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Cardiolipins and biomembrane function

Frederic L. Hoch

Departments of Internal Medicine and Biological Chemistry, The University of Michigan Medical School, Ann Arbor, MI (USA)

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Contents

Abbreviations: BAT, brown adipose tissue; BMR, basal metabolic rate (oxygen); C_i, flux control coefficient (as defined and measured in Ref. 228); CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; C_{MH^+}, effective proton conductance; Δp, protonmotive force (in mV, defined as in Refs. 396 and 438 as $\Delta p = \Delta\tilde{\mu}_{H^+}/F = \Delta\psi - 59\Delta pH$), where $\Delta\psi$ and ΔpH are the transmembrane differences in electrical potential and pH; EFA, essential fatty acid(s); FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; P_{H^+}, apparent proton permeability; PGP_H, 2,3-diphytanyl-*sn*-glycerol-1-phospho-*sn*-glycerol-1′-phosphate; T4, thyroxine; T3, triiodothyronine.

I Introduction

"Almost all the cardiolipin of the (eukaryote) cell is in the inner mitochondrial membranes, making up about one-fifth of the total lipid. It is its intensely

Correspondence: F.L. Hoch, 3455 Woodland Road, Ann Arbor, MI 48104-4257, USA

hydrophobic, lipid character that gives the membrane a coherent structure, determines its permeability, and accounts for the properties of its enzymes" [614].

Cardiolipins are unique to biomembranes which have coupled phosphorylation and electron-transport: bacterial plasma membranes, chromatophores, chloroplasts, mitochondria [500]. Chemiosmotic mechanisms of oxidative phosphorylation [433–438,462] most readily explain the roles of membrane phospholipids and phospholipid-embedded proteins that carry electrons, protons and metabolites. Correlations between lipid composition and oxidative phosphorylation have been developed in a series of reviews: on lipids of subcellular particles [190], regulation of membrane enzymes [569], lipids of mitochondria [136] and chemistry and metabolism of cardiolipins [305,320].

Recent reviews on lipids and thyroid hormones [284,285] summarize evidence that thyroid hormones control genetic expression of the enzymes that synthesize and desaturate fatty acyls-CoA. The products of up- or down-regulation change mitochondrial phospholipid compositions and alter oxidative phosphorylation, with the result that thyroid levels relate directly with respiration in State 4 and to a lesser degree State 3. These changes reflect abnormal rate-temperature profiles typical of altered membrane lipid compositions. A special role was ascribed to the relative amounts of cardiolipins and to their very high $18:2(n-6)$ fatty acyl contents in thyroid-sensitive tissues. Such a causal sequence is consistent with observations that dietary EFA-deficiency, without involving genetic mechanisms, specifically depletes $(n-6)$-unsaturated fatty acyls in mitochondrial phospholipids, especially cardiolipins, and according to many studies increases proton leakage under State 4 conditions.

The present review examines more widely the roles of cardiolipins in membranes and reconsiders some previously reviewed data in the light of mechanisms of proton permeability in phospholipid membranes (see section II-A), and cardiolipin-protein interactions (section II-B). A number of conditions that alter membrane phospholipids, some selective for cardiolipins, increase or slow bacterial (section III) and mitochondrial (section IV) respiration. Because changes in phospholipids affect temperature-dependence of respiration and transfer rates of many substrate carriers, temperature and substrate are here specified together with respiratory and membrane potential data; sometimes temperature and substrate are crucial for demonstrating effects.

II. Phospholipids and membrane energy transduction

In the original scheme of delocalized chemiosmosis, the closed cyclic property of the proton circuits requires that the efflux $(\partial(\rightarrow H^+)/\partial t$, to the outer phase in mitochondria) through the respiratory chain (o/r) equal the total return flux $(\partial(\leftarrow H^+)/\partial t$ to the inner phase) through the ATP-synthase (reversible ATPase) (h/d), by diffusion (D) and by exchange (X) across the membrane [433,437]; the balance, modified from Ref. 437, Eqn. 25, is:

$$\left(\frac{\partial(\rightarrow H^+)}{\partial t}\right)_{o/r} = \left(\frac{\partial(\leftarrow H^+)}{\partial t}\right)_{h/d} + \left(\frac{\partial(\leftarrow H^+)}{\partial t}\right)_{D} + \left(\frac{\partial(\leftarrow H^+)}{\partial t}\right)_{X} \qquad (1)$$

II-A. State 4 respiration: proton leak

Under State 4 conditions the potentially great proton passage through the ATP-synthase term (h/d) is minimal, as shown by the failure of oligomycin to inhibit (but see IV-C.2.d). Proton reflux then depends on the lesser conductance by diffusion and exchange – the 'proton leak'. The 'diffusion' term implied a mechanism of passive transport of protons across a relatively ion-impermeable non-aqueous phase (the phospholipid bilayer) in the coupling membrane [437] – a phospholipid leak. (The uncoupling protein of the inner mitochondrial membrane in brown adipose tissue (BAT) is a special mechanism for an externally regulated exchange leak under State 4 or State 3 conditions (section IV-C.2.c).) Proton reflux completely governs State 4 respiration [53,228,433,434,438,744]. For example, when KCN is added to rat liver mitochondria to block electron transport and eliminate possible proton pump slippage under State 4 conditions (succinate, P_i and oligomycin, 25°C), $\Delta\psi$ dissipates with a $t_{1/2}$ of 1–3 s; $-\Delta\psi \cdot t^{-1}$ is therefore attributed to only passive leak of ions [744]. Because $-\Delta\psi \cdot t^{-1}$ and State 4 respiration $(-\Delta O_2 \cdot t^{-1})$ have identical relationships to absolute values of $\Delta\psi$ down-titrated with malonate, these workers conclude that State 4 respiration depends on passive leak of protons.

We need to understand the roles of individual phospholipids in proton leak mechanisms under State 4 conditions. First, how much of the proton leak is mediated by membrane phospholipids and how much by exchange-carrier-proteins? Minimization of exchange-leaks by removing ions from the media [466] or adding specific inhibitors of carriers [62] does not consistently slow State 4 proton leakage [228,673]. Nevertheless, exchanger cycling by Ca^{2+}/H^+ [462,464] or $Ca^{2+}/Na^+/H^+$ [68] is assigned a role in mitochondrial State 4 proton circuits. However, phospholipids appear to dominate in proton conductance. This conclusion is supported by similarities in permeability of intact mitochondria and protein-free membranes. Proton permeability is usually in the range 10^{-3}–10^{-4} cm s^{-1} in

mitochondria, in membranes made of their extracted phospholipids, and in some membranes formed from stable synthetic phosphatidylcholines or phosphatidylethanolamines that usually contain two saturated fatty acyls or one saturated and one monounsaturated fatty acyl [146,167,238,371,468]. Nichols and Deamer [468] conclude that the proton-leak via the inner mitochondrial membrane phospholipids can account for the passive proton flux in State 4 and that it is not necessary to postulate an X-leak.

Three mechanisms for proton conduction in phospholipid bilayers are, in increasing applicability to a leak under State 4 conditions: (i) along the surface of anionic phospholipid headgroups, (ii) through protonophoric non-bilayer phospholipid domains and (iii) across the bilayer.

(i) Haines [251] proposes that anionic headgroups of lipids in biomembranes conduct protons 10^5-fold faster than water does, whereby protons pumped from the other side of the membrane are confined to a surface and conducted laterally. The anionic polar headgroups of PGP_{II}, the diphytanyl ether analogues of phosphatidylglycerol phosphate (from archaebacterial membranes, see section III), readily conduct protons laterally in highly condensed monolayers [522,647]. Cardiolipins are virtually the only anionic phospholipid in the inner membrane of mitochondria [307]. Cardiolipins are the most acidic of eubacterial and mitochondrial membrane phospholipids. Cardiolipin phosphoryl-groups are the most extended and rigidly fixed; the free hydroxyl group of the charged -phosphoryl-CH_2-CHOH-CH_2-phosphoryl- backbone, as well as the ester carbonyl groups, contribute to the stability of an intra- and intermolecular, bidimensional, hydrogen-bonded network that includes water molecules of the hydration layer [313,631]. Cardiolipin fatty acyl saturation modulates surface conduction of protons. *Escherichia coli* cells that have saturated cardiolipins eject respiratory-chain-generated protons from the cytosol across the membrane to the exterior; cells with cardiolipins containing mostly monounsaturated fatty acyls retain some of these pumped protons by conducting them laterally back to the cytosol [221] (see section III). Such a network of cardiolipin polar groups is thought to conduct protons laterally in membranes and vesicles of extracted phospholipids of *E. coli* [516,517,648], chloroplasts [21] and mitochondria [313].

Hydrogenated beef heart cardiolipins, which contain 86% saturated fatty acyls and 9.4% 18:1 acyls, form bilayers in aqueous dispersions and thereby conduct protons on their surfaces [313]. In contrast, natural highly unsaturated beef heart cardiolipins form nonbilayer H_{II} phases (see below), obviously because of different acyl packing. To conduct surface protons, they have to be embedded in an ordered lipid bilayer, provided in mitochondria by phosphatidylcholines and phosphatidylethanolamines. This interplay may explain a special connection of cardiolipin unsaturation with proton conduction, together with a dependence on the other mitochondrial phospholipids.

However, a surface route does not readily explain proton reflux in mitochondrial State 4, since net vectorial proton flux requires a route perpendicular to the membrane, and most evidence eliminates reentry through an exchanger protein. Surface routing might be important under State 3 conditions where active exchangers cotransport protons (see next section).

(ii) A role as protonophores has been suggested for nonbilayer structures inside the inner membrane of mitochondria that are identified through combinations of measurements by small-angle X-ray diffraction, electron microscopy, and ^{31}P-NMR. They are depicted as an hexagonal H_{II} lipid phase with interior polar groups surrounding an aqueous channel, or as other nonlamellar lipid structures [125,139,141,582]. These structures are thought to disturb the lamellar bilayers that preserve the membrane permeability barrier [514] and thereby would promote transmembrane proton leakage, or to conduct protons within the membrane, which would shunt protons away from the leak.

A bilayer → hexagonal H_{II} transition occurs rapidly and readily (low E_a) in aqueous suspensions of phosphatidylethanolamines with fatty acyls in liquid-crystalline phase. The H_{II} form is favored by increased fatty acyl unsaturation and carbon chain length [570], protonation of the phosphatidylethanolamines and high ionic strengths. The headgroup and acyl chain compositions of cardiolipins modulate phase changes in model membrane systems. Neutralization of cardiolipins with cations (Ca^{2+} and Mg^{2+}) [529] including protons, or high [NaCl], induces the H_{II} phase, more readily as the fatty acyls are changed from *tetra*-14:0-cardiolipin to *tetra*-18:1-cardiolipin to the natural beef heart mitochondrial cardiolipins with high 18:2 contents [570]. Thus, if an H_{II} phase does conduct protons intramembranally, shifts from high polyunsaturated to high monounsaturated or saturated fatty acyl contents might promote transmembranal proton flow. Membrane proteins also affect H_{II} phase formation, but differently in model systems and intact mitochondria. Cytochrome *c*, which is cationic but not naturally embedded in the mitochondrial inner membrane, specifically induces the H_{II} phase when liposomes contain cardiolipins plus a nonspecific variety of other phospholipids [138]. A cardiolipin-cytochrome *c* complex may facilitate direct transfer of electrons to cytochrome *c* oxidase [141]. In contrast, the presence of some intrinsic proteins, e.g., cytochrome *c* oxidase favors the lamellar bilayer phase. Ca^{2+} [543] or NaCl [514] override this preference and induce the H_{II} phase in an oxidase-cardiolipin system.

On the contrary, little if any H_{II} phase exists in intact mitochondria, and the proteins apparently over-

ride ion effects [140]. Almost all the endogenous phospholipids in rat liver mitochondria in State 4 (> 98%) and State 3 (> 95%), even with Ca^{2+} added, maintain a bilayer structure over the range 0–37°C. It remains conceivable that undetected domains of H_{II} phase (< 2–5% of phospholipids) are a limited route for proton flux. Seddon et al. [582] suggest that proton- or cation-neutralized cardiolipins together with associated proteins might conduct protons to couple electron-transport to the ATP-synthase, i.e., in State 3. Such structures, and the surface networks of H-bonded cardiolipins (see (ii) above), might be a physical basis for postulated intramembrane coupling mechanisms [555–557,704] (see section II-B).

(iii) Proton passage across phospholipid portions of bilayer membranes thus seems most likely under State 4 conditions. Protons do not move by classic diffusion across bilayer membranes made from pure phosphatidylcholines or phosphatidylethanolamines. Increased $[H^+]$ would accelerate flux by a diffusion mechanism but proton conductance increases only about 10-fold over the pH range 11 to 2 [238] and is relatively constant in liposomes over 2–3 pH units around neutrality [69,145,239–241,452,506,507]. Further, simple diffusion does not account for protons being about 10^7-fold more permeable than K^+ and Na^+ [145]. Therefore, proton-selective transport is proposed to proceed via trace amounts of natural protonophores or transient chains of hydrogen-bonded water molecules.

Suggested protonophores include (undetectable) traces of (i) free fatty acids, (ii) O_2^- superoxide anions and (iii) carbon dioxide and its hydrates.

(i) Cascal oxidation of manufactured lipid bilayers or doping with free fatty acids does increase apparent P_{H^+} [60], and serum albumin (which strongly binds free fatty acids) can lower conductance [239,240]. However, albumin does not diminish uncoupling in mitochondria biologically depleted of phospholipid unsaturated fatty acyls, indicating that the more saturated fatty acyl chains, not free fatty acids, conduct the accelerated proton leakage [263] (see section IV-C.4). Further, free fatty acids are said to be ineffective protonophores [241,557] because their specific activities are too low for trace amounts to be effective, their flip-flop rates in the membrane are too low, they uncouple maximally at high pH where they are completely anionic, and their pK_a values are much lower than required for transmembrane H^+-shuttling. Contrariwise, the pK_a of the carboxylic group of 18:1 in the polar surfaces of egg phosphatidylcholine multilayers is 6.2, which is 1.3 units higher than in aqueous phase [571].

(ii) Carbonera and Azzone [77] propose that one-electron reactions in the electron transport chain reduce $O_2 \rightarrow O_2^-$ superoxide anion which attacks proteins and unsaturated lipids to create water-channels that increase P_{H^+}. Protective mechanisms include conversion of superoxide radical $\rightarrow H_2O_2$ through superoxide dismutase action in the matrix; H_2O_2 and glutathione oxidation through glutathione peroxidase action completes the cycle. Formation of autoxidized protonophoric side-products by mitochondrial oxidations would depend in part on fatty acyl unsaturation. Cardiolipins are the most highly unsaturated of the mitochondrial inner membrane phospholipids in liver and heart, in the sense that they contain the least proportions of saturated fatty acyls (other phospholipids often have greater unsaturation indices), and so have been thought to be the most likely source of protonophoric products of autoxidations. Protective mechanisms against free radical actions may have special significance for cardiolipins [173].

(iii) Norris and Powell [475] present evidence that, in large unilamellar vesicles of di-18:1-phosphatidylcholine, carbon dioxide, carbonic acid and bicarbonate could act as proton carriers in addition to passive proton flux. P_{H^+} is about 10^{-4} cm s^{-1} at 25°C even when these carbonates are removed to below the detection limit of 20 μM. They note that oxidative decarboxylations in the mitochondrial matrix produce carbon dioxide in quantity, question why millimolar concentrations of carbonates at the inner surface of the inner membrane do not dissipate Δp, and propose that one mechanism might involve acidic phospholipids (in mitochondria these would be cardiolipins) that limit the concentrations of carbonates at the surface and retard movements of HCO_3^-.

Proton permeation and water permeation are interrelated in phospholipid membranes; evidence includes the similarities in their permeability coefficients [146], high E_a values at temperatures above the phase transition temperature [167] and temperature-dependence [51]. Ice conducts protons faster, through increased order. In phospholipid membranes, external water is thought to extend perpendicularly into the nonpolar phase as a single chain of water molecules transiently stabilized both by hydrogen bonding and the ordering effect of the surrounding parallel (i.e., saturated) hydrocarbon chains [146,463]. Movement of protons by successive turning and hopping defects in the water strand can explain why H^+ conductance is constant with ΔpH but supralinear with $\Delta\psi$ [452] and is so much faster than that of Na^+ or K^+ (protons can move along hydrogen bonds). Against this model are: the implausibility of an ordering of water molecules into extended hydrogen-bonded chains in a nonpolar environment [145]; the absence of detectable water; and the failure of D_2O to slow conductance across pure phospholipid membranes although conductance of D_2O is 100-fold less than H_2O in ice. The water-chain model implies that fatty acyl saturation and increased order promote proton permeability. In Träuble's mech-

anism [52,660,661], water (and protons) permeates phospholipid bilayers via β-coupled configurations (2_{gl}-kinks) that move along extended saturated acyl chains. The presence of carbon-carbon double bonds which do not allow free rotation about their axes would block the progress of 'kink wave'. Thereby, bilayers of di-18:1-phosphatidylcholine conduct protons more slowly than bilayers of di-18:0-phosphatidylcholine [146].

Cardiolipins play a special role among mitochondrial phospholipids in conducting protons. Mitochondrial inner membrane integrity depends specifically on cardiolipins. Enzymatic digestion of cardiolipins but not phosphatidylethanolamines or phosphatidylcholines correlates with the disruption of structure [23], and hypo-osmatic swelling that produces an uncoupling proton-leak decreases cardiolipin/protein and cardiolipin/phospholipid by 30%, but phosphatidylethanolamine/phospholipid by only 8% [349].

Proton permeability in natural membranes is inverse to phospholipid fatty acyl unsaturation, sometimes in an individual phospholipid. Some cardiolipins are not only the 'most unsaturated' of membrane phospholipids but also the most responsive to regulatory influences that alter their amounts, decrease their unsaturation, and change proton leak rates (see sections III, IV).

Interplay between properties of the apolar hydrocarbon bilayer interior and the hydration and charge of the phospholipid surfaces determines function of the lipid portion of membranes. Increased unsaturation of the acyl chains augments surface binding of phospholipids to cations [82] and binding of cardiolipin acyl chains to proteins [576,577,620]. Cardiolipins, among the mitochondrial inner membrane phospholipids, are not only the least saturated and most acidic, the most rigidly fixed in a H-bonded network that orients surface water molecules and cationic residues of proteins [633], and when neutralized are the most favorable to the formation of nonbilayer, proton-conducting structures [570,582], but they are also the most localized to one (the inner) face. This concentration counters in part the dilution of these special properties by the paucity of cardiolipins. Cardiolipins thereby can conduct protons and chelate cations. They may also act as effectors or specific receptors, and orient proteins (see section II-B).

Bilayer membrane stability depends on the molecular geometry and 'shapes' of the phospholipids [700]. Cardiolipin headgroups are important in determining the shape of the molecule, and their interactions with ions alter lipid phase behavior [515]. The 'monolayer intrinsic radius of curvature' distinguishes bilayer from nonbilayer phospholipids [233]. The presence of unsaturated fatty acyl groups in membrane phospholipids shortens mean end-to-end hydrocarbon chain length to a minimum, in order of effectiveness 18:3($n-3$) > 18:2($n-6$) > 18:1($n-9$) acyls [60]. The extended cardiolipin polar head group is associated with very high proportions of 18:2($n-6$) acyls, less 18:1($n-9$) acyls and few saturated fatty acyls in cardiolipins from liver, heart and kidney. Unsaturated fatty acyls in these cardiolipins would widen the hydrophobic ends to form a truncated cone, and their monolayer positioning should emphasize the effects of this structure. Conversely, cardiolipins that contain less polyunsaturated fatty acyls and more monounsaturated fatty acyls and saturated fatty acyls would have closer packed hydrocarbon chains and a cylindrical or inverted cone structure. The extended hydrophilic group of highly unsaturated cardiolipins, with its four kinked long-chain hydrophobic moieties, creates regions of small curvature in the membrane [427]; these cardiolipins find their way to the inner surface of liposomes formed from mixtures of phospholipids [447]. Cardiolipins containing four 16:0 fatty acyl groups form bilayer liposomes [526], and the mostly saturated cardiolipins of bacteria are situated in both faces of the plasma membrane (see section III). However, the naturally unsaturated cardiolipins of beef heart are also on both sides of the lamellae in sonicated vesicles [377], perhaps because cardiolipins are more than 50% of the total phospholipids.

Highly unsaturated cardiolipins from mitochondria are not usable alone in studies of proton flux in protein-free vesicles because they do not form bilayers; they must be studied in bilayers formed by other phospholipids [313] (see above). Hydrogenated beef heart cardiolipins (18:0-cardiolipin) [313] and synthetic cardiolipins with four similar saturated fatty acyl groups form bilayer membranes that have thermotropic phase transitions characteristic of phospholipid bilayer assemblies [526,570]. Bilayer membranes of even the relatively saturated bacterial cardiolipins (see section III-A) are also too unstable for measurements of proton conductance, in contrast to phosphatidylglycerols and phosphatidylethanolamines [295].

Artificial membranes that contain relatively saturated phosphatidylcholines + phosphatidylethanolamines leak protons as fast as natural membranes that contain unsaturated phosphatidylcholines + phosphatidylethanolamines + cardiolipins [468]; if unsaturation does not matter, the cardiolipins are inert. But unsaturation affects proton movement. Phospholipid fatty acyl unsaturation, chain length and thermal disorder all influence membrane proton permeabilities, but not always the same way in natural and artificial membranes. P_{H^+} increases linearly when fatty acyl unsaturation increases from 1 to 8 double bonds in unilamellar vesicles of phosphatidylcholines [506]. Given a fixed degree of unsaturation, increasing thermal disorder of fatty acyls raises P_{H^+} in single lamellar vesicles of

di-14:0-phosphatidate [167] and in bilayer liposomes made from horse bean phospholipids [553]. In contrast, decreased mitochondrial unsaturation, almost completely confined to cardiolipins, accelerates proton leakage, e.g., in EFA-deficiency [263,284] (see section IV).

Perkins and Cafiso [507] suggest that fundamentally different mechanisms might operate in sonicated vesicles and planar bilayers. From the disparate effects of fatty acyl unsaturation on proton permeability and bilayer structures, mechanisms also differ between model membrane systems and mitochondrial inner membrane bilayers.

II-B. State 3 respiration: cardiolipin–protein interactions; orientation

In contrast to the regulation of State 4 respiration by Δp and the very slow reflux proton leak, the more rapid rate of State 3 respiration is governed by Δp and the balance between proton ejection and reflux [247,248,435]. The generators of Δp operate under both State 3 and State 4 conditions. They are proteins: substrate carriers (plus the P_i/H^+ symporter with dicarboxylates), dehydrogenases and electron-carrier proton-ejectors. In State 3, the rapid rate of proton ejection may elicit limitation by some electron-transport step(s), in contrast to the lesser demands made by the slower State 4 rate. In State 3, rapid proton reflux is initiated by proteins: the ADP/ATP carrier exchanges external ADP for matrix ATP, the P_i/H^+ symporter acts, and increased matrix ADP/ATP ratio and $[P_i]$ activate massive proton reflux through the ATP-synthase. Augmented proton current accelerates respiration 5- to 10-fold over the rate allowed by proton reflux across phospholipids under State 4 conditions, and is regulated by the relative capacities of proton ejectors and reflux pathways. The contributions of these processes have been formulated and measured as flux control coefficients, C_i, and $\Sigma C_i = 1.0$ [228].

To discern roles of cardiolipins we must know to what degree carrier-proteins or phospholipids mediate State 3 proton flux, and then consider cardiolipin interactions with those proteins for regulatory properties. Proton flux under State 3 conditions stops the cytosol → matrix proton leak across the membrane phospholipids. That the leak may stop because inner membrane ultrastructure changes in the State 4 → State 3 transition does not seem to have been considered. Electron micrographs of isolated and in situ rat liver mitochondria show that the discrete cristae of the inner membrane condense into irregular folds [243], which should alter packing of inner face cardiolipins that normally seek concave surfaces. Two mechanisms for cessation of the leak are put forward. One observes that Δp is high in State 4 and becomes lower in State 3, and that proton conductance across phospholipids drops exponentially with Δp in this range [459]. This mechanism leaves the proteins to conduct protons. The other proposes that protons evolved by the electron transport chain circumvent the transmembranal proton leak by gaining direct intramembranal access to the F_1-ATPase [555,556]. Little direct evidence exists that these intramembranal protons are conducted laterally by phospholipids, e.g., via surface headgroups of cardiolipins or their analogues (sections II-A and III). Minimal ^{31}P-NMR signals exist in rat liver mitochondria in States 3, 4 or 3u for nonbilayer H_{II}-phase protonophoric lipid structures that involve cardiolipins and might also serve this function, and the phospholipids are more than 95% in bilayer structure [140]. Thus, proton passage through mitochondrial carrier proteins appears to limit State 3 respiration.

Mitochondrial proteins, almost all lipophilic and/or basic [320], bind most strongly to acidic phospholipids, particularly to cardiolipins among the inner membrane phospholipids. Many but not all are carrier proteins in oxidative phosphorylation and their contribution to State 3 regulation differs according to tissue and age of the eukaryote (see Table I and Refs. 284, 285).

At least several of these mitochondrial proteins are genetically related. One gene for the ADP/ATP carrier is expressed in heart, another in intestine, and both of these together with a third in liver [119]; all are homologous with the carriers of P_i, of α-ketoglutarate malate, and of dicarboxylates, as well as with the protonophoric uncoupling protein in BAT mitochondria (see IV-C.2.c). This suggests a common evolutionary origin [16,363,564]. The primary structures of an ADP/ATP carrier, the P_i transporter, and the uncoupling protein reveal that each has three 100-residue positively charged homologous domains; each domain comprises two homologous α-helical sections separated by a more hydrophilic segment [363]. Most of the hydrophilic portion faces the matrix, where most of the cardiolipins are. All these carriers function through common mechanisms that may involve H^+-anion cotransport, except for the uncoupling protein [363].

The high affinity of cardiolipins, usually from heart mitochondria, for many basic or hydrophilic membrane proteins makes it difficult to characterize cardiolipin–protein interactions that regulate State 3 respiration. However, beef heart cardiolipins are the most effective phospholipids that modulate the secondary structure of inner membrane proteins and modify their kinetic parameters [418]. We know that diet-induced alterations of fatty acyl compositions of membrane phospholipids change the cooperativity of several effector-enzyme systems [182]. In one case, State 3 respiration slows when rats incorporate fed linelaidic acid (18:2(*n* − 6)*tr,tr*) into heart mitochondrial phosphatidylcholines and phosphatidylethanolamines but not cardiolipins;

State 4 proton leakage, like the cardiolipins, does not change [115] (see section IV-C).

Reconstitution experiments on isolated inner membrane proteins have been used to study the role of cardiolipins. These pose several problems of interpretation for State 3 regulation, as will be detailed. The criteria for reconstitution are variously the strength of cardiolipin-binding to proteins in situ or isolated and perhaps delipidated, catalytic activity and proton-ejection activity. Cardiolipins are difficult to remove completely from some inner membrane proteins, perhaps because some cardiolipins are buried in the proteins, e.g., the cardiolipins in intact rat liver mitochondria are less accessible than phosphatidylcholines and phosphatidylethanolamines to reduction by a Pd-complex [577]. Despite the known retention of phospholipids, many purified and 'delipidated' protein complexes are reconstituted without lipid analyses. To test for non-specific effects of phospholipids, asolectin (soybean phospholipids) vesicles are used, but asolectins contain cardiolipins as 10% [454] or 25% [447] of total phospholipid P.

TABLE I

Carrier-protein flux control coefficients (C_i) in state 3 respiration, their responsivity to altered thyroid states, and interactions with cardiolipins (summarized in part from Ref. 284)

Abbreviations: CL, cardiolipin; CP, carnitine palmitoyl

Carrier-protein: mitochondria	C_i	CL-interaction		Ref.
		Binding	Activity	
Monocarboxylate [†]: heart *	(0.37) [a]	specific	+	494
Dicarboxylate [†b]: liver *	0.33	specific	:	673
liver, fetal *	0.12			25
Tricarboxylate [†]: liver *		specific	+	495
CPTransferase I [†]: liver *		specific	– [c]	see 284
CPTranslocase [†]: liver *		specific	+ [d]	see 284
Dehydrogenase [†], NADH: heart *	0.37 [a]	specific	+ [d]	155
substrates [e]: liver		none		55
β-hydroxybutyrate: liver *		specific	– [c]	36
Cytochrome *c* [†]: liver *		non-spec	[f]	157
Cytochrome bc_1 [†]: liver *	0.03	specific	+	673
Cytochrome aa_3 [†]: liver	0.17	specific	+	673
heart	0	specific?	+	513, 678
yeast	0.55	non-spec	+	421, 690
ADP/ATP [‡b]: liver *	0.29	specific	+	673
(P_i 3 mM)	0.45			374, 375
(P_i 10 mM)	0.19			
liver, fetal *	0.15			25
heart	0.0	specific		155, 355, 356
yeast (P_i 0.5 mM)	0.2			421
(P_i 7.7 mM)	0			
P_i [‡b]: liver *	0	specific	+	497, 673
liver, fetal *	0.13	specific	+	25
yeast (P_i 0.5 mM)	0			30, 421
(P_i 7.7 mM)	0.5			
Proton leak [‡]: yeast (P_i 0.5 mM)	0.4			421
ATP-synthase [†]: liver (P_i 10 mM)	0	specific	+	47, 228
(P_i 3 mM)	0.41	specific	+	374, 375
(P_i 10 mM)	0.17			
liver, fetal *	0.6	specific	+	25
heart	0.46	non-spec	+	155
Ca^{2+} [‡]: heart *		specific	+	159
K^+ [‡]: liver *				599
Uncoupling protein [‡b]: BAT *	1.0			465
NAD(P)H): liver *		non-spec	–	566

* Responds to altered thyroid state; [†] generates or [‡] consumes Δp [248,435];

[a] rat cardiac pyruvate carrier limits State 3 [596] and is included in NADH dehydrogenase C_i [155];

[b] common evolutionary origin [16,564];

[c] makes activity latent;

[d] orients;

[e] soluble isocitrate, glutamate, and malate dehydrogenases, and membrane-bound succinate [c] and glycerol-3-phosphate dehydrogenases;

[f] promotes H_{II}-phase.

The following mitochondrial proteins and complexes, and their relevance to State 3 regulation, will be discussed: (a) substrate carriers; (b) NADH dehydrogenase; (c) cytochrome bc_1; (d) cytochrome *c* oxidase; (e) ATP-synthase; (f) ADP/ATP carrier; and (g) P_i carrier. In addition, involvement of peripheral cardiolipins in binding certain peptides and proteins especially exemplifies orienting effects.

(a) Substrate carriers. Cardiolipins are the only phospholipid that keeps the following mitochondrial substrate transporters active during purification and reactivates them after delipidation: those for mono-, di- and tri-carboxylates, α-ketoglutarate, aspartate/glutamate, and the palmitoylcarnitine transferase plus (acyl)carnitine translocase system (for references, see Ref. 284 and Table I). The dicarboxylate carrier is a major regulator of State 3 respiration ($C_i = 0.33$) in mitochondria from livers of adult rats and less so in fetal rats. The pyruvate carrier plus the NADH dehydrogenase contribute a $C_i = 0.37$ toward regulation in heart but not in liver mitochondria [596]. Succinate dehydrogenase apparently contributes a $C_i < 0.14$ in rat liver mitochondria [228].

(b) NADH dehydrogenase (and/or the pyruvate carrier) is one of the two dominant regulators of State 3 ($C_i = 0.37$) in rat heart mitochondria [155] but regulates in no other mitochondria studied. Phospholipase A hydrolysis of cardiolipin, but not phosphatidylcholine or phosphatidylethanolamine, solubilizes the NADH dehydrogenase of intact mitochondria of beef heart, which implicates cardiolipin in its binding on the matrix side of the inner membrane [23]. Reconstitution of the lost activity of extensively delipidated (0.2% phospholipids) NADH dehydrogenase (NADH-ubiquinone reductase from beef heart mitochondria) requires cardiolipin specifically, other phospholipids non-specifically as dispersants or orienters [199].

(c) The cytochrome bc_1 segment in delipidated complexes is activated by several phospholipids but only in the presence of cardiolipins; in mitochondria it is inhibited by adriamycin (which reacts with cardiolipins) (see Ref. 284). Beef heart mitochondrial ubiquinol-cytochrome *c* reductase, purified and delipidated by ammonium sulfate and cholate fractionations, retains 10% of the original phospholipids, which in turn are 65% cardiolipins; removal of residual phospholipids by more drastic extractions denatures the complex [739]. A quinone-like inhibitor of electron transport in the undelipidated reductase binds to an acyl group of a structurally essential cardiolipin molecule in the quinol oxidizing site of the bc_1 complex [732]. This cardiolipin is retained even in 'delipidated' reductase. The bc_1 segment regulates minimally in rat liver mitochondria [228] but significantly when cardiolipins are altered [673] (see section IV-C.2.d); it does not regulate in heart mitochondria [155]. The yeast mitochondrial ubiquinol-cytochrome *c* reductase, when delipidated like the beef heart complex or by hexane extractions, loses almost all electron transport activity and its normal 1 : 1 antimycin binding [663]. Chloroform-methanol extracts of native and hexane-delipidated complex contain similar amounts of c diolipin, phosphatidylcholine and phosphatidylethanolamine; further addition of these phospholipids in 1 : 1 : 2 ratios restores 90% of activity, asolectin or cardiolipin restores 60%, and cardiolipin also brings back antimycin binding.

