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MODIFICATION OF LIVER MITOCHONDRIAL LIPIDS AND OF ADENINE NUCLEOTIDE TRANSLOCASE AND OXIDATIVE PHOSPHORYLATION BY COLD ADAPTATION

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The decrease in respiration rate following thyroidectomy is preceded by changes in the lipid composition of the mitochondrial membrane (Hoch, F.L., Subramanian, C., Dhopeshwarkar, G.A. and Mead, J.F. (1981) Lipids 16, 328–334) and in concert, changes in the kinetic parameters of the adenine nucleotide translocase (Mak, I.T., Shrago, E. and Elson, C.E. (1981) Fed. Proc. 40, 398). To demonstrate that physiological adaptation also involves this sequence of events, rats were housed at 8°C for 3–4 weeks. Cold adaptation resulted in a modest (5%) increase in the unsaturation index for the mitochondrial fatty acids comprised of a significant increase in arachidonic acid and a reciprocal decrease in linoleic acid. Phospholipid analysis indicated that cold adaptation increased the mitochondrial phosphatidylethanolamine and reciprocally decreased the phosphatidylcholine content. Concomitantly, cold adaptation resulted in 25–30% increases in rat liver mitochondrial respiratory activities without changing the respiratory control or ADP/O ratios. The kinetic parameters of the adenine nucleotide translocase were determined by the back-exchange method (Pfaff, E. and Klingenberg, M. (1968) Eur. J. Biochem. 6, 66–79). At 0–4 and 10°C, the V_{max} and K_m of the cold-adapted rat liver adenine nucleotide translocase were not distinguishable from the control values. The K_i values determined by Dixon plot studies for atractylate and palmitoyl-CoA were also comparable between the two groups. However, at 25 and 37°C, cold-adapted rat liver adenine nucleotide translocase exhibited a 20% increase in V_{max} and a 20% decrease in K_m for external ADP. The results suggest that one adaption to a cold environment involves hormone-mediated changes in the lipid composition in the mitochondrial membranes which in turn modulate the adenine nucleotide translocase and subsequent respiratory activities.

Introduction

Prolonged exposure to low environmental temperature initiates in the rat and other species an adaptable, nonshivering thermogenic response, one mediator of which is an increase in thyroid hormone activity [1,2]. The important role played by thyroid hormones in cold defense was demonstrated by studies [3,4] which showed that hypothyroid rats were unable to survive beyond a few days at 4°C.

Parallel with the increase in whole body heat production are increases in oxygen consumption by hepatic and other tissues [5,6]. Earlier reports [7,8] suggesting that the molecular action of the thyroid hormones was to uncouple oxidative phosphorylation must be discounted in light of recent reports that respiratory control ratios calculated for hepatic mitochondria from cold-adapted rats

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are equal to those of normal rats [5,9,10].

ADP, one of the rate-limiting substrates for coupled mitochondrial respiration, enters the mitochondrial matrix via an exchange transport process mediated by the membrane-bound adenine nucleotide translocase [11]. The increased respiratory activity of the cold-adapted state may reflect an increase in the cytosolic level of ADP, an action compatible with the suggestion that the treatment increases plasma membrane $(Na^+ + K^+)$-ATPase activity [6]. Alternatively, the adenine nucleotide translocase might be rate limiting for cellular respiration [12]. Changes in the physical characteristics of the lipid matrix of the inner membrane lipids might influence the adenine nucleotide translocase activity [11]. Hoch et al. [13] showed that thyroid hormone could affect both the fatty acid and phospholipid distributions in mitochondrial lipids. Our recent study [14] indicated an impaired adenine nucleotide translocase activity in the hypothyroid rat. We have therefore considered the possibility that prolonged cold exposure involves hormone-mediated modulations in the physical properties of the mitochondrial membrane lipid which, in turn, affect the ADP-ATP translocation and the subsequent overall rate of oxidative phosphorylation.

