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The influence of hypothyroidism on the transport of phosphate and on the lipid composition in rat-liver mitochondria

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The influence of hypothyroidism on the transport of phosphate and on the lipid composition in rat-liver mitochondria was examined. It was found that the rate of phosphate transport is reduced (around 40%) in mitochondria from hypothyroid rats compared to that obtained in mitochondria from normal rats. Treatment of hypothyroid rats with thyroid hormone reverses this effect completely. Kinetic analysis of the phosphate transport indicates that only the V_{max} of this process is affected, while there is no change in the K_m values. The lower rate of phosphate transport in mitochondria from hypothyroid rats is also demonstrated by swelling experiments. There is no significant difference either in the respiratory control ratios or in the ADP/O ratios between these two types of mitochondria. The hepatic mitochondrial lipid composition is altered significantly in hypothyroid rats. The total cholesterol increases, the phospholipids decrease and the cholesterol/phospholipid molar ratio increases (around 40%). Among the phospholipids, cardiolipin shows the greatest alteration (30% decrease in the hypothyroid rats). The phosphatidylethanolamine/phosphatidylcholine ratio also decreases. Alterations were also found in the pattern of fatty acids. These changes in lipid composition may be responsible, at least in part, for the depression of the phosphate carrier activity in mitochondria from hypothyroid rats.

Introduction

Although the mechanism is still not completely defined, the thyroid hormones are known to play an important role in the regulation of hepatic metabolism. Mitochondria are considered to be likely subcellular targets of thyroid hormone action in view of their central role in energy metabolism.

The transport of anionic substrates across the mitochondrial inner membrane may represent an important point in the regulation of several cytosolic and mitochondrial pathways (for reviews see Refs. 1–3). Thyroid hormones have been shown to influence the activity of several mitochondrial anion carrier proteins [4–10]. The rates of both substrate oxidation and oxidative phosphorylation are decreased in intact mitochondria from the livers of hypothyroid rats as compared with mitochondria from normal rats [11–13]. Hypothyroidism appears to affect oxidative phosphorylation through an altered activity of the adenine nucleotide carrier [6,13].

The synthesis of ATP during oxidative phosphorylation requires the uptake of both ADP and inorganic phosphate. The translocation of phosphate across the mitochondrial membrane is mediated by a specific carrier (for reviews see Refs. 1,14). The phosphate carrier protein has been purified to homogeneity from bovine and rat-liver mitochondria [15–17]. The activity of the isolated phosphate carrier, reconstituted in artificial membranes such as liposomes, appears to be strongly influenced by the lipid composition of these artificial organelles. A specific requirement of cardiolipin has been demonstrated in these reconstitution experiments [18–20].

Changes in lipid content, lipid composition and in the lipid-protein interaction have been reported in mitochondrial membranes under different thyroid states [21–24]. These changes have been considered at

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least in part responsible for the changes in the activity of certain anion carrier proteins [6,8–10].

We have recently reported stimulation of phosphate transport in liver mitochondria from hyperthyroid rats [25]. In this paper the influence of the hypothyroid state on the translocation of phosphate and on the lipid composition in rat-liver mitochondria was studied.

Materials and Methods

Chemicals

The radioactive [^{32}P]orthophosphate, $^{3}H_2O$ and [U-^{14}C]sucrose were obtained from Amersham (U.K.). 5,5-Dimethyl[^{14}C]oxazoline-2,4-dione was obtained from New England Nuclear. All other reagents were purchased from Sigma.

Animals

Male Wistar rats (200–250 g), housed at a temperature of 22°C and fed ad libitum with a standard diet were used throughout these studies. Animals were made hypothyroid by adding 6-*n*-propyl-2-thiouracil (PTU; 0.05%, w/v) to their drinking water for 3–4 weeks [26]. The treatment with PTU resulted in a decrease in the level of circulating 3,3',5,5'-tetraiodo-L-thyronine from 55.9 ± 2.2 pmol/ml ($n = 5$) to 4.8 ± 0.8 pmol/ml ($n = 5$). For thyroid hormone treatment of rats pretreated with PTU, a dose of T_3 (3,3',5-triiodo-L-thyronine) of 25 μg/100 g body weight was injected intraperitoneally for 2 days. Control animals received only the solvent for the same period. Animals were killed 24 h after final administration.

