increased proton permeability of the energy-coupling membranes uncouples phosphorylation from electron transfer, at least to some extent.

As the parts of intermediary metabolism were "solved", interest shifted back to the whole. It was realized that even if the component parts (each corresponding to an enzyme-catalyzed reaction) behaved as independent units solely connected through the concentration of metabolites, the whole was appreciably more complex than the simplest sum of the parts. Several methods to evaluate the proper "sum" of independent parts, such as nonequilibrium thermodynamics and metabolic control theory (reviewed in Reference 1), have been developed and applied to oxidative phosphorylation.

The notion that the enzyme-catalyzed reactions behave as independent units was merely the result of the application of Occam's razor (the simplest explanation is preferred) and the success of reconstituting metabolic pathways with the isolated enzymes. Initially, the available structural information suggested that the cell consisted of a water-like phase within which organelles are suspended. Further electron microscopic evidence was consistent with the inside of the metabolically active organelles also being a water phase. Glycolytic- and Krebs-cycle enzymes could be purified to homogeneity while retaining their activity. Thus, the concept of the cell as a number of bags of enzymes dissolved in saline, with metabolite translocators in the walls of the bags, arose. We find it important to confirm that, indeed, if the enzymes were organized in this fashion, metabolism could proceed. On the other hand, it is important to note that it really is Occam's razor that led to this view of dispersed metabolism, not strict experimental verification.

Observations not directly in line with this picture of metabolism as occurring in a soup[3] have been available for a long time. Ling[4] has given an elegant though perhaps one-sided, overview. A striking example is that upon rupture of a plant or protozoan cell, the cytoplasm can be squeezed out like toothpaste out of its tube[4,5] and is osmotically active.[6] However, it was only the increased resolution of the electron microscope, as well as the development of new procedures of fixation, that demolished the structural basis for the "broth" view of

metabolism (see, e.g., References 7 and 8, and see, however, Reference 9) and replaced it with a picture of vermicelli soup.[3] It turned out that the water phases of the cell do not solely consist of saline with dissolved enzymes. They are home to an array of filaments, including microfilaments and microtubules. Strikingly, organelles such as mitochondria are not always simply dissolved in the aqueous phase, but may be bound to and propelled along microtubules.[10] None of this is controversial. What remains controversial is the question whether there exists in vivo an even finer fibrous network, called the "microtrabecular lattice",[7,11] and whether the enzymes are bound to, or perhaps even constitute, the threads of such a lattice (the vermicelli in the soup[3]), rather than float individually between the microfilaments and microtubules. In the former, but perhaps even in the latter case, the standard view of metabolism as the nonsimple sum of independent enzyme-catalyzed reactions must be modified. Different reactions could sense each other in ways other than through the concentrations of the intermediary metabolites.

In the other chapters of this book and in Reference 12, much can be found on this issue. It is our purpose to discuss it for the case of membrane-linked free-energy transduction. This process is special in that (1) most of the participating enzymes are not freely dissolved in the water phase anyway (though they may be dissolved in the two-dimensional membrane phase), (2) the intermediary "metabolite" (the proton) is extremely mobile, (3) there are energetic limitations in addition to the usual kinetic requirement, and (4) readily delocalizing electrical potentials are involved.

Indeed, although historically the tendency has been to assume that the cell is a bag of homogeneously dispersed enzymes (or a set of such bags), this is not a very realistic assumption whenever one is interested in the fine tuning of even glycolytic metabolism (see Chapters 2, 4, and 9). Below we review data indicating that such an assumption is even less realistic for membrane-linked free-energy transduction.

The total amount of recent information obtained in all relevant bioenergetic systems is too large to be reviewed within the present limitations. We shall, therefore, limit ourselves to aspects of free-energy transduction in mitochondrial membranes. Elsewhere[13] we shall discuss other membrane systems. As this chapter was being submitted, another review of biological free-energy transduction appeared.[14]

II. THE STRUCTURAL ORGANIZATION OF OXIDATIVE PHOSPHORYLATION: THE SIMPLE VIEW

A. Electron Transfer

The electron-transfer complexes reside in the inner membrane of the mitochondria. Three complexes are involved in the overall electron-transfer reaction between NADH and O_2. Electron transfer between the first two is catalyzed by ubiquinone, a lipid-soluble molecule where the redox active head, which may oscillate between the two sides of the membrane,[15] is connected to a long hydrophobic tail. The water-soluble cytochrome *c* shuttles electrons between cytochrome c_1 and cytochrome oxidase. Ubiquinone[16] and cytochrome *c*[17] have been shown to diffuse, the latter, at high and physiological ionic strengths, in three dimensions.[17,18]

It has been proposed[19] that electron transfer from NADH to O_2 involves random collisions between the participating enzymes (the traditional "complexes" I, III, and IV) and coenzymes ubiquinone and cytochrome *c*. Hackenbrock and colleagues[16,17,19] determined the diffusion coefficients for lateral diffusion of all the components of the electron transfer chain in fused inner mitochondrial membranes. From the turnover times observed in that system, one can calculate the mean square displacement of the enzymes and coenzymes during their average turnover at active (state 3) rates of respiration (Table 1, see also References 13 and 21). Reduced complex I, for instance, has an average lifetime of only some 0.3 msec. In

Table 1
EXPECTED DELOCALIZATION OF ELECTRON-TRANSFER CHAIN COMPONENTS

Redox[a] complex	State[b]	D[c] (10^{-10} cm²/sec)	Content[d] (nmol/mg prot.)	Conc.[e] (10^{10}/cm²)	S[f] (10^3 nm²)	(%)	τ[g] (msec)	Δx^2 [h] (nm²)	S_d[i] (10^3 nm²)	p_{det} [j]	r (nm)
I	tot	4	0.014	2.8	4	(1.2)					4
	ox	4		2.7	3.7		5.9	31	3.0	0.80	
	red	4		0.14	72		0.30	7	0.15	0.002	
II	tot	4	0.027	5.4	2	(0.08)					0.7
	ox	4		5.1	2		11	42	5.6	1.8	
	red	4		0.27	37		0.59	10	0.31	0.008	
Q	tot	30	0.86	169	0.059	(0.2)					0.2
	ox	30		152	0.066		340	639	1300	20 000	
	red	30		17	0.59		37	105	35	59	
III	tot	4.4	0.041	8.1	1.2	(1.6)					2.5
	ox	4.4		6.8	1.5		15	52	8.5	5.7	
	red	4.4		1.3	7.7		1.4	16	0.80	0.10	
c	tot	19	0.122	24	0.41	(1.7)					1.5
	ox	19		21.5	0.46		24	134	56	122	
	red	19		2.7	3.7		2.9	47	6.9	1.9	
IV	tot	3.7	0.095	19	0.53	(3.7)	21				2.5
	ox	3.7		15	0.67		17	50	7.8	12	
	red	3.7		3.8	2.6		4.3	13	0.50	0.2	
V	tot	8	0.2	40	0.25	(20)	18	76	18	72	4

[a] The different components of the electron-transfer chain involved in mitochondrial oxidative phosphorylation complexes I (NADH:Q oxidoreductase), II (succinate: Q oxidoreductase), III (QH_2: *c* oxidoreductase), IV (*c*: O_2 oxidoreductase), ubiquinone ('Q') and cytochrome *c* ('*c*'). Complex V is the ATP synthase.

[b] The fraction to which the other data apply (total, oxidized, reduced).

[c] Diffusion coefficient from Reference 17. It is assumed that there is no dependence of D on the redox state of the molecule or complex.

[d] Content in mitochondria from Reference 17.

[e] Concentration in number of molecules or complexes per unit surface area of the inner mitochondrial membrane; from Reference 19, calculated from column 4 assuming a surface area (one side) of 305 cm²/mg protein. To calculate the concentrations of the reduced and oxidized fractions, I, II, Q, III, *c* and IV were assumed to be 5, 5, 10, 16, 11 and 20% reduced, respectively as in Reference 17.

[f] The surface area per molecule, calculated by taking the inverses of the value in column 5. Between brackets the percentage of this surface area that is taken by the proteins' mass is indicated.

[a] Average lifetime, calculated by using a turnover time, of cytochrome oxidase of 48 electrons per second per aa_3, the fact that, I, II, III, c and IV catalyze 2, 2, 2, 1, 1, and 1 electron transfers, respectively and the relative concentrations of the complexes (column 4). For instance, for complex I red: $0.3 = \frac{1000}{48} \cdot \frac{0.14}{19} \cdot \frac{2}{1}$.

[b] The mean square displacement during the average lifetime of the molecule in its reduced or oxidized state. From $\Delta x^2 = 4D\tau$, taking D from column 3 and τ from column 7.

[i] The area over which 60% of the molecules delocalize during their average lifetime, calculated as $\pi \cdot \Delta x^2$.

[j] ρ_{del} is the delocalization ratio, i.e., the ratio of column 9 to column 6. This is an approximate measure of the number of molecules of a complex that mix during average lifetime; r is the assumed radius of the complex.

that time it will diffuse up to a mean square displacement of only 7 nm; i.e., 60% of the complexes would be within a circle of 154 nm^2 around the point at which they were reduced. The average area per reduced complex I is approximately 72,000 nm^2,[17] so that the delocalization of reduced complex I during its lifetime is only over some 0.002 complex I molecules (cf. Table 1 and Reference 13). Both in its oxidized and its reduced state, coenzyme Q would be largely delocalized. During its lifetime, Q would diffuse through an area that contains approximately 20,000 other molecules of Q. For QH_2 this number is 59 (cf. Table 1). In its oxidized state, cytochrome *c* would also delocalize. For reduced cytochrome *c*, the two-dimensional delocalization is rather limited (only over an area containing some two molecules of reduced cytochrome *c* (cf. Table 1). However, at physiological ionic strengths diffusion of cytochrome *c* in three dimensions may give rise to additional delocalization.[18]

The respiratory-chain complexes, especially when in their reduced state, do not delocalize. The extreme examples are the reduced complexes I and II, which in their average lifetime, diffuse only over an area less than 1% of the average area per respective complex. Oxidized complex III and oxidized complex IV may delocalize to some extent. Rich[21] has expressed the delocalization of QH_2 in terms of the number of bc_1 complexes residing in the area through which the former would diffuse during its turnover. This number amounted to 80[21] (see also 22).

Another property that indicates to what extent molecules delocalize is the τ_{coll}, i.e., the average time between collisions with another molecule of the same kind or with a molecule with which they are expected to react. In three dimensions the rate of collisions between molecules A and B is[23,24]

$$\nu/[A] = [B] \cdot 4 \cdot \pi \cdot (r_A + r_B) \cdot (D_A + D_B) \tag{1}$$

where r_A and r_B are the molecular radii of A and B, D_A and D_B are their diffusion coefficients. For enzymes in solution at a concentration of 10 μM with radii of 5 nm and with diffusion coefficients of $2 \cdot 10^{-7}$ $cm^2 sec^{-1}$, this leads to collision frequencies of 15,000 per sec; $\tau_{coll} \simeq 66$ μsec (see also Reference 25). Clearly, in three dimensions with unhindered diffusion, collision rates would be high enough for enzymes to collide many times per turnover.

To examine this point for the two-dimensional diffusion of electron-transfer catalysts, an equation[24] was used[16,17,20] which is based on the conjecture that one can obtain the collision rate between independently diffusing species by calculating their collision rate if one of the species would be considered fixed in space and adding that to the collision rate if the other species would be considered fixed:

$$\begin{aligned} 1/\tau_{coll} = \nu/[A] = \\ - 2 \cdot \pi \cdot [B] \cdot \{D_A/(\ln\{(r_A + r_B) \cdot \sqrt{(\pi \cdot [B])}\}) \\ + D_B/(\ln\{(r_A + r_B \cdot \sqrt{\pi \cdot [A]})\}) \end{aligned} \tag{2}$$

We took the ratio of the reactive lifetime of each component to the inverse of the collision frequency with some of the potentially relevant reaction partners. This number of diffusion encounters per average reactive lifetime is given in Table 2.

The diagonal elements of Table 2 give the number of times per reactive lifetime components of the electron-transfer chain encounter "themselves", i.e., another molecular complex of the same constitution. Again, one may conclude that in their reduced states, none of the redox components, except QH_2, delocalizes during turnover. Of the oxidized components only QH_2, *c*, and, to a limited extent, III and IV, delocalize. That the mobility of the complexes would not be sufficient to allow for random-collision electron-transfer between the complexes themselves is illustrated by the corresponding off-diagonal elements of Table

Table 2
NUMBER OF COLLISION ENCOUNTERS PER AVERAGE LIFETIME (ρ_{coll})

I	I_o	I_r	II_o	II_r	Q	QH_2	III_o	III_r	c_o	c_r	IV_o	IV_r	V
I_o	0.3												70
I_r		0.000			11		0.06						3
II_o			0.5										300
II_r				0.001	4		0.08						1.3
Q		11		4	4000								11000
QH_2						30	26						4000
III_o		0.06		0.08		26	2						80
III_r								0.02	2.2		0.8		6
c_o								2.2	40				300
c_r										0.4	5		32
IV_o								0.08		5	6		90
IV_r												0.2	20
V	30	0.6	14	0.3	500	90	36	3	300	13	80	7	300

Note: Using Equation 2, and the data listed in Table 3 of reference 17 (cf. Table 1), collision rates were calculated and converted to average times between collisions (τ_{coll}) for each partner (through division by its concentration). This Table lists the rates of the reactive lifetime to the collision time for each component for interaction with relevant other components. To obtain values on the diagonal the self-collision rate as calculated from Equation 2 was divided by 2.

2. For instance, reduced complex I only meets 0.06 oxidized complexes III in its lifetime, and reduced complex III only meets 0.8 complexes IV in its lifetime. Even if every collision were to lead to electron transfer, this would not allow for sufficiently rapid catalysis of the reduction of oxygen by $NADH_2$.

The emerging picture is that of a limited random collision model[19] of electron transfer, limited in the sense that the electron-transfer complexes are essentially localized (i.e., "stay at the same place") during turnover, whereas the coenzymes, ubiquinone and cytochrome *c*, are delocalized. Provided that a sizable percentage of the collisions results in electron transfer, the electron flux from NADH to oxygen may be accounted for by the collision rate between ubiquinone and cytochrome *c* and their redox partners.

B. The Standard View of the Organization of Mitochondrial Oxidative Phosphorylation

In the standard view of mitochondrial oxidative phosphorylation, the mitochondrion is seen as essentially identical to the swollen and fused mitoplasts with which Hackenbrock and colleagues performed many measurements of lateral diffusion; a single, continuous, inner mitochondrial membrane, separating a homogeneous aqueous matrix space from a homogenous external space. The outer mitochondrial membrane is considered a sieve that only retains macromolecules. Within each space, enzymes and metabolites are conceived to be present at uniform concentration, maintained through random diffusion. As one consequence, there is only a single transmembrane electrochemical potential difference for protons, $\Delta\tilde{\mu}_H$, per mitochondrion, and it is this $\Delta\tilde{\mu}_H$ that is the sole energetic intermediate between electron transfer and phosphorylation.

There is ample reason for assuming a structurally continuous water phase to be homogeneous in its proton electrochemical potential. To take a "worst possible" case, we shall consider that a proton pumped out by an electron-transfer complex would have to be replenished by a proton let into the matrix by an ATP synthase diametrically located on the supposedly spherical inner membrane (the sphere's diameter would be approximately 1.5 μm[26]). The proton flux per unit surface area could amount to approximately $8 \cdot 10^{13} H^+/cm^2/sec$ (from Table 1, assuming an H^+/O of 8 and turnover time of 20 msec). From the first law of Fick:

$$J = D \cdot \Delta c/\Delta x \qquad (3)$$

with a Δx of $1.5 \cdot 10^{-4}$ cm and a proton diffusion coefficient of 10^{-4} $cm^2 sec^{-1}$, one obtains a concentration difference of approximately 0.1 μM. At pH, 7 this amounts to a pH difference of only approximately a 0.1 unit. In reality, protons pumped out of the matrix space by an electron-transfer-driven proton pump may reenter through ATP synthases that are much closer-by than the ones diametrically apposed in the mitochondrion. This will further reduce heterogeneity in the proton electrochemical potential. Using the diffusion constant for OH^- for diffusion on the outside of thylakoid grana, Junge and Polle[27] estimated that there might be a pH difference of up to 0.3 units between the center of a chloroplast granum and its sides. In intact cells, dimensions are larger, and significant pH gradients have been calculated and found.[28,29] In chloroplasts, indirect experimental evidence for small lateral pH gradients exists.[30]

This standard view has been fruitful in directing much of the research towards the mechanism, thermodynamics, and control of energy coupling in mitochondrial oxidative phosphorylation (for reviews see References 1 and 31 to 33). However, the information which is available, but rarely reviewed, suggests that the structure of and around the inner mitochondrial membrane is much more complex than the homogeneous spherical surface of the standard view.[34] After reviewing some of this information, we shall discuss what the implications might be of the actual structure for the kinetic, thermodynamic, and control behavior of oxidative phosphorylation.

III. THE STRUCTURE OF MITOCHONDRIAL MEMBRANES

The paradigm for a biological membrane is a lipid bilayer forming a two-dimensional sea in which membrane proteins float.[35] Although this picture corresponds well to what is seen in electron microscopic pictures of eukaryotic plasma membranes, most energy-transducing membranes appear to have more complicated structures.[36] They often consist of more than 50% protein, and their staining pattern in the electron microscope often deviates from the canonical railroad track (in the case of Osmium staining). Moreover, the secondary structure of the inner mitochondrial membrane is not the spherical surface frequently drawn in schemes of energy coupling (e.g., Figure 1A).

Numerous electron microscopic studies[37,38] have indicated that the inner mitochondrial membrane is highly folded. Other experimental methods, such as light-scattering and gravimetric methods, have confirmed this. For instance, from the data of Massari et al.,[39] it can be estimated that the surface area available for water permeation can increase more than fivefold upon swelling of the matrix space beyond the point where the outer membrane, if present, would break. This suggests that in rat liver mitochondria with an intact outer membrane, the inner mitochondrial membrane is folded at least to the extent that the surface area of the sphere it occupies is less than 20% of the total surface area of the membrane, In 0.10 M KCl this may even be less than 4%. For condensed rat liver mitochondria recent stereological analyses revealed that the surface area of the inner membrane approximately exceeds that of the outer membrane by a factor of 3.5.[26]

Already early on[37,38] it was shown that the folding of the inner membrane was not random (Figure 1B), as in an incompletely inflated paper bag, but such that part of the inner membrane would appear to be maximally expanded so as to cover the outer membrane from the inside. This part would thus enclose a sphere, or, more often, a cylinder rounded on both ends. The excess inner membrane would consist in involutions from this cylinder (Figure 1C). These involutions were called the mitochondrial *cristae*.[37] In principle, these could form (inverted) spherical saclets; rather, they appeared to be flat pockets.

