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Minireview: Cardiolipins and Mitochondrial Proton-Selective Leakage[2]

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The proton-selective leak (State 4 respiratory rate) but not $\Delta\psi$, in mitochondria from thyroid-sensitive tissues, responds to *in vivo* stimuli in unique correlation with changes in cardiolipins, saturated and mono-unsaturated (extended) fatty acyl contents, cardiolipins/phospholipids ratios, and/or membrane outer-sidedness. Liver mitochondrial State 4 respiration, basal in fasted rats, contributes little to resting metabolic rate in fed rats, where State 3 depresses Δ_ψ. In a proposed model, an essential inner-membrane outer-surface proton antenna collects protons and donates them, via a water-shuttle, to transmembrane porters: transient water-molecule-chains between extended phospholipid acyls; protonophores, and uncoupling proteins. Only cardiolipin microdomains can donate, from an anomalously-dissociating phosphate group in each headgroup; unadapted cardiolipins have few conducting water chains. Thyroid states regulate each cardiolipin property, and are permissive, via the proton antenna, for proton leaks, including those through adapted and possibly constitutive BAT and ectopic uncoupling proteins. Slow leakage in liposomes may reflect insufficient cardiolipin proton antennas.

KEY WORDS: Mitochondria; proton-leak; cardiolipins; membrane sidedness; phospholipids; fatty acyls; thyroid hormone actions; uncoupling proteins.

INTRODUCTION

A proton-selective leak is required for State 4 respiration in isolated mitochondria (Mitchell and Moyle, 1967; Brand, 1990a; Nicholls and Ferguson, 1992; Brand *et al.*, 1994). Evidence that cardiolipins (CLs) are involved in the regulation of this leak, and a model for their role, are presented here. In chemiosmotic processes (Mitchell and Moyle, 1967; Nicholls and Ferguson, 1992), the PL bilayer of the inner membrane maintains the oxidatively generated protonmotive force at a high level by preventing Δp from driving back almost any of the pumped protons and any external K^+ and Na^+. Some protons can leak back to the matrix, via a proton-collecting antenna (buffering headgroups of inner membrane outer-surface mtPLs) that donates protons to transmembrane porters: water interpolated among extended acyl chains of mtPLs, proteins, and protonophores. The return flux is slow under physiological, State 4 conditions, and depends on the antenna, the number of transporter molecules, and Δp. Protons return to the matrix 5 to $\geq$20 times faster in State 3 via the F0F1 and in State 3u via a protonophore, and up to 3 times faster via water among the excess extended FA in mitochondrial PLs, especially CLs, from EFA-deficient animals (see Hoch, 1988).

THE SIGNIFICANCE OF STATE 4 RESPIRATION

State 4 respiration is the major physiological basis of the BMR, according to early, mostly spectrophoto-

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[2] *Abbreviations.*BAT: brown adipose tissue; CL(s), ΣCL, CLo, CLi: cardiolipin(s), total, outside-surface, inside-surface cardiolipins; FA, SFA, MUFA, PUFA: fatty acyls (acids), saturated, monounsaturated, polyunsaturated fatty acyls; PE, PC: phosphatidylethanolamine, -choline; EFA: essential fatty acid(s); CPT, CPTo (outer surface), CPTi (inner surface): carnitine palmitoyltransferase; DNP: 2,4-dinitrophenol; PLA2: phospholipase A2; T4: L-thyroxine; T3: L-triiodothyronine; tHBC: transient hydrogen-bonded chain of single-file water molecules; UCP: uncoupling protein; UI: unsaturation index [Σ(%FA/ΣFA x number of double bonds)].

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metric, studies on fasted animals (reviewed by Hoch, 1971). It is a minor contributor, according to recent studies on fed animals, mostly from effects of respiratory inhibitors. It seemed logical to examine correlations between induced changes in fasted animals' mitochondrial respiration and BMR, by definition measured in fasting animals. Animals were fasted overnight once Rubner (see Kleiber, 1961) demonstrated that feeding proteins or amino acids raise the metabolic rate above the basal, fasting rate by about 30% of their catabolizable energy, the "specific dynamic effect." We fasted rats axiomatically (e.g., Hoch and Lipmann, 1954; Hoch, 1967).

Chance (1967a) and coworkers defined the resting state of cells: "From the biochemical standpoint, rest is a situation of maximal potential for functional activity. From the viewpoint of oxidative phosphorylation we define the state of complete rest as the maximal value obtainable by the ratio of ATP/ADP . . . These conditions of maximal ATP/ADP in the mitochondria cause a slowing of electron transport characteristic of the resting state." The mitochondrial protonmotive force Δp is maximal under State 4 conditions (Mitchell and Moyle, 1967; Nicholls and Ferguson, 1992), like the high resting membrane potential of neurons. Quantitation of mitochondrial respiration in whole-cell preparations, necessary to determine contributions to whole-body respiration, have been questioned because of limited permeability of tissue slices, cells, and perfused organs to oxygen, substrates, and effectors, and changes in effector specificity. Noninvasive optical measurements on whole cells and organs eliminated most of these problems, and showed considerable reduction of mitochondrial flavoproteins and pyridine nucleotides characteristic of State 4 conditions in resting cells (Chance *et al.*, 1962). In the livers of fed rats, incomplete reduction of mitochondrial NAD^+ indicated State 3 conditions (Chance, 1967b), which is consistent with the specific dynamic effect that raises BMR. Intact cells show respiratory control, i.e., ADP+Pi stimulated respiration above resting State 4 levels. A protonophore (DNP) accelerates mitochondrial respiration 2- to 3-fold in vitro and up to 4-fold in vivo (as measured by the metabolic rate; see Ref. Hoch, 1971); DNP does not accelerate respiration (by increasing proton reflux, not generally understood at that time) in State 3 much, if at all, because respiration is already rapid (Hoch, 1992). For example, perfusates 0.14 mM in DNP raise oxygen consumption of skeletal muscle of fed rats only ~30% (Rolfe and Brand, 1996). The contributions of organs (slices) to total oxygen consumption were measured in fasted rats by Field *et al.* (1939; data cited by Hoch, 1971; Rolfe and Brown, 1997).

Many investigators have accepted State 4 as at least a significant contributor to the BMR, e.g., 80–90% in 'resting' isolated liver cells (Harper and Brand, 1993), and 60% in perfused skeletal muscle of rats (Rolfe and Brand, 1996), or somewhat less (Brand, 1990a,b; Rolfe *et al.*, 1994). Liver mitochondrial State 4 rates have been a basis for comparisons of BMRs in evolutionary adaptations (Brand *et al.*, 1991) and especially in rats in abnormal thyroid states (Hafner *et al.*, 1988) or in EFA-deficiency (Alfin-Slater *et al.*, 1968), where BMR changes are a major sign. The slowed leak of mitochondrial protons is the basis of the low BMR in hypothyroid rats, from top-down kinetics analyses (Harper and Brand, 1993).

Recent reports that mitochondria in whole-cell preparations (hepatocyte suspensions, tissue slices, and perfused organs and tissues) respire slowly in State 4, conclude that this State contributes little to whole-body oxygen-consumption. However these protocols minimize State 4 respiration by using fed rats. Under a number of different titles comprising "Standard Metabolic Rate" (Rolfe and Brand, 1996; Rolfe and Brown, 1997), the metabolic rate of fed rats is dubbed "Resting" - although Standard and Basal signify the postabsorptive phase (Kleiber, 1961; Brand, 1990b). Feeding puts liver mitochondrial respiration into State 3, which is known to slow State 4 respiration.

Hepatocytes prepared from fed rats ('fed hepatocytes') remain viable in suspension when put into another "resting" state in vitro, viz., minus glucose, but those from starved animals do not (Nobes *et al.*, 1990a) - which is why fed rats were used (Nobes *et al.*, 1990b). Hepatocytes from fasted rats, on the other hand, gave similar values as fed hepatocytes for Ci for the proton leak and all respiratory components (Brown *et al.*, 1990). Brand and coworkers measure and manipulate mitochondrial Δp and respiratory rates in order to characterize control of respiration. The manipulations crucially depend on the specific inhibitory action of oligomycin on the ATP synthase to block proton reflux, in isolated mitochondria and in mitochondria *in situ* in whole-cell preparations. In isolated mitochondria oxidizing an activated substrate, oligomycin specifically blocks proton reflux through the ATP synthase; the remaining respiration is in State 4, controlled by proton leakage through membrane PLs or proteins, as discussed below. Oligomycin inhibits respiration of various tissue slices only slightly (<20–

30%), although the feeding states were not mentioned (see Tobin and Slater, 1965, and Refs. therein), which indicates that State 4 predominated. However the use of oligomycin on intact cells to determine Ci for the proton leak has been questioned. In hepatocytes, interpretation of oligomycin effects on respiration suffers from lack of definition of the endogenous substrate, which may vary with the presence of metabolites determined by pre-feeding or -fasting. Side effects of the oligomycin-induced depression of cell [ATP] blunt oligomycin specificity for determining the respiratory State. Oligomycin inhibits whole-cell State 4 oxidations of FA, which are substrates in fasted animals after glycogen is exhausted, because low [ATP] depresses activation of FA to the acyl-CoA substrate for β-oxidation, and oxidation of acetyl-CoA through the Krebs cycle, in hepatocytes (Berry *et al.*, 1983) and in BAT (Nicholls and Locke, 1984), and produces the low respiratory rate of State 5, not State 4 conditions.

If depriving fed hepatocytes of glucose is meant to mimic the fasted condition, it should be noted that fasting *in vivo* does more than decrease blood [glucose]: it changes hormone levels, especially insulin and glucagon, that have widespread metabolic effects. Besides, periportal hepatocytes (see below) β-oxidize FA from triglyceride stores rather than glucose because starvation exhausts glycogen stores. Such resting cells respire at rates between State 3 and State 4, at a Δ_ψ around 160 mV at 37 C (Nobes *et al.*, 1990). Liver mitochondria from that laboratory behave similarly in suspension and in hepatocytes (Brown *et al.*, 1990). Under State 4 conditions in isolated mitochondria (succinate, 37 C), $\Delta\psi$ = 216 mV (Hafner *et al.*, 1988), while in State 3 (+ADP+Pi), 155 mV (Harper *et al.*, 1990); cf. 220 mV and 154 mV, respectively, reported by Nicholls and Ferguson (1992), and 166 mv for State 3 (Crespo-Armas and Mowbray, 1987). From these data, one can adduce an extra proton leak via F0F1 (but not an UCP; see below) that drops $\Delta\psi$. The "resting" hepatocytes from fed rats respire in State 3, and are really "working" hepatocytes that convert ATP → ADP-which accounts for the specific dynamic effect. The low $\Delta\psi$ is taken as the "resting" value that drives the leak (State 4). After adding oligomycin to eliminate proton flux via F0F1 (and perhaps a part of the leak), remaining respiration is down-titrated with myxothiazol to the resting value of $\Delta\psi$ - where it is taken as State 4. The need for down-titration means, and direct titrations show, that oligomycin raises the value of $\Delta\psi$ - further proof that these cells from fed rats respire in State 3. The titration being in the steep zone of the $\Delta\psi$ vs. respiration-rate curve, the leak slows and contributes little to tissue respiration and the metabolic rate. Nicholls (1974) demonstrated suppression of the mitochondrial proton leak and State 4 respiration under State 3 conditions, and attributed it to the lowered $\Delta\psi$ caused by massive reflux of protons through F0F1. Evidence against the possibility of 'redox slip' of proton pumps at high $\Delta\psi$ - failure of proton ejection during electron transport - is discussed by Brand (1990a).

