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Thyroid Status Is a Key Regulator of Both Flux and Efficiency of Oxidative Phosphorylation in Rat Hepatocytes

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Thyroid status is crucial in energy homeostasis, but despite extensive studies the actual mechanism by which it regulates mitochondrial respiration and ATP synthesis is still unclear. We studied oxidative phosphorylation in both intact liver cells and isolated mitochondria from in vivo models of severe not life threatening hyper- and hypothyroidism. Thyroid status correlated with cellular and mitochondrial oxygen consumption rates as well as with maximal mitochondrial ATP production. Addition of a protonophoric uncoupler, 2,4-dinitrophenol, to hepatocytes did not mimic the cellular energetic change linked to hyperthyroidism. Mitochondrial content of cytochrome oxidase, ATP synthase, phosphate and adenine nucleotide carriers were increased in hyperthyroidism and decreased in hypothyroidism as compared to controls. As a result of these complex changes, the maximal rate of ATP synthesis increased in hyperthyroidism despite a decrease in ATP/O ratio, while in hypothyroidism ATP/O ratio increased but did not compensate for the flux limitation of oxidative phosphorylation. We conclude that energy homeostasis depends on a compromise between rate and efficiency, which is mainly regulated by thyroid hormones.

KEY WORDS: Mitochondria; adenine nucleotide carrier; Pi carrier; protonmotive force; cytochrome; phosphate potential; control analysis; uncoupling; rat body mass.

INTRODUCTION

Thyroid status is crucial for energy homeostasis and its physiological role in growth as well as in organ and cell differentiation has been extensively studied (Werner and Nauman, 1968; Zhang and Lazar, 2000). Since the first description of an increased rate of oxygen consumption by thyroid extracts, thyroid hormones are well known to exert profound effects on energy metabolism (Rohrer, 1924; Schryver, 1905). The thermogenic effect of these hormones was demonstrated by an increase in oxygen consumption associated with heat production and weight reduction. Such an effect is characterized by a marked oxidation of the redox status in both cytosol and mitochondrial spaces (Kalderon *et al.*, 1992), while changes in phosphate potential appear to be variable (Kalderon *et al.*, 1992; Seitz *et al.*, 1985). The increased respiration in mitochondria isolated from hyperthyroid rats indicates that in addition to extramitochondrial effects, thyroid hormones are directly involved in mitochondrial metabolism (reviewed in Goglia *et al.*, 1999; Soboll, 1993). Several reports describe the influence of thyroid status on oxidation rate, mitochondrial membrane potential ($\Delta\Psi_m$), and ADP phosphorylation (Crespo-Armas and Mowbray, 1987; Hafner and Brand, 1988; Harper and Brand, 1993; Horrum *et al.*, 1991;

Key to abbreviations: $\Delta\Psi_m$, electrical potential difference across the mitochondrial inner membrane; ATP/O, adenosine triphosphate synthesis (nmol/min/mg protein)/oxygen consumption rate (10^{-9} atom/min/mg); PTU, 6-*n*-propyl-2-thiouracil; T_3, 3,3′,5-triiodo-L-thyronine; AcAc, acetoacetic acid; βOHBu, β-hydroxybutyric acid; $TPMP^+$, triphenylmethylphosphonium ion; TMPD, N,N,N',N'-tetramethyl-1,4-phenylenediamine; DNP, 2,4-dinitrophenol; Δp, protonmotive force; ΔpH, pH difference across the mitochondrial inner membrane; DMO, 5,5-dimethyloxazolidine-dione; $\Delta G'_p$, Gibbs free-energy of ATP hydrolysis reaction (phosphate potential); $\Delta E'_h$, Gibbs free-energy difference in oxidation reaction (redox potential); ANC, adenine nucleotide carrier; CAT, carboxyatractylate; *J*, metabolic flux (μmol/min/g dry cells).

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Nishiki *et al.*, 1978). Although a change in the yield of ATP synthesis seems linked to thyroid status, whether hyperthyroidism or hypothyroidism actually affect oxidative phosphorylation efficiency is still a unresolved, Indeed a decrease in mitochondrial proton leak with a concomitant increase in mitochondrial membrane potential has been consistently reported in most studies using isolated liver mitochondria or cells from hypothyroid rats (Bobyleva *et al.*, 1998; Hafner *et al.*, 1988), conversely to other studies where $\Delta\Psi_m$ has been reported to be either unaffected (Crespo-Armas and Mowbray, 1987; Harper and Brand, 1993) or decreased (Horrum *et al.*, 1991). Moreover, in hypothyroidism, ATP/O ratios have been reported to be unaffected (Hafner and Brand, 1988; Nishiki *et al.*, 1978). Similar contradictory results exist for hyperthyroidism as reviewed in Soboll (1993) and Goglia *et al.* (1999).