The view that the *cristae* are not simple involutions of the inner surface membrane, but unconnected vesicular structures,[38] is almost as old as the view that they are.[37] Much of the issue was resolved when Daems and Wisse[40] (see also References 41 to 43) observed that

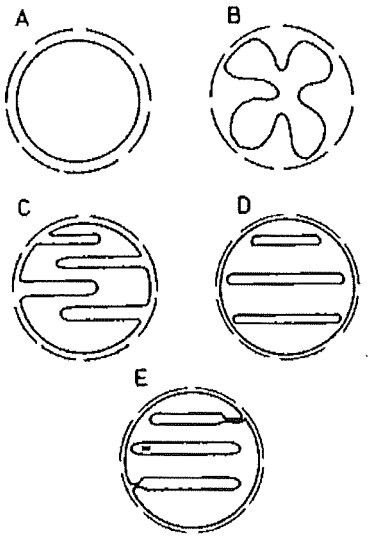

FIGURE 1. Five views on mitochondrial structure. The broken outer circle is the outer membrane. The views differ with respect to the structure of the inner mitochondrial membrane(s). (A) The diagram common in discussions of chemiosmotic coupling;[1] a homogeneous inner membrane as a sperical shell (or equivalently as essentially a flat membrane). (B) The view behind the diagram (A);[1] the single inner membrane may be folded (because its surface area exceeds that of the outer membrane), but this does not produce any essential diffusion limitations, or membrane inhomogeneities. (C) The view[37] that the inner mitochondrial membrane is continuous, where a part takes the form of a spherical shell just below the outer membrane, whereas the rest consists of involutions (the '*cristae*') taking the form (in the orthodox conformation) of flat surfaces. (D) The view[38] that the inner mitochondrial membranes are discontinuous, such that they consist of an 'inner surface membrane' covering the outer ('surface') membrane from the inside, separate from saclets within the matrix space (the *cristae*). (E) The now-accepted structure[40] in which the *cristae* membranes are clearly distinct from the inner surface membranes, but are connected to them through stalk-like structures called *pediculi* ('footlets'); through these *pediculi* the inside of the *cristae* would be in limited connection with the space between the two surface membranes. Note that in this figure, single, not double lines refer to membranes.

tubular structures, approximately 30 nm in diameter and called *pediculi*, connect the *cristae* to the inner surface membrane, making the contents of the *cristae* continuous, though not necessarily homogeneous, with the space between the inner and outer (surface) membrane (Figure 1E).[34,43] The total surface area of the *cristae* relative to that of the unconvoluted part of the inner membrane (i.e., the extent of folding) varies from tissue to tissue, with the hormonal regime,[43,44] with the osmotic strengths of the various compartments,[45] and with the metabolic state.[46-48] Thus, in heart mitochondria the inner membrane is much more convoluted than in liver mitochondria, approximately in correlation with the respective rates of respiration.[49] In fact, the form of the *cristae* also varies greatly between tissues and species; in some they even form long narrow trigonal channels.[50] The number of *pediculi* per mitochondrion may be small (in the order of ten); estimated from data reported in Reference 43 and an assumed mitochondrial diameter of 1.5 μm,[26] but tends to be increased by thyroid hormone, such that the communication between intra*cristal* space and intermembrane space may well improve under those conditions.[43,44]

Using various methods to prepare the sample for electron microscopic analysis, Sjöstrand and colleagues have also progressed to the above picture,[34,38,41,48,51-53] although they certainly emphasize different aspects. They did confirm[52] the view that exchange between the space between the inner and outer surface membranes, on the one hand, and the intra*cristal* space, on the other hand, does not involve the matrix space. They distinguish three, rather than two, types of mitochondrial membrane: the outermost surface membrane (the outer membrane), the innermost surface membrane (corresponding to what others consider the non-involuted inner membrane), and the inner membranes (corresponding to the *cristae*). The

discontinuity and compositional difference between the innermost surface membrane and the inner membranes is stressed. Moreover, at least in the earlier work of these authors, the inner membranes were treated as single, rather than two, sandwiched membranes; their backbone (corresponding to the intra*cristal* space in other views) is protein, which is covered on either side by a rather continuous phospholipid bilayer. The basis for this view appears to be[29,51] (1) the absence of staining of the middle of the 12-nm-thick *cristae* in mitochondria in vivo (which suggests that that space is closely packed, perhaps with protein molecules) in freeze-sectioned mitochondria (see, however, Reference 53); (2) the less-intense staining of the *cristae* compared to the matrix in low-denaturation-embedding electron microscopy and the higher electron scattering of the *cristae* (indicating a higher mass density of the *cristae*) in dark-field-illuminating electron microscopy of the same type of preparation; (3) the observation in freeze-fractured mitochondria that the *cristae* consist of arrays of particles larger than 4 nm with a rather heterogeneous size distribution, whereas, the inner surface membranes would harbor a rather narrow size distribution of particles around 11 nm. One should note, however, that the absence of stain from the *cristae* depended strongly on the precise procedure of preparation of the sample, purportedly because, otherwise, protein denaturation would occur.

In Sjöstrand's view[34,51] not only is the intra*cristal* space absent (see, however, Reference 53), but also the intermembrane space (the space between the innermost and the outmost surface membrane). Indeed, in most electron microscope pictures of his[34,38,51] and other[43,46,47] groups, such spaces are absent, whereas a matrix space is always clearly observable. In some conditions, however, deviations from this so-called orthodox conformation are observed. One such condition is upon isolation of mitochondria in 0.25 *M* sucrose,[47,52,54] a frequently used isolation medium. An OsO_4-stained clot was then seen in the otherwise unstained compartment bounded by the outer membrane. Within the clot, smaller unstained volumes were visible, which may correspond to intra*cristal* spaces. This "condensed" conformation reverted to the "orthodox" conformation when (1) respiratory substrate was added in the absence of ADP,[47,48,52] (2) *immediately after* the mitochondria were resuspended in 0.10 *M* sucrose (even in the presence of an inhibitor of endogenous respiration), or (3) when osmotic swelling of the mitochondria was induced.[45] In the presence of ADP and phosphate[47] or uncoupler[52,55,56] (see, however, Reference 47) the mitochondria attained the condensed conformation.

The changes in mitochondrial conformation that occur during transitions between respiration in the absence of ADP (or, respiration when added ADP has been phosphorylated, "state 4") and state 3 (respiration in the presence of ADP) are still a matter of debate. The difference in interpretation focuses on state 3, where Hackenbrock,[47] considering thin sections of OsO_4-fixed mitochondria, observed the same overall volume as in state 4, but the intramembrane space now taking up to 50% of that volume, and swollen *cristae*, most often not obviously connected with the intermembrane space, taking up half of the rest. Muscatello and colleagues,[55] when negatively staining rat liver mitochondria, found state 3 mitochondria to be 30% reduced in volume and observed a dense stain in the center. These authors also concluded that the mitochondria had undergone a transition to a condensed configuration, although of a somewhat different kind than that proposed by Hackenbrock.[47] Candipan and Sjöstrand,[52,57] largely reproducing and extending these observations by using various sample-processing techniques, did not observe highly swollen *cristae* in state 3. They interpreted their finding that the ammonium molybdate did not stain the *cristae*, but did stain the matrix (though only in state 3 and only in less than 50% of the mitochondria) to indicate that the connection between the *cristal* space and the intermembrane space does not lead through the matrix, but rather through the *pediculi*. They stress that they disagree with Hackenbrock[47] in that in state 3 there is no free communication between the intra*cristal* spaces and the space between the two surface membranes.

That a significant fraction of mitochondrial lipids is in the bilayer structure is suggested by their ^{31}P peak in NMR experiments.[58] On the other hand, mitochondrial lipids are prone to form nonbilayer structures such as hexagonal tubes and stacked bilayers, especially in the presence of Ca^{2+}.[59] Thus, it has been speculated[60] that a minority of the lipids form nonbilayer structures. Of note is the observation[60] of multilamellar structures in the mitochondrial matrix, when mitochondria are incubated in the presence of Ca^{2+} or Mn^{2+}, but in the absence of respiratory substrate.

In the usual experimental set-up (for an exception, see Reference 54) the colloid osmotic pressure outside the mitochondria is smaller than that of the space between the two surface membranes and of the intra*cristal* space. Consequently, the space between the inner and outer surface membranes will tend to swell relative to the extramitochondrial space and in state 3, also relative to the matrix space.[54,61] This then may account for observations that the overall mitochondrial volume would be unaffected by the state 3 to state 4 transition;[47] (negative staining[52,55] is not a sensitive assay for this volume), whereas the *cristae* and the space between the surface membranes would swell. Because in vivo the extramitochondrial colloid osmotic pressure is higher than in the usual in vitro experiments, the slightest attractive interactions between the inner and outer surface membranes and between the membranes bordering a crista, or an excess colloid osmotic pressure of the cytosol, might keep these membranes together. Then the state 4 to state 3 transition would be accompanied by a change in overall mitochondrial volume, without much swelling of the intra*cristal* or intermembrane spaces.[54]

There are two potentially necessary addenda to this picture. One considers the possibility that diffusion of salt through the *pediculi* is restricted. In that case salt leaking out of the matrix space during state 3 respiration will accumulate in the intra*cristal* space and will cause this to swell much more extensively than the intermembrane space. This situation would be consistent with the observations of Cardipan and Sjöstrand[52] (see also Reference 62), but not so much with the huge increase in space between the two surface membranes, as observed by Hackenbrock.[47]

The second addendum considers the interaction between the two surface membranes. Hackenbrock[63] observed contact points between the two membranes in thin-sectioned mitochondria. In freeze-fracture studies, the fracture plane alternates between following the inner and following the outer surface membranes.[42,60,64] The number of contact points has been found to be higher at 37°C than at room temperature,[60] higher in the presence of 1 m*M* Ca^{2+}, or Mn^{2+} (which, it should be mentioned, are unphysiologically high concentrations), or apocytochrome *c*,[60] and higher in state 3 than in state 4, but much lower in the presence of uncoupler, and somewhat lower in osmotically shrunken mitochondria.[62]

Van Venetië and Verkley[60] have speculated that at the contact points, the inner and outer surface membranes might be continuous through an inverted micellar or hexagonal tube structure. However, the data seem equally compatible with close apposition of the two membranes, perhaps with an extra electrostatic interaction.

Sjöstrand and Candipan[65] speculated that the connections between the inner and outer membranes constitute a proteinaceous diffusion pathway from the extramitochondrial space directly into the *cristae* located below (Figure 2A). Brdziczka and Reith,[43] on the other hand, speculate that the connections between the inner and outer surface membranes delineate subcompartments of the intermembrane space, which are connected on the one side to the extramitochondrial space through the porin and connected on the other side to the intra*cristal* space through a *pediculus* (Figure 2B).

The structure of the outer membrane itself is of interest because it is traditionally assumed to act simply as a sieve which is completely permeable to molecules smaller than mol wt 10,000. Yet, the outer surface membrane would seem to be a candidate for the Singer-Nicolson model (it has a lower protein:lipid ratio than the inner mitochondrial membranes[66]).

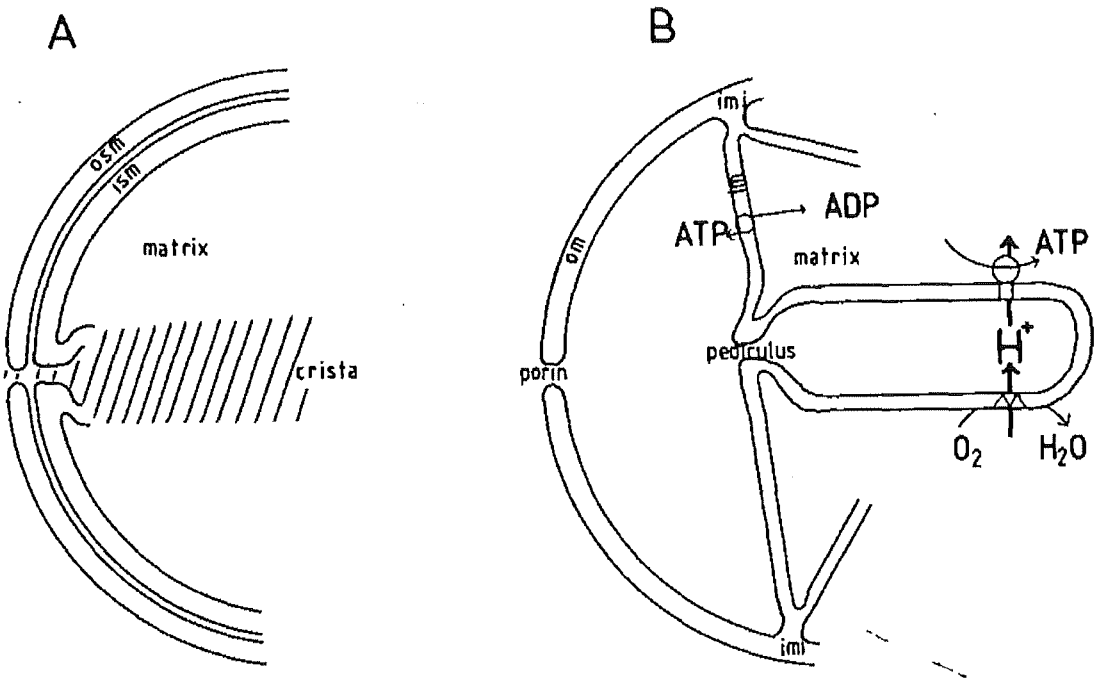

FIGURE 2. Two views with respect to the relative structural organization of pores in the outer mitochondrial membrane, contact points between the two surface membranes, *pediculi*, and *cristae*. (A) The contact points between the two surface membranes, the porin, and the *cristae* are supposed to be adjacent, such that they constitute a proteinaceous channel (the dashed area) connecting the intra*cristal* space through the *pediculus* and the porin directly with the extramitochondrial space.[65] (B) The contact points are supposed to consist of inverted micellar structures and are located away from the porins and the *pediculi*, such that extra compartments are formed between the two surface membranes.[43] In the *cristae*, protons pumped by electron-transfer complexes may be confined to the neighboring ATP synthase. 'osm', and 'ism' refer to outer and inner surface membrane respectively, 'om' and 'im' refer to outer membrane and inner membrane, respectively. 'imi' refers to the inverted micellar structure supposedly constituting the contact points between the inner and outer mitochondrial membranes.[60] Note that in this figure membranes are denoted by double lines.

This paradox was resolved by the discovery of a pore-forming protein in the outer membrane of mitochondria[67] that was essential for the outer membrane transport of ADP. In brain and under some conditions in other tissues, hexokinase binds to porin,[69] thus accounting for channeling of intramitochondrially synthesized ATP to glucose.

At this point, we should perhaps interject that most of the structural information we have on mitochondria has been gathered through electron microscopy. Thus, its validity for the mitochondrion *in situ* depends on the assumption that the preparation of the sample for the various forms of electron microscopy has not distorted the structure.[57] Since water may be much of the substance that separates mitochondrial membranes, and water is either removed, replaced, or frozen in the usual preparation procedures, one can always make the objection that the change in water (medium) properties has altered the arrangement of the membranes. Since we are not electron microscopists, we cannot decide how grave this objection is. We shall have to leave much of the critical judgment to the reader, or to electron microscopists willing to review this matter.

The fact that the connections between the intra*cristal* space and the intermembrane space may be narrow or rare, suggests that their content may not be homogeneous with that of the space between the two surface membranes, with the extramitochondrial space, or even with that of other intra*cristal* spaces.[43,70] When the convolution of the inner membrane is high, even the matrix space may behave as a number of relatively independent subcompartments. One way to examine these possibilities would be to ask whether the inner mitochondrial membrane and the aqueous compartments it separates behave as an ideal osmometer.

Several studies have established a linear relationship between the volume of the mitochondrial matrix space and the inverse of the externally applied osmotic pressure.[71] However, such demonstrations depended on preincubation of the mitochondria below approximately 80 mOsm.[39] If the preincubation was at higher osmolarities, the shrinking induced by increasing the osmolarity of the medium was much less.[39,61] Since around 80 mOsm Stoner and Sirak[45] observed a disorganization of the outer mitochondrial membrane, the ideal osmotic behavior most probably requires the rupture of the outer mitochondrial membrane, which would happen at a matrix volume of some 2 $\mu\ell$/mg protein.[39,72]

There are several explanations other than inhomogeneity of the compartments, for the apparently nonideal osmotic behavior when the outer membrane is still intact.[13] One is that the detection method used (light scattering plus gravimetric analysis[39]) fails to reflect (solely) volume changes when the outer membrane is intact.[72-75]

The more direct question of whether the intra*cristal* space plus the intermembrane space and the extramitochondrial space behave each as a single homogeneous phase is difficult to address. However, from the work of Massari et al.[39] on water permeability of mitochondrial membranes, we may be able to deduce a provisional answer. Presumably after lesion of the outer membrane, water permeability per mitochondrion varied with the $^2/_3$ power of the mitochondrial matrix volume. This would suggest that the surface area of the inner membrane involved in *cristae* formation does not contribute to water permeation; it is as if the intra*cristal* space does not allow rapid water flow, or is not at the same osmotic strength as the intramembrane space. When, again in the absence of an outer membrane, the volume is less than 2 $\mu\ell$/mg protein (i.e., in the physiological range), the apparent surface area available for water permeation depends even more strongly on the volume, suggesting[39] that at small mitochondrial volumes, even the nonfolded parts of the inner membrane have a reduced water permeability. At a volume of 1 $\mu\ell$/mg protein the membranes appear to become completely impermeable to H_2O (see, however, Reference 2). This may be the point where the intramitochondrial water is little more than crystal water for the proteins.[76]

Garlid and Beavis,[77] accounting for deviations between light-scattering results and actual volume measurements, found that the permeability of the inner mitochondrial membrane to erythritol did not vary with the mitochondrial volume. It may be that the equilibration of substances with the intra*cristal* space is slow relative to the rapid permeation of water, but fast relative to the slower permeation of erythritol.

At this point, our interpretation of the electron microscopic- and other other observations of mitochondrial structure is that

1. The outer membrane is a standard biological membrane with approximately 50[69] pores.
2. The inner surface membrane and the *cristae* membranes are continuous, though not necessarily all the way as a single bilayer. The *pediculi* that connect the intra*cristal* space with the space between the two surface membranes may consist of alternative membrane structures and may be rather narrow and perhaps dynamic (they may even be temporally absent).
3. *In situ* and in state 4 in vitro the intra*cristal* space and the space between the two surface membranes is narrow (approximately 10 nm) and probably protein rich.
4. In state 3 the *cristae* are swollen, but it is not clear if the *pediculi* have increased or decreased in diameter or number.
5. There are contact points between the two surface membranes.
6. The structural data are compatible with subcompartmentation of (a) the space between the two surface membranes,[43] (b) the *cristal* space (such that each *crista* or even parts thereof would constitute its own compartment[43,51,70]) and (c), especially in heart mitochondria, the matrix space.

