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CALORIMETRIC AND FREEZE FRACTURE ANALYSIS OF LIPID PHASE TRANSITIONS AND LATERAL TRANSLATIONAL MOTION OF INTRAMEMBRANE PARTICLES IN MITOCHONDRIAL MEMBRANES

CHARLES R. HACKENBROCK, MATTHIAS HÖCHLI and RAYMOND M. CHAU

The Department of Cell Biology, The University of Texas Health Science Center, Southwestern Medical School, Dallas, Texas 75235 *(U.S.A.)*

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SUMMARY

Differential scanning calorimetry combined with freeze fracture electron microscopy reveals that thermotropic lipid phase transitions and lateral translational motion of intramembrane particles occur in both membranes of whole, intact rat liver mitochondria and in isolated inner and outer membranes. The onset temperature of the liquid crystalline to gel state lipid phase transition in whole mitochondria and in the isolated outer membrane fraction is biphasic with an initial transition exotherm occurring at 9 °C. The onset temperature of the transition exotherm of the isolated inner membrane occurs at −4 °C. The onset temperature of the lipid transition endotherm is −15 °C for whole mitochondria, the inner membrane, and the outer membrane fractions. These calorimetric analyses reveal that the bilayer lipid in the inner, energy transducing membrane is more fluid than in the outer membrane. Mitochondrial membranes cooled to temperatures in the region of their transition exotherms and then frozen reveal striking lateral separations between smooth, intramembrane particle-free regions (rich in gel state lipid) and particle-dense regions (rich in integral proteins) in their hydrophobic fracture faces. Such thermotropic lipid-protein lateral separations are completely reversible. These freeze fracture observations suggest that both mitochondrial membranes are naturally fluid to the extent that the integral membrane proteins can diffuse laterally in the bilayer lipid.

INTRODUCTION

Freeze fracture studies have revealed that various purified integral proteins, e.g., rhodopsin [1, 2], glycophorin [3, 4], microsomal ATPase [4], and human myelin N-2 protein [5], appear as intramembrane particles when reconstituted in the bilayer of synthetic phospholipid vesicles. Studies combining freeze fracture electron microscopy with spin labeling have further revealed that thermotropic lateral separations can occur between the bilayer lipid and integral proteins of such well characterized model membrane systems [3, 4]. These separations occur at the characteristic temperatures at which liquid crystalline to gel state phase transitions occur in the

bilayer lipid and are expressed in the freeze fractured membranes as lateral separations between smooth, intramembrane particle-free regions (rich in gel state lipid), and intramembrane particle-dense regions (rich in integral proteins) [2–4]. It is generally believed that the low temperature-induced gel state bilayer lipid excludes the integral membrane proteins laterally as exothermic cooperative ordering occurs in the phospholipids of the bilayer [6].

Similar observations and interpretations have been made in studies on various bacterial cell membranes using freeze fracturing combined with either spin labeling, differential scanning calorimetry, or X-ray diffraction [7–11]. However, the use of these techniques indicates that thermotropic lipid phase transitions and lateral separations of membrane components do not occur in some mebranes, notably various cholesterol-rich mammalian lymphoid cell plasma membranes [12], and erythrocyte [13] and nerve myelin membranes [14]. Of particular interest is the recent finding that lateral separations between lipid- and protein-rich regions cannot be detected in some *Bacillus* and *Staphylococcus* membranes by freeze fracture electron microscopy, although lipid phase transitions can be detected by differential scanning calorimetry [9]. Such a result may be accounted for by the presence of branched chain lipids in these membranes [9, 11]. In some natural membranes, lateral translational motion of integral proteins and/or lipids may tend to be restrained through a continuous protein lattice. Alternatively, cooperative lipid phase transitions may occur in microdomains which are not sufficiently extensive to influence the distribution of integral proteins in the plane of the membrane.

Although several reports indicate that thermotropic lipid phase transitions may occur in the membranes of mitochondria [15–20], only one report, which has emerged from this laboratory, suggests that lateral separations occur between integral membrane proteins and the gel state bilayer lipid during such transitions [21]. In addition, the independent contributions of the inner and the outer mitochondrial membranes to the broad lipid phase transition observed in the whole mitochondrion have not been determined. Of primary interest in this regard is whether or not integral proteins of the inner, energy transducing membrane can undergo lateral translational diffusion of any consequence. A popular inference is that the metabolic proteins of this protein-rich membrane are stabilized through a continuous rigid protein lattice [22–24], which appears consistent with the rapid protein-protein interactions that occur in this membrane.

We report here observations on thermotropic lipid phase transitions and the influence of such transitions on the lateral motional freedom of integral proteins in the membranes of the whole, intact mitochondrion, as well as in isolated inner and outer mitochondrial membranes. Our observations show that the bilayer lipid is fluid in both mitochondrial membranes, with a significantly lower and narrower lipid phase transition occurring in the energy transducing membrane. The data suggest that the bilayer lipid imparts a considerable potential for lateral translational motion to the integral proteins of both membranes.

METHODS

Mitochondria and membrane preparations

Liver mitochondria were isolated from male Sprague-Dawley rats in 250

mosM sucrose according to the method of Schneider [25] and used in studies on intact, whole mitochondria. Liver mitochondria were also isolated from male Sprague-Dawley rats in an isolation medium containing 70 mM sucrose, 220 mM mannitol, 2 mM HEPES (*N*-2-hydroxyethyl-piperazine *N'*-2-ethanesulfonic acid), 0.5 mg bovine serum albumin/ml, and KOH to pH 7.4. This medium is designated H medium and is 300 mosM. From these mitochondria, subsequent removal of the outer membrane and purification of the inner membrane-matrix (mitoplast) fraction was carried out by use of a controlled digitonin incubation [26, 27]. For a purified inner membrane-matrix fraction showing a condensed configuration, 0.75 % digitonin in the 300mosM H medium was used for 15 min. The purified condensed inner membrane-matrix fraction was resuspended in the 300 mosmolar H medium at 100 mg/ml at 0 °C. The condensed inner membrane-matrix fraction was converted to an inner membrane-matrix fraction showing a completely spherical configuration by washing and resuspending in a 7.5 times diluted 300 mosM H medium to give a spherical inner membrane-matrix fraction at 100 mg/ml of 40 mosM H medium at 0 °C [27]. An outer membrane fraction was purified after swelling the sucrose-prepared mitochondria in 20 mM phosphate buffer, pH 7.4 [28].