(d) Cytochrome *c* oxidase in mitochondria catalyzes reduction of molecular oxygen to water while it transports (pumps) one proton from matrix → cytosol for each electron transferred. Cytochrome oxidase is catalytically active when isolated from beef heart mitochondria by methods that may involve mild solvent extractions, and retains approx. 50 phospholipids including cardiolipins, presumably as an exterior shell (see Ref. 413); however, some cardiolipins seem to be buried in the protein complex and resist such extractions (see below). These phospholipids have been more or less cleared by (i) further more vigorous extractions, which pose risk of protein denaturation, or by (ii) exchange with di-14 : 0-phosphatidylcholine, which preserves the proteins. Effects of added phospholipids on catalytic activity have determined specificity, but results of these two procedures have evoked different conclusions.

(i) Serial extractions involving Triton X-100 and glycerol delipidate isolated enzyme [24,198,218,549, 678]. Cytochrome oxidase so stripped of all but 2–3 mol of cardiolipin per mol enzyme, and dispersed with lyso-phosphatidylcholine, had the molecular activity of unextracted enzyme at 25°C, about 160 s^{-1} [549] or 400 s^{-1} [678]; the latter workers stressed the point that assays that allow only half-maximal activity could mask or change specific lipid requirements. Further extractions partly reduced activity and removed all but about 1 cardiolipin per mol; reconstitution required 2–3 beef heart cardiolipins to restore full catalytic activity [678]; phosphatidic acid reconstituted in one study [218] but not in another [198]; all other phospholipids were ineffective although they bound to the delipidated oxidase. Thus, cardiolipins appear to be specific for full catalytic activity of this cytochrome oxidase when it already contains one molecule of unextractable cardiolipin. Cardiolipins have been described as a prosthetic group of such preparations, involved in cyclic transport (pumping?) of monovalent cations (although H^+ is omitted from the list) [197]. Vik et al. [678] concluded that the cardiolipins bind the substrate molecule cytochrome *c* at the low-affinity site on the oxidase through electrostatic interaction of their anionic groups with several cationic groups around the heme edge, which activates by orienting the two molecules for electron transfer. Beef heart cardiolipins with the natu-

ral 92% 18:2 + 8% 18:1 acyl content or with substituted 47% 18:2 + 52% 18:1 acyls activate equally effectively, cardiolipin substituted with 100% 6:0 acyls is half as effective, and removal of two of the four natural acyls abolishes catalytic activation [131].

(ii) Substitution with di-14:0-phosphatidylcholine produces a beef heart cytochrome oxidase that contains 0.19–0.06 mol cardiolipin per mol and has the activity of the original enzyme, approx. 160 s^{-1} [2]: cardiolipins are not essential for catalysis. By EPR measurements of competitive displacement of bound fatty acyl spin-labeled phosphatidylcholines and cardiolipin, cardiolipins are the most highly specific for about 50 enzyme sites; presumably cardiolipin polar sites interact with lysyl residues at the polar-apolar interfaces on subunits III, V, VI and VII [413,512,513], and three or four acyl chains are necessary for optimal binding [513]. Activation of lipid-substituted oxidase was thought to facilitate interconversion between two oxidase conformations [2].

Purified cytochrome oxidase from beef heart, when reconstituted into phospholipid vesicles, also pumps protons while transferring electrons (H^+/e^- ratios are 0.5–1.0) and changing conformation (see Refs. 84, 407, 617). Modifications of the enzyme, among them depletion of subunit III that is the 'H^+-channel' and may bind cardiolipins (see above), lower H^+/e^- stoichiometry without much change in catalytic rate (uncouple; see Refs. 392, 617), which is ca. 200 s^{-1} at 25°C [469]. The lipid contents of the enzyme preparations, some of them detergent-treated, used in the cited studies have not been documented. (Symmetrically, the above cited studies on the certifiedly lipid-depleted and lipid-reconstituted oxidase present no data on proton-pumping; some may be uncoupled and so incompletely 'reconstituted'.) presumably, the purified enzyme retains at least the most firmly bound cardiolipins. In addition, the reconstituting vesicles are asolectins which naturally contain cardiolipin (see above). A possible role for headgroups of a H-bonded network or H_{II}-phase of cardiolipins, starting in the ligand-binding Cu center of the membrane-embedded oxidase, is suggested by a diagram of P. Mitchell [436] that depicts a 'proton conductor' to the outer aqueous domain.

The apparent disagreements on the requirement for cardiolipins for beef heart cytochrome oxidase activity may be academic with regard to State 3 regulation, in two senses. First, the finding that di-14:0-phosphatidylcholine provides a low level of activity indicates that cardiolipins are not chemically specific for oxidase catalysis, but since the oxidase in the inner membrane sees no di-14:0-phosphatidylcholine, and phosphatidylcholines and phosphatidylethanolamines do not reconstitute partly delipidated enzyme, the cardiolipins seem to be 'biologically specific'. Second, the notion that this oxidase may be 'the major regulatory component of the electron-transport system' [2] cites Owen and Wilson [487], who assumed that kinetic control generally operates only at the reduced cytochrome aa_3 interaction with oxygen because all proximal electron flow rate steps are very rapid in comparison. In rat heart mitochondria the cytochrome oxidase C_i cannot be > 0.17 since measured C_i values (pyruvate/malate, 25°C) of the ATP-synthase plus the NADH dehydrogenase (and pyruvate carrier) amount to 0.83 [155]. In rat liver mitochondria C_i is minimal [47,203] or equal to 0.17 [228,673] but altered cardiolipin amounts and fatty acyl composition do not affect C_i (see Ref. 284).

Yeast cytochrome oxidase basal catalytic activity depends on the binding of 9 mol of cardiolipin per mol enzyme that resist exchange with detergent, and can be enhanced by either phosphatidylcholine or phosphatidylethanolamine [679]. Activity of purified yeast aa_3 does not absolutely require cardiolipins and can use substituted di-14:0-phosphatidylcholine, but these results accomodated a specific role for membrane cardiolipins in orienting cytochrome aa_3 for electron transfer [690]. In *Saccharomyces cerevisiae* mitochondria respiring in State 3, cytochrome aa_3 $C_i = 0.5–0.6$ (Table I). Yeast mitochondrial cardiolipins (unlike heart cardiolipins) contain 96% monounsaturated fatty acyls and no 18:2 acyls (see section IV-C.4), which may impart to cytochrome oxidase a rate or an orientation that limits O_2 reduction rate.

(e) ATP-synthase catalysis (ATPase) is cardiolipin-dependent. Peroxidation of rat liver mitochondrial lipids removes unsaturated fatty acyls from all phospholipids, but only the disappearance of cardiolipin 18:2 acyls correlates with loss of ATPase activity [395,572]. Ernster et al. [174] suggest that the embedment of the protonophoric F_0-ATPase in cardiolipins is necessary for ATPase function. A highly purified, active, oligomycin-sensitive ATPase contains two molecules of cardiolipin and less phosphatidylcholine and phosphatidylethanolamine [163]. A delipidated preparation of beef heart mitochondrial ATPase is best activated and made oligomycin-sensitive by cardiolipins among the naturally available phospholipids, which was attributed to effective orientation of F_1F_0 and the oligomycin binding site [129]. From reconstitution experiments with liposomes, phospholipids are not just a residential matrix but also modify ATPase catalytic properties [130]. Oligomycin-sensitive ATPase V_{max} increases linearly with the negative charge on individual phospholipids, except for beef heart cardiolipins that activate most effectively and supralinearly perhaps because they are highly unsaturated and the other phospholipids were not. Reconstitution of beef heart mitochondrial F_0 in cardiolipin-containing asolectin vesicles requires a protein component, $F_0$1, for proper alignment of F_0 for proton-translocation and binding to the F_1 catalytic complex [237]; endogenous phospholipids

do not appear to have been measured. ATP-synthases are the major regulators of State 3 in adult and fetal rat heart mitochondria (Table I).

(f) The ADP/ATP carrier of rat liver mitochondria loses activity but not capacity to bind adenine nucleotides when cardiolipins or phosphatidylethanolamines are partly removed by selective phospholipases A [625]. A complex of protein-cardiolipin-phosphatidylethanolamine is proposed as the nucleotide translocator. The carrier is a major regulator of State 3 respiration: in isolated rat-hepatocytes $C_i = 0.26$ [161], and in liver mitochondria $C_i = 0.29$ and changes concomitantly with cardiolipin amounts and fatty acyls (see Ref. 284). The beef heart mitochondrial ADP/ATP carrier is activated by cardiolipins, phosphatidylcholine and phosphatidylethanolamine equally [370]; it binds all these phospholipids [158], cardiolipins the most strongly. Dimeric carrier isolated from these mitochondria binds six molecules of cardiolipins (with fatty acyl compositions similar to total mitochondrial cardiolipins) of which only two exchange with spin-labeled cardiolipins [37,303,575]. From studies of binding, cardiolipin fatty acyl groups need not be unsaturated to interact strongly with this ADP/ATP carrier when it is already bound to di-14:0- or di-16:0-phosphatidylcholines, but only the natural highly unsaturated beef heart cardiolipins produce significant cardiolipin-carrier aggregation. From these binding studies, the four unexchangeable cardiolipins were proposed to participate in nucleotide transport function of the carrier [303] but transport was not measured. Rat and rabbit heart mitochondrial ADP/ATP carriers contribute a $C_i = 0$ for regulation of full State 3 respiration [155,355,356] (Table I) although C_i for the rabbit carrier (succinate, 37°C) is about 0.7 when respiration is partly inhibited [355,356].

(g) P_i transporter protein purified from heart mitochondria and reconstituted in asolectin vesicles is inhibited when adriamycin binds the cardiolipins [447]. The transporter loses activity when cardiolipins are removed, and phosphatidylcholine-vesicles do not reconstitute unless some cardiolipins are added (see Ref. 284). The P_i carrier does not regulate heart mitochondrial State 3 respiration, but in yeast mitochondria a significant C_i diminishes with ΔpH [30].

In summary, it appears by deductions from reconstitution experiments on isolated proteins from the inner membrane that cardiolipins play a constitutive role in mitochondrial State 3 respiration. Cardiolipins bind and activate just about all inner membrane regulatory and nonregulatory (State 3) protein complexes. Fatty acyl composition affects both binding and activation. Cardiolipin reconstitution of one complex or protein applies directly to State 3 rate only if that complex regulates. But specific inhibitor titrations of these single steps in intact mitochondria, interpreted through the concepts of metabolic control analysis [228,673], show that State 3 respiration is regulated at a higher level of organization where two or more protein-mediated processes contribute according to their rates relative to each other. Cardiolipins may participate at this more organized level. Several reconstitution and binding experiments suggest that cardiolipins orient proteins for optimal transport of electrons (ubiquinol → cytochrome c, cytochrome $c \rightarrow aa_3$) or metabolites, and align F_0F_1 for proton passage.

From 10% to 25% of cardiolipins are in the outer layer of mitochondrial inner membranes and face the intermembranal space and the outer membrane in rat liver, bovine heart and pig heart [261] (see Ref. 136). In apparent disagreement, 23% of total cardiolipins of rat liver mitochondria are isolated as a 'true component' of the rat liver mitochondrial outer membrane [307]. However, the enzymes and especially the lipids of the outer membrane so resemble those of the endoplasmic reticulum that the membranes have been thought to be one [172]. Reconciling these views are studies on the contact sites between the inner membrane outer face and the outer membrane seen in electron micrographs of State 3 mitochondria by Hackenbrock [243]. Contact sites isolated from mouse liver mitochondria as microdomains are greatly enriched in cardiolipins (27% of phospholipids) [19] – it seems moot to assign these shared 'peripheral' cardiolipins to one surface. These cardiolipins clearly orient some proteins secondarily involved in oxidative phosphorylation. Several properties of contact sites pertain to State 3 conditions and protein orientation. (i) Contact sites exist mostly under State 3 conditions; peripheral cardiolipins act as specific receptors for (ii) selected phosphotransferases and (iii) leader peptides of import proteins, and (iv) bind liver mitochondrial carnitine palmitoyltransferase.

(i) Contact sites, as estimated from electron micrographs of freeze-fractured mitochondria of rat livers, exist transiently and depend on energy state [57,243, 365]. Their number is minimal when mitochondria are fixed in State 3u (uncoupled); the ratios in mitochondria in State 3u : State 4 : State 3 are, respectively, 1:6:23 or 1:3.3:8 [3]. If the cardiolipins aggregated in the sites are recruited transmembranally from the inner face, the shift in sidedness might be involved in the loss of the proton leak under State 3 conditions, given a role of cardiolipins in State 4 proton leakage (section II-A).

(ii) Contact sites under State 3 conditions specifically bind and orient hexokinase, nucleotide diphosphate kinase and creatine kinase, but not adenylate kinase [57,365]. Thereby, hexokinase reacts more readily with ATP evolved from the ADP/ATP carrier on the inner membrane periphery than with added ATP. Outer cardiolipins are cited as a specific receptor in rat heart for the mitochondrial isoenzyme of creatine

phosphokinase, similarly aligning access to this carrier [448]. Mitochondrial creatine kinase concentrates at contact sites [3]. The blocking effects of adriamycin, specific for cardiolipins, show that this kinase binds to cardiolipins of intact mitochondria of rat heart or liver, and to (beef heart) cardiolipin in liposomes containing other phospholipids [448]. The kinase from rat heart binds to beef heart liposome surfaces [100]. Mitochondrial creatine kinase isoenzymes bind preferentially to monolayers of cardiolipin but also to other anionic phospholipids as well as to phospholipid extracts from either inner or outer membranes of mitochondria [552].

(iii) Peripheral cardiolipins interact with signal peptides of precursors of proteins synthesized in the endoplasmic reticulum and destined for mitochondria. These cardiolipins seem strategically concentrated for import through the contact sites. In vesicles prepared from phosphatidylcholines or phosphatidylethanolamines plus a cardiolipin, the two or three positively charged amino acid residues in the N-terminal portion of the peptide bind specifically at the outer surface cardiolipin -phosphoryl-glycerol-phosphoryl- headgroup [486]. The degree of saturation of the fatty acyl of the cardiolipin does not affect binding. Binding to cardiolipins as well as to surface protein groups is thought to mediate the targeting of the precursor protein complex to mitochondria; the translocation of the precursor across the inner membrane requires unfolding of the precursor protein [674] which also seems to involve binding to the cardiolipin [171]. Introduction of newly synthesized mitochondrial proteins that are active in oxidative phosphorylation should increase only State 3 respiration, which depends on electron-chain proton-pumping, rather than State 4 respiration which depends only on the proton-leak.

(iv) The balance between the active-form outer-surface carnitine palmitoyltransferase and the latent-form inner-surface carnitine palmitoyltransferase regulates β-oxidation in mammalian mitochondria; outer-face cardiolipins may orient and activate carnitine palmitoyltransferase as well as the translocase in rat liver mitochondria, to a degree regulated by thyroid hormone levels (see Ref. 284). Recent reconstitution experiments on the transferase reveal a detergent-soluble 'inner membrane material', most active in fasted animals, that is involved in binding and sensitivity to inhibition by malonyl-CoA, perhaps through orientation of catalytic and malonyl-CoA-binding subunits [208].

Evidence for the existence of an H-bonded network of cardiolipins in the inner face of the inner membrane [261] (see Ref. 136) is presented elsewhere in this review (e.g., sections II-A, IV). Since the anionic headgroups are all alike, these cardiolipin fatty acyl groups must be presumed to interact more specifically with lipophilic portions of cationic proteins in the membrane. Cardiolipin fatty acyls contain considerable information. The three significant diacylglycerol molecular species in beef heart cardiolipins are 79% 18:2-18:2, 11% 18:2-18:1 and 6% 18:2-18:3; yeast cardiolipins have a more even distribution of eight 16-C and 18-C saturated and monounsaturated acyls [575]. Rat liver, heart and kidney cardiolipins include at least eight molecular species that alter proportions in response to a fat-free diet [718,719,721]. Cardiolipins were first characterized through their antigenic properties, implying their specific binding to protein. But the cardiolipin network is not fixed by covalent bonds, and no known mechanism can be evoked that would form a coded cardiolipin sequence as an orienting template for oxidative phosphorylation proteins. Thus, the information for sequential reactions must lie in the protein structures, and intervening cardiolipins appear to orient at least some protein–protein interactions.

Reconstitution experiments on model systems require complementary approaches to evaluate structural roles of membrane lipids [535]. Reconstitutions of oxidative phosphorylation, using criteria of both catalysis and vectorial proton passage, and several protein complexes integrated with various cardiolipins (including cardiolipins with fatty acyl profiles altered in vivo), complemented with measurements of C_i values, would be a (challenging) start in deducing possible regulatory roles of cardiolipins in State 3 respiration from synthetic experiments. Until such systematic experiments test possible regulatory effects of altered cardiolipin amounts and fatty acyl compositions, the rest of this review attempts to recognize, by induction from correlated changes in biomembrane function, regulatory roles of cardiolipins altered in situ under biological influences.

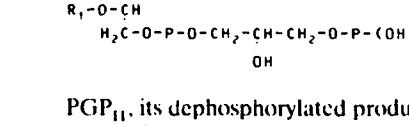
III. Cardiolipins in prokaryotes

Two prokaryote kingdoms, archaebacteria and eubacteria, are distinguished by different rRNA and protein sequences [715]. Although the archaebacteria live under apparently primitive biotic environments at very low [oxygen], high [H^+] or [salt] or [methane], and high temperature, they are more closely related to the eukaryotes (the third kingdom) than to the eubacteria, which appear to be the most primitive (see Ref. 511).

Membrane ether-lipids are chemical markers for archaebacteria. All contain branched-chain isoprenoid alcohols (e.g., C_{20} phytanyls) as diethers of 2,3-di-*O*-*sn*-glycerol or a more complex branched nonitol [143,144,379]. Biphtanyl-glycerol is the cell membrane lipid of *Methanopyrus*, which grows optimally at 110°C [312]. Some of the diethers in methanogens and thermophiles are joined covalently at the hydrocarbon ends to form glycerol tetraethers. The glycerol-OH on one end is substituted with carbohydrate, on the opposite

end with phosphate, in some thermophilic archaebacteria. This molecule, with carbohydrates facing outward, is thought to form the lipid phase of a unilamellar membrane, rather like bilayer membranes but with the median hydrophobic space fused. These membranes are rigid (archaebacteria have no peptidoglycan cell wall) and provide a temperature-stable barrier against a gradient of 4 to 5 pH units that drives the diffusion of protons into the cell. The archaebacterium *Thermoplasma acidophila*, which grows optimally at 59°C and pH 2 but not at all above pH 4, maintains an internal pH between 6.4 and 6.9; $\Delta\psi$ is about 120 mV (positive inside) and ΔpH is 290 mV, so that Δp is approx. 170 mV [309]. Neither protonophores nor inhibitors of electron transport change $\Delta\psi$ or internal pH; increase of external pH correlates linearly with decrease in $\Delta\psi$: acidophiles need no proton pump to disequilibrate protons across their membranes [489]. Hsung and Haug [309] conclude that both $\Delta\psi$ and ΔpH are maintained passively. The proton influx that drives ATP synthesis appears to come from the low external pH and its partial negation by $\Delta\psi$, which is thought to be a Donnan potential generated by charged macromolecules that do not permeate the cell membrane.

Both energy-transducing membranes in extremely halophilic archaebacteria contain diphytanyl ether-linked phospholipids and glycolipids. PGP_{II} (2,3-diphytanyl-*sn*-glycero-1-phospho-*sn*-glycerol-1'-phosphate; R_1 = phytanyl chain) is 65% of the total polar lipid of *Halobacterium cutirubrum*; it is the diphytanyl ether analogue of phosphatidylglycerol phosphate [522,647].

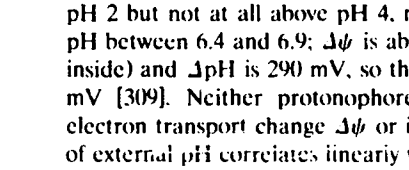


PGP_{II}, its dephosphorylated products and the halophile glycolipids form bilayers in aqueous dispersions that are in liquid crystalline state from −30°C to 80°C. Their possible role in a membrane-surface hydrogen-bonded network that conducts protons laterally is discussed in section II-A.

The archaebacterium *Thermoplasma acidophilum* grows optimally at 56°C, pH 2, and adapts its membrane lipids for growth at 37°C by increasing their fluidity [733]. The main lipids are two repetitively methyl-branched, saturated C_{40} side-chains, ether-linked to two glycerol molecules; serine and phosphate groups on some of the glycerol-OH sites provide acid polar groups. The shift from growth at 56°C → 37°C diminishes lipid phosphorus by 10%, halves serine moieties and doubles acyl cyclization. The resultant increased membrane fluidity alters membrane-dependency (Arrhenius profile) of ATPase activity: activities are lower over the temperature range of measurements and transition temperatures decrease by 7.5°C.

III-A. Cardiolipin / phospholipid ratios and fatty acyl composition

The eubacteria and the eukaryotes branch fairly early from the archaebacteria in a universal phylogenetic tree determined from comparisons of 16S-rRNA sequences [715]. The phospholipids of eubacteria prokaryotes are fatty acyl esters of 1,2-*sn*-glycerol-3-phosphate, like those in eukaryotes [217]. Cardiolipins (diphosphatidylglycerol; R_2COO- = fatty acyl ester) resemble a portion of PGP_{II}:



Cardiolipins in eubacteria are synthesized from two molecules of phosphatidylglycerol [188,305]. Phosphatidylglycerols comprise 10–35% of the total phospholipid phosphorus and are the major phospholipid (50–80% of P) in some eubacteria [305].

As shown in Table II, cardiolipin per total phospholipid contents are up to 15% in *M. lysodeikticus*, *S. aureus*, *Salmonella typhimurium*, *B. cereus*, up to 40% in the Actinomycetes [346], and 80% in a form of *S. aureus* [305]. Fatty acyl compositions of eubacteria vary widely (Table II). Some eubacteria thereby maintain plasma membranes in environments as extreme as those resisted by the lipids of archaebacteria. The fatty acyl composition of cardiolipins and phosphatidylglycerols is usally similar, and in prokaryotes sometimes differs from that of other phospholipids [217].

Cardiolipins and phosphatidylglycerols, as well as other phospholipids and phosphoglycolipids, are almost equally distributed in the inner and outer faces (2:3, respectively) while the gucosylglycerides are completely outside in *A. laidlawii* membranes [554]. Cardiolipins are equally in and out, phosphatidylglycerols all outside, phosphatidylinositols all inside in *M. lysodeikticus*. From the similar polar backbone glycerol groups of cardiolipins and PGP_{II}, it seems that either could participate in lateral conduction of protons via H-bonded networks. The backbone glycerol in bilayer membranes made from cardiolipins purified from *E. coli* in stationary-phase, 37°C, is more rigid than other phospholipid headgroups and remains so in membranes that contain 20% cardiolipins and 80% phosphatidylcholines [8]. The corresponding glycerol in phosphatidylglycerols has free motion: the conversion of phosphatidylglycerols → cardiolipins would increase order in membrane surface, and even minor amounts of cardiolipins could contribute to proton movements.

Both fatty acyl composition and relative proportions of bacterial cardiolipins (usually uniquely or more markedly among the phospholipids present) vary with, and respond to, growth stage, presence of oxygen,

temperature, illumination, proton reflux. The changes induced suggest that cardiolipins participate in energy transduction.

Growth phase: the growth phase of bacterial cells affects cardiolipin/phospholipid ratios, cardiolipin and phospholipid fatty acyl composition and membrane function. In exponential growth phase at a given temperature, cardiolipins of *E. coli* contain 70% 18:1($n-7$) (*cis*-vaccenoyl) acyl groups (Table II); unsaturated fatty acyl contents of cardiolipins > phosphatidylglycerols > phosphatidylethanolamines [360]. When exponential shifts to stationary phase, 18:1 acyl content in

TABLE II

Fatty acyl composition of cardiolipins and cardiolipin / total phospholipid ratios, prokaryotes

Abbreviations: UI, unsaturation index; CL P, cardiolipin P; ΣPL P, total phospholipid P; *b*, branched; *i*, *iso*-; *a*, *anteiso*-; *c*, ω-cyclohexyl fatty acyl; *cy*, cyclopropane methylene component; *t*, tuberculostearic acyl (10-methyl-19:0). Various unspecified unsaturated fatty acyl groups were omitted in these studies because they were minor components.

Cell/Rx	Fatty acyl (% of total fatty acyls in cardiolipins)										UI	%CL P / ΣPL P	Refs.
	14:0	14:1	15:0	16:0	16:1	17:0	18:0	18:1	18:2	19:0			
Acholeplasma													
laidlawii	31.5		0.2	54.3	2.9	0.2	0.9	1.3			4	50.0	616
Bacillus													
acidocaldarius 60°C pH3	0.9		15.8*b*	2.3		18.1*b*				5.2	0	66.1	380
				1.8*b*		54.4*c*							
Bacillus													
subtilis exponential 37°C	1.6		49.9*b*	6.5		16.0	2.4	6.2	1.0		2		671
				5.4*i*		15.1*a*							
Caulobacter													
crescentus 30°C	7.6	10.1		21.6	12.6		10.4	32.7			55	10.1[d]	389
+ 18:2	10.0	8.1		20.4	13.7		6.8	27.4	11.9		73		
Escherichia													
coli exponential 40°C	4			17	7	2.0*a*	2	66.0($n-7$)		1.0*cy*	73	15.2	8, 121, 346, 360
30°C	4			13	5	2.0*a*	1	71.0($n-7$)		2.0*cy*	76		
20°C	3			9	5	2.0*a*	2	77.0($n-7$)		1.0*cy*	82	9.1	
stationary 40°C			4	50	1	13.0*a*		6.0($n-7$)		12.0*cy*	7	19.6	
30°C			4	41	2	11.0*a*	4	22.0($n-7$)		9.0*cy*	24		
Hemophilus													
parainfluenzae 37°C				32.0	22.9		4.7	3.2			26	3.0	698
Methylosinus													
trichosporium 21°C, CH_4					13			87			100	0.7	691
Micrococcus													
lysodeikticus 37°C	2.0		2.0	8.1	2.9	5.9*a*					3	38.7	333
	1.0*a*		65.8*a*	12.1*i*									
+ pantoyl lactone	4.0		2.0	16.1	4.8	2.1*a*					5	52.7[c]	
	2.0*i*		46.3*a*	21.1*i*									
Mycobacterium													
butyricum 40°C				13	24			22		19.0*t*	46		478
20°C				2	38			44		11.3*t*	82		
Nocardia													
coeliaca 37°C			8.9*i*	30.1		3.6*t*	3.6			4.4	0	15.0	734
				31.2*i*		6.3*a*	4.6*t*						
Paracoccidioides													
brasiliensis 37°C				6	2		1	41	50		143	10.7	411
Rhodospirillum													
rubrum 30°C dark	2.4			11.8	27.2		2.8	48.9($n-7$)			76	12.2	565
light	1.0			17.6	20.8		9.9	42.3($n-7$)			63	11.3	
Staphylococcus													
aureus $+O_2$			40.9*a*	1.8	0.1	9.7*b*	10.0			9.9*b*	0	21.9	195, 603, 699
$-O_2$			23.6*a*	5.4	2.1	2.0*b*	13.4			3.4*b*	2	23.8	
Streptococcus 37°C	5.6	0.6		32.7	14.0		17.4	26.3		3.2*cy*	50	58.7	189
Thermophilic													
bacterium PS3 70°C	1.1*i*		54.3*i*	2.1		27.7*i*	5.5				0	19.2	342, 343
				9.3*i*									

[d] diphosphatidylglycerol/ΣPL = 78%;

[c] *lyso*-CL = 20.4% in addition.

cardiolipins decreases strikingly to as low as 6%, while saturated fatty acyls content (especially 16:0 but also nonlinear 17:0 and 19:0 acyls) rise to 86%, cardiolipin/phospholipid increases and relative amounts of phosphatidylglycerol, the cardiolipin parent compound, decrease stoichiometrically. Analogously, *Thiobacillus thiooxidans* growing at 30°C increases cardiolipin/phospholipid proportions linearly from 7% (day 1) to 21% (day 8) whilst phosphatidylglycerol/phospholipid decreases [601]. In *E. coli*, at the same time cardiolipins increase, an enzyme methylates 18:1 acyls across the unsaturated bond to form *cyclopropane*-19:0, a saturated fatty acyl [123]. In the exponential → stationary phase transition, the fatty acyl compositions of phosphatidylglycerols and phosphatidylethanolamines change but little: the fatty acyl contents of cardiolipins are the most sensitive to growth phase.

Growth phase may affect analytical recovery of cardiolipins. In stationary growth phase, routine chloroform-methanol extractions recover as little as 5% of the cardiolipins and all the phosphatidylglycerols and phosphatidylethanolamines from whole cells of several Gram-positive bacteria, but in early exponential phase all cardiolipins are extracted [187]. Cardiolipin/phospholipid ratios are up to 57% when the mucopeptide-teichoic acid components of the thick cell walls of stationary phase cells are removed, e.g., by lysozyme; exponential phase cells have thinner walls that leave cardiolipins accessible. It is not clear if the altered fatty acyl compositions of the cardiolipins in the different growth phases contribute to cardiolipin-cell wall association.

Electron-transport rate increases with growth rate, as exemplified by *Klebsiella aerogenes* [456]. In early, exponential stages of growth, microbial cells oxidize carbon substrates efficiently, to transport solutes and synthesize biomass. When growth stops, cells are thought to oxidize less efficiently by uncoupling oxidative phosphorylation or turning ATP over rapidly in a futile metabolic cycle [271,649]. Uncoupling mechanisms in the membrane (see Eqn. 1) include decreases in overall H^+/e^- ratios (e.g., through elimination of proton-translocating loops in electron-transport chains [338]), and dissipation of Δp by reflux of extruded protons through a phospholipid-leak or an F_0-leak. One might add that conversion of State 3 → State 4 respiration, when diminished ATP-utilization depresses [ADP] and stops phosphorylation, increases Δp and does not decrease efficiency of proton cotransport through exchangers. Intact bacterial cells show no respiratory control when ADP + P_i are added (see Ref. 200), nor should they since bacterial plasma membranes have no (and need no) ADP/ATP carrier [331]. State 3 and State 4 are distinct in everted particles from bacterial membranes (see below).

An O_2-pulse given to anaerobic *E. coli* cells grown at 37°C produces a 2- to 3-fold greater proton efflux in stationary-phase cells than in exponential-phase cells [221]: exponential-phase cells with cardiolipins containing high monounsaturated fatty acyls seem to retain protons; stationary cells with cardiolipins repleted in saturated fatty acyls lose protons (see section II-A). In stationary cells the O_2-pulse is followed by lusty respiration but proton efflux is much slower [221], apparently reflecting a secondary slow 'leak' of protons that equilibrates external $[H^+]$ with protons in or on the outer aspect of the membrane. The rest of the protons extruded by electron-transport do not appear to leave the cell membrane but to be laterally conducted, to return to the cytosol via proton-driven exchange transport or ATP-synthase.

Apparent proton conductance across bacterial membranes has been measured by ΔpH/time after an H^+-pulse under anaerobic conditions [408,409]. With Δp assumed to be 200 mV and protein contents distributed as per Neidhart [455], $C_{M^{H^+}}$ in *E. coli* cells can be calculated to be 0.08 ng ion H^+ min^{-1} (mg total protein)$^{-1}$ mV^{-1}; if membrane protein is 15% of total protein, $C_{M^{H^+}}$ is 0.5 ng ion H^+ min^{-1} mg^{-1} mV^{-1}, a value in the range of mitochondrial conductances [462,464]. $C_{M^{H^+}}$ is 0.7 ng ion H^+ min^{-1} (mg membrane protein)$^{-1}$ mV^{-1} under similar assumptions in *Streptococcus lactis* in early stationary phase.

Real proton conductances must be compared at the same Δp because proton current has a supralinear and shifting relationship with high values of Δp, as pointed out by Brand and coworkers (section IV-C.2.d). Membrane ion conductance adapts to growth rate in photoheterotrophic cultures of *Rhodobacter capsulatus*, an α-purple eubacterium, grown at 30°C, pH 7 [645,646]. Dissipative ion current (clearly protons) increases disproportionally and non-linearly with $\Delta\psi$. As growth is limited by [carbon source] or by decreased illumination, conductance increases; current/$\Delta\psi$ is greater when substrate limitation is more severe. Current is greater in stationary than exponential cells for similar values of $\Delta\psi$ above threshold. Most of the current at maximal $\Delta\psi$ is via the ATP-synthase, i.e., State 3 respiration. When the F_0-channel in intact cells is blocked by venturicidin, rapid proton flux persists: the high $\Delta\psi$ augments another conducting pathway(s). A proton-leak (perhaps similar to that in State 4) appears, as it does in mitochondrial vesicles stripped of F_1 when F_0 is blocked by oligomycin. Taylor and Jackson [645,646] dismiss dielectric breakdown as an explanation for a new proton leak because $\Delta\psi$ is still at physiological levels, and the phenomenon is reversible, like the cycle State 4 → 3 → 4. These workers propose that a membrane gated-protonophore has a threshold at slightly greater values of $\Delta\psi$ than does the F_0, and that decreased growth rate (somehow) depresses this threshold. Stationary-phase cell membranes, compared

with log-phase, leak more protons; their membranes have a greater proportion of cardiolipins and those cardiolipins are the most markedly depleted in unsaturated fatty acyls. As will be seen, fatty acyl composition of cardiolipins is analogously connected with proton leakage in many energy transducing membranes.

