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Decreased Activity of the Pyruvate Translocator and Changes in the Lipid Composition in Heart Mitochondria from Hypothyroid Rats

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A study of the transport of pyruvate in heart mitochondria from normal and hypothyroid rats has been carried out. Heart mitochondria from hypothyroid rats translocate pyruvate via the α-cyanocinnamate sensitive carrier much more slowly than do mitochondria from normal rats. Kinetic analysis of the pyruvate transport shows that the V_{max} of this process is decreased while there is practically no change in the K_m values. Neither a decrease in the transmembrane ΔpH value nor a decrease in the total number of the pyruvate carrier molecules, titrated with labeled α-cyanocinnamate, account for the decreased rate of pyruvate transport. The lower activity of the pyruvate translocator in mitochondria from hypothyroid rats is associated with a parallel decrease of the rate of pyruvate supported oxygen uptake. There is, however, no difference in either the respiratory control ratios or in the ADP/O ratios between these two types of mitochondria. The heart mitochondrial lipid composition is significantly altered in hypothyroid rats. Cardiolipin, particularly, was found to decrease by around 36%. In addition the pattern of fatty acids was found to be altered in mitochondrial membranes from hypothyroid rats. It is suggested that the decreased activity of the pyruvate translocator in heart mitochondria from hypothyroid rats can be ascribed to changes in the lipid environment which surrounds the pyruvate carrier molecule in the mitochondrial membrane.

Thyroid hormones have been recognized as one of the major factors involved in the regulation of cardiac function. Hyperthyroidism causes tachicardia, whereas hypothyroidism induces bradicardia. In hypothyroidism the rate of oxidation of many substrates is decreased compared with that found in mitochondria from normal rats (1, 2). Since most substrate oxidation takes place in mitochondria, many studies on the mechanism of action of thyroid hormones have involved these organelles (for review see Ref. (3)).

The transport of substrates across the mitochondrial membrane is an important point in the regulation of the mitochondrial energy metabolism. Thyroid hormones have been shown to influence the transport of substrates in mitochondria (4-8). The activity of the adenine nucleotide translocase has been shown to increase in the mitochondria from hyperthyroid rats (4) and decrease in the mitochondria from hypothyroid rats (6, 7).

Pyruvate is an important fuel for energy in the heart. The transport of this substrate in mitochondria is mediated by a specific transporting system (9, 10). The kinetic properties, substrate specificity, and sensitivity to specific inhibitors of the pyruvate carrier have been studied in detail (11-15). Several molecular aspects of this system have also been elucidated (16-18) and more recently purification of

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the pyruvate carrier molecule has been achieved (19).

The kinetic parameters of the pyruvate carrier have been observed to change in mitochondria isolated from animals under different metabolic conditions such as hormone treatment (20, 21) or in pathological conditions such as diabetes (22) or in different tumor states (23, 24).

In the present work a comparative study of the transport of pyruvate in heart mitochondria from normal and hypothyroid rats has been carried out. The results obtained indicate that the transport of pyruvate and the pyruvate supported oxygen uptake are both depressed in mitochondria isolated from hypothyroid rats as compared with mitochondria isolated from normal rats.

MATERIALS AND METHODS

Chemicals. The radioactive [2-^{14}C]pyruvate, [^{14}C]-acetate, and 6,6'-[^{3}H]sucrose, were obtained from the Radiochemical Centre, Amersham. Radioactive pyruvate was treated as follows. It was dissolved in water, divided into 5 μCi samples, freeze-dried, and stored in sealed tubes at −20°C. α-Cyanocarboxyl[^{14}C]cinnamic acid was synthesized at the Radiochemical Centre. Its specific activity was 19.2 mCi/mmol and its purity was 98%. 5,5-Dimethyl[^{14}C]oxazoline-2-4-dione was obtained from New England Nuclear. All other reagents were of reagent grade purity and 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 PTU[2] (0.05%, w/v) to the drinking water for 3–4 weeks (25). The treatment with PTU resulted in a decrease in the level of circulating T_4 from 55.5 ± 2.1 pmol/ml (n = 5) to 4.7 ± 0.6 pmol/ml (n = 4).

Rat heart mitochondria were prepared essentially as described in Ref. (16). Mitochondria were resuspended in 0.25 M sucrose, 5 mM Tris–HCl, pH 7.0, and stored in ice.