The purities of the inner membrane-matrix and outer membrane fractions were determined by assessing monoamine oxidase and cytochrome *c* oxidase contents after solubilization of the fractions with Lubrol [26]. Specific activities of monoamine oxidase were determined spectrophotometrically in nmoles benzylamine oxidized $\cdot$ min^{-1} $\cdot$ mg^{-1} protein. Specific activities of cytochrome *c* oxidase were determined polarographically in natoms oxygen reduced $\cdot$ min^{-1} $\cdot$ mg^{-1} protein. Protein was determined by a biuret method [29].

Differential scanning calorimetry

Whole mitochondria (in 250 mosM sucrose, 10 mM Tris $\cdot$ HCl buffer, pH 7.4), the condensed inner membrane-matrix fraction (in 300 mosM H medium), and the spherical inner membrane-matrix fraction (in 40 mosM H medium) were sedimented at 22 000 $\times$ *g* for 15 min in their respective media plus 50 % ethylene glycol, 25 % ethylene glycol, or 30 % glycerol. The outer membrane fraction was sedimented in 125 mosM sucrose plus 50 % ethylene glycol at 106 000 $\times$ *g* for 15 min. Of the thick pellets, 75 mg were weighed into Perkin-Elmer 75 μl, matched-weight, stainless steel specimen capsules using an analytical balance and then sealed with O-ring covers. The reference samples contained 75 mg of the appropriate membrane suspension medium mixed with 2 % swollen Sephadex G-200 beads to prevent convection [30]. Thermograms were obtained with a Perkin-Elmer DSC-2 differential scanning calorimeter, programmed for cooling and heating at 5 or 10 °C $\cdot$ min^{-1} and full scale sensitivity ranges of 0.5 or 0.2 mcal $\cdot$ s^{-1}. Glass distilled water and Perkin-Elmer indium were used as standards for temperature and power calibration of the DSC-2. Sequential cooling and heating runs were repeated at least twice on all membrane samples, and at least two different samples of each membrane fraction were analyzed.

Freeze fracture electron microscopy

For freeze fracture studies, whole mitochondria, the spherical inner membrane-matrix fraction, and the outer membrane fraction were incubated at room temperature for 5 min in their respective media to which 30 % glycerol, buffered at

pH 7.4 with 10 mM Tris · HCl, was added. In a limited number of experiments, glycerol was replaced by ethylene glycol, or cryoprotectors were eliminated altogether. Samples were then pelleted at 25 °C. The pelleted samples were subsequently equilibrated at 30, 25, 10, 8, 7, 0, −6, −8, or −13 °C, transferred to gold-nickel specimen holders which were equilibrated to corresponding temperatures, and then rapidly frozen in Freon 22 precooled by liquid nitrogen. For reversibility studies, specimens equilibrated at −10 °C were returned quickly to 25 °C and then rapidly frozen.

Fracturing of specimens, vacuum sublimation and platinum-carbon replication were carried out at −100 °C at $2 \cdot 10^{-6}$ Torr in a Balzers BA360 freeze-etching apparatus equipped with electron guns. Thin sections were made from epoxy-embedded samples and stained as reported earlier [31, 32]. Electron micrographs were taken with a Philips 300 Electron Microscope, operated at 80 kV for platinum replicas and at 60 kV for thin sections.

RESULTS

Membrane preparations and purity

Preparations used in this study are demonstrated in Figs. 1 through 4. Figs. 1 and 2 show the typically condensed configuration of freshly isolated whole liver mitochondria [31, 32], and the condensed configuration of the purified inner membrane-matrix fraction after removal of the outer membrane by controlled digitonin treatment [33]. Since the membrane topography of the inner membrane-matrix fraction is exceptionally complex [33, 34], it does not offer an adequately large surface area for observation by freeze fracture electron microscopy [27]. For purposes of observation by freeze fracturing, therefore, a spherical configuration (Fig. 3) was produced in the purified inner membrane-matrix preparation in a 40 mosM medium [27]. The purified outer membrane preparation is shown in Fig. 4.

The relative purity of the inner and outer membrane preparations was determined by the specific activities of monoamine oxidase, an enzyme marker for the outer membrane, and cytochrome *c* oxidase, an enzyme marker for the inner membrane (Table I). The outer membrane fraction was enriched approximately 30-fold and contained an insignificant contamination of inner membrane. The inner membrane-matrix fraction, which is composed of 75 % matrix protein [35], proved

TABLE I

RELATIVE PURITY OF FRACTIONATED MITOCHONDRIAL MEMBRANES

	Specific activity*	
	Monoamine oxidase	Cytochrome *c* oxidase
Whole mitochondria	12.8	720
Condensed inner membrane-matrix fraction	0.257	1060
Spherical inner membrane-matrix fraction	0.014	1200
Outer membrane fraction	352.0	150

* Specific activity: for monoamine oxidase in nmol benzylamine oxidized · min^{-1} · mg^{-1} protein; for cytochrome *c* oxidase in natoms oxygen reduced · min^{-1} · mg^{-1} protein.