Rat-liver mitochondria were prepared by differential centrifugation of liver homogenates as previously described [27]. The mitochondrial pellet was washed three times with the medium. The yield of mitochondrial protein within each group of animals was consistent (31.5 ± 2.8 and 30.3 ± 3.1 mg/g liver wet wt. from normal and hypothyroid rats, respectively) suggesting minimal variation in the preparation of the mitochondrial fractions. The ratio of mitochondrial cytochrome aa_3:protein was essentially the same in the two mitochondrial preparations, the values being 231 ± 24 and 222 ± 23 pmol/mg protein in mitochondria from normal and hypothyroid rats, respectively.

Mitochondrial phosphate transport

The transport of exogenous phosphate into mitochondria was measured at 0°C by the "inhibitor stop method", essentially as described in Ref. 28. Mitochondria (around 2 mg of protein/ml) were preincubated in a reaction medium that contained in a final volume of 1 ml: 100 mM sucrose, 50 mM KCl, 20 mM Tris-HCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 1 mM *n*-butylmalonate (to inhibit the dicarboxylate carrier which is also able to transport phosphate) and 2 μg/ml rotenone (final pH 7.4). After a period of equilibration of 2 min, phosphate transport was initiated by adding radioactive phosphate and stopped after time t by rapid addition of 0.2 mM mersalyl. The tubes were then rapidly centrifuged at 12000 × g for 2 min. The pellets were washed several times in 0.25 M sucrose and then the radioactivity was counted in a scintillation counter. Phosphate transport was considered as the difference between noninhibited and mersalyl-treated samples (in the latter case mersalyl was added in the preincubation phase 2 min before radioactive phosphate). When mersalyl was added to the incubation before starting the assay with radioactive phosphate, the amount of phosphate bound was the same in the mitochondria isolated from normal and hypothyroid rats. This indicates that the hypothyroid state had no effect on non-specific phosphate binding.

The transport of phosphate in mitochondria was also measured as phosphate-[^{32}P]phosphate exchange. In these experiments mitochondria from both control and hypothyroid rats were first preloaded with unlabelled phosphate as follows. Aliquots of mitochondria (40–50 mg protein) were incubated at 0°C in 20 ml of the buffer containing 100 mM sucrose, 50 mM KCl, 20 mM Tris-HCl, 1 mM EDTA, 3 μg/ml rotenone and 2 mM unlabelled phosphate (final pH 7.4). After a period of equilibration of 5 min, mitochondria were centrifuged at 9000 × g for 10 min. The mitochondrial pellet was resuspended in 0.25 M sucrose. The rate of phosphate-[^{32}P]phosphate exchange was then measured as described above.

Mitochondrial swelling

Mitochondrial osmotic volume changes were measured by the apparent absorbance changes at 540 nm with a spectrophotometer linked to a suitable recorder. The reactions were carried out at 25°C in 3 ml of the appropriate isoosmotic medium as indicated in the legends to figures.

Transmembrane ΔpH measurements

The transmembrane ΔpH values in mitochondria were determined essentially as described in Ref. 9.

Mitochondrial respiration

Reactions were carried out in a 1-ml water-jacketed closed chamber with magnetic stirring at 25°C. O_2 uptake was measured polarographically with a Clark oxygen electrode connected to a suitable recorder.

High-pressure liquid chromatography analysis of lipids

Phospholipids, fatty acids and cholesterol were analyzed by HPLC, using a Beckman 344 gradient liquid chromatograph. Extraction and analysis of phospholipids, fatty acids and cholesterol were carried out as described in Ref. 24.

