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Effect of hyperthyroidism on the transport of pyruvate in rat-heart mitochondria

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A comparative study of the transport of pyruvate in heart mitochondria from normal and triiodothyronine-treated rats has been carried out. It has been found that the rate of carrier-mediated (α-cyanocinnamate-sensitive) pyruvate uptake is significantly enhanced in mitochondria from triiodothyronine-treated rats as compared with mitochondria from control rats. The kinetic parameters of the pyruvate uptake indicate that only the V_{max} of this process is enhanced whilst there is no change in the K_m value. The enhanced rate of pyruvate uptake is not dependent on the increase of the transmembrane ΔpH value (both mitochondria from normal and triiodothyronine-treated rats exhibit the same ΔpH value) neither does it depend on the increase of the pyruvate carrier molecules (titration of these last with α-cyanocinnamate gives the same total number of binding sites). The pyruvate-dependent oxygen uptake is stimulated by 35–40% in mitochondria from hyperthyroid rats when compared with mitochondria from control rats. 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 phospholipid composition is altered significantly in hyperthyroid rats; in particular, negatively charged phospholipid such as cardiolipin and phosphatidylserine were found to increase by more than 50%. Minor alterations were found in the pattern of fatty acids with an increase of 20:4/18:2 ratio. It is suggested that the changes in the kinetic parameters of pyruvate transport in mitochondria from hyperthyroid rats involve hormone-mediated changes in the lipid composition of the mitochondrial membranes which in turn modulate the activity of the pyruvate carrier.

Introduction

It has been well established that the transport of pyruvate across the mitochondrial membrane is mediated by a specific transporting system which brings about both a pyruvate/pyruvate exchange and a net movement of the anion across the inner membrane, the latter process being accompanied by the cotransport of H^+ (or counter transport of hydroxyl ions [1–3]. The kinetic properties, substrate specificity and sensitivity to specific inhibitor of the pyruvate carrier have been studied in detail [4–10]. Several molecular aspects of this system have also been elucidated by following the binding of radiolabelled α-cyanocinnamate to mitochondria [11,12]. Recently partial purification of the pyruvate carrier molecule from bovine-heart mitochondria has been achieved [13].

Abbreviations: α-CC, α-cyanocinnamate; EDTA, ethylenediaminetetracetic acid; Tris, tris(hydroxylmethyl)aminomethane; DMO, 5,5-dimethyloxazoline-2,4-dione; TMPD, tetramethylphenylenediamine; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone.

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It has long been known that an increase in metabolic rate follows administration of sufficient dose of thyroid hormone. Animals treated with large doses of thyroxine manifest an increased rate of oxygen consumption, a rise in body temperature and a loss in weight, all consequent to an increase in the rate of oxidation of substrates [14]. Since most substrate oxidation takes place in mitochondria, many studies on the mechanism of action of thyroid hormone have involved these organelles (for a review, see Ref. 15).

A crucial point in the regulation of mitochondrial energy metabolism is represented by the transport of metabolites across the mitochondrial membrane. Thyroid hormones have been shown to influence anion transport across the mitochondrial membrane [16–20]. The activity of adenine nucleotide translocase has been shown to be stimulated in mitochondria from hyperthyroid rats [16] and decreased in mitochondria from thyroidectomized rats [18].

The kinetic parameters of the pyruvate-transporting system have been observed to change in conjunction with different metabolic situations such as hormone treatment [21–23] or several pathological situations such as diabetes [24] or different tumour states [25,26].

Hyperthyroidism has a profound effect on cardiac performance. As pyruvate plays a central role in energy metabolism, the possibility that thyroid hormone may influence the metabolism of pyruvate at the level of the mitochondrial pyruvate translocation has been considered. We have therefore undertaken a comparative study of the transport of pyruvate in heart mitochondria from normal and triiodothyronine treated rats.