From our studies of the liver adenine nucleotide translocase, we conclude that the elevation in respiration observed during cold-adaptation can be related to 20% increases in both the V_{max} and the binding affinity of the adenine nucleotide translocase for ADP. Changes in the lipid matrix of the mitochondrial membrane consisting of increased unsaturation index and proportion of phosphatidylethanolamine contribute to the adaptative response.

This work has been presented in part at FASEB, New Orleans, LA, April 15–23, 1982 (Fed. Proc. 41, 402).

Materials and Methods

Male Sprague-Dawley rats (190–200 g) purchased from ARS-Sprague-Dawley (Madison, WI) were housed individually in the Animal Care Unit, at either 28°C (control) or 8 ± 2°C (cold adapted). The animals were maintained on a 12 h dark-light cycle with free access to food (Purina rat chow) and water for 3–4 weeks.

The rats were weighed and killed by blows to the head. The livers were immediately removed, washed in a chilled medium of 250 mM sucrose, 4 mM Tris-HCl (pH 7.4) and 1 mM EDTA and homogenized in 8 vol. of medium with a Potter Elvehjem-type homogenizer. Liver mitochondria were prepared by differential centrifugation according to the method of Schneider [15]. Mitochondrial respiration rates, with succinate or β-hydroxybutyrate as substrate, were determined polarographically as described by Estabrook [16].

Adenine nucleotide translocase activity was measured by the 'back exchange' technique of Pfaff and Klingenberg [17]. Mitochondria (about 60 mg mitochondrial protein) were loaded with [^{14}C]ATP by incubating at 0–4°C for 45 min with 0.50 μCi of [^{14}C]ATP in 5 ml of medium consisting of 250 mM sucrose, 4 mM Tris-HCl (pH 7.4) and 1 mM EDTA. The [^{14}C]ATP-loaded mitochondria were washed twice and suspended in the above medium at a concentration of 10 mg protein/ml. The back exchange was carried out with 1 mg [^{14}C]ATP-loaded mitochondrial protein (greater than 6000 dpm) which was preincubated for 5 min at the reaction temperature (0–4, 10, 25 or 37°C) in 1 ml of medium consisting of 100 mM KCl, 40 mM Tris-HCl (pH 7.4) and 1.0 mM EDTA. The exchange was started by adding specific quantities of ADP and stopped after specified periods of incubation by the addition of 50 μM atractylate. The reaction mixture was then centrifuged, the pellet dissolved in Soluene 100 (Packard Co., Downers Grove, IL) and the radioactivity in the pellet in 10 ml of Bray's scintillation fluid was determined. Each assay was accompanied by a control sample in which the addition of atractylate preceded that of ADP at time zero which provided a correction for the atractylate-insensitive efflux of the internal labelled adenine nucleotides. The transport rate is expressed as net percent efflux of mitochondrial ^{14}C-labelled adenine nucleotides according to the equation:

$$\% \text{ transport} = 100(\text{dpm}_{\text{control}} - \text{dpm}_{\text{assay}})/\text{dpm}_{\text{control}}$$

where dpm represents the radioactivity in the pellet. The kinetic parameters for the reciprocal plots were calculated within the initial apparent linear

rates of transport which were, respectively, 10 min, 1 min, 30 s and 15 s for 0–4, 10, 25 and 37°C.

K_i values for atractylate and palmitoyl-CoA were determined according to the method of Dixon [18]. The inhibitors were added to the incubation mixtures and preincubated with the [^{14}C]ATP-loaded mitochondria for 5 min at 0–4°C. ADP was added to initiate the transport, the reaction allowed to proceed at 0–4°C, stopped by the addition of 50 μM atractylate, centrifuged, and radioactivity in the pellet determined. Concentrations of the inhibitors and ADP are presented on the appropriate figures.

Lipids were extracted from freeze-thawed mitochondrial preparations according to the method of Ames [19] in which methanol, chloroform and aqueous suspensions of mitochondria are in the proportions 2 : 1 : 0.8 (v/v/v).