Protein concentration was measured by the usual biuret method using bovine serum albumin as standard.

The standard medium used in the measurements of respiratory activity, binding experiments, ΔpH measurements, and substrate transport, contained the following basic reagents: 100 mM sucrose, 50 mM KCl, 20 mM Tris-HCl, 1 mM $MgCl_2$, and 0.5 mM EDTA.

Measurements of respiration. Rates of oxygen consumption by mitochondria were measured in a thermostatically controlled oxygraph vessel with a Clark-type electrode (YSI Model 53, oxygen monitor, Yellow Springs Instrument Co., Yellow Springs, OH).

Measurements of binding. The binding of α-cyano[^{14}C]cinnamate to mitochondria was assayed essentially as described in (16). Mitochondria (0.8–1.2 mg mitochondrial protein/ml) were incubated at 25°C in the standard reaction medium described above. After 3 min of preincubation labeled α-cyanocinnamate was added and 5 min later the reaction was terminated by rapid centrifugation of the mitochondria suspension in the cold at 15,000g. The supernatant was decanted and the pellet rinsed with 15% (w/v) $HClO_4$. The binding was determined by measuring the radioactivity in the sediment and in the supernatant by liquid scintillation counting. The radioactivity of the pellet was corrected for the α-cyanocinnamate in the extramitochondrial space. The latter was determined in separate samples by the distribution of [^{3}H]sucrose.

Pyruvate transport. The rate of pyruvate transport by mitochondria was measured by the stop inhibitor method, using α-cyanocinnamate as inhibitor (10). The experiments were conducted in plastic centrifuge tubes (1.5 ml capacity). Each reaction mixture contained in 1 ml of the reaction medium described above: 0.5 mM sodium arsenite, 5 μg/ml rotenone, 0.5 μg/ml antimycin, 3 mM ascorbic acid, 0.05 mM TMPD, and 0.8–1.2 mg of mitochondrial protein. Final pH 6.8; T, 10°C. After 3 min of preincubation of mitochondria, radiolabeled pyruvate was added and 30 s later the reaction was stopped by the addition of 1 mM α-cyanocinnamate. The 30-s time point was used to calculate the kinetic constants in that pyruvate uptake was linear for the first 60 s. Immediately after the addition of the inhibitor, the tubes were rapidly centrifuged at 20,000g for 1 min. The pellets were washed with 0.25 M sucrose and acidified with 15% (w/v) $HClO_4$. The centrifugation of the mitochondrial pellets and all the subsequent operations of washing the pellets were made at 4°C to avoid pyruvate metabolism. Both radioactive and enzymatic assays of pyruvate gave identical results. The vials were then recentrifuged. Solubilized mitochondria were transferred to a 10-ml scintillation counter. The amount of radiolabeled pyruvate associated with the mitochondria was calculated from the amount of radioactivity in the mitochondrial pellet and the specific activity of the [^{14}C]pyruvate. The amount of [^{14}C]pyruvate present in the fluid outside the matrix or absorbed to the mitochondria, was estimated in reactions in which α-

[2] Abbreviations used: α-CC, α-cyanocinnamate; DMO, 5,5-dimethyloxazoline-2,4-dione; TMPD, tetramethylphenylenediamine; PTU, 6-n-propyl-2-thiouracil; T_3, L-3,3',5-triiodothyronine; T_4, L-3,3',5,5'-tetraiodothyronine; FCCP, carbonylcyanide-p-trifluoromethoxyphenylhydrazone.

TABLE I

KINETIC PARAMETERS OF PYRUVATE UPTAKE IN MITOCHONDRIA ISOLATED FROM NORMAL AND HYPOTHYROID RATS

Animals	K_m (μM)	V_{max} (nmol/min/mg of protein)
Normal	214 ± 19	6.78 ± 0.59
Hypothyroid	220 ± 21	4.12 ± 0.45[a]

Note. The rate of pyruvate uptake by mitochondria was measured essentially as described under Materials and Methods. The K_m and V_{max} values were calculated from double-reciprocal plots of the rates of pyruvate uptake versus pyruvate concentrations (ranging from 0.05 to 1 mM). Each value represents the mean ± SE obtained for six experiments. Each experiment consisted of two preparations of heart mitochondria isolated from eight hypothyroid and eight control rats.