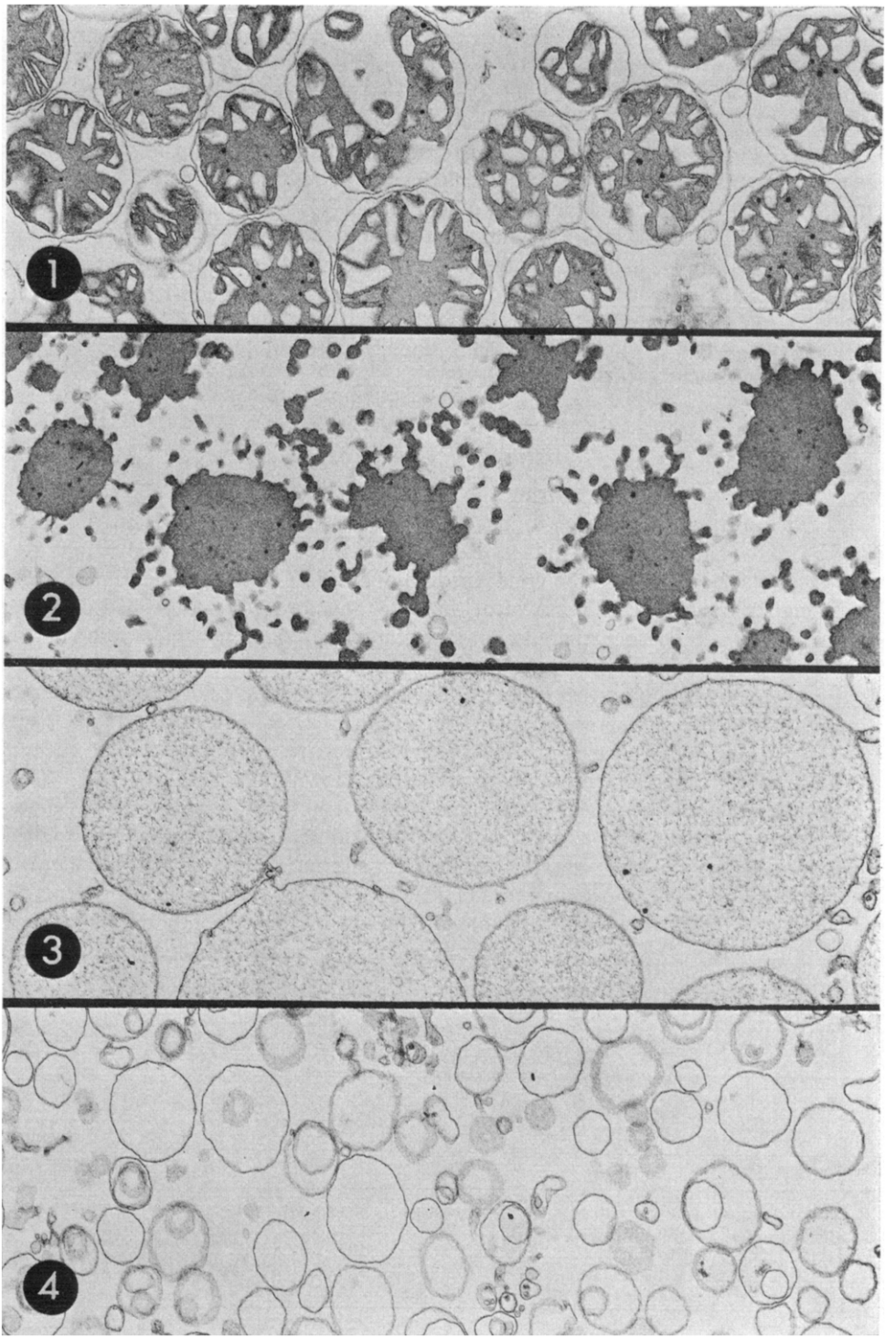


Fig. 1. Whole mitochondria freshly isolated from rat liver. ×20 000.
Fig. 2. Purified condensed inner membrane-matrix fraction. ×20 000
Fig. 3. Purified spherical inner membrane-matrix fraction. ×20 000
Fig. 4. Purified outer membrane fraction. ×20 000

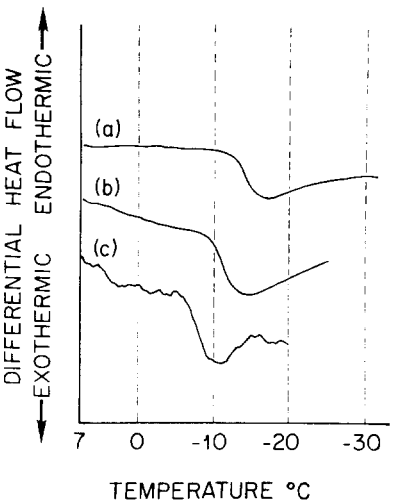


Fig. 5. Calorimetric cooling curves of the spherical inner membrane-matrix fraction in 50 % ethylene glycol (a), 25 % ethylene glycol (b), and 30 % glycerol (c). Cooling rate, 10 °C · min^{-1}; sensitivity, 0.5 mcal · s^{-1}.

TABLE II

RELATIVE LIPID PHASE TRANSITION TEMPERATURES OF WHOLE MITOCHONDRIA AND FRACTIONATED MEMBRANES

All temperatures given are averages of 2 or 3 heating or cooling runs and are ±1 °C.

	Onset temperature in 50 % ethylene glycol (°C)		Onset temperature corrected (°C)*		Extent of transition region (°C)
	Exothermal	Endothermal	Exothermal	Endothermal	
Whole mitochondria	+3, −5	−21, −14	+9, +1	−15, −8	24
Condensed inner membrane-matrix fraction	−10	−21, −14	−4	−15, −8	11
Spherical inner membrane-matrix fraction	−10	−21, −14	−4	−15, −8	11
Outer membrane fraction	+3, −5	−21	+9, +1	−15	24

* Corrected for 6 °C depressions induced by 50 % ethylene glycol.

to be essentially free of outer membrane. The added increase in the specific activity of cytochrome *c* oxidase in the spherical inner membrane-matrix fraction occurred owing to a 20 % loss of matrix protein and residual outer membrane during the preparatory swelling procedure. Both the condensed and spherical inner membrane-matrix preparations are active in electron transport and oxidative phosphorylation [36].

Differential scanning calorimetry and lipid phase transitions

In the calorimetric analysis, the point of deflection from the baseline trace was taken as the onset temperature of the lipid phase transition. The upper transition temperature was determined by the onset temperature on cooling while the lower transition temperature was determined by the onset temperature on heating. The difference between these two onset temperatures was used to define the extent of the transition region as is customary in studies on mixtures of pure lipids [37, 38]. Neither the peak maximum nor the point of return to the baseline was used in determining transitions. Since it was observed that transition temperatures were generally low in mitochondrial membranes, it was necessary to include 50 % ethylene glycol in the membrane samples to adequately depress the water freezing exotherm. This permitted cooling runs down to approximately −30 °C. It was determined, however, that 50 % ethylene glycol depressed the transition temperatures by 3 °C compared to 25 % ethylene glycol, and by 6 °C compared to 30 % glycerol (Fig. 5). Transition temperature depressions of 3 to 5 °C induced by 50 % ethylene glycol have been previously noted in studies on bacterial cell membranes [39]. In the present study, the 6 °C depression induced by 50 % ethylene glycol has been corrected for in the calorimetric traces and in the text to compare more directly these data with the freeze fracture results in which 30 % glycerol was the cryoprotector of choice. Table II contains both the corrected and uncorrected onset temperature values for all membrane preparations.

Since the freeze fracture electron microscopy in this study was performed on membrane samples cooled to various temperatures, the lipid transition exotherms were generally of greater value than endotherms for correlation with ultrastructural details. A definite pattern appeared in the relative onset temperatures in the exotherms of the various mitochondrial membrane samples. The main onset temperature for the whole mitochondrion, as well as for the purified outer membrane, occurred at 1 °C

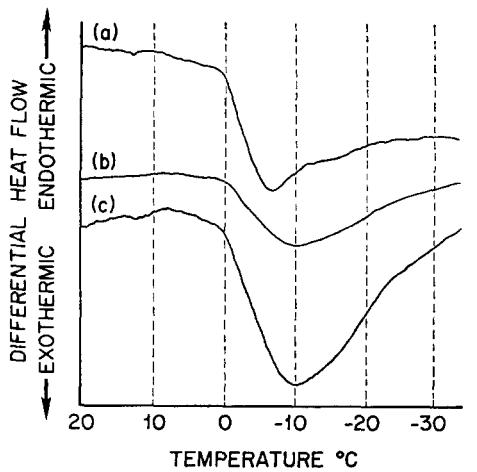


Fig. 6. Calorimetric cooling curves of whole mitochondria (a), and the purified outer membrane fraction (b, c) in 50 % ethylene glycol. Cooling rate, 5 °C · min^{-1}; sensitivity, 0.5 mcal · s^{-1} (a, b) and 0.2 mcal · s^{-1} (c).