The methyl esters of fatty acids were obtained by transesterification of a portion of the extracted lipid in CH_3OH/HCl. For each sample, 5.0 ml of 0.5 M HCl in methanol were added to the dry lipid extract, refluxed at 75–80°C for 2–3 h, then cooled to room temperature. The fatty acid methyl esters were extracted and concentrated in petroleum ether.

Gas liquid chromatographic analyses of the fatty acid methyl esters were done on a Hewlett-Packard gas chromatograph Type 5730 A with a column of 10% DEGS on Chromosorb W (80/100 mesh). The instrument, equipped with a hydrogen flame ionization detector and coupled with an integrator, was operated at a temperature gradient programmed at 150°C for the initial 2 min followed by 3°C increments per min for 15 min to 195°C which was then held for another 15 min. The fatty acid methyl esters were identified by comparisons with retention times of standards.

The phosphatidylcholine (PC), phosphatidylethanolamine (PE) and neutral lipids in another aliquot of the mitochondrial lipid extracts were separated and quantified by a rapid method of high-performance liquid chromatography (HPLC) according to the method of Hanson et al. [20] with some modifications. The separation was performed with a Water Associates liquid chromatographic system comprising an Ultrasil-NH_2 column (Altex) with a 5 μm packing in a stainless-steel tube (250 × 4.6 mm inner diameter) coupled with a Perkin-Elmer LC-75 Spectrophotometric Detector and Spectra-Physics (SP 4100) Computing Integrator. The lipids were eluted using an isocratic mixture of *n*-hexane/2-propanol/water (6 : 8 : 1, v/v/v) at a flow rate of 0.7 ml/min and were detected at 206 nm. The separation was completed within 30 min. The PC, PE and neutral lipids and their relative weight distributions were identified and calculated against known quantities of bovine liver PC, egg yolk PE and triolein.

Protein was determined by the procedure of Lowry et al. [21] using bovine serum albumin as the standard.

Results are expressed as mean values ± S.D. Statistical significances were determined by Student's *t*-test. Regression lines were calculated by the least-squares method [22]. The validity of each regression line was tested by Student's *t*-test.

[^{14}C]ATP (44.8 mCi/mmol) was purchased from New England Nuclear, Boston, MA; ADP and palmitoyl-CoA from P-L Biochemicals, Milwaukee, WI; and atractylate, bovine liver PC, egg yolk PE and triolein from Sigma, St. Louis, MO. All the HPLC solvents were purchased from Burdick and Jackson Laboratories, Muskegon, MI.

Results

Cold-induced modification of rat growth and mitochondrial oxidative phosphorylation

Although body weights of cold-acclimated rats were significantly lower, their liver weights were significantly higher than the control values (Table I). Both State 3 and State 4 respirations in response to either succinate or β-hydroxybtyrate were elevated 25–30% by cold adaptation. However, neither the respiratory control ratio nor the ADP/O ratio was altered which indicates that the efficiency of the mitochondrial respiratory functions was not affected by the experimental conditions (Table I). The mitochondrial pool of adenine nucleotides was also not affected by the cold adaptation. During the 45 min incubation with [^{14}C]ATP, cold-adapted mitochondria loaded 105 ± 8% ($n = 5$) radiolabel compared to control.

Adenine nucleotide transport kinetics

The kinetics of the ADP-[^{14}C]ATP back exchange, based on the initial linear rates of percent

TABLE I

EFFECT OF COLD ADAPTATION ON RAT GROWTH AND OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA

Mitochondrial respiration rates were determined polarographically at 30°C and are expressed in ngatom oxygen consumed/min per mg protein. Values are means ± S.D., $n = 6$. RCR, respiratory control ratio.