These conflicting results are mainly related to two problems: (i) the changes induced by thyroid hormones are multiple and (ii) the constraints linked to the experimental models. Firstly, multiply effects of thyroid hormones on oxidative phosphorylation have been well described. Indeed, it is noteworthy that increasing or decreasing thyroid hormone concentration affects membrane lipid composition (Brand *et al.*, 1992; Hulbert *et al.*, 1976; Ida Chen and Hoch, 1977) and that mitochondrial lipid composition affects enzyme activity (Dabbeni-Sala *et al.*, 1981; Paradies and Ruggiero, 1991) and oxidative phosphorylation efficiency (Brand *et al.*, 1991; Brookes *et al.*, 1997, 1998). Moreover (i) adenine nucleotide carrier (Dummler *et al.*, 1996; Gregory and Berry, 1991; Luciakova and Nelson, 1992; Verhoeven *et al.*, 1985), (ii) Mg^{2+}-dependent ATPase activity of isolated mitochondria (Clot and Baudry, 1982; Maddaiah *et al.*, 1981), and (iii) mitochondrial respiratory chain activity and dehydrogenases (Clot and Baudry, 1982; Dummler *et al.*, 1996; Gregory and Berry, 1991; Horrum *et al.*, 1985; Luciakova and Nelson, 1992; Maddaiah *et al.*, 1981; Nishiki *et al.*, 1978; Verhoeven *et al.*, 1985) are affected by thyroid status. Secondly, assessing in situ the oxidative phosphorylation pathway and its regulation is difficult. Indeed the number of accessible quantitative parameters is limited when using integrated experimental models, like intact cells (e.g., net ATP synthesis rate is not available). Conversely, in isolated mitochondria where the access to these quantitative parameters is easier, the relevance of the findings is limited as compared to intact living systems. In the present work we have investigated the effect of profound, but life compatible, thyroid status changes in both hyper- and hypothyroidism in intact liver cells. In this intact cellular model we found that thyroid status was responsible for dramatic changes in energy metabolism. The changes in respiration rate appear to be, at least partly, linked to a change in the overall efficiency of the redox proton-pumps (slipping) rather than to a pure protonophoric effect. By using isolated mitochondria, we have confirmed this mechanism of slipping at the level of cytochrome oxidase. Moreover, hyperthyroidism was responsible for an increase in ATP synthesis capacity at the expense of the yield of oxidative phosphorylation pathway, and conversely, hypothyroidism led to an increase in ATP/O efficiency associated with a dramatic decrease in oxidative phosphorylation capacity.

MATERIALS AND METHODS

Studies were conducted on male Wistar rats fed for 5 weeks with a standard diet consisting in 72% of total energy as carbohydrate, 22% as protein and 6% as lipid. Rats (28 days old, 80–100 g) were divided in three groups: euthyroid (control), hypothyroid (PTU-treated), and hyperthyroid (T_3-treated) groups. In the hypothyroid group, 0.05% (wt/vol) 6-*n*-propyl-2-thiouracil (PTU) was given to the rats in drinking water. Hyperthyroidism was induced by daily intraperitoneal injections of 3,3′,5-triiodo-L-thyroinine (15 μg T_3/100 g body weight) for 10 days before killing. Solutions of T_3 dissolved in 0.05 M NaOH were diluted with 0.9% (wt/vol) NaCl. Animals had access to food and water ad libitum.

Hepatocytes were isolated by the method of Berry and Friend (1969) as modified by Groen *et al.* (1982), from rats fasted for 20–24 h. Liver cells (15 mg dry cells/mL) were incubated with 20 mM glucose as substrate. After 25 min incubation, myxothiazol-sensitive (noncompetitive inhibitor of Complex 3, Thierbach and Reichenbach, 1981) oxygen consumption was measured. At the same time, 300 μL of the cell suspension were withdrawn, and mitochondrial and cytosolic contents were separated by using the digitonin fractionation method (Zuurendonk and Tager, 1974). ATP and ADP were measured by HPLC as described previously (Argaud *et al.*, 1993) and Pi was measured as described by Summer (1944). Volumes and membrane electrical potential difference measurements were determined in intact cells as described in Espié *et al.* (1995). The intramitochondrial NADH/NAD^+ ratio was determined by the metabolite indicator method (Akerboom *et al.*, 1979) assuming the reaction catalyzed by β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) is in near-equilibrium:

$$K_{app} = \frac{[\text{AcAc}] \times [\text{NADH}]}{[\beta\text{OHBu}] \times [\text{NAD}^+]},$$

i.e., 4.93×10^{-2} (Williamson *et al.*, 1967).