[a] $P < 0.001$.

cyanocinnamate was added before [^{14}C]pyruvate. The difference between the amount of pyruvate associated with mitochondria in the absence of α-cyanocinnamate and that associated with the organelles in the reactions in which the inhibitor was added before the radioactive pyruvate, was defined as [^{14}C]pyruvate uptake.

Transmembrane ΔpH measurements. The transmembrane ΔpH was measured as follows. Mitochondria (0.8–1.2 mg of protein) were preincubated in the standard medium described above plus 0.05 mM TMPD and 3 mM ascorbate. Final pH 6.8; T, 10°C. After 3 min of preincubation 15 μM radiolabeled DMO was added and 3 min later, mitochondria were separated from the medium by rapid centrifugation. The external pH was determined potentiometrically on the supernatant obtained after centrifugation of the mitochondrial suspension. The intramitochondrial pH (matrix space) was calculated on the basis of the distribution of [^{14}C]DMO between the matrix space and the medium by the equation of Addanki *et al.* ((26); see also Ref. (9)).

High-pressure liquid chromatography analysis of phospholipids and fatty acids. Phospholipids and fatty acids were analyzed by HPLC, using a Beckman 344 gradient liquid chromatograph. Lipids from heart mitochondria were extracted with chloroform/methanol by the procedure of Bligh and Dyer (27). Phospholipids were separated by the HPLC method previously described (28, 29) with an Altex Ultra-Si column (4.6 × 250 mm). The chromatographic system was programmed for gradient elution using two mobile phases: solvent A, hexane/2-propanol (6/8, v/v) and solvent B, hexane/2-propanol/water (6/8/1.4, v/v/v). The percentage of solvent B in solvent A was increased in 15 min from 0 to 100%. Flow rate was 2 ml/min and detection was at 206 nm.

To analyze fatty acids, heart mitochondria were saponified with 5% KOH in 50% aqueous methanol for 40 min at 90°C. After acidification, the solution was first extracted with chloroform, next dried, and then esterified with *m*-methoxyphenacylbromide for HPLC analysis (30). For this analysis, the column was an Altex Ultrasphere-ODS reverse phase (4.6 × 250 mm). The mobile phase was tetrahydrofuran:acetonitrile:water (45/25/35, v/v/v) at a flow rate of 2 ml/min.

RESULTS

The kinetic constants of the pyruvate transport in mitochondria from normal and hypothyroid rats, obtained from six different experiments, are reported in Table I. Note that while the affinity of the carrier for pyruvate is practically the same in these two types of mitochondria, the maximal velocity of the pyruvate uptake is significantly depressed (around 40%) in mitochondria from hypothyroid rats when compared with that obtained in mitochondria from control rats, the V_{max} values being, respectively, 4.12 ± 0.38 and 6.78 ± 0.59 nmol/min/mg of mitochondrial protein.

The uptake of pyruvate by mitochondria is very sensitive to the transmembrane ΔpH (9, 10). Thus changes in the mitochondrial pH gradient may, in principle, account for the change in the pyruvate uptake. The transmembrane ΔpH gradient was measured by the distribution of DMO (26). The results reported in Table II show

TABLE II

TRANSMEMBRANE ΔpH VALUES IN HEART MITOCHONDRIA FROM NORMAL AND HYPOTHYROID RATS

Animals	ΔpH ($pH_{in} - pH_{out}$)
Normal	0.88 ± 0.06
Hypothyroid	0.87 ± 0.07

Note. The transmembrane ΔpH values in mitochondria were determined as described under Materials and Methods. Each value represents the mean ± SE obtained for four experiments with five rats each.

that there is no change in the transmembrane ΔpH values in mitochondria from normal and hypothyroid rats. In addition, in conditions under which the uptake of pyruvate was decreased by around 40% in mitochondria from hypothyroid rats, no change in the uptake of acetate, an anion which enters mitochondria as free acid independently of pyruvate carrier (9), was observed (results not shown).

The reduced activity of the pyruvate translocator in mitochondria from hypothyroid rats may also be a consequence of a decrease of the pyruvate carrier molecules in the mitochondrial membrane, induced by the hypothyroid state. In order to verify this, the pyruvate carrier molecules were titrated with labeled α-cyanocinnamate. In fact, as previously shown (16), α-cyanocinnamate can be used as a molecular marker of the pyruvate carrier in that its binding to heart mitochondria parallels the inhibition of pyruvate transport by the same inhibitor.