The results obtained indicate that the transport of pyruvate and the pyruvate-dependent oxygen uptake are both stimulated in mitochondria from hyperthyroid rats as compared with mitochondria isolated from control 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 in the Radiochemical Centre, Amersham. 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 ± 1 °C, were used throughout these studies. Animals were made hyperthyroid using 3,3′,5-triiodo-L-thyronine (30 μg per 100 g body weight) dissolved in 0.9 NaCl/propyleneglycol (40 : 60, v/v) which was injected intraperitoneally with a single daily injection for 5 consecutive days [27]. In the same way control animals received only the solvent for the same period. The drug dose and the treatment duration were chosen to obtain a variation of the haematic triiodothyronine level without significantly changing the body weight of the animals. Animals were killed 24 h after final administration.

Rat-heart mitochondria were prepared as described in Ref. 11. Mitochondria were resuspended in 0.25 M sucrose, with protein being determined by the usual biuret method.

The standard medium used in the measurements of respiratory activity, binding experiment and anion transport usually contained 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 as previously described [11,12].

Measurements of binding. The binding of α-cyanocinnamate to mitochondria was assayed essentially as described in Refs. 11 and 12.

Pyruvate transport. The initial rate of pyruvate transport by mitochondria was measured at 10 °C by the Halestrap inhibitor stop method, using α-cyanocinnamate as inhibitor [2]. The reactions 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. After 3 min of preincubation of mitochondria, radiolabelled pyruvate was added and at appropriate

times to be described in the legends to figures, the reaction was stopped by the addition of 1 mM α-cyanocinnamate. The tubes were rapidly centrifuged at 20000 × *g* for 5 min. The pellets were washed with 0.25 M sucrose and dissolved in $HClO_4$. The centrifugation of mitochondrial pellets and all the subsequent operations of washing of the pellets were made at 4° C to avoid pyruvate metabolism. Both radioactive and enzymic assays of pyruvate gave similar results. The vials were then recentrifuged. Solubilized mitochondria were transferred to 10 ml scintillation counter. The amount of radiolabelled pyruvate, expressed as nmol per mg of mitochondrial protein, 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-cyanocinnamate was added before [^{14}C]pyruvate. The difference between the amount of pyruvate associated with the 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.

pH measurements. 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 5,5-dimethyl[^{14}C]oxazoline-2,4-dione between the matrix space and the medium by the equation of Addanki et al. (Ref. 28, see also Ref. 1).

High pressure liquid chromatography (HPLC) analysis of phospholipids and fatty acid. 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 [29]. Phospholipids were separated by the HPLC method previously described [27] with an Altex ultrasil-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 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

Pyruvate translocator kinetics

A representative experiment of the pyruvate translocase activity of heart mitochondria from normal and from triiodothyronine-treated rats, assayed by monitoring uptake of [^{14}C]pyruvate is shown in Fig. 1. At 10° C and external pyruvate concentration of 250 μM, uptake of [^{14}C]pyruvate

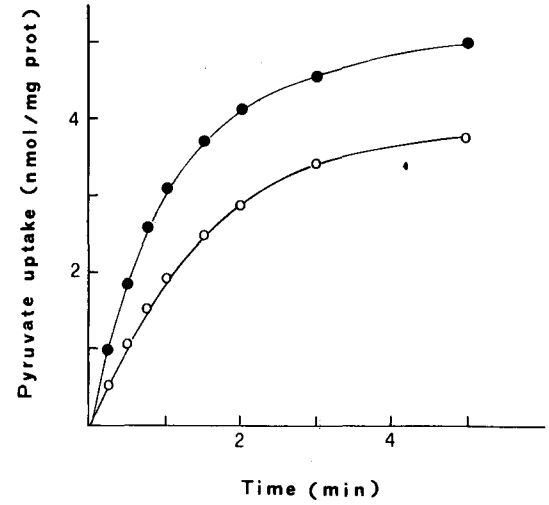

Fig. 1. Time-course of [^{14}C]pyruvate uptake by mitochondria from normal and hyperthyroid rats. The rate of pyruvate uptake was followed as described in Materials and Methods. Mitochondria (1 mg protein (prot.)/ml) were added to the reaction medium described under Materials and Methods. Final pH 7.0. After 3 min of preincubation 250 μM [^{14}C]pyruvate was added. 1 mM α-cyanocinnamate was added after various times to stop the reaction. ○, mitochondria from normal rats; ●, mitochondria from hyperthyroid rats.