Temperature: membrane fatty acyl unsaturations vary inversely with environmental temperatures in prokaryotes and the simpler eukaryotes, which maintains membrane lipids above their melting points and retains appropriate membrane barrier and matrix properties [267,268,399,527]. Eubacteria in exponential-phase adapt to decreased temperatures by increasing unsaturated fatty acyls contents in membrane phospholipids [122,123,142,201,480], either without changing the proportions of phospholipid-subclasses or changing them slightly in *E. coli* so that cardiolipin/phospholipid ratio halves, phosphatidylglycerol/phospholipid doubles and phosphatidylethanolamine/phospholipid shifts from 84% to 74% [480]. Specific enzymatic desaturations, elongations and transacylations mediate phospholipid adaptations to low temperatures. Cardiolipins contain 66% *cis*-18:1($n-7$), 17% 16:0 acyls in *E. coli* growing at 40°C (Table II). The *cis*-3-unsaturated bond derives from the anaerobic dehydration of D-β-hydroxy-10:0-acyl-carrier-protein, an intermediate in the fatty acid synthesis pathway, to form either *cis*-10:1($n-7$)-acyl-carrier-protein or *trans*-10:1($n-8$)-acyl-carrier-protein (the conventional step in further elongation by malonyl-acyl-carrier-protein to form 16:0-acyl-carrier-protein). Elongations convert *cis*-10:1($n-7$) → *cis*-16:1($n-7$). In cells growing at 40°C, cardiolipins incorporate (transacylate) this 16:1 acyl minimally (7%; Table II), phosphatidylglycerols 18%, and phosphatidylethanolamines 24% [360]; decreases in temperature do not affect these 16:1 acyl contents. Elongation of 16:1($n-7$) → 18:1($n-7$) chains increases 15 s after temperature is decreased from 42°C → 24°C, through the activation of existing β-ketoacyl-acyl carrier protein synthase II, that catalyzes condensation of malonyl-acyl-carrier-protein with the growing fatty acyl chain [142]. This cold-activated enzyme is a translation product different from the less active, thermally insensitive synthase I. Transacylation of 18:1 acyls increases: in cardiolipins 18:1 acyl content rises to 77% at 20°C, displacing 16:0 acyls to 9% (Table II); 18:1 acyls also accrue in phosphatidylglycerols (41% → 54%) and phosphatidylethanolamines (24% → 33%). Thus, cardiolipins play the most prominent but not the exclusive role in *E. coli* exponential-phase adaptation to low temperatures, by increasing their already high contents of 18:1 acyls. Cardiolipins in stationary-phase *E. coli*, although much less unsaturated (7%) than in exponential-phase when growth is at 40°C, triple their 18:1 acyl content at 30°C [360] (Table II). The low 18:1 acyl contents of phosphatidylethanolamines and phosphatidylglycerols in stationary-phase cells grown at 40°C (5%) increase even more strikingly in cells grown at 20°C (to 40–60%).

Changes in membrane physical and barrier properties accompany growth-temperature-induced adaptations in phospholipid fatty acyl compositions. Permeability (estimated from osmotic swelling) of liposomes prepared from extracted phospholipids is inverse to growth temperatures between 40°C and 20°C when *E. coli* is grown to the end of exponential-phase [244]. Mean surface area of films made from total phospholipids of *E. coli* [244] or from phosphatidylethanolamines extracted from *Pseudomonas fluorescens* cells grown at 22°C is greater than in films from cells grown at 5°C [124]. Thermal acclimation counteracts the enhanced molecular cohesion in monolayers at the lower temperatures [266].

When grown to stationary-phase at 40°C or at 20°C, *Mycobacterium butyricum* changes mainly the fatty acyl contents of cardiolipins [478] (Table II) but with less emphasis on elongations and desaturations than in *E. coli*. At either temperature, cardiolipin percentage of 16:1 = 18:1; at 40°C 16:1 + 18:1 = 46% of fatty acyls, at 20°C, 82% of fatty acyl at the expense of 16:0 and 19:0-branched fatty acyls. At 40°C, the phosphatidylethanolamines contain half as much monounsaturated fatty acyls (23%) as cardiolipins; at 20°C, phosphatidylethanolamine monounsaturated fatty acyls rise to only 32%.

Ester-linked fatty acyl phospholipids are in plasma membranes of eubacterial thermophiles that remain stable at high temperatures, as do membranes with ether-linked phytanyl glycerols in archaebacterial thermophiles. The eubacterial moderate thermophile *Bacillus stearothermophilus* grows optimally at 65°C, the extreme thermophile *Thermophilus aquaticus*, at 90°C. Cardiolipins in *B. stearothermophilus* cells in late exponential-phase are 30–40% of total phospholipids and 50% of membrane phospholipids; phosphatidylglycerol/phospholipid and phosphatidylethanolamine/phospholipid ratios are each about 25% [78]. Thermophile total phospholipids contain all or almost all saturated, branched-chain, *iso*- or *anteiso*-15-carbon and 17-carbon fatty acyls. They contain no unsaturated fatty acyls [484] or 11.5% *iso*-17:1 [600]. The fatty acyls of the cardiolipins of *B. stearothermophilus* [78] and the extreme thermophilic bacterium PS3 [343] are all saturated (Table II); PS3 cardiolipin/phospholipid ratio is 19%, phosphatidylglycerol/phospholipid, 13%. The resistance of the saturated phospholipids of PS3 to heat and oxidation makes them useful in reconstituting the stable PS3 F_0F_1-ATPase complex [343,618,619]. Saturated cardiolipins are only 3% of total phospholipids of *T. aquaticus* in late exponential-phase or stationary-phase; 75% of total phospho-

lipids is an unidentified phospholipid that contains three saturated fatty acyls, one glycerol and an unsaturated long-chain amine [532].

Cardiolipins are 66% of the completely saturated membrane phospholipids of *Bacillus acidocaldarius*, an extreme thermo-acidophile eubacterium [380] (Table II); proton conductance is in the range seen in other bacteria [372]. Right-side-out membrane vesicles pump respiratory-chain protons outward and maintain an internal pH of 5.8–7.1 (external pH being 2–3) as well as a reversed $\Delta\psi$, positive inside. Protonophores abolish most of the ΔpH.

Cell membranes of *T. aquaticus* and *T. thermophilus* leak protons, more rapidly as in vivo and in vitro temperatures are increased. A pH gradient generated in anaerobic *T. thermophilus* cells by an oxygen-pulse decays by proton leakage: when measured at (50°C) the $t_{1/2}$ is 45 s in cells adapted by growth at 60°C, 19 s in cells adapted at 79°C; when measured at 79°C, $t_{1/2}$ is ≤ 8 s [425]. (Rat liver mitochondrial $t_{1/2}$ measured at 25°C is 86–140 s, and 5 s in the presence of FCCP [437,438,459].) Despite the great proton leakage, rapid cell respiration maintains Δp at 197 mV (assayed at 70°C, pH 7.5), but growth efficiency is one third that of mesophilic bacteria that have an equivalent respiratory system (see Ref. 184). *T. thermophilus* cells at 70°C maintain an adenine nucleotide phosphorylation potential of 43.5 kJ/mol, and added FCCP depresses it to only 38.0 kJ/mol: a high H^+/O quotient must accompany the rapid oxidative generation of Δp.

ATPase-containing vesicles made from extracted saturated phospholipids of PS3 can attain a Δp of 310 mv at 45°C; the $\Delta\psi$ component decays with a half-life of 55 s. Half-life is 17 s (54°C) in less stable vesicles made from the naturally unsaturated soybean phospholipids.

Thermophilic bacteria grown to late exponential-phase at increased temperatures adapt by lengthening their phospholipid carbon-chains and synthesizing *anteiso-* or *iso*-fatty acids that have higher melting points [78]; the fatty acyl composition of phospholipid subclasses are not reported. With cardiolipins containing saturated fatty acyls the membranes of thermophilic bacteria seem to support energy metabolism at high temperatures through a compromise between high stability and inefficient proton retention.

Effects of $[O_2]$: transition from anaerobiosis to aerobiosis leads to formation of the electron-transport chain while it increases cardiolipins and other phospholipids by 60% in *Staphylococcus aureus* [699] (Table II). However, only the cardiolipins reconstitute activity in the delipidated bacterial D-lactate oxidase, and (when they are not supplemented with Q_{10}) in NADH oxidase and succinate oxidase [177,424]. Limiting $[O_2]$ during *E. coli* growth at 30°C markedly decreases efficiency of energy conversion, as the cells synthesize a_1a_2 cytochrome oxidases which may be associated with non-phosphorylating respiratory chains [338,429].

ADP phosphorylation: colicin K, 2,4-dinitrophenol, cyanide, penicillin or ultraviolet radiation [572] diminish cell phosphorylating ability while they increase the cardiolipin/phosphatidylglycerol ratio [629].

Protonophores: cardiolipins accumulate in bacteria grown in the presence of a protonophore. Cardiolipins in *B. subtilis* so grown comprise up to 70% of total phospholipids when extracted with adequately acidic solvents, but otherwise large amounts of phosphatidylglycerol are present as a cardiolipin breakdown product [376]. Phosphatidylglycerols are also a product of incomplete extraction in *Streptococcus* [189]. Cardiolipins accumulate in plasma membranes of *Hemophilus parainfluenzae* grown with CCCP while the highly active cardiolipin-specific phospholipase D is blocked [481]. Since CCCP does not inhibit the isolated enzyme, the proton leak appears to depress cardiolipin catabolism. Conversely, blockage of any F_0-leak with oligomycin enhances cardiolipin hydrolysis.

Growth of the *E. coli* UV6 strain in the presence of 0.25 mM CCCP, as compared with its parent strain -CCCP, halves the phospholipid content per mg protein of the inner cell membrane; leaves proportions of cardiolipins, phosphatidylethanolamines and phosphatidylglycerols unchanged; and increases unsaturated fatty acyls (16:1($n-7$) + 18:1($n-7$)) from 25% (-CCCP) to 45% with reciprocal decrease in saturated fatty acyls (plus *cis*-9,10-methylene-17:0) [272,583]. ATP/ADP ratios are maintained while content of ATP + ADP rises 50% and P_i rises 13-fold. Increase in unsaturation of the membrane (we do not know fatty acyl composition of cardiolipins) might be viewed as an adaptative attempt to retain an effective Δp.

Mitochondrial origins: the present mitochondrion is thought to be derived from a successful symbiosis of an eukaryotic cell that performed anaerobic glycolysis, and a prokaryotic cell that had developed oxidative phosphorylation in a membrane, based on homologies between mitochondrial and prokaryotic DNAs, ribosomes and proteins [614]. From rRNA and cytochrome sequences, the most likely prokaryote endosymbiont is among the α-subdivision of the photosynthesizing, purple, nonsulfur, Gram-negative bacteria [715,731]. Some α-purple bacteria still associate intracellularly with various eukaryotes either as pathogens (e.g., agrobacteria for plants, Rickettsiae for animals) or as essential intracellular components, e.g., Rhizobacteriae for nitrogen-fixation in legumes [715]. Aerobic metabolism arose a number of times in α-purple bacteria and early mitochondria may have metabolized dissolved O_2 even before photosynthesis produced high atmospheric $[O_2]$ [581].

Micrococcus denitrificans, in the α3-subgroup of purple bacteria [715], has more mitochondrial features

than other aerobic bacteria [331]: membrane phospholipids include an unusally high 31% of phosphatidylcholines, 52% are phosphatidylglycerols and 3.2% cardiolipins [701]; phospholipid fatty acyls are all straight-chain; its cytochrome *c* reacts with mitochondrial aa_3; its P_i-carrier is sensitive to sulfhydryl reactants. Everted particles from *M. denitrificans* plasma membrane behave like mitochondria: respiration (NADH, 30°C) is stimulated by addition of ADP + P_i or FCCP, and depressed by the ATPase inhibitor venturicidin; H^+/O ratios are comparable [329,330]. *Rhodospirillum rubrum*, in the α1-subgroup of purple bacteria, has cardiolipins with relatively unsaturated fatty acyl groups that become slightly less unsaturated when photosynthesis is established (Table II).

Fatty acyl composition of cardiolipins is distinct from other phospholipids in several phyla: the saturated cardiolipins of *N. coeliaca* are characterized by very high contents of 16:0 acyls [734]; cardiolipins of *E. coli* and *M. butyricum* are more unsaturated than phosphatidylethanolamines and phosphatidylglycerols under similar growth and temperature conditions (see above); cardiolipins of *S. cerevisiae* mitochondria are distinctively repleted in 16:1 and 18:1 fatty acyls (section IV-C.4); and cardiolipins from heart, liver or kidney mitochondria are enriched in 18:2 acyls (section IV-A). Thus, phospholipid fatty acyl compositions have undergone an evolutionary shift from completely endogenous saturated and monounsaturated fatty acyls in prokaryotes, to significant amounts of exogenous EFA (especially 18:2($n-6$) in cardiolipins) and their ($n-6$)- and ($n-3$)-polyunsaturated fatty acyls derivatives in animal mitochondria. *C. crescentus* can even incorporate into its cardiolipins 18:2($n-6$) fatty acyls added to the growth medium (Table II).

IV. Cardiolipins in eukaryotes

Cardiolipins are synthesized in the mitochondrial inner membrane from one molecule of a phosphatidylglycerol and one molecule of a CDP-diacylglycerol. The liponucleotides are produced from phosphatidate by either mitochondrial or microsomal CTP-specific transferases [305,320]. Cardiolipin linoleoyl acyls turn over faster than in other phospholipids in liver mitochondria [378], probably as part of a remodeling that keeps the 18:2 content higher in cardiolipin than in its precursors. This remodeling appears to be a mitochondrial cycle not found in microsomes, that comprises phospholipase-A-catalyzed monodeacylation of newly synthesized cardiolipin, and a specific 18:2-CoA: monolysocardiolipin transfer(ase?) [578]. In contrast to the 18:2 acyls, the -P-glycerol-P- grouping turns over much more slowly in rat liver cardiolipins than the glycerol backbone of other phospholipids [378] and probably persists as long as the mitochondrial membrane itself, since cardiolipin and mitochondrial half-lives are comparable [191,232].

A corollary of the concept that cardiolipins exist in membranes that perform oxidative phosphorylation would be that cardiolipins do not exist in obligatively anaerobic eukaryotes that have no mitochondria. Some protozoa have evolved without mitochondria [80]: Archamoebae, Metamonada, Microsporidia and Parabasalia. *Giardia lamblia*, an archezoan metamonad with no mitochondria, two nuclei and DNA of early evolutionary origin, is a recently proposed candidate for the anaerobic eukaryote that was the first host for an aerobic prokaryote among the α-purple eubacteria [340]. It now lives parasitically in animal and human intestinal tracts and depends on preformed biliary phospholipids and cholesterol for phospholipids and fatty acids, which it does not readily synthesize. Bile phospholipids do not include cardiolipins [183]. Giardia phospholipids incorporate saturated fatty acids and 18:1 acid from the growth medium [327,344], but when external 18:2 acid is 56% of fatty acids the phospholipids contain only 4.5% 18:2 acyls [344]; this would be consistent with the absence of cell cardiolipins. No cardiolipins are reported in *G. lamblia* membranes [42,327,344] or in another amitochondrial anaerobe, *Entamoeba invadens* [426,667], in phospholipid analyses that usually detect small quantities of cardiolipins, although some unidentified phospholipids were seen. Presumably mtDNA codes for the enzymes that synthesize cardiolipins. Descendent eukaryotes of such amitochondrial anaerobes would thereby have no cardiolipins in their plasma membrane phospholipids.

IV-A. Cardiolipin / phospholipid ratios and fatty acyl composition

Analyses for tissue cardiolipins and phospholipids should separate phospholipid subclasses completely and non-destructively. However, the usual extraction methods may not recover all the cardiolipins of beef heart [612,716] or the cardiolipins of some stationary-phase bacterial cells (section III-A). Some mitochondrial cardiolipins can readily be peroxidized, polymerized or deacylated. Cardiolipins in various vertebrate tissues, especially heart, are preferentially deacylated by an endogenous phospholipase A [242]. On the other hand, liver cardiolipins are not especially sensitive to oxidation with ascorbate, which decreases unsaturated fatty acyls in all phospholipids [395,572].

Tissue concentration of cardiolipins (per amount of tissue, DNA, total phospholipids or protein) measures the cell content of mitochondrial inner membranes [326] and therefore correlates with tissue total oxidative capacity. The ratio cardiolipin per mitochondrial total phospholipids, and the percentage distribution of

TABLE III

Fatty acyl composition of cardiolipins and mitochondrial cardiolipin / total phospholipid ratios: eukaryotes

Abbreviations: as in Table II. Because various unspecified unsaturated fatty acyl groups were omitted in these studies as minor components, the UI values may not correspond to the percentages of unsaturated fatty acyls shown.

Tissue Cell Diet/Rx	Fatty acyl (% of total fatty acyls in cardiolipins)									UI	%Cl./ΣPL mitochondria		Refs.
	16:0	16:1	18:0	18:1	18:2	18:3	20:3	20:4	22:6		per ΣP	Δ% vs. control	
Saccharomyces													
cerevisiae stationary	4.0 [f]	7.6		48.3						96	15.6		324, 690
exponential											9.4		
Tetrahymena													
pyriformis 35–39°C	3.6 [f]	2.8	2.1	4.0	39.7	41.6($n-6$)				211	14.8		200, 337, 730
15°C	1.3 [f]	2.6	2.0	4.0	38.2	44.4($n-6$)				216	11.2	−24%	
Acanthamoeba													
castellanii 21°C	6.0 [f]	0.8	1.8	50.1	5.9	6.8	4.7($n-6$)	23.5		185	8.1		664
Chlorella													
vulgaris dark	31.7 [f]	13.0 [f]	3.9	18.9	29.0	6.4($n-3$)				109			467
light	37.2 [f]	16.5 [f]	2.2	25.6	31.5	5.4($n-3$)				121			
Spinacia													
oleracea 18°C leaves	1.6			7.0	38.0	53.0				242	19.8		166
petioles	1.4			5.0	56.0	38.0				231	21.4		
Mung bean 25°C	11.0		16.0	8.0	30.0	35.0($n-3$)				173	23.0		202, 422
mitochondria	3.0		0.5	6.5	39.0	50.0				235	14.0		43
Sycamore													
mitochondria 25°C $-O_2$	5		1	25	44	24.0($n-3$)				185	14.0		44
$+O_2$	7		1	5	62	25.0($n-3$)				204	14.0	0%	
Bufo													
arenarum: oocyte	1.9	6.2	1.5	28.3	55.9	4.1				159	6.6		11, 48
blastula	1.8	6.1	0.6	19.1	54.5	4.3				147			
gastrula	1.7	7.2	1.1	25.6	48.3	4.1				142	8.0		
Liver													
rat: corn oil	5.4	3.9	3.4	17.9	61.8	0.5	1.9	2.0	0.9	165	20.3		110, 113, 154, 291, 349, 390, 441, 492, 502, 563, 717, 729
18:2($n-6$)	2.2	7.4	0.5	15.0	67.2	2.5	2.2	2.5		186			728
fat-free	4.7	27.2	2.0	41.2	12.3	0.4	2.3	1.9	2.2	118			154, 335, 628, 703
hydrog. fat	5.8	23.5	3.5	44.5	12.1		6.3	2.9		123			154
18:3($n-3$)	9.9	14.8	5.3	40.8	3.0	13.5	0.7		10.7	176			658, 728
22:1($n-9$)	5.4	10.9		41.9 [c]	25.1		2.0		4.5	13[c]	5.9	0%	735
fish oil	5.7	3.3	11.5	29.6	24.4	1.2		2.1	10.2 [a]	180			729
fetal	5.1	15.2	2.1	32.8	41.9	0.3		2.9		145	13.5		325, 707
1-day-old	0.9	5.9	2.6	26.6	58.6	0.7	1.7	0.9	1.7	173	11.3	−48%	354, 441
hypothyroid	8.0		6.1	17.8	53.6		1.3	3.6		143	20.5	+72%	291
hyperthyroid	33.0	7.4	16.5	14.2	20.1		3.4	3.0	2.4	98	30.5	+29%	563
hypophysectomized	7.3	4.9	4.8	15.3	67.4		0.4			157	14.9	−26%	110

+ growth hormone	7.2	5.9	4.8	20.6	57.0		2.6			156	24.1	+62%	
ethanol-fed	7.9	1.2	5.1	25.8	53.0		2.6	2.2		148	16.1	−7%	128, 168, 574, 654, 686
Mouse	3.5	1.6	1.5	9.3	69.9	1.4	2.6	1.8	2.7	186	4.1		457
ethanol-fed	30.1 [a]	3.8	10.0	8.8	45.0			2.5		113	2.6	+226%	430
Guinea-pig	4.6	0.6	1.6	6.8	75.4	3.0	6.8	0.7		188	22.5		498, 500
Beef	1.7		1.8	8.1	70.0	15.5				195			400
Hamster: corn oil	3.4		1.0	11.2	84.4					180			33
fat-free	4.1	13.3	0.7	47.8	30.1		1.4			126			
Pig	1.5	1.3	2.7	9.7	77.6	2.3				173			314
Pigeon	1.0	3.7	1.1	10.4	78.5	1.0	0.7	0.9		180	11.9		150
Heart													
Rat: corn oil	0.9	0.4	0.9	6.5	82.5		0.9	2.4	2.6	200	14.5		101, 283, 318, 367, 491, 494, 496, 533, 639, 670, 717
fat-free		16.4		46.3	10.0		7.0	6.0		128	15.0	0%	41
hydrog. fat	12.4	2.9	7.5	42.6 [b]	24.2		2.0	0.1	2.5	126			308
olive oil	2.6		2.3	22.4	51.7			7.5	5.2	204	8.4	−5%	367
soybean oil	5.9	0.5	9.0	9.7	51.0	1.2		3.2	2.0	140	9.8	+23%	318
tr,tr-18:2($n-6$)	1.5		1.5	18.0	70.0			2.0		166			115
22:1($n-9$)	5.6	2.0	2.0	19.3	62.1	0.8	1.3	1.2	0.8	155	16.7	−2%	45, 151, 152, 321, 735
fish oil	7.9	0.7	2.7	22.9	25.8	0.8	0.7	5.0	33.5 [a]	300	9.0	−2%	90, 729
menhaden oil	1.8	1.5	0.5	12.6	57.6		1.5	3.3	15.4 [a]	247	15.4	−22%	639
cod liver oil	6.6	1.5	4.4	9.8	67.5			1.1	3.4	172	12.4	+59%	234
3-month-old	9.7		3.1	51.0	32.8		0.9	0.5	0.6	120	14.4		358
24-month-old	12.3		5.2	42.3	32.0		1.8	2.3	3.3	141	13.9	−3%	
norepinephrine	3.3	1.9	3.0	7.7	74.5			2.0	8.6 [a]	213	9.9	−4%	170
hypothyroid	3.1		5.6	7.4	55.4			3.4	1.9	171	14.5	−6%	283
hyperthyroid 3 d	2.8		3.7	11.6	64.0			3.9	1.4	178	14.9	−4%	
5 d											27.5	+37%	494
hypertrophy				7.1	83.5			1.3	2.1	192	11.6	−11%	533
Human	4.3	4.7	1.9	13.8	73.8	2.1				169	16.9		213, 214, 479
male	5.3	2.7	5.9	9.7	64.1	0.8	0.3	5.4	1.2	177	12.5		550
female	4.4	2.6	5.2	8.9	68.4	0.7	0.3	4.8	1.0	180	14.5		
Dog				9.6	90.4					190	15.9		669, 693
Rabbit, control	11.5		6.9	12.7	62.4			3.7	1.5	161	20.2		382
K^+-depleted	8.9		3.8	12.5	71.0			3.5	1.0	176	21.1	+5%	
Pig: control	11.0	3.6	6.5	11.5	63.2	1.7		2.6		157	19.6		116, 369, 584
22:1($n-9$)	6.5		10.8	22.6	48.1	1.5		0.7		136	18.7	−5%	369
ischemic	1.6	9.8	1.6	17.0	68.1					163	14.5	−24%	593
Beef	0.9	2.3	1.2	7.1	83.8	5.0				192	14.0		190, 696
purified	0.4	0.6		6.1	92.0	0.7				193	35.0		306, 612, 720
Chick: sunflower oil	8.7				76.5	12.0($n-6$)		2.9		200			537
22:1($n-9$)	8.1			28.4 [c]	47.1	9.3($n-3$)		7.2		179			
Frog	1.2	1.9	0.8	4.2($n-9$) 8.2($n-7$)	69.8	11.5($n-3$)	0.2	0.6		191	9.3		[d]
Kidney cortex													
Rat: corn oil	2.7	0.8	1.0	10.8	76.3		2.7	4.1	0.8	197	8.0		107
diabetic	2.1	0.8	1.1	10.9	79.7		1.9	2.2	0.3	193	8.6	+8%	
fat-free		21.2		55.1	4.7		3.7	4.2		114			41

TABLE III (continued)

Tissue Cell Diet/Rx	Fatty acyl (% of total fatty acyls in cardiolipins)									UI	%CL/Σ PL mitochondria		Refs.
	16:0	16:1	18:0	18:1	18:2	18:3	20:3	20:4	22:6		per Σ P	Δ% vs. control	
Pig	5.8	3.1	1.5	15.6	74.0					167			224
18:3($n-3$)				10.0	52.0	12.0($n-3$)	13.0($n-3$)	2.0[a]		197	5.7	0%	585
18:1($n-9$)				12.5	57.0	9.0($n-3$)	10.5($n-3$)	2.5[a]		195	5.7	0%	
Skeletal muscle													
Rat	6.5	1.2	3.3	8.6	77.6	1.3	0.5	1.3		176	1.4 to 10.6		63–65, 166, 476, 606
Mouse [c] 18 C	19.7[a]	1.4	21.7	12.8	20.7	2.6		12.2		112	8.7		179, 607
5 C	34.0[a]	0.3	40.6	2.6	10.7		5.1		1.3	46	10.5	+21%	
Mouse [b], normal	10.3	7.9	3.5	24.8	36.8		2.5	1.6	12.7	199	5.4		503
dystrophic	7.3	5.9	3.5	22.5	40.4		2.3	1.8	16.3	221	4.5	−10%	
Rabbit	5.5	0.8	4.0	8.6	76.0	4.6	0.9	0.5		174			373
Human > 2 mos	3.5	1.4	3.8	13.7	73.3	2.0	1.1	1.6		172	9.5		63, 64, 373
fetal		10.0		35.0	40.0					125	2.5	−74%	
Monkey	1.8	1.2	0.9	14.8	80.9					178			417
Pig	22.8[a]	3.5	10.0	24.4	32.3	0.9		3.2		108			584
Pigeon breast	0.4	0.7	2.2	12.8	84.0					180	9.0		224
Chicken	5.5	1.6	6.6	21.8	61.0	1.4		2.1		158			373
Trout	19.1	4.1	13.5	44.6		6.5($n-3$)			1.7[a]	77	4.0		224
Carp 32°C	2.0	2.0	2.0	5.0	64.6	2.6($n-3$)	4.3($n-6$)	3.5	8.0[a]	227	14.9		713, 714
10°C	11.3	2.2	1.0	17.9	39.0	1.0($n-3$)	3.4($n-6$)	2.1	7.5[a]	184	15.3	+3%	
Locusta migratoria	6.6	2.7	2.0	8.7	63.0	16.8($n-3$)				188			[i]
Hyalophora cecropia	2.5	3.4	1.6	2.2	9.2	71.4($n-3$)				238	15.4		653
Adipose, brown													
Rat: 28°C	13.9	16.1	5.4	38.0	19.8			6.8		121	13.5		591
22°C	10.4	7.3	8.5	30.3	39.0	0.7		4.1		134	15.5		74, 540, 541
5°C	15.8	3.4	7.2	24.7	44.3			4.2		134	12.3	−15%	
28°C: hydrog. fat	14.6	19.6	5.6	51.5	2.8		3.4($n-6$)	2.5		97	13.3		591
5°C: hydrog. fat	19.6	15.2	5.5	46.5	7.0		4.6($n-6$)	1.3		93	12.4	−7%	
22°C: + T4	10.4	9.8	6.4	30.5	40.1			2.8		132	9.3	−16%	540, 541
Hamster: 22°C	12.5	(20.8)	23.6	12.5	22.2	2.8		5.6		109	7.0		74
4°C	11.1	4.9	6.2	24.7	49.4	3.7				140	8.0	+14%	
Mouse	12.6	4.0	11.9	22.2	31.7	3.0	6.5			118	13.6		626

Lung: Pig	6.3	2.1	4.9	13.8	67.1		1.5	2.5		165			224
Rat	22.5		43.8	15.4	15.2					46	5.7		118
Adrenal cortex													
Rat	23.4 [f]	3.3	11.7	30.0	15.0		3.3	13.3		126	9.1		111
Beef	41.5 [f]	1.3	24.4	12.8	11.9		0.2	0.5		45	6.0		253
Dog	13.0	2.0	4.8	20.0	45.2	1.4	2.3	4.3	4.0	165	5.5		88
Gastrointestinal mucosa													
Rat: corn oil	3.2	3.2	1.1	17.4	67.6			2.0		164	2.0		740
hydrog. fat		17.9		40.3	30.8					120	2.0		
fat-free	5.8	16.8	3.1	42.2	3.5		4.4	2.1		88			41
Pig	19.9		7.1	23.6	41.1			7.2		131	5.6		590
Rabbit	24.0		8.5	17.3	26.1			2.9	10.0	141	7.3		
Frog	14.4	4.4	5.7	24.0	41.1	8.2				131	5.8		
Locusta migratoria	4.0	3.3	1.6	10.7	70.4	10.0($n-3$)				185			[i]
Testis: rat	55.1		15.7	14.5	13.2					41	6.0		118
mouse	57.0		2.0	12.0	11.0			3.0	7.0 [d]	84	3.0		229
Spleen: rat	12.8		21.6	15.4	48.6					113	7.1		118
Cartilage, epiphyseal:													
chick	25.4 [f]	10.0	22.6	26.4	5.6		0	0.5	(3.2)	71	1.9		725
Brain													
Rat: corn oil	4.6	3.4	2.5	38.6	13.1		2.3	20.5	13.4	235			729
fish oil	4.7	5.4	2.5	43.5	6.6		2.3	19.8	14.8	237			
fat-free	9.5	6.3	10.2	42.7			2.2	5.5	11.1	144			41
Lymphocytes: quiesc.	33.6 [f]		17.0	7.3	5.6		1.2	15.2	3.9 [d]	127	5.3		14
transf.	33.9 [f]		15.5	10.6	9.4	1.5	0.9	12.6	3.7 [d]	128	5.4	+ 2%	
Jensen sarcoma	7.1	2.9	5.5	26.1	54.6	1.7		0.4		145	2.2		162
Hepatoma 7288C	16.0	1.2	25.1	26.0	16.8			7.3	4.3	116	4.7	− 11%	444, 536, 722, 743
host liver	6.1	1.0	3.3	14.3	70.6			1.0	0.9	166	5.3	− 29%	

[a] Plus about 5% 20:5($n-3$)+22:5($n-3$). Contains
[b] 21.1% *cis*-18:1, 21.5% *trans*-18:1;
[c] 4.1%-6% 22:1;
[e] Comprises 17.4% 20:1($n-11$)+11.0% 22:1($n-9$).
[f] 10:0+12:0+14:0+16:0;
[g] Arctic mice *Microtus pennsylvanicus* (fatty acyls/ca. diolipin), *Clethrionomus rutilus* (cardiolipin/phospholipid);
[h] phenotypically normal and dystrophic mice, Bar Harbor strain C57BL/6J.
[i] 22:5;
[r] 14:1+16:1.
[s] R.L. Wolff, personal communication.

fatty acyl groups in cardiolipins, measure inner membrane composition, and appear to correlate with specific oxidative activity, based on evidence discussed here and elsewhere. Cardiolipin P per total phospholipids P shows the cardiolipin contribution to the total fatty acyl groups of the membrane, since there are two acyls for each P atom of either cardiolipins or other phospholipids. Mol-fraction of cardiolipin/total phospholipids denotes the contribution of the -P-glycerol-P- grouping of cardiolipins to the polar groups of the membrane.

By the usual chloroform/methanol extractions, rat liver mitochondria contain some 300–500 nmol of phospholipids per mg protein [386]. We found 190 nmol of total phospholipids per mg protein, including 11 nmol of cardiolipin P per mg [291]. Cardiolipin P per phospholipid P ratios are 10–25% in most tissues [136,190,305,697] (Table III).

As much as 35% cardiolipin P per phospholipid P can be recovered from beef heart [612]. Contents of cardiolipins are of the same order as matrix adenine nucleotides, pyridine nucleotides and perhaps divalent cations, and several orders greater than the amounts of matrix proteins. Little attention has been given to variations in the small amounts of cardiolipin on the outside layer of the inner membranes, which determine specific binding to certain proteins.

IV-B. Natural variation

Animal cardiolipins vary widely in fatty acyl distribution, contrary to statements that cardiolipin normally is very homogeneous, with 60–80% linoleoyl chains [89,582]. Some physiological variations in amounts of cardiolipins per amount of tissue [136] and in percentage fatty acyl distributions [320] have been reviewed. Several in vivo factors are associated with distinctive patterns of cardiolipin fatty acyl compositions and cardiolipin/phospholipid ratios (Table III): development and aging, tissues and species, diet fatty acids, ethanol feeding, Mg-deficiency, environmental temperature, altered hormonal states and pathological conditions. In some cases, mitochondrial respiration varies with cardiolipin compositions.