The subcompartmentation of the cristal space would imply that the space between the surface membranes and the intra*cristal* space are distinct. It is likely that the "borders" between the various subcompartments would not be strictly impermeable, but rather consist of areas of hindered diffusion leading to inhomogeneities in terms of metabolite, ion, and perhaps even proton concentrations (or rather electrochemical activities) in cases where there is a flux of these substances. The electron-microscopic structure of mitochondria suggests inhomogeneity of the membrane and the enclosed compartments.

IV. POTENTIAL IMPLICATIONS OF STRUCTURE FOR THERMOKINETICS AND CONTROL OF CHEMIOSMOTIC COUPLING

One research strategy compares experimental data to the predictions made by the reigning theory. If the data and predictions do not correspond, then inductive reasoning is applied so as to amend the theory and make it consistent with the experimental observations. Two of us have partaken this inductive approach in rather recent reviews.[2,78] Other, more analytic reviews also exist.[31,32] Here we wish to accomplish what we call a deductive review. That is, we start from the relatively incomplete structural information concerning mitochondria and deduce what sort of kinetic and thermodynamic (or, in short, "thermokinetic") properties energy coupling would have in such a system if it were organized according to the basic principles of chemiosmotic coupling. These basic principles are, we repeat, two proton pumps with a proton gradient in between. Whether that proton gradient is homogeneous remains to be seen.

We shall limit ourselves to four possible implications of the observed structure of inner mitochondrial membranes. This limitation is inspired both by considerations of space and by the fact that these four are the ones that are best understood at the theoretical level. The first is that the energy-dependent electric-potential profile may be special, the second is that proton movement may be retarded due to buffer groups, the third is that the region close to the membrane surface constitutes an extra compartment which does not always equilibrate with the adjacent aqueous bulk phase, and the last is that there are, in fact, many such compartments which are thermokinetically independent. Our approach will be to first review the concept behind the implication and then review experimental data that may suggest its relevance or irrelevance.

A. The Membrane Surface: Energy-Dependent Surface Potentials Causing Protons to Stick to the Membrane

For a simple phospholipid bilayer across which protons are pumped into a medium at physiological ionic strength, the energy-dependent surface potential and the capture of the protons by the surface, will be negligible.[79,80] The reason is that the medium ions will redistribute between the bulk phase and the surface layer to the extent that the membrane surface will carry an excess number of positive charges virtually equal to the number of protons pumped. In the case of 100 m*M* KCl as the medium, these charges will consist for over 99.99% of K^+ rather than H^+ ions.

As reviewed above, the mitochondrial membranes are much more complex than a phospholipid bilayer. For a number of model systems that share properties with the inner mitochondrial membranes, calculations have been completed recently.[80] In one of these, the system was that of a planar hydrophobic membrane bounded on both sides by a medium of high buffer capacity, typically such that the concentration of buffer-bound protons would be the same order of magnitude as the concentration of freely mobile other ions. In such a condition, the charge compensation for the protons moving away from the "positive" side of the membrane would be by protons bound to the buffer, as well as free-salt ions moving towards the membrane surface. The energy-dependent surface potential would still be of negligible magnitude, but the number of protons appearing in the bulk aqueous phase would be lower than the number of protons pumped across the membrane.

The fact that both the inside and the outside of the inner membrane are bounded by protein-rich solutions, plus the recognition that a 5-nm-thick biological membrane might best be described by a 2-nm-thick hydrophobic layer, sandwiched by two more hydrophilic layers consisting of phospholipid headgroups and proteins (both transmembrane and peripheral), makes us realize that this model for the mitochondrial inner membrane may be somewhat realistic.[51,81] The recent understanding that the outer membrane is not really a sieve, but that its permeability is based on the presence of a limited number of pores, which may be partly occluded by bound glycolytic enzymes, as well as the proposal[43] that the intermembrane compartment may be small and subdivided into subcompartments by contact between the inner and the outer membranes, contribute to this. For instance, assuming that 5% of the amino acids have a protonatable group at its pK_a and assuming a specific weight of the protein bordering the inner hydrophobic phase of the membrane of 1 kg/ℓ, one calculates that the concentration of protein-bound protons in the membrane's surface layer may be as high as 0.25 *M*. At an ionic strength of approximately 0.25 *M*, this would have the effect that approximately 50% of the pumped protons would be occluded from a measuring device in the bulk aqueous phase.

A second model system for energy-transducing membranes[80] was that in which a salt-impermeant layer, approximately 10 nm thick, would border the hydrophobic core of the membrane. In this case, the charge of protons moving away from the membrane surface after proton pumping could not be compensated by ions other than protons. Provided that the total proton concentration (bound plus free) in the region bordering the membrane would be of the order of 0.1 *M* or higher, the net number of protons appearing in the bulk aqueous phase after proton pumping would be considerably less than the number of protons pumped across the membrane.[80] One piece of evidence for the relevance of these considerations may be the energy-dependent surface potential of -10 mV observed in mitochondria.[82]

In bacterial chromatophores an energy-dependent surface potential of approximately +7 mV was reported.[83] These results might reflect that approximately 10% of the transmembrane electric potential difference in these systems would drop across the surface layer. This would be in line with the results of theoretical calculations,[80,84] where it should be noted that this is the surface potential at low ionic strength of the external medium. If buffer groups coated the membrane surface, then this type of surface potential could have quite strong implications for the tendency of pumped protons to move into the bulk aqueous phase.[80,84] That Tb^{3+} did not exhibit energy-dependent binding may reflect the fact that there is no surface potential after all[85] (the measurements with the ESR probes have been frought with difficulties[82,85]), or that the surface is not accessible to that ion. Using various probes, Kraayenhof and co-workers have long since made the point that near membrane surfaces the electric potential will strongly depend on position.[86]

Perhaps the most physiological line of evidence for the relevance of the above considerations derives from experiments in which oxygen "pulses" have been administered to anaerobic mitochondria or bacteria. Since the earliest experiments of this type, it has been realized that few of the expected protons actually enter the external phase of mitochondria unless special precautions are taken. One important reason for this is that the proton pumps are electrogenic, so that their action generates a transmembrane electric potential difference. Because the electric capacitance of the membrane is small relative to the buffer capacities of the phases bordering the membrane, the electric potential difference will build up relatively rapidly and then cause back-leakage of protons, reduction in the respiratory rate, and perhaps even slip[1,87-90] in the proton pumps. To prevent the back pressure[1] on the pumped protons, it has become customary to add membrane-permeant ions, or ionophores in experiments of this type, and indeed, this increases the observed number of protons.

It is not certain whether the transmembrane electric potential difference between the bulk aqueous phases is solely responsible for the reduction in the number of protons observed in

the aqueous bulk phase when no such permeant ions are added. By lowering the amount of oxygen added per unit membrane surface area to an amount that would not cause a transmembrane electrical potential difference of more than 30 mV, Hitchens and Kell[91,92] eliminated the possibility that the potential difference between the aqueous bulk phases would cause the protons to flow back before they could be detected by their pH electrode in the external phase. Yet, they observed that an insufficient number of protons appeared in the aqueous bulk phase, unless chaotropic ions like SCN^- were added.

Conover and colleagues[93,234] inspected the number of protons ejected by mitochondria upon the addition of small pulses of oxygen with simultaneous measurement of some electric potential by use of the probe merocyanin 540. They observed that while the indicated electric potential was submaximal, the proton per electron stoichiometry was lower than expected, unless the ionic strength of the medium was increased. Higuti and colleagues[94] found that positively charged anisotropic inhibitors like TPA^+, increased the ejection of protons by ATP or succinate-energized mitochondria, whereas they did not increase state 4 respiration. However, since this experiment was carried out at a concentration of TPA^+ near 20 μM, one would expect a steady-state accumulation of approximately 200 nmol/mg protein, which might take approximately 30 sec to be achieved. It is not clear whether a transient increase in state 4 respiration might have escaped these authors. A traditional interpretation in terms of a permeant ion lowering the membrane potential until it is accumulated would then still be possible.

Other experimental observations that might reflect retention of protons in a proteinaceous layer covering the hydrophobic core of the membrane, are reviewed in Reference 13. Although some of the experimental evidence may suggest that not all the pumped protons do enter the aqueous bulk phase, it by no means constitutes sufficient proof that this is due to the reasons described above.

To summarize this subsection, we note that the structure of the membrane and its environment would be expected to lead to a specific profile of the local, energy-dependent electric potential and pH. One effect could be the retention of protons close to the membrane surface under energized conditions.

B. Retardation of Local pH Relaxation by Buffers

Junge and co-workers[27,95] have pointed at another, kinetic, consequence of the structure of energy-transducing membranes; the presence of a high concentration of immobile buffer groups will cause protons ejected by a proton pump to bind to the buffer, rather than diffuse away to the bulk aqueous phase.[27,78,96] Consequently, they would escape detection, at least for some time. As noted by various authors,[27,78,97,98] the effect of buffers is somewhat subtle; at steady-state, protons binding to the buffer and, thus, exempt from free diffusion, will be replaced by protons dissociating from the buffer and the steady-state proton diffusion across a given pH gradient will be independent of (or, if the groups themselves are mobile, enhanced by) the presence of buffer groups. Thus, whereas the surface-potential effects discussed in the previous section would persist for as long as the electric field would persist and ions would not access the surface, the effect discussed here would disappear at steady-state. Effectively, the buffer reduces the apparent diffusion coefficient as it appears in the second diffusion law of Fick, but not the true diffusion coefficient that appears in the first law of Fick (Equation 3). In fact, if the buffer groups are mobile, buffering will tend to increase, rather than decrease, the true diffusion coefficient.[98]

An effect one does expect, however, is that a high local buffer capacity will decrease the absolute magnitude of the pH change at the site of proton pumping due to proton pumping and will also increase the half-time with which that local pH-change relaxes. Similarly, the appearance of protons in the bulk phase after a single turnover of the proton pumps will be retarded. Junge and colleagues found indications for this phenomenon in chloroplast prep-

arations in which the grana membranes are stacked. Proton disappearance from the external phase was slow when Photosystem II was excited in the presence, but not in the absence of $MgCl_2$.[99] That $MgCl_2$ would cause stacking of the grana membranes was suggested by the concomitant reduction in Photosystem II fluorescence. When Photosystem I was excited in the presence of excess electron acceptor, proton disappearance was rapid,[100] which is suggestive in view of the preferential localization of Photosystem I in the nonappressed membranes.[101] In this subsection we reviewed how the presence of buffer groups may lead to kinetic retardation, without leading to a heterogeneity in the electrochemical potential for protons.

C. An Extra Proton Compartment Due to Restricted Proton Diffusion

1. The Concept

The limited number of pores in the outer membrane, the possibly limited space between the two mitochondrial surface membranes, and the narrowness of the *pediculi*, as well as the flatness of the intra*cristal* spaces themselves, might be considered so extreme as to even prevent rapid proton diffusion from the sites to which protons are pumped by the primary proton pumps to the aqueous bulk phase outside the mitochondria.[43,70,81] One may then wonder whether the electrochemical potential for protons measured outside the mitochondria would be representative for the proton electrochemical potential at the external side of the electron-transfer chains and ATP synthases.[102] In contrast to Sections IV.A and IV.B, we consider here the possibility that there is a resistance for proton diffusion between a local proton space and the adjacent aqueous bulk phase that would result in a difference in electrochemical potential for protons under steady-state conditions. The presence of a high concentration of buffer groups (which would not reduce the diffusion constant in the first diffusion law of Fick) or the effects of limited accessibility of the surface for ions and ensuing surface potentials cannot be responsible for such a resistance.

It has been proposed[70] that when the osmotic strength of the medium surrounding the mitochondria is hypotonic, the matrix would swell to the maximum volume it can assume within the limits of the outer membrane. This would imply a compression of the intra*cristal* space and of the space between the outer and inner surface membranes. Also, the *pediculi* might narrow down. As a consequence, there might be a diffusion barrier for protons diffusing from the intra*cristal* spaces to the phase outside the mitochondrion. The (many) electron transfer complexes and ATP synthases present in each *crista* would form their own microcosmos with chemiosmotic coupling. The proton electrochemical potential within the intra*cristal* spaces could well differ substantially from the proton electrochemical potential outside the mitochondrion, provided (see below) that the proton back-leakage and the proton pumping would not be distributed symmetrically over the mitochondrial membranes.[102-105] The relationship between the transmembrane electrochemical potential difference for protons between the two surfaces of the membrane near the proton pumps ($\Delta\tilde{\mu}_H$) and that between the two aqueous bulk phases ($\Delta\tilde{\mu}_H$) has been expressed as the ratio of the resistance of the membrane vs. proton leakage to the sum of the resistance of the membrane vs. proton leakage and the resistance for proton movement between the membrane surface and the bulk aqueous phase.[102] Stressing that there might also be a resistance for proton diffusion between the electron-transfer complexes and the ATP synthases, and allowing the resistance for lateral proton diffusion between the proton pumps and the proton leaks to be finite, more complete, electric,[106,107] or mosaic nonequilibrium thermodynamic[108] networks have been described.

From its first quantitative description,[102] the localized chemiosmotic-coupling model has assumed that the proton resistance between the proton domains and the bordering aqueous bulk phase would be approximately an order of magnitude *smaller* than the resistance between the two aqueous bulk phases. Thus, the discrepancy between bulk phase $\Delta\tilde{\mu}_H$ and local $\Delta\tilde{\mu}_H$ would only become large when the proton permeability of the mitochondrial membrane

would be increased, or the imbalance between the two proton gradients be increased in some other way. A consequence is that the proton resistance between the aqueous bulk phase and the local proton domains would hardly impede ATP synthesis driven by an artificially generated proton potential difference between the aqueous bulk phases.

This specific proposal for "localized chemiosmotic coupling" had been preceded, paralleled, and followed by quite a few others, for instance, one where there would be only a single extra proton space inside the membrane,[109] or where the two extra proton spaces would consist of proton wires running along the membrane surfaces and connecting the proton pumps[2,110-113] (for further review see Reference 78).

2. Predicted Nonunique Relationships between Pumping Rates and $\Delta\tilde{\mu}_H$

Were agents employed that differentially affect the relationship between the local proton potential ($\Delta\lambda_H$) and the bulk phase potential ($\Delta\tilde{\mu}_H$), the variation of the rate of electron transfer with $\Delta\tilde{\mu}_H$ could be different, even though the variation of electron transfer with the local proton potential would have to be the same. An example would be the comparison between the effects of ADP, which would stimulate respiration by primarily affecting $\Delta\lambda_H$ and, hence, not increase the disequilibrium between $\Delta\lambda_H$ and $\Delta\tilde{\mu}_H$, to the effects of a protonophore that would increase the proton permeability of the membrane, largely in areas away from the *cristae*, and strongly reduce $\Delta\tilde{\mu}_H$ without much affecting $\Delta\lambda_H$. One would expect the variation of the rate of electron transfer with $\Delta\tilde{\mu}_H$ to be steep when ADP would be added, but shallow when the protonophore would be used.

Padan and Rottenberg confirmed this prediction.[114] Similarly, the variation of the rate of ATP synthesis with $\Delta\tilde{\mu}_H$ appeared to be different, depending on whether one would vary the proton permeability of the membrane, or inhibit electron transfer. The older references may be found in earlier reviews.[78,115] Here, we review some pertinent experimental results obtained since then.

Woelders and colleagues[116] have redetermined the relationship between $\Delta\tilde{\mu}_H$ and respiration, as well as the relationship between $\Delta\tilde{\mu}_H$ and ATP synthesis in rat liver mitochondria. They found no significant difference in the relationship of flux to $\Delta\tilde{\mu}_H$ between the two different methods of variation (titration of the other pump vs. uncoupler titration). In beef heart submitochondrial particles, Sorgato and colleagues[117] found an identical relationship between $\Delta\tilde{\mu}_H$ and the rate of ATP synthesis, independent of whether they titrated with the uncoupler FCCP, or inhibited respiration with malonate.

In contrast, it was confirmed that in rat liver mitochondria, the rate of ATP synthesis varied differently in the two cases.[118] Controls were carried out for a suggested[117] artifact of inaccurate measurement of $\Delta\tilde{\mu}_H$ at high respiratory rates[118] due to a deficiency in the centrifugation method used. Moreover, the rate of ATP synthesis driven by a $\Delta\tilde{\mu}_H$ generated through K^+-efflux also depended differently on $\Delta\tilde{\mu}_H$, when compared to ATP synthesis driven by respiration and titrated by malonate,[119] especially at the higher rates of ATP synthesis. This would be consistent with a finite resistance for proton movement between bulk phase and ATP synthase. It is noteworthy that Zoratti and colleagues use $TPMP^+$ distribution as a probe for the transmembrane electric potential difference. Woelders and colleagues reported that under identical conditions, K^+ and $TPMP^+$ gave different readings of the electric potential.[116]

An explanation for the nonunique dependence of rates on $\Delta\tilde{\mu}_H$ that does not require microcompartmentation of protons is a presumably indirect, allosteric interaction between the electron-transfer complexes and the ATP synthases, as proposed by Melandri et al.[120] (see also References 237, 121 and 122). An interesting aspect of the ATP synthase in this regard is the inhibitor protein.[123] Perhaps the most convincing observation in favor of these allosteric interaction models and against both the localized and the simple chemiosmotic coupling models, consisted of the observation that in *Rhodopseudomonas sphaeroides*, il-

lumination-activated alanine uptake while *decreasing* $\Delta\psi$.[124] This has been interpreted in terms of an influence of the redox state of an electron-transfer component on the activity of the alanine carrier.[121,124] An important candidate for the messenger involved in this type of allosteric interaction between electron-transfer chains and secondary transport systems is natural redox mediators such as glutathione. The activity of bacterial solute carriers has, indeed, been shown to be affected by changes in the redox state of thiol groups in them.[125]

The observation of increased alanine transport at a decreased $\Delta\tilde{\mu}_H$ can ge explained in terms of a localized chemiosmotic-coupling model, if it is assumed that protonmotive-force-consuming processes other than alanine transport would be directly activated by an increased light intensity, for instance, through a locally oscillating electric field (see below). Alternatively, there might be a locally activated $TPMP^+$ pump.[126]

3. Predicted Energetic Incompetence of $\Delta\tilde{\mu}_H$

Allosteric interaction between electron-transfer complexes and the ATP synthase could not push the ATP synthesis reaction away from equilibrium. If there is a local proton domain, then this implies that $\Delta\tilde{\chi}_H$, i.e., the proton electrochemical potential difference between the domain and the other side of the membrane (e.g., between the intra*cristal* space and the mitochondrial matrix), should always contain sufficient free energy compared with the free energy of hydrolysis of ATP (ΔG_p), for any ATP synthesis to arise. If there is a resistor between the proton domain and the aqueous bulk phase, then — especially if the $\Delta\tilde{\mu}_H$ would be drained by a proton leak in the inner surface membrane — $\Delta\tilde{\mu}_H$ might be much lower than $\Delta\tilde{\chi}_H$. Thus, a comparison of $\Delta\tilde{\mu}_H$ to ΔG_p could suggest that there would be insufficient free energy in the proton gradient to account for the synthesis of ATP.