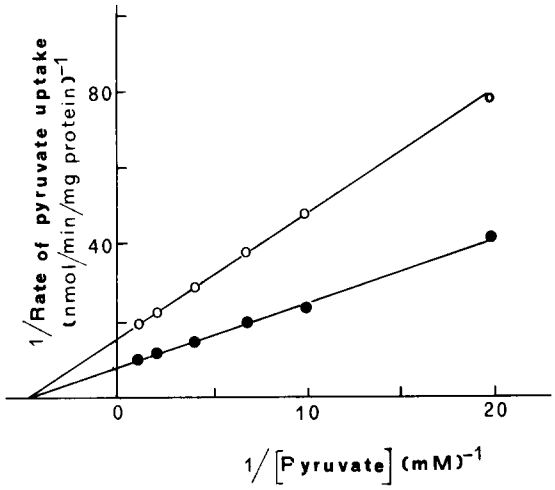

Fig. 2. Double reciprocal plots of pyruvate uptake by heart mitochondria from normal and hyperthyroid rats. The rate of pyruvate uptake was followed as described under Materials and Methods and in the legend to Fig. 1. Mitochondria (1 mg protein/ml) were added to the reaction medium described in Materials and Methods. After 3 min of preincubation, [^{14}C]pyruvate, at the concentrations indicated in the figure, was added. 30 s later 1 mM α-cyanocinnamate was added to stop the reaction. ○, mitochondria from normal rats; ●, mitochondria from hyperthyroid rats.

followed first-order kinetics for the first 45–60 s. When the pyruvate carrier inhibitor α-cyanocinnamate was added to the incubations before starting the assay with labelled pyruvate, the amount of pyruvate bound was the same in the two preparations of mitochondria. This indicates that the triiodothyronine treatment was without effect on non-specific pyruvate binding. In contrast at each subsequent time, pyruvate uptake by mitochondria from triiodothyronine-treated rats was significantly higher than the controls. This clearly demonstrates that the rate of pyruvate uptake in mitochondria from hyperthyroid rats is stimulated as compared with mitochondria from untreated rats.

Fig. 2 illustrates the kinetic of pyruvate uptake in mitochondria isolated from controls and hyperthyroid rats. Double reciprocal plots of net pyruvate uptake followed saturation kinetics in both types of mitochondria. It can be noted, however, that while the affinity of the carrier for pyruvate remained practically the same, the maximal velocity of the pyruvate uptake was significantly increased in mitochondria from triiodothyronine treated rats. The statistical evaluation of the kinetic parameters of pyruvate uptake in heart mitochondria from normal and hyperthyroid rats, obtained from six experiments, gave the following results; $K_m = 210 \pm 14$ and 208 ± 12 μM and $V_{max} = 6.7 \pm 0.42$ and 11.3 ± 0.98 nmol/min per mg of mitochondrial protein in mitochondria from control and hyperthyroid rats, respectively.

Transmembrane Δ pH in mitochondria

It has been well established that the transport of pyruvate by mitochondria is driven by the transmembrane ΔpH [1,2]. Thus the enhancement of pyruvate uptake by mitochondria from hyperthyroid rats may, in principle, be due to an increase of the mitochondrial transmembrane ΔpH. In order to verify this possibility, a direct measurement of the transmembrane ΔpH, monitored by the distribution of DMO (5,5-dimethyloxazoline-2,4-dione), was made. The results obtained showed that there was no change in the transmembrane ΔpH values in mitochondria from normal and hyperthyroid rats; the ΔpH values were 0.88 ± 0.05 and 0.89 ± 0.04 (mean ± S.E.M. for 6 experiments), respectively.