A representative experiment of the binding of radioactive α-cyanocinnamate to mitochondria from normal and hypothyroid rats is reported in Fig. 1. Note that the binding curves of α-cyanocinnamate are practically similar in these two types of mitochondria. Scatchard plots of these binding data, obtained from six different experiments, gave the same total number of binding sites (48 ± 5 pmol/mg of mitochondrial protein) and the same value for the apparent dissociation constants (K_d = 0.098 ± 0.012 μM).

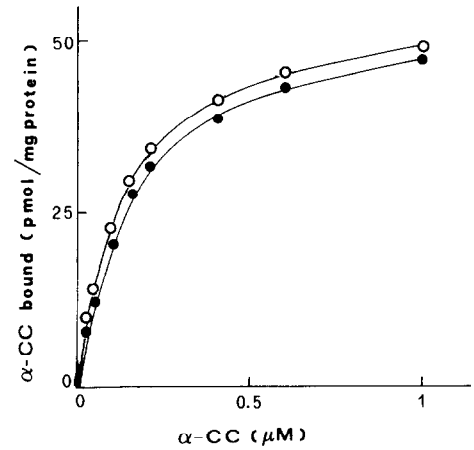

FIG. 1. Titration of α-cyanocinnamate binding sites in mitochondria from normal and hypothyroid rats. Mitochondria (1 mg of protein/ml) were preincubated in the standard reaction medium described under Materials and Methods. Final pH 7.0; *T*, 25°C. After 3 min of preincubation increasing concentrations of labeled α-cyanocinnamate were added and 5 min later mitochondria were separated from the medium by rapid centrifugation. The binding of α-cyanocinnamate was determined as described in (16). (○) Mitochondria from normal rats; (●) mitochondria from hypothyroid rats.

It has been demonstrated that the transport of pyruvate in heart mitochondria is the rate-limiting step for pyruvate oxidation (31). Thus, changes in the rate of pyruvate transport can be associated with parallel changes in the rate of pyruvate-dependent oxygen consumption. The results reported in Table III show that the rate of pyruvate supported oxygen uptake in mitochondria from hypothyroid rats is significantly depressed (around 32%) as compared with that obtained with mitochondria from control rats. Similar results were obtained when an uncoupler (FCCP) was used instead of ADP to stimulate pyruvate oxidation (not shown). No such decrement was observed when pyruvate was used at higher concentration (2.5 mM) to make the carrier less limiting. Neither the respiratory control ratio nor the ADP/O ratio were altered in these two types of mitochondria (see also Refs. (32, 33)).

Thyroid hormones have been shown to alter the lipid composition of the mitochondrial membrane (34, 35). On the other hand the activity of several anion transporting proteins is influenced by the lipid composition of the inner mitochondrial membrane (6, 36, 37). We have therefore analyzed the lipid composition of heart mitochondrial membrane from normal and hypothyroid rats.

A typical mitochondrial phospholipid separation carried out by our modified HPLC method is reported in Fig. 2. The order of elution in this system is neutral lipids (as a single peak), cardiolipin, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and phosphatidylcholine. The gradient separation we used has

TABLE III

THE RATES OF PYRUVATE-DEPENDENT OXYGEN UPTAKE IN HEART MITOCHONDRIA FROM NORMAL AND HYPOTHYROID RATS

Animals	Pyruvate oxidation (ng-atom O/min/mg protein)	Respiratory control ratio	ADP/O
Normal	205 ± 18	9.85 ± 0.92	2.83 ± 0.21
Hypothyroid	140 ± 15[a]	9.78 ± 0.95	2.81 ± 0.24

Note. The pyruvate-dependent oxygen uptake was measured with a Clark-type electrode. Mitochondria (0.8-1.2 mg of protein/ml) were preincubated in the standard reaction medium described under Materials and Methods. Final pH 7.2; *T*, 25°C. When a steady state of oxygen consumption was obtained, 0.5 mM pyruvate was added. One minute later respiration was stimulated by the addition of 2 mM ADP. For the determination of the RCR and ADP/O values pyruvate was added at a concentration of 2.5 mM together with 2.5 mM malate. Each value represents the mean ± SE obtained for seven experiments with six rats each.

[a] $P < 0.01$.

been found to be successful in separating five phospholipid components.