Animals	Body weight	Liver weight per 100 g	Rate of oxidation		RCR	ADP/O
			+ADP	−ADP		
(β-hydroxybutyrate)						
Control	310 ± 13	4.07 ± 0.29	51.4 ± 3.2	9.4 ± 1.2	5.49 ± 0.44	2.71 ± 0.16
Cold adapted	245 ± 26 [b]	4.70 ± 0.44 [b]	66.1 ± 4.9 [b]	12.2 ± 1.9 [a]	5.50 ± 0.57	2.69 ± 0.13
(succinate)						
Control			99.1 ± 8.9	23.8 ± 1.6	4.16 ± 0.21	1.73 ± 0.15
Cold adapted			127 ± 10.4 [b]	31.7 ± 3.5 [b]	4.02 ± 0.25	1.70 ± 0.12

[a] $P < 0.05$ compared with values for controls.
[b] $P < 0.01$ compared with values for controls.

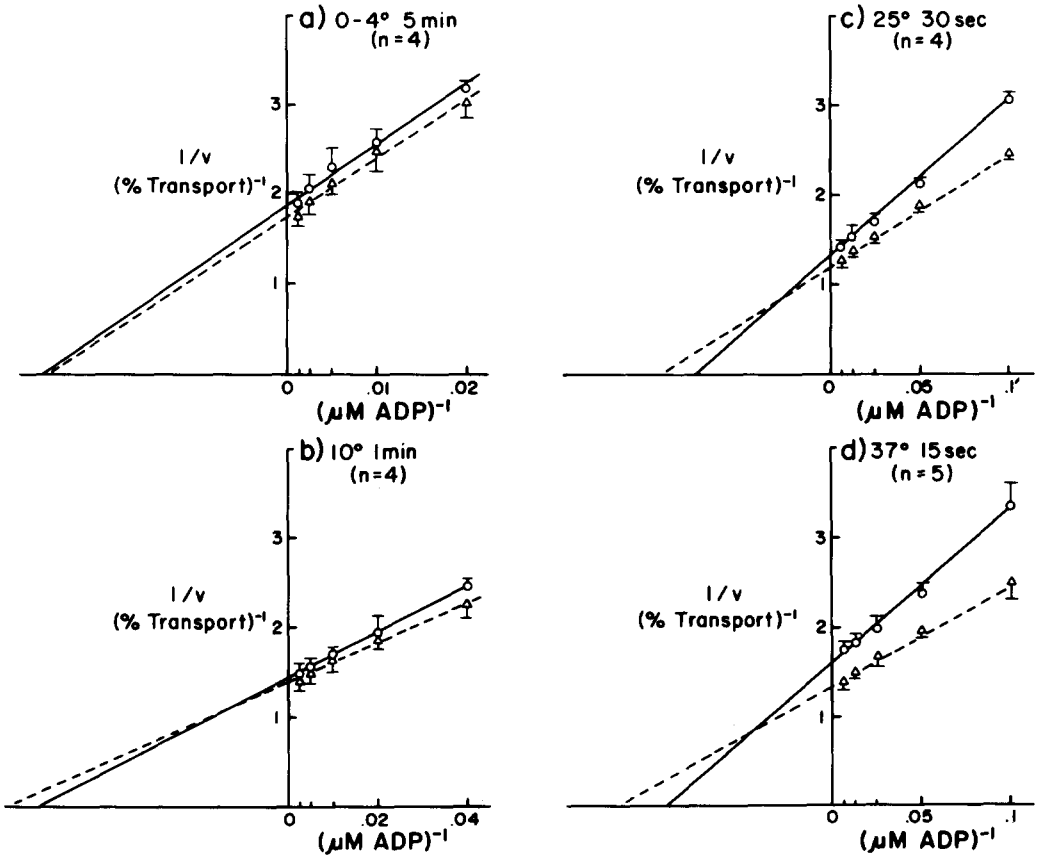

Fig. 1. Determinations of K_m and V_{max} for ADP/[^{14}C]ATP transport in control (solid lines) and cold-adapted (broken lines) rat liver mitochondria. [^{14}C]ATP-loaded mitochondria (1 mg protein) in 1 ml of incubation medium (pH 7.4) were preincubated for 5 min at the reaction temperature. The transport was started by adding specific quantities of ADP and stopped after specified periods of incubation by the addition of 50 μM atractylate.