Because the stoichiometry of $\vec{H}^+$/ATP at which the ATP synthase operates is incompletely known, this comparison by itself is not quite sufficient; if ATP would be made at a phosphate potential of 50 kJ/mol while the $\Delta\tilde{\mu}_H$ would only be 5 kJ/mol, this might just reflect that the $\vec{H}^+$/ATP stoichiometry is as high as 10. However, if one allows the ATP synthase to operate in reverse and then measures the ratio of the phosphate potential to $\Delta\tilde{\mu}_H$, such high values for the stoichiometry (if constant) can be excluded.[1,127-132] Consequently, $\Delta\tilde{\mu}_H$ must exceed a significant thermodynamic threshold value before ATP synthesis can become measurable (e.g., References 133 to 136). A noteworthy exception to this was reported for the ATP synthase of a thermophilic cyanobacterium, reconstituted in extremely proton-impermeable liposomes.[137] Also, in this system independent of whether the reaction involved ATP hydrolysis or ATP synthesis, a potassium-ion diffusion potential would lead to a steady-state phosphorylation potential of some nine times the transmembrane electric potential. Since the pH gradient would be negligible or reverse, the authors concluded that this ATP synthase would have a proton translocation stoichiometry of close to 9.[137] It would be particularly illuminating to see if (1) the transmembrane electric potential *generated* by this ATPase could exceed 80 mV, (2) if an electric-potential-dependent myokinase activity can be excluded in these experiments, and (3) if similar observations are possible with other ATP synthases in similar experiments (which will require extremely tight liposomes).

At an external pH of 10.5 and an internal pH of approximately 8.5, *B. alcalophilus* can still maintain a significant (i.e., >40 kJ/mol) phosphate potential, whereas $\Delta\tilde{\mu}_H$ does not amount to more than 6 kJ/mol (for review see Reference 138). To exclude the possibility that the ATP was derived from glycolysis, Krulwich and colleagues have also performed experiments with inside-out vesicles of this bacterium and made essentially the same observations.[139] Interestingly, at normal pHs an artificial pH gradient was not effective in driving ATP synthesis in alcalophilic cells,[140,141] suggestive of a barrier between bulk phase and membrane and eliminating the explanation of an extreme $\vec{H}$/ATP ratio.

Measurements of the electric potential in phosphorylating mitochondria by means of

microelectrode impalement,[142-144] revealed tiny, if not inversely oriented, electric potentials. The pH gradients did not compensate.[145] These observations are not in agreement with transmembrane electric potentials measured with redistributing probes in the absence of microelectrode impalement or uncouplers.[104,114,127,128,146-148] Possible interpretations include that (1) the electrodes crossed the inner membranes an even number of times, (2) the electrode impalement short-circuited the spaces between which it measured the electric potential difference (but apparently left the electric potential between spaces relevant for ATP synthesis intact), or (3) the redistributing probes are not valid as indicators of the transmembrane electric potential difference (which may be difficult to believe when K^+ is the probe ion in the presence of the selective ionophore valinomycin). In these cases it would seem important that the transmembrane electric potential be measured with redistributing ions in the impaled mitochondrion, and that the capacity of the system to drive ATP synthesis under conditions where the potential is clamped as a diffusion potential, is compared to the capacity in the absence of such a diffusion potential, but at similarly measured magnitudes of the transmembrane electric potential difference.

If the ATP synthase is a reasonably well-coupled enzyme, then under state 4 conditions (i.e., in a situation where there is a preset $\Delta\tilde{\mu}_H$ and the phosphate potential is allowed to build in time until net phosphorylation ceases) the phosphate potential generated should be approximately equal to the electrochemical potential difference for protons multiplied by the proton translocation stoichiometry; if the enzyme slips, it should be smaller. If one takes the ratio of the phosphate potential to $\Delta\tilde{\mu}_H$, then this should correspond to that proton translocation stoichiometry. By increasing the proton permeability of the membrane,[127,128,146,148,149] or by inhibiting electron transfer,[104] one may repeat this experiment at different magnitudes of $\Delta\tilde{\mu}_H$. The simplest version of the delocalized chemiosmotic coupling theory would predict that this ratio would remain constant, or decrease (i.e., at least not increase above the stoichiometry obtained in the hydrolysis direction), whereas the insight that the energy-coupling protons may be localized to special domains would predict that ratio to increase when $\Delta\tilde{\mu}_H$ is drained by uncoupler.

All early experimental results for mitochondria demonstrated that this ratio increased as more and more protonophore or electron transfer inhibitor was added. More recently, a decrease in temperature was shown to have a similar effect.[150] In these experiments the dynamic nature of the steady-state was assured by approaching it from either side. In submitochondrial[151,152] and sub-bacterial[153,154] particles, changes in the ionic composition of the medium led to similar phenomena.

Upon increasing the accuracy with which they had measured $\Delta\tilde{\mu}_H$ (compromised by volume changes of the mitochondrion upon the addition of uncoupler and by probe binding) and the phosphate potential (compromised by hydrolysis of ATP and ADP upon quenching of the sample) and upon reducing the interference of the adenylate kinase reaction by adding the adenylate kinase inhibitor AP5A, van Dam and colleagues[155] observed a constant ratio between phosphate potential and $\Delta\tilde{\mu}_H$. This ratio amounted to 3 in intact mitochondria, consistent with an $\overrightarrow{H}^+$/ATP stoichiometry of 2 for the ATP synthase and 1 for the combination of the adenine nucleotide and the phosphate translocator.

Although Woelders et al.[155] definitely demonstrated that at least some of the earlier reports that the ratio varied with $\Delta\tilde{\mu}_H$ had to be reexamined, the issue has not been closed. Zoratti and colleagues,[156] repeating and extending their earlier experiments with extra care, continued to observe an increased ratio between phosphate potential and $\Delta\tilde{\mu}_H$ at low magnitudes of the latter. These authors also demonstrated actual ATP synthesis at the insufficiently high magnitudes of $\Delta\tilde{\mu}_H$.

Rottenberg[130] recently examined the same question by use of phosphorous NMR in order to circumvent the problem of the measurement of the phosphate potential in quenched samples. He observed that at both high and low magnitudes of $\Delta\tilde{\mu}_H$, the state 4 phosphate

potential varied linearly with $\Delta\tilde{\mu}_H$. However, at high $\Delta\tilde{\mu}_H$ this variation was considerably less than at low $\Delta\tilde{\mu}_H$; again, the ratio of ΔG_p to $\Delta\tilde{\mu}_H$ went up as the latter was decreased. The intriguing feature was that at high magnitudes of $\Delta\tilde{\mu}_H$, the variation of the phosphate potential with $\Delta\tilde{\mu}_H$ was suggestive of an $\vec{H}^+$/ATP stoichiometry of only 1, the same number as found with submitochondrial particles.

Earlier, Ogawa and Lee[129] had studied the intramitochondrial phosphate potential by NMR and compared this to $\Delta\tilde{\mu}_H$. During an ATP synthesis/ATP hydrolysis cycle, they found that the ratio between the phosphate potential and $\Delta\tilde{\mu}_H$ was rather independent of whether there was ATP synthesis or ATP hydrolysis (cf. Reference 131). The ratio tended to increase towards lower magnitudes of $\Delta\tilde{\mu}_H$.

Much of the other evidence of free-energy transduction in the absence of sufficiently high $\Delta\tilde{\mu}_H$ has been reviewed before[31,78,115] and will be reviewed elsewhere.[13]

From the above, it appears that the structure of free-energy-transducing membranes would be expected to lead to inhomogeneities in the spatial profiles of pH, electric potential, and perhaps even of proton electrochemical potential. Some of the available experimental evidence suggests that this leads to significant difficulties in measuring, or perhaps even defining $\Delta\tilde{\mu}_H$.

D. A Multitude of Extra Proton Domains?

1. Structure May Imply that the Coupling Units Are Extremely Small

The compression of the intra*cristal* space in mitochondria may limit proton diffusion with the effect of increasing the likelihood that the proton pumped by an electron-transfer chain complex is captured by the ATP synthase that is nearest to it. In effect, coupling units of an electron-transfer complex plus neighboring ATP synthases, would arise.[43,70,78,96,157]

In the simplest version of the chemiosmotic-coupling hypothesis protons pumped by any of the approximately 20,000 electron-transfer cytochrome oxidases of a rat liver mitochondrion can drive any of its approximately 15,000[26] ATP synthases to the synthesis of ATP; the coupling unit is as large as an entire mitochondrion. To energize a mitochondrion in the sense of generating a membrane potential of 0.2V, one would have to translocate approximately one million protons. To increase its internal pH by a unit, the mitochondrion would have to extrude approximately 10 million protons (using an internal buffer capacity of approximately 20 m*M*[158]). Although not particularly large, these particle numbers still satisfy the condition for the system to be described by the thermodynamic limit of statistical mechanics;[1] the square root of 10^6 (i.e., the standard deviation in the number of particles) is only 0.1% of that number. In the hypotheses of localized chemiosmotic coupling where there is only a single extra proton space introduced (or two, one for each side of the membrane), the size of the coupling unit is still quite considerable; it still contains all proton pumps, and approximately 1 million protons may be required to energize it.

If the delocalization of the protons would be limited because of the limited communication of the intra*cristal* spaces with the intermembrane space and, hence, with each other, e.g., because of the narrowness of the *pediculi*, or if even within each *crista*, the close apposition of the two membranes would slow down proton diffusion considerably, then one should propose an extra proton space for every single *crista* or part thereof. Inspection of electron micrographs suggests that rat liver mitochondria may contain up to 25 *cristae*, whereas heart mitochondria may contain many more. This may imply that each *crista* contains approximately 500 cytochrome oxidases and ATP synthases. From an estimated surface area of some 0.01 μm^2, one may expect an electric capacitance of some 10^{-16}F. Consequently, approximately 100 charges would have to be translocated across the *crista* membrane to generate a significant electric potential difference. In the case of a Poisson distribution, this already causes a relative standard deviation of approximately 10% between membrane potentials across the different *cristae*. Similarly, a decrease in the pH of an isolated intra*cristal*

space by a single unit would require the introduction of only approximately 1000 protons (assuming a buffer capacity of approximately 20 m*M* and a width of approximately 10 nm). In this respect, the statistical fluctuation would already amount to approximately 3%. We would suspect, however, that this effect would be too small to be detectable.

It would also be possible that proton diffusion in the *cristae* themselves would be so limited that a proton pumped by one cytochrome oxidase would have a much higher probability of being translocated back by the proximal ATP synthase than by any distal ATP synthases within the same *crista*. In that case, the effective coupling unit would consist of only a single cytochrome oxidase and one or a few ATP synthases that are close by. It is this latter size of coupling unit that was proposed in the coupling-unit elaboration of localized chemiosmotic coupling theories,[159] as well as in earlier proposals concerning the organization of membrane-linked free-energy transduction.[160]

2. The Implication that the Average Proton Gradient May Not Be Relevant

If only the matrix space (or "the" intra*cristal* space) of the mitochondrion were subdivided in a large number of small proton domains between which proton equilibration would be slow, one could readily understand that one would find a high phosphate potential at low average magnitudes of $\Delta\tilde{\mu}_H$. First, the proton domains may be so small as to have the average number of protons per domain as small as two or three. In such a case, the relative statistical variation of the number of protons between the various proton domains may be as high as 70%.[1] As a consequence, the rate of ATP synthesis can no longer be accurately predicted by inserting the average concentration of protons into the rate equation for ATP synthesis. Since over a large range of $\Delta\tilde{\mu}_H$ values the rate of ATP synthesis depends nonproportionally (note that this does not imply nonlinearly) on the proton concentration or $\Delta\tilde{\mu}_H$,[1,135] the proton domains with more than the average number of protons will generate more extra ATP synthesis than the proton domains with fewer than the average number of protons catalyzing ATP hydrolysis.[161] The rate of ATP synthesis would depend on the distribution of the protons over the proton domains at a given average number of protons per domain. This would explain that the relationship between the rate of ATP synthesis and the average $\Delta\tilde{\mu}_H$ may be different, depending on whether one varies the rate of electron transfer or the rate at which protons leak back across the membrane; at the same average $\Delta\tilde{\mu}_H$, the distributions could differ.[161,162]

3. Implications of the Small Size of the Coupling Units for the Control of Free-Energy Transduction

If the coupling units were to be extremely small, e.g., such that they comprise only one primary and one secondary proton pump, or (as analyzed in detail by Kahn[163] and Rich[21]) just a few more, then that would cause the energetic intermediate of the system not to behave as a pool kinetically. The essence of pool behavior is that protons pumped by any of the cytochrome oxidases could drive any of the ATP synthases into making ATP. Systems with pool behavior follow the familiar, as well as some less familiar rules concerning the control of the pathway flux and the concentration of the metabolites at steady-state[164,165] (for reviews see References 1 and 166).

One such rule of thumb was that the effect of an inhibitor of the primary proton pump on the pathway flux would be decreased by partial inhibition of the secondary pump (through the addition of an inhibitor specific to that pump); the latter inhibitor would make the primary pump less and, hence, the secondary pump more rate limiting. In the case where the proton leaks are the output system, one would expect that inhibition of electron transfer would decrease the effect of uncouplers on respiration. In systems with coupling units, each comprising only a single electron-transfer complex and a single ATP synthase at a time, one would expect that elimination of some of the primary pumps would not affect the titer of an inhibitor of the secondary pumps with respect to the pathway flux.

The experimental evidence in submitochondrial particles pointed in the latter direction; the inhibitors tended to exhibit no or positive, rather than negative, cooperativity. Lee and colleagues[167] observed that respiratory inhibitors of electron transfer did not reduce the effect of protonophore on respiration of submitochondrial particles. Oligomycin enhanced or did not reduce the effect of rotenone on ATP-energized reverse electron transfer in submitochondrial particles[1,168-170] (cf. Reference 171). Hitchens and Kell[172,173] have expanded the rationale of these experiments and carried them out with electron-transfer-driven phosphorylation in bacterial chromatophores. They also found positive or no, rather than negative, cooperativity between inhibitors of electron transfer and ATP synthase. Thus, it was concluded from the results of these so-called "double-inhibitor titrations" that the coupling unit in these systems must be quite small.[2,78,174] Since similar results have been obtained titrating oxidative phosphorylation by intact mitochondria with malonate and ADP[175] and in related experiments with thylakoid membranes,[176] this may not be a mere consequence of an artificially small size of the coupling unit in submitochondrial particles and bacterial chromatophores.[174]

It has been proposed[177] that another expectation from small coupling units would be that the apparent K_m for ADP in oxidative phosphorylation by submitochondrial particles would be independent of the rate of electron transfer. This expectation was *not* borne out; the apparent K_m for ADP in oxidative phosphorylation in submitochondrial particles was *increased* by inhibitors of electron transfer. Similarly, partial inhibition of the ATP synthases switched oxidative phosphorylation from low to high K_m kinetics.[177]

Baum et al.[168,169] and Hitchens and Kell[178] also introduced the so-called "uncoupler-inhibitor titrations". Here the tenet was that partial inhibition of the secondary proton pumps should, if anything, *increase* $\Delta\tilde{\mu}_H$ and, hence, reduce the effectiveness by which added uncoupler would abolish overall free-energy transduction. In experimental practice the opposite was observed.[1,168,169,178,179] Again, this led to the conclusion that the usual interpretation of chemiosmotic coupling in terms of one coupling unit per organelle might not be right.

We have recently reviewed the interpretation of double-inhibitor titrations at length[174] and can therefore be brief in our present analysis. Roughly two theoretical approaches have been applied to this question. First, several authors[180-183] found parameter choices in simple kinetic models of free-energy-transducing systems that would produce positive cooperativity between inhibitors of primary and inhibitors of secondary pumps, as well as positive cooperativity between inhibitors of the secondary pump and uncouplers. This proved that it was still possible that, if allowed arbitrary choices for the kinetic parameters of the enzymes in the system, systems reflecting small pool size would have a coupling unit of large size. Indeed, an experimental study with ATP synthase and bacteriorhodopsin co-reconstituted into liposomes, did not obey all but one of the rules expected for double inhibitor titrations in delocalized systems[184,185] (see, however, Reference 186).

The other approach used metabolic control theory[1,164-166,187] to examine what would be predicted for the effect of an inhibitor of the primary pumps on the flux control coefficient (i.e., the extent to which they are rate limiting) of the secondary proton pumps. It turned out that if the dependencies of the pumping rates and the proton leakage rate on $\Delta\tilde{\mu}_H$ would be linear or virtually linear over the range of the experiments, inhibition of the primary proton pumps should decrease the flux control by the secondary pump[1,96] and [174,185] vice versa. Thus, in this case, the rule for the delocalized systems was confirmed theoretically. Importantly, the rule of thumb for the uncoupler inhibitor titration was not confirmed by the control theoretic analysis; partial inhibition of the secondary proton pump was predicted to increase the flux control by the proton leak, just as was observed experimentally (the end-point of these titrations remained a valid argument, however). Also in this analysis, if the $\Delta\tilde{\mu}_H$ dependencies would be taken arbitrarily, then any result could be obtained in the double-inhibitor titrations. An extensive analysis, which took note of what we do know

about dependencies of rates of proton pumping on $\Delta\tilde{\mu}_{H^+}$.[174] suggested that the results of the double-inhibitor experiments cannot be explained in a coupling scheme with a pool of protons as the intermediate. Pietrobon and colleagues came to a similar conclusion.[188]

Metabolic control theory also suggests another approach to the question of the size of the coupling units. If the coupling unit size is large, then the normal control theorems should be obeyed. For instance, the sum of the flux-control coefficients should equal 1. Conversely, if the coupling unit would comprise only a single copy of the primary proton pump and a single copy of the secondary proton pump, then the sum of the flux control coefficients should equal 2.[2] Herweier and colleagues compared the effect of a covalent inhibitor of the ATP synthase on (1) oxidative phosphorylation with NADH as the substrate, (2) reverse electron transfer, and (3) ATP-P_i-exchange to the effect on uncoupler-stimulated ATPase.[171] Their results revealed that for the first two reactions the ATP synthase had flux-control coefficients of 1, whereas for the second reaction it had a flux-control coefficient of 2. In NADH-energized phosphorylation, complex I was similarly shown to have a flux-control coefficient of 1. These results suggest that the sum of the flux-control coefficients might equal 2, rather than 1, in both oxidative phosphorylation with NADH as the substrate and in ATP-P_i exchange; the coupling unit might be quite small. Alas, the argument was not completely closed. The proton leak is a third element in the system, which might have a flux-control coefficient on any output reaction of -1 (adding protonophore would reduce ATP synthesis or ATP-P_i exchange).[1] Then the sum of the flux-control coefficients would still add up to 1, and the results would be consistent with a delocalized coupling scheme.