The analysis of the phospholipid composition of heart mitochondrial membrane is significantly different in hypothyroid rats as compared with normal rats (see Table IV). In particular, cardiolipin and phosphatidylethanolamine were found to decrease whereas phosphatidylinositol, phosphatidylserine, and phosphatidylcholine were found to increase in mitochondrial membrane from hypothyroid rats.

Alterations of the fatty acid distribution were also observed in mitochondria membrane from hypothyroid rats (see Table V).

DISCUSSION

The present study shows that the rate of pyruvate transport in heart mitochondria from hypothyroid rats is significantly decreased as compared to that obtained from mitochondria of normal rats. The transport of pyruvate in mitochondria is driven by the transmembrane ΔpH. Thus, a decrease in the mitochondrial pH gradient could, in principle, account for the depressed rate of pyruvate transport. However, as reported in Table II, no change in the transmembrane ΔpH values was observed in mitochondria from normal and hypothyroid rats.

The analysis of the kinetic parameters of pyruvate transport, reported in Table I, indicates that the V_{max} of this process is depressed in mitochondria isolated from hypothyroid rats as compared with that obtained in mitochondria from normal rats, while the affinity of pyruvate for its carrier remains unchanged in both these types of mitochondria. These observations can be interpreted to indicate that while the nature of the pyruvate carrier appears unaffected, the number of carrier molecules is lower in mitochondria from hypothyroid rats. However, as reported in Fig. 1, the number of specific α-cyanocinnamate binding sites, which expresses quantitatively the total number of the pyruvate carrier molecules (16), is not changed in mitochondria from hypothyroid rats when compared with mitochondria from normal rats. Thus, the reduced V_{max} value for the pyruvate transport observed in mitochondria from hypothyroid rats cannot be due to a decrease in the total number of pyruvate carrier molecules. Rather it can be ascribed to a change in the turnover of the pyruvate carrier.

The transport of pyruvate in heart mitochondria is rate limiting for pyruvate oxidation (31). The data reported in Table III show that the rate of pyruvate-dependent oxygen uptake decreases in mitochondria from hypothyroid rats. This decrease in the pyruvate oxidation is well correlated with the decrease of the pyruvate transport. The different temperature at which these two functions were measured precludes a direct quantitative comparison.

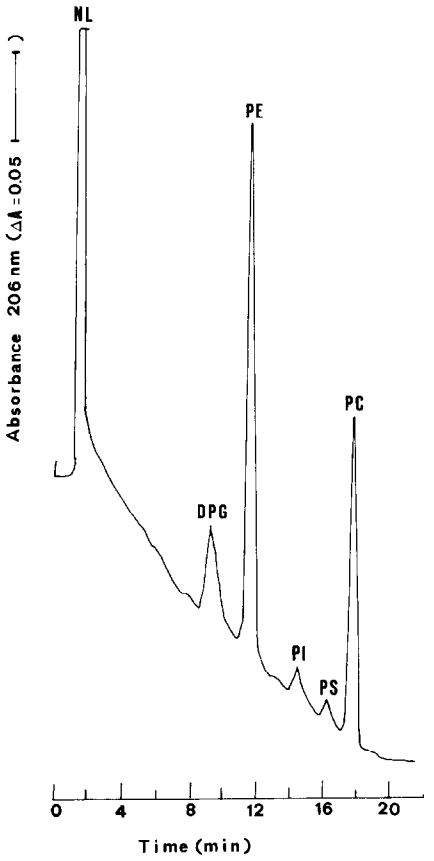

FIG. 2. Chromatogram of phospholipid extract from heart mitochondria of normal rats. NL, neutral lipids; DPG, cardiolipin; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine. The chromatographic system was programmed for gradient elution using two mobile phases: solvent A, hexane:2-propanol (6:8, v/v) and solvent B, hexane:2-propanol:water (6:8:1.4, v/v/v). The percentage of solvent B in solvent A was increased in 15 min from 0 to 100%. Flow rate was 2 ml/min and detection was at 206 nm. Chart speed 30 cm/h.

No difference in the respiratory control ratio or in the ADP/O ratio in mitochondria from normal or hypothyroid rats was detected (see also Refs. (32, 33)). This indicates that the hypothyroid state affects the pyruvate transport and the pyruvate-dependent oxygen respiration without affecting the efficiency of the mitochondrial respiratory functions.