Titration of the α-cyanocinnamate binding-sites in heart mitochondria

As stated above, the enhancement of pyruvate transport in mitochondria from triiodothyronine-treated rats is characterized by an apparent increase in the V_{max} value. This may reflect an increase in either the number or the mobility of the pyruvate carrier molecule in the mitochondrial membrane. The pyruvate carrier had to be specifically labelled in order to verify whether their number increased in mitochondria from hyperthyroid rats. α-Cyanocinnamate was used as molecular marker of pyruvate carrier molecules, since it has been previously demonstrated that the binding of labelled α-cyanocinnamate to heart mitochondria parallels the inhibition of pyruvate transport by this inhibitor [11,12]. Fig. 3 shows the results of a representative experiment of the binding of radioactive α-cyanocinnamate by mitochondria from normal and triiodothyronine-treated rats. It should be noted that the binding curves of α-cyanocinnamate are practically similar in these

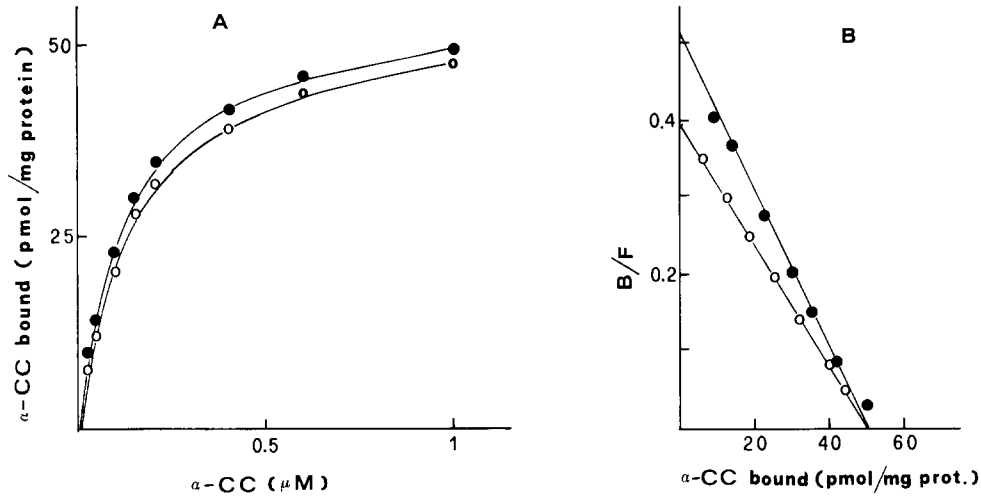

Fig. 3. Titration of α-cyanocinnamate binding sites in mitochondria from normal and hyperthyroid rats. (A) Mitochondria (1 mg protein (prot.) per ml) were preincubated in the standard reaction medium described in Materials and Methods. Final pH 7.0; *T*, 25 °C. After 3 min of preincubation increasing concentrations of labelled α-cyanocinnamate (α-CC) were added and 3 min later mitochondria were separated from the medium by rapid centrifugation. The binding of α-cyanocinnamate was determined as described in Ref. 11. ○, mitochondria from normal rats; ●, mitochondria from hyperthyroid rats. (B) Scatchard plots of the binding of α-cyanocinnamate. Data from Fig. 1A.

two types of mitochondria. Scatchard plots of these binding data give the same total number of binding sites (48.4 ± pmol per mg mitochondrial protein) in both these types of mitochondria, whilst there is a slight increase in the affinity for α-cyanocinnamate binding in mitochondria from hyperthyroid rats ($K_d = 0.096 \pm 0.012$ μM) with respect to mitochondria from normal rats ($K_d = 0.120 \pm 0.015$ μM).

Pyruvate oxidation

The transport of pyruvate in mitochondria can be followed as well by measuring the rate of pyruvate-dependent oxygen uptake in the presence of ADP. Under this experimental condition the transport of pyruvate by heart mitochondria has been shown to be a rate-limiting step for its oxidation [31]. We have also confirmed this result under our experimental conditions, by titrating

TABLE I

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

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 medium described in 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. 1 min later respiration was stimulated by the addition of 2 mM ADP. State-3 respiration refers to the rate of oxygen uptake in the presence of added ADP. State-4 respiration refers to the rate of oxygen uptake in the absence of added ADP. Each value represents the mean ± S.E.M. obtained for six experiments with five rats each.