Fasting or feeding animals affects the metabolic patterns of their hepatocytes in situ and in vitro, and differently in parenchymal cells of the periportal and perivenous zones of the liver lobule. These cells in fed rats differ in structure and function (Jungermann, 1986; Thurman *et al.*, 1986; Gumucio, 1989). Periportal cells, in the oxygen-rich zone, depend more on mitochondrial oxidative metabolism and have more and larger mitochondria with a greater area of inner membranes, and distinctive mtPLs (see next section). They are equipped for β-oxidation of FA. Perivenous cells depend on anaerobic mechanisms, like glycogen metabolism. Starvation for 24 h slows periportal hepatocytes' oxygen consumption without changing the respiratory chain (as in the State 3 → State 4 transition), and increases activity of their gluconeogenic enzymes (Jungermann, 1986).

Whatever the quantitative contribution of the mitochondrial proton-selective leak and State 4 respiration to the BMR may be, and evidence speaks for a significant one (see also Rolfe and Brand, 1997), this paper concerns the regulatory and adaptive influences of cardiolipins on the leak, and proposes a model mechanism. Protons may leak back into the mitochondrial matrix via several mechanisms: (a) via lipids, i.e., mtPL(s); (b) via introduced protonophores; and (c) via proteins, e.g., pore proteins, proton symporters and exchangers, and uncoupling proteins.

Mitochondrial mechanisms of transmembrane proton transport, usually in liver, can be resolved in part through examination of the permissive effects of thyroid state, which are based on sequential thyroid regulation of lipogenic enzyme activation, lipid remodeling, and protein synthesis (reviewed in Hoch, 1988, 1992). A dose of the hormone, given to hypothyroid rats, sets off numerous changes in liver mitochondria: it stimulates the depressed respiration in State 4 hours later, and the depressed protein synthesis (labeled amino acid incorporation) days later (Tata *et al.*, 1963). The differences in the latent periods of thyroid hormones' successive in vivo stimulatory actions and effects on lipid and protein synthesis seem to reflect

the activation of the mitochondrial proton leak, first via PLs and then perhaps via UCPs (but not in liver). T4, injected into hypothyroid rats, rapidly and progressively (in 2–180 min) stimulates liver mitochondrial depressed respiratory rates in State 4 and 3u (Hoch, 1967). As rapidly, and accompanying the leak changes, in 60–150 min a T3-injection lowers mitochondrial ΣPL contents of 18:2, 20:4 and 22:6 FA by as much as 50%, and replaces them with SFA + MUFA, but we do not know CL compositions (Hoch, 1988). Injected T3 activates hepatic acetyl-CoA carboxylase and FA synthetase, first by their phosphorylation, then by their synthesis. In hypothyroid rats, a $mRNA_{S14}$ that is involved in lipogenesis appears in 10 min (Narayan *et al.*, 1984). Albumin, which sequesters FFA, blocks the increased proton leak that appears 30 min after LT4-injection (Hoch, 1967), as it would if this early leak were via a (constitutive, at this early time) UCP. But as discussed below, livers have no UCPs, constitutive or induced, so the early leak still appears to be via altered mtPLs.

The lipid mechanisms (1.) seem to be primary, because they are induced faster and because in vivo non-lipid routes (2; protonophores) depend on them, and a non-physiological route (pore proteins in the mitochondrial permeability transition; see (3)) involves them. The primacy of lipid mechanisms would lie in the need for a membrane outer-surface proton antenna (concentrated buffer) as a source of enough protons to account for the observed rates of their transmembrane leakage via any route, because an outer aqueous $[H^+]$ of 10^{-7} M is not sufficient (Haines, 1983; Deamer, 1996; Marrink *et al.*, 1996); see section '*proton antenna*'. CLo-headgroup microdomains in the outer surface of the inner membrane have the properties to serve as such a source.

Mitochondrial phospholipids and State 4 respiration (proton leakage).

Cardiolipins in eukaryotes are found consistently only in mitochondrial inner membranes. A review of data on cardiolipin FA compositions and relative amounts per mitochondrial PLs, and mitochondrial State 4 respiration and proton leakage (Hoch, 1992), leads to the hypothesis: amounts of SFA and/or MUFA per cardiolipin, and/or amounts of CLs per total inner membrane PLs, relate directly to State 4 respiration, and thereby to the rate of the mitochondrial proton leak. In this paper, we show that published effects of in vivo agents on resolved mitochondrial CLs, PCs and PEs, and mitochondrial State 4 respiration, mostly confirm or support this hypothesis. Roles of CLs can be assigned from studies on mechanisms of proton-selective conductance in membrane PLs, and from the special properties of CLs: organization into surface headgroup microdomains, asymmetry in bilayer membranes, ability to bind and donate protons at physiological pH, and propinquity to proton acceptors in mitochondrial microstructure. These properties are relevant for a model in which outer-surface CL microdomains act as a competitive antenna for protons and as an unique donor of collected protons for translocation by a proton leak, protonophores, or UCPs.

Table I compiles data from published experiments on measured in-vivo-induced changes in FA compositions, relative amounts of mitochondrial CLs, PCs, and PEs, membrane sidedness of CLs, and State 4 respiration (proton leakage). The experiments are grouped according to the degree of correlation, that supports or denies the proposed role for CLs. Dietary fatty acids, altered thyroid states, cold-adaptation, ethanol-feeding, or aging and injection of acetyl-L-carnitine, all induce *positive* data in rat mitochondria. *Diet*: A fat-free diet, or one high in MUFA and low in 18:2 acid, produces EFA-deficiency. It repletes liver mtPLs with endogenous SFA and MUFA, heart mtPLs with exogenous MUFA, and both with the desaturation product 20:3ω9. In the mtPCs and mtPEs, 20:3ω9 acyls (not normally present) maintain unsaturation by substituting for 18:2, 20:4, and 22:6 acyls. In the mtCLs, SFA and/or MUFA, but not 20:3ω9 acyls, replace 18:2ω6 acyls, and UI decreases; CL/mtPL remains unchanged. EFA-deficiency in MUFA-fed rats' liver mitochondria accelerates State 4 respiration and the proton leak but leaves a normal Δp (Rafael *et al.*, 1984). Dietary erucate, 22:1ω9, which goes specifically to rat heart mitochondria, selectively halves CL 18:2 content and raises CL SFA+MUFA 2.5-fold, but changes composition of PCs and PEs minimally; it increases State 4 respiration markedly.

Thyroid States

Thyroid-treatment of euthyroid rats (hyperthyroids) (Ruggiero *et al.*, 1984) increases the liver mitochondrial CL P/mtPL P ratio and SFA content in CLs, while halving CL 18:2 acyl content, but does not affect PCs+PEs; State 4 respiration rate increases by 65% (see Hoch, 1988). In hypothyroid rats, injected hor-

Table I. Fatty Acyl Compositions of Mitchondrial Cardiolipins, Phosphatidylcholines, and -Ethanolamines, Cardiolipin Content, and Sidedness Correlation with the Proton-Selective Leak (State 4 Respiration)[a]

Mitochondria PL		Diet/Rx	% FA/PL S	% FA/PL MU	Unsaturation index ω6	Unsaturation index ω3	Unsaturation index ω9	% CL P mtPL P	State 4 Δ%, °C	References
					Positive correlations[b]					
Rat liver	CL	18:2ω6	6	15	97					Tischer and Glenn, 1965; Wolff,
		Fat-free	10	74	42			Δ = 0	+64, 30	1988; Hoch, 1992
	PC	18:2ω6	50	4	92	126				
		Fat free	41	13	24	33	102			
	PE	18:2ω6	47	2	92	150				
		Fat free	44	16	44	42	63			
Rat liver	CL	18:2ω6	11	36	107				+30, 25	Rafael *et al.*, 1984; Divakaran and
		MUFA[c]	11	68	55					Venkataraman, 1977
	PC	18:2ω6	47	11	121	28				
		MUFA	48	15	54	23	61			
	PE	18:2ω6	45	6	119	70				
		MUFA	48	15	95	16	51			
Rat heart	CL	18:2ω6	4	19	152					
		MUFA[c]	20	46	64				+23, 37	Hsu and Kummerow, 1977;
		22:1ω9[d]	22	36[e]	83	11			+76, 37	Clandinin, 1978
	PC	18:2ω6	33	20	90	31				
		MUFA	43	40	31	19	11			
		22:1ω9	42	39	33	29				
	PE	18:2ω6	44	21	32	86				
		MUFA	43	39	36	51	7			
		22:1ω9	29	39	49	95				
Rat liver	CL	Euthyroid	19	23	113	14		12[f]		Hoch and Lipmann, 1954; Hoch,
		Euthyroid + T3	50	22	52	25		18[f]	+56, 30	1968a; Hoch *et al.*, 1981;
		Hypothyroid	14	18	126			21[f]	−26, 25	Ruggiero *et al.*, 1984;
	PC	Euthyroid	37	23	142	25				Crespo-Armas and Mowbray,
		Euthyroid + T3	38	22	152	27				1987
		Hypothyroid	37	19	112	10				
	PE	Euthyroid	35	20	138	36				
		Euthyroid + T3	37	15	157	41				
		Hypothyroid	37	19	115	26				
Rat heart	CL	Euthyroid + ω6	9	6	168	4		19		Pehowich, 1995
		Hypothyroid + ω6	10	3	165	2		13	−45, 30	
		Euthyroid + ω3	15	7	128	31		18		
		Hypothyroid + ω3	10	8	143	27		12	−39, 30	
	PC	Euthyroid + ω6	48	2	155	13				
		Hypothyroid + ω6	46	2	147	10				
		Euthyroid + ω3	50	6	95	79				
		Hypothyroid + ω3	47	7	87	85				
	PE	Euthyroid + ω6	43	3	162	64				
		Hypothyroid + ω6	36	4	183	59				
		Euthyroid + ω3	48	5	44	200				
		Hypothyroid + ω3	43	5	57	204				
Rat liver	CL	Host	9	15	145	4		5.3		
		Hepatoma	41	27	63	26		4.7	+61, 30	Wood, 1975; Morton *et al.* 1976
	PC	Host	18	18	159	38				
		Hepatoma	23	37	97	6				
	PE	Host	38	6	140	80				
		Hepatoma	23	35	94	17				
Rat BAT	CL	28°C	19	54	85			14		Goubern *et al.*, 1990;
		5°C	27	31	94			12	−27, 25	Senault *et al.*, 1990
	PC	28°C	39	38	72					
		5°C	42	19	107					