It should be noted that the lower values for the rates of pyruvate transport and pyruvate oxidation observed in mitochondria from hypothyroid rats were both completely restored to normal levels by treat-

TABLE IV

PHOSPHOLIPID COMPOSITION IN RAT HEART MITOCHONDRIA AS DETERMINED BY HPLC

Phospholipid	Distribution (mol %) Normal	Hypothyroid
Cardiolipin	13.0 ± 1.0	8.3 ± 0.9[a]
Phosphatidylethanolamine	41.9 ± 1.6	36.4 ± 1.5[a]
Phosphatidylinositol	1.4 ± 0.2	1.9 ± 0.2[b]
Phosphatidylserine	2.5 ± 0.7	3.0 ± 0.5
Phosphatidylcholine	41.2 ± 2.0	50.4 ± 1.7[a]

Note. For phospholipid extraction and analysis, see Materials and Methods. Each value represents the mean obtained for six experiments with six rats each ± SE.

[a] $P < 0.01$ vs normal.

[b] $P < 0.02$ vs normal.

TABLE V

PATTERN OF FATTY ACIDS IN RAT HEART MITOCHONDRIA AS DETERMINED BY HPLC

Fatty acid	Distribution (mol %) Normal	Hypothyroid
16:0	14.9 ± 0.9	17.6 ± 0.8[a]
16:1	5.7 ± 0.4	5.4 ± 0.5
18:0	17.4 ± 0.6	16.0 ± 0.5[b]
18:1	8.1 ± 0.7	7.7 ± 0.6
18:2	27.2 ± 1.2	24.6 ± 1.0[a]
20:3	2.5 ± 0.3	2.7 ± 0.4
20:4	23.4 ± 0.8	25.1 ± 0.7[b]
22:6	0.8 ± 0.04	0.9 ± 0.05
U.I.	174.1 ± 2.4	176.2 ± 2.5
20:4/18:2	0.86 ± 0.07	1.02 ± 0.07[b]

Note. Extraction and analysis of fatty acids were carried out as described under Materials and Methods. Each value represents the mean obtained for six experiments with six rats each ± SE. The unsaturation index (U.I.) is defined as Σ mol% of each fatty acid × number of double bonds of the same fatty acid.

[a] $P < 0.01$ vs normal.

[b] $P < 0.02$ vs normal.

ing the hypothyroid animals with L-3,3',5-T_3 (25 μg/100 g body weight) injected intraperitoneally for 2 days (results not shown).

Thyroid hormones have been shown to alter the lipid composition of mitochondrial membrane (6, 34, 35). On the other hand the lipid composition of the inner mitochondrial membrane appears to influence the activity of anion transporting proteins. For example, it has been shown that the activity of the adenine nucleotide translocator is stimulated in mitochondria from hyperthyroid rats (4, 38) and depressed in mitochondria from hypothyroid rats (5, 7). These changes have been associated with modifications in the physicochemical characteristics of the inner membrane lipids. It has been shown that phospholipids are required for isolation and reconstitution of the activity of certain mitochondrial anion carrier protein. Cardiolipin, in particular, appears to be specifically required for isolation and reconstitution in the liposomes of the transport activity of the phosphate (39, 40) and tricarboxylate carrier (41). More recently, the reconstituted transport activity of the isolated pyruvate carrier in the liposomes has also been shown to be dependent on the presence of cardiolipin and on a more or less defined lipid environment (19). If this *in vitro* requirement of phospholipids reflects somehow the *in vivo* situation, then the analysis of the mitochondrial membrane lipids may throw light on the molecular mechanism underlying the depressed activity of the pyruvate translocase observed in mitochondria from hypothyroid rats. The data reported in Tables IV and V document significant alterations in the phospholipid and fatty acid compositions of heart mitochondrial membrane from hypothyroid rats. Cardiolipin level, in particular, was reduced by 36% in mitochondria from hypothyroid rats.

It can therefore be proposed that one of the possible factors responsible for the decreased activity of the pyruvate carrier in mitochondria from hypothyroid rats is the modification of the lipid environment surrounding the carrier molecule. This modification would lead in turn to a change in the mobility of the carrier molecule in the membrane.

Pyruvate, together with free fatty acids, is the major energy source in the heart. Thus, the decreased activity of the pyruvate translocator observed in mitochondria from hypothyroid rats may account, at least in part, for the lower energy metabolism of the heart in hypothyroid animals.

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