Since increased proton permeability should stimulate respiration, the flux-control coefficient of the proton leak on *respiration* is likely to be positive. Thus, it is of extreme interest that Mereno-Sanchez and colleagues[189] recently reported that in submitochondrial particles the sum of the flux-control coefficients with respect to respiration greatly exceeded 1. In mitochondrial oxidative phosphorylation Groen and colleagues[190] did not observe such an excessive sum of the limited number of flux-control coefficients they measured.

That the tight control[191] observed in submitochondrial particles and bacterial chromatophores is not confined to systems where the vesicular units are extremely small, is demonstrated by the results of double-inhibitor-type experiments in intact rat liver mitochondria. Krasinskaya and colleagues[175] reported that an inhibitor of the adenine nucleotide translocator did not affect the titer of malonate with respect to state 3 respiration. Interestingly, this work also suggests why it may sometimes be difficult to observe the implications of the complex in vivo structure of free-energy-transducing membranes; the tight control was observed in hypo- and isoosmotic media, but not in hyperosmotic media (where state 3 respiration was lower anyway). This would be in line with the suggestion[70,175] that the hypertonic state of the mitochondrial matrix, by pressing the inner mitochondrial membranes onto each other and onto the outer mitochondrial membrane, might be responsible for subcompartmentation of the free-energy-transducing protons. It is not certain whether differences in experimental conditions affecting the mitochondrial structure are responsible for the fact that Stoner[192] in double-inhibitor titration studies found evidence for delocalization of the high energy intermediate.

Krasinskaya and colleagues also showed that the P/O ratio of mitochondria decreased with increasing tonicity of the medium.[175] If indeed, at higher tonicities of the medium, proton domains would become connected to the bulk aqueous phase, then this would suggest that, as expected,[157] the free-energy-transduction pathway through the intact proton domain would allow higher yields of oxidative phosphorylation and, hence, higher thermodynamic efficiencies. Interestingly, in the isoosmotic medium a 50% inhibition of respiration by malonate led to approximately 70% inhibition of phosphorylation at a decrease in membrane potential by only 12 mV. This and similar observations[193] in submitochondrial particles can be explained by assuming a very strong dependence of phosphorylation rate on $\Delta\tilde{\mu}_{H^+}$. How-

ever, the observation[175] that in *hyperosmotic* media malonate induced much stronger reductions in membrane potential at similar inhibitions of respiration and phosphorylation, would suggest that the phenomenon is a consequence of proton compartmentation.

As an additional caveat in the interpretation of the results of double inhibitor titrations, we remind the reader that the appearance of small coupling units does not have to refer to the proton domains and the pathway of the free-energy transduction. It could equally well reflect direct regulatory interaction between electron-transfer chains and ATP synthases, under conditions where the proton pathway would be delocalized.[194]

We would summarize this section by pointing out that there are various extents to which there may be heterogeneity in proton electrochemical potentials. Conclusive experimental evidence allowing us to decide where reality lies between complete delocalization and effective supercomplexes of electron transfer components and ATP synthases, is not available. At the moment, one can only choose on the basis of *assumed* proton mobilities in the complex mitochondrial structure.

V. THE VARIOUS RECENT MODIFICATIONS OF THE CHEMIOSMOTIC COUPLING HYPOTHESIS

A. The Minimal Hypothesis in 1984

In 1983 [96] and 1984 [78] we examined what would be the minimal addition to the traditional chemiosmotic coupling scheme that would make it account for the empirical knowledge of the moment. The minimal addition comprised the notion that the effective coupling unit might be small enough as to allow only limited pool behavior of the energized protons. At the same time it incorporated extra proton spaces which under steady-state conditions would not be at equilibrium with the aqueous bulk phases at their side of the membrane. One or a few primary proton pumps and one or a few secondary proton pumps would share a small proton space, which would be separated from the bulk aqueous phase at the same side of the membrane by some as of yet undefined resistance against proton movement. An entire mitochondrion would then contain approximately up to 20,000 [26] such coupling units, each consisting of a proton domain (or perhaps two, one on either side of the membrane), one or a few primary proton pumps, and one or a few secondary proton pumps. It is not likely that every coupling unit would permanently contain only a single primary and only a single secondary proton pump, because the relative concentrations of electron-transfer complexes and ATP synthases[26] would not lend themselves to that. What is also relevant here is that inhibition of ATP-P_i exchange in submitochondrial particles by azido-ATP was not linear with the inhibition of uncoupler-stimulated ATPase, suggesting that more than a single ATP synthase complex was effective involved in that exchange reaction[171] (cf. Reference 32).

The minimum hypothesis left open the question of whether the complexes between electron-transfer complexes and ATP synthases would have a long or a short lifetime. In view of the lateral mobility of electron-transfer complexes and ATP synthases in flattened inner mitochondrial membranes, it may seem likely that the complexes are not irreversible. Radiation inactivation experiments with *M. leisodeikticus* membranes[195] indicated that the effective target size for NADH dehydrogenase is equivalent to the monomeric form of the complex.

B. The Parallel-Coupling Hypothesis

Our formulation of a minimal hypothesis in 1983 [78,96] was not meant to imply that the ideas were particularly novel. In fact, as reviewed in Reference 78, various alternatives to the simplest version of the chemiosmotic coupling hypothesis had preceded this minimal formulation. An example is the parallel-coupling hypothesis,[114,115] which stated that in addition to the free-energy transduction via bulk-phase proton movement and monitorable

through $\Delta\bar{\mu}_{H}$, there be a more direct mechanism of energy transfer. This concept is similar, though not identical, to the concept of localized chemiosmotic coupling and has not been explicit with regards to the size of the coupling unit. At present, the parallel-coupling hypothesis seems to stand near the dynamic aggregate model for free-energy transduction in oxidative phosphorylation (cf. Section V.D.).[115]

C. Collisional Models

Slater and colleagues[14,196] generalized the collisional mechanism of electron transfer into what one may call the collisional model of free-energy transduction. In this hypothesis, the proton pumped by the electron-transfer protein would not dissociate from that primary proton pump until the protein would, due to lateral diffusion, bump into an ATP synthase. Then direct proton transfer would occur between the electron-transfer-linked proton pump and the ATP synthase. This model goes beyond the minimum hypothesis described above in proposing that the coupling units have a lifetime shorter than the average turnover time of the ATP synthase.

In Table 2 it can be seen that based on the diffusion coefficients observed by Hackenbrock and colleagues in fused mitochondrial inner membranes,[20,197] the collision-frequency between the electron-transfer complexes (especially if the relevant collision would occur while they are in their oxidized state) and the ATP synthase is sufficient to account for the observed rates of oxidative phosphorylation in intact mitochondria,[196,197] assuming that a significant fraction of the collisions would lead to proton transfer. Thus, in fused inner mitochondrial membranes this collisional mechanism of oxidative phosphorylation would seem feasible.

Yet, in experiments in which the phospholipid to protein ratio was varied by fusing liposomes to inner membranes, the diffusion coefficient of complex III depended strongly on the lipid to protein ratio of the membranes, suggesting that its diffusion would be strongly reduced if further constraints would be placed on its mobility. Also, we recall that Hochman et al.[198] reported a diffusion coefficient for cytochrome oxidase that was threefold lower than the one reported by Hackenbrock and colleagues. Because the concentration of cytochrome oxidase might also be lower than previously assumed, these authors concluded that the diffusion of cytochrome *c* might not be fast enough to reach cytochrome oxidase once each turnover.[198,199]

D. The Dynamic Aggregate Model

The diffusion rates on which the calculations of Tables 1 and 2 are based had been measured[20,197] in fused, inner mitochondrial membranes and inner membranes of megamitochondria: for the experimental technique used, the membrane had to be flat. The inner mitochondrial membrane *in situ* is highly folded; adjacent to it on either side there is an environment that is rich in protein. The space between the outer and inner mitochondrial surface membranes, as well as the intra*cristal* space, has been shown to be very narrow.

It must, therefore, be considered that the lateral diffusion rates of proteins in the inner mitochondrial membranes *in situ* may be much lower than those observed in the flattened inner membrane preparations. Indeed, it has been reviewed[200] that contact between membrane proteins and proteins outside the membranes may greatly reduce the diffusion rate of the former. In the case of mitochondria such contacts have indeed been proposed,[201] partly on the basis of affinity of enzymes for one another which has been demonstrated in vitro. Then, there is the possibility of reduced diffusion rates of membrane proteins due to the curvature of the membrane. Also, in principle, crowding could increase tremendously the effective aggregation of proteins.[202]

The above caveats, plus the observation of a rather slow diffusion rate of cytochrome *c*,[198,199] have led Ferguson-Miller and co-workers to stress that the electron-transfer complexes may form a dynamic aggregate on the time-scale of turnover; these aggregates would stay together for at least a number of turnovers; thus, electron transfer from NADH to O_2 would

not critically depend on the diffusion of the electron-transfer complexes, cytochrome *c*, and ubiquinone.

For coupling-unit hypotheses of mitochondrial free-energy transduction, this also seems a possibility. The electron transfer complexes and the ATP synthases may form dynamic aggregates which are stable over times longer than two turnovers, but may rearrange dynamically at longer time-scales.[199] This nonminimum model for coupling of oxidation to phosphorylation may be called the "dynamic aggregate" model for chemiosmotic coupling. It is an extension of the 1984 minimum hypothesis that differs from the collisional model described in the previous section. Here, the lifetime of the coupling units significantly exceeds the turnover time of the ATP synthase.

Finding that the degree of coupling of mitochondrial oxidative phosphorylation depended on the temperature, Rottenberg[203] suggested that temperature may affect the formation of aggregates between electron-transfer complexes and ATP synthases. The decreased coupling at high temperatures was not the result of a lower $\Delta\tilde{\mu}_H$, but was accompanied by a lower phosphate potential.[150] In submitochondrial particles the fluorescence anisotropy decay of a label attached to F_1, revealed a 60% subpopulation of rotationally frozen ATP synthases.[204]

To our knowledge, oxidative phosphorylation has not been reported in inner mitochondrial membranes diluted by fusion with liposomes, nor in megamitochondrial inner membranes under the conditions of the fluorescence recovery after photobleaching experiments. Dilution of the concentration of the electron-transfer complexes and the ATP synthases by approximately 30% would not be expected to have such a strong effect on the collision frequency between the complexes; the effect on the rate of electron transfer between electron-transfer complexes and ubiquinone was approximately only 20%.[19] In the coupling-unit model, the dilution of the proteins in the membrane could well reduce their association or destroy the required barrier for proton diffusion between the proton domain and the aqueous bulk phase. The standard chemiosmotic coupling theory would, of course, explain the observations by simply stating that the fusion had resulted in an increase in the passive leakage of protons through the membranes.

In a random diffusion model one would expect that the participating proteins and the participating coenzymes could form "pools". Indeed, ubiquinone in energy-coupling membranes has been shown to kinetically exhibit pool behavior.[205] Even if one postulates direct proton transfer from the primary proton pump to the secondary proton pump, then one might expect kinetics reflecting pool behavior, because the enzymes themselves would constitute pools. Typically, elimination of a primary proton pump through the action of an irreversible inhibitor would not have the effect of simultaneously eliminating an equivalent number of secondary proton pumps. If for some reason there would be an excess capacity in the primary proton pumps (e.g., because the turnover of the ATP synthase would be rate limiting), then one would not even expect the inhibitor of the primary proton pump to have any effect at all.

A quantitative model for the collision hypothesis (cf. References 13 and 235) suggested that to accommodate the results of the double-inhibitor titrations indicative of the absence of pooling, the collision model must assume that diffusion is rate limiting. This implies that for each turnover, an electron-transfer chain only collides once with a single ATP synthase, at least under the conditions where the double inhibitor titrations have been carried out. Based on the diffusion data of Hackenbrock and colleagues, this is not obviously the case. If there were free diffusion, then one would expect multiple collisions to take place per turnover (cf. Table 2). Of course, one may here again point at the presumably reduced diffusion rates of the complexes in the inner mitochondrial membrane, but doing so may take away supportive character of the results of Hackenbrock et al. If the diffusion rates in the inner mitochondrial membrane would have to be assumed to be slower anyway, then they may just as well be assumed to be too slow to allow for one collision per turnover, so that the dynamic aggregate version of the coupling unit model could just as well be valid.

E. Coupling Models Not Requiring Extra Proton Barriers

Quite a few studies have appeared in the literature suggesting that membrane surfaces may rapidly conduct protons,[111,206] thus providing a simple mechanism for the direct transfer of protons from electron-transfer complexes to ATP synthases. A series of studies by Teissié and co-workers[206-208] and an interpretation thereof,[209] deserve some comment in this respect. These authors used a Langmuir trough, in which the subphase (1 m*M* phosphate at pH 6.8 initially) was divided into three parts by two glass plates. After sudden acidification of the left-hand compartment, the appearance of surface protons at the monolayer in the right-hand compartment was monitored either through the fluorescence of fluorescein attached to phospholipids, or[208] by electrodes above them. The results indicated a diffusion coefficient, apparently along the surface, approximately 400 times faster than in normal water. For the questions we are asking, however, it is more important whether it follows from the observations that the diffusion between the surface and the bulk phase is indeed "slow", as concluded by Selwyn.[209] This slowness may be suggested by the facts that (1) the conduction occurred through the middle compartment, even though the bulk phase there remained at pH 6.8, and (2) for an appreciable time, there remained a pH difference of approximately 4 units between the surface and the bulk phase in the left-hand compartment. However, this observation would be expected if the diffusion of protons between the surface and the bulk phase would proceed at its normal rate. Since this diffusion coefficient is approximately 400 times lower, only a fraction of the proton current would escape into the bulk phase and, because the bulk phase is buffered by the 1-m*M* phosphate, this fraction would not be expected to cause a significant reduction in bulk phase pH.

We continue[78,102,170] to stress that to understand that there could be rapid proton transfer between the proton pumps is not the problem. Even with the diffusion constant for protons in water, proton delocalization along the surface would be fast enough for protons to move from electron-transfer complexes to ATP synthases. The problem is to understand why, at steady state, the protons would not equilibrate electrochemically with the aqueous bulk phase bordering the local proton domains (cf. Section II.B).[210,211] The experiment discussed in the previous paragraph does not achieve such a steady state.

The collision model also requires some type of proton barrier. From what we known of proton binding groups on proteins, their dissociation rate constants are generally in the order of 40,000 sec^{-1} (see below). Thus, the protons pumped by the primary proton pump would be expected to dissociate into the neighboring aqueous phase, unless there would be some barrier. Assuming a diffusion-limited association rate constant of approximately $4 \cdot 10^{11}$ M^{-1} sec^{-1} (calculated from the Debye-Smoluchowski Equation) and a pK_a around 7, this gives a dissociation rate constant of approximately 40,000 sec^{-1}, which is too fast for the proton to be effectively retarded on an inert protein on the time-scale of turnover (for experimental see References 210, 238, 239).

Thus, in both the static coupling unit hypothesis and in the collisional hypothesis an extra proton barrier is needed, for which there is little existing empirical basis; it is little more than an *ad hoc* postulate. We considered this a weak point of the various localized coupling hypotheses.

More recently, however, theoretical considerations suggested that proton barriers may not really be required to make the inner mitochondrial membrane plus its *cristae* function as a set of coupling units. During turnover, most membrane enzymes, and certainly ion pumps, generate a varying electric field[212-215] which might activate a secondary proton pump (e.g., an ATP synthase), temporarily or permanently residing in the same region.[216-218] Since periods of decreased local electric potential are also implied by the phenomenon of the oscillation, it seemed that the oscillating electric field would not be able to shift the equilibrium, i.e., allow the ATP synthase to synthesize ATP at a phosphate potential significantly higher than the average $\Delta\tilde{\mu}_H$ multiplied by the proton translocation stoichiometry. A passive pore (as

proposed in Reference 215) would always work in the direction of dissipating even the local electrochemical potential difference for protons.

The demonstration that such ATP synthesis might be possible came both from experimental and theoretical work. First, it was shown that an oscillating electric field across the erythrocyte membrane can induce uptake of K^+ against its electrochemical gradient, catalyzed by the Na^+-K^+-ATPase, probably in the absence of a free-energy source other than that oscillating electric field.[219] Subsequent theoretical considerations of the thermokinetic properties of membrane enzymes revealed that harvest of free energy from an oscillating, or fluctuating (in the nonequilibrium sense, for details refer to Reference 218) field should be quite a general phenomenon, although the quantitative importance compared to other mechanisms of free-energy transduction could not yet be assessed.[218,220,221] Thus, it was shown in model calculations that if electron transfer and ATP synthesis would both involve cyclic transfer of charge across the membrane, or a cyclic variation in the dipole moment of the enzyme, then close proximity of an electron transfer complex and an ATP synthase[213] could well induce the latter to synthesize ATP against a phosphate potential in the absence of any direct physical contact between the two enzymes and, at a zero-time average difference in electric potential and pH gradient across the membrane.[191,213,218] In an additional theoretical study, it could even be shown that when $\Delta\tilde{\mu}_H$ was zero at all times, but the transmembrane electric potential and pH fluctuated in a compensatory manner, an ATP synthase could, in principle, make ATP against the free energy of hydrolysis of that molecule.[191]

As a result of these studies, it has been proposed[119,217,236] that energy coupling is organized in coupling units consisting of primary and secondary proton pumps that are close enough to each other so as to make them sense changes in the electric field generated by one another. Under normal operating conditions, there would be a significant $\Delta\tilde{\mu}_H$ between the bulk phases. However, if the average electrochemical potential difference for protons between the two bulk aqueous phases would be greatly reduced, free-energy transduction through the locally oscillating electric field might become quantiatively important and might continue to drive ATP synthesis, even though the time and space averaged $\Delta\tilde{\mu}_H$ would predict ATP hydrolysis.

Although this proposal[191] is speculative, it offers the advantages that it no longer requires the presence of a proton barrier. Yet, it explains many, if not all, the anomalies that seemed to be in conflict with the standard interpretation of the chemiosmotic coupling theory without challenging chemiosmotic properties that have become widely (if passively) accepted. The enzymes are still proton pumps, the delocalized electrochemical proton gradient will normally contribute significantly to free-energy transduction, and ATP synthesis induced by artificial electrochemical potential differences for protons are explained as usual.

As with the other models, a lack of clarity remains with respect to some of the quantitative aspects. If the inner mitochondrial membrane were an ideal lipid bilayer, then the electron-transfer complexes and the ATP synthases would have to be as close together as 1 nm in order to make electrostatic interactions relevant.[213,240] As we reviewed in Section IV.A, however,[80,84,191] electric potentials may extend much wider if the accessibility of the membrane surface to ions is limited, or slow. At present, information on this ion accessibility in intact mitochondria is simply unavailable.