Table I. Continued

Mitochondria PL	Diet/Rx	% FA/PL S	% FA/PL MU	Unsaturation index ω6	Unsaturation index ω3	Unsaturation index ω9	% CL P mtPL P	State 4 Δ%, °C	References
PE	28°C	33	36	117					
	5°C	38	14	171					
Rat liver cells:	Control	5	15	161	6	0	8.1		Castro *et al.*, 1991; Garcia-Ruiz
p.portal CL	+ethanol[g]	16	31	106	4	0	8.3	+35, ?	*et al.*, 1994a,b
PC	Control	34	13	155	21	0			
	+ethanol	40	16	128	19				
PE	Control	34	13	144	47				
	+ethanol	41	14	120	48				
p.venous CL	Control	11	23	140	9				
	+ethanol	22	36	86	4			+191, ?	
PC	Control	40	50	129	22				
	+ethanol	46	16	101	20				
PE	Control	41	14	121	52				
	+ethanol	48	14	99	47				
Rat heart CL[h]	Young rat	14	9	152	13		14		Paradies *et al.*, 1994
	Aged rat	14	10	151	11		10	−21, 25	
	Ag + AcC[i]	13	11	147	14		15	−3, 25	
				Supportive correlations[b]					
Rat heart CL	18:2ω6[j]	4	6	197	6				Astorg and Chevalier, 1991
	+18:3ω3[k]	5	6	171	36			−5, 30	
PC	18:2ω6	56	5	178	6				
	+18:2ω3	44	6	136	47				
PE	18:2ω6	39	7	208	30				
	+18:2ω3	38	7	96	152				
Rat liver CL	18:2ω6	8	5	178	8				Astorg and Chevalier, 1991
	+18:2ω3	16	14	147	47			+5, 30	
PC	18:2ω6	44	5	181	11				
	+18:2ω3	42	5	136	65				
PE	18:2ω6	41	6	188	26				
	+18:2ω3	45	5	110	111				
Rat heart CL	18:2ω6	1	7	177	7				Yamaoka *et al.*, 1988, 1990
	−18:2ω6[l]	1	13	157	16	0	Δ = 0	−6, 25	
PC	18:2ω6	42	44	176	16				
	−18:2ω6	42	13	124	48	0			
PE	18:2ω6	39	42	145	19				
	−18:2ω6	37	7	77	228	0			
Rat heart CL	18:2ω6	5	20	70					Combe *et al.*, 1980
	+*t*, *t*18:2ω6	4	18	72				−5, 25	
PC	18:2ω6	48	8	92	28				
	+*t*, *t*18:2ω6	7	9	141	18				
PE	18:2ω6	46	11	64	118				
	+*t*, *t*18:2ω6	7	10	79	142				
Rat heart CL	Euthyroid	4	9	170	14		16		Shaw and Hoch, 1977; Hoch,
	Euthyroid+ T3	7	12	148	19		15	0, 25	1982
	Hypothyroid	9	7	137	26		15	−8, 32	
PC	Euthyroid	41	19	114	10				
	Euthyroid + T3	40	20	116	6				
	Hypothyroid	44	20	99	14				
PE	Euthyroid	38	17	96	78				
	Euthyroid + T3	30	19	108	89				
	Hypothyroid	29	16	139	57				
Rat muscle CL	Control	4	9	178			13		Cardellach *et al.*, 1991
	+ethanol[g]	6	8	175			15	−8, 27	

Table I. Continued

Mitochondria PL	Diet/Rx	% FA/PL S	% FA/PL MU	Unsaturation index ω6	Unsaturation index ω3	Unsaturation index ω9	% CL P mtPL P	State 4 Δ%, °C	References
PC	Control	34	12	155	17				
	+ethanol	34	13	150	16				
PE	Control	35	6	112	163				
	+ethanol	34	6	124	132				
Rat liver CL	Control	9	27	112	25		15		Spach *et al.*, 1979; Cunningham
	+ethanol[g]	12	26	93	43		14	+8, 30	*et al.*, 1982; Spach and
PC	Control	40	14	135	115	30			Cunningham, 1987
	+ethanol	40	16	120	31				
PE	Control	44	12	115	53				
	+ethanol	41	15	99	55				
				Negative correlations[b]					
Rat liver cells: CL	P. venous	11	23	140	9		8.0		Castro *et al.*, 1991; Garcia-Ruiz
	P. portal	5	15	161	6		8.0	+21,?	*et al.*, 1994a,b
Rat heart CL	18:2ω6	1	7	177			13		Yamaoka *et al.*, 1988, 1990
	ω3PUFA[m]	9	23	50	261		12	−19, 30	
	MUFA[n]	2	18	138	49		13	−13, 25	
PC	18:2ω6	42	44	176	16				
	ω3PUFA		6	76	154				
	MUFA	34	20	127	50	0			
PE	18:2ω6	39	42	145	19				
	ω3PUFA	7	9	94	239				
	MUFA	34	18	67	152	0			
Chick heart CL	18:2ω6	9		165	36				Renner *et al.*, 1979
	22:1ω9[o]	8	28[p]	123	28			(−2, 37)[q]	
PC	18:2ω6	44	16	119	1				
	22:1ω9	40	17	138	3				
PE	18:2ω6	59	14	58					
	22:1ω9	51	25	55					

[a] S, saturated; Mμ, unsaturated ($c - t$ 16:1 + 18 acyls); unsaturation index of ω6-, ω3-, and ω9 (only 20:3ω9)-polyunsaturated fatty acyls.

[b] *Positive*, necessary and sufficient to accept correlation with cardiolipins: the inducing agent changes State 4 respiration rate and one, two, or all of the ratios (SFA + MUFA)/CL, CL/mtPL, CLo/ΣCL, in the same direction. *Supportive*, necessary but not sufficient to accept: the inducing agent does not change (SFA + MUFA)/CL, CL/mtPL, or State 4 respiration rate, but does alter unsaturation of PCs or PEs. *Negative*, necessary and sufficient to reject correlation: CLs change but State 4 rate does not, or CLs do not change but State 4 does.

[c] Fed hydrogenated coconut oil (70% c + tMUFA, 27% SFA, 3% 18:2 ω6) for 6 weeks (Hsu and Kummerow, 1977).

[d] Fed rapeseed oil (14% 18:2 ω6, 18% 18:3; 49% 22:1 ω9 (*ibid*).

[e] 5–9% 20:1 + 22:1 ω9.

[f] A sidedness-marker enzyme (see Harb *et al.*, 1981), CPTo, (Stakkestad and Bremer, 1982, 1983; Hoch, 1992; Power *et al.*, 1994) gives CLo/ΣCL ratios of 11% in euthyroids, 21% in hyperthyroids, and 6% in hypothyroids.

[g] Rats fed ethanol (36% of total cal) chronically, 31 d (Cardellach *et al.*, 1991; Castro *et al.*, 1991; Garcia-Ruiz *et al.*, 1994a,b) or 35–70 d (Spach *et al.*, 1979).

[h] FA in mtPCs, PEs, PSs, and PIs were not altered in any group (Paradies *et al.*, 1994).

[i] Acetyl-L-carnitine 0.3 mg/g bw injected ip 3 h before killing (*ibid*).

[j] Diets fed to dam and to weanling (source of mitochondria) contained 10% oils: control oils are 69% 18:2 ω6, 0.32% 18:3 ω3 fatty acids.

[k] +18:3 ω3-diet oils are 37% 18:2 ω6, 32% 18:3 ω3 (Astorg and Chevalier, 1991).

[l] No 18:2 ω6, 40% SFA, 60% MUFA, fed 10 d.

[m] Fed sardine oil (30% ω3PUFA, 6% 18:2 ω6).

[n] Fed hydrogenated corn oil (24% SFA, 69% c + tMUFA, 2% 18:2 ω6) for 10 d (Yamaoka *et al.*, 1988, 1990).

[o] Fed rapeseed oil (15% 18:2 ω6, 7% 18:3, 12% 20:1, 37% 22:1 ω9 (Renner *et al.*, 1979).

[p] 17% 20:1 + 11% 22:1 ω9.

[q] Measured in chick heart mitochondria prepared in the presence of heparin: controls respire in State 4 (pyruvate + malate, 37°C) at 90 ng atom O/min/mg, +22:1ω9 at 88 (Renner *et al.*, 1979), whereas chick heart mitochondria prepared without heparin respire (pyruvate + malate, 30.5°C) at 0–3 ng atoms O/min/mg protein (Toth *et al.*, 1986).

mone increases the proton leak, extrapolated from FCCP-titration, by 43% (Horrum *et al.*, 1990). The lipid changes are ascribable to the T3-induced stimulations of several enzymatic steps in lipid metabolism: hepatic microsomal de novo synthesis of 16:0 and 18:0 fatty acids (Oppenheimer *et al.* (1987); activities of Δ9-desaturase that produces 16:1 and 18:1 acyls, and Δ5-and Δ6-desaturases that further unsaturate 18:2 and 18:3 acyls (Hoch *et al.*, 1980; Hoch, 1988); and mitochondrial cardiolipin-synthase (Hostetler, 1991). In heart mitochondrial inner membranes from hypothyroid (propylthiouracil-induced) rats, CLs are depleted by 32% (Ruggiero *et al.*, 1984; Pehowich, 1995), while SFA + MUFA in CLs and the FA profiles of PCs+PEs hardly change; State 4 at 30 C decreases by about 45%. However, in liver mitochondria from hypothyroid (^{131}I-induced) rats (Hoch *et al.*, 1981), CL/mtPL was 70% above control ratios and (SFA+MUFA)/CL and ω6-PUFA in PCs+PEs decreased minimally. Nevertheless, State 4 respiration was about 26% (and down to 47%) less than in normals (Hoch, 1968b; see Hoch 1988). In hepatocytes from hypothyroid rats, the decreased mitochondrial proton leak is caused by decreased "permeability" (really proton supply), $\Delta\psi$ being constant, and is reckoned to account for all of the respiratory deficit (Harper and Brand, 1993). Here, the sidedness of CLs (see below), not the unsaturation, is associated with the proton leak; the implications for the role of CLs are discussed below (see *proton antenna*).

Cold-Adaptation

Cold adaptation in rats decreases State 4 respiration (+GDP to block proton leakage via UCP1) in BAT mitochondria by 27% (Table I); mitochondria from 5 C-adapted rats have CLs with 21% less SFA+-MUFA and 15% lower proportions of CLs/mtPLs (Goubern, 1990; Senault, 1990). The PCs+PEs *gain* ω6-unsaturation at the expense of their MUFA. Although the changes in (SFA+MUFA)/CL correlate with those in proton leakage and are thereby consistent with the hypothesis, it should be noted that BAT mitochondrial CLs of room-temperature-adapted rats are not like those in mitochondria of other tissues, and that they adapt to cold oppositely. Rat BAT mitochondrial CLs normally (un-induced) have a higher (SFA+-MUFA)/CL ratio, 73%, than rat liver mitochondria, 30% (Table I) - a ratio like that in hyperthyroid rats' liver mitochondria, 72%. Cold-adaptation slows rat BAT mitochondrial State 4 respiration, but in contrast, accelerates State 4 in liver and/or skeletal muscle mitochondria of rats, mice, hamsters, and seals (see Hoch, 1992). Hyperthyroid liver mitochondria leak protons 56% faster than normals, and their CLo/ΣCL is twice normal. In BAT mitochondria, this ratio is lower than in the hyperthyroids, but their CL-sidedness has not been reported. If sidedness depends, at least in part, on FA/CL profiles, CLo/ΣCL might also be high, producing a large proton antenna and increasing the supply of protons for translocation (see below).

Experiments on poikilotherms' adaptations to cold, and estivation (not shown in Table I because they lack State 4 measurements) implicate CLs as a target lipid. Skeletal muscle mitochondria of cold-adapted carp show opposing changes in CLs and PCs (Wodtke, 1981) like those in rat BAT mitochondria: the carp PCs (the major PL) become more unsaturated ('homeoviscous adaptation' (Sinensky, 1974) that maintains membrane fluidity?), which covers up the decreased UI of the CLs in mtΣPL assays. When trout adapt from 20 C → 5 C, the liver CLs' ω3-UI rises 4-fold and the CL content of liver ΣPLs diminishes by 47%, while liver ΣPLs/g liver remain unchanged, indicating a decrease in mitochondrial CL contents (CL/mtPLs) and/or a decrease in the number of mitochondria per g liver (Hazel, 1979); in trout gills, CLs/ΣPLs rise by 42% (Hazel, 1985).