Determination of phosphate and protein

The endogenous level of phosphate was determined chemically [29] in perchloric acid extracts. Protein concentration was measured by the usual biuret method, using serum albumin as standard.

Statistical analysis

Results are expressed as mean values ± standard error. Statistical significances were determined by the Student's *t*-test.

Results

Fig. 1 illustrates the results of six separate experiments on the time-course of phosphate uptake by liver mitochondria from control, hypothyroid and hypothyroid rats treated with T_3. Both the rate and the final extent of phosphate uptake by mitochondria from hypothyroid rats were significantly lower than those obtained with mitochondria from normal rats. The lower values for the rate of phosphate uptake observed in the mitochondria from hypothyroid rats were completely restored to normal levels by treating the hypothyroid rats with T_3. Very similar results were obtained when liver mitochondria from both normal and hypothyroid rats were first preloaded with cold phosphate and then the activity of the phosphate carrier was measured as rates of phosphate-[^{32}P]phosphate exchange (results not reported).

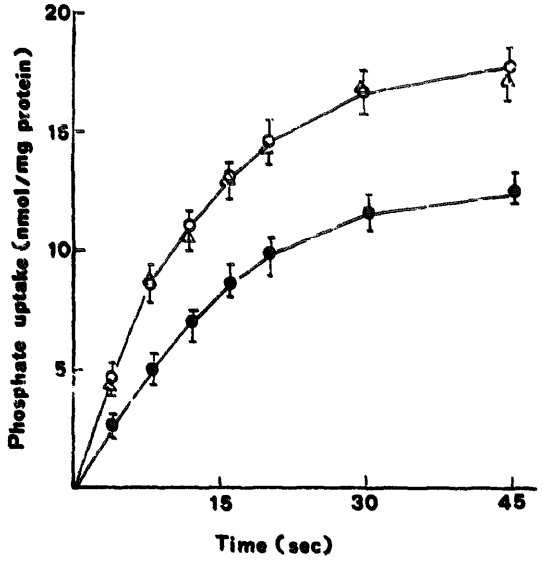

Fig. 1. Time-course of phosphate uptake by liver mitochondria from normal, hypothyroid and hypothyroid + T_3 rats. The transport of phosphate in mitochondria was measured as described under Materials and Methods. Mitochondria (around 2 mg protein/ml) were preincubated in the standard reaction medium at pH 7.4 and 0°C. After 2 min of preincubation 2 mM radioactive phosphate was added. The reaction was stopped at the times indicated by adding 2 mM mersalyl. Values are expressed as the means ± S.E. for six separate experiments. ○, Mitochondria from normal rats; ●, mitochondria from hypothyroid rats; △, mitochondria from hypothyroid rats treated with T_3.

TABLE I

Kinetic parameters of the phosphate transport in liver mitochondria isolated from normal and hypothyroid rats

The rate of phosphate transport by mitochondria was measured essentially as described under Materials and Methods and in the legend to Fig. 1. The K_m and V_{max} values were calculated from double-reciprocal plots of the rates of phosphate uptake vs. phosphate concentrations (ranging from 0.250 to 4 mM). Each value represents the mean ± S.E. obtained for six separate experiments. Each experiment consisted of two preparations of liver mitochondria from four hypothyroid and four control rats. [a] $P < 0.01$.

Animals	K_m (mM)	V_{max} (nmol/min per mg of protein)
Normal	1.75 ± 0.20	174 ± 21
Hypothyroid	1.64 ± 0.22	103 ± 15 [a]

The kinetic parameters of the phosphate transport by mitochondria from normal and hypothyroid rats were determined by studying the dependence on substrate concentration of the rate of phosphate uptake. The values, reported in Table I, indicate that the maximal velocity of the phosphate transport was significantly depressed (around 40%) in mitochondria from hypothyroid rats when compared with that obtained in mitochondria from control rats. There was practically no change in the affinity of phosphate for its carrier system in both of these types of mitochondria. It should be noted that the values obtained for the kinetic constants of the phosphate carrier in mitochondria from control rats are in good agreement with previous data reported in the literature [30–32].