$\Delta E'_h$, the span of the redox potential between the donor electron couple ($NADH/NAD^+$) and the final electron acceptor couple (O/H_2O) across the respiratory chain was calculated from:

$$\Delta E'_h = -\Delta E^{\circ\prime}_h - 61.5 \log\left(\frac{[NADH]}{[NAD^+]}\right)$$

$$\text{with } \Delta E^{\circ\prime}_h = 1.2 \text{ V}.$$

Liver mitochondria were prepared according to Klingenberg and Slenczka (1959) in the following medium: 250 mM sucrose, 1 mM EGTA, 20 mM Tris–HCl (pH 7.2). Mitochondrial protein was estimated by the biuret method using bovine serum albumin as standard (Gornall *et al.*, 1948).

The rate of mitochondrial oxygen consumption was measured polarographically at 37°C using a Clark electrode. Respiration medium contained 125 mM KCl, 1 mM EGTA, 5 mM Pi–Tris, 20 mM Tris–HCl (pH 7.2), supplemented with 5 mM succinate–Tris/0.5 mM malate–Tris plus 1.25 μM rotenone and oligomycin (1.25 μg/mg protein). State 3 respiration was obtained after the addition of 1 mM ADP–Tris, but in absence of oligomycin. Cytochrome oxidase activity was assessed in the respiratory medium supplemented with 1 mM TMPD, 5 mM ascorbate, 3.6 mM myxothiazol, and 75 μM DNP.

Protonmotive force (Δp) measurements were performed as previously described (Devin *et al.*, 1996) in the same medium as described above.

ATP/O ratios with succinate as respiratory substrate were determined from the ATP synthesis rate versus respiratory rate with both an ADP regenerating system based on hexokinase (EC 2.7.1.1) *plus* glucose, or at saturating ADP concentrations as previously described (Nogueira *et al.*, 2001).

Respiratory-chain cytochrome concentrations, control coefficients of phosphate and adenine nucleotide carriers, and ATPase activity were determined on rat liver mitochondria as previously described (Nogueira *et al.*, 2001).

ATP, ADP, Pi, glucose 6-phosphate, β-hydroxybutyrate dehydrogenase, and hexokinase were purchased from Roche (Meylen, France). Succinic acid, rotenone, trizma base, EGTA, oligomycin, glucose, myxothiazol, DNP, carboxyatractylate, and malonic acid were from Sigma (L'Isle d'Abeau, France), HCl, malic acid, and $MgCl_2$ from Merck (Nogent, France), white labeled compounds were from Amersham (Les Ulis, France).

Results are expressed as mean ± SEM. Statistical significant differences were assessed by ANOVA followed by Fisher's protected least significant difference (PLSD) post hoc test or by unpaired Student *t* test (Stat View®, Abacus concepts, Inc., Berkeley, CA, 1992). Nonlinear fitting of the experimental curves was performed by using Kaleidagraph® software (Abelbeck software, 1994).

RESULTS

Influence of Thyroid Status on Animal Growth

Body mass was monitored during the 5-week experimental period (Fig. 1). Twenty-five days after the beginning of the experiment, the hyperthyroid group received T_3-treatment for 10 days. This treatment induced a decrease in mass gain as compared to controls (final mass 247 ± 9 vs. 315 ± 12 g, $p < 0.01$). Hypothyroidism was induced by treatment with PTU during the entire course of the study (35 days). Hypothyroid status was responsible for a marked decrease in growth and at the end of the study, the body mass of the PTU-treated rats was greatly reduced as compared to control and to the hyperthyroid group (143 ± 3 g). Hence both hyper- and hypothyroidism were responsible for a reduction in growth although to a different extent, hypothyroid rats being much more affected.