Estivation

The mitochondria of the hepatopancreas of active terrestrial snails have about half the CL content seen in rat liver mitochondria, but the CL UI is about 50% higher; the (SFA+MUFA)/CL is only 4%; and 18:2ω6+18:3ω3 content is ~30% higher (Stuart *et al.*, 1998a,b). Estivation, induced by drought, does not change snail temperature or water content but lowers their BMR a smashing 85%, without significantly decreasing hepatopancreas weight or mitochondrial proteins; the respiratory contribution of these mitochondria to BMR is not reported. Estivation changes the mitochondria drastically, too: mtPL/mt protein drops 72%; CL/mtPL falls 35%; CL saturation, (SFA-+MUFA)/CL rises 9.5-fold, replacing half the 18:2ω6+18:3ω3 contents. The mt(PC+PE) lipids change only slightly, mainly from a halving of 18:2ω6 + 18:3ω3 contents. Although the depletion of these EFA and the accretion of CL SFA+MUFA resemble effects of EFA-deficiency in rat liver mitochondria and

may reflect starvation, the specific attenuation of CL is unique. Estivation depresses cytochrome oxidase activity 89% ([*aa3*] is not reported). Compare this with heart mitochondria of hypothyroid rats where activity drops 37% vs. normals, [*aa3*] only 15%; CL/mtPL 63%, and CL outside is low but FA/ΣCL are normal; cytochrome oxidase activity is restored by addition of CL (Paradies *et al.*, 1993). The induced changes in snail mitochondria, according to the model for CL function to be developed subsequently, would have opposing effects on the proton leak.

Ethanol-Feeding

Ethanol-feeding of rats selectively increases State 4 rates and CL-saturation in mitochondria prepared from different zones of the liver lobule (discussed above). In periportal mitochondria, control SFA+-MUFA/CLs ratios are 20%, in rats fed ethanol, 47%, and State 4 is 35% faster. In mitochondria from perivenous areas, control CLs contain 34% SFA+MUFA, vs. 58% +ethanol, and State 4 is almost twice as fast. Ethanol-feeding has minor effects on PCs and PEs.

Aging in rats does not alter their heart mitochondrial SFA+MUFA/CLs ratios, or the FA in other PLs, but does depress CL contents by 29%, while it slows State 4 by 21%. Injection of aged rats with acetyl-L-carnitine only 3 h before killing restores both CL contents and State 4 rates to youthful values (Paradies *et al.*, 1994); perhaps the binding of CPTo to CLo is involved.

Supporting data were obtained mostly from studies on muscle mitochondria from heart or skeleton, in rats fed ω3-unsaturated FA, *t,t*-18:2ω6, or ethanol, or altered in thyroid state (Table I). Diets supplemented with 18:3ω3 also contained 37% 18:2ω6; they induced partial replacement of 18:2 with 18:3ω3 in heart mitochondrial CLs, PCs, and PEs; but no change in (SFA+-MUFA)/CL or in State 4 respiration. Fed *t,t*-18:2ω6 does not enter rat heart CLs or change their FA distribution, but does replace almost all the SFA in mtPCs+PEs; State 4 is not affected. Dietary ethanol partly replaces rat liver mitochondrial 18:2 with 18:3 acyls in mtCLs, PCs and PEs but has no effect on (SFA+MUFA)/CLs or on State 4; in skeletal muscle mitochondria, FA/CLs and State 4 remain unaffected while in mtPEs ω6-unsaturated acyls partly replace ω3-acyls. A 3-day T3-treatment has little or no effect on rat heart mitochondrial State 4 respiration, CL SFA+MUFA, or CL/mtPLs (although it raises these parameters in liver mitochondria).

The 5 negative experiments shown in Table I do not actually reject the CL-hypothesis, because of special natural or experimental conditions. The reader will have noticed in Table I that mitochondria from normal rat periportal hepatocytes have CLs with 20% SFA+MUFA, compared with 33% in CLs from perivenous hepatocyte mitochondria. Both sets somehow have about half the CL/mtPL ratio seen in rat liver (mixed) mitochondria. Not shown in Table I, the periportal mitochondria respire 21% *faster* in State 4 at a Δp = 176 mV, *cf.* 181 mV in perivenous mitochondria (Garcia-Ruiz *et al.*, 1994a,b) - also low levels. The greater area of the periportal mitochondrial inner membranes (Jungermann, 1986) would explain this experimental result.

Feeding rats excess ω3PUFA 30 days alters their heart mitochondrial CLs: ω3PUFA appear and SFA + MUFA content rises 4-fold, replacing most of the 18:2ω6 acyls, but in PCs+PEs only a small amount of ω3PUFA and no 20:3ω9 acyls appear; State 4 respiration (glutamate/malate, 25 C) slows by 28% (Yamaoka *et al.*, 1988). Feeding rats excess MUFA for 10 days increases their heart mitochondrial SFA+-MUFA/CL ratios to 20%, yet slows State 4 respiration (25 C) by 12% (Yamaoka *et al.*, 1990), and changes FA in PCs+PEs like ω3PUFA-feeding does. However, studies by other investigators produce positive results. Feeding excess MUFA 6 weeks increases content of CL SFA+MUFA almost 3-fold and replaces some 18:2 ω6 acyls; in PCs + PEs, MUFA replace half the 18:2 acyls and a trace of 20:3ω9 appears; State 4 respiration *at 37 C increases* by as much as 32% (Hsu and Kummerow, 1977). The disparity apparently arises from the thermotropic properties of altered lipids in rat heart mitochondria (Shaw and Hoch, 1977). State 4 rates (glutamate) are normal in hypothyroid rats ± T3 when measured at ≤25 C, and significantly decrease vs. controls only at ≥32 C.

Chicks were fed the 22:1ω9 fatty acid (Renner *et al.*, 1979), which targets CLs in heart mitochondria: 22:1 became 11% of FA/CLs, its derivative 20:1 = 17%; both displaced some 18:2ω6 and 18:3ω3 acyls; CL SFA and the natural MUFA remained at control levels. PCs+PEs changed FA profiles very little. State 4 respiration (pyruvate/malate, 37 C) stayed at control levels. However, control State 4 was 90 ng atom O/min/mg (Renner *et al.*, 1979). Rat or human heart mitochondria respire in State 4 at 37 C at 9-24 ng atom O/min/mg (Sordahl, 1984). State 4 was measured

after ADP-cycling (Lyons *et al.*, 1974), which accelerates State 4 in rat liver mitochondria to 80 ng atom O/min/mg (Royce and Holmes, 1984). There are objections to calling an increased proton leak 'improved' respiration (Hoch, 1988). Chick heart mitochondria prepared and assayed without ADP-cycling respire in State 4 (30.5 C) at 0–3 ng atom O/min/mg (Toth *et al.*, 1986); their CLs contain only 8.7% SFA and no MUFA, while their PCs+PEs contain 61% and 73% SFA+MUFA, respectively (Renner *et al.*, 1979). Chick heart mtPC+PE tHBCs can't be conducting many protons.

The absence of many expected experiments that convincingly rule out a role for CLs in the proton leak is telling in ruling out the recently proposed unique adaptive roles for protein-mediated proton leakage. Therefore, from the positive and supporting data on mitochondria from rat liver, heart, skeletal muscle, and BAT, CLs are involved in proton leakage. But this conclusion need not apply to tissues of other rat organs, or of other species or phyla. The only complete data - on resolved mtPLs and their FA, the sidedness of CLs, together with State 4 respiration - come from mitochondria of tissues that are thyroid-sensitive. Rat tissue thyroid-receptor count and mitochondrial proton-leakage response to thyroid levels correlate with mitochondrial CL contents and degree of unsaturation (mainly 18:2 acyl content) (Hoch, 1988). Mitochondria from thyroid-insensitive tissues still need correlated measurements.

Protons leak slowly across the mitochondrial inner membrane, driven into the matrix by a high $\Delta\psi$. In the few reports which mtPL compositions are also measured, $\Delta\psi$ remains relatively constant even though State 4 changes. Thus, although State 4 does alter, $\Delta\psi$ does not: in EFA-deficiency (Rafael *et al.*, 1984), in cold-adaptation of rat BAT mtPLs (Goubern *et al.*, 1990; Senault *et al.*, 1990), or in hyper- or hypothyroidism (see Hoch, 1988 for refs.). Changes in State 4 rates thus appear to be connected with observed changes in a mtPL. The mechanisms whereby inner membrane mtPLs mediate and regulate proton translocation are therefore relevant to the role of CLs.

CARDIOLIPINS IN PROTON-SELECTIVE MEMBRANE-CONDUCTANCE

Cardiolipins have been recognized to have an unique, albeit as yet undefined, role among mitochondrial PLs in oxidative phosphorylation (Divakaran and Venkataraman, 1977; Ruggiero *et al.*, 1984; Astorg and Chevalier, 1991). Mitochondria, probably evolved from a prokaryote endosymbiont with CLs in its cell membranes, persevered in synthesizing, remodeling, and retaining CLs in their inner membranes. Mitochondrial CLs have distinctive chemical and physical properties (see Hostetler, 1982; Hoch, 1992). Cardiolipin molecules have 4 fatty acyl groups while other PLs have 2. Different molecular acyl species of CLs contain a much more limited variety and proportion of fatty acyls than other PLs: CL acyls are almost all 16C and 18C, and normally have no more unsaturated bonds than 18:3 (Schlame *et al.*, 1991); 18:2ω6 is their chief and in some cases almost only FA, which makes CLs depend on an external supply of this EFA. CL species vary among different tissues of eukaryotes, and among different eukaryotes, suggesting that CLs are adapted for special roles. CL organization in membranes is of a higher order than that of other PLs, at several levels. CL intramolecular H-bonding enables proton binding at neutral pH; intermolecular CL-CL H-bonding in membranes forms microdomains that include water; CLs are asymmetrical across membrane bilayers; and CLs localize strategically in morphological structures in mitochondrial membranes.

The mechanism of proton conduction across PL membranes is generally thought to resemble that in ice: via hydrogen bonds between water molecules (Teissie *et al.*, 1993). In PL membranes, in contrast to ice, the H-bonded water pathways are confined and conduct protons vectorially. Water molecules permeate the hydrophilic and hydrophobic portions of a membrane bilayer, from molecular dynamics simulations on (16:0,16:0)-PC (Marrink and Berendsen, 1994). Protons are conducted (i) transmembranally into the matrix via water molecules among extended acyl chains (tHBCs). The tHBCs receive protons from (ii) protonated anionic headgroups of PLs on the outer surface (PLo)-the proton antenna, and (iii) from lateral conduction along the PLo headgroup surface.