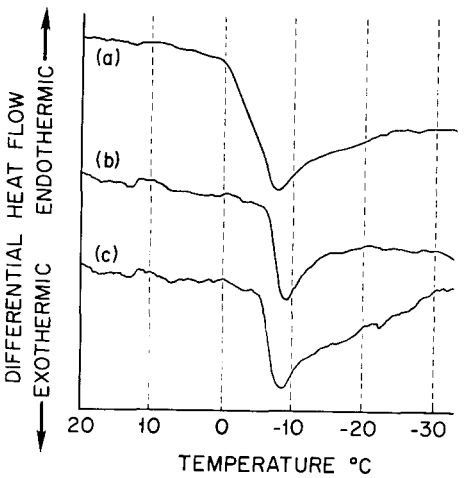


Fig. 7. Calorimetric cooling curves of whole mitochondria (a), the condensed inner membrane-matrix fraction (b), and the spherical inner membrane-matrix fraction (c) in 50 % ethylene glycol. Cooling rate, 5 °C · min^{-1}; sensitivity, 0.5 mcal · s^{-1}.

(Fig. 6). However, what appeared as a slight exothermal deflection at approximately 9 °C at a recording sensitivity of 0.5 mcal · s^{-1} was more adequately resolved at a sensitivity of 0.2 mcal · s^{-1}. The higher sensitivity traces clearly revealed the biphasic nature of the exotherm with the first transition at 9 °C, and the second major transition at 1° C (Fig. 6c).

The onset temperature in the exothermal transition of both the condensed and spherical inner membrane-matrix preparations occurred at −4 °C (Fig. 7). This represented a considerably lower transition temperature, compared to that of the

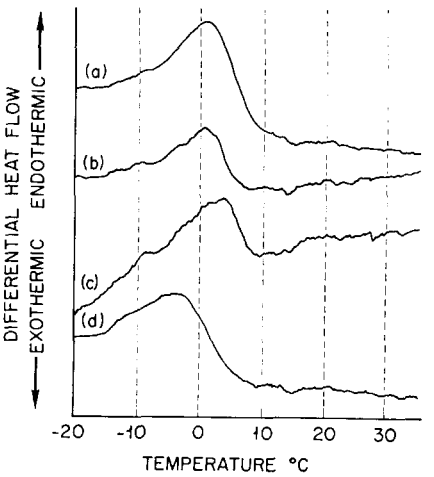


Fig. 8. Calorimetric heating curves of whole mitochondria (a), the condensed inner membrane-matrix fraction (b), the spherical inner membrane-matrix fraction (c), and the outer membrane fraction (d) in 50 % ethylene glycol. Cooling rate, 5 °C · min^{-1}; sensitivity, 0.5 mcal · s^{-1}.

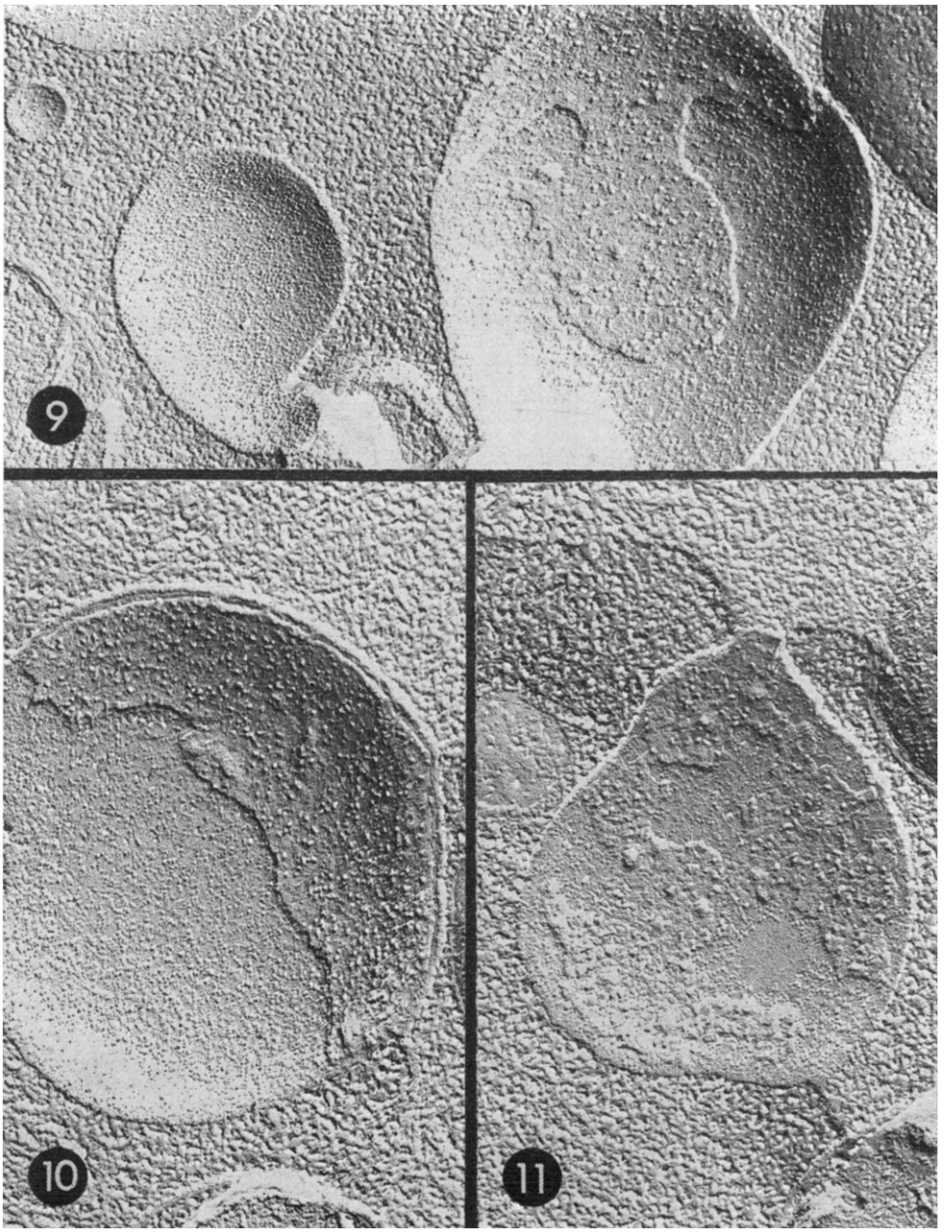


Fig. 9. Concave fracture faces of both membranes of whole mitochondria equilibrated at 30 °C, then frozen. Outer membrane (left); outer and inner membranes (right). ×67 500.