Effects of Hyper- and Hypothyroidism on Oxidative Phosphorylation in Intact Hepatocytes

Table I shows that thyroid hormone treatment led to a significant increase in the respiratory rate of hepatocytes (+76%) as already described (Bobyleva *et al.*,

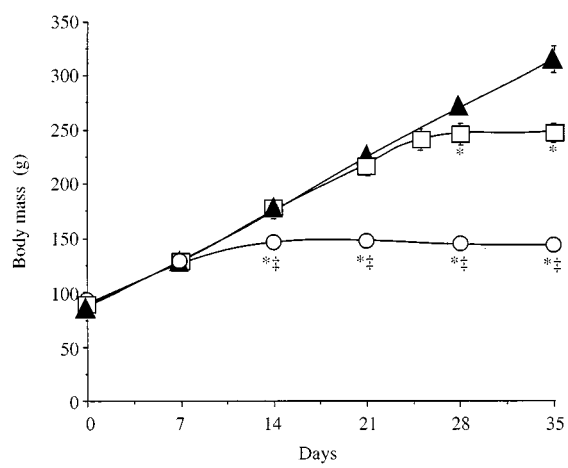

Fig. 1. *Evolution of rats' mass during the study.* Body mass data were monitored when hypothyroid treatment was initiated (Day 0) and thereafter weekly until rats were killed. In the hyperthyroid group weight was recorded daily during the 10 days of treatment (Days 25–35). Results are mean ± SEM from at least eight different rats in each group. $p < 0.05$; *vs. control; ‡vs. hyperthyroid. Control (▲), hypothyroid (○), and hyperthyroid (□).

Table I. Effect of Thyroid Status on Cellular Energetic Parameters: Comparison With DNP-Uncoupling

	JO_2 (nmol O_2/min/ mg dry mass)	Volume (μL/mg dry mass)		ATP/ADP		Pi (mM)		$-\Delta G'_p$ (kJ/mol)		$-\Delta\Psi$ mito (mV)
		Cyto	Mito	Cyto	Mito	Cyto	Mito	Cyto	Mito	
Control	12.9 ± 0.6	1.55 ± 0.09	0.45 ± 0.05	11.6 ± 0.6	2.0 ± 0.2	2.0 ± 0.2	24.8 ± 2.3	55.4 ± 0.3	44.3 ± 0.3	151 ± 2
Hyperthyroidy	$22.7 \pm 1.1^{*}$	$1.83 \pm 0.06^{*}$	$0.36 \pm 0.02^{*}$	$4.8 \pm 0.7^{*}$	1.9 ± 0.2	1.6 ± 0.1	$44.6 \pm 7.2^{*}$	$53.6 \pm 0.4^{*}$	$43.1 \pm 0.7^{*}$	$142 \pm 1^{*}$
Hypothyroidy	$9.8 \pm 0.6^{*\ddagger}$	$1.45 \pm 0.05^{\ddagger}$	$0.45 \pm 0.04^{\ddagger}$	$9.7 \pm 1.1^{*\ddagger}$	1.9 ± 0.2	$2.1 \pm 0.1^{\ddagger}$	$36.1 \pm 2.7^{*}$	$54.5 \pm 0.3^{*}$	$43.4 \pm 0.2^{*}$	$151 \pm 2^{\ddagger}$
Control + DNP	$22.7 \pm 0.8^{*}$	$1.50 \pm 0.09^{\ddagger}$	$0.43 \pm 0.03^{\ddagger}$	$4.1 \pm 0.7^{*}$	$0.8 \pm 0.1^{*\ddagger}$	1.7 ± 0.2	$27.2 \pm 3.2^{\ddagger}$	$52.9 \pm 0.4^{*}$	$41.7 \pm 0.4^{*\ddagger}$	$137 \pm 3^{*}$
Hypothyroidy + DNP	$12.5 \pm 1.2^{\$}$	1.45 ± 0.05	0.43 ± 0.02	$3.7 \pm 0.8^{*\$}$	$0.6 \pm 0.1^{*\$}$	2.2 ± 0.1	$39.4 \pm 4.0^{*}$	$51.7 \pm 0.6^{*\$}$	$40.2 \pm 0.3^{*\$}$	147 ± 2

Note. Hepatocytes from control, hyper-, and hypothyroid rats were incubated in a Krebs-bicarbonate medium containing 20 mM glucose. DNP concentrations used were 75 or 20 μM, respectively for control and hypothyroid group. After 25 min of incubation, myxothiazol sensitive oxygen uptake (JO_2) was determined. At the same time, mitochondrial and cytosolic adenine nucleotide Pi contents were measured and phosphate potentials ($\Delta G'_p$) were calculated from ATP, ADP, and Pi. In parallel experiments, cell and mitochondrial matrix volumes were determined by subtracting respectively [^{14}C]-carboxymethylinulin or [^{14}C]-mannitol spaces from water space determined by ^3H_2O. Mitochondrial $\Delta\Psi$ was determined from the [^{3}H]-TPMP$^+$ distribution. Results are mean $\pm$ SEM ($n = 8$ from four different rats). $p < 0.05$: *vs. control (all groups); ‡vs. hyperthyroidy (hypothyroidy, control + DNP); $^{\$}$vs. hypothyroidy (hypothyroidy + DNP).