IV-B.1. Development and aging

The life-cycles of various prokaryotes involve lipid changes. During embryogenesis in Amphibia, from oocyte → gastrula, mitochondrial cardiolipin/phospholipid ratios increase minimally while cardiolipin fatty acyl composition and mitochondrial function do not change. After gastrulation, State 4 respiration and mitochondrial replication accelerate. In fetal → postnatal development of rats, and perhaps during maturation to adults (4 months), cardiolipins and mitochondrial oxidative phosphorylation change greatly. Changes in senescence (> 30 months in rats) have elicited much less agreement among observers.

Unfertilized eggs of *Xenopus laevis* accumulate fuel and metabolite precursors and store 10^7 mitochondria per cell to be used during embryogenesis – the oocytes contain 10^5 more mitochondria than somatic cells [91]. The egg cell contributes all the mtDNA to the fertilized zygote: mitochondrial inheritance is maternal in *Xenopus* [137] and in the higher animals including humans [212,602]. Most of the human mtDNA codes for polypeptides in complex I and cytochromes *b* and aa_3 (see Ref. 75). Embryogenesis in nonmammalian species involves rapid cell division of the large fertilized oocyte, without increase in total mass. The fertilized egg divides to 64 cells in 4 h, 10^4 cells at 6 h (blastula) and $3 \cdot 10^4$ cells at 10 h (gastrula) [6]. Oocyte mitochondria are diluted among daughter cells until gastrulation, when new mitochondrial rRNA synthesis starts [91,692]; oocyte cardiolipins are conserved in embryogenesis.

Cardiolipins in mitochondria of unfertilized eggs of the toad *Bufo arenarum* are about 7% of total phospholipids and have characteristically high contents of 18:2 (55%) and 18:1 (28%) acyls [11,48] (Table III). Cardiolipin/phospholipid proportions increase slightly while cardiolipin fatty acyl composition remains constant during development from fertilized egg to gastrula stage. Mitochondrial cardiolipins increase $^{32}P_i$-uptake minimally up to gastrulation, whereas phosphatidylcholines, phosphatidylethanolamines and phosphatidylinositols turn over progressively faster [11]. Mitochondrial function up to gastrulation has adult characteristics in terms of relative rates of substrate oxidation, and inhibitor patterns [385,568], but calculated State 4 respiration (25°C) is only about 2 ng atom O min^{-1} (mg mitochondrial protein)$^{-1}$. State 4 accelerates 4-fold after gastrula stage: the new mitochondria leak protons faster than their maternal precursors.

Ryuzaki [567] reports that relative amounts of phosphatidylcholines (50%), phosphatidylethanolamines (20%) and phosphatidylinositols (1–2%) remain constant in the post-gastrula embryonal development of *Rana nigromaculata* to the tadpole stage. Chromatograms of egg and embryo lipids show progressively greater amounts of unidentified frontal phospholipids (usually cardiolipins and phosphatidate) which seem to be some 25% of total phospholipids in early tadpole stages.

Development: the liver, its membranes and its lipids restructure remarkably during natural or thyroid-induced Anuran metamorphosis. Hepatic synthesis of phospholipids in endoplasmic reticulum and mitochondria becomes rapid in > 4-day-old *Xenopus* larvae, when T3-binding appears [641–643]. In endoplasmic reticulum, the phospholipid-subclass ratios persist.

The phospholipid/neutral lipid ratio is 0.5 in livers of *R. catesbiana* tadpoles, 3 in frogs [282,287] and 1.5 in *R. esculenta* frogs [709]. Organelle membranes are vesicular in tadpoles, bilamellar in frogs [641]. Tadpole liver total lipids contain 30% polyunsaturated fatty acyls, 45% monounsaturated fatty acyls; heart lipids have 30% polyunsaturated fatty acyls, 25% monounsaturated fatty acyls. Frog livers have high polyunsaturated fatty acyl contents [29,282,287]. The linoleoyl acyls in *Rana esculenta* livers are exogenous, since 18:2 is essential [29]. Both tadpole and frog livers convert 18:2 to $(n-6)$-polyunsaturated fatty acyl derivatives very actively, but during metamorphosis phospholipids are synthesized that retain the polyunsaturated fatty acyls produced – perhaps cardiolipins; we have no measurements. Cardiolipin/phospholipid ratios are 2% in frog liver and skeletal muscles, 5% in kidney and 9.3% in heart [561,606,709]. To the extent that the cardiolipin fatty acyl composition of oocytes represents that of somatic cells, adult *Bufo* cardiolipins have high 18:2 acyl contents like rat liver mitochondrial cardiolipins, but higher contents of 18:1 and significant amounts of an 18:3 acyl.

Cardiolipins in insect flight muscles increase linearly and strikingly over the 5–9 days when pupae develop into adults: from 7% to 20% of mitochondrial phospholipids in tobacco horn worms [85] and from 1.3% to 12% in *H. cecropia* [653]. The highly unsaturated cardiolipins of *Cecropia* adults contain 71% $18:3(n-3)$ acyls derived from the phytophagous habits of these insects, plus 9% 18:2 acyls and 6% monounsaturated fatty acyls (Table III).

Cardiolipin contents of livers [325] and kidneys [394] of fetal rats are half of adult levels, and in fetal human skeletal muscles, one quarter [63–65]: fetal cells contain few mitochondria. In addition, the cardiolipin/phospholipid ratio in liver mitochondria of fetuses is less than half that at 3 weeks after birth [390,441]: fetal mitochondria have low cardiolipin contents. The relative rates of cardiolipin synthesis are, respectively, 1:4 in heart mitochondria from adult and neonatal rats, 11:22 in liver mitochondria of neonatals and adults [635].

Fetal tissues also have few 18:2 acyl groups. The 6% of 18:2 acyls in liver phospholipids of 1- and 5-day-old rats doubles by day 15 and triples by day 25 and in adults [589]. Fetal rat liver cardiolipins contain 40–60% 18:2 acyls and 30–50% monounsaturated fatty acyls, adult liver cardiolipins have 60–80% 18:2 and 20% monounsaturated fatty acyls [354,441,707]. Even more strikingly, skeletal muscle cardiolipins of human fetuses are 40% in 18:2 and 45% in monounsaturated fatty acyls, which shifts to 90% 18:2 and less than 10% monounsaturated fatty acyls by 2 months after birth [63–65]. Low fetal 18:2 contents reflect a cardiolipin-deficit or an inability to enrich cardiolipins with 18:2 acyls. Although the pattern resembles that in EFA-deficiency, phosphatidylcholines and phosphatidylethanolamines contain as much 18:2 in fetal as in adult livers [441]; adult EFA-deficiency depletes 18:2 acyls in these fractions (see section IV-C.1.a). In liver mitochondrial cardiolipins of 1-day-old rats, 18:2 acyls are 48% of total fatty acyls, 58% on day 3, 63% on day 15 and 79% (adult level) on day 25; 18:1 acyl contents decline reciprocally [441]. Thus, linoleoyl acyls replace monounsaturated fatty acyls during suckling more slowly than fed 18:2 acid reverses EFA-deficiency in adult rats (Table III), perhaps because the necessary enzymes must develop.

Mitochondria from livers of fetal (term) or 1-day-old rats leak protons under State 4 conditions up to twice faster than do those from adult rats, and still phosphorylate ADP efficiently [25,510]. State 4 respiration halves between day 2 and 9; slow proton-leakage and the adult cardiolipin high-18:2, low-monounsaturated fatty acyls profile develop in parallel. In contrast to adults, the major regulators of State 3 respiration in liver mitochondria of term fetuses are ATP-synthase and P_i-carrier, and the dicarboxylate carrier and ADP/ATP carrier are less important [25] (see section II-B). Mitochondria from maturing rat brains also leak protons, but transiently. State 4 respiration is very slow in the first week postpartum, 6–9-fold faster in weeks 2 and 3, and less rapid thereafter [296]. Maturation of rat brain depends on adequate thyroid hormone levels, and fetal hypothyroidism produces damage that is not reversed by thyroid administration after week 2 postpartum [225].

Chondrocytes develop in epiphyseal growth plates both pre- and postnatally. The fatty acyl patterns of membrane phospholipids of growth plate cartilage cells from young chicks, fetal calves or newborn pigs resemble those of livers from EFA-deficient animals, although the phospholipids of their muscles, liver, bones and sera show normal fatty acyl patterns [4,5]. Chondrocyte membrane levels of $18:2(n-6)$ and $20:4(n-6)$ acyls are very low, amounts of monounsaturated fatty acyls are high, and Mead acid $20:3(n-9)$ appears in quantity in phosphatidylethanolamines and phosphatidylinositols. With aging, the cartilage of weight-bearing joints linearly accrues $(n-6)$-unsaturated fatty acyls that displace the $20:3(n-9)$ acyls. An apparent localized EFA-deficiency in developing cartilage has been attributed to (i) a limited access of plasma EFA-albumin complexes to cartilage cells caused by the dense impermeable matrix of proteoglycans and collagen that also seems to slow amino acid exchanges; (ii) the observed rapid metabolism of 18:2 and 20:4 acyls in growth plate cells; and (iii) the high activity of chondrocyte $\Delta 9$-desaturase. Cardiolipins of isolated epiphyseal chondrocyte membranes prepared with collagenase digestion comprise only 1.9% of phos-

pholipid P [725,726]: chondrocytes contain few mitochondria. Cardiolipin fatty acyls in chick chondrocyte membranes are mostly monounsaturated fatty acyls (36%) and saturated fatty acyls (48%), whilst 18:2 acyls are only 5.9%; unsaturation index is 71 and no 20:3(n - 9) fatty acyls are present [725] (Table III). This pattern is seen in cardiolipins of other developing cells in fetal liver and in hepatomas (Table III); in contrast, in EFA-deficiency 18:2 acyls are depleted, only monounsaturated fatty acyls are high, and liver cardiolipin/phospholipid ratios are normal.

Oxidative phosphorylation in chondrocyte mitochondria apparently depends on the preparative methods, which are difficult because cartilage is dense and has few mitochondria. Chick chondrocyte mitochondria prepared by high-speed homogenizations and differential sedimentations do not leak protons excessively: State 4 respiration (succinate, 26°C) is 40–53 ng atoms O min^{-1} mg^{-1} [383,384], as in normal liver mitochondria (Table VIII). Thus, these low-linoleate-content cardiolipins do not accelerate proton leakage, perhaps because they are so sparse – which implies that normal proton leakage can depend on phosphatidylcholines and phosphatidylethanolamines. Mitochondrial Ca^{2+} in cartilage is 10–30-fold that in soft tissues like liver; mitochondria from the hypertrophic zone of the growth plate have about 80 nmol/mg protein and those from the calcifying zone about 300 nmol/mg [594]. In the naturally Ca^{2+}-loaded cartilage mitochondria, ADP + P_i produce State 4 → 3 → 4 transitions. In liver mitochondria loaded with this much Ca^{2+}, and in mitochondria from Ehrlich ascites tumor cells, ADP + P_i stimulate a continued rapid respiration (proton cycling); hepatoma cardiolipins also have low 18:2 and high monounsaturated + polyunsaturated fatty acyl contents (see Table III).

Mitochondria have also been prepared from calf growth plate chondrocytes by pressure-disruption, sequential digestions with EGTA, trypsin, hyaluronidase and collagenase, and centrifugations [627]. Mitochondria in these cells are 4% as many, and have about the same Ca^{2+} content, as in guinea pig hepatocytes. Rates of respiration (ng atoms O min^{-1} mg^{-1}) plus succinate (rotenone, P_i?, 37°C) are 58; +ADP (State 3), 70; +oligomycin (i.e., State 4), 34; +Ca^{2+} or uncoupler, about 125. Thus, blockage of the H^+-ATPase inhibits the apparent State 4 proton-leak by 40% – as it did in the guinea pig liver mitochondria these workers used for comparisons. However, the State 3 rate in the liver mitochondria was 226 and the uncoupled rate was 290. These chondrocyte mitochondrial phospholipids do not appear to leak protons excessively but the H^+-ATPase does; the mitochondria were thought to be specialized for Ca^{2+}-transport rather than ADP phosphorylation.

Maturation in rats fed a high-fat diet (20–40% of calories as soy bean oil) from age 21 days to 4 months is accompanied by increased heart mitochondrial cardiolipin P per total phospholipids P (18% before, 22% after) and diminished cardiolipin 18:2 content [319] (Table III). These changes in inner membrane lipids are attributed to aging rather than diet and are thought to account for observed alterations in thermotropic properties and decreases in State 3 respiration and ATP-P_i exchange [106].

Senescence in laboratory rats is accompanied by depletions: in membrane phospholipids, in unsaturation of fatty acyls, and in cardiolipins and phosphatidylethanolamines among the mitochondrial phospholipids. Such changes have been measured in liver, kidney and heart phospholipids, plasma membranes, mitochondria and microsomes [227,270,471,473,545, 592,640,682,683]. Usually saturated fatty acyl contents increase reciprocally, monoene acyls remain unchanged, and 20:4 acyls decrease; 18:2 and 22:6 acyl contents are repleted or depleted in different reports. Because 20:4 acyls are a desaturation product of 18:2(n - 6), and 22:6 acyls derive from fed 18:3(n - 3), the observed decrease in fatty acyl-CoA Δ6-desaturase activity with aging [59] has been proposed as an important aging target [297]. A defect in Δ9-desaturations [196,204,205] is not accompanied by diminished membrane contents of 18:1 acyls.

Liver, heart and kidney mitochondria of senescent rats contain 25% less cardiolipin per total phospholipids than those from young rats. In liver, less cardiolipin may be synthesized because activity of the mitochondrial CTP:phosphatidate cytidyltransferase decreases [682]. According to Jakovcic et al. [326], decreased cardiolipins are associated with lessened inner membrane area. Liver mitochondria from old rats have smaller, sparser cristae in situ [644], and increased lipid structural order [682,683] and fragility [694].

Cardiolipin fatty acyl compositions in senescence have been described in two studies on rat heart mitochondria [359,391] (see Table III). In one report, young-rat cardiolipins contain 1.3% 18:2 acyls (*sic*) and 50% saturated fatty acyls while old-rat cardiolipins have 19.6% 18:2, 40% saturated fatty acyls; the other phospholipids are similarly very low in polyunsaturated fatty acyls and high in saturated fatty acyls [391]. In the other study, mitochondrial cardiolipins from either young or old rats contain 32% 18:2 acyls and about 50% 18:1 acyls [359]. The 23% diminution in 18:2 acyls of total mitochondrial phospholipids with aging is not reflected in the resolved cardiolipins, phosphatidylcholines, or phosphatidylethanolamines: some 18:2 acyls have been lost. Comparisons with heart cardiolipins of adult rats in Table III, which contain more than 80% 18:2 fatty acyl groups and more than 50% of the total mitochondrial 18:2 acyls, indicate that cardiolipins in both these studies have been extensively oxidized

during extraction and analysis. It seems likely that senescence does deplete 18:2 acyls in heart cardiolipins, from the 15–37% loss of 18:2 acyls in mitochondrial phospholipids [359,474,496] – but we do not know what acyls replace them. Loss of cardiolipin 18:2 acyls has been attributed to increased peroxidative reactions with senescence [270,473], which should selectively destroy membrane polyunsaturated fatty acyls through mitochondrial and microsomal byproducts of O_2 metabolism. However, although inner mitochondrial membranes from 14-month-old rat hearts generate more superoxide radicals than those from 3-month-old rats, no further increase occurs at 24 months [451]. Another mechanism might be a progressive loss of preventive reactions that scavenge free radicals [256].

Senescence generally slows both State 4 and State 3 respiration in various mitochondria (Table IV). State 4 and State 3 do not decrease equally in different studies; Hansford [258] finds no change in State 4 respiration. Even though these disparities make resultant respiratory control ratios irrelevant to senescence mechanisms, some workers conclude that oxidative phosphorylation is not grossly impaired in mitochondria from senescent animals because respiratory control ratios do not decrease [216,257,412]. The constancy or decrease of State 4 respiration with advanced age indicates that proton leakage does not increase, or even decreases. The variability in findings on State 3 respiration in senescent animals may well reflect the sensitivity of control coefficient values to experimental conditions [228], and the fact that different carriers regulate in liver and heart (and probably other) mitochondria, which may be why senescence has tissue- and substrate-specific effects on State 3 respiration (Table IV). A decrease in activities of carriers depresses respiration, not through changes in carrier molecules or amounts but by decreased matrix pools of exchangeable metabolites and by (perhaps related) altered inner membrane phospholipids [256,257].

In liver mitochondria, senescence decreases the ADP/ATP carrier V_{max} (ADP, 4°C) by 32%, which seems attributable to the 28% diminution in matrix content of ATP + ADP (the exchangeable nucleotides) and the 20% decrease in the rate of actual ATP exchange; concomitantly, the carrier C_i gains a value of 0.5, compared with 0.28 in mitochondria from young rats [135,228,358]. State 3 respiration (succinate, 30°C) slows by 20%. Cytochrome aa_3 is a minor regulator of State 3 oxidation, with $C_i = 0.11$, in liver mitochondria from either old or young rats [135]; $C_i = 0.17$ in young rats [228]. This is consistent with the constancy of [heme aa_3] in liver mitochondria from old and young rats. Livers from old rats contain about 30% less aa_3 than those from young rats [683], which indicates fewer mitochondria. Although the dicarboxylate carrier is the other major regulator of State 3 respiration in young rat liver mitochondria, with $C_i = 0.33$ [228], no measurements are available of its contribution to slowed succinate oxidation in senescence.

In heart mitochondria, senescence alters sedimentation behavior [449] and Arrhenius profiles of the F_1F_0-ATPase, the dehydrogenases specific for succinate, glu-

TABLE IV

Effects of senescence on mitochondrial cardiolipin / phospholipid ratios and respiration in state 4 and state 3 (25° - 35°C)

Abbreviations as in Tables I and II. Data are shown as means of percentage values versus young controls (Δ%).

Mitochondria	CL/ΣPL		Respiration			
	Δ%	Refs.	substrate	Δ% State		Refs.
				4	3	
Liver	− 23	358, 682	succinate	3	− 16	135, 301, 302, 358, 682
			glutamate/malate	− 9	− 32	12, 362
			β-hydroxybutyrate	− 25	− 12	216
Heart	− 26	227, 359, 391, 474, 496	succinate	− 12	+ 5	135
			glutamate/malate	− 13	− 10	12, 96, 102, 451
			pyruvate	− 20	− 22	359, 496
			β-hydroxybutyrate	− 20	− 18	216
			palmitoylcarnitine	0	− 27	96
Kidney	− 20	227	β-hydroxybutyrate	− 24	− 21	216
Muscle			glutamate/malate	− 8	− 19	96
Brain			succinate	− 9	− 4	96, 148, 149, 256, 681
			glutamate/malate	− 41	− 30	
			pyruvate/malate	− 23	− 27	

tamate, and β-hydroxybutyrate, and the succinate oxidase [471,472]. In vitro application of detergent restores normal activity–temperature relationships and (thereby) restores depressed rates, which further indicates that altered membrane lipids, rather than proteins, are responsible [471]. Senescence depresses State 4 respiration when glutamate/malate, pyruvate or β-hydroxybutyrate (but not palmitoylcarnitine) is oxidized (Table IV). With succinate, O_2-pulsing under State 4 conditions produces similar H^+/O ratios (proton-slip) in young and old [412], indicating that proton permeability diminishes with age.

In heart mitochondria from old rats, as compared with young rats, respiration under State 3 conditions slows when glutamate/malate, pyruvate, β-hydroxybutyrate or palmitoylcarnitine (but not succinate) are substrates (Table IV). Depressed activities of several carriers in the inner membrane are involved in this down-regulation. (i) ADP/ATP carrier rate depression should affect all substrate oxidations. Without measurements of C_i it is difficult to assess how important observed 40% decreases in senescent rat heart mitochondrial ADP/ATP carrier activity at 6°C [474] are in the slowing of State 3 respiration at ≥ 25°C. This carrier does not regulate in hearts of adult rabbits [355,356] (see Table I), although it does in liver mitochondria. Old-heart mitochondria have as many available ADP/ATP carrier sites as young mitochondria, but matrix ATP + ADP is 20% less [359,474]. Nohl and Krämer [474] attribute decreased activity to decreased cardiolipin/phospholipid and phosphatidylethanolamine/phospholipid ratios that depress membrane fluidity. Kim et al. [359] downplay any role of matrix ATP + ADP concentrations because adenine nucleotide depletion is less severe than in liver mitochondria, and the progress of the depletion does not correlate with the depression of myocardial respiration.

(ii) Monocarboxylate carrier. Senescence slows pyruvate oxidation under State 3 conditions (25°C) in heart mitochondria (Table IV). The pyruvate transporter in heart mitochondria from young rats contributes to the State 3-regulating C_i value of 0.37 measured from rotenone titrations of the NADH dehydrogenase [155], and pyruvate transport limits pyruvate oxidation in rat heart (but not liver) mitochondria [596]. Senescence slows pyruvate uptake at all temperatures between 4°C and 28°C, and shifts the transition temperature in Arrhenius profiles from 16°C → 21°C, without changing the number of available carrier sites or ΔpH (this transporter responds sensitively to ΔpH across inner membrane). These findings suggest that the observed changes in fatty acyl composition of phospholipids and decrease in cardiolipin/phospholipid affect the carrier activity through specific binding to cardiolipins. (iii) The palmitoylcarnitine-carnitine translocase V_{max} (5°C) decreases by 35%, and State 3 oxidation diminishes by 27% (Table IV) in heart mitochondria of senescent rats [257]. Translocase first-order rate constant for exchange remains constant, but matrix contents of carnitine and exchanged-carnitine are 35% and 22%, respectively, of the youthful values; the carrier molecules do not change, the concentration gradient for exchanges falls. This translocase is quite specific for cardiolipins (see Table I; Ref. 284).

(iv) Two mitochondrial carriers of Ca^{2+}, the uniporter for uptake and the $Ca^{2+}/2Na^+$ antiporter for egress, operate in senescent-heart mitochondria at rates 30% below those in mitochondria from hearts of young rats [258]. Some workers think that State 4 respiration involves $Ca^{2+}/Na^+/H^+$ cycling, which might account for slowed State 4 rates, but should extend to all substrates; no one has shown that Ca^{2+} normally regulate State 3 rates significantly.

In brain mitochondria oxidizing glutamate/malate (25°C), decreases in respiration (Table IV) are accompanied by sharply diminished (by 68%) glutamate uptake, probably involving the electroneutral glutamate/OH^- antiporter [681]. The authors believe that this may be a specific effect of senescence-induced membrane lipid changes which do not alter succinate and pyruvate oxidation rates – but they did not measure succinate or pyruvate transport. With succinate as substrate, plots of $\Delta\psi$ vs. proton leakage rates calculated from State 4 respiration are similar in young and old; calculated C_{H^+} are 1.37 and 1.22 ng ion H^+ min^{-1} mg^{-1} mV^{-1}, respectively. For glutamate/malate oxidation, C_{H^+} corresponding values are 0.70 and 0.42.

Attempts to delay senescence are interestingly related to phospholipid and EFA metabolism. Repeated cold-induced hibernation, which starves the animal while decreasing caloric output, prolongs the life of Turkish hamsters by 50% [398]. Hibernation in ground squirrels increases heart membrane 18:2 content of total lipids 3.4-fold (8.5% → 28.6%) at the expense of halved 16:0 and polyunsaturated fatty acyls contents [528]; no cardiolipin fatty acyl composition was presented but because most heart 18:2 acyls are in cardiolipins, involvement of cardiolipins seems likely. Caloric restriction (by −40%) extends life span ~40% in laboratory rats [186,416,423,737,738]; 21 days of food restriction stimulates the Δ6-desaturase 3-fold and the Δ9 enzyme by 36% [180]. Recent publicity on anti-senescence effects of growth hormone might be considered here in the light of studies showing that rat liver mitochondrial cardiolipin/phospholipid ratios vary directly with growth hormone levels (Table III; see section IV-C.2.d).

IV-B.2. Tissues, species

An early study of rat tissues [118] showed that cardiolipin/phospholipid ratios were 15–20% and cardiolipin 18:2 acyl contents are more than 75% of fatty

acyl groups in heart and liver, less in skeletal muscle and spleen, and lowest in testis and brain (about 1–2%), even though rat brain contains 0.6 μmol of cardiolipins per g wet weight [104]. In all these cardiolipins, saturated fatty acyls contents were inverse to 18:2 acyl contents. Improved methods [612,720,729] confirm the differences in cardiolipin contents, but reveal polyunsaturated fatty acyls instead of saturated fatty acyls in several tissues. Beef heart cardiolipins contain hardly any saturated fatty acyls [720]. Rat brain cardiolipins have saturated fatty acyl contents as low as liver cardiolipins, little 18:2, and high contents of 20:4($n-6$) and 22:6($n-3$) polyunsaturated fatty acyls [729].

Intratissue heterogeneity has recently been described. Cardiolipins differ in mitochondria of hepatocytes of the periportal and perivenous zones of rat liver lobules [79]. Percentages of 18:2 acyls, monounsaturated and saturated acyls are, respectively, 75:15:5.4 in periportal cardiolipins and 63:20:11 in perivenous cardiolipins. Cardiolipin P contents per mitochondrial phospholipid P are 16.1% and 15.4%.

Species: purified cardiolipins from beef hearts contain more than 90% 18:2 fatty acyl groups and about 7% other unsaturated acyls [612,720]. Beef heart mitochondria respire slowly in State 4: 6.7 and 26.6 ng atom O min^{-1} mg^{-1} (succinate and pyruvate/malate, respectively; 30°C) [613]. Mitochondria from the hearts of chicks contain cardiolipins with 91% polyunsaturated fatty acyls and 9% saturated fatty acyls [537]; they respire in State 4 (pyruvate/malate, 30.5°C) not at all in many cases, or at a rate of 3 ng atom O min^{-1} mg^{-1} while State 3 is 115-fold faster [659]. Thus, these highly unsaturated cardiolipins are associated with slow proton leaks (low P_{H^+}), but without corresponding measurements of Δp we can not be certain that this is due to low $C_{M^{H^+}}$.

Rat skeletal muscle cardiolipins are heterogeneous. The amounts of cardiolipins (a measure of mitochondrial content) in white muscle, intermediate muscle, red muscle and heart muscle, are 1:2.5:5:10; mitochondrial cardiolipin/phospholipid ratios (which register membrane composition) are 1:2.5:2.5:3.5, respectively [476]. This accounts for the great range of cardiolipin/phospholipid ratios in various studies, but 18:2 acyl contents are almost 80% (Table III). (The mitochondrial electron transport and α-glycerophosphate dehydrogenase components of rat skeletal muscle fast-twitch and slow-twitch fibers are also heterogeneous in their response to exercise [523] and thyroid state [705,706].) Cardiolipins of human muscle and pigeon breast muscle also have very high 18:2 acyl contents. Skeletal muscle cardiolipins of mice (the Arctic mouse *Microtus pennsylvanicus* and Bar Harbor strain C57BL/6J) have 20–50% 18:2 acyls (Table III). The Bar Harbor mouse cardiolipins are repleted in 22:6($n-3$) acyls (their muscle phosphatidylcholines and phosphatidylethanolamines even contain 2 to 3-fold more 22:6) [503], like heart muscle cardiolipins from rats fed diets containing fish oils. The fatty acids of a mouse laboratory chow include 4.3%($n-3$)-unsaturated fatty acids [431] which are absent from rat chows; 3 months on this diet doubles 22:6 acyl contents in liver and kidney total phospholipids. Pig muscle cardiolipins have relatively low 18:2 acyl contents, slightly higher monounsaturated fatty acyls, and definitely higher saturated fatty acyls that include, uniquely, significant amounts of 14:0, 12:0 and 10:0 acyls (Table III). (Pig muscle phospholipids are all unusually saturated and lack ($n-3$)-polyunsaturated acyls [116,127,369,485,585], probably because fed 18:3($n-3$) fatty acid competes poorly with fed 18:2($n-6$); these pigs are sensitive to the malignant hyperthermia syndrome, see section IV-C.2.c.) Fish (trout) skeletal muscle cardiolipins apparently lack 18:2($n-6$) and have few 18:3($n-3$) acyls but have high contents of saturated and 18:1 fatty acyls (80%) [224].

Hamster BAT mitochondrial cardiolipins are less unsaturated than rat BAT cardiolipins, and hamster BAT mitochondria have half the cardiolipin/phospholipid ratio (see section IV-C.2.c).

Plant cardiolipins are all in the inner membrane of mitochondria, where they comprise 15–25% of mitochondrial phospholipids [43,44]. Plant cardiolipins include 65–94% 18:2($n-6$) + 18:3($n-3$) fatty acyls, but no longer or more unsaturated fatty acyls (Table III). The unsaturation index under some conditions is more than 235, which is greater than in mitochondrial phosphatidylcholines and phosphatidylethanolamines, and exceeds the unsaturation index of cardiolipins in animal mitochondria, except for those from heart and brain of rats fed fish oils. These high unsaturations may all denote phospholipid adaptations to low environmental temperatures. Plant lipid fatty acyl compositions are regulated by temperature and illumination [202], and the mitochondrial phospholipids adjust to the appropriate metabolic role. The unsaturation index in cardiolipins results mostly from the ratio of 18:2/18:3 acyls. Spinach petioles (leaf stalks) do not perform photosynthesis; their mitochondria have high contents of respiratory chain components; the cardiolipins (together with phosphatidylcholines and phosphatidylethanolamines) contain more 18:2 than 18:3 acyls [166] (Table III). In spinach leaves, that perform photosynthesis, cardiolipin 18:3 > 18:2, and mitochondrial respiratory components are fewer. Cardiolipins from mung beans and their mitochondria also have more 18:3 than 18:2 acyls.

Cardiolipins from mitochondria of sycamore cells cultured at very low (10 μM) [O_2] contain 25% 18:1, 44% 18:2 and 24% 18:3 acyls [43,44] (Table III); phosphatidyl-cholines, -ethanolamines, -inositols and -glycerols contain 55–80% unsaturated fatty acyls: the

necessary Δ9-, Δ12- and Δ15-desaturations must require little O_2. When these cells grow in the presence of 250 μM O_2, cardiolipin/phospholipid ratios do not change, but in cardiolipins 18:2(*n* − 6) progressively replaces most of the 18:1 acyls, while 18:3(*n* − 3) remains constant; in mitochondrial phosphatidylcholines and phosphatidylethanolamines, both 18:2 and 18:3 increase. The stoichiometric shift from 18:1 acyls to 18:2(*n* − 6) and 18:3(*n* − 3) acyls probably results from increased oxygen-dependent Δ12- and Δ15-desaturations. However, saturated fatty acyl content of each phospholipid changes minimally. Growth in oxygen also fails to alter mitochondrial respiratory rates in State 4, State 3 and State 3u, or the thermotropic properties of States 3 and 3u. Therefore, State 4 proton leakage seems to correlate with the constant saturated fatty acyl contents of cardiolipins (and other phospholipids) rather than the altered distributions of unsaturated fatty acyls – which is consistent with the direct relationship between saturated fatty acyl contents in cardiolipins and State 4 proton leakage observed in other mitochondria. These plant mitochondria oxidize very rapidly: State 4 respiration (succinate, 25°C) is 130 ng atom O min^{-1} mg^{-1} (cf. about 40 in rat liver mitochondria, see section IV-C.2.d), and State 3 four times faster; and the Arrhenius profile of uncoupled respiration remains inflected like that of State 3.

However, comparisons with animal mitochondria may not be valid. Plant (turnip and pea leaf) mitochondria transport additional electrons through bypasses that do not pump protons, around complex I and the oxidase (see Ref. 442), which can contribute considerably to State 4 respiration. In State 4 (malate, 25°C), pea mitochondria can be calculated to conduct at a $C_{M^{H^+}}$ of about 18 ng ion H^+ min^{-1} mg^{-1} mV^{-1}. State 4 in turnip mitochondria is not regulated by carrier proteins; State 3 is regulated mostly by cytochromes bc_1 and aa_3, and less by the ATP-synthase [490].

Chlorella vulgaris cardiolipin unsaturation is relatively low, and increases slightly after illumination, through accumulation of monounsaturated fatty acyls (Table III). *Tetrahymena pyriformis* mitochondrial cardiolipins contain few saturated fatty acyls (7%) and mostly unsaturated fatty acyls (83%), like heart cardiolipins; these cells must biosynthesize 18:2(*n* − 6) and 18:3(*n* − 6) from 18:1 fatty acyls by Δ12- and Δ6-desaturations, as plants do. However, *T. pyriformis* seems to have no Δ15-desaturase, as judged from the absence of (*n* − 3)-polyunsaturated fatty acyls in any of its phospholipids [337]; the fatty acyl profiles of the phosphatidylcholines + phosphatidylethanolamines (50% of phospholipids) are similar to those of the cardiolipins. *Acanthamoeba castellani* cardiolipins contain few 18:2 or 18:3 fatty acyls, 50% 18:1, and, uniquely, about 30% of 20:3(*n* − 6) + 20:4(*n* − 6) acyls [664] (Table III).