The lower activity of the phosphate carrier in hepatic mitochondria from hypothyroid rats with respect to normal rats is further documented by swelling experiments. Nonrespiring mitochondria swell when suspended in isoosmotic ammonium phosphate due to the entrance of NH_3 and $H_2PO_4^-$ transported into mitochondria with a proton by the phosphate carrier [33]. A typical experiment reported in Fig. 2 shows that mitochondria from normal rats underwent large-amplitude swelling when suspended in isoosmotic solution of NH_4PO_4. However, it should be noted that both the rate and the final extent of the swelling in ammonium phosphate were markedly decreased in mitochondria from hypothyroid rats as compared to the values obtained with mitochondria from normal rats. Treatment of hypothyroid rats with T_3 reversed this effect almost completely. These results were compared with the rate of swelling of the same preparations of mitochondria when suspended in ammonium acetate. No change in either the rate or the final extent of the swelling in ammonium acetate could be observed between these two types of mitochondria.

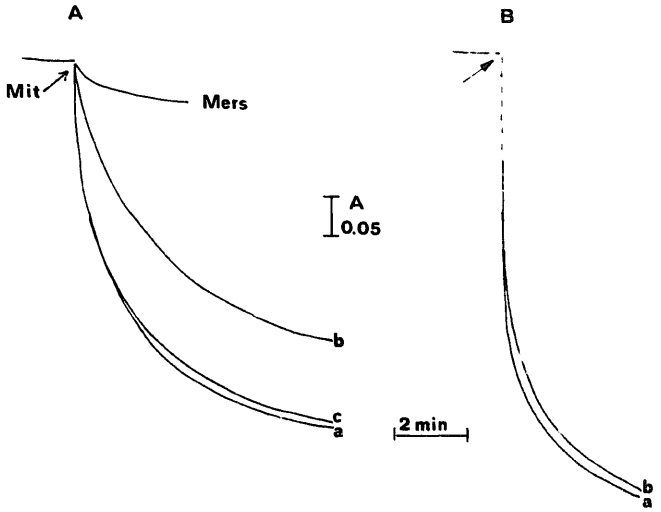

Fig. 2. Swelling of liver mitochondria from normal, hypothyroid and hypothyroid + T_3 rats in isoosmotic solution of ammonium phosphate (A) or ammonium acetate (B). Mitochondrial swelling was monitored as described under Materials and Methods. Mitochondria (approximately 0.7 mg of protein) were suspended in a 3-ml solution of 125 mM NH_4PO_3 (A) or 125 mM ammonium acetate (B) containing in addition 5 mM Hepes, 0.5 mM EDTA and, only in (A), 6 μg of rotenone, pH 7.4, 25 °C. When present, mersalyl was 0.2 mM. The reaction was initiated by the addition of mitochondria to the medium. Trace (a): mitochondria from normal rats; trace (b): mitochondria from hypothyroid rats; trace (c): mitochondria from hypothyroid rats treated with T_3. The experiment shown is representative of five different experiments.