1998; Gregory and Berry, 1991; Harper and Brand, 1993; Seitz *et al.*, 1985). Conversely hypothyroidism was responsible for a significant decrease in respiratory rate (−25%) (Bobyleva *et al.*, 1998; Gregory and Berry, 1991; Harper and Brand, 1993; Seitz *et al.*, 1985). These modifications in respiratory rates could be due to a change in a protonophoric leak through the inner mitochondrial membrace (Brand *et al.*, 1992; Harper and Brand, 1993). Therefore, we have tried to mimic these hormone-related changes by addition of adequate amounts of the uncoupler DNP, and proper addition (75 μM) to control cells allowed the reaching of a similar respiratory rate was observed in hyperthyroidism. Similarly, adequate addition of DNP (20 μM) to liver cells from hypothyroid rats resulted in a similar respiratory rate to controls. Prior to DNP addition, $\Delta\Psi_m$ was significantly lower in hyperthyroidism while it was unaffected in hypothyroidism. DNP addition to control cells, mimicking the respiration in hyperthyroidism, resulted in a decrease in $\Delta\Psi_m$ in such a way that it was identical to that of the hyperthyroid group. When liver cells from hypothyroid rats were treated with DNP in order to match the respiratory rate of control cells, $\Delta\Psi_m$ was not significantly affected. Hypothyroidism and DNP addition did not affect cytosolic nor mitochondrial volume conversely to hyperthyroidism, which was responsible for an increase in the cytosolic volume while mitochondrial space decreased.

Remarkably, ATP/ADP ratio was affected neither in hyper- nor in hypothyroidism in the matrix, while in the cytosol both conditions were responsible for a significant decrease, although to a different extent since the effect was much more pronounced in hyperthyroidism. The addition of DNP in the hypothyroid group resulted in a dramatic decrease of this ratio in both cytosol and mitochondria. DNP-uncoupling in control cells resulted in a decrease in ATP/ADP ratio, both in cytosol and in mitochondria, but when compared to hyperthyroid cells, ATP/ADP ratio was significantly lowered only in the matrix. The resulting effect of changes in ATP/ADP ratio as well as in Pi concentration, as expressed as $\Delta G'_p$, was a decrease in hyper- and hypothyroidism, both in cytosol and mitochondrial spaces. It is of interest to note that we did not find a significant difference between hyper- and hypothyroid groups in mitochondrial $\Delta G'_p$.

Hence, hyperthyroidism was characterized by a twofold increase in respiration rate while $\Delta\Psi_m$ and both cytosolic and mitochondrial $\Delta G'_p$ were decreased, a picture compatible with a protonophoric uncoupling effect. Indeed, when compared to control cells adequately uncoupled in order to match the respiratory rate of hyperthyroid cells, $\Delta\Psi_m$ and cytosolic $\Delta G'_p$ were not different. But mitochondrial $\Delta G'_p$ was lower in uncoupled control cells indicating that protonophoric uncoupling per se cannot explain the totality of the effects of hyperthyroidism. When compared to control cells, the hypothyroid state was characterized by a significant decrease in the respiratory rate, $\Delta\Psi_m$ was unchanged and both cytosolic and mitochondrial $\Delta G'_p$ decreased. This whole picture cannot be explained by a decrease in the protonophoric leak across the mitochondrial membrane. Indeed, when uncoupled, in order to match the respiratory rate of control cells, hypothyroid cells exhibited, for a similar $\Delta\Psi_m$, a dramatic decrease in both cytosolic and mitochondrial $\Delta G'_p$.

Hence hyper- and hypothyroidism led to opposite changes concerning the cellular energy metabolism only at the level of respiration. Moreover, these changes seemed not to be simply the result of an effect limited to a change in the rate of protonophoric leak.