Transbilayer Conduction

Protons are conducted across PL bilayer membranes, according to the most plausible evidence available (Deamer, 1987; Nagle, 1987; Paula *et al.*, 1996), via defects in acyl packing that are transients, produced by dynamic properties of the bilayer structure. The defects contain water that is hydrogen-bonded and so can conduct protons selectively as compared with

larger cations. The only transient defects of a length long enough to span a monolayer would be between pairs of extended acyl groups ≥ 16-18C-i.e. SFA or MUFA; their methyl ends in inner and outer monolayers abut transiently to traverse the bilayer. More unsaturated FA (PUFA) kink and bend dynamically and are shorter. Protons shift position by sequential transport of a hopping defect and a turning defect along the HOHOHO . . . hydrogen-bonded water molecule chain - the tHBC or 'proton wire'. Extended acyls between two adjacent PLs could confine tHBCs. In CLs the saturated and monounsaturated fatty acyls normally bind at positions 2(2″) in glycerol (Wolff and Entressangles, 1991). Thus, saturated and monounsaturated chains of adjacent peripheral CL molecules might pack together, and allow water molecules between the headgroups to contribute to the formation of a tHBC. Water penetrates between the lipid polar heads in bilayer membranes formed from di-14:0-PC and cholesterol (Warren, 1987).

Mechanisms proposed for transmembrane proton flux across PLs must accommodate several observations. The relationship between $J_H^+ / \Delta p$ at high Δp is exponential (non-ohmic and superlinear) (Nagle, 1987; Deamer and Nichols, 1989). Proton flux is almost independent of ΔpH. One mechanism prevails over a wide temperature range, as shown by the linearity of Arrhenius profiles, i.e. constant E_a: for Δp (15–50 C) (Rossignol *et al.*, 1982) and for State 4 respiration in heart mitochondria (15–37 C) (Hoch and Hoijer, 1981). The profile for State 3 respiratory rate inflects, indicating a phase transition that depends on the overall FA unsaturation (i.e. fluidity). But that transition in membrane fluidity does not alter the E_a of the proton-selective leak in State 4.

The kinetics of proton conduction by tHBCs are most consistent with a model in which a tandem pair of end-to-end half-tHBCs spans the bilayer; most of the time each tHBC only goes across one layer (Nagle, 1987); pairing need last <1 sec (Deamer, 1987) and frequency of coincidence of the 2 half-tHBCs' methyl ends limits proton translocation rate; and computer plots are superlinear when $\Delta\psi$ drives. Although the turning defects in the half-tHBCs could also limit proton flux, the observation of a single E_a value speaks against a contribution of a second controlling process. This model is best accommodated by the anatomy of biomembranes. An alternative model requires that 2 extended, parallel FA chains of PLs be long enough to span both monolayers, 4–5 nm (Nagle, 1987; Jain, 1988). No FA chain found in liver or heart mitochondrial PLs is long enough. More likely, each of a pair of extended FA chains from 1 or 2 PL molecules supports a half-spanning tHBC; 1 pair is in each monolayer; coincidence of a methyl end in each monolayer establishes a full course of links—an all-the-way tHBC—for transmembrane flux of protons.

Proton Antenna

A membrane outer-surface proton collector- and-donor is theoretically necessary for proton translocation. From molecular dynamics simulations of proton transport across a bilayer membrane, the computed value for proton permeation fits experimental data only if proton delivery is not rate-limiting, but the assumption that concentrated fixed adjacent buffer molecules ("suitable lipid headgroups") donate protons, and mobile buffers deliver them directly (almost) solves the delivery problem (Marrink *et al.*, 1996). At pH 7, $[H^+]$ is too low, by a factor of 10^8, to account for the observed rate of translocation, but protonated buffer ions can deliver enough H^+ to the heads of tHBCs (Deamer, 1996). Haines (1983) proposed that a membrane outer-surface collection of anionic PL headgroups acts as a trap or buffer that concentrates protons and passes them directly to a "proton-translocating pore" (a transport protein using the proton gradient). The observed aggregation of the buffering CLo headgroups into a surface microdomain should further promote eventual proton delivery to a CL or an adjacent tHBC or other porter for translocation. Marrink *et al.* (1996) and Deamer (1996) suggested that a buffer shuttle (Benz and McLaughlin, 1983) mediated these proton transfers to FCCP; later experiments with Δ[buffer] and protonophore P13 indicated that water hydrolysis supplied protons (Kasianowicz *et al.*, 1987).

In State 4, tHBCs would translocate protons across the membrane bilayers. From ^{1}H-NMR measurements on microvesicles of PC+PE (Lange *et al.*, 1975; Ralph *et al.*, 1985), as construed by Tocanne and Teissie (1990), a proton deficit induced by translocation would be replenished by a cooperative effect of 10^4 phospholipids surrounding the tHBC and *acting as an antenna to collect protons from the bulk phase.* From evidence that will be discussed, a proton antenna forms more effectively - even uniquely - in a surface CL-microdomain rather than among the more weakly interacting and less anionic PCs+PEs. As a result, CLs could divert protons from the PCs+PEs - 'proton-channeling'. Proton movement via tHBCs of all PLs

would be alike; then the rate would be determined by the antenna's proton-collecting and -donating power, and the number of tHBCs accessible for proton donation.

More readily than other mtPLs, CLs can establish intermolecular H-bonds to form a microdomain. CLs have larger head groups that can pack more closely and maintain hydrogen bonding in spite of the loose packing and separation of molecules that CLs' high content of unsaturated FA promotes (as unsaturated FA do among lysyl-phosphatidylglycerols (Boggs, 1987)). CLs from thyroid-sensitive tissues contain few fatty acyls that are not unsaturated - only 20% in rat liver mitochondria and 10% in rat, beef, or chick heart mitochondria. Cardiolipins have the most extended headgroups of the inner membrane phospholipids: a CL molecule that has either saturated fatty acyls or mostly unsaturated acyls has a surface area of 1.2 nm^2 (Goormaghtigh *et al.*, 1990), as compared with 0.689 nm^2 for a (16:0,16:0)-PC molecule (Deamer, 1987).

Sidedness

PL headgroups in the inner-membrane outer-monolayer (mtPLo, the 'front-9' of the course) collect pumped-out protons and donate them to the vestibule of a transmembrane porter. CLs can be very asymmetrical between the bilayers. CLo/ΣCL ≅ 0.2 in rat liver and bovine heart mitochondrial inner membranes (see Daum, 1985); 0.11 in rat liver mitochondria by endogenous CPTo measurements (see Table I Footnote f-perhaps CPTo binds 2 CLo molecules); 0.07 in CL-PC liposomes (Müller *et al.*, 1984); 0 in mtPL liposomes (Cheneval *et al.*, 1985). The forces that determine sidedness of CLs are incompletely understood. Because unsaturated CLs are almost completely in the inner leaflet of artificial PL vesicles that have no proteins, a property of the PL determines sidedness, e.g. CL shape as determined by headgroup and acyl dynamic volumes. In CLs of liver and heart, the high proportions of unsaturated FA make for loose packing in the hydrophobic region and a conical shape that predisposes toward positioning in concave areas of a membrane surface (see Hoch, 1992 for references): on the inner monolayers of the mitochondrial inner membrane and of artificial PL vesicles. Molecular species of PEs with saturated and mono-unsaturated fatty acyl groups are on the outer convex surface of artificial vesicles and curved natural membranes (Hullin *et al.*, 1991). The preference of CL molecular species with SFA+MUFA for the outer surface of the monolayer may cause sidedness. However, other, unknown factors also seem to operate. In liver mitochondria from hypothyroid rats, the slowed proton leak can be ascribed to a CLo diminished because the thyroid-dependent CL-synthase fails to maintain the CL/mtPL ratio; the synthase is in the matrix side of the inner membrane (Schlame and Haldar, 1993), which may also contribute to the low CLo/ΣCL, since the FA/CL profile is normal (Hoch *et al.*, 1981).

The high surface charge-density of bilayer anionic PLs produces a negative electrostatic surface potential (Tocanne and Teissie, 1990) that attracts protons. The differences between surface and interior polarity shift the acid-base equilibrium, both forces raising apparent pK′ values of their ionized groups above the pK's of similar groups in aqueous solution. e.g. the pK of dimethylhydrogenphosphate is 1.2, while the phosphate pK's of PCs and PEs in membranes are 2–3 (Boggs, 1987; Marsh, 1990). Properties of CL molecules further their proton-collecting capacities. Intramolecular (Hübner *et al.*, 1991) and intermolecular (Tocanne and Teissie, 1990) H-bonding shortens the distance between CL phosphoryl groups, which electrostatically inhibits further proton dissociation. CL phosphoryls H-bond extensively to form a microdomain that includes water molecules. That water seems a possible contributor to the few water molecules that permeate the hydrophobic phase of the bilayer.

Bilayer membrane CLs (beef heart CL and 18:0-CL) each have one phosphate group that is a strong acid, apparent pK = 2.8, that can not buffer protons at neutral pH (Kates *et al.*, 1993). The second phosphate is a very weak acid that titrates anomalously: an apparent pK_2 = 7.5 shifts upward to 9.5 during the titration. The anomaly is attributed to an intramolecular H-bonding, in which the central glycerol OH group participates in stabilizing the formation of a cyclic H-bonded monoprotonated form of CL, in the bilayer plane (Hübner *et al.*, 1991). Thus, only CLs among mtPLs can function as a strong proton antenna and donor at neutral pH. Proton acceptors include transmembrane porters: tHBCs of mtPLs; protonophores; proteins. Direct transfer to CLs seems possible. Some of the 18:0 ester C=O groups also H-bond to the glycerol OH and the C=O bonds orient more towards the perpendicular to the bilayer plane, which suggests a direct return path for pumped protons to a water-column head of a CL-tHBC. The water-shuttle can also transfer protons from the antenna to proteins, protonophores, and tHBCs of other mtPLs.

CLo groups are relatively sparse, and only one phosphate (P*) on each CLo protonates: CLo P/ΣCL P = 20%; CLo P*/CLo P = 50%; ΣCL P/mtPL P ≅ 20% in rat liver mitochondria; thus, effective CLo/mtPL ≅ 2%. CLo aggregation into microdomains (Tocanne and Teissie, 1990; Hübner *et al.* 1991) would counter some of its paucity by increasing the negative surface charge-density and concentrating protonated buffer for local donations. Does this make CLo buffer capacity high enough and can the CLo high pK and concentration compete for protons with the many, but more dispersed PEs+PCs? This problem may be circumvented when the CLo protons are removed in a vectorial process, the proton leak, through CLo-tHBCs. Normally, CLs have few extended FA, but many extended FA are induced by various agents (Table I), that could mediate proton removal via the antenna's donation to adjacent mtPLs' tHBCs, in a reversal of the 'proton-steal'. This apparently occurs in liver mitochondria from hypothyroid rats (see above), where a decreased CLo antenna slows the leak.

Can the even smaller proportions of CLs/mtPLs in mitochondria from thyroid-insensitive tissues attract and donate enough protons and do CLs in all mitochondria participate in the proton-selective leak? Cardiolipins should mediate the leak universally, by stealing protons from mtPCs+mtPEs. Pig heart mitochondria have no CLo. State 4 respiration is 9 ng atoms O/min/mg at 25 C (Godinot *et al.*, 1969), 15 at 30 C (Vial *et al.*, 1978), and much slower than in mitochondria from rat liver and heart. Adult rat brain mitochondria are thyroid-insensitive; their CL P/PL P = 1.6%, 18:2/CL = 13%, and SFA+MUFA/PL = 49% (cf. rat liver mean values of, respectively, 20%, 62% and 31% (Hoch, 1992). They should have a small proton-antenna. On the other hand, the many saturated FA in brain mitochondria should raise CLo/ΣCL; perhaps that accounts for State 4 (succinate, 30 C) being 34 ng atoms O/min/mg (my calculation from Higgins, 1968). However, protons apparently can leak back into some mitochondria with minimal CLs, and across some membranes with no CLs. PCs and PEs in membrane surfaces do not form intramolecular H-bonds that enable them to store and donate enough protons at neutral pH, although they do form intermolecular H-bonded structures that include water molecules (Eibl and Woolley, 1979) and rapidly conduct protons laterally (Prats *et al.*, 1987). CLs aggregate and have a high-pK phosphate group that can act as a proton antenna, but few SFA+MUFA that can form tHBCs. PCs+PEs aggregate less and have low-pK phosphates that do not collect protons readily, but their high content of SFA+MUFA could form many tHBCs. In the outer monolayer, CLo headgroups would collect protons better or even uniquely; in the inner monolayer, PC+PE tHBCs would translocate protons better.