TABLE II

REGRESSION EQUATIONS, $\hat{Y} = a + bx$, AND KINETIC PARAMETERS OF THE ADENINE NUCLEOTIDE TRANSLOCASE ACTIVITY IN HEPATIC MITOCHONDRIA ISOLATED FROM CONTROL AND COLD-ADAPTED RATS

The conditions for the assays are given in Fig. 1. n.s., not significant

Fig.	Linear Regression				K_m (μM)			V_{max} (%·s^{-1})		
	Intercept		Coefficient		Control	(P)	Cold	Control	(P)	Cold
	Control	Cold	Control	Cold						
1a	1.87	1.77	68.3	66.3	36.7 ± 4.8	(n.s.)	36.5 ± 6.0	0.178 ± 0.010	(n.s.)	0.188 ± 0.008
1b	1.44	1.40	25.3	27.1	17.7 ± 1.6	(n.s.)	15.8 ± 2.5	1.16 ± 0.06	(n.s.)	1.19 ± 0.10
1c	1.30	1.20	17.2	13.3	13.2 ± 0.1	(0.05)	11.2 ± 1.1	2.55 ± 0.03	(0.001)	2.78 ± 0.06
1d	1.61	1.36	17.1	11.1	10.6 ± 1.4	(0.05)	8.2 ± 1.6	4.13 ± 0.12	(0.001)	4.90 ± 0.11

^{14}C-labelled adenine nucleotide egress at 0–4, 10, 25 and 37°C, are shown in Fig. 1 and Table II. At the lower temperatures, the mitochondrial adenine nucleotide translocase of the cold-adapted groups exhibited 5–10% higher levels of activity, but the V_{max} and K_m calculated were not distinguishable from those of the control groups (Table II). According to the Dixon plots (Fig. 2), neither the K_i for atractylate (control, 0.209 μM; cold, 0.221 μM) nor that for palmitoyl-CoA (control, 1.26 ± 0.15 μM; cold, 1.18 ± 0.18 μM) was affected by cold adaptation. These studies using the well-characterized inhibitors were done at 0–4°C.

At assay temperatures of 25 and 37°C, the kinetic parameters were significantly influenced by the cold adaptation (Fig. 1c and d). At the assay temperature near body temperature, the adenine nucleotide translocase activity of the cold-adapted group was characterized by a 19% higher V_{max} ($P < 0.001$) and a 23% lower K_m ($P < 0.05$) (Table II).

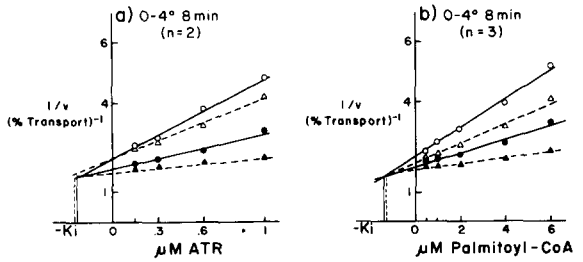

Fig. 2. Determination of K_i values for atractylate (ATR) (a) and palmitoyl-CoA (b) inhibitions of the ADP/[^{14}C]ATP transport in control (solid lines) and cold-adapted (broken lines) rat liver mitochondria. The inhibitors were added to the mixtures prior to the preincubations. Final ADP concentrations were either 50 μM (○, △) or 200 μM (●, ▲). Other conditions are described in Materials and Methods and in Fig. 1.

Mitochondrial lipid fatty acid pattern.

Fatty acid profiles of the lipid extracts of the hepatic mitochondria are shown in Table III.

TABLE III

FATTY ACID PATTERNS OF LIVER MITOCHONDRIA FROM CONTROL AND COLD-ADAPTED RATS (%)

The unsaturation index is the average number of double bonds per fatty acid molecule. Values are mean ± S.D., $n = 6$.