IV-C. Experimental and pathological variation

The fatty acyl composition of cardiolipins varies with (i) input of 18:2(*n* − 6), an essential fatty acid not synthesized by animal cells, in the diet or culture medium; (ii) input of fatty acids that affect the metabolism of 18:2; and (iii) physiological or pathological alterations in the metabolism of fatty acyls and phospholipids. Diets do not generally change cardiolipin/phospholipid ratios, altered metabolism does, both alter cardiolipin fatty acyl compositions. Metabolic changes have been observed during ethanol-feeding, cofactor (Mg^{2+}) deficiency, environmental temperature variations, altered hormonal states, aging and development, tissue ischemia, cell transformation and in some specific disease states, as is discussed below. Obviously, mitochondrial fatty acyl composition can be more precisely and extensively manipulated, either through fatty acyl supply or induced metabolic changes, in cultured aerobic cells than in living animals. This advantage must be weighed against the uncertainty of equating viability of cell cultures with normal cell function and compatibility in whole organisms. For this reason, insights from whole animal experiments seem valuable in interpreting events in cell suspensions. Some changes in cardiolipin fatty acyl unsaturation and in cardiolipin/phospholipid ratios are associated with altered mitochondrial inner membrane proton permeability (State 4 respiration) and ADP phosphorylation (State 3).

IV-C.1. Dietary manipulation, whole animal

IV-C.1.a. Essential fatty acid (18:2(n − 6))-deficiency. Dietary requirements of 18:2 or other (*n* − 6)-polyunsaturated fatty acyls are probably in the range of 1% of total calories [7] but vary with the clinical or metabolic criterion used for physiological normality [546,547]. Varied dietary input of hydrogenated or 18:2 fatty acids does not change cardiolipin/phospholipid ratios in liver [41,658], heart [41,45,90,368 548,729], skeletal muscle [41] and intestinal mucosa [740].

Several workers have noted that 'homeostatic' mechanisms keep mitochondrial total lipid and phospholipid unsaturation/saturation ratios constant when diets contain as few 18:2 acyls as 9% of total fatty acyls, and fat contents are 4–16% [105,211]. It should be noted that rat liver mitochondria normally contain about 20% of cardiolipin/phospholipid and the cardiolipins contain some 60% 18:2 per total fatty acyls (Table III) or up to 80% [716]; the other phospholipids contain only 9% 18:2 but are 80% of total phospholipids. Ergo, two thirds of the 18:2 acyls in total phospholipids of liver mitochondria are in cardiolipins.

Depletion of total phospholipids 18:2 acyls by EFA-deficiencies thus reflects mostly the cardiolipin compartment. However, examination of individual phospholipids is still necessary to determine the extent of dietary or metabolic effects, and opposing changes in 18:2 acyl contents of cardiolipins and the other phospholipids can make measurements of 18:2 contents of total phospholipids misleading (see sections IV-C.2.a and c).

Diets low enough in $(n-6)$-unsaturated fatty acyls evoke compensatory metabolic mechanisms. First, rapidly activated and induced hepatic enzymes of de novo biosynthesis and Δ9-desaturation produce saturated fatty acyls-CoA and monounsaturated fatty acyls; the monounsaturated fatty acyls replace 18:2 acyls in cardiolipins but not in other phospholipids [716]. Diets that contain lipids with only hydrogenated fatty acids also displace some 18:2 acyls with endogenous or exogenous monounsaturated fatty acyls, in the pattern seen with fat-free feeding. After a few weeks, increased hepatic elongations and Δ6- and Δ5-desaturases [58] convert endogenous and exogenous monounsaturated fatty acyls-CoA → 20:3$(n-9)$-CoA, and some 20:3$(n-9)$ acyls are esterified into phosphatidylcholines and phosphatidylethanolamines but not cardiolipins.

Diets that are fat-free, or that contain hydrogenated fats, replace most of the 18:2 acyls in rat liver, heart, kidney and intestinal mucosa cardiolipins with monounsaturated fatty acyls (Table III). They raise monounsaturated fatty acyl (including *trans*-monounsaturated fatty acyls found in hydrogenated fats) proportions in rat liver cardiolipins from 10–20% of total fatty acyls in controls to as much as 70%. A fat-free diet removes all 18:2 and most 20:4 acyls from rat brain cardiolipins, which normally contain only 13% 18:2, and slightly raises contents of saturated fatty acyls, monounsaturated fatty acyls, and a 20:3 acyl [41] (Table III).

Fatty acyl composition of phospholipids has been measured as early as 1 day after the start of an EFA-deficient diet, but for some reason oxidative phosphorylation has been examined after at least 4 weeks and mostly after 8 weeks. Changes in mitochondrial morphology and enzyme activities appear as early as 1–2 weeks and make mitochondria the most sensitive indicator of EFA-depletion [7,265]. Hayashida and Portman [265] found that 1 week of a fat-free- or saturated fatty acyls-diet depresses liver mitochondrial total lipid 18:2 content from 23% (control) to 7%, and begins to release succinate dehydrogenase activity from its partly crypticized, membrane-bound state.

EFA-deficiency linearly depletes 18:2$(n-6)$ acyl groups in cardiolipins of rat liver mitochondria, from 79% of total fatty acyls to less than half in 7 days and to a quarter in 66 days [716], see Table V.

TABLE V

Time-course of changes in fatty acyl composition of cardiolipins and other phospholipids in rat liver mitochondria during dietary essential fatty acid-deficiency and -repletion (from Wolff [716])

Fatty acyl	% of total fatty acyls in cardiolipins				
	fat-free diet			+ EFA diet [a]	
	day 0	day 2	day 7	day 8	day 12
16:0	3	4	4	3	3
16:1(n − 7)	1	8	18	13	7
18:0	1	1	1	1	1
18:1(n − 9)	4	12	18	4	4
18:1(n − 7)*cis*	8	13	18	15	14
18:2(n − 6)	79	57	33 [b]	47	62
Other (n − 6)	4	5	5	5	4
20:3(n − 6) [c]	0	1	1 [d]	1	0
22:6(n − 3)	1	1	1	1	1
	% of total fatty acyls in other phospholipids				
16:0	24	26	25	22	23
16:1(n − 7)	1	3	3	1	1
18:0	22	16	16	23	20
18:1(n − 9)	4	8	17	14	7
18:1(n − 7)*cis*	2	6	5	2	2
18:2(n − 6)	9	3	2	8	10
Other (n − 6)	21	16	17	23	25
20:3(n − 9)	0	3	4 [e]	1	0
22:6(n − 3)	16	8	7	10	10

[a] + EFA = 4% fat in diet, containing 50% 18:2(n − 6) and 3.7% 18:3(n − 3) fatty acids;
[b] + approx. 4% 18:2(n − 7) [721];
[c] identified by R.L. Wolff [716]; in 66 days on the fat-free diet.
[d] 0.8% 20:3(n − 9) acyls appear in PLs, and
[e] 13% 20:3(n − 9), in other PLs.

The 18:2 content in cardiolipins and in other mitochondrial phospholipids (where 18:2 acyls are only 9% of total fatty acyls) starts to fall in 2 days, when 18:2 acyls in cardiolipins become 73% of the 18:2/total phospholipids. Given a cardiolipin/phospholipid ratio of 12.7% (Table III), calculated 18:2 content of total phospholipids of liver mitochondria is 18%, and EFA-deficiency depresses it to 10% on day 2, 6% on day 7, and 4% on day 66 (Table V); a fat-free diet replaces 18:2 acyls in total phospholipids with monounsaturated fatty acyls even faster in rats prepared metabolically by fasting (see below). Resupply of EFA raises 18:2 acyl contents in cardiolipins by 50% in 1 day. EFA-deficiency-induced monounsaturated fatty acyls (16:1(n − 7), 18:1(n − 9), and *cis*-18:1(n − 7)) rapidly and linearly replace 18:2 acyls, but only in cardiolipins, to reach 54% of total fatty acyls. Repletion with EFA replaces some cardiolipin monounsaturated fatty acyls rather slowly, and *cis*-18:1(n − 7) acyls persist tenaciously. Saturated fatty acyls, other $(n-6)$-polyunsaturated fatty acyls, and 22:6$(n-3)$ acyls are normally minimal in cardiolipins and major components in other phospholipids; EFA-deficiency or -feeding does not affect these acyls in either locus. Unexpectedly, the

20:3($n-6$) fatty acyl group appears in liver (and kidney) cardiolipins within 2 days of EFA-deficiency. This desaturation derivative of the deficient dietary 18:2 fatty acid strongly binds cardiolipins [716] and may accumulate progressively through EFA-deficiency-induced Δ6-desaturations [58]; 20:3($n-6$) biosynthesis should further deplete hepatic 18:2 acyls. The diagnostic fatty acid of prolonged EFA-deficiency, 20:3($n-9$), appears in cardiolipins only at 9 weeks, but accumulates much earlier in other phospholipids (4% in 7 days, 13–21% in 9 weeks [265,716], where it is also quickly replaced when EFA are fed. These findings confirm a central role of rat liver mitochondrial cardiolipins in EFA metabolism, and leave the altered fatty acyl composition of cardiolipins to account for observations that EFA-deficiency increases proton leakage.

EFA-deficient diets increase State 4 respiration and proton leakage; see Ref. 284 for a critique of apparently contrary findings. In recent studies, saturated fatty acyl-diets accelerate rat liver mitochondrial State 4 respiration (25°C) by 25–50% in 6–14 weeks [147,525]. By the criteria of the State 3:State 4 ratio rather than the absolute rates, together with the ADP/O ratio, Rafael et al. [525] and some other workers (see Ref. 284) conclude that EFA-deficiency has no effect on oxidative phosphorylation. State 3 respiration at 25°C increases 27% [525] or not at all [147], or 37% at 37°C (glutamate) where the P/O ratio decreases significantly at 9 weeks [153,154]. The BMR rises 25% [525] or 16% [736], in units of rate of O_2 consumption per (kg body weight)$^{0.74}$, the appropriate power term [361]. The accelerated mitochondrial respiration accounts for the thermogenesis (see section IV-C.2.c); others disagree [525].

The membrane potential under State 4 conditions (succinate) in mitochondria from control rats is 186 mV, from EFA-deficient rats, 187 mV [525], and the effective proton conductance can be calculated to be 0.66 and 0.90 ng ion H^+ min^{-1} mg^{-1} mV^{-1}, respectively. An increased State 4 proton leak rate with unchanged Δp means that EFA-deficiency increases C_M, the effective proton conductance of the inner membrane, in rat liver mitochondria. Presumably, EFA-deficiency exerts its effects via the altered fatty acyl compositions, which are most extreme in the cardiolipins, and most compensated in the phosphatidylcholines and phosphatidylethanolamines, of mitochondrial inner membranes. The simplest mechanism is an increase in the normal proton phospholipid-leak, but abnormal mechanisms might involve altered phospholipid–protein interactions that introduce an F_0-leak or X-leaks, or increased H^+/O-slip (see section II-A).

Hamster liver mitochondrial cardiolipins are even more highly unsaturated than rat cardiolipins (85% 18:2 + 11% 18:1 acyls) and have almost no saturated fatty acyls, like beef heart cardiolipins (Table III). EFA-deficiency brings 18:2 content down to 30% and substitutes 60% monounsaturated fatty acyls plus 1.4% 20:3 acyls; I find no studies on liver mitochondrial respiration in EFA-deficient hamsters.

Based on studies of EFA-deficient rats, Divakaran and Venkataraman [154] originally proposed that the function of cardiolipins in the liver mitochondrial membrane differs from that of the other phospholipids. From this review of later findings, the great changes in cardiolipins might be responsible for increased proton leakage and State 3 respiration. The bulk phosphatidylcholines and phosphatidylethanolamines would affect bulk physical properties like the effects of temperature on rate processes and membrane fluidity, although phosphatidylcholine and phosphatidylethanolamine vesicles leak protons (see section II-A); their repletion with 20:3($n-9$) fatty acyl might account for normal properties measured in EFA-deficiency.

Either a hydrogenated-fat-diet, or T4-treatment (0.1 $\mu g\ g^{-1}\ d^{-1}$ for 7 days) of EFA-sufficient rats, accelerates liver mitochondrial State 4 respiration at 25°C. T4 given to EFA-deficient rats accelerates State 4 rates (to State 3u levels) and linearizes Arrhenius profiles [147]. Hyperthyroidism depletes 18:2 acyls severely in cardiolipins and less in other phospholipids, but raises cardiolipin saturated fatty acyls and ($n-6$)-polyunsaturated fatty acyls contents rather than monounsaturated fatty acyls (see section IV-C.2.d), it would be interesting to see the fatty acyl composition of liver cardiolipins from EFA-deficient hyperthyroid rats.

Feeding rats starved 1–2 days a fat-free diet (i.e., carbohydrates and proteins) produces the acutest EFA-deficiency, probably by first introducing metabolic changes that accentuate and hasten changes due to absence of 18:2 fatty acid input. Starvation signals glucagon and catecholamines, and protein phosphorylation. Subsequent fat-free feeding signals insulin release and dephosphorylation of the extensively phosphorylated proteins, which stimulates hepatic fatty acyl synthesis [311,650] and Δ9-desaturations [317] within minutes or hours. Fatty acid synthetase activity rises 10-fold in 12 h, 50-fold in 48 h; newly synthesized enzyme appears in 12 h and increases less markedly [192]. Within a few hours the monounsaturated fatty acyls begin to replace 18:2 acyls in total phospholipids of rat liver mitochondria and 18:2 content falls from 23% to reach 5.3% in 48 h [9]; a simple fat-free diet acts more slowly. Neither fatty acyl contents of cardiolipins nor oxidative phosphorylation have been reported under these conditions, to my knowledge. However, from Wolff's study [716] it seems likely that changes in fatty acyl/cardiolipins predominate, except for the (late) appearance of 20:3($n-9$) acyls in the other phospholipids.

In humans, total parenteral feeding with fat-free fluids that contain glucose suppresses adipose tissue

lipolysis and makes 18:2($n-6$) acyls stored in triacylglycerols unavailable: biochemical signs of EFA-deficiency appear within 1 week in infants, with their extra demands for growth (see Ref. 546).

A fat-free diet given for 14 weeks depletes rat heart cardiolipins of 18:2 acyls, substitutes monounsaturated fatty acyls (63%) and ($n-6$)-polyunsaturated fatty acyls (13%) synthesized in the liver, and maintains a cardiolipin free of saturated fatty acyls [41]. Cardiolipins are the most resistant among cardiac phospholipids against changing fatty acyl contents when rats are fed diets high in saturated fatty acyls [368]. Feeding hydrogenated oils introduces 6–39% *trans*-18:1($n-9$) into heart mitochondrial phospholipids [308]; *trans*-monounsaturated fatty acyls are structurally like saturated fatty acyls [716].

Cardiolipins in heart mitochondria from rats so fed for 6 weeks contain somewhat less 18:2 than corn oil controls (40% vs. 72%); their saturated fatty acyls are only slightly elevated (11% vs. 6%), their monounsaturated fatty acyls are more markedly higher (16:1 + 18:1 are 27% vs. 13%) and *trans*-18:1($n-9$) appears (17%) [308]. These cardiolipin changes are less than in liver mitochondria but are still accompanied by 15–30% rises in State 4 respiration (37°C, pyruvate or 18:1-carnitine, but not glutamate) and no decrease in phosphorylation efficiency.

Fed linelaidic acid (*trans,trans*-18:2($n-6$)) appears in rat heart mitochondrial phosphatidylethanolamines (5.5%) and phosphatidylcholines (4.5%), but not in cardiolipins [115] (Table III). State 4 respiration (succinate or glutamate/malate, 25°C) remains unchanged while State 3 (and thereby the respiratory control ratio) decreases (see section II-B). These findings are consistent with the fatty acyls of cardiolipins regulating the proton leak; the altered mitochondrial phosphatidylcholines and phosphatidylethanolamines leave the leak unchanged, but may be involved in down-regulating State 3 respiration by changing C_i values of the rate-limiting transporter proteins.

It is striking that these dietarily-induced EFA-deficiencies do not compensatorily increase the trace percentage contents of 20:3($n-9$) fatty acyls in liver, heart and kidney cardiolipins. The 20:3 acyls partly displace 20:4 acyls in all the other phospholipid fractions (phosphatidyl-choline, -ethanolamine, -inositol and -serine), more in liver phospholipids than in renal phospholipids [580]. EFA-deficiency produces loose-coupling of liver mitochondrial oxidative phosphorylation despite the maintenance of relatively high total phospholipid unsaturation, perhaps because the low 18:2 and high 16:1 + 18:1 contents in the cardiolipins persist – given the special relationship between cardiolipins and proton conductance adduced here.

IV-C.1.b. Feeding ($n-3$)-unsaturated fatty acids. Linolenic acid, 18:3($n-3$), is biosynthetically essential because vertebrates lack a Δ15-desaturase [58–60]. At adequate levels of ($n-6$)-polyunsaturated fatty acyls, ($n-3$)-polyunsaturated fatty acyls are satisfactory substitutes in vertebrates: arctic marine animals, and humans that subsist on them, as well as dogs fed fish oils [126], have high ($n-3$)-polyunsaturated fatty acyls levels and relatively low ($n-6$) levels in membrane phospholipids [547,608] but exhibit no classical signs of ($n-6$)-EFA-deficiency. Fed ($n-3$)-unsaturated acids readily replace a part of the ($n-6$)-unsaturated acyls in heart phospholipids and neutral lipids [236], and ($n-3$)-acylCoA molecules are preferred substrates for desaturases [117]. Tissue ($n-3$)-polyunsaturated fatty acyls content is biologically conserved to such an extraordinary degree that two generations of rats must be fed a purified 18:3($n-3$)-deficient diet to deplete the ($n-3$) polyunsaturated fatty acyls, especially 22:6 acyls, in brain, heart, muscle, retina and liver [657]; 22:6($n-3$) acyls are replaced mainly by 22:5($n-6$) acyls – successfully, since the animals survived. The only signs of verified 18:3($n-3$) deficiency in humans are neurological abnormalities [294] which may be connected with the high percentage of 22:6($n-3$) acyls in brain cardiolipins (Table III).

Feeding diets supplemented with fish oils partly replaces cardiac 18:2 acyls with the fed ($n-3$)-polyunsaturated fatty acyls and derivatives. In rats fed a diet containing 10% cod liver oil for more than 3 months, heart cardiolipin/phospholipid ratio rises from 7.8% (control) to 12.4%, and the cardiolipins are not depleted of 18:2 acyls but the phosphatidylcholines and phosphatidylethanolamines are [234]. On the other hand, sardine or tuna [90] oil in diets does not change cardiolipin/phospholipid proportions. Heart mitochondria of rats fed sardine oil for 10 days contain cardiolipins very low in 18:2($n-6$) acyls, as in EFA-deficient rats; fed or derived ($n-3$)-polyunsaturated fatty acyls, especially 22:6($n-3$), rather than saturated fatty acyls (which appear in amounts of 9% of total fatty acyls) or monounsaturated fatty acyls, replace cardiolipin 18:2 acyls, raising the unsaturation index to 316 [729]. No Mead acid, 20:3($n-9$), appears in the cardiolipins, or (not shown) in the phosphatidylcholines and phosphatidylethanolamines, although they too are depleted in 18:2($n-6$) and 20:4($n-6$) fatty acyls. It is striking that, in the face of the ($n-6$)-polyunsaturated fatty acyl depletions, the proton leakage rate in the heart mitochondria from sardine oil-fed rats, as expressed by State 4 respiration (succinate or glutamate/malate, 25°C), is unchanged or decreases.

It should be noted that the total fatty acyls in 'fish oil-diets' contain up to 13% 18:2($n-6$) fatty acid [90,126,729], either because they supplement an ordinary chow or because the total fatty acyls of sardine oil, for example, include 6% 18:2 acid, enough to avoid absolute deficiency. Actual substitution of 18:3($n-3$)

fatty acid for 18:2 in an otherwise fat-free diet (fed for 12 weeks) strips rat liver cardiolipins of just about all 18:2 acyls, elevates monounsaturated fatty acyls, 18:3($n-3$) and 22:6($n-3$) contents in cardiolipins (Table III), and evokes the appearance of the 20:3($n-9$) acyls typical of EFA-deficiency in all the phosphatides except cardiolipins, but does not change cardiolipin/phospholipid ratios [658]. Such rats rapidly develop clinical signs of EFA-deficiency, but oxidative phosphorylation does not seem to have been measured in their liver mitochondria.

Pigs have been fed diets containing 8% fat, which in controls contained 14% of 18:1($n-9$), 55% of 18:2($n-6$) and 6% of 18:3($n-3$) per total fatty acids [485]. Diets enriched in 18:3($n-3$) (progressively up to 31%), high in 18:1 (31%) and low in 18:2($n-6$) (19%) introduce 18:3($n-3$), 20:3($n-3$), and 20:5($n-3$) acyls that are not found in renal cardiolipins of pigs on the control diet. These ($n-3$)-unsaturated acyls displace some 18:2 and 18:1 acyls [585] (Table III). The high-18:3 diets lead to impaired feed utilization for weight gain (mitochondrial dysfunction was mentioned), enlarged heart, liver and kidneys, anemia, and 'yellow fat disease' that may arise from deposits containing polyunsaturated fatty acyl peroxidation products. These toxic effects were attributed in part to the known competitiveness of 18:3($n-3$)-CoA for Δ6-desaturase. Diets enriched in 18:1($n-9$) fatty acid (to 43%) and constant in 18:2($n-6$) (19%, a low value) and 18:3($n-3$) (20%, high) raise pig kidney cardiolipin 18:1 acyl content only slightly, deplete 18:2 acyls somewhat, and substitute about 20% of 18:3($n-3$) + 20:3($n-3$) acyls.

IV-C.1.c. Feeding erucate. Erucate-feeding tests how fatty acyl composition of cardiolipins affects cardiac oxidative phosphorylation, because rats incorporate fed 22:1($n-9$) fatty acid into heart [45,308,537,735] and liver [735] mitochondrial cardiolipins but not into phosphatidylcholines or phosphatidylethanolamines, without altering cardiolipin/phospholipid ratios, and exhibit in vivo and morphological signs of myocardial damage. In contrast, feeding hydrogenated fats changes fatty acyl in heart cardiolipins least, see (a) above. In heart mitochondrial cardiolipins from rats fed erucate diets for $\geq$ 28 days (but not less), 18:2 drops to 40–55% (cf. 68% in rats fed corn oil diets), 4%–8.5% 22:1 + 20:1 acyls appear, saturated fatty acyls increase to 10–25% (6% in controls) and monounsaturated fatty acyls to 27% (13% in controls) to replace the 18:2 acyls (Table III). These heart mitochondria respire faster in State 4 (37°C, with glutamate or 18:1-carnitine) and slower in State 3 [308]. Compared with soybean-oil-fed control rats, 22:1-feeding for at least 28 days accelerates State 4 respiration 60%–100% with several substrates [105]. In contrast, in heart mitochondrial cardiolipins from chicks fed 22:1 fatty acid for 24 days, the decreased 18:2 acyls (77% → 47%) are replaced by 22:1 (11%) and 20:1($n-11$) acyls (17%), with no change in 16:0 and no appearance of 18:0, 16:1 or 18:1 acyls (that are absent from safflower-oil-fed controls) [537]. Here State 4 respiration remains unchanged (pyruvate/malate, 37°C) while State 3 and the ADP/O ratios decline slightly. These findings suggest again that the substituting saturated fatty acyls and/or monounsaturated fatty acyls, rather than the decrease in cardiolipin 18:2 acyls, correlate with proton leakage. Findings that dietary 22:1 fatty acid fails to alter respiratory rates or phosphorylation in heart mitochondria [156] were obtained in rats fed 3 days (*sic*), were not accompanied by fatty acyl analyses of the phospholipids, and are to be expected from the studies cited above.

Observations that accumulated saturated fatty acyls and monounsaturated fatty acyls in cardiolipins obtained from EFA-deficient correlate with increased proton leakage seem to be contradicted by the effects of unsaturated fatty acyls-deficiency in yeasts (see section IV-C.4), where monounsaturated fatty acyls decrease proton leakage, and substituted saturated fatty acyls increase it. But the cardiolipins of the yeasts studied contain 96% monounsaturated fatty acyls and no 18:2 acyls. If the differences between rat and yeast mitochondria do not preclude a comparison, monounsaturated fatty acyls make yeast mitochondria more leaky to protons than do 18:2 acyls in rat mitochondria.

If, as much of the evidence cited above suggests, the fatty acyl composition of cardiolipins is connected with mitochondrial proton leakage, the thermotropic properties of State 4 respiration should change with fatty acyl substitutions, especially saturated fatty acyls and monounsaturated fatty acyls for polyunsaturated fatty acyls. Surprisingly little attention appears to have been given to the measurement of Arrhenius profiles of rate processes in oxidative phosphorylation in the long-known states of EFA-deficiency. These would be important in determining mechanisms, and because altered profiles make the temperature of assay crucial for demonstrating rate changes [98,99].

IV-C.2. Metabolic manipulation, whole animal

IV-C.2.a. Feeding ethanol. Ethanol-feeding raises 18:2 and depresses 20:4 acyl proportions in rat liver mitochondrial total phospholipids [194,430], which has been ascribed to an accompanying decrease in activities of microsomal fatty acyl-CoA Δ5- and Δ6-desaturases [458]. A similar fatty acyl redistribution, plus an accumulation of 22:6($n-3$) acyls, occurs in rat liver microsome phospholipids, and because the activities of Δ9- and Δ5-desaturases are increased and that of the Δ6-desaturase remains normal, the effects of desaturases on membrane fatty acyl changes were dis-

counted [672]. 30 days of feeding ethanol as 36% of total calories augments 18:2 and depletes 20:4 in phosphatidylcholines and phosphatidylethanolamines [128] but ethanol as 14% of calories leaves phosphatidylcholines and phosphatidylethanolamines unchanged [686]. However, in cardiolipins ethanol-feeding depletes 18:2 content by 17–25% [128,686] and reciprocally increases saturated fatty acyls and 18:1 acyls (Table III), which does not seem attributable to any accumulation of 18:2-CoA molecules available to mitochondria. Cardiolipins of periportal and perivenous zone mitochondria contain less 18:2 and more monounsaturated and saturated acyls than controls (see section IV-B.2); the percentages in periportals are 47:31:16 and in perivenous, 38:36:22; and cardiolipin P per phospholipid P ratios are 16.6 and 17.2 [79]. Overall mitochondrial cardiolipin/phospholipid ratios decrease minimally in rat livers (7%) [128,168, 574,654] and baboon livers (3%) [17], but may increase in mouse livers [430]. By measurements of total cardiolipins, ethanol-feeding does not produce very large differences from control cardiolipins yet hepatic 'ethanol-cardiolipins' exert some specific effects on membrane function. The zonal heterogeneity of these cardiolipins implies that the perivenous hepatocyte fraction, which is more abnormal than the mean value, may be the most effective; studies on its power to make mitochondria tolerant to ethanol (see below) should be informative.

Ethanol-feeding (i) depresses liver mitochondrial respiration and changes its Arrhenius profiles in a way that is seen when inner membrane phospholipid fatty acyl compositions are altered; and (ii) induces adaptations of membrane structure stability against ethanol applied in vitro that are specifically due to altered anionic phospholipids – in mitochondria, the cardiolipins.

(i) Rat liver mitochondrial respiration is altered by prolonged feeding of ethanol as follows. In six studies using succinate as substrate at 30°C, State 4 rates decrease by an average of 16%, State 3 by 34% [81,304,562,574,622,623]. Of course the greater depression of State 3 respiration decreases the mean respiratory control ratio, but this does not signify 'loose-coupling', a term that describes decreased control ratios caused by protonophorically increased State 4 respiration. State 4 and 3u respiration rates (NADH, 25°C) are also slowed in inner membrane particles [651]. State 4 respiration (malate/glutamate, 25°C) in intact mitochondria decelerates while Δp stays at control levels and State 3 decreases even more [558]. Changed Arrhenius profiles of rates in both respiratory states diminish the ethanol effect at higher temperatures of measurement [555,557]. An opposing view, based on the lack of changes in fatty acyl composition of mitochondrial total phospholipids [219], has been refuted by pointing out that the fatty acyl changes in the relatively small cardiolipin fraction are obscured when total phospholipids are measured [560]. Several analogous instances are pointed out in this review.

The mechanisms that retard State 4 and State 3 respiration must differ. Slowed State 4 indicates a slower transmembrane proton-leak; the constant Δp indicates that ethanol-feeding diminishes $C_{M^{H^+}}$. Altered C_i values appear responsible for the slowed State 3 rate. Although Spach and Cunningham [622] do not present a complete profile of control coefficients, their titrations with specific inhibitors indicate that cytochrome aa_3 limits State 3 respiration more than in control mitochondria ($C_i = 0.17$) when dicarboxylate substrates are oxidized. Although ethanol-feeding results in lower mitochondrial general synthesis of proteins, and in depressed content and activity of cytochrome aa_3 [17,114,562,651], the amount of cytochrome oxidase apoprotein does not decrease, but only half of it is in active form with bound heme-aa_3 [652]. The mechanism of this ethanol effect is not known; Thayer and Rubin [652] suggest that altered membrane lipids may interfere with heme-protein assembly, or that holoenzyme is degraded. The specific association of cytochrome oxidase and cardiolipins is well known (see section II-B). These authors also note that other mitochondrial components limit State 3 respiration, especially the ATP-synthase and the NADH dehydrogenase. Neither of these regulates normally in liver mitochondria [228], but decreased activities of mitochondrial ATPase and succinate dehydrogenase are reported [562,651]. Effects of ethanol on the major regulators of normal rat liver mitochondrial State 3 respiration, the carriers of ADP/ATP and dicarboxylate substrates, do not seem to have been studied. Chronic ethanol-feeding of rats produces a partially 'hyperthyroid' metabolic pattern in liver [322,323]: decreased glycogen content; increased β-adrenergic sensitivity; increased microsomal glucose-6-phosphatase, NADPH: cytochrome c reductase, NADH oxidase, and α-glycerophosphate dehydrogenase activities; increased mitochondrial size; and accelerations of respiration by 30% (at 37°C) in liver slices [677] and 23% in perfused livers [322] of ethanolized rats. Propylthiouracil, in dosage that corrects hyperthyroidism in human subjects, is said to protect their livers against the lethal toxic effects of chronic excessive ethanol intake [483]. However, these findings are not accompanied by characteristic 'hyperthyroid' increases in cytochrome oxidase amounts and activity in isolated mitochondria, or the typical changes in liver phospholipid fatty acyl profiles, especially in unresolved cardiolipins, and seem paradoxical in view of the decreased mitochondrial respiration. Studies on periportal and perivenous mitochondria might settle these inconsistencies; cardiolipins from mitochondria of perivenous

hepatocytes of ethanol-fed rats show an abnormal fatty acyl pattern (see above and Ref. 79) almost as extreme as that in hyperthyroids (Table III). The apparent paradox may also be explained by observations that ouabain returns the high respiratory rates of liver slices to control levels [677]: the hypermetabolic effects of ethanol may reflect in part the plasma membrane ouabain-sensitive NaK-ATPase, not mitochondria. That ATPase activity shows altered lipid-dependency [304] but the nature of the membrane lipid changes is not yet clear. Plasma membrane anionic lipids include phosphatidylinositols (6.7% of total phospholipids) and phosphatidylserines (4.7%), and ethanol feeding does change their properties in conferring membrane adaptation.

(ii) Continued administration of ethanol blunts in vitro responses to ethanol, in liver mitochondria through changes in cardiolipins. Ethanol, 0.5–1.0 M (150–300 μmol/mg mitochondrial protein) produces real loose-coupling (State 4 increases by 63% at 25°C and 80% at 40°C) much less effectively in liver mitochondria from ethanolized rats than in those from normals: a 'tolerance' [559]. Membranes prepared from mouse brain synaptosomes, rat erythrocytes, liver mitochondria or microsomes, or pancreatic acini, adapt to the continued presence of ethanol. Fed ethanol produces blood levels around 50 mM in 4–5 weeks, when in vitro exposure to [ethanol] = 50–100 mM (about 20 μmol/mg) disorders membrane structure little or not at all, although it disturbs bilayer structure in membranes from sober controls. Ethanol-feeding itself does not consistently increase the order of the fatty acyl chains. Membrane tolerance resides in the phospholipids of the membrane and not in the proteins, and appears in protein-free liposomes of phospholipid extracted from the various membranes. Although Hoek and Taraschi [292] note that it is "not essential to maintain the physiological asymmetric distribution of membrane lipids, which is lost in reconstituted liposomes", cardiolipins maintain their asymmetric distribution by finding the concave inner surface of liposomes [427,447].

In mixed-phospholipid liposomes prepared from liver mitochondria of ethanol-fed rats, only the cardiolipin fraction of the extracted phospholipids confers membrane tolerance to added ethanol; phosphatidylinositols from microsomes and phosphatidylserines from synaptosomes act similarly in the corresponding mixed-phospholipid vesicles. We do not know the changes in the phosphatidylinositols or phosphatidylserines, but we do know that fatty acyls change in the cardiolipins. The mitochondrial phospholipids contain cardiolipins as 10% of total phospholipids (and 46% phosphatidylcholines, 42% phosphatidylethanolamines, 2% phosphatidylinositols + phosphatidylserines – about the proportions in preparations from control-fed animals); the ethanol-tolerance persists when the cardiolipin/phospholipid ratio is made as low as 3%. It may be relevant that ethanol interacts directly with the headgroup region of anionic phospholipids and exerts an ordering effect on membrane surface H-bonded networks [292] in which cardiolipins may play a role (see section II-A).