Proton permeability coefficients (in cm/s), measured at pH 7 under a similar driving force (ΔpH or Δψ) in artificial bilayer membrane preparations, are 10^{-4} – 10^{-6} with pure PCs or PEs; cf. $\sim 10^{-3}$ in mitochondria (Deamer, 1987). However, PC or PE bilayers preparations are bathed in buffers of different pH; mitochondrial protons are ejected by discrete respiratory assemblies, which allows two paths to compete for protons: a lateral, surface conduction path versus a perpendicular, transmembrane conduction path.

Lateral conduction

A membrane surface restricts water molecules to essentially 2 dimensions among acidic headgroups of PLs, and a kinetic barrier separates that bound-water from bulk-water molecules (Antonenko *et al.*, 1993), so that protons are conducted laterally. The lateral flux of protons on a surface of a PL membrane is linear with the area of the polar headgroup and the packing of diacyl PLs, but not with their charge, and the tetraacyl CLs do not follow this linearity (Prats *et al.*, 1987; Teissie *et al.*, 1993). PLs can H-bond heteromolecularly with other lipids (Boggs, 1987); PE, PS, or PA can H-bond homomolecularly but PC does not. The phosphate in PC and PE does not get completely protonated, perhaps because of H-bonding between the acid and anion forms. Proton capture (see (i) above), on the other hand, depends on headgroup negative-charge density, which would be greater in CL microdomains.

The high order of organization of CL headgroups in membrane surfaces may explain several observations. In membranes containing only PCs and/or PEs, some intermolecular H-bonds form between headgroups (Boggs, 1987), and protons permeate. Mitochondrial membranes with CLs that can form few tHBCs (i.e. have few SFA+MUFA), and with PCs+PEs that can form many tHBCs, leak few if any protons (e.g. chick heart mitochondria). The proton leak across PLs is minimized under State 3 conditions (Nicholls, 1974), which not only partly discharge Δψ but also change surface organization: inner-membrane outer-surface CL-domains (CLo) condense with contact sites on the

outer membrane (Adams *et al.*, 1989; Ardail *et al.*, 1990). State 3 conditions alter, not CL headgroups, but some mitochondrial proteins (e.g.F0F1, the carriers of ATP/ADP or Pi, some electron transporters). Therefore, protein-protein interactions probably drive these contacts. Several enzymes are specifically bound and oriented by CLo to react more readily with ATP generated from the proton reflux through the ATP-synthase and the ADP/ATP translocase; both these protein complexes bind cardiolipins strongly (see Hoch, 1992). This attachment of CLo may interrupt the surface-network → tHBCs proton route, donate protons even more exclusively (another proton-channeling) to the newly activated, higher capacity, protein proton-sinks like F_0, and thereby contribute to the observed cessation of proton leakage.

CLo is implicated in the proton-selective leak by the effects of several specific or surface-active agents: phospholipase A2, local anesthetics, or adriamycin. PLA2 act on an exposed membrane surface by interfacial catalysis (Van den Bosch, 1982; Gelb *et al.*, 1995), and participate in the remodeling of FA compositions of membrane PLs. Remodeling must be especially extensive in CLs, because FA/CL in some tissues differ strikingly from the FA in CL precursors; in phosphatidylglycerol of rat liver mitochondria, 18:2 acyls are about 20% of ΣFA (but 77–94% in CLs), and 16:0 acyls are 12% (1–3% in CLs) (Hostetler, 1982). CLs are enriched with 18:2 acyls in a specific deacylation-reacylation cycle, mediated by monolyso-CL (Schlame and Rüstow, 1990). The deacylation is specific for CLs newly synthesized by the CL-synthase in the inner face of the inner membrane (Schlame and Haldar, 1993).

Endogenous PLA2's are involved in the slowing of State 4 respiration in hibernation, e.g. in liver mitochondria of hamsters (Chaffee *et al.*, 1961) or ground squirrels (Brustovetsky *et al.*, 1989). In hamsters, changes in membrane-dependency of the rate of State 4 respiration reflect inner membrane FA/mtPL compositions. In squirrels, endogenous mitochondrial PLA2 activity drops by about 60%, suggesting that hibernation regulates State 4 through a change in the state of the inner membrane caused by decreased FA-remodeling of PLo. PLA2 activity is sensitive to thyroid hormones in vivo and in vitro (see Marzoev *et al.*, 1983, for Refs.): in rats pretreated 48 h with T4, liver mitochondrial PLA2 activity is 3 times control levels. These physiologically induced activations of PLA2 would appear to involve the proton-selective leak. Endogenous PLA2's attack CLo, sometimes specifically. PLA2 activated by hypotonicity decreases rat liver mitochondrial CL/mtPLs ratios by 30% and lyso-CLs/mtPLs (only ~1–3%) by 60%; mtPE/mtPLs decreases much less (Kargapolov, 1979). Endogenous PLA2 activated by Ca^{2+} added to rat liver mitochondrial mitoplasts deacylates only 23% of the mtCLs (CLo?) but all the mtPC+PE (De Winter *et al.*, 1987).

Mitochondrial Microstucture: Propinquity

Electron microscopy and fractionation of rat liver mitochondrial fragments (see review by Brdiczka, 1991, and references therein) show that the PLs and proteins in mitochondrial inner membrane convoluted cristae differ from those of the inner boundary membrane, the portion of inner membrane that faces the outer membrane and the cytosol. Crista membranes contain twice as much CL/ΣPL and PE/ΣPL, and more cytochrome aa_3 and succinate dehydrogenase, indicating that this zone of membrane transports electrons and pumps protons, i.e., generates Δp. CL-synthase in the cristae cosediments with aa_3 in fragmented inner membranes (Schlame and Haldar, 1993). Inner boundary membranes have much more PC/ΣPL, and embed proteins that transfer and synthesize, i.e. disperse Δp. Tubular membranous structures connect cristae and inner boundary membrane; they proliferate 5-fold after 5–15 days of T3-treatment, which also augments the cristae and boundary membranes by 50%, and amounts of CLs (CL/mtPLs) and the SFA+MUFA/CLs by 75% (Ruggiero *et al.*, 1984). Thus, T3 increases inner membrane area per amount of protein (see also Jakovcic *et al.*, 1978), which should increase the proton leak. Measured area of inner membrane of liver mitochondria from hypothyroid rats treated with T3 is 2–3 times that in untreated hypothyroids, but this accounts for less than half the acceleration of the leak, and the rest is ascribed to the 2- to 3-fold increase in proton permeability observed in extracted mtPLs (Brand *et al.*, 1992).

Cardiolipins per mtPLs ≅ 10% in inner boundary fractions and ~20% in cristae fractions of rat liver mitochondria. Several fractions of mouse liver mitochondria that comprise contact sites between outer and inner membrane (Ardail *et al.*, 1990) contain more CLs/ΣPLs (24%) than outer (4%) or inner (18%) membranes, and the CLs are fully unsaturated. More saturated CLs might then find the matrix-side of the cristae and contribute to proton leakage. Such observations suggest that structural factors could 'channel' metabolites in linked reactions and processes, in the sense

used by Srere (1987). E.g., ADP-induced, State 3 contact-sites between inner and outer mitochondrial membranes (Adams *et al.*, 1989) place kinases near the ADP/ATP carrier and facilitate the transfer of newly synthesized ATP toward anabolic pathways and away from the catabolic ATPases - an 'ATP-channeling'. The CLo-channeling of protons proposed here may involve the Clo proton-antenna located in the crista membrane next to proton-extruding electron-transport chains.

PROTON LEAKAGE

A model for cardiolipin mechanisms in proton leakage

Figure 1, constructed from data on State 4 conditions of mitochondria from thyroid-responsive tissues of rats, depicts two adjacent unprotonated CL molecules in a CL-microdomain, an H-bonded cardiolipin-water-headgroup network in the outer surface of the inner membrane bilayer. In this microdomain, one phosphate group of each CL molecule can bind protons from the exterior water phase at physiological pH and also divert them competitively from the protonated headgroups of PCs+PEs, as into an antenna. Even small microdomains of CLo, as in chick heart mitochondria, would then appear to be a 'black-hole' for protons in the absence of other acceptors of protons. In this snapshot, protonated CLo's donate protons to their own (normally few) tHBCs of water that is intercalated between two extended (saturated or monounsaturated) acyl chains bound at an *sn*-1(1′) position on each adjacent cardiolipin molecule, and penetrate the outer monolayer. Transient contact of methyl ends with any tHBC in the inner monolayer would complete proton conductance, driven by $\Delta\psi$, via this lipid route. Non-lipid routes for proton conductance, that could accept protons from the antenna and the water-shuttle, are not shown, but could be protonophores or proteins, including the F0F1 under State 3 conditions.

Possible regulators of the reflux proton leak are: the ratio of CLo SFA+MUFA per mtΣPLs (which includes the amounts of these FA per CL molecule; the amounts of CL molecules per total mtPLs, and the CL sidedness, CLo/ΣCL); the proton donor (CLoH) and its propinquity; and $\Delta\psi$. Fick's law does not apply to the tHBC or UCP mechanisms (Nicholls and Locke, 1984; Deamer and Gutknecht, 1986).

CLs in at least liver and heart mitochondria set the proton-selective leak at a rate determined by the balance between the amount of proton antenna (CLo/mtPL and CLo/ΣCL) and the number of CL tHBCs (SFA+MUFA), a function of saturation - whereby the high content of CL 18:2 acyls is a proton stopper (Hoch, 1988). That rate is relatively slow (cf. State 3 or 3u) because those CLs have few SFA+MUFA and therefore few tHBCs to remove protons. The antenna would then donate excess protons to other mtPLs, proteins, or protonophores. Single or multiple changes in CLs and their relationship with mtPLs can up- or down-regulate the proton leak. An increase in CL SFA+MUFA contents (we do not know CL sidedness) in EFA-deficiency accelerates the leak in rat liver mitochondria, and the rat's BMR. A decrease in CLo amount and sidedness (ΣCL/mtPL and CLo/ΣCL) decreases the leak in liver mitochondria from hypothyroid rats. In liver mitochondria from hyperthyroid, 3 parameters increase to accelerate the proton leak and the BMR. In estivating snails' mitochondria (see above), the sharp decreases in mtPL/mt protein and in CL/mtPL would deplete the outer-surface CL proton-antenna, and thereby decrease the leak, in consistence with the low BMR. The primacy of the antenna would override the promotion of the leak expected from increased (SFA+MUFA)/CL, that would provide more CL tHBCs - perhaps a compensation that raises respiratory contribution to maintain a viable BMR.