Fatty acid	Control	Cold adapted
16:0	17.7 ± 2.1	17.1 ± 1.4
18:0	17.6 ± 1.4	19.1 ± 1.7
18:1	9.2 ± 2.4	7.6 ± 0.4
18:2	21.7 ± 2.8	17.9 ± 1.2 [a]
20:3	1.0 ± 0.1	0.9 ± 0.2
20:4	21.3 ± 1.6	24.1 ± 1.9 [a]
22:5	1.5 ± 0.2	1.4 ± 0.1
22:6	6.3 ± 0.8	7.3 ± 0.6
20:4/18:2	1.00 ± 0.12	1.34 ± 0.05 [b]
Mean carbon length	18.42	18.55
Unsaturation index	1.93	2.03

[a] $P < 0.05$ compared with values for controls.
[b] $P < 0.005$ compared with values for controls.

Comparisons of the two patterns reveal similar proportions of saturated, monounsaturated and polyunsaturated fatty acids. Although the sum of the fatty acids arising from dietary linoleic acid in each fatty acid profile is 51.7%, a critical inspection of the polyunsaturated fatty acid profile reveals that products of elongation and desaturation activities are more predominant at the expense of the precursor in the lipids of the cold-adapted mitochondria. These adjustments underlie calculated mean chain lengths of 18.42 and 18.55 carbons and of unsaturation indices of 1.93 and 2.03 double bonds for lipids of the control and cold-adapted groups. The ratio of arachidonic to linoleic acid increased ($P < 0.005$) from 0.98 to 1.35 with cold adaptation.

PE, PC and neutral lipid distributions.

The relative proportions of PC and PE, the two phospholipids containing about 80% of the mitochondrial membrane-bound lipid phosphorus and of neutral lipid, predominantly cholesterol [23,24], are listed in Table IV. The patterns are calculated from detector-integrator generated counts/g lipid, e.g., 400 counts/μg triacylglycerol, 300 counts/μg PC and 280 counts/μg PE. With cold adaptation, the proportion of neutral lipid remained unchanged, the PE increased and reciprocally, PC decreased. The changes in the phospholipid pattern accompanying cold adaptation forced a change ($P < 0.05$) in the PC/PE ratio from 1.15 to 0.90.

The presence of more than one species may be indicated by shoulder formation on both PE and the PC peaks with the shoulders representing differences in fatty acid chain length and unsaturation (Fig. 3).

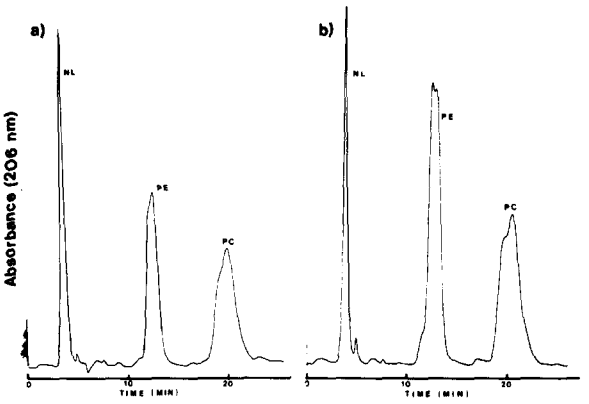

Fig. 3. HPLC separation of the lipid extracts from mitochondria of control (a) and cold-adapted (b) rats. The three major peaks represent, respectively, neutral lipids (NL), PE and PC. Chromatographic conditions: Column, Ultrasil-NH_2; eluent solvent, hexane/2-propanol/water (6:8:1, v/v/v); flow rate, 0.7 ml per min; pressure, 500–600 lb/inch^2; temperature, ambient; detector, ultraviolet absorbance at 206 nm.