Fig. 10 Concave fracture faces of both membranes of a whole mitochondrion equilibrated at 10 °C, then frozen. ×67 500.

Fig. 11. Concave fracture faces of both membranes of a whole mitochondrion equilibrated at 7 °C, then frozen. ×67 500.

composite two-membrane system of the whole mitochondrion or of the purified outer membrane. The reproducibility of the onset temperature in the exothermal transition was indicated by comparing samples of the two different inner membrane-matrix preparations which showed the same onset temperature as well as the same peak maximum (Figs. 7b, c). One inner membrane preparation consisted of the condensed configuration in high osmolar medium, while the other preparation was of the spherical configuration in low osmolar medium, and in addition contained 20 % less matrix protein. It may also be noted that superimposable exothermal deflections occurred above noise level at 21, 12, and 1 °C in the two inner membrane preparations (Figs. 7b, c).

Upon heating from −30 °C, the onset temperature in the endothermal transition of the whole mitochondrion appeared somewhat biphasic, with a first transition at approximately −15 °C, and a second transition at −8 °C (Fig. 8). These two transitions in the endotherm of the composite two-membrane system of the whole mitochondrion clearly correlated with an onset temperature of −15 °C in the endotherm for the purified outer membrane, and an onset temperature of −8 °C in the endotherm for both of the inner membrane-matrix preparations (Fig. 8). In addition, a slightly lower transition temperature appeared in the inner membrane preparation, at approximately −15 °C, but could not be clearly resolved at all baseline slopes. Table II summarizes the onset temperatures of both the exotherm and endotherm transitions as well as the extents of the transition regions for the various mitochondrial membrane preparations.

Freeze fracture electron microscopy and lateral translational motion of intramembrane particles

Whole mitochondria frozen rapidly from 30 °C contain a random distribution of intramembrane particles embedded in a smooth continuum of the hydrophobic interior of both membranes, as revealed by freeze fracture electron microscopy (Fig. 9). The intramembrane particles have a frequency size distribution peak of 8 nm and 10 nm in the outer and inner membranes, respectively [40, 41]. Whole mitochondria cooled slowly to 10 °C prior to rapid freezing, i.e., cooled to just above the onset temperature of their first transition exotherm (Fig. 6a), revealed an identical random distribution of intramembrane particles (Fig. 10). However, when cooled to 7 °C, or just below the first onset temperature of their transition exotherm, whole mitochondria revealed slight lateral separations between smooth, particle-free regions and particle-dense regions in the hydrophobic fracture faces of the outer membrane (Fig. 11). Thus, at approximately the onset temperature of the transition exotherm for whole mitochondria, a slight lateral displacement of intramembrane particles occurred in the outer membrane, presumably owing to a limited degree of exothermal ordering in the bilayer lipid (small smooth patches) restricted to this membrane.

When mitochondria were cooled slowly to 0 °C, which is just below the second or major onset temperature of their exothermal transition (Fig. 6), more obvious lateral separations occurred between the smooth, intramembrane particle-free regions and intramembrane particle-dense regions in the outer membrane (Fig. 12). Again, no such lateral separations were observed in the inner membrane (Fig. 12). However, mitochondria slowly cooled to −8 °C, which is slightly below the onset temperature of the exothermal transition of the inner membrane (Figs. 7b, c), exhibited dramatic

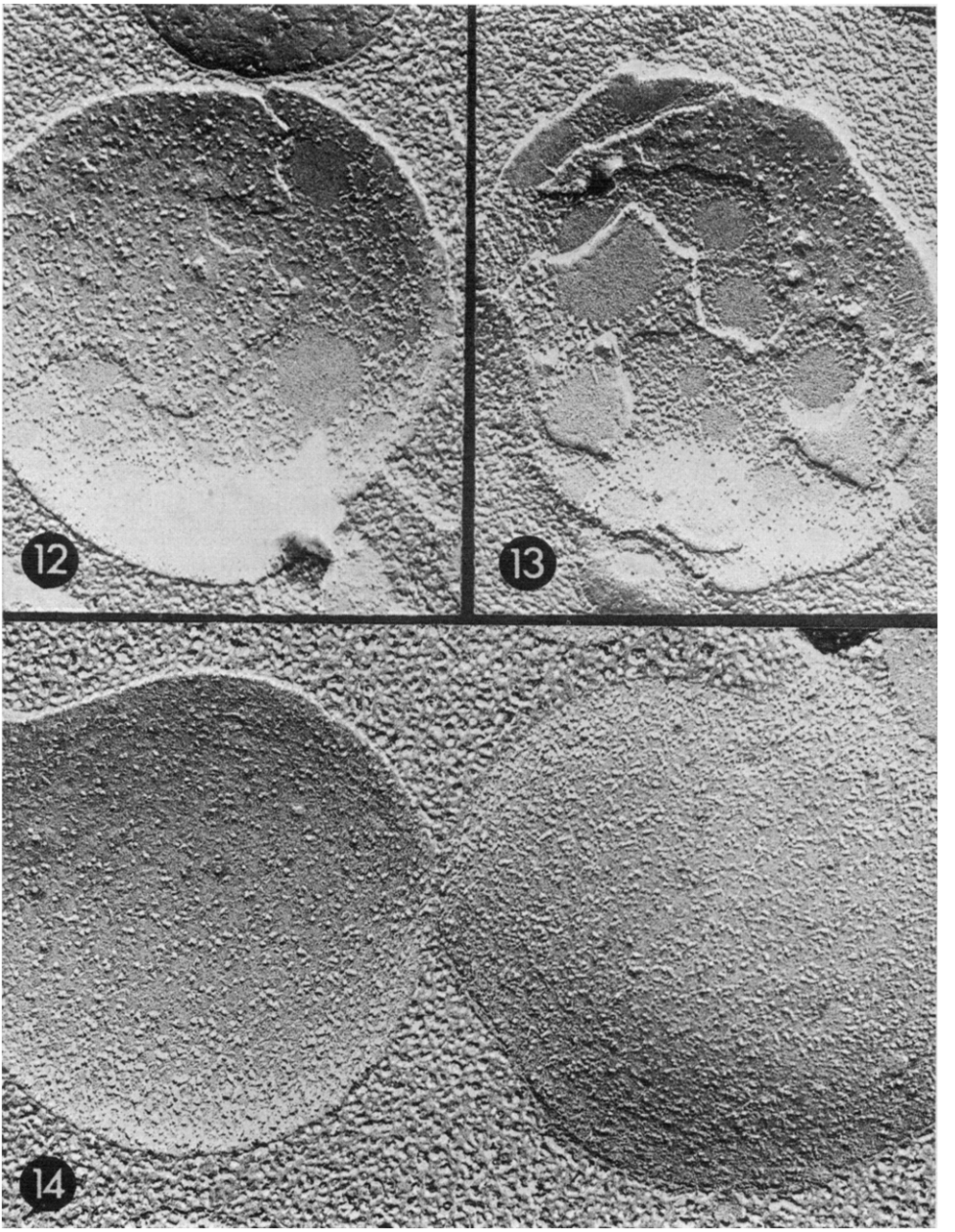


Fig. 12. Concave fracture faces of both membranes of a whole mitochondrion equilibrated at 0 °C, then frozen. ×67 500.