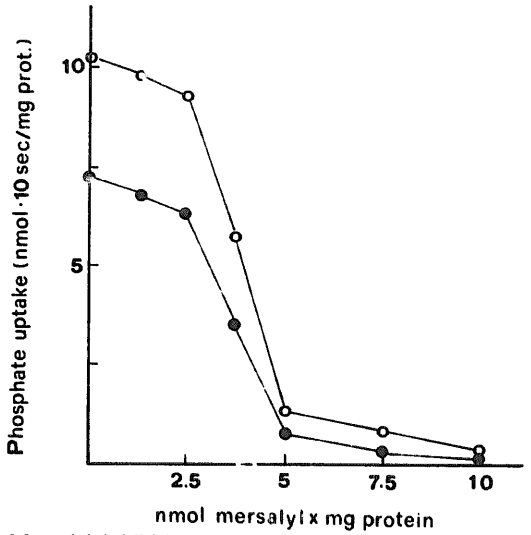

Fig. 3. Mersalyl inhibition of phosphate transport in mitochondria from control and hypothyroid rats. Experimental conditions as in Fig. 1. Mitochondrial protein was 2 mg/ml. Mersalyl was added, at the concentrations indicated, during the preincubation phase. The data represent the results of one of four experiments which gave similar results. ○, Mitochondria from normal rats; ●, mitochondria from hypothyroid rats.

The decreased activity of the phosphate carrier in mitochondria from hypothyroid rats could be due to a lower content of functional phosphate carrier protein. To assess this, inhibitor titrations of phosphate transport with mersalyl were carried out in mitochondria from both normal and hypothyroid rats. The minimal amount of this inhibitor required to obtain maximal inhibition of the phosphate transport should give a measure of the amount of the phosphate carrier protein in mitochondria [25]. Total inhibition of the phosphate transport was achieved by approximately the same concentration of mersalyl in both mitochondrial preparations from control and hypothyroid rats (see Fig. 3). This suggests that the hypothyroid status makes no difference to the amount of functional phosphate carrier in mitochondria.

Respiratory activities of hepatic mitochondria from normal and hypothyroid rats are reported in Table II. Consistent with published data [34,35] it was found that mitochondria isolated from hypothyroid rats and utilizing succinate as substrate exhibited a decline in both State 3 and State 4 respiration compared to normal mitochondria. This decline was more pronounced in the case of State 4 respiration. The mechanism by which the hypothyroid status induces a decline in both State 3 and State 4 respiration in mitochondria remains uncertain and is still a matter of debate [34–38]. There was no significant difference in the ADP/O ratios between mitochondria from normal and hypothyroid rats.

The effect of the hypothyroid state on the rat-liver mitochondrial phospholipids and total cholesterol content is shown in Table III. From this Table, total cholesterol can be seen to increase by 24% and, inversely, phospholipids decrease by 14% in mitochondria from hypothyroid rats. Consequently, the inverse relationship between the change in the total cholesterol and phospholipid content observed in these

TABLE II

Respiratory activities of hepatic mitochondria from normal and hypothyroid rats

The succinate-dependent oxygen consumption was measured polarographically, as described under Materials and Methods. Mitochondria (1.2–1.5 mg of protein) were incubated at 25°C in a reaction medium containing: 100 mM sucrose, 50 mM KCl, 20 mM Tris-HCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 5 mM P_i (final pH 7.2). When a steady state of oxygen consumption was obtained, 3 mM succinate was added. 2 min later respiration was stimulated by the addition of 0.4 mM ADP. State 3 refers to the rate of oxygen uptake in the presence of ADP. State 4 refers to the rate of oxygen uptake in the absence of ADP. Each value represents the means ± S.E. obtained for five separate experiments. [a] $P < 0.01$; [b] $P < 0.025$.

Animals	Respiratory activity (ngatom O/min per mg protein)		Respiratory control ratio	ADP/O
	State 3	State 4		
Normal	185 ± 15	30 ± 2.7	6.2 ± 0.64	1.78 ± 0.11
Hypothyroid	151 ± 14 [a]	22 ± 2.4 [b]	6.7 ± 0.68	1.75 ± 0.12

TABLE III

Cholesterol and phospholipid content in hepatic mitochondria from normal and hypothyroid rats

For cholesterol and phospholipid extraction and characterization, see Materials and Methods. Each value represents the mean ± S.E. obtained for six experiments. Cholesterol is expressed as nmol/mg protein and phospholipids as nmol lipid P_i/mg protein. [a] $P < 0.01$; [b] $P < 0.001$.