Discussion

Consistent with the reports of others [5,9,10] is our observation that cold adaptation imposes on hepatic mitochondria a 25–30% increase in the rate of oxygen uptake. Contrary to those reports [7,8] was our demonstration of tightly coupled oxidative phosphorylation (Table I). Cold adaptation increased both State 3 and State 4 respirations supported by either an NAD- or FAD-dependent substrate. Since the respiratory control ratio and ADP/O ratio were not influenced by the treatment, the increased rate of oxygen consumption must support increased rates of ADP phosphorylation at both sites I and II of ATP synthesis. One of

TABLE IV

EFFECT OF COLD ADAPTATION ON MITOCHONDRIAL NEUTRAL LIPIDS, PE AND PC DISTRIBUTIONS

Values are expressed as wt%. Neutral lipids are expressed as triolein equivalent in weight.

Rat groups (n)	Neutral lipids	PE	PC	PC/PE
Control (6)	21.4 ± 4.6	36.6 ± 3.1	42.0 ± 2.1	1.15 ± 0.08
Cold adapted (5)	19.0 ± 1.8	42.6 ± 4.2 [a]	38.4 ± 2.6 [a]	0.90 ± 0.15 [a]
(% of control)	(89.0)	(116)	(91.4)	(78.2)

[a] $P < 0.05$ compared with values for controls.

the controlling factors for the increased respiration is the increased availability of ADP [12]. Associated with the increase in mitochondrial respiratory activity were increases of 20% both for the V_{max} and for the substrate affinity of the adenine nucleotide translocase, increases which would underlie an increased matrix availability of ADP. These differences in the kinetic parameters of the adenine nucleotide translocase were apparent only at temperatures above the phase transition temperature for the membrane lipids (i.e., 15–20°C [25]). Palmitoyl-CoA may act as a natural effector of adenine nucleotide translocase activity [26]. The K_i for this competitive inhibitor must be determined at a temperature which depresses mitochondrial acyl-CoA : carnitine acyltransferase activity in order to prevent oxidation of the acyl-CoA. At this temperature, neither the K_i for palmitoyl-CoA nor that for atractylate was affected by the cold adaptation. The data presented thus far are entirely consistent with the proposal that adenine nucleotide translocase activity is rate limiting for oxidative phosphorylation [12].

The analyses of the mitochondrial membrane lipids yield insight into the molecular mechanism underlying the difference in the adenine nucleotide translocase activity subsequent to cold adaptation. Subtle changes in the membrane composition might influence adenine nucleotide translocase activity. Changes of two types were noted, the first being a modest increase (5%) in the unsaturation index of the constituent fatty acids, a change underscored by an increase in arachidonic acid with a reciprocal decrease in linoleic acid. The second change involved a reciprocal increase in the PE and decrease in the PC contents of the membranes, a change which has also been observed in hepatic mitochondrial lipids of goldfish adapted to low temperature [27].

The thyroid hormones, possibly interacting with catecholamines, mediate the nonshivering thermogenic response to cold adaptation [1,2]. One action of the thyroid hormones is to increase the conversion of linoleic to arachidonic acid [28–30] and concomitantly, the unsaturation index of mitochondrial lipids [31]. In thyroid hormone deficiency, the opposite effects have been noted [13]. Another action of thyroid hormones appears to be on the phospholipid pattern of the mitochondrial membrane lipids [13]. Associated with these changes in mitochondrial lipids in response to the hormone deficiency is an impaired adenine nucleotide translocase activity consisting of a lower V_{max} and higher K_m for substrate [14].

We propose that cold adaptation initiates a temporal response, the first of which is a hormone-mediated change in the lipid matrix of the mitochondrial and presumably, other cellular membranes. The changes in the lipid composition of the mitochondrial membrane are parallel to those which in vitro destabilize the lipid bilayer [32,33]. The adenine nucleotide translocase embedded in the less stable bilayer is both more assessible to the cytosolic ADP and has a higher V_{max} allowing more rapid transport of ADP to the phosphorylation sites. Similar changes in the plasma membrane, we predict, underlie the increased $(Na^+ + K^+)$-ATPase activity [6] which maintains the cytosolic pool of ADP required for the exchange transport of mitochondrial ATP.

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