Animals	Pyruvate oxidation (ng-atom O per min per mg protein)		Respiratory control ratio	ADP/O
	State 3	State 4		
Normal	202.1 ± 16.4	20.5 ± 1.6	9.71 ± 0.83	2.82 ± 0.18
Hyperthyroid	278.3 ± 21.6 [a]	28.6 ± 2.2 [a]	9.79 ± 0.85	2.84 ± 0.21

[a] $P < 0.01$.

TABLE II

PHOSPHOLIPID COMPOSITION IN RAT HEART MITOCHONDRIA AS DETERMINED BY HPLC

For phospholipid extraction and analysis, see the Materials and Methods section. Each value represents the mean ± S.E. obtained from six different experiments with five rats each.

Phospholipid	Distribution (mol%)	
	Normal	Hyperthyroid
Cardiolipin	12.6 ± 1.4	19.0 ± 1.0 [a]
Phosphatidylethanolamine	42.0 ± 1.9	37.9 ± 1.5 [c]
Phosphatidylinositol	1.9 ± 0.2	2.4 ± 0.2 [c]
Phosphatidylserine	2.1 ± 0.6	3.5 ± 0.4 [b]
Phosphatidylcholine	41.4 ± 1.2	37.2 ± 1.1 [b]

[a] $P < 0.001$.
[b] $P < 0.01$.
[c] $P < 0.02$.

the pyruvate dependent oxygen uptake with α-cyanocinnamate (not shown). The Dixon plot for α-cyanocinnamate was linear, indicating that pyruvate transport was rate limiting for pyruvate respiration. The results reported in Table I show that the rate of pyruvate-dependent oxygen uptake by mitochondria from hyperthyroid rats is increased by 38% when compared with mitochondria from control rats. Under identical experimental condition the rate of succinate-supported oxygen uptake was stimulated by only 20% (not shown). However, neither the respiratory control ratio nor the ADP/O ratio were altered in these two types of mitochondria.

TABLE III

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

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

Fatty acid	Distribution (mol%)	
	Normal	Hyperthyroid
16:0	16.4 ±0.8	14.8 ±0.6 [b]
16:1	5.7 ±0.5	6.2 ±0.6
18:0	18.3 ±0.9	21.3 ±1.0 [a]
18:1	8.8 ±0.6	10.2 ±0.7 [b]
18:2	25.7 ±1.3	21.1 ±1.0 [a]
20:3	3.1 ±0.4	4.7 ±0.6 [a]
20:4	22.0 ±1.2	21.7 ±1.3
U.I.	163.2 ±2.5	159.5 ±2.1
20:4/18:2	0.85 ± 0.07	1.03 ± 0.06 [a]

[a] $P < 0.01$.
[b] $P < 0.02$.

Mitochondrial phospholipids and fatty acids patterns

The lipid composition of the inner mitochondrial membrane appears to play a role in regulating the activity of anion transporting protein [32,33]. On the other hand thyroid hormones have been shown to alter the lipid composition of the mitochondrial membranes [34,35]. Phospholipid composition of heart mitochondria is significantly different in normal and triiodothyronine-treated rats (see Table II). In particular an increase was found in the negatively charged phospholipids such as cardiolipin, phosphatidylserine and phosphatidylinositol with a decrease of phosphatidylethanolamine and phosphatidylcholine. Chemical changes in membrane lipids were further investigated by analyzing the fatty acids composition in these mitochondrial membranes (see Table III). Alterations of fatty acids distribution were observed in mitochondrial membrane from hyperthyroid rats. In particular, there is an increase of stearic (18:0), oleic (18:1) and eicosatrienoic (20:3) acids with a decrease of palmitic (16:0) and linoleic (18:2). The desaturating activity, measured as the ratio of the unsaturated fatty acids, 20:4/18:2, is well above the control value.

Discussion

The results presented in this paper demonstrate that the transport of pyruvate by heart mitochondria from hyperthyroid rats is significantly increased when compared with mitochondria isolated from control rats. Kinetic analysis with radioisotopic tracer shows that the maximal velocity of pyruvate uptake is enhanced whilst there is no change in the affinity of pyruvate for its transporting system in these two types of mitochondria.