Effect of Thyroid Status on the Respiratory Chain

We next studied the efficiency of the respiratory chain by investigating the relationship between oxygen consumption rate and the overall thermodynamic driving force expressed as $2\Delta E'_h - n\Delta_p$. This relationship was established experimentally by modulating the protonmotive force (Δp) in intact cells with small additions of DNP. $\Delta E'_h$ is the difference in redox potential across the respiratory chain (see Material and Methods section) and n is the H^+/O stoichiometry of the overall respiratory chain. It is accepted that n is equal to 10 for substrates giving their electrons to Complex I (Espié *et al.*, 1995; Stoner, 1987); however, the actual value of n does not play any role in the shape of the relationship. Thyroid status directly affected the respiratory chain (Fig. 2): whatever the thermodynamic driving force, the respiratory rate was higher in hyperthyroidism and lower in hypothyroidism as compared to controls.

Since thyroid hormones affect the transcription of the mitochondrial glycerol 3-phosphate oxidase (EC 1.1.99.5), whose activity plays a key role in the regulation of the glycerol phosphate shuttle (Lee and Lardy, 1965; Muller and Seitz, 1994), it may affect the ratio of substrates to Site 1 or to Site 2 of the respiratory chain, that is, between NADH or $FADH_2$ (see Discussion section). The respiratory chain efficiency was studied independently of the nature of the respiratory substrates by investigating the relationship between oxygen consumption rate and the protonmotive force in nonphosphorylating (State 4) isolated mitochondria. This was achieved by modulating the respiratory rate with malonate (an inhibitor of succinodehydrogenase) in the presence of succinate. Since the classical observation by Nicholls (1974), it is well known that such a relationship is ohmic (linear) at low protonmotive forces

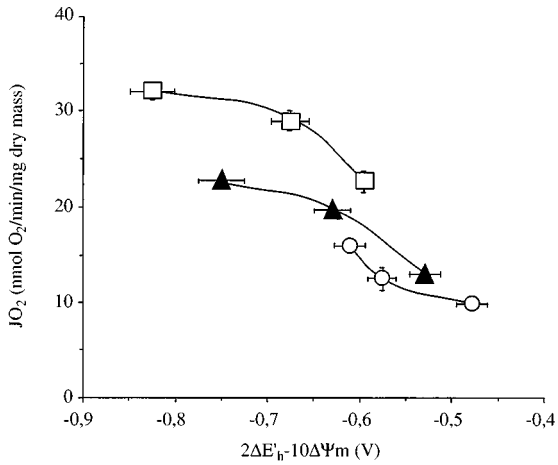

Fig. 2. *Relationships between oxygen consumption rates and overall thermodynamic forces at the level of the respiratory chain in hepatocytes from control, hypothyroid, and hyperthyroid rats.* Rat liver cells (15 mg/mL) were incubated as described in Table I, the respiratory rate being titrated in each group by different DNP concentrations (20–75 μM). $\Delta E'_h$ is the difference in redox potential across the electron transport chain and 10 is the proton/2 electrons stoichiometry of the respiratory chain. Data are mean ± SEM ($n = 8$ from four different rats). Control (▲), hypothyroid (○), and hyperthyroid (□).

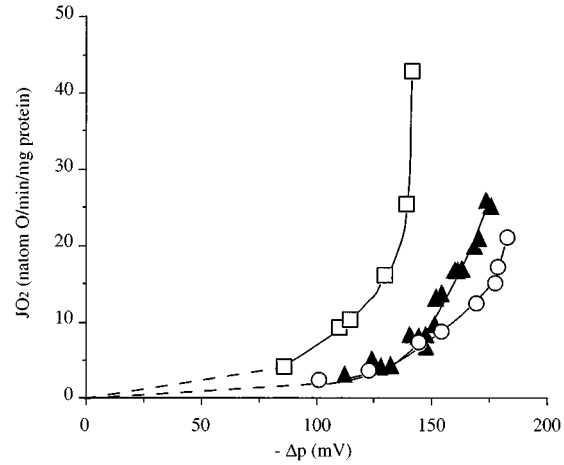

Fig. 3. *Relationships between oxygen consumption rates and protonmotive forces in isolated mitochondria from control, hypothyroid, and hyperthyroid rats.* Rat liver mitochondria (4 mg/mL) were suspended at 37°C in the respiratory medium (125 mM KCl, 1 mM EGTA, 20 mM Tris–HCl, pH 7.2) supplemented with 5 mM Tris–succinate, 0.5 mM Tris–malate, 5 mM Tris–Pi, 1.25 μM rotenone and oligomycin 1.25 μg/mg protein. The respiration rate was modulated by the addition of Tris–malonate. Δp measurements were performed in parallel experiments in the same conditions except that the medium was supplemented with [^{3}H]-TPMP$^+$ for $\Delta\Psi$ determinations, with [^{14}C]-DMO for ΔpH determinations, or with ^3H_2O and [^{14}C]-mannitol for matrix volume determinations. Each determination was performed in duplicate. Control (▲), hypothyroid (○), and hyperthyroid (□).