In the above discussion of hypotheses of free-energy transduction, we have limited ourselves to the discussion of proton-mediated coupling. Other possible modes of free-energy transduction have been reviewed elsewhere.[222-224]

Reviewing most often tantalizes one to draw a single conclusion about how the system is organized. For membrane-linked free-energy transduction, such a single conclusion is still not possible. In 1984 [78,157] we escaped the dilemma by refraining from complete concepts and limiting ourselves to minimal models. This time we would like to point at the five possible ways of viewing proton-mediated free-energy transduction outlined above and the

many possibilities that lie between them. Without further experimentation directed at the quantitative and thermodynamic properties of mitochondrial proton movement, as well as the structural organization of the enzymes involved in the intact mitochondrion, it is a futile effort to try to decide between these possibilities. Yet, the five existing views may serve as the inspiration for such continued experimentation. In view of the quantitative nature of the differences, we feel that theoretical modeling studies should accompany such experimentation. By the standards of biochemistry and molecular biology, the question of how proton-mediated free-energy transduction is organized, is still unsolved.

VI. CONSEQUENCES OF THE UNSUSPECTED COMPLEXITY OF CHEMIOSMOTIC COUPLING: UNCOUPLERS, SLIPPERS, AND DECOUPLERS

When new mechanistic features are proposed, this implies that there might be as-yet unknown ways in which the behavior of the system can be modified, in particular, uncoupled.

Ramirez and colleagues focused on fluorescamine derivatives and distinguished three classes.[225-227] These compounds do not uncouple much in the most traditional sense, i.e., that of decreasing the P/O ratio,[226] but they do interfere with mitochondrial proton extrusion upon addition of ATP or respiratory substrate. Rottenberg identified general anesthetics as a new type of uncoupler, called "decoupler"; they reduce the P/O ratio without causing much of a decrease in $\Delta\tilde{\mu}_{H}$. These agents increased state 4, but not state 3, respiration and increased the rate of ATP hydrolysis both in the presence and in the absence of FCCP.[228] Rottenberg and Hashimoto[229] found that free fatty acids uncouple in a fashion that is again somewhat different. Importantly, free fatty acids did not inhibit ATP synthesis driven by an artificial pH gradient in submitochondrial particles.[230] Thus, these agents would not cause slip in the ATP synthase, but specifically interfere with the intramembrane proton conductance.[229]

Azzone et al.[231] obtained similar, though not identical, results comparing the effects of chloroform (a general anesthetic), oleic acid, and FCCP. The main difference was that oleic acid did increase the membrane conductance to protons and did decrease $\Delta\tilde{\mu}_{H}$ while reducing the P/O ratio. These authors concluded that both FCCP and oleic acid uncouple through more than one mechanism.

Higuti and colleagues[94,215,232] have studied two groups of so-called "anisotropic" inhibitors of oxidative phosphorylation. The two groups differ in that they are effective only from the cytosolic side of the membrane (the positively charged ones) or only from the matrix side. Higuti and colleagues propose that the inhibitors act by interacting with defined[215] membrane proteins called chargerins, that are involved in the proton pumping action of the electron transfer complexes and of the ATP synthase. These may be the materialization of the proposed protoneural proteins.[2,112]

Some, but probably not all, of the above-described effects of the novel uncouplers may be explained by making quite a different modification to the simplest version of the chemiosmotic hypothesis, i.e., by assuming that the compounds cause the pumps to slip.[226,227,231,233] On the other hand, the observations from which it has been concluded that mitochondrial proton pumps slip, can also be explained by assuming that they do not slip, but rather that there is proton microcompartmentation.[78]

VII. CONCLUSIONS

We conclude that effects on the kinetics and thermodynamics of oxidative phosphorylation, expected from the complex structure of the mitchondrion, are indeed observed. A simple interpretation of most results is that there is an effective microcompartmentation of protons. This need not require the presence of proton barriers. Yet, the enormous complexity of the

structure of free-energy-transducing membranes prevents any definite conclusions on to what extent such microcompartmentation is occurring. As yet, it is not possible to exclude the possibility that the protons are effectively delocalized and that a number of other modifications of the simplest version of the chemiosmotic coupling hypothesis (such as slip and allosteric regulation) are solely responsible for the experimental observations.

VIII. SUMMARY

A review of the structure of energy-transducing membranes with a focus on the mitochondrion, reveals that there is a wide gap between the actual structure and the structure depicted in the usual diagrams illustrating the chemiosmotic coupling concept. Potential implications of the actual structure for chemiosmotic coupling are considered. These include increased localization of the energy-coupling protons near membranes, apparent direct interaction between electron transfer and ATP synthesis, and an apparent "slip" of the proton pumps. A review of the relevant experimental information suggests that at least some of these may be relevant for free-energy coupling in the physiological state. To understand physiological membrane-linked energy transduction, a new chemiosmotic coupling concept is needed which considers various kinds of heterogeneity. The various attempts to develop such a concept are reviewed.

ACKNOWLEDGMENTS

We thank Drs. R. D. Astumian, G. F. Azzone, J. S. Clegg, T. E. Conover, D. P. Jones, D. Pietrobon, H. Rottenberg, F. Sjöstrand, E. C. Slater, and G. R. Welch for discussions, and Drs. T. E. Conover and H. Rottenberg for allowing perusal of unpublished information. This work was supported in part by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.). Douglas B. Kell thanks the SERC for financial support.

REFERENCES

1. **Westerhoff, H. V. and Van Dam, K.**, *Thermodynamics and Control of Biological Free-Energy Transduction*, Elsevier, Amsterdam, 1987.
2. **Kell, D. B. and Westerhoff, H. V.**, Catalytic facilitation and membrane bioenergetics, in *Organized Multienzyme Systems: Catalytic Properties*, Welch, G. R., Ed., Academic Press, New York, 1985, 63.
3. **Westerhoff, H. V.**, Organization in the cell soup, *Nature*, 311, 489, 1985.
4. **Ling, G. B.**, *In Search of the Physical Basis of Life*, Plenum Press, New York, 1984.
5. **Kuroda, K.**, in *Primitive Motile Systems in Cell Biology*, Allen, R. D. and Kamiya, N., Eds., Academic Press, New York, 1964, 3.
6. **De Vries, H.**, *Arch. Neerl. Sci.*, 6, 117, 1871.
7. **Porter, K. R.**, Structural organization of the cytomatrix, in *The Organization of Cell Metabolism*, Welch, G. R. and Clegg, J. S., Eds., Plenum Press, New York, 1986, 9.
8. **Fey, E. G. and Penman, S.**, New views of cell and tissue architecture: embedment-free electron microscopy and biochemical analysis, in *The Organization of Cell Metabolism*, Welch, G. R. and Clegg, J. S., Eds., Plenum Press, New York, 1986, 89.
9. **Ris, H.**, The cytoplasmic "microtrabecular lattice" — reality or artifact, in *34th Annu. Proc. Electron Microscopy Society of American*, Bailey, G. W., Ed., Claitors Publishing, Baton Rouge, La., 1980, 812.
10. **Vale, R. D., Schnapp, B. J., Reese, T. S., and Sheetz, M. P.**, Movement of organelles along filaments dissociated from the axoplasm of the squid giant axon, *Cell*, 40, 449, 1985.
11. **Wolosewick, J. J. and Porter, K. R.**, The microtrabecular lattice of the cytoplasmic ground substance: artifact or reality, *J. Cell. Biol.*, 82, 114, 1979.
12. **Welch, G. R. and Clegg, J. S.**, *The Organization of Cell Metabolism*, Plenum Press, New York, 1986.

13. **Westerhoff, H. V.**, The organization of chemiosmotic coupling, *Microbiol. Rev.*, 1989, in press.
14. **Slater, E. C.**, The mechanism of the conservation of energy in biological oxidations, *Eur. J. Biochem.*, 166, 489, 1987.
15. **Stidham, M. A., McIntosh, T. J., and Siedow, J. N.**, On the localization of ubiquinone in phosphatidylcholine biolayers, *Biochim. Biophys. Acta*, 767, 423, 1984.
16. **Hackenbrock, C. R., Chazotte, B., and Gupte, S. S.**, Lateral diffusion of coenzyme Q and its redox partners in the control of mitochondrial electron transport, in *Biomedical and Clinical Aspects of Coenzyme Q*, Vol. 5, Folkers, K. and Yamamura, Y., Eds., Elsevier, Amsterdam, 1986, 25.
17. **Hackenbrock, C. R., Gupte, S. S., and Gazotte, B.**, The random collision model of mitochondrial electron transport, in *Achievements and Perspectives of Mitochondrial Research*, Vol. 1, Quagliariello, E. et al., Eds., Elsevier, Amsterdam, 1985, 83.
18. **Gupte, S. S. and Hackenbrock, C. R.**, Multidimensional diffusion of cytochrome *c* in mitochondrial electron transport, in *Integration of Mitochondrial Function*, Lemasters, J. J. et al., Eds., Plenum Press, New York, 1988.
19. **Hackenbrock, C. R.**, Lateral diffusion and electron transfer in the mitochondrial inner membrane, *Trends Biochem. Sci.*, 6, 151, 1981.
20. **Gupte, S. S., Wu, E.-S., Hoechli, L., Hoechli, M., Jacobson, K., Sowers, A. E., and Hackenbrock, C. R.**, Relationship between lateral diffusion, collision frequency, and electron transfer of mitochondrial inner membrane oxidation-reduction components, *Proc. Natl. Acad. Sci. U.S.A.*, 81, 2606, 1984.
21. **Rich, P. R.**, Electron and proton transfer through quinones and cytochrome bc complexes, *Biochim. Biophys. Acta*, 768, 53, 1984.
22. **Kell, D. B.**, On the lateral mobility of proteins in prokaryotic membranes, *Biochem. Soc. Trans.*, 14, 825, 1986.
23. **Von Smoluchowski, M.**, Versuch einer mathematischen Theorie der Koagulationskinetik kolloider Lösungen, *Z. Phys. Chemie*, 92, 129, 1917.
24. **Hardt, S. L.**, Rates of diffusion controlled reactions in one, two and three dimensions, *Biophys. Chem.*, 10, 239, 1979.
25. **Hess, B. and Bolteux, A.**, Heterologous enzyme-enzyme interactions, in *Protein-Protein Interactions*, Springer-Verlag, Berlin, 1972, 271.
26. **Schwerzmann, K., Cruz-Orive, L. M., Eggman, R., Sanger, A., and Weibel, E. R.**, Molecular architecture of the inner membrane of mitochondria from rat liver: a combined biochemical and stereological study, *J. Cell Biol.*, 102, 97, 1986.
27. **Junge, W. and Polle, A.**, Theory of proton flow along appressed thylakoid membranes under both non-stationary and stationary conditions, *Biochim. Biophys. Acta*, 848, 265, 1986.
28. **Slavík, J. and Kotyk, A.**, Intracellular pH distribution and transmembrane pH profile of yeast cells, *Biochim. Biophys. Acta*, 776, 679, 1984.
29. **Slavík, J.**, The pH gradient across the plasma membrane — does it really exist?, *EBEC Rep.*, 4, 336, 1986.
30. **De Kouchkovsky, Y., Sigalat, C., Haraux, F., and Phung Nhu Hung, S.**, Proton currents and local energy coupling in thylakoids: a survey, in *Ion Interactions in Energy Transfer Biomembranes*, Papageorgiou, G. C. et al., Eds., Plenum Press, New York, 1986, 119.
31. **Ferguson, S. J.**, Fully delocalised chemiosmotic or localised protons flow pathways in energy coupling? A scrutiny of experimental evidence, *Biochim. Biophys. Acta*, 811, 47, 1985.
32. **Hatefi, Y.**, The mitochondrial electron transport and oxidative phosphorylation system, *Annu. Rev. Biochem.*, 54, 1015, 1985.
33. **Harold, F. M.**, *The Vital Force: A Study of Bioenergetics*, W. H. Freeman, New York, 1986.
34. **Sjöstrand, F. S.**, The diversity of function and structure of cellular membranes, *Subcell. Biochem.*, 9, 335, 1983.
35. **Singer, S. J. and Nicolson, G. L.**, The fluid mosaic model of the structure of cell membranes, *Science*, 175, 720, 1972.
36. **Crane, F. L. and Hall, J. D.**, Diversity in membrane structure: from unit to binary, *Ann. N.Y. Acad. Sci.*, 195, 24, 1972.
37. **Palade, G. E.**, The fine structure of mitochondria, *Anat. Rec.*, 114, 427, 1952.
38. **Sjöstrand, F. S.**, Electron microscopy of mitochondria and cytoplasmic double membranes. Ultrastructure of rod-shaped mitochondria, *Nature*, 171, 30, 1953.
39. **Massari, S., Frigeri, L., and Azzone, G. F.**, Permeability to water, dimension of surface and structural changes during swelling in rat liver mitochondria, *J. Membr. Biol.*, 9, 57, 1972.
40. **Daems, W. Th. and Wisse, E.**, Shape and attachment of the *cristae mitochondriales* in mouse hepatic cell mitochondria, *J. Ultrastruct. Res.*, 16, 123, 1966.
41. **Andersson-Cedergren, E.**, Ultrastructure of motor end plate and sarcoplasmic components of mouse skeletal muscle fiber as revealed by 3-dimensional reconstructions from serial sections, *J. Ultrastruct. Res.*, Suppl. 1, 1, 1959.

42. **Brdiczka, D. and Kolb, V.**, Contact sites between the outer and inner boundary membranes of rat-liver mitochondria: electron microscopic morphology and possible function, *J. Ultrastruct. Res.*, 63, 94, 1978.
43. **Brdiczka, D. and Reith, A.**, Functional and structural heterogeneity of the inner mitochondrial membrane, in *The Organization of Cell Metabolism*, Welch, G. R. and Clegg, J. S., Eds., Plenum Press, New York, 1986, 277.
44. **Reith, A.**, Grenzen und Swierigkeiten bei Struktur- und Funktionsanalysen in der Biomedizin durch Korrelation stereologisch- biochemischer Daten, *Gegenbaurs Morph. Jahrb. Leipzig*, 126, 327, 1980.
45. **Stoner, C. D. and Sirak, H. D.**, Osmotically induced alterations in volume and ultrastructure of mitochondria isolated from rat liver and bovine heart, *J. Cell Biol.*, 43, 521, 1969.
46. **Hackenbrock, C. R.**, Ultrastructural basis for metabolically linked mechanical activities in mitochondria. I. Reversible ultrastructural changes with change in metabolic state in isolated rat-liver mitochondria, *J. Cell Biol.*, 30, 269, 1966.
47. **Hackenbrock, C. R.**, Ultrastructural bases for metabolically linked mechanical activity in mitochondria. II. Electron-transport linked ultrastructural transformations in mitochondria, *J. Cell Biol.*, 37, 345, 1968.
48. **Candipan, R. C. and Sjöstrand, F. S.**, Freeze-fracture analysis of isolated liver mitochondria in different metabolic states, *J. Ultrastruct. Res.*, 89, 274, 1984.
49. **Lehninger, A. L.**, *The Mitochondrion*, Benjamin, New York, 1964.
50. **Revel, J. P., Fawcett, D. W., and Philpott, C. W.**, Observations on mitochondrial structure. Angular configurations of *cristae*, *J. Cell Biol.*, 16, 187, 1963.
51. **Sjöstrand, F. S.**, The structure of mitochondrial membranes. A new concept, *J. Ultrastruct. Res.*, 64, 217, 1978.
52. **Candipan, R. C. and Sjöstrand, F. S.**, Permeability changes in isolated liver mitochondria during different metabolic states, *J. Ultrastruct. Res.*, 89, 212, 1984.
53. **Candipan, R. C. and Sjöstrand, F. S.**, Water movement from intra*cristal* spaces in isolated liver mitochondria, *J. Ultrastruct. Res.*, 89, 249, 1984.
54. **Baakeva, L. R., Chentsov, Y. S., Jasaitis, A. A., and Skulachev, V. P.**, The effect of oncotic pressure on heart muscle mitochondria, *Biochim. Biophys. Acta*, 275, 319, 1972.
55. **Muscatello, U., Guarriera-Bobyleva, V., and Buffa, P.**, Configurational changes in isolated rat liver mitochondria as revealed by negative staining. II. Modifications caused by changes in respiratory state, *J. Ultrastruct. Res.*, 40, 235, 1972.
56. **Blair, P. V. and Munn, E. A.**, Regulation of mitochondrial oxidations by induction of morphological changes. I. Dinitrophenol initiated concomitant transitions in inner membrane matrix structure and succinate oxidation, *Biochem. Biophys. Res. Commun.*, 49, 727, 1972.
57. **Candipan, R. C. and Sjöstrand, F. S.**, An analysis of the contribution of the preparatory technique to the appearance of condensed and orthodox conformations of liver mitochondria, *J. Ultrastruct. Res.*, 89, 281, 1984.
58. **De Kruijff, B., Nayar, R., and Cullis, P. R.**, ^{31}P NMR studies on phospholipid structure in membranes of intact, functionally active rat-liver mitochondria, *Biochim. Biophys. Acta*, 684, 47, 1982.
59. **Cullis, P. R., De Kruijff, B., Hope, M. J., Nayar, R., Rietveld, A., and Verkleij, A. J.**, *Biochim. Biophys. Acta*, 600, 625, 1980.
60. **Van Venetië, R. and Verkleij, A. J.**, Possible role of non-bilayer lipids in the structure of mitochondria. A freeze-fracture electron microscopy study, *Biochim. Biophys. Acta*, 692, 397, 1982.
61. **Lemasters, J. J.**, Possible role of the mitochondrial outer membrane as an oncotic regulator of mitochondrial volume, *FEBS Lett.*, 88, 10, 1978.
62. **Knoll, G. and Brdiczka, D.**, Changes in freeze-fractured mitochondrial membranes correlated to their energetic state. Dynamic interactions of the boundary membranes, *Biochim. Biophys. Acta*, 733, 102, 1983.
63. **Hackenbrock, C. R.**, Chemical and physical fixation of isolated mitochondria in low-energy and high-energy states, *Proc. Natl. Acad. Sci. U.S.A.*, 61, 598, 1968.
64. **Wrigglesworth, J. M., Packer, L., and Branton, D.**, Organization of mitochondrial structure as revealed by freeze etching, *Biochim. Biophys. Acta*, 205, 125, 1970.
65. **Sjöstrand, F. S. and Candipan, R. C.**, Connections between the *cristae* and the surface of rat heart muscle mitochondria, *J. Ultrastruct. Res.*, 91, 38, 1985.
66. **Lehninger, A. L.**, *Biochemistry*, Worth Publishers, New York, 1975.
67. **Zalman, L. S., Nikaido, H., and Kagawa, Y.**, Mitochondrial outer membrane contains a protein producing nonspecific diffusion channels, *J. Biol. Chem.*, 255, 1771, 1980.
68. **Roos, N., Benz, R., and Brdiczka, D.**, Identification and characterization of the pore-forming protein in the outer membrane of rat-liver mitochondria, *Biochim. Biophys. Acta*, 686, 204, 1982.
69. **Fiek, C., Benz, R., Roos, N., and Brdiczka, D.**, Evidence for identity between the hexokinase-binding protein and the mitochondrial porin in the outer membrane of rat liver mitochondria, *Biochim. Biophys. Acta*, 688, 429, 1982.
70. **Van Dam, K., Woelders, H., Colen, A.-M., and Westerhoff, H. V.**, A structural basis for mosaic protonic coupling, *Biochem. Soc. Trans.*, 12, 401, 1984.