The driving force for pyruvate uptake, i.e., the mitochondrial transmembrane ΔpH [1,2], is practically the same in mitochondria from untreated

and hormone-treated rats. This rules out the possibility that the enhancement of pyruvate transport is simply due to an increase in the transmembrane ΔpH. This conclusion is further supported by the lack of stimulation of acetate uptake, an anion which moves across the mitochondrial membrane as function of ΔpH, independently of pyruvate carrier [1,2]. In fact, in condition under which the uptake of pyruvate was stimulated by 60% in mitochondria from triiodothyronine-treated rats, no stimulation of the uptake of acetate was observed (results not shown).

The number of specific α-cyanocinnamate binding-sites, which expresses quantitatively the number of pyruvate carrier molecules in the mitochondrial membranes [11,12], does not change in mitochondria from hyperthyroid rats as compared with mitochondria from control rats. This indicates that the increase in the V_{max} of pyruvate uptake in mitochondria from hormone treated rats, is not dependent on the increase in the total number of pyruvate carrier molecules.

Evidence has been presented that pyruvate transport is the rate-limiting step for pyruvate oxidation in rat-heart mitochondria [31]. We have found (see Table I) that the rate of pyruvate supported oxygen uptake is enhanced in mitochondria from hyperthyroid rats. Similar stimulation was obtained when the pyruvate-dependent oxygen uptake was followed with mitochondria incubated with an uncoupler (FCCP) rather coupled with ADP (results not reported). This indicates that the stimulation of pyruvate respiration observed in mitochondria from triiodothyronine-treated rats cannot simply be ascribed to the enhancement of mitochondrial ADP uptake [16]. The increase in the pyruvate dependent oxygen uptake is well correlated with the increase in the pyruvate transport. However, the difference in the temperature at which these two functions were measured precludes a direct quantitative comparison of their absolute velocities. It should be noted that neither the respiratory control ratio nor the ADP/O ratio (see also Refs. 36 and 37) were changed by triiodothyronine treatment. This indicates that, at doses given, this hormone administered in vivo increased both the pyruvate-dependent respiration and the pyruvate transport without affecting the efficiency of the mitochondrial respiratory functions. In addition, triiodothyronine, administered in vitro, had no effect on the rate of pyruvate uptake. Rather a slight decrease of this process was observed (results not shown).

Membrane fluidity is known to play a role in determining receptor activity, transport and enzyme activities in mammalian systems. Studies have been made which show that thyroid hormones can modulate this fluidity by changing the fatty acid composition of membrane lipids [18,38–41]. Lipids appear to be required for isolation, purification and reconstitution of the activity of certain mitochondrial anion-transporting proteins [13,33, 42–43]. Evidence concerning the role of phospholipids in the activity of adenine nucleotide translocator has been derived from reconstitution experiments with the isolated carrier protein [33]. The activity of the adenine nucleotide translocator is decreased in mitochondria from hyperthyroid rats. This decrease has been associated with changes in the physicochemical characteristics of lipid matrix of the inner membrane lipids [18].

A recent report in the literature has indicated that the transport activity of the isolated pyruvate carrier appears to be dependent on the presence of cardiolipin and on a more or less defined lipid environment [13]. The analysis of the mitochondrial membrane lipids (see Table II) shows that there is a significant increase (around 50%) of cardiolipin and other negatively charged phospholipids in the mitochondrial membrane from hyperthyroid rats when compared with mitochondrial membrane from control rats. This indicates an increase in lipid fluidity of the bulk phase of membrane lipids. It may therefore be suggested that one of the possible factors responsible for the enhancement of the activity of the pyruvate carrier in mitochondria from hyperthyroid rats is the change in the physicochemical characteristics of the lipid matrix of the inner mitochondrial membrane. This in turn might modify the mobility of the carrier molecule in the membrane. This hypothesis requires, of course, a systematic examination of the exact role of lipids in the activity of the reconstituted pyruvate carrier.

Whatever is the molecular mechanism of the thyroid hormone-induced stimulation of the

pyruvate transport, it is clear that this stimulation, together with other factors, may account for the increased mitochondrial oxidative capacity typical of the hyperthyroid state.

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