Fig. 13. Concave fracture faces of both membranes of a whole mitochondrion equilibrated at −8 °C, then frozen. ×67 500.

Fig. 14. Concave (left) and convex (right) fracture faces of the spherical inner membrane equilibrated at 0 °C, then frozen. ×67 500.

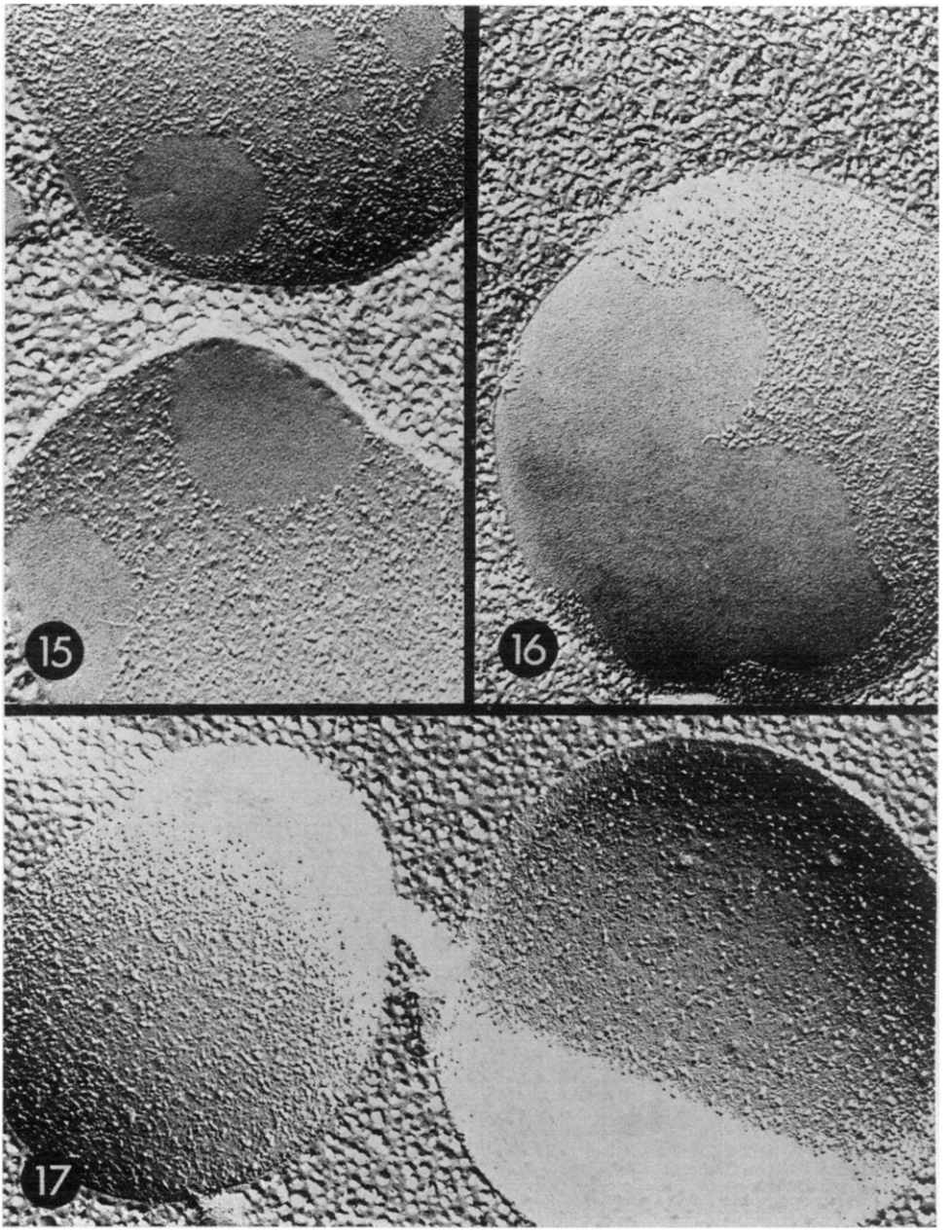
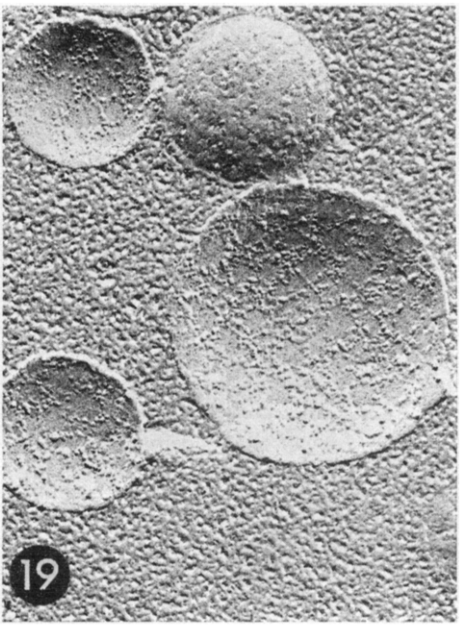
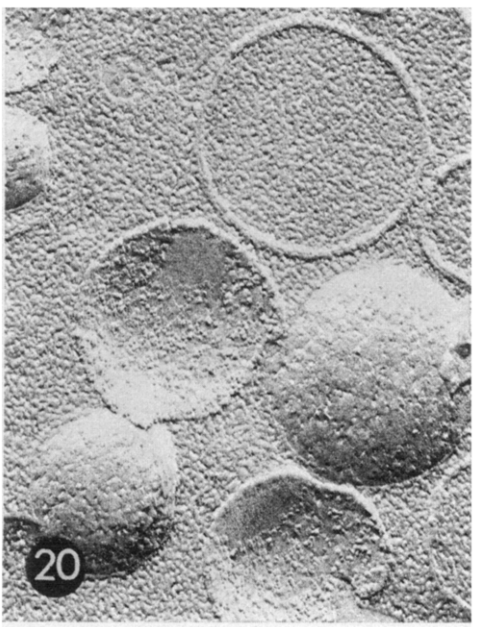


Fig. 15. Concave (lower) and convex (upper) fracture faces of the spherical inner membrane equilibrated at −8 °C, then frozen. ×67 500.