IV-C.2.b. Magnesium deficiency. Mg-deficiency decreases liver microsomal Δ6-desaturase activity and (thereby?) raises the 18:2 acyl content in the phospholipids of microsomes [403–405] and mitochondria [509]. Mg^{2+} ions associate with mitochondrial cardiolipins [526] and are cofactors in phosphatidylcholine biosynthesis. Therefore, reports that dietary Mg-deficiency uncouples oxidative phosphorylation [680] seem worth examining here. Heart mitochondria isolated from young rats were uncoupled in 4–8 days of feeding a Mg-deficient diet, with P/O ratios 1.4-1.0 (α-ketoglutarate, temperature not specified). Oxidative phosphorylation in liver or kidney mitochondria was minimally affected, but both liver and heart mitochondria from Mg-deficient rats swelled faster at 37°C (but not at 20°C) than did mitochondria from controls [453]. However, P/O ratios in heart mitochondria were normal under similar conditions although serum [Mg] was as low as 20% of normal; no respiratory rates were shown [32]. And Hegsted and coworkers [453] found normal amounts of Mg in heart and liver mitochondria of Mg-deficient rats.

Mg-deficiency damages mainly the mitochondria of rat hearts; electron microscopy shows decreased matrix density, swelling and myelin figures [269,638]. Damaged mitochondria in situ also appear in EFA-deficiency and thyrotoxicosis (see Ref. 284) even though some in vitro assays of oxidative phosphorylation are within normal ranges. Mitochondrial defects in Mg-deficiency are still invoked; for example, ethanol-feeding leads to hypomagnesemia, which is partly blamed for heart lesions of mitochondrial origin [56].

Even though mitochondria contain normal amounts of Mg, Mg-deficiency-induced alterations in mitochondrial phospholipids would persist. The enzymatic conversion of phosphorylcholine → phosphatidylcholine requires Mg^{2+}. By the 4th day of Mg-deficiency, synthesis (measured by ^{32}P incorporation and lipid phosphorus) of heart mitochondria total phospholipids and resolved phosphatidyl-ethanolamine, -serine, -choline, -inositol and sphingomyelin (?) decreases by some 50% [66]. Because the specific activity of total phospholipids was low although relative specific activities of these individual phospholipids was not much decreased, synthesis of some other undetermined lipid-soluble fraction was thought to be susceptible to Mg-deficiency – phosphatidic acid, an intermediate in phospholipid synthesis, was proposed but cardiolipins were also not analyzed in this study and seem more likely to accumu-

late as an end-product. This idea is indirectly supported by observations that liver mitochondrial lipids from Mg-deficient rats, compared with controls, contain 12% more 18:2 acyls [509], and microsomes contain 25% more 18:2, 50% more 20:3(n - 6), and 10% less 20:4 acyls. These findings are attributed to a decreased Δ6- and/or Δ5-desaturase activity [see 403,404]. No direct information is available on cardiolipin composition or content in liver or heart mitochondria in Mg-deficiency, but in kidney cells grown on low-[Mg] media, phosphatidylcholine increases while phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and sphingomyelin decrease [405].

Effects of Mg-deficiency should be difficult to detect in in vitro assays of oxidative phosphorylation. Mg^{2+} is required in the reaction mixture; the presence of 22.5 μmol of $MgSO_4$ [32,680] should reverse any deficit. Non-invasive in situ measurements by saturation transfer ^{31}P-NMR (see section IV-C.2.d) could be more definitive.

IV-C.2.c. Temperature. The eukaryote basic strategies for coping with decreases in environmental temperatures involve keeping biomembranes functional (e.g., so that they do not congeal) and generating more heat (so that cell temperature does not get low enough to congeal membranes). These strategies converge in mitochondrial inner membranes, which both mediate energy transductions and generate basal and extra heat. We know much less about metabolic adaptations to above thermoneutral environments than to below thermoneutrality. Cold-activated hormonal mechanisms mediated by local metabolites regulate mitochondrial function; in contrast, thermosensitive enzymes regulate membrane function and heat production in prokaryotes (section III-A).

Living cells generate heat almost completely from highly exergonic oxidative reactions. 'Thermogenesis' denotes extra heat production above basal levels [519]. Researchers disagree over thermogenic mechanisms. Some early notions, including those of the reviewer [278], and some current ideas [132,193,363,450,525] attribute extra heat production to uncoupling and the diversion of the free energy change of oxidations from driving the endergonic phosphorylation reaction, ADP + $P_i \rightarrow$ ATP. Others point out that the caloric yield due to substrate oxidation is independent of the reaction sequence: "... any ATP synthesized by mitochondria within a cell is immediately recycled and does not appear in the final equation of reactants and products ..." [465] (see also Refs. 38,273–275,280,518,519). Therefore, respiration rate is the source and index of thermogenesis, and the chemical mechanism that sets respiratory rate can be considered the mechanism that regulates heat production [273–275,465,518,519]. This argument seems the sounder, and is productive in depicting mechanisms whereby membrane phospholipids, especially cardiolipins, may participate in extra heat generation by mitochondria.

TABLE VI

Heat production from altered mitochondrial respiratory rates

(For references, see the text.)

Heat production (respiratory state [a])	Tissue	Δ Proton-flux mechanism	Rate
Basal heat:			
State 4	any	proton leak	+ +
State 3	any	C_i values	+ + +
Extra heat:			
loose-coupled (4)	any	protonophore	+ + + +
uncoupled (3u)	any	protonophore	+ + + +
cold: shivering (3)	muscle	+ ATPase	+ + + +
cold: metabolic (4)	liver, muscle	+ PL-leak	+ + + +
	rat BAT	+ uncoupling protein	+ + + +
	rat BAT	− PL-leak	+
EFA-deficiency (4)	rat liver	+ PL-leak	+ + + +
	rat BAT	− PL-leak	+
hyperthyroid (4)	liver	+ PL/F_0-leak	+ + + +
malignant hyperthermia	muscle	+ X-leaks?	+ + + +
Less heat:			
hypothyroid (4)	liver	− PL-leak	+
diving (5 [b])	seal muscle	vasoconstriction	0

[a] As defined by Chance and Williams [86].

[b] State 5, defined as $[O_2] = 0$, here also includes [S] = 0, in muscles.

Table VI summarizes some mechanisms whereby altered mitochondrial respiratory rates (proton flux) change heat production. Basal heat is produced from mitochondrial State 4 respiration (nonphosphorylating) at the slow rate set by the proton leak, and from the more rapid (phosphorylating) State 3 oxidation rate regulated by the C_i values of certain steps. Basal proton leakage and C_i values differ among the mitochondria of various tissues; so do the phospholipids and cardiolipins; perhaps the two are connected. Different mitochondria also vary in thermogenic responses to temperature adaptations; perhaps this is also not unconnected with their phospholipid compositions.

A crucial test for a respiratory thermogenic mechanism is the existence of loose-coupling [381], the acceleration of State 4 respiration with continued synthesis of ATP, i.e., without uncoupling. By an 'uncoupling' mechanism, loose-coupling should not be thermogenic – but loose-coupling is observed in mitochondria from thyrotoxic human and animal subjects [641–643] that are in a veritable thermogenic state, i.e., with elevated BMR (oxygen). Loose-coupling produces extra heat when proton leakage is increased almost maximally but not enough to dissipate Δp tellingly. Uncoupled oxidative phosphorylation (with Δp insufficient for phosphorylation) produces extra heat at this faster, maximal rate set by a protonophoric mechanism; uncoupling-thermogenesis from State 3 respiration is usu-

ally minor, since respiration is already near maximal because of the large influx of protons through the ATP-synthase. Thermogenesis starting from State 4 respiration ranges from 2-fold to 20-fold, depending on the mechanism that increases proton leakage. This, and the fact that protonophores like dinitrophenol and the BAT uncoupling protein can triple the BMR in vivo, strongly implies that most resting cells generate basal heat from State 4 respiration rather than from State 3 [278,280,597].

Ectotherms, with body temperatures that rapidly near changed ambient temperatures, adapt to cold by increasing fatty acyl unsaturation in their membrane phospholipids to maintain appropriate function [266, 268,399,527].

Unsaturation of mitochondrial lipids correlates inversely with growth temperature among yeasts [20,687]. The mesophilic (growing between 0°C and 36°C) yeast *Candida lipolytica* has mitochondrial phosphatidylcholines that are more unsaturated when growth is at 10°C than at 25°C, and the phosphatidylinositol, phosphatidylserine and phosphatidylethanolamine fractions show lesser increases in unsaturation; little cardiolipin is present and its fatty acyl composition is not reported; unsaturation changes through a reciprocal relationship between percentages of 18:1 and 18:2 acyl groups [350,351,655]. A psychrophilic *Candida* growing at 0–17°C shows little change in fatty acyl composition. *Saccharomyces cerevisiae* grown at 15°C or 30°C has phospholipids of similar fatty acyl composition, and cardiolipin/phospholipid ratios of about 4%, but phosphatidylcholine/phospholipid is higher at the lower growth temperature [315]. Unsaturation index of total lipids of *Saccharomyces* grown at 40°C is 6.3, at 26°C, 45 [87]. Mitochondrial phospholipids from the thermophilic yeast *Torulopsis bovina* include 25% cardiolipins; in psychrophile mitochondria cardiolipin/phospholipid is only 8% [20]. No fatty acyl compositions of cardiolipins are reported in these studies but we know that monounsaturated fatty acyls are 96% of the fatty acyl in mitochondrial cardiolipins of (presumably warm) *Saccharomyces* (see section IV-C.1.a).

Cold-adaptation (37–40°C → 15°C) induces some comparable alterations in the phospholipids of the fungus *Neurospora crassa* [1] and the protozoan *Tetrahymena pyriformis* [200,730]. The relative amount of cardiolipins in *Tetrahymena* total phospholipids decreases from 14.5% to 9.9% (Table III) and in *Neurospora* halves, which indicates that there are fewer mitochondria. In *Neurospora* and *Tetrahymena* mitochondria the cardiolipins per phospholipids are about halved, indicating that membrane composition changes; *Tetrahymena* cardiolipins are replaced by phosphatidylcholines and phosphatidylethanolamines. The unsaturation index rises in *Tetrahymena* total phospholipids from 127 to 160, and in *Neurospora* mitochondrial phospholipids from 159 to 216 as 18:3 acyls substitute for 18:2 acyls. Cold acclimation does not change *Tetrahymena* cardiolipin fatty acyls, which are quite unsaturated (unsaturation index 209; see Table III). In warm-cell mitochondria the phosphatidylethanolamines [200] and glyceryl 2-aminoethylphosphonolipids [337] are even more unsaturated; cold-exposure increases the unsaturation of these phospholipids rapidly and linearly, and overall unsaturation index increases from 139 to 166 over the first 10 h [730]. Cold makes the endothelial reticulum membrane lipids more rigid, which activates existing Δ9-desaturase enzyme and rapidly supplies more unsaturated fatty acyls; no new desaturase is induced, nor does the increase in dissolved $[O_2]$ accelerate desaturations the way it does in bacteria [609,610]. Cold adaptation does not alter the fluidity of extracted mitochondrial lipids, but removal of cardiolipins from the phospholipids of cold (but not warm) mitochondria increases phospholipid fluidity; Yamauchi et al. [730] believe that cardiolipins decrease fluidity in these mitochondria while the other phospholipids increase fluidity, and that cardiolipin reacts preferentially with other phospholipids when they are more highly unsaturated, to counter overfluidization. An interaction between cardiolipins and the bulk phosphatidylcholines and phosphatidylethanolamines might also affect membrane barrier properties and account for the apparent predominance of cardiolipin compositions in regulating State 4 proton leakage across mitochondrial phospholipids (see section II-A).

Flight muscle mitochondria from blowflies acclimated at 9°C, as opposed to 24°C, show increased fatty acyl unsaturation of total phospholipids; faster State 4 (proton leak) and State 3 (C_i values) respiration (+50–90%, pyruvate/proline, measured at 10–30°C) [133]; shifted inflections in Arrhenius profile of some electron carriers demonstrate altered lipid-dependence.

Early studies on poikilothermic animals found no consistent effects of temperature changes on the unsaturation index of total phospholipids from mitochondrial membranes and mitochondrial respiration, although altered Arrhenius profiles of respiratory rates implicated the lipids [666]. The apparent discrepancies are resolved by consideration of individual phospholipids rather than total phospholipids, as suggested by various workers [266,432,666]. When the environmental temperature of goldfish shifts from 30 → 10°C, mitochondria from various tissues increase contents of phospholipid monounsaturated fatty acyls and polyunsaturated fatty acyls. Hazel [266] tests the ability of these temperature-altered phospholipids to reactivate delipidated succinate dehydrogenase from goldfish skeletal muscle mitochondria. Reactivation depends on whether the mitochondrial phospholipids, but not the enzyme, are from 25°C- or 5°C-adapted goldfish: total

phospholipids from 5°C-mitochondria activate to 42% of the in situ V_{max}, total phospholipids from 25°C-mitochondria, to 28%. The efficacy of an isolated phospholipid subclass depends on its acidity: phosphatidylinositol > cardiolipin > phosphatidylserine > phosphatidylethanolamine > phosphatidylcholine.

Wodtke [710-713] measures effects of cold-acclimation (26–32°C → 10°C) on the resolved phospholipids of liver and skeletal muscle mitochondria of carp. In liver mitochondria from warm-adapted carp, the unsaturation index of phosphatidylcholines and phosphatidylethanolamines is less than that of total phospholipids [712], so the cardiolipins (not measured) must be more highly unsaturated. Cold-adaptation does not alter cardiolipin/phospholipid, phosphatidylcholine/phospholipid or phosphatidylethanolamine/phospholipid; comparisons of fatty acyl contents in total phospholipids with those in phosphatidylcholines and phosphatidylethanolamines indicates that the cardiolipins must lose 18:2 and 18:1 acyls, and gain saturated fatty acyls. In muscle mitochondria, cold-acclimation does not change subclass phospholipid/total phospholipids ratios, including cardiolipin/phospholipid, or the unsaturation of the total phospholipids. However, measured cardiolipin unsaturation drops sharply (unsaturation index 214 → 160; Table III) while phosphatidylcholine unsaturation index rises as markedly (203 → 249); in total phospholipids these opposing changes cancel out and thereby fatty acyl analyses of total phospholipids and resolved phospholipids lead to contradictory conclusions [714]. In frozen and thawed mitochondria from cold-acclimated carp, succinoxidase activity is faster by 30%, and cytochrome oxidase specific activity is about 50% greater while $[aa_3]$ and K_m for cytochrome *c* remain unchanged [713]; activation of cytochrome oxidase is attributed to phosphatidylcholine-induced augmentation of membrane fluidity rather than altered cardiolipin–oxidase interaction.

Tissues of goldfish kept at 10°C show the following differences from those kept at 30°C. (i) Mitochondria prepared from gills contain total phospholipids that are more unsaturated and include more cardiolipins and phosphatidylethanolamines [71]; (ii) activities of their NADH-cytochrome-*c* reductase, succinate-cytochrome-*c* reductase, and cytochrome oxidase are higher when measured at 20°C, while concentrations of cytochromes aa_3, *-b* and *-c* are unchanged [70]. (iii) Cytochrome oxidase activity (mitochondrial oxidative capacity) is increased at all assay temperatures from 10–40°C in homogenates of gills, brain or muscle, but is higher in liver homogenates only when measured at 30–40°C [70]. (iv) Liver mitochondria have unchanged State 3 respiratory rates (20°C) but decreased efficiency of phosphorylation [347]. Cold-acclimation (22°C → 12°C) of eels slows liver cell mitochondrial respiration (succinate, measured at 25°C) in State 4 although ADP/O ratios also decrease [710,711]. However, the temperature of the assay is crucial for showing cold-adaptation effects on mitochondrial respiration (as it is in demonstrating thyroid-state effects [99], see section IV-C.4.d). Arrhenius profiles of State 4 and State 3 respiration change with cold-acclimation in such a way that rates are higher only when assayed at < 20°C, in tench liver and muscle mitochondria [521] and in frog skeletal muscle mitochondria [520].

Shivering is an early thermogenic response in chilled homeotherms. Skeletal muscle contracts without doing work, maintains a high extramitochondrial steady-state [ADP] and $[P_i]$, and thereby accelerates respiration in the State 4 → State 3 transition. Later responses to cold include thermogenic metabolic changes; in skeletal muscles these may amplify or replace shivering thermogenesis. Skeletal muscle mitochondrial cardiolipins are relatively low in 18:2 and high in saturated fatty acyls contents in the Arctic mouse *Microtus pennsylvanicus* kept at 18°C (Table III), as compared with other Arctic mice. Cardiolipin saturated fatty acyls increase further while monounsaturated fatty acyls + polyunsaturated fatty acyl contents decline reciprocally when *Microtus* adapts to a 5°C environment; these muscle cardiolipins resemble the liver cardiolipins of thyroid-treated rats in a typically thermogenic state. Skeletal muscles of cold-adapted rats have more mitochondria, repackaged to contain less cytochrome oxidase [34]; nevertheless State 3 specific respiratory activity (pyruvate/malate, 37°C) rises 30% in both State 4 and State 3 [276]. Muscle mitochondria from cold-adapted (6°C) seals are loose-coupled with equal State 3 and 4 rates (succinate, 25°C); in mitochondria from 20°C-adapted seals State 4 rates are much slower than State 3 rates [223]. Cold-exposure of hamsters, but not of rats, increases liver mitochondrial respiration (succinate, 37°C) in State 4 and State 3, as well as oxidative capacity (cytochrome oxidase) [83]. However, liver mitochondria from cold-adapted rats respire 30% faster in both State 4 and State 3 (succinate, 30°C); V_{max} of the ADP/ATP carrier is above warm-adapted levels, but only when measured at 25 or 37°C; and 20:4 acyls replace a small fraction of 18:2 acyls in mitochondrial total lipids [406].

Seals that dive for long periods arrest oxidations in skeletal muscles, probably by constricting arterial supply and thereby shutting down O_2 and substrate supplies [579]. Reduced heat production in hypothyroidism, and thermogenesis in thyrotoxicosis, are discussed in section IV-C.2.d.

Major metabolic thermogenic adaptations of mammals to low environmental temperatures occur in BAT. BAT raises body temperature far out of proportion to its limited localization and amount; a norepinephrine infusion increases BAT respiration 30-fold in normal rats and 80-fold in cold-adapted animals where it con-

tributes almost two-thirds of the tripled BMR. Special features account for its thermogenic capacity: a proton-exchanger 'uncoupling' protein is found only in the numerous large BAT mitochondria; the mitochondria are situated and equipped for fatty acid catabolism. BAT mitochondrial cardiolipins and phospholipids adapt to cold and diet but, from one-temperature measurements, do not consistently contribute to thermogenesis.

The activated uncoupling protein mediates thermogenesis by accelerating proton reflux (Table I); 'uncoupling' is not necessary (see above). BAT mitochondria are rapidly and intensely thermogenic because a high content of uncoupling protein confers great protonophoric capacity. Uncoupling protein characteristics and roles in adaptation of animals to cold environments, the neonatal state, or overeating, have been reviewed [73,274,463,465,605]. The uncoupling proteins of rats, hamsters, guinea-pigs and rabbits crossreact immunologically. Although uncoupling protein is genetically related, ADP/ATP carriers of beef heart and rat liver mitochondria do not crossreact with uncoupling protein antibodies [539].

The uncoupling protein, the ADP/ATP carrier and the P_i carrier have repeated and related amino acid sequences, exist in dimer form in the membrane, and seem to share a common evolutionary origin, low hydrophobicity, three-dimensional structure, and transport mechanisms [16,363,564]. Uncoupling protein net positive charge is the lowest of the three, so it would presumably interact electrostatically least with cardiolipins; the P_i and ADP/ATP carriers react specifically with cardiolipins (see section II-B). Uncoupling protein purified from BAT mitochondria of cold-adapted hamsters retains 0.06 g of phospholipids per g protein [393], which would correspond to 1.2 mol of cardiolipins/mol. Solubilized pure uncoupling protein has been reconstituted by incorporation into liposomes made from phosphatidylcholines, phosphatidylethanolamines and cardiolipin (49.5 : 49.5 : 1) [634], or from egg yolk phospholipids [364] which probably include some cardiolipin because they also reconstitute the cardiolipin-dependent ADP/ATP carrier. No systematic study of retained phospholipids, or of reconstitution efficiencies of various phospholipids, seems to be available.

Extramitochondrial purine nucleotides and protons are negative effectors, and fatty acids are positive effectors, for uncoupling protein exchange of protons [465]. GDP, GTP, ATP and ADP bind non-covalently and reversibly, without modifying (e.g., phosphorylating) the protein. One specific, high-affinity, saturable binding site on the protein dimer is on the outer face of the inner membrane where it is exposed to purine nucleotides in the cytosol and to whatever amounts of cardiolipins are on the outer face. Protons increase purine nucleotide binding to the protein site. Free fatty acids in the order 16 : 0 > 18 : 1 > 18 : 2 > 8 : 0 compete with MgATP-binding to override the depressed proton conductance. There is no evidence that observed up-regulation of proton flux by hydrophobic sulfhydryl reagents [538] is physiological.

Proton exchange across the active uncoupling protein is nearly independent of ΔpH, and is too slow to proceed via a proton channel mechanism [363]. The amount of activated uncoupling protein regulates proton flow [463,465]. Klingenberg [363] proposes that the BAT uncoupling protein evolved late (i.e., in mammals) as an amputated H^+/anion cotransporter (or OH^-/anion antitransporter) similar to a portion of the related carriers of ADP/ATP and P_i, and shares features of their proton cotransport. We do not know if the similarity extends to a specific uncoupling protein interaction with cardiolipins that affects proton transport.

BAT is specialized for thermogenic fatty acid catabolism. Its profuse sympathetic innervation and capillary vasculature promote activation of hormone-sensitive lipase that supplies fatty acids from local and distant triacylglycerol stores. BAT mitochondria possess a very active set of β-oxidation enzymes; the standard enthalpy change for oxidation of 2 fatty acid carbons → acetyl-CoA is twice that of 2 glucose carbons → acetyl-CoA. The mitochondria contain little ATP-synthase and so do not support a futile cycle that involves the plasma membrane NaK-ATPase [605].

BAT mitochondria contain specialized phospholipids as compared with liver mitochondria. BAT cardiolipins from rats, hamsters, and mice housed at 22°C are low in 18 : 2(n − 6) acyls and repleted with saturated fatty acyls (Table III). BAT mitochondria from rats and hamsters acclimated to different temperatures, and deprived of dietary EFA, are compared in Table VII.

Cardiolipins of rats kept at 22°C contain about 20% saturated fatty acyls, 40% monounsaturated fatty acyls and 40% polyunsaturated fatty acyls; phosphatidylcholines and phosphatidylethanolamines have around 40% saturated fatty acyls and 30% each of polyunsaturated and monounsaturated fatty acyls; unsaturation indices in phosphatidylethanolamines > phosphatidylcholines. Cold-adaptation from 28°C (thermoneutral) → 5°C increases polyunsaturated/monounsaturated fatty acyls ratios without changing the saturated fatty acyl groups of, any phospholipid in rat BAT mitochondria. Cardiolipins respond most, their polyunsaturated plus monounsaturated fatty acyls being greater than in phosphatidylcholines and phosphatidylethanolamines: polyunsaturated fatty acyls increase 80% (18 : 2 acyl content doubles) in cardiolipins, 70% in phosphatidylcholines and 50% in phosphatidylethanolamines. In addition, phosphatidylcholine/phos-

phatidylethanolamine ratios drop from 1.06 → 0.76; since phosphatidylcholines are less unsaturated, inner membrane overall unsaturation index increases further. Phosphatidylcholines and phosphatidylethanolamines are the major phospholipids, and thus seem responsible for the observed accompanying increase in membrane fluidity [72,73,591], a bulk property. State 4 respiratory rate (25°C, GDP-insensitive) decreases while a high Δp persists: $C_{M^{H^+}}$ drops by 26% (Table VII). In the absence of Arrhenius plots to determine thermotropic properties, it appears that a diminished proton leak accompanies the cold-induced substitution of polyunsaturated fatty acyls for monounsaturated fatty acyls in mitochondrial phospholipids, especially cardiolipins: the rat BAT mitochondrial phospholipid-leak does not seem to become thermogenic in the adaption to a cold environment. Cold-induced changes in BAT and other mitochondrial phospholipids may preserve bilayer milieu properties rather than barrier properties.

In all rat BAT mitochondrial phospholipids, EFA-deficiency markedly depletes 18:2($n-6$) and 20:4($n-6$) acyls when rats are kept at 28°C or 5°C (Table VII). This loss of unsaturation is relieved partly by sharp increases in monounsaturated fatty acyl contents, partly by the appearance of 20:3 acyls, much less in cardiolipins than in phosphatidylcholines and phosphatidylethanolamines (as in liver mitochondria, see section IV-C.1.a). GDP-insensitive State 4 respiration (i.e., the phospholipid-leak) in BAT mitochondria decreases with EFA-deficiency: $C_{M^{H^+}}$ falls by 18% in 28°C-rats and 5% in 5°C-rats; inhibitor titrations reveal corresponding down-shifts in the relationship between proton flux and Δp [220]: the rat BAT mitochondrial phospholipid-leak does not become thermogenic in the adaptive responses to EFA-deficiency. GDP-sensitive respiration increases. BAT mitochondria prepared from EFA-deficient rats respire 80% faster than controls under 'State 4' conditions (pyruvate/malate, −GDP, 25°C) [736] because uncoupling protein content accrues by 46% and EFA-deficiency augments the protonophore-evoked respiratory chain capacity of rat BAT mitochondria by 60%. The rat BAT mitochondrial uncoupling protein becomes thermogenic in response to cold-adaptation or EFA-deficiency.

BAT mitochondrial cardiolipins of hamsters kept at 22°C, as compared with those of rats at 22°C, contain 50% less 18:2 acyls, 30% less polyunsaturated fatty acyls and twice the saturated fatty acyls [74] (Tables III and VIII). Hamster BAT mitochondrial phosphatidylcholines have about twice the polyunsaturated fatty acyls and less monounsaturated fatty acyls. Cold-adaptation increases cardiolipin unsaturation by doubling the content of 18:2 acyls and polyunsaturated fatty acyls, and halving saturated fatty acyls, but does not change fatty acyls in phosphatidylcholines or phosphatidylethanolamines. BAT mitochondria from cold-adapted hamsters respire under State 4 conditions (glycerol-3-phosphate, 23°C) at 61 ng atom O min^{-1} mg^{-1}, Δp is < 10 mV, and proton conductance via the

TABLE VII

Rat and hamster brown adipose tissue and liver mitochondria: fatty acyl unsaturations in cardiolipins, phosphatidylcholines, and phosphatidylethanolamines; State 4 respiration and $C_{M^{H^+}}$; effects of temperature adaptation and dietary essential fatty acid deficiency (calculated from data of references shown in parentheses)

Abbreviations: BAT, brown adipose tissue; CLs, cardiolipins; EFA, essential fatty acids; PCs, phosphatidylcholines; PEs, phosphatidylethanolamines; PUFA, MUFA, SFA, polyunsaturated, monounsaturated, and saturated fatty acyls; UI, unsaturation index.

	Acclimation temperature (°C) (± dietary EFA)										
	rat BAT [74,220,540,591]					hamster BAT [74,460]		rat liver [154,525]		hamster liver [33]	
	28°(+)	22°(+)	5°(+)	28°(−)	5°(−)	22°(+)	5°(+)	22°(+)	22°(−)	22°(+)	22°(−)
CLs: PUFA	26.6	43.8	48.5	8.7 [a]	12.9 [a]	30.6	53.1	53.4	21.3 [a]	84.4	31.5
MUFA	54.1	37.6	28.1	71.1	61.7	33.3	29.6	35.9	68.0	11.2	61.1
SFA	19.3	18.9	23.0	20.2	25.4	36.1	17.3	10.0	9.3	4.4	4.8
UI	121	134	134	97	95	109	140	153	123	180	126
PCs: PUFA	22.9	20.3	39.2	12.1 [b]	13.8 [b]	36.1	34.2	41.7	40.8 [b]	38.8	33.3 [c]
MUFA	38.2	34.0	19.2	52.3	47.7	24.8	29.1	11.0	11.0	10.5	23.8
SFA	38.9	45.7	41.6	35.6	38.5	39.0	36.7	46.0	48.2	50.7	43.6
UI	110	91	126	90	87	106	127	160	140	153	120
PEs: PUFA	31.2	28.9	47.4	24.0 [a]	34.4 [b]	33.1	34.7	49.0	37.2 [b]	23.9	20.2 [a]
MUFA	35.8	28.5	14.3	47.0	31.2	30.9	30.5	6.3	15.1	22.8	45.8
SFA	33.0	42.8	38.3	29.0	34.4	36.3	33.2	43.7	45.7	53.0	33.0
UI	153	132	185	128	148	116	127	133	137	90	110
State 4	44 [d]		32 [d]	30 [d]	28 [d]		48 [d]	20 [c]	28 [c]		
Δp (mV)	228		224	190	206		220	186	187		
$C_{M^{H^+}}$ [f]	1.16		0.86	0.95	0.82		1.31	0.66	0.90		

20:3 acyls are about [a] one third or less, [b] half or [c] four fifths, of these PUFA; [d] substrate glycerol-3-phosphate, +GDP, +serum albumin, 25°C (ng atom O min^{-1} mg^{-1}); [e] substrate succinate, 25°C; [f] calculated using $H^+/O = 6$ [220] (ng ion H^+ min^{-1} mg^{-1} mV^{-1}).

uncoupling protein is 35 ng ion H^+ min^{-1} mg^{-1} mV^{-1} ($H^+/O = 6$) [461]. However, when the uncoupling protein is blocked with GDP and free fatty acids are sequestered, State 4 respiration via the phospholipid-leak is 48 ng atom O min^{-1} mg^{-1}, $\Delta p = 220$ mV, and $C_{M^{H^+}} = 1.31$ ng ion H^+ min^{-1} mg^{-1} mV^{-1}. Although no BAT mitochondrial conductance is presented for hamsters kept at 22°C, $C_{M^{H^+}} = 0.32$ (succinate, 23°C) in liver mitochondria from unadapted rats. Hamster BAT mitochondrial phospholipid-leak seems to become thermogenic as part of cold-adaptation.

Rat liver mitochondrial cardiolipins contain 20–75% more polyunsaturated fatty acyls, about the same proportion of monounsaturated fatty acyls, and half the saturated fatty acyls found in BAT mitochondria (Tables III and VII). Phosphatidylcholines and phosphatidylethanolamines have about the same saturated fatty acyl content in liver and BAT mitochondria, but in the liver organelle phosphatidylcholine and phosphatidylethanolamine polyunsaturated/monounsaturated fatty acyl ratio is 4–7, whilst in BAT it is 0.7–1. Fatty acyl compositions of phospholipids do not seem to have been reported in liver mitochondria of cold-adapted rats. EFA-deficiency leaves rat mitochondrial phospholipid saturated fatty acyls unaffected, partly substitutes monounsaturated fatty acyls and only few 20:3 acyls for cardiolipin polyunsaturated fatty acyls, but exerts little effect on the unsaturation of phosphatidylcholines and phosphatidylethanolamines (see section IV-C.1.a). This constancy of the bulk phospholipids might account for the minor changes reported for bulk physical properties of liver mitochondria from EFA-deficient rats [284] and leaves the cardiolipin changes to account for effects on respiration. EFA-deficiency results in striking accelerations of rat liver mitochondrial State 4 phospholipid-leak (Table VII): $C_{M^{H^+}}$ rises 36%. Cold-exposure of hamsters, but not of rats, increases liver mitochondrial respiration (succinate, 37°C) in State 4 and State 3, as well as oxidative capacity (cytochrome oxidase activity) [83]. Hibernation in hamsters diminishes State 4 and State 3 respiration measured at 7°C, but augments both rates when measured at 37°C, and also cytochrome oxidase activity; these findings emphasize the need for thermotropic data on temperature adaptations. No information seems to be on hand on liver mitochondrial phospholipids in cold-exposed hamsters, or fatty acyl compositions of any mitochondrial phospholipid subclass in BAT of hamsters that go on to hibernate [10].

BAT responds to altered thyroid hormone levels. BAT cells have approximately the same specific T3-receptor binding capacity [39,40,193] as white adipose tissue cells [67] and hepatocytes [482]. In rat BAT, unlike other cells, an intracellular 5′-deiodinase that converts T4 → T3 generates 55% of the nuclear-bound T3, which makes the BAT receptor relatively insensitive to plasma [T3] [39,40]. The deiodinase is stimulated by extramitochondrial signals that include α_1-agonists and other hormones. Receptor occupancy determines expression of the uncoupling protein and thereby BAT adaptation to cold environments.

Low temperatures evoke thyroid hormone secretion necessary for cold-adaptation; hypothyroid rats do not survive in cold environments [587]. Many steps in the sequence of events that controls the uncoupling protein are thyroid-responsive in BAT and other tissues (see Ref. 284): the number of β-adrenergic receptors and their accesibility to norepinephrine; coupling of occupied receptors, via G-proteins, to adenylate cyclase (hypothyroidism increases the sensitivity of the G_i-protein in BAT that couples inhibitory agonists (e.g., adenosine) to adenylate cyclase 1723]); adenylate kinase activity; cytosol [cAMP]; protein kinase-mediated phosphorylation of lipase; conversions of triacylglycerol-fatty-acyl → free fatty acid → fatty acyl-CoA that regulates [free fatty acids]; the rate-limiting steps in de novo acetyl-CoA → fatty acid; and the desaturations of fatty acyl-CoA that lead to unsaturated free fatty acids that are less effective in activating the uncoupling protein.