71. **Tedeschi, H. and Harris, D. L.**, The osmotic behavior and permeability to ion-electrolytes of mitochondria, *Arch. Biochem. Biophys.*, 58, 52, 1955.
72. **Beavis, A. D., Brannan, R. D., and Garlid, K. D.**, Swelling and contraction of the mitochondrial matrix. I. A structural interpretation of the relationship between light scattering and matrix volume, *J. Biol. Chem.*, 260, 13424, 1985.
73. **Koch, A. L.**, Some calculations on the turbidity of mitochondria and bacteria, *Biochim. Biophys. Acta*, 51, 429, 1961.
74. **Harris, E. J. and Van Dam, K.**, Changes of total water and sucrose space accompanying induced ion uptake and phosphate swelling of rat liver mitochondria, *Biochem. J.*, 106, 759, 1967.
75. **Knight, V. A., Wiggins, P. M., Harvey, J. D., and O'Brien, J. A.**, The relationship between the size of mitochondria and the intensity of light that they scatter in energetic states, *Biochim. Biophys. Acta*, 637, 146, 1981.
76. **Srere, P.**, The structure of the mitochondrial inner membrane-matrix compartment, *Trends Biochem. Sci.*, 7, 375, 1982.
77. **Garlid, K. D. and Beavis, A. D.**, Swelling and contraction of the mitochondrial matrix. II. Quantitative application of the light scattering technique to solute transport across the inner membrane, *J. Biol. Chem.*, 260, 13434, 1985.
78. **Westerhoff, H. V., Melandri, B. A., Venturoli, G., Azzone, G. F., and Kell, D. B.**, A minimal hypothesis for membrane-linked free-energy transduction. The role of independent, small coupling units, *Biochim. Biophys. Acta*, 768, 257, 1984.
79. **Mitchell, P.**, *Chemiosmotic Coupling and Energy Transduction*, Glynn Research Laboratories, Bodmin, England, 1968.
80. **Kamp, F., Chen, Y., and Westerhoff, H. V.**, Energization induced redistribution of charge carriers near membranes, *Biophys. Chem.*, 1988.
81. **Williams, R. J. P.**, Mitochondrial compartments and chemi-osmosis, *Trends Biochem. Sci.*, 8, 48, 1983.
82. **Hartzog, G., Mehlhorn, R. J., and Packer, L.**, A reevaluation of the surface potential of the inner mitochondrial membrane using ESR techniques, in *Ion Interactions in Energy Transfer Biomembranes*, Papageorgiou, G. C., Barber, J., and Papa, S., Eds., Plenum Press, New York, 1986, 39.
83. **Rottenberg, H.**, The determination of the surface potential of biological membranes, *Methods Enzymol.*, in press.
84. **Kamp, F., Chen, Y., and Westerhoff, H. V.**, Spatial charge distribution and electric potential profile across energy coupling membranes, *EBEC Rep.*, 4, 323, 1986.
85. **Rottenberg, H.**, Surface potential in energized mitochondria, in *Ion Interactions in Energy Transfer Biomembranes*, Papageorgiou, G. C., Barber, J., and Papa, S., Eds., Plenum Press, New York, 1986, 29.
86. **Van Walraven, H. S., Marvin, H. J. P., Koppenaal, E., and Kraayenhof, R.**, Proton movements and electric potential generation in reconstituted ATPase proteoliposomes from the thermophilic cyanobacterium *Synechococcus* 6716, *Eur. J. Biochem.*, 144, 555, 1984.
87. **Pietrobon, D., Azzone, G. F., and Walz, D.**, Effect of Funiculosin and Antimycin A on the redox-driven H^+-pumps in mitochondria: on the nature of "leaks", *Eur. J. Biochem.*, 117, 389, 1981.
88. **Pietrobon, D., Zoratti, M., Azzone, G. F., Stucki, J. W., and Walz, D.**, Non-equilibrium thermodynamic assessment of redox-driven H^+ pumps in mitochondria, *Eur. J. Biochem.*, 127, 483, 1982.
89. **Pietrobon, D., Zoratti, M., and Azzone, G. F.**, Molecular slipping in redox and ATPase H^+ pumps, *Biochim. Biophys. Acta*, 723, 317, 1983.
90. **Zoratti, M., Favaron, M., Pietrobon, D., and Azzone, G. F.**, Intrinsic uncoupling of mitochondrial proton pumps. 1. Non-ohmic conductance cannot account for the nonlinear dependence of static head respiration on $\Delta\tilde{\mu}_H$, *Biochemistry*, 25, 760, 1986.
91. **Kell, D. B. and Hitchens, G. D.**, Proton-coupled energy transduction by biological membranes. Principles, pathways and praxis, *Faraday Discuss. Chem. Soc.*, 74, 377, 1982.
92. **Hitchens, G. D. and Kell, D. B.**, On the effects of thiocyanate and venturicidin on respiration-driven proton translocation in *P. denitrificans*, *Biochim. Biophys. Acta*, 766, 222, 1984.
93. **Conover, T. E. and Azzone, G. F.**, *Mitochondria and Microsomes*, Lee, C. P., Schatz, G., and Dallner, G., Eds., Addison-Wesley, Reading, Mass., 1981, 481.
94. **Higuti, T., Arakaki, N., Kotera, Y., Takigawa, M., Tani, I., and Shibuya, M.**, Triphenyltetrazolium and its derivatives are anisotropic inhibitors of energy transduction in oxidative phosphorylation in rat-liver mitochondria, *Biochim. Biophys. Acta*, 725, 1, 1983.
95. **Hong, Y. Q. and Junge, W.**, Localized or delocalized protons in photophosphorylation? On the accessibility of the thylakoid lumen for ions and buffers, *Biochim. Biophys. Acta*, 722, 197, 1983.
96. **Westerhoff, H. V.**, Mosaic Non-Equilibrium Thermodynamics and the Control of Biological Free-Energy Transduction, Academisch proefschrift, University of Amsterdam, 1983.
97. **Junge, W. and McLaughlin, S.**, The role of fixed and mobile buffers in the kinetics of proton movement, *Biochim. Biophys. Acta*, 890, 1, 1987.

98. **Gutman, M., Nachliel, E., and Gershon, E.,** Effect of buffer on kinetics of proton equilibration with a protonatable group, *Biochemistry*, 24, 2937, 1985.
99. **Polle, A. and Junge, W.,** The slow rise of the flash-light-induced alkalinization by Photosystem II of the suspending medium is reversibly related to thylakoid stacking, *Biochim. Biophys. Acta*, 848, 257, 1986.
100. **Polle, A. and Junge, W.,** The slow rate of proton consumption at the reducing side of PSI is limited by the slow rate of redox reactions of extrinsic electron acceptors, but not by a diffusion barrier for protons, *Biochim. Biophys. Acta*, 848, 274, 1986.
101. **Anderson, J. M. and Andersson, B.,** The architecture of photosynthetic membranes: lateral and transverse organization, *Trends Biochem. Sci.*, 7, 288, 1982.
102. **Van Dam, K., Wiechmann, A. H. C. A., Hellingwerf, K. J., Arents, J. C., and Westerhoff, H. V.,** Functioning of the mitochondrial ATP-synthesizing machinery, *Fed. Eur. Biochem. Soc. Symp.*, 45, 121, 1978.
103. **Mitchell, P.,** *Chem. Br.*, 17, 14, 1981.
104. **Westerhoff, H. V., Simonetti, A. L. M., and Van Dam, K.,** The hypothesis of localized chemiosmosis is unsatisfactory, *Biochem. J.*, 200, 193, 1981.
105. **Nagle, J. F. and Dilley, R. A.,** Models of localized energy coupling, *J. Bioenerg. Biomembr.*, 18, 55, 1986.
106. **De Kouchkovsky, Y., Haraux, F., and Sigalat, C.,** Effect of hydrogen-deuterium exchange on energy-coupled processes in thylakoids, *FEBS Lett.*, 139, 245, 1982.
107. **De Kouchkovsky, Y., Haraux, F., and Sigalat, C.,** A microchemiosmotic interpretation of energy-dependent processes in biomembranes based on the photosynthetic behaviour of thylakoids, *Bioelectrochem. Bioenerg.*, 13, 143, 1984.
108. **Ferrer, F. and Jou, D.,** Non-local thermodynamic effects and efficiency of oxidative phosphorylation, *J. Theor. Biol.*, 115, 153, 1985.
109. **Williams, R. J. P.,** Possible functions of chains of catalysts. II, *J. Theor. Biol.*, 3, 209, 1962.
110. **Kell, D. B., Clarke, D. J., and Morris, J. G.,** On proton-coupled information transfer along the surface of biological membranes and the mode of action of certain colicins, *FEMS Microbiol. Lett.*, 11, 1, 1981.
111. **Kell, D. B.,** On the functional proton current pathway of electron transport phosphorylation. An electrodic view, *Biochim. Biophys. Acta*, 549, 55, 1979.
112. **Kell, D. B. and Morris, J. G.,** Proton-coupled membrane energy transduction: pathways, mechanisms and control, in *Vectorial Reactions in Electron and Ion Transport*, Palmieri, F. et al., Eds., Elsevier, Amsterdam, 1981, 339.
113. **Laszlo, J. A., Baker, G. A., and Dilley, R. A.,** Nonequilibration of membrane-associated protons with the internal aqueous space in dark-maintained chloroplast thylakoids, *J. Bioenerg. Biomembr.*, 16, 37, 1984.
114. **Padan, E. and Rottenberg, H.,** Respiratory control and the proton electrochemical gradient, *Eur. J. Biochem.*, 40, 431, 1973.
115. **Rottenberg, H.,** Proton-coupled energy conversion: chemiosmotic and intramembrane coupling, *Mod. Cell Biol.*, 4, 47, 1985.
116. **Woelders, H., Putters, J., and Van Dam, K.,** Flow-force relationships in mitochondrial oxidative phosphorylation, *FEBS Lett.*, 204, 17, 1986.
117. **Sorgato, M. C., Lippe, G., Seren, S., and Ferguson, S. J.,** Partial uncoupling or inhibition of electron transport rate have equivalent effects on the relationship between the rate of ATP synthesis and proton-motive force in submitochondrial particles, *FEBS Lett.*, 181, 323, 1985.
118. **Zoratti, M. and Petronilli, V.,** Multiple relationships between rate of oxidative phosphorylation and $\Delta\tilde{\mu}_H$ in rat liver mitochondria, *FEBS Lett.*, 193, 276, 1985.
119. **Zoratti, M., Petronilli, V., and Azzone, G. F.,** ATP synthase-mediated proton fluxes and phosphorylation in rat liver mitochondria: dependence on $\Delta\tilde{\mu}_H$, *Biochim. Biophys. Acta*, 851, 123, 1986.
120. **Melandri, B. A., De Santis, A., Venturoli, G., and Baccarini-Melandri, A.,** The rates of onset of photophosphorylation and of the protonic electrochemical potential difference in bacterial chromatophores, *FEBS Lett.*, 95, 130, 1978.
121. **Elferink M. G., Hellingwerf, K. J., Van Dijl, J. M., Robillard, G. T., Poolman, B., and Konings, W. N.,** The role of electron transfer and dithiol-disulfide interchange in solute transport in bacteria, *Ann. N.Y. Acad. Sci.*, 456, 361, 1985.
122. **Kadenbach, B.,** Regulation of respiration and ATP synthesis in higher organisms: hypothesis, *J. Bioenerg. Biomembr.*, 18, 39, 1986.
123. **Chernyak, B. V. and Kozlov, I. A.,** Regulation of H^+-ATPases in oxidative- and photophosphorylation, *Trends Biochem. Sci.*, 11, 32, 1986.
124. **Elferink, M. G. L., Hellingwerf, K. J., Van Belkum, M. J., Poolman, B., and Konings, W. N.,** Direct interaction between linear electron transfer chains and solute transport systems in bacteria, *FEMS Microbiol. Lett.*, 21, 293, 1984.

125. **Konings, W. N. and Robillard, G. T.,** Physical mechanism for regulation of proton solute symport in E. coli, *Proc. Natl. Acad. Sci. U.S.A.*, 79, 5480, 1982.
126. **Midgley, M.,** An efflux system for cationic dyes and related compounds in *E. coli*, *Microbiol. Sci.*, 4, 125, 1987.
127. **Nicholls, D. G.,** The influence of respiration and ATP hydrolysis on the proton-electrochemical gradient across the inner membrane of rat-liver mitochondria as determined by ion distribution, *Eur. J. Biochem.*, 50, 305, 1974.
128. **Wiechmann, A. H. C. A., Beem, E. P., and Van Dam, K.,** The relation between H^+ translocation and ATP synthesis in mitochondria, in *Electron Transfer Chains and Oxidative Phosphorylation*, Quagliariello, E. et al., Eds., North-Holland, Amsterdam, 1975, 335.
129. **Ogawa, S. and Lee, T. M.,** The relation between the internal phosphorylation potential and the proton motive force in mitochondria during ATP synthesis and hydrolysis, *J. Biol. Chem.*, , 259, 10004, 1984.
130. **Rottenberg, H.,** The relationship between the proton electrochemical potential and phosphate potential in rat liver mitochondria. Re-evaluation using ^{31}P NMR, *Ann. N.Y. Acad. Sci.*, 508, 507, 1987.
131. **Van Dam, K., Westerhoff, H. V., Rutgers, M., Bode, J. A., De Jonge, P. C., Bos, M. M., and Van den Berg, G.,** Comparison of reaction stoichiometry to differential importance of thermodynamic forces for reaction rates, in *Vectorial Reactions in Electron and Ion Transport*, Palmieri, F. et al., Eds., Elsevier, Amsterdam, 1981, 389.
132. **Westerhoff, H. V., Plomp, P. J. A. M., Groen, A. K., Wanders, R. J. A., Bode, J. A., and Van Dam, K.,** On the origin of the limited control of mitochondrial respiration by the adenine nucleotide translocator, *Arch. Biochem. Biophys.*, 257, 154, 1987.
133. **Thayer, W. S. and Hinkle, P. C.,** Kinetics of ATP synthesis in beef heart submitochondrial particles, *J. Biol. Chem.*, 250, 5336, 1975.
134. **Maloney, P. C.,** Energy coupling to ATP synthesis by the proton-translocating ATPase, *J. Membr. Biol.*, 67, 1, 1982.
135. **Gräber, P.,** Phosphorylation in chloroplasts: ATP synthesis driven by $\Delta\psi$ and by ΔpH of artificial or light generated origin, *Curr. Top. Membr. Transp.*, 16, 215, 1982.
136. **Kell, D. B.,** Localized protonic coupling: overview and critical evaluation of techniques, *Methods Enzymol.*, 86, 538, 1986.
137. **Van Walraven, H. S., Haak, N. P,. Krab, K., and Kraayenhof, R.,** Evidence for a high proton translocation stoichiometry of the H^+-ATPase complex in well coupled proteoliposomes reconstituted from a thermophilic cyanobacterium, *FEBS Lett.*, 208, 138, 1986.
138. **Krulwich, T. A.,** Bioenergetics of alkalophilic bacteria, *J. Membr. Biol.*, 89, 113, 1986.
139. **Guffanti, A. A., Bornstein, R. F., and Krulwich, T. A.,** Oxidative phosphorylation by membrane vesicles from *bacillus alcalophilus*, *Biochim. Biophys. Acta*, 635, 619, 1981.
140. **Guffanti, A. A., Fuchs, R. T., Schneider, M., Chiu, E., and Krulwich, T. A.,** A transmembrane electrical potential generated by respiration is not equivalent to a diffusion potential of the same magnitude for ATP synthesis by *B. firmus* RAB, *J. Biol. Chem.*, 259, 2971, 1984.
141. **Guffanti, A. A., Finkelthal, O., Hicks, D. B., Falk, L., Sidhu, A., Garro, A., and Krulwich, T. A.,** Isolation and characterization of new facultatively alkalophilic strains of *Bacillus* species, *J. Bacteriol.*, 167, 766, 1986.
142. **Maloff, B. L., Scordilis, S. P., and Tedeschi, H.,** Analysis of metabolic viability of single giant mitochondria. Experiments with intact and impaled mitochondria, *J. Cell Biol.*, 78, 214, 1978.
143. **Giulian, D. and Diacumakos, E. G.,** The electrophysiological mapping of compartments within the mammalian cell, *J. Cell Biol.*, 72, 86, 1977.
144. **Tedeschi, H.,** The mitochondrial membrane potential, *Biol. Rev.*, 55, 171, 1980.
145. **Campo, M. L. and Tedeschi, H.,** Protonmotive force in giant mitochondria, *Eur. J. Biochem.*, 141, 5, 1984.
146. **Azzone, G. F., Pozzan, T., Massari, S., Bragadin, M., and Pregnolato, L.,** Proton electrochemical gradient and rate of controlled respiration in mitochondria, *Biochim. Biophys. Acta*, 501, 296, 1978.
147. **Van Dam, K., Westerhoff, H. V., Krab, K., Van der Meer, R., and Arents, J. C.,** Relationship between chemiosmotic flows and thermodynamic forces in oxidative phosphorylation, *Biochim. Biophys. Acta*, 591, 240, 1980.
148. **Wilson, D. F. and Forman, N. G.,** Mitochondrial transmembrane pH and electrical gradients: evaluation of their energy relationships with respiratory rate and ATP synthesis, *Biochemistry*, 21, 1438, 1982.
149. **Mandolino, G., DeSantis, A., and Melandri, B. A.,** Localized coupling in oxidative phosphorylation by mitochondria from *Jerusalem artichoke*, *Biochim. Biophys. Acta*, 723, 428, 1983.
150. **Rottenberg, H., Robertson, D. E., and Rubin, E.,** The effect of temperature and chronic ethanol feeding on the proton electrochemical gradient and phosphate potential in rat liver mitochondria, *Biochim. Biophys. Acta*, 809, 1, 1985.