Fig. 16. Convex fracture face of the spherical inner membrane equilibrated at −13 °C, then frozen. ×67 500.

Fig. 17. Convex (left) and concave (right) fracture faces of the spherical inner membrane equilibrated at −10 °C, returned to 25 °C, and then frozen. ×67 500.





Figs. 18–21. Concave and convex fracture faces of the purified outer membrane equilibrated at 25 °C (Fig. 18), 8 °C (Fig. 19), 0 °C (Fig. 20), −6 °C (Fig. 21), and then frozen. ×67 500.

lateral separation between large, smooth regions and intramembrane particle-dense regions in the inner as well as in the outer membrane (Fig. 13).

Similar close relationships exist between thermotropic lipid phase transitions and the occurrence of lateral separations between smooth, intramembrane particle-free regions and intramembrane particle-dense regions in purified inner and outer mitochondrial membranes. In cooling experiments spherical inner membrane-matrix preparations, which showed an exothermal onset temperature of −4 °C (Fig. 7c), routinely exhibited randomly dispersed intramembrane particles at 4° C above the exothermal onset temperature (Fig. 14), and extensive lateral separations between large, smooth regions and intramembrane particle-dense regions at 4 °C below the onset temperature of the exothermal transition (Fig. 15). At −13 °C, which is approximately the lower end of the lipid phase transition region of the inner membrane (Table II), exothermal ordering in the bilayer lipid was near maximal, while lateral separations of membrane components were impressive as revealed by freeze fractured inner membranes (Fig. 16). These lateral separations, as well as those observed in the membranes of the whole mitochondrion, were completely reversed within a few seconds when the decreased temperature was returned to levels above the onset temperature (Fig. 17). Such reversibility is a strong indication that free lateral translational diffusion of integral proteins can occur in the bilayer lipid of both mitocondrial membranes.

Studies on purified outer membrane revealed a pattern of thermotropic lateral separation of membrane components similar to that of the outer membrane of the whole mitochondrion (Figs. 18–21). In cooling experiments, aggregation of intramembrane particles in the purified outer membrane was often apparent at 8 °C (Fig. 19), i.e., just below the first onset temperature of the exothermal transition (Fig. 6c). More definitive separations between smooth, intramembrane particle-free regions and intramembrane particle-dense regions were exhibited at 0 °C (Fig. 20), i.e., just below the second or major onset temperature of the exothermal transition (Fig. 6c). Such separations increased at − 6 °C (Fig. 21).

DISCUSSION

Results of the combined calorimetric and freeze fracture study reported here reveal that thermotropic lipid phase transitions and lipid-protein lateral separations can occur in both mitochondrial membranes and that these related events are totally reversible. The temperature at which the first sign of an exothermic liquid crystalline to gel state phase transition occurs, as revealed by differential scanning calorimetry, is observed to be in agreement with the temperature at which thermotropic lipid-protein lateral separation occurs, as revealed by freeze fracture electron microscopy. Such correlation occurs at approximately 9 °C in the outer membrane and at approximately − 4 °C in the inner membrane. The extent of the thermotropic transition region is quite narrow in the inner membrane (11 °C), indicating a higher degree of cooperative lipid transition as compared to the outer membrane (24 °C). The less cooperative lipid transition in the outer membrane appears to account for the wide lipid phase transition region exhibited by the composite two-membrane system of the whole mitochondrion.

The difference between the two membranes in the onset temperatures of exo-

thermal and endothermal lipid phase transitions, in the extents of the transition regions, and in the lipid-protein lateral separations, is apparently related to the distinct lipid and protein composition of the two membranes. However, it is difficult to appraise these differences in terms of the relative influence of the lipid and protein components. Data derived from less complex model membrane systems may be utilized in attempting such appraisal. Our observations are generally consistent with the predictions made from such model systems. Phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, and cardiolipin comprise 97 % of the phospholipid of rat liver mitochondrial membranes, and are considerably more saturated in the outer than in the inner membrane [42, 43]. Phosphatidylcholine plus phosphatidylethanolamine account for 80 % of the phospholipids of both membranes of the mitochondrion. These phospholipids are collectively 53 % unsaturated in the inner membrane and 45 % unsaturated in the outer membrane. Cardiolipin essentially represents the remainder (20 %) of the phospholipid of the inner membrane. The cardiolipin is 90 % unsaturated, with oleic (18 : 1) and linoleic (18 : 2) acids representing 80 % of the unsaturated fatty acids. Phosphatidylinositol accounts for about 10 % of the phospholipid of the outer membrane. It is 65 % saturated with palmitic (16 : 0) and steric (18 : 0) acids representing 62 % of the saturated fatty acids. Thus, that the inner mitochondrial membrane exhibits a lower lipid phase transition than the outer membrane is consistent with the finding that unsaturated phospholipids of model bilayer systems have lower characteristic transition temperatures than saturated phospholipids [44].

The two mitochondrial membranes differ also in their cholesterol content [42]. In model systems cholesterol tends to decrease the heat of transition between the gel and liquid crystalline phase and also to decrease the fluidity of phospholipids in the liquid crystalline state [13, 45]. Although the molar ratio of cholesterol to phospholipid-*P* is low in the outer membrane (0.13), the inner membrane is essentially free of the sterol. Thus, the cholesterol content of mitochondrial membranes shows an inverse relationship to the content of unsaturated phospholipids. A similar relationship has been pointed out for plasma membranes [42, 46]. The absence of cholesterol combined with the high content of unsaturated phospholipids in the inner membrane is consistent with the configurational plasticity exhibited by this membrane during change in respiratory state [31, 32]. As determined in the present study, the cholesterol content of the outer membrane is not sufficiently high to eliminate a lipid phase transition in this membrane. However, the cholesterol content may be high enough in the outer membrane to contribute to the physical parameters which effect the broader, less cooperative lipid phase transition observed in this membrane compared to the inner membrane. *A. laidlawii* plasma membranes containing only 7.6 % cholesterol exhibit a 31 % reduction in the energy content of the lipid phase transition compared to sterol-free membranes [47].