Hyperthyroidism in rats kept at room temperatures affects the following processes in BAT. (i) Occupancy of BAT T3-receptors rapidly stimulates lipogenesis, which is already intense [339]; lipogenic enzyme activities double [215,508]; mitochondrial α-glycerophosphate dehydrogenase activity, normally some 60-fold higher than in liver, further increases by 50% [362]. (ii) Fatty acid β-oxidation and succinate oxidation in State 4 (25°C) rise 4-fold [215]; the mitochondrial outer carnitine long-chain-acyl-CoA transferase that regulates β-oxidation in liver and other tissues is thyroid-sensitive and cardiolipin-dependent (see Ref. 284). (iii) BAT proliferates (white adipose tissue atrophies [702]) but cytochrome oxidase and cardiolipin/mg BAT remain unchanged [540], which denotes a constant content of mitochondria. (iv) Although T3-treatment (0.3 $\mu g\ g^{-1}\ day^{-1} \times 5$ d) increases the percentages of 18:2 and 20:4 acyls in total phospholipids of BAT mitochondria but not BAT microsomes [215], more vigorous and prolonged T4-treatment of rats (0.5 $\mu g\ g^{-1}\ day^{-1} \times 40$ days) does not change BAT fatty acyl/cardiolipin composition or cardiolipin/phospholipid ratios [446] (Table VII). The low-18:2, high saturated fatty acyl pattern of BAT cardiolipins characterizes tissues that do not respond thermogenically to thyroid hormone levels [284]. (v) Thyrotoxicosis depletes BAT uncoupling protein in warm rats [637,662], and blocks cold-exposure from inducing uncoupling protein: it is a condition with negative survival value in rats at low environmental temperatures [83].

Brown adipocytes from hypothyroid rats contain 3-fold more $mRNA_{S14}$ (an intermediate in lipogenesis

initiation) than euthyroids. Increased activities of BAT fatty acid synthase, ATP-citrate lyase and glycolytic flux lead to an 11-fold rise in fatty acyl synthesis [26,193]. White adipose tissue from the same animals has only 20% of normal levels of $mRNA_{S14}$ and shows no increase in fatty acyl synthesis; liver lipogenesis decreases. It is surprising that both hypothyroidism and hyperthyroidism augment lipogenesis in BAT. Hypothyroidism doubles or triples activity of the BAT peroxisomal non-phosphorylating β-oxidation apparatus plus catalase [210]. This system normally contributes 1% to 30% of the thermogenesis of overall fatty acid oxidation, and also accelerates during cold-exposure [72].

Malignant hyperthermia is a lethal syndrome of excessive mitochondrial heat production accompanied by skeletal muscle rigidity, seen rarely in sensitive humans and more often in pigs of the German Landrace strain, when 'stressed' or exposed to halocarbon anesthetics [92–94,230,280]. Respiration of skeletal muscle mitochondria from malignant-hyperthermia-sensitive pigs is abnormally stimulated by Ca^{2+}. Involvement of the unusually saturated muscle cardiolipins (Table III) and phospholipids (see section IV-B.2) of this and other strains of pigs does not seem to have been considered, although the increased mitochondrial Ca^{2+}/H^+ exchange-leak shows abnormal lipid-dependency.

Hypothyroidism is accompanied by changes in BAT of 25°C-adapted rats, and mice [316], that resemble the effects of cold exposure: increased BAT weight, DNA, and mitochondrial cytochrome oxidase (see Ref. 465); the last two are also unusual in hypothyroidism. However, hypothyroidism abolishes the increased respiration of BAT normally effected by norepinephrine or by exposing mice to the cold [293]. Cold exposure is lethal, and produces no lipolysis in BAT or increase in BAT mitochondrial uncoupling protein. Low, maintenance dosage of T4 allows cold-induced synthesis of uncoupling protein. BAT is also unreactive in hypophysectomized rats and *ob/ob* mice. Combined maintenance of hypophysectomized rats with T4 and corticosterone permits cold-adaptation and production of mitochondrial uncoupling protein. Although T4-treatment increases uncoupling protein in *ob/ob* mice, it does not permit cold-induced thermogenesis in BAT or save the cold mice [274]. Mitochondrial phospholipid fatty acyl changes in cold-adaptation are not consistently thermogenic in BAT but may be in liver (see above). Therefore, observed defects in hepatic fatty acyl-CoA desaturative mechanisms may contribute to the hypothyroid's cold-sensitivity [284]; normal cats also fail to acclimate [46], and they have no Δ6-desaturase and a relatively inactive Δ5-desaturase [546]. It is generally concluded that thyroid hormone is necessary but not sufficient for cold adaptation.

IV-C.2.d. Hormonal state. Thyroid: thyroid hormone levels and mitochondrial respiration (especially State 4) correlate directly in rat liver, heart and kidney [277,278], and human skeletal muscle [632], tissues in which mitochondria have high cardiolipin/phospholipid ratios, cardiolipins have high 18:2 contents, and nuclei possess many T3-receptors [284,285]. A uniform hypothyroid state is readily achieved by arresting hormone synthesis, but the effects of hormone administration or excess depend on several variables.

In the studies of Withers and Hulbert [708] on liver mitochondria of hypothyroid rats, fatty acyl compositions of total lipids were close to normal and did not change when the rats were fed a control diet plus saturated or unsaturated fatty acids; they concluded that the hormone acts at least in part directly on mitochondrial membrane lipids.

In our hypothyroid rats, liver mitochondrial phospholipids show an abnormally high-18:2, low-20:4 fatty acyl pattern [97–99,291] that reflects in part a 72% increase in cardiolipins with normally high 18:2 contents and no 20:4 acyls, in part the abnormal compositions of the phosphatidylcholines and phosphatidylethanolamines [291]. Raederstorff et al. [524] have recently confirmed this hormone-responsive pattern of abnormality in $(n-6)$-unsaturated fatty acyls in the phosphatidylcholines and phosphatidylethanolamines of mitochondria from livers of hypothyroid rats, and also find significantly decreased contents of $22:6(n-3)$ acyls. Lack of thyroid hormone usually raises levels of metabolic intermediates by slowing their removal more than their synthesis. It is therefore of interest that cardiolipin accumulates in plasma membranes of *H. influenzae* when activity of a cardiolipin-specific phospholipase D is inhibited (see section III-A). Phospholipase D enzymes exist in rat liver endoplasmic reticulum [665].

The data are not sufficient to decide if cardiolipins, or phosphatidylcholines and phosphatidylethanolamines, or both, contribute to the slowed phospholipid-leak that characterizes hypothyroidism. Studies on induced changes in mitochondrial phospholipids (section IV-C) indicate a specific role for cardiolipins. The sidedness of the extra cardiolipins in the inner membranes of mitochondria from hypothyroids (or hyperthyroids) has not been examined, but the unsaturation of cardiolipins influences sidedness. The normal fatty acyl profiles of the excess cardiolipins should favor inner-sidedness. Supporting this inference are the following observations on the outer-face mitochondrial carnitine palmitoyltransferase: its activity and susceptibility to physiological inhibitors have characteristics of lipid-dependency [366,741]; cardiolipins specifically activate the purified enzyme and may orient the mitochondrial enzyme [470]; activity is abnormally low in hypothyroids (see Ref. 284).

In hypothyroidism, 18:2 acyl contents rise and 20:4

contents fall reciprocally not only in liver mitochondria but also in the phospholipids of microsomes and nuclear envelopes [181,291,595]. The pervasiveness of these alterations has been attributed to the observed defects in the desaturation steps of the conversion of 18:2-CoA → 20:4-CoA [284]. The diminished incorporation of administered 18:2 fatty acid into liver phospholipids [291] does not seem to limit 18:2 acyl accumulation in liver organelle phospholipids. Nor does the failure of the fasted-refed hypothyroid rat to induce hepatic Δ9-desaturase [288] deplete 16:1 and 18:1 fatty acyls.

To examine effects of thyroid hormone, several dose-time schedules have evolved. Originally, treatment of normal euthyroid rats with a very high dose of DL-T4, equivalent to 70 μg of the effective component L-T4 per g body weight, given over 4 days, increased liver mitochondrial State 4 respiration (30°C) and abolished phosphorylation of ADP [290,414]. Later, one much smaller dose of T3 given to a hypothyroid rat was observed to stimulate mitochondrial State 3 respiration and synthesis of liver phospholipids, RNA, and proteins after 2 days [642,643]. Even more rapid effects on respiration are reported: one injection increases State 4 respiration in 2–15 min in hypothyroid rats, and in 6–24 h (but not at 48–72 h) in euthyroid rats [279]; perfusion of hypothyroid rats' livers with T3 progressively increases State 3 respiration to a maximum at 60 min [586]; T3 affects cultured hepatocytes in 2 h [563]. 'Hyperthyroidism' is now often produced by 10–21 daily injections into euthyroid rats. Only a few of the later treatment schedules can be integrated with studies on phospholipid metabolism and resolved mitochondrial phospholipids.

Thyroid-treatment very rapidly leads to increased activities of lipogenic enzymes [482] and fatty acyl-CoA Δ9-, Δ6- and Δ5-desaturases, and to altered mitochondrial composition and function [284,285]. After one injection into a hypothyroid rat, T3-occupancy of T3-depleted nuclear receptors in lipogenic tissues begins to activate in minutes, and then induce synthesis of, the depleted fatty acid synthetase, acetyl-CoA carboxylase, and supporting $NADP^+$-reductases [482]. Saturated fatty acyls, perhaps those newly synthesized, substitute for many of the 18:2 and 20:4 acyl groups in total phospholipids of liver mitochondria by 1–2.5 h [284] but there is no information on cardiolipins this early. In contrast, in normal rats fasted and refed carbohydrates, monounsaturated fatty acyls replace liver mitochondrial total phospholipids 18:2 and 20:4 acyls even more severely by 2 days [9]. T3-treatment of euthyroids on each of 5 days substitutes saturated fatty acyls only in cardiolipins; mitochondrial phosphatidylcholines and phosphatidylethanolamines become slightly more unsaturated because 20:4 acyls replace some of the 18:2 acyls [378] (Table III). This hyperthyroid pattern of cardiolipins with low 18:2 and high saturated fatty acyl contents is unique among agents that change liver cardiolipin fatty acyl distributions. Microsomal phospholipid fatty acyls remain unchanged after 5-days treatment [563] but show the mitochondrial pattern after a 21-days schedule [180]. To the present, we do not know the mitochondrial phospholipid compositions that accompany uncoupling proton flux after high doses of hormone.

Conversion of 18:2 → 20:4 acyls, which would account for the low 18:2 and high 20:4 pattern by accelerated microsomal Δ6- and Δ5-desaturations, occurs transiently in 1–4 h in T3-treated hypothyroid rats, and after 3 days of treatment in euthyroid rats. The Δ9-desaturase conversion of 18:0 → 18:1 acyls intensifies but seems not to affect cardiolipin or other phospholipid compositions, probably because 18:1 acid is so available from the diet [99,181].

Hyperthyroidism in rats also stimulates almost all the successive steps in hepatic metabolism of fatty acids and phosphoglycerols, and mitochondrial cardiolipin synthesis [284]. Cardiolipin/phospholipid ratios are abnormally high (+50%) in liver and heart mitochondria after normal rats are treated with T3 for 5 days [563]; a 3-day schedule did not alter heart cardiolipin/phospholipid proportions [283]. Increased cardiolipin/phospholipid ratios could result from a greater increase in cardiolipin synthesis than in phosphatidylcholine and phosphatidylethanolamine synthesis. Another possible mechanism, activation of a mitochondrial phospholipase A_2 by elevated levels of thyroid hormone [443] and cardiolipins [387], would remove phosphatidylcholines and phosphatidylethanolamines with high polyunsaturated fatty acyls contents but leave cardiolipins unlysed [576].

The decreased unsaturation of the cardiolipins from hyperthyroids should remove the conical shape that makes cardiolipins seek concave hydrophobic surfaces. Amounts of cardiolipin in the outer face of mitochondrial inner membranes do indeed seem to be increased in hyperthyroids, as judged from activity of the liver mitochondrial 'outer' carnitine palmitoyltransferase (see Ref. 284).

Recent studies define thyroid effects on mitochondrial State 4 respiration in terms of proton conductance, driving force Δp [54,246–249] and inner membrane area [249]. Hypothyroidism depresses proton leakage estimated from either FCCP-titrations [228,673] or State 4 rates (succinate, 25 or 37°C) multiplied by the H^+/O quotient (Table VIII).

The slowed leak results from decreased proton-conductance of the membrane, since hypothyroidism does not alter Δp in correlation with State 4 rates. $C_{M^{H^+}}$ for euthyroid rat liver mitochondria (succinate, 25°C) is typically 0.3–1 ng ion H^+ min^{-1} mg^{-1} mV^{-1} [464]. $C_{M^{H^+}}$ is 0.63–1.62 in Table VIII, perhaps because

$C_{M^{H^+}}$ tends to increase with temperature. $C_{M^{H^+}}$ for mitochondria from hypothyroid rats (0.74–0.9) is consistently lower than in euthyroids and does not vary much with temperature in the few measurements shown. Brand and coworkers [248,450] conclude that the slowed proton leak in hypothyroidism accounts for the entire diminished proton permeability, making it redundant to postulate a decreased H^+/O-slip.

The effects of hormone administration depend on the initial thyroid state, the dose-time schedule, and perhaps the conditions of assay. In liver mitochondria from hypothyroid rats given one dose of T3 1 day before killing, proton leakage increases 30–40% as estimated from FCCP-titrations or State 4 respiration (25°C) [298–300] (Table VIII). The excess leak is not via the F_0: oligomycin actually increases State 4 by 26%. Treatment raises the proton driving force Δp mainly by increasing ΔpH to 81 mV (an unusually high level considering the presence of P_i); the resultant $C_{M^{H^+}}$ rises to normal levels. Introduction of protonophores would increase proton leakage; one proposed source is autoxidation of unsaturated fatty acyl groups in membrane phospholipids (see section II-A). Products of mechanisms that protect against superoxides (e.g. oxidized glutathione and malondialdehyde) do not increase 24 h after hypophysectomized (and so also hypothyroid) rats are injected with T3, although the proton leak (State 4 respiration) accelerates 2.2-fold [401]. Thus, lipid peroxidation does not seem to cause this augmented proton leak.

Treating normal rats with T3 for 10 days ('hyperthyroids') increases liver mitochondrial State 4 respiration (37°C) 64% in the studies of Hafner et al. [249]. The increased leak, as measured by State 4 respiration, is not inhibited by oligomycin and so does not proceed across F_0; the leak was not measured by FCCP-titration. $C_{M^{H^+}}$ for all mitochondria from hyperthyroids is in the range 0.84–2.38 ng ion H^+ min^{-1} mg^{-1} mV^{-1}, consistently greater than control values (Table VIII). The major proton drive is $\Delta\psi$ (which treatment diminishes by 14 mV), since the presence of nigericin and P_i collapses most of the ΔpH (only 15.9 mV in euthyroids

TABLE VIII

Liver mitochondria: effects of thyroid state on proton leak (State 4, + oligomycin); $\Delta\psi$, -59ΔpH and Δp during State 4 and State 3 respiration (succinate or succinate/malate); calculation of effective proton conductance [462].

$$C_{M^{H^+}}\ (ng\ ion\ H^+\ min^{-1}\ mg^{-1}\ mV^{-1}) = \frac{respiration\ (ng\ atom\ O\ min^{-1}\ mg^{-1}) \times 6\ ng\ ion\ H^+/ng\ atom\ O}{\Delta p\ (mV)}$$

Abbreviations: ET, thyroid-treated euthyroids; HT, thyroid-treated hypothyroids; oligo, oligomycin.

Thyroid state	Proton leak (nmol FCCP/mg)	Respiration (ng atom O min^{-1} mg^{-1})		$\Delta\psi$ (mV)	-59ΔpH (mV)	Δp (mV)	$C_{M^{H^+}}$	Reaction mixture, additions (Refs.)
		− oligo	+ oligo					
Euthyroid		20		181	14	195	0.63	State 4, 30°C, P_i, valinomycin [598]
ET (T4sc 8 $\mu g\ g^{-1}$ + 1 d)		30		194	17	211	0.84	
Euthyroid		106		103	10	113	5.61	State 3, + ADP
ET (T4sc 8 $\mu g\ g^{-1}$ + 1 d)		141		103	8	111	7.62	
Euthyroid	11.8	53						State 4, 26°C, P_i, ATP [673]
Hypothyroid	4.5	33	29	139			(< 1.42)	
HT (T3ip 0.4 $\mu g\ g^{-1}$ + 1 d)	5.8	46	35	146			(< 1.89)	
ET (T4ip 8 $\mu g\ g^{-1}$ + 1 d)		62						
Euthyroid	6.8	38	42	131	73.7	205	1.08	State 4, 25°C, P_i, ATP [298–300]
Hypothyroid	5.1	29	28	125	73.7	199	0.89	
HT (T3sc 0.4 $\mu g\ g^{-1}$ + 1 d)	7.3	35	44	127	81.0	208	0.98	
ET (T4sc 0.15 $\mu g\ g^{-1}\ d^{-1}$ × 10 d)	6.6	53	46	133	80.4	214	1.27	
Euthyroid			49	200	15.9	216	1.22	State 4, 37°C, P_i, nigericin [249]
Hypothyroid			30	214	11.8	232	0.74	
ET (T3 0.15 $\mu g\ g^{-1}\ d^{-1}$ × 10 d)			80	188	18.7	202	2.38	
Euthyroid		187	(52) [a]	166	25.5	192	5.84	State 3, 37°C, ADP [120]
Hypothyroid		95	(29) [a]	179	11.8	191	2.98	
HT (T3iv 0.15 $\mu g\ g^{-1}$ + 15 m)		108	(28) [a]	180	18.7	199	3.26	

[a] State 4 respiration calculated from values shown for State 3 and respiratory control ratios.

and 2.8 mV higher after treatment). Because Δp is in the order hypothyroid > euthyroid > hyperthyroid, while respiration is in the order hyperthyroid > euthyroid > hypothyroid, these workers ascribe the differences in State 4 rates at least in part to changes in the area of the inner membrane (per mg mitochondrial protein). By stereological electron micrographic analyses, the area of cristae was found to be 25% below normal in mitochondria of hypothyroid rats, as was the ratio of cardiolipin/mitochondrial protein [326]. We found a mitochondrial cardiolipin/phospholipid ratio 72% higher than normal [291]; the increased normal cardiolipins might increase proton retention in addition to the effects of decreased conductance area. Jakovcic et al. [326] made rats hyperthyroid by administering T4 over 21 days, enough time to induce a new population of mitochondria with augmented cristael area, and perhaps comparable with the 10-day treatments shown in Table VIII. It seems doubtful though that 1-day to 3-day treatments produce new mitochondria, which leaves the proton permeabilities rather than membrane area to account for such early stimulations of State 4 respiration.

Horrum et al. [298–300] find that 10 daily T4 injections accelerate State 4 respiration (25°C) in the absence of oligomycin, although they do not increase State 4 or the degree of proton leakage measured by FCCP-titrations in the presence of oligomycin. Oligomycin inhibits only the increased portion of State 4 respiration; ergo, an F_0-leak accounts for the rise in proton flux above the persisting basal proton-leak. As calculated from Δp, the hyperthyroid state raises $C_{M^{H^+}}$ from 1.08 (control) to 1.27 ng ion H^+ min^{-1} mg^{-1} mV^{-1}. Despite the presence of 10 mM P_i, ΔpH is relatively high in euthyroids (74.7 mV) and 6 mV greater in the hyperthyroid mitochondria, whilst $\Delta\psi$ hardly changes. Thus, a T3-induced F_0-leak appears to be selectively driven by $\Delta[H^+]$. If the lowered unsaturation of the cardiolipins observed at 5 days of T3-treatment [563] continues to 10 days, it is not associated with an increased phospholipid-leak, which contrasts with findings in EFA-deficiency and other conditions. If FCCP-titrations do index the proton leak, which is disputed [62], these observations also seem to conflict with those of Nicholls [459] that proton passage through the F_0-channel in the presence of ADP + P_i decreases ΔpH enough to eliminate proton leaks; further, hyperthyroidism raises ΔpH (Table VIII). The alternative mechanisms, whereby T4-treatment of normal rats initiates an F_0-leak when ΔpH is allowed to contribute to proton drive, or T3-treatment increases other leak routes when $\Delta\psi$ is made to predominate [249], should be resolved by treatment of rats with either T4 or T3, and measurements at 25 and 37°C, ± oligomycin.

A significant F_0-leak in hyperthyroid mitochondria under State 4 conditions might be considered an acceleration of a normal proton route through F_0 [415]. These studies report an impressive degree of F_0-leak in the very slow respiration of normal rat liver mitochondria (+substrate, O_2 and P_i, 25°C) (see Table VIII). Oligomycin inhibits State 4 respiration by approx. 50% and raises $\Delta\psi$ to 187 mV from a level of 176 mV. The corresponding effective proton conductances can be calculated to be < 0.20 (since ΔpH was not measured) ng ion H^+ min^{-1} mg^{-1} mV^{-1} plus oligomycin and < 0.43 minus oligomycin. Oligomycin inhibits State 3 by 90%, to leave a nonphosphorylating respiratory rate that is 30% below the uninhibited State 4 rate. Masini et al. [415] concluded that a large portion of State 4 respiration is normally linked to rephosphorylation of intrinsic ATP used in matrix reactions like pumping Mg^{2+} and changing membrane conformation. However, the mitochondrial concentrations in these studies were 3–3.5 mg of protein per ml, and the State 4 rates are as low as 25% of those measured at 25–26°C by other workers cited in Table VIII, who used 0.13–2 mg mitochondrial protein per ml (cf. Estabrook [178]: 1.7 mg/ml) and saw no oligomycin inhibition of State 4 respiration in controls. Similarly, Shears and Bronk [598], who used 4 mg/ml, reported a State 4 rate at 30°C that is half that found by the others at 25°C. It thus appears that $[O_2]$ may have limited respiration in Masini et al.'s measurements, and thereby activated ATPase.

Altered thyroid states induce much larger changes in State 4 respiration than in Δp, apparently by altering the supralinearity of proton conductance at the high Δp values characteristic of State 4 respiration [53,54,246]. Supralinearity has been ascribed to several mechanisms: dielectric breakdown of membrane resistance to proton current [459]; intramembranal proton circuits not mediated by Δp [488,556]; lateral proton conductance mediated by cardiolipins and PGP_H on surfaces of bacterial membranes (see section II-A); and properties of membrane lipids whereby (saturated) phospholipids pass protons transmembranally along a transient hydrogen-bonded chain of water molecules (see section II-A). Mitochondrial $C_{M^{H^+}}$ is ohmic in euthyroids to about 180 mV, in hypothyroids to 200 mV, and in hyperthyroids to 160 mV. Thus, the susceptibility of inner membrane to breakdown at high proton pressure – if that is the mechanism – is in the order hyperthyroid > euthyroid > hypothyroid.

Evidence is discussed in section II-A that unsaturated cardiolipins contribute to proton retention at high Δp under State 4 conditions, whereas the uncoupling protein in BAT leaks protons and disperses Δp. Increased amounts (cardiolipin/phospholipid) of abnormally saturated liver cardiolipins are associated with proton-leakiness and thermogenesis in mitochondria of hyperthyroid animals. The amount of uncoupling pro-

tein is up-regulated by decreases in ambient temperatures and the resulting proton leakage is thermogenic, like the effects of thyrotoxicosis. Hypothyroidism augments liver mitochondrial cardiolipin/phospholipid ratios and the cardiolipins are normally unsaturated; by their normal role in proton-retention, these cardiolipins should decrease proton leakage and thermogenesis; it is not clear if the phosphatidylcholines and phosphatidylethanolamines, which also become less unsaturated, participate in proton-retention. It seems pertinent that chilled subjects secrete more thyroid hormone, and warmed subjects, less. Elevation of temperature also down-regulates amount of BAT mitochondrial uncoupling protein, proton-leakage and thermogenesis.

Thyroid-state affects State 3 respiration rates. Although cardiolipins are specific for many of the regulated steps, mechanisms of thyroid effects are not proven to be mediated by the known cardiolipin changes. State 3 Δp is about 110 (30°C) or 190 mV (37°C) in liver mitochondria from euthyroids, and untreated or treated hypothyroids [120] (Table VIII). Effective proton conductance at 37°C is 5.84 ng ion H^+ min^{-1} mg^{-1} mV^{-1} in mitochondria from euthyroids and half that in hypothyroids. The proton routes initiated or augmented in mitochondria of hypothyroid rats by induction of State 3, namely the ADP/ATP carrier, ATP-synthase, P_i/H^+ symport, dicarboxylate carrier, cytochrome bc_1 and cytochrome oxidase, would be involved in these proton conductances, since the proton leak disappears in State 3.

The ADP/ATP carrier activity is sensitive to effects of altered thyroid states on: (i) the membrane environment of the carrier; (ii) amounts and exchangeability of ADP + ATP in the matrix; (iii) ratios of [free ATP]/[free ADP] in matrix and cytosol; and perhaps (iv) the proportions of ΔpH and $\Delta\psi$ (see above; this carrier discharges $\Delta\psi$ [155]). All these factors determine the carrier rate and, together with rates of other regulators, its C_i contribution to State 3 control. (i) An altered membrane environment of the ADP/ATP carrier in liver mitochondria from hypothyroid rats is indicated by the linearity of the Arrhenius profile (0–37°C) of external ADP translocation [281]; the profile in normals inflects sharply at 17°C. As a result, at 0°C (a usual temperature for assay) hypothyroid mitochondria translocate external ADP at a rate 66% less than normal but at 25°C the rate is normal. The abnormal thermotropic properties are typical of effects of altered membrane lipids. (ii) Content of ADP + ATP in liver mitochondria of hypothyroid rats is 60% greater than in normals, and pyridine nucleotides are 40% above normal [281,341]. Of the 13.8 nmol of ATP + ADP per mg protein, hypothyroids exchange only the normal content of 9.6 nmol/mg via the ADP/ATP carrier [281,586]. A similar compartmentation occurs when ATP + ADP accumulates after glucagon treatment [255]. One LT4-injection corrects, in 3 days, the nucleotide compartmentation and temperature-rate relationship. Accumulation of ATP can proceed through an ATP-Mg/P_i carrier that moves ATP passively down a concentration gradient, but that would not account for abnormal compartmentation [15]: loaded nucleotides exchange completely, as shown by the linearity of adenine nucleotide translocase velocity with matrix [ATP + ADP] even at high concentrations.

(iii) The ATP/ADP ratio in the matrix of liver mitochondria from hypothyroid rats is subnormal [281] because ADP is phosphorylated slowly [99]; the ratio in the cytoplasm is high because ATP is utilized slowly (e.g., Ref. 165). Increased matrix ATP + ADP and ADP/ATP ratio should accelerate translocation of external ADP, but the compartmentation leaves the abnormal lipid dependency of the carrier in hypothyroid mitochondria to account for the slowed transport.

Claims that an identity of the ADP/ATP carrier with a rat liver mitochondrial T3-receptor accounts for in vitro and in vivo hormonal acceleration of State 3 respiration [630] are difficult to accept conceptually [245,284] and are contradicted experimentally [530].

The cardiolipin-specific carriers of monocarboxylates, dicarboxylates and tricarboxylates, as well as acylcarnitines (palmitoylcarnitine transferases plus (acyl) carnitine translocase), are all thyroid-responsive [284] (Table I). In heart mitochondria of hyperthyroid rats (5-day T3-treatment), as compared with euthyroids, the pyruvate carrier V_{max} at 10°C is 70% higher, K_m for pyruvate is unchanged, oxidation of pyruvate in State 3 at 25°C is accelerated 40%; cardiolipin/phospholipid is 50% greater but fatty acyl composition is not reported [494]. Cardiolipin per phospholipid ratios also rise in liver mitochondria, where cardiolipin 18:2 acyls are extensively replaced with saturated fatty acyls [563] (Table III). The tricarboxylate carrier in liver mitochondria does not seem to have been tested for State 3 regulation; its V_{max} at 4°C increases 62% in hyperthyroid rats, K_m for citrate remains unchanged, and activity increases across the range of 1–27°C while transition temperatures in Arrhenius profiles shift from 18°C → 13°C 1495]. Mitochondrial ΔpH remains at 55 mV, and carrier amount also stays constant, suggesting that the hormone activates carrier through either increased general membrane fluidity or specific interactions between cardiolipins and carrier.

Liver mitochondria from hyperthyroid rats (5 days of T3) take up or exchange P_i 50% faster than normals, as measured by V_{max} at 0°C [497]. The amount of P_i-carrier, the K_m for P_i, the initial intramitochondrial [P_i], and the ΔpH, are all close to normal. Paradies and Ruggiero [497] conclude that the increase in cardiolipin/phospholipid ratio accelerates P_i-carrier activity, which may contribute to an increased mitochon-

drial State 3 respiration because P_i transport is obligatory for malate, citrate and α-ketoglutarate transport, and closely linked to ADP and Ca^{2+} transport. However, the great capacity of the P_i-carrier usually precludes a regulatory role. Selwyn [588] cites a report of Gainutdinov et al. that thyroid hormone treatment increases a heat-stable, cytoplasmic factor that accelerates equivalent proton influx along the P_i/OH antiporter portion of a P_i cycle that includes a pH-dependent anion-conducting channel in liver and heart mitochondrial membranes.

The ATP-synthase in heart is thyroid-dependent in situ and in vivo [353], and is the major regulator of State 3 in adult and fetal heart mitochondria. The ATP-synthase in adult liver mitochondria is also thyroid- and cardiolipin-dependent, but does not regulate (Table I). In skeletal muscle, ATP takes up $^{*}P_i$ more slowly in thyrotoxics than in normals [334]. Ernster et al. [174] suggest that the embedment of the protonophoric F_0-ATPase in cardiolipins is necessary for ATPase function. A role for an abnormally conductive ATP-synthase (F_0-leak) in the augmented portion of State 4 proton leakage in liver mitochondria from hyperthyroid rats [298–300] is disputed (see section IV-C.2.d).

Insulin: cardiolipin fatty acyl distributions and cardiolipin/phospholipid ratios are normal in renal cortex total phospholipids of rats after 2 months of streptozotocin-induced diabetes [107] (Table III). Cardiolipin/phospholipid quotients in liver mitochondria are normal in 4- to 10-week diabetic rats [68,332,676] but slightly lowered in skeletal muscle mitochondria from chronically diabetic human patients [254]. Thus, from these few studies it appears that cardiolipins do not participate in the better-documented changes in polyunsaturated phospholipids from liver, heart, kidney and testis, and from liver mitochondria or microsomes, that are reported in diabetic rats 2–70 days after injection of alloxan or streptozotocin [61,68,107,164, 181,286,293,310,675]: 18:2($n-6$) and 22:6($n-3$) acyl contents increase, 20:4 acyls are depleted. These fatty acyl changes are seen in phosphatidylcholines and phosphatidylethanolamines, but only kidney cardiolipins have been analyzed [107]. If the normal kidney cardiolipins in diabetics indicate that liver cardiolipins are also normal (additional reasons are presented here to think they are), lipid-dependent abnormalities of mitochondrial function in diabetics are effects of altered fatty acyl compositions of phosphatidylcholines and phosphatidylethanolamines. Altered cardiolipins affect function differently.

Fatty acyl changes in lipids of diabetic subjects can be attributed to usual mechanisms: the fatty acyls of mitochondrial phospholipids are replaced in part by incoming fatty acyls, in this case mobilized from depot fat. Release of the hormone-sensitive lipase activity from insulin-inhibition in experimentally-induced diabetes mellitus rapidly mobilizes free fatty acids from white adipose tissue triacylglycerols into plasma. Triacylglycerol fatty acyls include approx. 25% 16:0, 40% 18:1, 25% 18:2($n-6$), and no 20:4($n-6$) acyls, in fasted rats [9] and in 3-week diabetic rats [310]. Albumin-bound fatty acids in plasma exchange with tissue fatty acyls.

When diabetes is induced by alloxan injection, free fatty acid content of rat liver mitochondria increases linearly from 5 μg (mg protein)$^{-1}$ at $t = 0$, at a rate of about 0.25 μg mg^{-1} h^{-1}, to a level of 18 μg mg^{-1} at 48 h; no fatty acid composition was presented [419] but one can be inferred. Diabetes also increases plasma [triacylglycerols] 4-fold; the liver esterifies free fatty acids, further desaturates some, and incorporates both saturated and unsaturated fatty acyls into triacylglycerols and phospholipids that are released to plasma. Normally, plasma triacylglycerols contain about 35% 16:0, 40% 18:1 and 7% 18:2 acyls, which indicates that fat partly retains stored essential 18:2($n-6$) acyls. In diabetic rats 21–30 days after streptozotocin administration, plasma triacylglycerol 18:2 acyl content is 24% at the expense of 16:0 and 18:1 acyls; plasma phospholipids contain twice the normal amount of 18:2 acyls and less than normal 20:4 acyls [310,724]; erythrocyte membrane phosphatidylcholines and phosphatidylethanolamines, which normally exchange with plasma lipids, mirror the high-18:2, low-20:4 acyl pattern in diabetics.

A high-18:2, low-20:4 acyl pattern in both mitochondrial and microsomal phosphatidylcholines and phosphatidylethanolamines, together with normal cardiolipins, occurs in mitochondria from hypothyroid rats, and is ascribed to observed decreases in hepatic Δ6- and Δ5-desaturations (see Ref. 284); fatty acid mobilization from triacylglycerols is suppressed in hypothyroidism. In contrast, fasting rats and refeeding them carbohydrates [9], or injecting hypothyroid rats with T3 [284], evokes newly biosynthesized saturated and monounsaturated fatty acids from lipogenic tissues, that partly replace liver organelle membrane fatty acyls. Most of the 18:2 acyls are displaced in cardiolipins from hyperthyroid rats (Table III) but any contribution of stored fatty acyls in the depleted triacylglycerols is uncertain.