but nonohmic (nonlinear) for higher values. Experimentally, it has been shown that protonophoric uncouplers result in a linear relationship (Brand *et al.*, 1994), whereas slipping of the proton pump shifts the relationship to the left (Luvisetto *et al.*, 1991; Pietrobon *et al.*, 1987). This relationship was shifted to the left by hyperthyroidism and to the right by hypothyroidism (Fig. 3), suggesting an increased physiological slipping of the respiratory chain by hyperthyroidism while hypothyroidism decreased it.

Such an effect could be due to changes in respiratory chain content (Piquet *et al.*, 2000), which was therefore investigated (Table II). We found a significant decrease in the total content of cytochromes in hypothyroidism while it increased in mitochondria from hyperthyroid rats. Cytochromes aa_3 and b were significantly decreased in mitochondria isolated from PTU-treated rats as compared to controls while cytochrome cc_1 was not affected. Hyperthyroidism led to an increase in all of the determined cytochromes. The variations in cytochrome aa_3 content according to thyroid status were consistent with maximal cytochrome oxidase activity (uncoupled respiration

Table II. Influence of Thyroid Status on Mitochondrial Cytochrome Contents

	Quantity of cytochrome (nmol/mg protein)			
	aa_3	b	cc_1	Total
Control	0.097 ± 0.010	0.188 ± 0.015	0.209 ± 0.016	0.495 ± 0.033
Hyperthyroid	0.224 ± 0.005*	0.276 ± 0.015*	0.320 ± 0.010*	0.820 ± 0.021*
Hypothyroid	0.020 ± 0.001*‡	0.106 ± 0.007*‡	0.215 ± 0.024‡	0.341 ± 0.021*‡

Note. Isolated mitochondria (5 mg/mL) were incubated in water and the cytochrome content was determined by spectrophotometry. The differences between fully reduced (sodium dithionite) and fully oxidized (H_2O_2) spectra were analyzed. The wavelength pairs and absorption coefficients used were cytochrome $a + a_3$ (605–630 nm) with $\varepsilon = 24\ mM^{-1} \times cm^{-1}$, cytochrome b (563–575 nm) with $\varepsilon = 18\ mM^{-1} \times cm^{-1}$ and cytochrome $c + c_1$ (550–540 nm) with $\varepsilon = 18\ mM^{-1} \times cm^{-1}$. Each determination was performed in triplicate from at least four different rats in each group. Results are mean ± SEM. $p < 0.05$; *vs. control; ‡vs. hyperthyroidy.

rate in the presence of TMPD-ascorbate as substrate for Complex IV). Indeed oxygen uptake was significantly increased in mitochondria isolated from T_3-treated rats while it was decreased in hypothyroidism (1114 ± 31, 393 ± 21, and 704 ± 13 10^{-9} atom O/min/mg protein, respectively for hyper, hypo, and euthyroid groups, $n = 9$ in each group, $p < 0.01$ vs. controls).

Influence of Thyroid Status on Oxidative Phosphorylation

In nonphosphorylating mitochondria (State 4), the respiratory rate was higher (+58%) in hyperthyroid rats compared to controls (Table III) and this was associated with a significant decrease in protonmotive force (−38 mV). In State 3 (phosphorylating condition), the protonmotive force was decreased also in hyperthyroid mitochondria (−20 mV) and the respiratory rate was increased (+127%). In hypothyroidism, the respiratory rate was lowered by 30% in State 4 and 40% in State 3 and the protonmotive force was significantly increased both in State 4 and in State 3. The increase in Δp observed in hypothyroid mitochondria was due to a significant increase in ΔpH, which was present both in State 4 and in State 3 (+10 mV), while the decrease in Δp observed in hyperthyroidism was due to a significant decrease in $\Delta\Psi$ both in State 4 and in State 3. Conversely to intact cells, in isolated mitochondria, matrix volumes were not affected by thyroid status.