Although more difficult to appraise, the distinct protein composition of the two mitochondrial membranes may also be expected to contribute to the differences in lipid phase transitions and lipid-protein lateral separations exhibited by the two membranes. The inner and outer membranes contain 75 % and 63 % protein respectively [42, 43]. Approximately 50 % of the inner membrane protein is integral [48, 49]. Since the apolar surfaces of globular integral proteins are in hydrophobic association with the acyl chains of membrane phospholipids, some fraction of the total phospholipid is most likely immobilized by the protein. This fraction of membrane

lipid may be as high as 15 to 25 % [39, 50–53]. The high content of integral protein in the inner membrane may therefore contribute to the membrane's lower transition temperature through a greater hindrance of the ordering efficiency of phospholipid acyl chains. In addition, possible electrostatic interactions between the large component of peripheral protein and the phospholipids of the inner membrane may influence the organization of the phospholipid polar head groups causing lower transition temperatures, as has been proposed from studies of lipid-protein charge interactions in model membrane systems [54]. Although a high protein content may hinder cooperative interaction of the phospholipid acyl chains, in the present study the extent of the region of the lipid phase transition was narrower and therefore of higher cooperativity in the inner than in the outer membrane. The relative extent of the lipid transition region of the two mitochondrial membranes, therefore, may be influenced more by the cholesterol in the outer membrane than by the protein in the inner membrane.

In the present study, a biphasic lipid transition exotherm occurring at approximately 9 and 1 °C was exhibited by the purified outer membrane. There was also suggestion of a biphasic transition endotherm occurring at −15 and −8 °C in the inner membrane preparations. In cooling experiments, freeze fractured outer membranes revealed a slight separation between smooth, intramembrane particle-free regions and intramembrane particle-dense regions at approximately 10 °C and a dramatically pronounced separation at 0 °C. Although not clearly understood, biphasic transitions may be explained by an immiscibility of bilayer lipids such that a heterogeneous lateral distribution of liquid crystalline lipid exists in the membranes above the transition temperatures. Heterogeneous lateral domains of distinct phospholipid composition in the inner membrane may result in more than one distinct onset temperature during thermotropic lipid phase transitions. Similarly, more than one onset temperature may occur in the outer membrane owing to heterogeneous lateral domains of distinct phospholipid-cholesterol composition.

Related to the idea of heterogeneous lipid domains in mitochondrial membranes, it should be noted here that our results using differential scanning calorimetry and freeze fracture electron microscopy did not reveal major lipid phase transitions or lipid-protein lateral separations in the inner membrane at temperatures between 8 and 27 °C. Several reports utilizing spin labeling and/or Arrhenius plots of enzyme activation energies present data which indicate phase transitions in the inner membrane of rat liver mitochondria in this temperature range [16, 17, 19, 20]. Thus, it appears that lipid phase transitions may occur in the highly unsaturated lipid-containing inner membrane at relatively high temperatures without the detection of a heat of transition in the purified membrane by differential scanning calorimetry and without the detection of lipid-protein separation by freeze fracture. Collectively, these findings may indicate that liquid crystalline to gel state transitions occur in microdomains in the inner membrane at temperatures between 8 and 27 °C, which are not sufficiently extensive to detect a heat of transition by differential scanning calorimetry or a lateral displacement of integral proteins by freeze fracture electron microscopy. Alternatively, in mitochondria particular enzymes may be confined within such lipid microdomains and thus exhibit thermotropic discontinuities in activation energies between 8 and 27 °C without lipid-protein lateral separations. Finally, some mitochondrial enzymes may undergo thermotropic conformational changes in the mem-

brane independent of the lipid phase [20]. Whether or not the slight exothermic deflections which occurred in the inner membrane at 21, 12, and 1 °C are related to phase transitions in lipid microdomains or to discontinuities in enzyme activation energies, is presently under investigation.

The data derived from the present study are consistent with the notion that both mitochondrial membranes are naturally fluid to the extent that the integral membrane proteins can diffuse laterally in the bilayer lipid. Previous findings are consistent with this idea. For example, it is well known that the energy-transducing membrane exhibits a rapid reversible configurational change at the initiation of oxidative phosphorylation [31, 32], a dynamic event which requires considerable membrane flexibility and plasticity. The inner membrane also shows an unordered lateral distribution of cytochrome *c* oxidase [35], a finding which is consistent with the existence of a fluid bilayer lipid. Although no distinction between the inner and outer membrane was made, previous studies utilizing X-ray diffraction and differential scanning calorimetry revealed that thermotropic phase transitions can occur in the lipids of whole mitochondria [15, 18]. As mentioned earlier, spin labeling [17, 19] and kinetic studies of various enzyme activities [16, 19, 20], also indicate that thermotropic transitions occur in the mitochondrial inner membrane. Carbon-13 NMR spectra reveal considerable mobility in the phospholipid acyl chains in mitochondrial membranes, greater mobility, e.g., compared to the acyl chains of the erythrocyte membrane [55].

We have recently determined that lipid-protein lateral separations induced in the energy transducing membrane by temperatures as low as −8 °C and their reversal, as revealed by freeze fracture electron microscopy, are not destructive to electron transport or oxidative phosphorylation [21]. This finding and the present results derived from the combined calorimetric and freeze fracture analysis, do not support earlier suggested molecular models which infer that the metabolic proteins of the energy tranducing membrane are stabilized through a rigid protein lattice [22–24]. Obviously some protein-protein interactions occur in the inner membrane during such rapid metabolic events as electron transport and energy conservation. However, there are a number of delays inherent in the mechanisms of electron transport and ATP synthesis which may be related to macromolecular diffusion prior to protein-protein interaction in the overall reaction of oxidative phosphorylation. In electron transport, delays in the half time of oxidation have been noted at the levels of the flavoproteins and cytochrome *b* [56]. Ubiquinone may diffuse between the dehydrogenases and cytochrome *b* to transfer reducing equivalents to the cytochrome chain [57, 58]. Also, cytochrome *c* may diffuse between the cytochrome *c* oxidase of more than one cytochrome chain [59]. Rate and half time of ATP synthesis also show delays compared to rates of oxidation in different segments of the respiratory chain [60, 61]. Whereas the half time of oxidation of cytochrome *c* oxidase is 0.5 ms [56], the half time of ATP synthesis coupled to this oxidation is approximately 100 ms [60]. That macromolecular diffusion may account for such delays in the metabolic events of the energy transducing membrane is consistent with the lateral translational mobility of integral proteins observed in the present study. That lateral translational diffusion of integral proteins can occur at rates within the time structure of electron transport and ATP synthesis is also consistent with the exceptionally low viscosity ($\approx$ 0.1 P at 30 °C) of the lipid phase of mitochondria [62], and the mean diffusion coefficient ($5 \cdot 10^{-9}$ $cm^2 \cdot s^{-1}$ at 20 °C) for integral proteins in biological membranes [63–65].

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