Thus, the augmented free fatty acids in liver mitochondria from diabetic rats would have a high proportion of 18:2 acyls. The fatty acid composition of these mitochondrial phospholipids reflects, first the influx of 18:2 acyls; secondly the preservation of 18:2 acyls through the known defect in hepatic Δ6-desaturation [196,204,205,428] that normally limits the conversion of 18:2($n-6$) $\rightarrow$ 20:4($n-6$) acyls; and thirdly, influx of 16:0 and 18:1 acyls (the slowed Δ9-desaturation in diabetics [204,317] is not accompanied by lowered 18:1

acyl contents in most studies). High 18:2 concentrations would protect the normal high 18:2($n-6$) acyl contents of cardiolipins. Improved mobilization of stored 18:2($n-6$) acyls and subsequent protection against desaturation may account for observations that an EFA-deficient diet depletes liver and heart phospholipid ($n-6$)-unsaturated fatty acyl groups less in diabetic rats than in control animals [544]. The desaturation defect and the excess of 16:0 + 18:1 acyls would remove 20:4 acyls where they normally abound, i.e. phosphatidylcholines and phosphatidylethanolamines. However, although the hepatic desaturative conversion of 18:2($n-3$) → 22:6($n-3$) acyls (lab diets contain no 22:6) slows as early as 2 days after alloxan injection [286], 22:6 acyl contents increase in mitochondria from diabetic rats – probably because phosphatidylcholines and phosphatidylethanolamines avidly retain 22:6 acyls, as shown by their 2.3-fold accumulation while 18:2 content is halved, in heart lipids of rats starved 7 days [727].

Altered mitochondrial membrane phospholipids in diabetic rats are accompanied by several changes. (i) Heart mitochondria and sarcoplasmic reticulum are enlarged in situ [534], which indicates that preparation artifacts do not account for the biochemical lesions. (ii) Activity of mitochondrial β-hydroxybutyrate dehydrogenase decreases because amount of enzyme diminishes [103]; activity responds abnormally to temperature changes because the enzyme binds phosphatidylcholines specifically and diabetic phosphatidylcholines have abnormal fatty acyl profiles [676].

(iii) Liver mitochondria from 2- to 3-day diabetic rats respire at normal rates at 37°C in both State 4 and State 3 [286]; State 4 respiration is normal at 30°C [259,499,675] but 25–45% faster than controls at 23–25°C [259,420]. At 30°C State 3 respiration together with ADP/ATP exchange rate slows by 45% [388]; uncoupling is reported [252]; skeletal muscle mitochondria from human chronic diabetics oxidize slowly in both State 3 and State 4 [254,397]. These data may reflect abnormal thermotropic properties seen in 2-day diabetics [286]: Arrhenius plots of State 4 rates are typically linear between 15 and 37°C [289] but E_a for diabetic mitochondria is 48 kJ/mol, for controls, 64 kJ/mol. The ratio of State 4 rate (proton leakage) in diabetics/controls rises as temperature is decreased: 1.3 at 37°C and 2.2 at 15°C. Increasing proton leakage uncouples; the ADP/O quotient diminishes as temperature is depressed. This unusual thermotropic property is associated with the abnormal phosphatidylcholines and phosphatidylethanolamines, and presumably normal cardiolipins.

(iv) Activity of anion transporters alters, as shown by measurements of specific activity and total activity of cardiolipin-dependent transporters (see section II-B) extracted in the presence of beef heart cardiolipin and reconstituted in soy bean phosphatidylcholines [348]: dicarboxylate carrier increases, as does pyruvate carrier after 7 days; phosphate carrier does not change; citrate carrier decreases progressively from 1 to 8 weeks after induction of diabetes. Intact mitochondra transport citrate (8°C) at subnormal rates [95]. Slowed adenine nucleotide transport (25°C) accompanies decreased State 3 respiration [388]. Transport of fatty acyl-CoA molecules from cytoplasm to mitochondrial matrix, and thereby β-oxidation, is accelerated. The transporter set includes mitochondrial outer-face carnitine palmitoyltransferase → (acyl)carnitine translocase → inner-face carnitine palmitoyltransferase and is cardiolipin-dependent (see Ref. 284) and thought to be oriented through cardiolipin-binding [470]. 10 days after rats are made diabetic, outer-face and inner-face carnitine palmitoyltransferase V_{max} doubles, in association with increased membrane fluidity as measured by a nonpolar-lipid probe; Arrhenius profiles of external-enzyme activity are almost linear in diabetics and controls, and converge toward 40°C (like plots of State 4 respiration; see above), while plots of inner-enzyme activity inflect similarly in control and diabetic preparations [50]. Outer-enzyme activity (37°C) increases 2.5-fold gradually from 48–120 h after insulin deprivation [222]. Activity of the liver mitochondrial (acyl)carnitine translocase at 0°C is 80% greater in diabetics than in controls, but the cause is an increase in mitochondrial matrix [carnitine] in the diabetics [501]. The inconsistency of timing and direction seems to eliminate cardiolipin-dependency as the common mechanism for all these changes in transporters.

Adrenergic agents: in rats injected daily for 2 weeks with norepinephrine (β_1-agonist), most of the 18:2 acyls in heart lipid phosphatidylcholines and phosphatidylethanolamines (obtained at 2 months) are replaced by 20:4($n-6$) and 22:6($n-3$) acyls [169,170]. The cardiolipin fatty acyl composition and cardiolipin/phospholipid ratios are not affected (Table III), but amounts of triacylglycerols are halved. Such rats are subject to acute myocardial necrosis when stressed by catecholamine administration. In cardiac lipids from rats treated with isoproterenol, a $\beta_1\beta_2$-agonist with similar sensitizing effects, 22:6 acyls partly replace 18:2 and 20:4 acyls [235]. Mitochondrial function was not reported.

Growth hormone: Maddaiah and coworkers [108–110,402] describe mitochondrial phospholipids and respiration in hypophysectomized rats, and the effects of treatment with growth hormone and T4. In liver mitochondria from hypophysectomized rats, the cardiolipin/phospholipid quotient is 26% less than in normals [110]. The fatty acyls of the cardiolipins are slightly repleted in 18:2 and lower in 18:1 contents; phosphatidylcholines and phosphatidylethanolamines contain less than normal proportions of 20:4($n-6$) and

22:6($n-3$) acyls, and phosphatidylethanolamines surprisingly have almost no 18:2 acyls. Seven daily injections of growth hormone raise cardiolipin/phospholipid 62% and normalize fatty acyl contents of all phospholipids. Liver mitochondrial respiration (glutamate/malate, 30°C) slows by about 35% in both State 4 and State 3, and 7 daily growth hormone treatments do not correct respiratory rates, but 7 days of T4 raises rates 114% and 143%, to levels well above normal [108]. The discrepancy between growth-hormone-induced correction of phospholipid abnormalities and the persistence of respiratory changes may only reflect the temperature and time chosen for measurements. Arrhenius profiles of the State 3 rates in these 7-day-treated animals diverge from normals at the lower range of temperatures. One injection of growth hormone accelerates State 3 (30°C) in 12 h and alters fatty acyls of total phospholipids in 4 h, suggesting that the fatty acyl changes mediate at least part of the early effect on State 3 [402].

Mitochondrial phospholipids from hearts of hypophysectomized rats have normal proportions of cardiolipins, and normal fatty acyl compositions of total lipids [109]; no analyses of fatty acyl contents of separated phospholipids were shown. A week of growth hormone injections increases total lipid 20:4 and decreases 22:6 acyl contents, while T4 has no effects thereon. Heart mitochondria from hypophysectomized rats respire faster than controls in State 3 and State 4 at temperatures above 20–25°C, and slower at temperatures below that range, i.e. the Arrhenius plots cross. Hypophysectomy linearizes the State 4 profile ($r = -0.978$), and T4-treatment even more ($r = -0.997$), to the degree seen in normal rat liver mitochondria [289]. Some of the lipid-dependent effects of hypophysectomy on mitochondrial respiration appear to be mediated through the hypothyroidism (see section IV-C.2.d) of TSH-deficiency.

IV-C.3. Localized ischemia and reperfusion

Localized tissue ischemia, produced by blocking arterial supply of oxygen, progressively removes a small portion of total phospholipids, mainly in the cardiolipin fraction, from animal heart [593] and from mitochondria of heart [668,669] and kidney [615] but not liver [440]. The cardiolipins remaining in pig heart after 40 min of ischemia (94% of control) have normal fatty acyl compositions (Table III), as do the other phospholipids [593]. Ischemic skeletal muscles of rats show no depletion of cardiolipins at 4 h; when they receive oxygen through reperfusion for 2 h, cardiolipin contents fall by 45% while phosphatidylcholines and phosphatidylethanolamines decrease by only 13% [621]. Cardiolipin fatty acyl compositions were not reported, but the loss of cardiolipins was ascribed to their peroxidation by free radicals produced from O_2^- metabolism, and the susceptibility of cardiolipins because of their 'unusually high content of unsaturated bonds'. Actually, the unsaturation index of cardiolipins in heart and muscle is usually less than that of the phosphatidylethanolamines that have high 22:6($n-3$) acyl contents, but almost all fatty acyls of cardiolipins have two or one unsaturated bonds. Presumably, peroxidation of fatty acyls potentiates removal of the headgroups of cardiolipins through the action of phospholipases C and/or D.

The oxygen free-radicals produced by ischemia/reperfusion appear to damage muscle, heart, liver, kidney and intestine tissues with cardiolipins that have high 18:2 + 18:1 acyl contents; inhibiting O_2^- production or scavenging O_2^- ameliorates damage. Ischemic damage exhibits as decreased mitochondrial fine structure [440,477] and diminished State 3 respiration with little change [439,440,604] or a rise [328] in State 4 respiration. Because $\Delta\psi$ and ΔpH in rat liver mitochondria remain normal [13,22], the maintenance of normal State 4 respiration means that the apparent $C_{M^{2+}}$ is decreased. The decrease in State 3 rate decrease is then due to altered C_i contributions, which might include the observed loss of CoQ [173] and cytochrome aa_3 (440) and decreased activities of NADH-ubiquinone reductase [134] and ADP/ATP carrier [604] but not the increased mitochondrial Ca^{2+} which was thought to dissipate Δp [134]. Since simple ischemia depletes mitochondrial cardiolipins only slightly and leaves the remaining heart cardiolipins with normal fatty acyls, the cardiolipins do not appear to be involved in the depression of State 3 respiration. Cardiolipins may be involved in the more severe changes during reperfusion: the oxidant activity of O_2^- added to cardiolipin-phosphatidylcholine-cytochrome c vesicles abolishes cardiolipin-cytochrome c binding [621]; ischemia depresses cytochrome aa_3 V_{max} in skeletal muscle mitochondria by −28%, and subsequent reperfusion lowers V_{max} by −62%; and an oriented cytochrome aa_3-cardiolipin-cytochrome c complex may transfer electrons [678]. And cardiolipins bind the ADP/ATP carrier (section II-B). It should be noted that despite the prolonged ischemia produced by local vasoconstriction in skeletal muscles of diving seals, which conserves oxygen [579], the reperfused muscles are not apparently damaged.

IV-C.4. Cultured cells: animal, yeast, plant

Several animal cell lines in culture require addition of an essential ($n-6$)-polyunsaturated fatty acyl to keep mitochondrial oxidative phosphorylation coupled. Such cells have not usually been analysed for cardiolipin/phospholipid or fatty acyl/cardiolipin compositions (except for transformed lines), but data on their total phospholipids or total lipids suggest that cardiolipins are involved. Rat-heart cells cultured in a fat-

free medium contain total lipids that are depleted of 18:2 and 20:4 acyls and repleted with 18:0 acyls. The cells stop growing and beating; oxidative phosphorylation is uncoupled, with decreased ADP/O and respiratory control ratios (250,260] but no State 4 and State 3 rates were shown. Added 18:2 or 20:4 fatty acid raises total lipid 18:2 + 20:4 acyl contents and partly removes 18:0 acyls, couples oxidative phosphorylation, but does not restore growth or contractility. Heart cells from neonatal rats grow better in serum than in added 18:2 acid, contain significant amounts of 22:6($n-3$) fatty acyls, and have cardiolipin P per total phospholipid P ratios about twice those in adult or neonatal rat-heart lipids; no fatty acyl/cardiolipin compositions were shown [551]. These cells are thought to retain much of the phospholipid fatty acyl composition of their mother tissue, and to metabolize rather than incorporate supplied fatty acids, unlike other cultured animal cells.

HeLa-S_3 cells also need 18:2($n-6$) or 20:4($n-6$) fatty acid for growth [206,207]. On 18:2($n-6$) fatty acid, their total lipid fatty acyl profiles run about 44% saturated fatty acyls, 20% monounsaturated fatty acyls, 9% 18:2($n-6$) and 12% 20:4($n-6$) fatty acyl groups. When no ($n-6$)-unsaturated fatty acids are supplied, monounsaturated fatty acyl contents increase 50–100% and 18:2 and 20:4 acyls almost disappear. Homogenates of deficient cells are uncoupled [206,207], with little or no respiratory control and decreased ADP/O ratios, but without stated State 4 rates we can not tell if the proton-leak increases. Either 18:2 or 20:4 acid supports growth, repletes 20:4 and 18:2 fatty acyls at least partly, and couples mitochondrial oxidative phosphorylation; the fatty acyl/cardiolipin composition with added 20:4 acid would be interesting to see.

Heart cells cultured longer than a day or so, the HeLa-S_3 cell strain, endothelial cells, skin fibroblasts, and many cultured cell lines [624] do not convert accumulated 18:2 to 20:4. A number of normal diploid cell lines perform Δ6- and Δ5-desaturations, and some transformed cells lack fatty acyl-CoA Δ9- and Δ6-desaturases [160,445]. Studies on the influence of transformation on biomembrane lipid contents and compositions of a variety of cell lines are often contradictory, perhaps because phospholipid compositions depend on malignancy of the tumor and diet of the host.

Mitochondria from hepatomas or ascites tumor cells contain about 50% more total phospholipid/mg protein and half the cardiolipin P per total phospholipid P found in rat or mouse liver mitochondria [444]; but see Ref. 536. Their total lipid fatty acyl compositions feature either lower [35] or higher [536] 18:1 acyl contents, and there is also disagreement on increased saturated fatty acyls and decreased 18:2 and 20:4 acyls. Cardiolipin fatty acyl contents differ from the expected liver cardiolipin patterns more consistently. The cardiolipins of hepatoma 7288C cells, as compared with the normal cardiolipins of the host liver, are severely depleted in 18:2 acyls and have 4.4-fold higher contents of 16:0 + 18:0 acyls and twice more 18:1 acyls [722,743] (Table III). In contrast, the 18:1 fatty acyls of the total phospholipids and the resolved phosphatidylcholines, phosphatidylethanolamines, and phosphatidylinositols are partly depleted [536]. These deviations in fatty acyl compositions are associated with altered differential scanning calorimetry profiles of phospholipids extracted from various hepatomas [185,636], increased proton leakage, and changed membrane-dependency of several enzymes and carriers, most strikingly the F_1-ATPase.

Mitochondria from tumor cells respire abnormally rapidly, especially in rapidly growing cells; State 4 in slow-growing hepatomas is 25% faster than in host liver, and 120% more rapid in fast-growing tumors, where State 3 also increases slightly [504]; State 4 is 50–400% above normal and State 3 up to 50% faster (loose-coupling) in hepatoma 7777 [536], hepatic islet-cell tumor [231] and in rats fed carcinogenic aminoazo dyes [18]. State 4 is normal in Ehrlich ascites tumor cells [573,656]; and State 4 is decreased while State 3 respiration increases in mouse hepatoma 98/15 [357,695] and in hepatomas of rats fed *N*-2-fluorenylacetamide [28].

In those tumor mitochondria where the proton leak increases, ADP/O ratios are not lowered. However, abnormal function and thermotropic properties of the F_1-ATPase characterize the mitochondria of many hepatomas [444]. Although addition of a protonophore or a 20°C increase in temperature of assay accelerates ATPase activity 5- to 10-fold in normal liver mitochondria, the ATPase of hepatoma mitochondria responds minimally [504]. In one proposed mechanism for the ATP-synthase [462], the normally high Δp drives protons through the F_0 units to protonate the F_1-ATP-synthase so that it binds added ADP + P_i more tightly and catalyzes a dehydration to form ATP. When $\Delta p = 0$ because a protonophore is present, added ATP should bind to the unprotonated F_1-ATPase and be hydrolyzed. Hepatoma F_1-ATPase under uncoupler-activated conditions either does not bind ATP or binds and does not hydrolyze. The loss of the normal ATPase heat activation suggests that the aberrant fatty acyls of the hepatoma phospholipids are involved. It may be pertinent that mitochondria from liver, kidney, heart (with 18:2-repleted cardiolipins), and skeletal muscle catalyze high uncoupler-activated ATPase activity, whereas mitochondria from brain and adrenal cortex have very low uncoupler-activated activity [505]. The cardiolipins of mitochondria of bovine adrenal cortex contain only 12% 18:2 and 65% saturated fatty acyls; cardiolipins of brain mitochondria have high

polyunsaturated fatty acyl and low 18:2 acyl contents (Table III). In hyperthyroid rats, liver cardiolipins with low contents of 18:2 and high contents of saturated fatty acyls (like cardiolipins in tumors) may leak protons through the F_0-ATPase (see section IV-C.2.d); we do not know their uncoupler-activated ATPase activity.

Thus, some tumor cells grow independently of a supply of the EFA 18:2($n-6$) and its derivatives, and perhaps thereby acquire rapid respiration that competes with host cells for substrates. Defective control mechanisms in the membranes of tumor mitochondria [684] and increased cytoplasmic [P_i] and [ADP] have been connected with the characteristically rapid rate of aerobic glycolysis in tumor cells [685]. Indeed, a large supply of 18:2 fatty acid (or, less so, 18:1) kills Ehrlich ascites carcinoma cells [742] and a variety of carcinoma cells cultured in vitro (see Ref. 742). Mice bearing the ascites tumor live longer when injected with linoleate, which elevates the 3% 18:2 content in total phospholipids of ascites cells to 45%. These findings suggest that the tumor cell abnormalities in fatty acyl content are primary adaptive changes.

Yeast mitochondria differ from animal mitochondria in several important respects. (i) *S. cerevisiae* cardiolipins have 96% monounsaturated fatty acyls and 4% saturated fatty acyls [690] (Table III), animal mitochondria contain mainly 18:2 and 18:1 fatty acyls. (ii) Yeast mitochondrial State 3 respiration is regulated by cytochrome oxidase and/or the P_i carrier and the proton leak (at low [P_i]) (Table I), in contrast to liver and heart mitochondria. The yeast cytochrome oxidase is catalytically active when di-14:0-phosphatidylcholine replaces its firmly bound cardiolipins [690] (IIB). (iii) Yeast mitochondrial ATP-synthase activity is optimal at both pH 6.2 and pH 9.4 [262] while animal enzyme has the lower pH maximum and binds cardiolipins. (iv) Yeast mitochondria oxidize external NADH; the NADH dehydrogenase of animal mitochondria oxidizes only matrix NADH, and requires cardiolipins for reconstitution and binding to the matrix face of the inner membrane (see section II-B). (v) Yeast mitochondria do not β-oxidize fatty acyl-CoAs but only incorporate the fatty acyls for structure of membranes [352]. The palmitoylcarnitine acyltransferase in animal mitochondria, which regulates β-oxidation, has cardiolipin requirements like the NADH dehydrogenase for positioning toward the matrix (see section II-B). (vi) Yeast mitochondrial inner membranes contain sterols [49]; sterols in animal mitochondria are considered by some to be a sign of contamination with endoplasmic reticulum. (vii) Yeast mitochondria biosynthesize more phospholipids autonomously. While both yeast and animal mitochondria synthesize all the cell's cardiolipins and phosphatidylglycerol, the yeast mitochondria can also form considerable amounts of phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine; phosphatidylcholine comes from enzymes in the endoplasmic reticulum in yeasts [112] and animal cells. Jakovcic et al. [324] conclude that high cardiolipin/phospholipid ratios characterize fully developed mitochondria of both yeasts and mammals.

The cardiolipin content of yeasts varies with species, genetic lesions [226], growth conditions (e.g., carbon source, temperature, [O_2], [inositol]) [410], and the degree of development of the mitochondrial inner membrane [325]. Stationary *S. cerevisiae* with glucose or galactose + lactate as carbon source contain more cardiolipin and have better developed mitochondria than repressed log-phase cells; so do aerobic cells versus anaerobic cells.

The phospholipids of many wild yeast strains contain ($n-6$)- and ($n-3$)-polyunsaturated fatty acyls, indicating the presence of Δ12- and Δ15-desaturation systems. Some yeasts, particularly the *Saccharomyces*, contain only monounsaturated fatty acyls: 14:1, 16:1, and 18:1 [175,345]. Saturated fatty acyls are important in membranes of a double-mutant strain of *S. cerevisiae* defective in both fatty acid synthetase and desaturase [176]. Optimal growth stringently requires saturated fatty acids with 14–16 carbons, plus an unsaturated fatty acid. With any given saturated fatty acyl, unsaturated fatty acid effectiveness is in the order 18:2($n-6$) > 18:1($n-9$) > 18:3($n-3$) > 20:1($n-9$). The growth response is not explained by the degree of fatty acid incorporation into total phospholipids or changes in membrane fluidity as measured by ESR of a 12-doxyl-18:0 probe at 30°C.

S. cerevisiae grown anaerobically or aerobically on media that contain glucose or galactose, ergosterol, Tween 80 (3% 14:1, 13% 16:1, 71% 18:1, 13% saturated fatty acyls), and no inositol, have total lipid fatty acyl compositions of about 80% monounsaturated fatty acyls and 20% saturated fatty acyls [336]. The anaerobic cells contain about 0.9 mg cardiolipin/g dry weight, and cardiolipin P per total phospholipid P = 5%. Aerobic cells contain 3.5 mg cardiolipin/g dry weight, and cardiolipin P per total phospholipid P = 12% – mitochondria proliferate about 4-fold (all the cardiolipins being mitochondrial in these yeasts) and more than double their cardiolipin/phospholipid ratios. Mitochondria from such aerobic yeasts (*S. cerevisiae* strain Yeast Foam or N.C.Y.C. 239) oxidize added NADH through a second dehydrogenase on the outer aspect of the inner membrane (a matrix-sided dehydrogenase oxidizes endogenous NADH, as in animal mitochondria), which averts limitation of oxidative rates by a substrate carrier [421]; the cardiolipins may be involved (see section II-B). At pH 6.5 or 6.7, 27°C, State 3 respiration (ng atom O min^{-1} (mg protein)$^{-1}$) with NADH is 740–800, State 4 is 270, State 3u (CCCP) is 900 (State 3 (succinate) is 185) [688]). These mitochondria leak protons at a rate equivalent to that

produced by 650 pmol of CCCP/mg protein as compared with 12 pmol of FCCP/mg in rat liver mitochondria [228]; the 55-fold disparity is not quite accounted for by the 33-fold greater protonophore action of FCCP over CCCP [611]. The rapid State 4 rate and the advent of a C_i for proton leakage that partly regulates State 3 also suggest that yeast mitochondrial membranes leak protons faster than rat liver mitochondria; the different substrates and low pH, and/or the fatty acyl composition of yeast cardiolipins, may be involved. Calculations from State 4 respiration (NADH) at 270 ng atom O min^{-1} mg^{-1}, $H^+/O = 9$ [462], and $\Delta p = 170$ mV [421], give a value for $C_{M^{H^+}} = 14.3$ ng ion H^+ min^{-1} mg^{-1} mV^{-1}, almost 15-fold the conductance calculated for rat liver mitochondria (see Table VIII).

Phospholipid subclasses in yeasts (as in mammals and bacteria) have distinctive and different fatty acyl compositions. In *S. cerevisiae*, cardiolipins are the most unsaturated (96% 16:1 + 18:1, 4% 16:0 + 14:0; see Table III) as compared with monounsaturated fatty acyl contents of 60% in phosphatidylinositols, 80% in phosphatidylethanolamines and 90% in phosphatidylcholines [689]. Separate fatty acyl-CoA pools do not seem to account for the distinct compositions in yeast cardiolipins and phosphatidylethanolamines, which presumably draw on a mitochondrial pool; more likely, specific mitochondrial enzymes acylate deacylated cardiolipins to favor monounsaturated fatty acyl incorporation.

The mutant strain KD115 of *Saccharomyces cerevisiae*, deficient in fatty acid desaturase and grown without inositol, can not biosynthesize monounsaturated fatty acyls but incorporates monounsaturated fatty acids from the medium into its mitochondria. Mitochondria with a monounsaturated fatty acyl content adjusted to about 80% of the total-lipid fatty acyls, and saturated fatty acyls to 20%, oxidize α-ketoglutarate (pH 6.5, 30°C) at a rate of 40 ng atom O min^{-1} mg^{-1} in State 4, 150 in State 3 with P/O ratios up to 2.5; cardiolipin/phospholipid ratio is 15.6% [262]. Rat liver mitochondrial values are comparable. In yeast mitochondria with approx. 20% monounsaturated fatty acyls and 80% saturated fatty acyls, the cardiolipin/phospholipid ratio remains at 15.5% but State 4 respiration increases 3.4-fold and P/O ratios are zero; the uncoupling results from increased permeability to protons, as shown by H^+-pulsing [263]. Proton leakage also abolishes the energy-dependent uptake of K^+ but not the normal degree of membrane impermeability to K^+. Raising the unsaturated fatty acyl content of the mitochondria to about 80% slows the proton leak, recouples oxidative phosphorylation and enables energy-supported K^+-accumulation; either mono- or polyunsaturated fatty acids are effective. Recoupling is insensitive to chloramphenicol and cycloheximide, indicating that all the proteins for oxidative phosphorylation are in the unsaturated-fatty-acyl-deficient mitochondria and that the unsaturated-fatty-acyl-deficiency itself causes reversible uncoupling. Free fatty acids are not responsible for the uncoupling; although free fatty acids can uncouple the recoupled mitochondria, free fatty acids do not accumulate in unsaturated fatty acyl-deficient mitochondria, and added albumin does not recouple (it does with added free fatty acids).

In yeasts, unsaturated-fatty-acyl-deficiency and/or reciprocal saturated-fatty-acyl-repletion increases the permeability of the lipids of the inner mitochondrial membrane to protons, apparently enough to dissipate the drive for energy-linked reactions in oxidative phosphorylation; without measurements of Δp we can not tell if proton conductance rises. In the liver mitochondria of EFA-deficient rats State 4 Δp is unchanged and so the increased rate of State 4 respiration should be due to increased proton conductance (see section II-A) – but we have no direct measurements by H^+-pulsing. Nor are analyses available to show if the fatty acyl changes are localized or general among the phospholipid subclasses in yeast. Selective defects in cardiolipins do occur in mitochondria from livers of EFA-deficient animals (section IV-C.1.a) and of ethanol-fed rats (section IV-C.2.a). The unsaturated fatty acyl-deficient yeast cardiolipins might conceivably retain their monounsaturated fatty acyls, since a content of 96% monounsaturated fatty acyls and a cardiolipin/phospholipid ratio of 15.5% could allow cardiolipins alone to contribute a ratio of 15% monounsaturated fatty acyls/phospholipids, and 20% monounsaturated fatty acyls are found. Cardiolipin analyses are needed.

Overall fatty acyl saturation varies directly with membrane proton permeability in mitochondria of yeasts and mammals. It should be noted that the proton permeability changes inversely with the degree of fatty acyl unsaturation; see section II-B for comparisons with model phospholipid vesicles.

The effect of unsaturated fatty acyl-deficiency in yeasts that increases mitochondrial proton leakage differs from the effect of inositol-deficiency that may include depressed mitochondrial respiratory capacity but certainly no increased proton leakage. A mutant *S. carlsbergensis* requires inositol for optimal growth [31,531]. Up to 90% of the inositol is in phospholipids. Lack of inositol is pleiotropic: it was originally said to diminish mitochondrial respiration, Krebs cycle activities, and contents of cytochromes and certain peptides [542]; lower total phospholipids per mg mitochondrial protein; lower phosphatidylinositol/total phospholipids (3.8%); decrease cardiolipin/mg protein and increase cardiolipin/total phospholipids (12.8% −inositol vs. 9.2% +inositol). The lost acidic phosphatidylinositols are replaced by acidic cardiolipins,

apparently because inositol depresses cardiolipin synthesis by inhibiting the mitochondrial phosphatidylglycerolphosphate synthetase [226]. The replacing cardiolipins support mitochondrial function or development. Later studies found equal respiration, mitochondrial respiratory chain components, and mitochondrial morphology in these yeasts grown with or without inositol [209,492] but reported abnormal cell walls due to accumulation of glucans [209].

Phosphatidylinositols do not seem to be as specific as cardiolipins for mitochondrial function. Mammalian intracellular membrane total phospholipids have a phosphatidylinositol content of about 8% [264]; in *S. cerevisiae* the phosphatidylinositol is 25% and cardiolipin is about 4% during anaerobic growth and 17% and 12%, respectively, during growth $+O_2$ [336]. In promitochondria, phosphatidylinositol/phospholipids = 25%, cardiolipin = 9%, when this yeast grows without added lipids, but the endoplasmic reticulum also has a high phosphatidylinositol/phospholipid ratio [493].

V. Summary

Evidence is discussed for roles of cardiolipins in oxidative phosphorylation mechanisms that regulate State 4 respiration by returning ejected protons across and over bacterial and mitochondrial membrane phospholipids, and that regulate State 3 respiration through the relative contributions of proteins that transport protons, electrons and/or metabolites.

The barrier properties of phospholipid bilayers support and regulate the slow proton leak that is the basis for State 4 respiration. Proton permeability is in the range 10^{-3}–10^{-4} cm s^{-1} in mitochondria and in protein-free membranes formed from extracted mitochondrial phospholipids or from stable synthetic phosphatidylcholines or phosphatidylethanolamines. The roles of cardiolipins in proton conductance in model phospholipid membrane systems need to be assessed in view of new findings by Hübner et al. [313]: saturated cardiolipins form bilayers whilst natural highly unsaturated cardiolipins form nonlamellar phases. Mitochondrial cardiolipins apparently participate in bilayers formed by phosphatidylcholines and phosphatidylethanolamines. It is not yet clear if cardiolipins themselves conduct protons back across the membrane according to their degree of fatty acyl saturation, and/or modulate proton conductance by phosphatidylcholines and phosphatidylethanolamines.

Mitochondrial cardiolipins, especially those with high 18:2 acyl contents, strongly bind many carrier and enzyme proteins that are involved in oxidative phosphorylation, some of which contribute to regulation of State 3 respiration. The role of cardiolipins in biomembrane protein function has been examined by measuring retained phospholipids and phospholipid binding in purified proteins, and by reconstituting delipidated proteins. The reconstitution criterion for the significance of cardiolipin–protein interactions has been catalytical activity; proton-pumping and multiprotein interactions have yet to be correlated. Some proteins, e.g., cytochrome *c* oxidase are catalytically active when dimyristoylphosphatidylcholine replaces retained cardiolipins. Cardiolipin–protein interactions orient membrane proteins, matrix proteins, and on the outer-face receptors, enzymes, and some leader peptides for import; activate enzymes or keep them inactive unless the inner membrane is disrupted; and modulate formation of nonbilayer H_{II}-phases. The capacity of the proton-exchanging uncoupling protein to accelerate thermogenic respiration in brown adipose tissue mitochondria of cold-adapted animals is not apparently affected by the increased cardiolipin unsaturation; this protein seems to take over the protonophoric role of cardiolipins in other mitochondria.

Many in vivo influences that affect proton leakage and carrier rates selectively alter cardiolipins in amount per mitochondrial phospholipids, in fatty acyl composition and perhaps in sidedness; other mitochondrial membrane phospholipids respond less or not at all. Cardiolipins with high contents of 18:2 acyls, less monounsaturated and very few saturated fatty acyls normally occur in liver, heart and kidney mitochondria, where State 4 respiration varies with thyroid hormone level; increase in proportions of normal cardiolipins, together with a high-18:2, low-20:4 acyl pattern in liver mitochondrial phosphatidylcholines and phosphatidylethanolamines, accompany slowed State 4 respiration in hypothyroidism. Cardiolipins with low 18:2 acyl contents are found in mitochondria of tissues that respire independently of thyroid state, e.g., brain, spleen, testis, adrenal cortex, brown adipose tissue; during cold-adaptation the 18:2 acyl content and unsaturation of brown adipose tissue mitochondria increase while State 4 respiration slows. Phospholipid-leak accelerates when mitochondrial cardiolipin 18:2 acyls are replaced by saturated or monounsaturated acyls, as in mitochondria prepared from some tissues of animals that are in postgastrulation embryonic state, fetuses, hyperthyroid, essential fatty acid-deficient, erucate- or ethanol-fed; and from cold-adapted fish, unsaturated fatty acid-deficient yeasts, and hepatoma cells. Phospholipids including cardiolipins in plasma membranes of thermophilic eubacteria contain only saturated and branched fatty acyls and are stable at high temperatures but leak protons. Conversely, diets or culture media that replace cardiolipin 18:2 acyls with other unsaturated fatty acyls (e.g., $(n-3)$-unsaturated or $22:1(n-9)$) without changing the low content of saturated fatty acyls, or that introduce a strange fatty acyl (linelaidoyl) into phosphatidyl-

cholines and phosphatidylethanolamines but not cardiolipins, do not increase mitochondrial proton leakage. Saturated fatty acyls of cardiolipins thus seem to be associated with the proton leak.

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