	Normal rats	Hypothyroid rats
Total cholesterol	16.0 ± 1.5	19.8 ± 1.6 [a]
Phospholipids	165.2 ± 9.8	142.7 ± 8.7 [a]
Ratio cholesterol/ phospholipid	0.1 ± 0.01	0.14 ± 0.01 [b]

organelles caused a 40% increase in the total cholesterol/phospholipid molar ratio.

The major phospholipids species in both mitochondrial membranes from normal and hypothyroid rats were quantitated following separation by high-performance liquid chromatography. The results of these analyses are given in Table IV. The most marked changes were the decrease in both the level of cardiolipin and in the ratio PE/PC in the mitochondrial membranes from hypothyroid rats.

Chemical changes in the mitochondrial membrane lipid composition were further investigated by analyzing the fatty acids composition in these mitochondrial membranes (see Table V). An alteration of fatty acids distribution was observed in mitochondrial membrane from hypothyroid rats. In particular, an increase in linoleic acid (18:2) and a decrease in arachidonic acid (20:4) occurred in the hypothyroid state.

TABLE IV

Phospholipid composition in liver mitochondria from normal and hypothyroid rats, as determined by HPLC

For phospholipid extraction and analysis, see Materials and Methods. Each value represents the mean ± S.E. obtained for six separate experiments. PE = phosphatidylethanolamine; PC = phosphatidylcholine. [a] $P < 0.001$; [b] $P < 0.01$.

Phospholipid	Distribution (mol%)	
	normal rats	hypothyroid rats
Cardiolipin	14.2 ± 1.0	10.1 ± 0.9 [a]
Phosphatidylethanolamine	29.7 ± 1.4	26.0 ± 1.5 [b]
Phosphatidylinositol	0.9 ± 0.3	0.6 ± 0.3
Phosphatidylserine	6.2 ± 1.0	5.8 ± 0.8
Phosphatidylcholine	49.0 ± 2.8	57.5 ± 2.0 [a]
PE/PC	0.61 ± 0.06	0.45 ± 0.08 [b]

TABLE V

Pattern of fatty acids in liver mitochondria from normal and hypothyroid rats as determined by HPLC

Extraction and analysis of fatty acids were carried out as described in Materials and Methods. Each value represents the mean ± S.E. obtained for six experiments. The unsaturation index (UI) is defined as Σ mol% of each fatty acid × number of double bonds of the same fatty acid. [a] $P < 0.001$; [b] $P < 0.01$.

Fatty acid	Distribution (mol%)	
	normal rats	hypothyroid rats
16:0	16.3 ± 1.2	17.1 ± 1.0
16:1	3.1 ± 0.4	2.6 ± 0.3
18:0	13.0 ± 0.8	13.2 ± 0.9
18:1	9.7 ± 0.7	8.6 ± 0.8
18:2	26.8 ± 1.4	31.5 ± 1.7 [a]
20:3	1.6 ± 0.3	1.8 ± 0.3
20:4	29.5 ± 1.7	25.2 ± 1.8 [b]
UI	189.2 ± 3.0	180.4 ± 3.2
20:4/18:2	1.1 ± 0.08	0.8 ± 0.05 [a]

Discussion

The results presented in this paper demonstrate that the activity of the phosphate carrier is decreased in mitochondria from hypothyroid rats. This decrease does not appear to be dependent on a change in the transmembrane ΔpH, towards which the phosphate carrier is very sensitive [14]. In fact, the swelling experiments reported in Fig. 2 show that while the uptake of phosphate is reduced in mitochondria from hypothyroid rats, that of acetate (an anion which, like phosphate, is transported in mitochondria as a function of ΔpH [39,40], is not affected. In addition no substantial changes in the transmembrane ΔpH value were observed in mitochondria from either control or hypothyroid rats, the values being 0.83 ± 0.08 and 0.81 ± 0.07 (means ± S.E. for five experiments), respectively.