Other proposed mechanisms have protons leak via membrane total lipids. Brand *et al.* (1991) conclude that, because the number of double bonds (UI) in PL acyl chains in the middle zone of the inner membrane of liver mitochondria from rats is 7-8 times that in liver mitochondria from lizards, the looser packing in the rat organelles explains their faster proton leak. The greater membrane fluidity is said to increase either the bilayers' permeability to protons (implying faster diffusion through lipids), or the activity of proton-transporter proteins, or both. However unsaturated bonds in acyl chains of (18:1,18:1)-PC membranes drastically inhibit ion permeation when compared with (18:0,18:0)-PC (Deamer and Bramhall, 1986); in mitochondria, EFA-deficiency replaces PL 18:2 and 20:4 acyls with more saturated acyls, yet it increases proton leakage (see Hoch, 1988). Protons do not leak across PL membranes by a partition-diffusion mechanism: (a) The rate fails to increase as expected when pH is decreased from 12 to 2; protons are driven mostly by $\Delta\psi$; Fick's law is not followed, and the Fick diffusion constant incorporates values of the viscosity of the

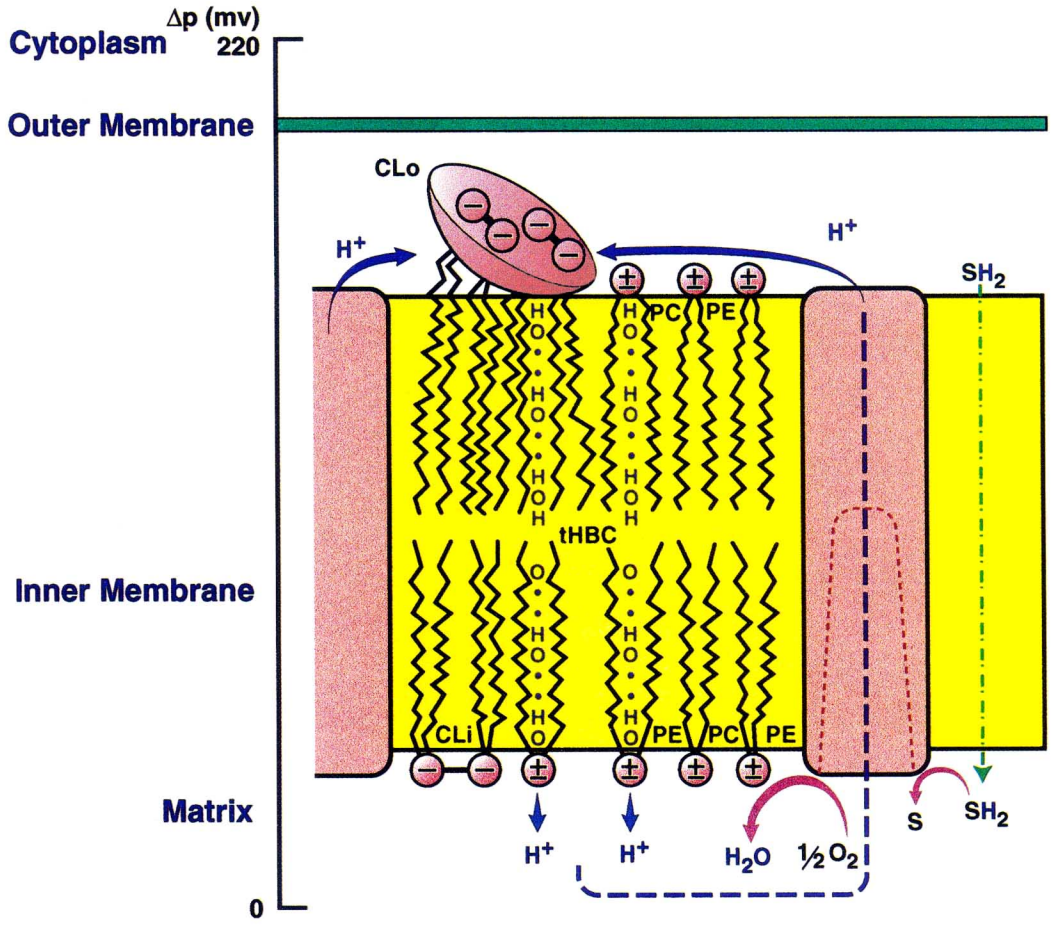

Fig. 1. A snapshot model for a lipid-mediated proton-selective leak under State 4 conditions in mitochondria from thyroid-responsive tissues of rats, that usually have high CL contents (Hoch, 1992; see text). The general circuitry of protons and electrons follows that in Nicholls and Ferguson (1992), Fig. 4–12. Inner membrane outer-surface cardiolipin (CLo) headgroups hydrogen bond to form a water-containing microdomain that acts as a strong proton antenna. In each CLo molecule, one of the phosphate groups, with a $pK = 7.5$, attracts protons from water and from the zwitterionic headgroups of PCs and PEs (a proton-channeling), and donates protons directly to CLo transient chains of H-bonded water molecules in single file between extended acyl chains that span the membrane outer monolayer (and, not shown, via water-shuttle to protonophores and proton-transporting proteins). Ordinarily, such CLs have few extended (i.e., saturated or monounsaturated) fatty acyl chains, and thereby retain protons by releasing them from antenna to water, which can not donate protons to water chains for translocation. Methyl-end transient contacts with inner monolayer water chains complete a proton pathway (a *wire* that excludes Na^+ and K^+) to the matrix. Transport is $\Delta\psi$ driven. A few water molecules are shown; the minimal complete tHBC needs about 20 (Nagle, 1987). The path shown for protons is thermogenic and can be an adaptive response (mediated by outer-surface CL amounts and fatty acyls) to stimuli cited in Table I.

membrane and suspending solution (Jain, 1988). (b) The leak is proton-selective; protons move faster than Na^+ or K^+ by many orders of magnitude, although the latter should diffuse readily. (c) In PL liposomes (with no proteins), results of experiments that use bilayer thickness effects on proton permeability coefficients to test between a solubility-diffusion mechanism and a transient-pore mechanism are consistent with the pore model, at UFA chain lengths of 14C-20C (Paula *et al.*, 1996). The UI of ΣPLs regulates thermotropic properties of membrane proteins (enzymes and carriers). Altered Arrhenius profiles of State 3 respiration (Chen, 1975; Hulbert et al., 1976) and other membrane processes (see Hoch, 1988) of liver and heart mito-

chondria from hypothyroid rats were diagnostic of membrane FA changes. They were probably examples of homeoviscous adaptation (Sinensky, 1974), and CLs, a minor component of mtPLs, would contribute little to this bulk-phase response.

In comparisons of proton permeability under $\Delta\psi$ values adjusted to be similar, rat liver mitochondria leak protons much faster than do liposomes (see Nichols and Deamer, 1980) of soy bean asolectins, or of PLs extracted from mitochondria of beef heart (Krishnamoorthy and Hinkle, 1984) or rat liver (Brookes *et al.*, 1997a). Brookes, Hulbert and Brand conclude that mtPLs do not conduct enough protons to account for the leak; Krishnamoorthy and Hinkle conclude that mitochondrial proteins alter the dielectric and accelerate proton leakage through the lipids; Deamer and Nichols conclude that integral proteins in the bilayer provide not only channels but also nonspecific conductance pathways.

A fourth explanation is possible. Liposomes have inadequate antennae. Liposomes contain CLs; even in asolectins, CLs are 10-25% of PLs (see Hoch, 1992). Liposomes show extreme PL-asymmetry, contradicting Brookes *et al.* (1997a); only 7% of CLs are in the outer leaflet of liposomes made from mixtures of CL+PC (Müller *et al.*, 1984). As noted above, adriamycin titrations of rat liver or heart mitochondria find CLo = 57%, twice the value found by other procedures, probably because it also reaches CLi; CLo was estimated from a first saturation level (Cheneval *et al.*, 1985). Titration of liposomes made from lipid extracts of mitochondria showed no intermediate saturation, and they bound adriamycin as strongly and completely as did whole mitochondria - showing there was very little CLo, as in the CL+PC liposomes. Deficiency in CLo would provide an insufficient proton antenna for the full proton leak. For a similar reason, the proton leak is slow in liver mitochondria from hypothyroid rats (Table I footnote f). An inadequate antenna in liposomes composed of the mtPLs from a variety of species would account for their limited proton permeability, that was reported by Brookes *et al.* (1997b) and used to rule out a lipid route for the leak.

Protonophore Mechanisms in Proton Leakage

An adsorbed protonophoric anion, e.g., P13, needs to receive protons from the proton antenna, via a membrane-water shuttle (Kasianowicz, 1987). Such a mechanism finally explains some old observations of permissive hormone effects on mitochondrial respiration that occur, surprisingly, in minutes (Hoch, 1965, 1968b). DNP-induced thermogenesis in fasted rats (an action on mitochondria) depends on thyroid state and responds to injected hormone. In hypothyroid rats, injected DNP raises the BMR by about 40% of its effect on normal rats; labeled DNP appears in the liver mitochondria in normal amounts and the $\Delta\psi$ has been reported to be normal. DNP added to the isolated liver mitochondria also fails to accelerate State 4 respiration (now interpretable as 'the leak') normally. The dose-sensitivity of respiration (leak), measured by slopes of linear plots of DNP-titrations that fitted the Hill equation, is subnormal in hypothyroids, above normal in hyperthyroids. The initial slopes of simple plots of respiration (leak) vs. [DNP] give analogous results. One injection of LT4 restores hypothyroid rats' mitochondrial DNP-sensitivity (leak) in 2 min, BMR response in 3–6 hrs. In hyperthyroid rats, DNP or salicylate injection raises BMR more than in euthyroids and becomes lethal; LT4 and either agent synergistically increase State 4 respiration and uncouple hyperthyroid mitochondria. FCCP-titrations of State 4 respiration provided extrapolated values of −pmol FCCP/mg protein at $J_{H+} = 0$ that were used as an indirect measure of the leak Ci (Groen *et al.*, 1982; Vehoeven *et al*, 1985; Horrum *et al.*, 1990), and also gave similar information, despite being dismissed as theoretically invalid (Brand *et al.*, 1988). Effects of thyroid state on CLo now seem to account for these findings: the deficient CLo (proton antenna) in hypothyroid mitochondria would decrease proton donation to the uncoupler. Thyroid hormone treatment raises CLo, in hypothyroids and in normals (Table I footnote f).

Protein Mechanisms in Proton Leakage

Mitochondrial membrane proteins have been cited as carriers for protons in the leak, even though Nichols and Deamer (1980) concluded that it is not necessary to postulate protein-mediated leaks because mtPLs leak enough protons. Nor does proton leakage via non-lipid routes account for the demonstrated rapid leak in EFA-deficient liver mitochondria of EFA-deficient rats (Rafael *et al.*, 1984 - although they called the oxidative phosphorylation normal because State 3/State 4 and ADP/O ratios were at control levels (see Hoch, 1992)), and acceleration of State 4 respiration in other lipid-treated animals (Table I). Minimization

of protein-mediated exchange leaks by removing ions from suspending media, or adding specific inhibitors of carriers, does not consistently slow State 4 proton leakage. Nevertheless, exchange proteins that are antiporters for protons and other cations, e.g. Ca^{2+}/H^+ or $Ca^{2+}/Na^+/H^+$, are assigned a role in State 4 proton circuits (see Hoch, 1992 for refs.). 'Pore proteins', in the sense of transmembrane porters of protons, not those in the permeability transition, have been suggested to be acceptors of protons from internal buffers (Haines, 1983).