151. **Branca, D., Ferguson, S. J., and Sorgato, M. C.,** Clarification of factors influencing the nature and magnitude of the protonmotive force in bovine heart submitochondrial particles, *Eur. J. Biochem.*, 116, 341, 1981.
152. **Sorgato, M. C., Galiazzo, F., Panato, L., and Ferguson, S. J.,** Estimation of translocation stoichiometry of mitochondrial ATPase by comparison of protonmotive forces with clamped phosphate potentials in submitochondrial particles, *Biochim. Biophys. Acta*, 682, 184, 1982.
153. **Kell, D. B., John, P., and Ferguson, S. J.,** The protonmotive force in phosphorylating membrane vesicles from *P. denitrificans*, *Biochem. J.*, 174, 257, 1978.
154. **Parsonage, D. and Ferguson, S. J.,** The puzzle of high phosphorylation potential and low protonmotive force generated by oxidative phosphorylation in *P. denitrificans* membrane vesicles in the presence of nitrate, *Biochem. Soc. Trans.*, 12, 456, 1984.
155. **Woelders, H., Van der Zande, W. J., Colen, A.-M. A. F., Wanders, R. J., and Van Dam, K.,** The phosphate potential maintained by mitochondria in state 4 is proportional to the proton-motive force, *FEBS Lett.*, 179, 278, 1985.
156. **Petronilli, V., Pietrobon, D., Zoratti, M., and Azzone, G. F.,** Free energy coupling between H^+-generating and H^+-consuming pumps. Ratio between output and input forces, *Eur. J. Biochem.*, 155, 423, 1986.
157. **Westerhoff, H. V., Colen, A.-M., and Van Dam, K.,** Metabolic control by pump slippage and proton leakage in delocalized and more localized energy-coupling schemes, *Biochem. Soc. Trans.*, 11, 81, 1983.
158. **Mitchell, P. and Moyle, J.,** Acid-base titration across the membrane system of rat-liver mitochondria, *Biochem. J.*, 104, 588, 1967.
159. **Westerhoff, H. V., Melandri, B. A., Venturoli, G., Azzone, G. F., and Kell, D. B.,** Mosaic protonic coupling hypothesis for free energy transduction, *FEBS Lett.*, 165, 1, 1984.
160. **Ernster, L., Junttl, K., and Asami, K.,** Mechanism of energy conservation in the mitochondrial membrane, *J. Bioenerg.*, 4, 149, 1973.
161. **Westerhoff, H. V. and Chen, Y.,** Stochastic free energy transduction, *Proc. Natl. Acad. Sci. U.S.A.*, 82, 3222, 1985.
162. **Westerhoff, H. V. and Kamp, F.,** Maxwell's demons in channeled metabolism: paradoxes and their resolution, in *The Organization of Cell Metabolism*, Welch, G. R. and Clegg, J. S., Eds., Plenum Press, New York, 1986, 339.
163. **Kahn, J. H.,** Absence of a common intermediate pool among individual enzyme chains of the energy-conservation pathway in chloroplasts of *Euglena gracilis*, *Biochem. J.*, 116, 55, 1970.
164. **Kacser, H. and Burns, J. A.,** The control of flux, in *Rate Control of Biological Processes*, Davis, D. D., Ed., Cambridge University Press, New York, 1973, 65.
165. **Heinrich, R., Rapoport, S. M., and Rapoport, T. A.,** Metabolic regulation and mathematical models, *Prog. Biophys. Mol. Biol.*, 32, 1, 1977.
166. **Westerhoff, H. V., Groen, A. K., and Wanders, R. J. A.,** Modern theories of metabolic control and their applications, *Biosci. Rep.*, 4, 1, 1984.
167. **Lee, C.-P., Ernster, L., and Chance, B.,** Studies of the energy-transfer system of submitochondrial particles. Kinetic studies of the effect of oligomycin on the respiratory chain of EDTA particles, *Eur. J. Biochem.*, 8, 153, 1969.
168. **Baum, H., Hall, G. S., Nalder, J., and Beechey, R. B.,** *Energy Transduction in Respiration and Photosynthesis*, Quagliariello et al., Eds., Adriatica Editrice, Bari, Italy, 1971, 747.
169. **Baum, H.,** *Molecular Biology of Membranes*, Fleischer, S. et al., Eds., Plenum Press, New York, 1978, 243.
170. **Westerhoff, H. V., Helgerson, S. L., Theg, S. M., Van Kooten, O., Wikström, M., Skulachev, V. P., and Dancshazy, Zs.,** The present state of the chemiosmotic coupling theory, *Acta Biochem. Biophys. Acad. Sci. Hung.*, 18, 125, 1983.
171. **Herweijer, M. A., Berden, J. A., Kemp, A., and Slater, E. C.,** Inhibition of energy-transducing reactions by 8-azido-ATP covalently bound to bovine heart submitochondrial particles: direct interaction between ATPase and redox enzymes, *Biochim. Biophys. Acta*, 809, 81, 1985.
172. **Hitchens, G. D. and Kell, D. B.,** On the extent of localization of the energized membrane state in chromatophores from *R. capsulata* N22, *Biochem. J.*, 206, 351, 1982.
173. **Hitchens, G. D. and Kell, D. B.,** Localized energy coupling during photophosphorylation by chromatophores of *R. capsulata* N22, *Biosci. Rep.*, 2, 743, 1982.
174. **Westerhoff, H. V. and Kell, D. B.,** A control theoretic analysis of inhibitor titration assays of metabolic channeling, *Comm. Mol. Cell. Biophys.*, in press.
175. **Krasinskaya, I. P., Marshansky, V. N., Dragunova, S. F., and Yaguzhinsky, L. S.,** Relationships of respiratory chain and ATP-synthetase in energized mitochondria, *FEBS Lett.*, 167, 176, 1984.
176. **Davenport, J. W.,** Double inhibitor titrations of photophosphorylation are consistent with delocalized coupling, *Biochim. Biophys. Acta*, 807, 300, 1985.

177. **Matsuno-Yagi, A. and Hatefi, Y.**, Kinetic modalities of ATP synthesis. Regulation by the mitochondrial respiratory chain, *J. Biol. Chem.*, 261, 14031, 1986.
178. **Hitchens, G. D. and Kell, D. B.**, Uncouplers can shuttle between localized energy-coupling sites during photophosphorylation by chromatophores of *R. capsulata* N22, *Biochem. J.*, 212, 25, 1983.
179. **Herweijer, M. A., Berden, J. A., and Slater, E. C.**, Uncoupler-inhibitor titrations of ATP-driven reverse electron transfer in bovine heart submitochondrial particles provide evidence for direct interaction between ATPase and NADH:Q oxidoreductase *Biochim. Biophys. Acta*, 849, 276, 1986.
180. **Harvey, R. J.**, Interaction of two inhibitors which act on different enzymes in a pathway, *J. Theor. Biol.*, 74, 411, 1978.
181. **O'Shea, P. S. and Thelen, M.**, On the logic of the application of double-inhibitor titrations for the elucidation of the mechanisms of energy coupling, *FEBS Lett.*, 176, 79, 1984.
182. **Pietrobon, D. and Caplan, S. R.**, Double-inhibitor and uncoupler titrations. II. Analysis with a nonlinear model of chemiosmotic energy coupling, *Biochemistry*, 25, 7690, 1986.
183. **Chen, Y.**, A theoretical study of double-inhibitor titration curves in free-energy-transducing networks, *Biochim. Biophys. Acta*, 850, 490, 1987.
184. **Van der Bend, R. L., Petersen, J., Berden, J. A., Van Dam, K., and Westerhoff, H. V.**, A critical appraisal of evidence for localized energy coupling. Kinetic studies on liposomes containing bacteriorhodopsin and ATP synthase, *Biochem. J.*, 230, 543, 1985.
185. **Westerhoff, H. V., Van der Bend, R. L., Van Dam, K., Berden, J., and Petersen, J.**, A model system for delocalized chemiosmotic coupling exhibited features thought diagnostic of localized coupling, *Biochem. J.*, 236, 932, 1986.
186. **Kell, D. B.**, Uncoupler titrations in co-reconstituted systems do not discriminate between localized and delocalized mechanisms of photophosphorylation, *Biochem. J.*, 236, 931, 1986.
187. **Westerhoff, H. V. and Kell, D. B.**, Matrix method for determining steps most rate-limiting to metabolic fluxes in biotechnological processes, *Biotechnol. Bioeng.*, 30, 101, 1987.
188. **Pietrobon, D., Petronilli, V., and Azzone, G. F.**, Analysis of free energy coupling and uncoupling by inhibitor titrations, in *Integration of Mitochondrial Function*, Lemasters, J. J., Ed., Plenum Press, New York, 1988.
189. **Moreno-Sanchez, R., Espinosa-Garcia, M. T., and Raya, J. C.**, Control of respiration and ATP hydrolysis in uncoupled mitochondria, in *Integration of Mitochondrial Function*, Lemasters, J. J. et al., Eds., Plenum Press, New York, 1987.
190. **Groen, A. K., Wanders, R. J. A., Westerhoff, H. V., Van der Meer, R., and Tager, J. M.**, Quantification of the contribution of various steps to the control of mitochondrial respiration, *J. Biol. Chem.*, 257, 2754, 1982.
191. **Westerhoff, H. V.**, The organization and control of protonic coupling, *EBEC Rep.*, 4, 8, 1986.
192. **Stoner, C. D.**, Steady-state kinetics of the overall oxidative phosphorylation reaction in heart mitochondria. Evidence for linkage of the energy-yielding and energy-consuming steps by freely diffusible intermediates and for an allosteric mechanism of respiratory control at coupling Site 2, *J. Bioenerg. Biomembr.*, 17, 85, 1985.
193. **Sorgato, M. C., Branca, D., and Ferguson, S. J.**, The rate of ATP synthesis by submitochondrial particles can be independent of the magnitude of the protonmotive force, *Biochem. J.*, 188, 945, 1980.
194. **Venturoli, G. and Melandri, B. A.**, The localized coupling of bacterial photophosphorylation. Effect of Antimycin A and DCCD in chromatophores from *R. sphaeroides*, Ga studied by single turnover analysis, *Biochim. Biophys. Acta*, 680, 8, 1980.
195. **Zinoveva, M. E., Kaprelyants, A. S., and Ostrovsky, D. N.**, Respiratory chain of bacterial membrane: assembly-mobile carriers equilibrium, *Mol. Cell. Biochem.*, 55, 141, 1983.
196. **Slater, E. C., Berden, J. A., and Herweijer, M. A.**, A hypothesis for the mechanism of respiratory-chain phosphorylation not involving the electrochemical gradient of protons as obligatory intermediate, *Biochim. Biophys. Acta*, 811, 217, 1985.
197. **Gupte, S. and Hackenbrock, C. R.**, A random collision model for proton transfer in mitochondrial ATP synthesis, in *The Mitochondrion, Abstr. Lehninger Mem. Symp.*, 1986.
198. **Hochman, J., Ferguson-Miller, S., and Schindler, M.**, Mobility in the mitochondrial electron transport chain, *Biochemistry*, 24, 2509, 1985.
199. **Ferguson-Miller, S., Hochman, J., and Schindler, M.**, Aggregation and diffusion in the mitochondrial electron transfer chain: role in electron flow and energy transfer, *Biochem. Soc. Trans.*, 14, 822, 1986.
200. **Jacobson, K.**, Lateral diffusion in membranes, *Cell Motility*, 3, 367, 1983.
201. **Srere, P. A.**, Complexes of sequential metabolic enzymes, *Annu. Rev. Biochem.*, 56, 89, 1987.
202. **Grasberger, B., Minton, A. P., DeLisi, C., and Metzger, H.**, Interaction between proteins localized in membranes, *Proc. Natl. Acad. Sci. U.S.A.*, 83, 6258, 1986.
203. **Rottenberg, H.**, Phase transitions and coupling in energy transducing membranes, *FEBS Lett.*, 94, 295, 1978.

204. **Silverstein, T. P. and Rottenberg, H.**, Dynamic protein aggregates in the mitochondrial inner membrane probed by time-resolved anisotropy measurements of erythrosin-labeled F_1, *Biophys. J.*, 51, 489a, 1987.
205. **Kröger, A. and Klingenberg, M.**, Quinones and nicotinamide nucleotides associated with electron transfer, *Vitam. Horm. (N.Y.)*, 28, 533, 1970.
206. **Teissié, J., Prats, M., Soucaille, P., and Tocanne, J. F.**, Evidence for conduction of protons along the interface between water and a polar lipid bilayer, *Proc. Natl. Acad. Sci. U.S.A.*, 82, 3217, 1985.
207. **Prats, M., Tocanne, J.-F., and Teissié, J.**, Lateral proton conduction at lipid-water interface. Its modulation by physical parameters. Experimental and mathematical approaches, *Eur. J. Biochem.*, 149, 663, 1985.
208. **Prats, M., Teissié, J., and Tocanne, J.-F.**, Lateral proton conduction at lipid-water interfaces and its implications for the chemiosmotic-coupling hypothesis, *Nature*, 322, 756, 1986.
209. **Selwyn, M. J.**, Surface conduction of protons, *Nature*, 322, 685, 1986.
210. **Kaslanowicz, J., Benz, R., and McLaughlin, S.**, How do protons cross the membrane-solution interface? Kinetic studies on bilayer membranes exposed to the protonophore S-13, *J. Membr. Biol.*, 95, 73, 1987.
211. **Gutman, M., Nachliel, E., and Fishman, M.**, Kinetic analysis of time resolved proton diffusion on phospholipid membrane, in *Ion Interactions in Energy Transfer Biomembranes*, Papageorgiou, G. C., Barber, J., and Papa, S., Eds., Plenum Press, New York, 1986, 93.
212. **Malpress, F. H.**, The coulombic hypothesis of mitochondrial energy transduction: an attempt to quantify relationships in the energized zones, *J. Theor. Biol.*, 111, 397, 1984.
213. **Westerhoff, H. V., Kell, D. B., and Astumian, R. D.**, The dynamics of electrostatic interactions between membrane proteins, *J. Electrostat.*, in press.
214. **Malpress, F. H.**, Electron transfer mechanisms and energy transduction n mitochondria, in *Electrochemistry, Sensors and Analysis*, Smyth, M. R. and Vos, J. G., Eds., Elsevier, Amsterdam, 1986, 179.
215. **Higuti, T., Takigawa, M., Kotera, Y., Oka, H., Uchida, J., Arakaki, R., Fujita, T., and Ogawa, T.**, Purified hydrophobic proteins, chargerins, are essential for energy transduction in mitochondria, *Proc. Natl. Acad. Sci. U.S.A.*, 82, 1331, 1985.
216. **Skulachev, V. P.**, The localized $\Delta\tilde{\mu}_H$ problem. The possible role of the local electric field in ATP synthesis, *FEBS Lett.*, 146, 1, 1982.
217. **Boyer, P. D.**, Correlations of the binding change mechanism with a new concept for proton translocation and energy transmission, in *H^+-ATPsynthase: Structure, Function, Regulation*, Papa, S., Ed., Adriatica Editrice, Bari, Italy, 1985, 329.
218. **Astumian, R. D., Chock, P. B., Tsong, T. Y., Chen, Y., and Westerhoff, H. V.**, Can free energy be transduced from electric noise?, *Proc. Natl. Acad. Sci. U.S.A.*, 84, 434, 1987.
219. **Serpersu, E. H. and Tsong, T. Y.**, Activation of electrogenic Rb^+ transport of (Na,K)-ATPase by an electric field, *J. Biol. Chem.*, 259, 7155, 1984.
220. **Tsong, T. Y. and Astumian, R. D.**, Absorption and conversion of electric field energy by membrane bound ATPases, *Bioelectrochem. Bioenerg.*, 15, 457, 1986.
221. **Westerhoff, H. V., Tsong, T. Y., Chock, P. B., Chen, Y., and Astumian, R. D.**, How enzymes can capture and transmit free energy from an oscillating electric field, *Proc. Natl. Acad. Sci. U.S.A.*, 83, 4734, 1986.
222. **Welch, G. R. and Kell, D. B.**, Not just catalysts-molecular machines in bioenergetics, in *The Fluctuating Enzyme*, Welch, G. R., Ed., Academic Press, New York, 1986, 451.
223. **Kamp, F. and Westerhoff, H. V.**, Molecular machines and energy channelling, in *The Organization of Cell Metabolism*, Welch, G. R. and Clegg, J. S., Eds., Plenum Press, New York, 1986, 357.
224. **Kell, D. B.**, Non-thermally excited modes and free-energy transduction in proteins and biological membranes in *Energy Transfer Dynamics*, Barrett, T. W. and Phol, H. A., Eds., Springer-Verlag, Berlin, 1987, 237.
225. **Ramirez, F., Shiuan, D., Tu, S.-I., and Marecek, J. F.**, Differential effects on energy transduction processes by fluorescamine derivatives in rat liver mitochondria, *Biochemistry*, 19, 1928, 1980.
226. **Ramirez, F., Tu, S.-I., Chatterji, P. R., McKeever, B., and Marecek, J. F.**, Amine fluorescamine compounds inhibit oxidative phosphorylation in rat liver mitochondria, *Arch. Biochem. Biophys.*, 230, 61, 1984.
227. **Tu, S.-I., Okazaki, H., Ramirez, F., Lam, E., and Marecek, J. F.**, Mutual regulation between mitochondrial ATPase and respiratory chain complexes, *Arch. Biochem. Biophys.*, 210, 124, 1981.
228. **Rottenberg, H.**, Uncoupling of oxidative phosphorylation in rat liver mitochondria by general anaesthetics, *Proc. Natl. Acad. Sci. U.S.A.*, 80, 3313, 1983.
229. **Rottenberg, H. and Hashimoto, K.**, Fatty acid uncoupling of oxidative phosphorylation in rat liver mitochondria, *Biochemistry*, 25, 1747, 1986.
230. **Rottenberg, H. and Steiner-Murdoch, S.**, Free Fatty acids decouple oxidative phosphorylation by dissipating intramembranal protons without inhibiting ATP synthesis driven by the proton electrochemical gradient, *FEBS Lett.*, 202, 314, 1986.

231. **Azzone, G. F., Pietrobon, D., and Luvisetto, S.,** Evaluation of protonophoric effects and of intrinsic uncoupling of oxidative phosphorylation. Comparison between experiments and computer modeling, in *Integration of Mitochondrial Function*, Lemasters, J. J. et al., Eds., Plenum Press, New York, 1988.
232. **Higuti, T.,** Conformational coupling in H^+ pumps and ATP synthesis. Its analysis with anisotropic inhibitors of energy transduction in oxidative phosphorylation, *Mol. Cell. Biochem.*, 61, 37, 1984.
233. **Tu, S.-I., Lam, E., Ramirez, F., and Marecek, J. F.,** Inhibition of the links between electron transfer and proton translocation in mitochondria, *Eur. J. Biochem.*, 113, 391, 1981.
234. **Conover, T. E.,** personal communication.
235. **Slater, E. C.,** personal communication.
236. **Westerhoff, H. V., Astumian, R. D., and Kamp, F.,** unpublished observations.
237. **Rigoulet, M., Guerin, B., and Denis, M.,** Modification of flow-force relationships by external ATP in yeast mitochondria, *Eur. J. Biochem.*, 168, 275, 1987.
238. **Pietrobon, D., Prod'hom, B., and Hess, P.,** Conformational changes associated with ion permeation in L-type calcium channels, *Nature*, 333, 373, 1988.
239. **Nachliel, E. and Gutman, M.,** Time-resolved proton phospholipid interaction methodology and kinetic analysis, *J. Am. Chem. Soc.*, 110, 2629, 1988.
240. **Kamp, F., Astumian, R. D., and Westerhoff, H. V.,** Coupling of vectorial proton flow to a biochemical reaction by local electric interactions, *Proc. Natl. Acad. Sci.*, U.S.A., 85, 1988.