A lower content of endogenous exchangeable phosphate may represent another possible factor responsible for the decreased activity of the phosphate carrier in mitochondria from hypothyroid rats. However, no difference in the content of endogenous phosphate was detected in the two preparations of mitochondria, the values being 12.8 ± 1.9 and 11.9 ± 1.7 nmol per mg of protein in mitochondria from normal and hypothyroid rats, respectively.

The lower activity of the phosphate carrier in mitochondria from hypothyroid rats was restored to normal level by treating the hypothyroid rats with thyroid hormone (see Figs. 1 and 2). This demonstrates that the inhibitory effect induced by the experimental hypothyroidism on the transport of phosphate is fully reversible, thus excluding any aspecific or irreversible effect of this pathological state on the phosphate carrier molecule itself or on the intactness of the mitochondrial membranes. This last point is also documented by the respiratory studies (see Table II) which show that neither the respiratory control ratios nor the ADP/O ratios were appreciably affected by the hypothyroid state.

The kinetic analysis of the phosphate carrier reported in Table I (similar K_m values and decreased V_{max} value in hypothyroid rats) indicates that the depressed translocating activity of the mitochondrial phosphate carrier in hypothyroid rats may be a result of changes in the amount or in the turnover number of the carrier rather than in the properties of the protein translocator molecule. Inhibitor titrations of the rate of phosphate transport with mersalyl (see Fig. 3) provide no evidence of a decreased content of functional phosphate carrier protein in mitochondrial membrane from hypothyroid rats. The quantity of functional adenine nucleotide carrier and that of pyruvate carrier was also found not to be changed by the hypothyroid state [6,9].

The decrease in the activity of the mitochondrial phosphate carrier in hypothyroid rats appears, therefore, to be a consequence of a modification of the lipid surrounding of the phosphate carrier molecule in the membrane, as proposed for other carrier-mediated processes [6,8,10,20,25,41,42]. The analysis of the mitochondrial membrane lipids (see Table III) reveals significant changes in the lipid composition in hypothyroid rats. Specifically, the total cholesterol and the cholesterol/phospholipid molar ratio increased and the PE/PC ratio decreased in mitochondrial membrane from hypothyroid rats. These changes are associated with an increase in membrane viscosity which, in turn, may reduce the mobility of the phosphate carrier molecule in the membrane and hence its functional activity.

The change in the membrane lipid composition may have a more specific effect on the activity of the phosphate carrier. In fact, it has been reported that cardiolipin, a phospholipid which is concentrated on the matrix side of the inner mitochondrial membrane, is required for full activity of the mitochondrial phosphate carrier, reconstituted in the liposomes [18–20]. A specific cardiolipin requirement has been demonstrated for other mitochondrial proteins and enzymes [43–44]. As reported in Table IV, the level of cardiolipin is markedly reduced (around 30%) in the mitochondrial membrane from hypothyroid rats. Thus, the lower content of cardiolipin could be directly responsible for the reduced activity of the mitochondrial phosphate carrier in the hypothyroid rats.

The transport of phosphate may be involved in regulating the supply of phosphate from the cytosol to the mitochondrial matrix for the reactions of oxidative phosphorylation. In addition, the transport of phosphate in mitochondria is closely linked to that of ADP and Ca^{2+} and is an obligatory step for the uptake of important anionic substrates such as malate, citrate and oxoglutarate, essential for the normal functioning of several cytosolic and mitochondrial metabolic pathways. A derangement of oxidative phosphorylation in intact liver mitochondria of hypothyroid rats has been reported [6,12,47]. An altered activity of the adenine nucleotide translocator has been reported to be, at least in part, responsible for such derangement. The depressed translocating activity of the phosphate carrier in hepatic mitochondria from hypothyroid rats, as found in the present study, may represent an additional factor responsible for the lower rate of oxidative phosphorylation and for the decrement in energy metabolism in hypothyroid animals.

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