MITOCHONDRIAL PORE PROTEINS AND THE "PERMEABILITY TRANSITION" LEAKS

The permeability transition of isolated mitochondria (reviewed by Zoratti and Szabò, 1995) is an increase much more drastic than the physiological mitochondrial State 4 proton-selective leak, and does not exclude K^+ and larger ions and molecules. Because the permeability transition leakage has an all-or-none character, is completely reversed by sequestration of Ca^{2+}, and is sensitive to low concentrations of inhibitors, and because of its electrophysiological properties, it is thought to be mediated by protein pores, not defects in the bilayer. However mtPLo are also involved. Addition of a purified, exogenous PLA2 doubles proton permeability in PL vesicles from beef heart mitochondria (Eriksson and Saris, 1989), and increases respiration under State 4 conditions in intact mitochondria from rat livers (Khole and Khole, 1985). PLA2 acts in vitro on PLo to induce a proton leak. PLA2 hydrolyzes the *sn*-2 fatty acyl of membrane PLo; *sn*-2 acyls are usually unsaturated and are almost all 18:2 acyls in most CLs (Schlame *et al.*, 1993). Permeability transition occurs when mitochondrial suspensions stand overlong, swell, uncouple, and release matrix contents (EM confirms that membrane is destructured) unless free Ca^{2+} are excluded, which implicates PLA2 actions. Permeability is rapidly induced by Ca^{2+} plus one of several inducers, among them the specific CL-binder, adriamycin. A role for CLs is also indicated by effects of N. naja PLA2 on beef heart mitochondrial cristae (Awasthi *et al.*, 1969). Membrane structure (on EM) persists and NADH dehydrogenase is retained as long as CLs, but not other mtPLs, remain; 31% of this inner membrane enzyme complex is released when PLA2 lyses 23% of the CLs (CLo?); cytochrome *c* is partially released only when a lyso-PLA2 is added.

Some local anesthetics perturb mitochondrial inner membrane/water interfaces by disrupting the outer-surface H-bonded PLo headgroup-water network, which further indicates that CLo are involved in the proton-selective leak. These agents loose-couple oxidative phosphorylation: the cation RNH_3^+ of bupivacaine accelerates rat liver mitochondrial State 4 respiration 7-fold but does not change Δp (Terada *et al.*, 1990); it binds outer-surface acidic phospholipids and may compete with Ca^{2+}-binding that activates endogenous PLA2 (see Zoratti and Szabò, 1995). In CL-PL liposomes, it acts on the CLs (Shimooka *et al.*, 1992).

Adriamycin, which specifically binds 2 CL headgroups (Nicolay *et al.*, 1984), induces the permeability transition (Zoratti and Szabò, 1995): it changes State 4 minimally but inhibits State 3 strongly (Gosalvez *et al.*, 1974), perhaps by disturbing CL-binding to electron-transport proteins. Titrations of liver or heart mitochondrial inner membranes suggest that adriamycin first binds CLo and then CLi, and that CLo/ΣCL is about 57% (Cheneval *et al.*, 1985), twice the ratio shown by other agents. However, adriamycin strongly deforms the mitochondrial structure and disrupts the inner membrane, so it seems possible that it binds CLo plus a portion of newly exposed CLi.

Uncoupling proteins (UCP)

Uncoupling proteins are likely mediators of adaptive proton-selective leakage. UCP1 (thermogenin) was found in BAT mitochondria. It transports protons or, equivalently, hydroxyl ions, and is analogous and partly homologous to other tripartite anion exchangers, e.g., the ADP/ATP translocase; it carries Cl^- as well (Nicholls and Locke, 1984). To accept protons from the external membrane-water shuttle, UCP1 requires FFA at its vestibule; the recoupling of BAT mitochondria requires blockage of proton reflux by both removal of the FFA and presence of external purine nucleotides. GDP is especially effective, but the concentrations of ADP + ATP used in converting State 4 $\rightarrow$ State 3 respiration, especially in ADP-cycling, can be also. Proton exchange across active UCP1 is nearly independent of pH, and is too slow to proceed via a proton-channel mechanism (Klingenberg, 1990).Thus, UCP1 proton transport may be contrasted with that of gramicidin, paired 15-unit polypeptides that encase a H-bonded water wire like the proton conductor among acyls of membrane PLs.

UCP1 proton transport depends on the number of UCP1 molecules, which is regulated by various agents. Norepinephrine rapidly accelerates UCP1 gene transcription, and more slowly, > 24 h, proliferation of BAT cells; it also acts more rapidly on adrenoceptors to increase local [cAMP], enzymatic lipolysis, and [FFA]. Other inducing agents include dietary factors (overfeeding, high FA contents), and cold-adaptation and arousal from hibernation that probably involve norepinephrine. Surprisingly, EFA-deficient diets decrease rat BAT mitochondrial State 4 (+GDP) proton leak (Table I) and are said to increase UCP1 in BAT (Yazbeck *et al.*, 1989; Goubern *et al.*, 1989; Senault *et al.*, 1990), perhaps by increasing the [GDP] necessary to block and measure UCP1. However, Nedergaard *et al.*, (1983) found a rise in BAT UCP1 content, not in EFA-deficient rats, but in rats fed excess EFA, which they thought to be a non-specific fat effect. An adequate thyroid state is completely permissive for cold-adaptation through the major thermogenic capacity of BAT, by several mechanisms (see Hoch, 1992). Hypothyroidism diminishes norepinephrine actions, mitochondrial protein synthesis, and the mitochondrial proton antenna that serves BAT UCP1. Cold kills hypothyroid rats.

UCP1 in BAT mitochondria has been identified by the use of anti-UCP1 antibodies in enzyme-linked immunoabsorbent assay, immunodiffusion, radioimmunoassay, or immunoprecipitation (reviewed by Ricquier *et al.*, 1992). UCP1 presence has been inferred from the detection of its mRNA. UCP1 has been quantitated specifically by its nucleotide affinity: e.g., by gel electrophoresis of inner membrane proteins treated with a nucleotide photoaffinity label made radioactive, which selects a 32 kD band. The amount of BAT UCP1 changes adaptively to external conditions, rising to 10–14% of inner-membrane proteins when animals are adapted to a cold environment, and disappearing from warm-adapted animals (Nicholls, 1983). However, norepinephrine added to BAT from room-temperature-adapted rats or hamsters accelerates respiration 10-fold within 2 min, which indicates that the UCP1 is constitutive under normal (noninduced) conditions. It should be noted that BAT, in addition to having mitochondria with a high-capacity proton-exchanger protein, is even more fully specialized for thermogenesis. BAT is extensively vascularized to supply FFA as substrates and exchange heat of their oxidation, and innervated by adrenergic fibers to respond quickly with lipolysis → FFA. Its mitochondria are repleted with carnitine and enzymes for FA-activation and β-oxidation (Nicholls and Locke, 1984), which evolves twice the free energy and heat change as, say, glucose oxidation; and they have little ATP synthase to compete for reflux of extruded protons.

Although Nicholls and Locke found no 32 kD protein peaks in mitochondria of tissues other than BAT, and concluded that UCP was unique there, newer evidence shows that BAT with its UCP1 can be induced in white adipose tissue and perhaps in slow-twitch skeletal muscle (Yoshida *et al.*, 1998) but not in liver (Ricquier *et al.*, 1992) or heart.

Ectopic, i.e., non-BAT, UCPs are also newly discovered. They leak protons and diminish the $\Delta\psi$ when their mRNAs are translated in yeast mitochondria (Gimeno *et al.*, 1997; Ricquier and Bouillaud, 1997), which is surprising because proton leakage through UCP1 in BAT mitochondria depends on the presence of FFA and stops when external purine nucleotides are present (Nicholls and Locke, 1984). It is not clear if ectopic UCPs function only adaptively (e.g., in chilled subjects), or constitutively, in normals where they would contribute to the BMR under unstressed, uninducing, conditions. Ectopic UCPs are tissue-specific. No BAT mitochondrial UCP1 was detected in 'normal' rat liver or beefheart mitochondria (Ricquier and Bouillaud, 1997). Non-BAT mitochondria had no detectable 32 kD protein peak with UCP1-reactivity, and exhibited no UCP characteristics of FFA-induced uncoupling that was recoupled by external nucleotides (Nicholls and Locke, 1984). No UCP1 or its mRNA was found in rat liver (Ricquier *et al.*, 1992); its homolog UCP2 was absent from hepatocytes but present in Kupffer cells (Larrouy *et al.*, 1997; Negre-Salvayre *et al.*, 1997).

UCP2 mRNA, from which the presence of UCP2 protein is inferred, is expressed strongly in rat spleen and skeletal muscle, and less in brain, WAT, macrophages, thymus, and bone marrow (Fleury *et al.*, 1997; Negre-Salvayre *et al.*, 1997). Interestingly, these tissues have few or no thyroid hormone receptors (see Hoch, 1992 for references). UCP2 mRNA is induced by: cold-exposure, in BAT and heart muscle; fasting, in skeletal muscle (which is inconsistent with its thermogenic role); and by thyroid hormones, in rat heart, although less in skeletal muscle, and not at all in liver or kidney (Lanni *et al.*, 1997). Endurance training suppresses it in skeletal muscle (Boss *et al.*, 1997a, 1998a,b). However, evidence is presented that UCP2 may not transport protons, or may use a different mechanism (Bienengraeber *et al.*, 1998).

UCP3 mRNA (70% like UCP1 and UCP2) is found in skeletal muscle in humans and in BAT and skeletal muscle in rodents (Vidal-Puig *et al.*, 1997); it is induced in skeletal muscle after fasting or cold exposure, and also suppressed by endurance training (Boss *et al.*, 1997b). UCP3 mRNA levels in muscle are also thyroid-dependent, being decreased 3-fold in hypothyroid rats and increased 6-fold in hyperthyroids (Gong *et al.*, 1997). UCP2 and UCP3 are thus proposed to mediate the hormone's late up- or down-regulation of thermogenesis.

An UCP induced by 15-day treatments of mice with a β3-adrenergic receptor agonist is found by antibodies and GDP-binding in white adipose tissue and slow-twitch skeletal muscle (which has lots of mitochondria) (Pico et al, 1998; Yoshida *et al.*, 1998). UCPH mRNA, another UCP1 homolog, is detected in human white adipose tissue and skeletal muscle (Gimeno *et al.*, 1997).

The question arises: with the small amounts of BAT in adults, are there enough ectopic UCPs induced or constitutive in tissues with enough of the thermogenic specialization of BAT to contribute significantly to the BMR? Recent science news reviews (Rawls, 1997; Gura, 1998) take proton leakage as a basis for the BMR, and manipulation of leakage via ectopic UCPs as a possible management of obesity. However, the dependence of the BMR in unfed, unchilled, unstimulated subjects upon a mitochondrial proton leak (respiration) that requires FFA and is inhibited by external nucleotides (even by the ADP concentrations that initiate State 3 when the phosphorylation ratio (ATP/(ADP x Pi) falls), does not seem a physiologically likely mechanism.

A minor PL among membrane lipids, the CLs, seems to have evolved to require an essential fatty acid, and to reside exclusively in the mitochondrial inner membrane, where it forms domains through lipid-lipid interactions. It is greatly asymmetrical without involving proteins, has the only PLo headgroup that can collect and donate pumped protons to transmembrane porters at physiological pH levels, and mediates an adaptively responsive proton-selective leak that regulates non-phosphorylating oxidations.

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