Pi uptake depends on ΔpH (Azzone *et al.*, 1976; Coty and Pedersen, 1974) and adenine nucleotide exchange is driven by $\Delta\Psi_m$; therefore, thyroid status might affect Pi and/or adenine nucleotide transport. This was investigated by the determination of flux control coefficients of ANC and of Pi carriers in the three groups. The experiment presented in Fig. 4(A) illustrates a typical titration of oxygen consumption in State 3 using mersalyl as inhibitor of the Pi carrier. As can by appreciated, hyperthyroidism but not hypothyroidism affected the flux control coefficient of the Pi carrier on State-3 respiratory flux (0.33 ± 0.05, 0.53 ± 0.02, and 0.53 ± 0.03 for hyperthyroidism, hypothyroidism, and controls respectively, $n = 4$ for each groups, $p < 0.05$ between hyperthyroidism and controls), an observation in agreement with the effect of thyroid status on ΔpH (see Table III). Furthermore, it must be noted that the mersalyl concentration required to completely inhibit Pi carrier activity was increased in hyperthyroidism and decreased in hypothyroidism (12.80 ± 0.21, 7.45 ± 0.15, and 10.50 ± 0.35 nmol mersalyl/mg protein, respectively for hyper, hypo, and euthyroid groups, $n = 4$, $p < 0.05$ vs. controls). This result indicates that Pi carrier content with higher in hyperthyroidism and lower in hypothyroidism as compared to controls.

The significant effect of thyroid hormone on phosphate potential gradient across the mitochondrial membrane could also be explained by a kinetic effect and/or by a thermodynamic change at the step of the adenine nucleotide carrier (ANC). Considering the electrogenicity of adenine nucleotide exchange through the ANC, the decrease in $\Delta\Psi_m$ occurring during hyperthyroidy (Table III) could provide an adequate explanation for the decreased gradient of ATP/ADP ratios (cytosol/matrix) across the mitochondrial membrane (2.48 ± 0.23 vs. 6.36 ± 0.81 for control groups, $n = 8$, $p < 0.05$). In addition, under these conditions the transport catalyzed by the ANC is out of equilibrium (Wanders *et al.*, 1981), and this step is probably controlled by kinetic rather than by thermodynamic parameters. Determination of ANC flux control coefficient was performed by using carboxyatractylate (CAT) as a quasi-irreversible inhibitor (Vignais *et al.*, 1973). Figure 4(B) shows the effect of thyroid status on CAT-inhibition of oxygen uptake under experimental conditions similar to those used for the Pi carrier. The control exerted by ANC on State-3 oxygen uptake was significantly increased in PTU-treated group (0.80 ± 0.04 vs. 0.32 ± 0.03 for the control group, $n = 4$, $p < 0.05$) while the decrease in hyperthyroid state was not significant (0.25 ± 0.04). Since the concentration of inhibitor required for maximal inhibition was increased by T_3-treatment and decreased by PTU-treatment (420 ± 9, 123 ± 3, and 177 ± 12 pmol carboxyatractylate/mg protein, respectively for hyper, hypo, and euthyroid groups, $n = 4$, $p < 0.05$), the thyroid status affected the mitochondrial content of the carrier, as already seen for the Pi carrier: ANC content was higher in hyperthyroidism and lower in hypothyroidism as compared to controls.

Thyroid Status and Efficiency of ATP Synthesis

Finally, we assessed oxidative phosphorylation efficiency by the determination of ATP/O stoichiometry either at maximal (State 3, Table III) or submaximal (hexokinase system, Fig. 5) ATP synthesis rates. Whatever the experimental conditions, hyperthyroidism decreased ATP/O while hypothyroidism increased it. The relationships between $J_{\text{glucose 6-phosphate}}$ assessing JATP and oxygen consumption rate were linear and almost parallel in the three groups (Fig. 5(A)). The relationship concerning the PTU-treated group was shifted to the left while that of T_3-treated group was shifted to the right compared to controls. The relation between oxidative-phosphorylation

Table III. Oxygen Consumption, Matrix Volume, and Protonmotive Force in Isolated Mitochondria of Control or Treated Rats

	JO_2 (10^{-9} atom O/min/mg protein)		Matrix volume (μL/mg protein)		Protonmotive force (mV)						JATP (nmol/min/mg protein)	ATP/O (nmol/10^{-9} atom)
					State 4			State 3				
	State 4	State 3	State 4	State 3	$-\Delta\Psi$	$-\Delta$pH	$-\Delta p$	$-\Delta\Psi$	$-\Delta$pH	$-\Delta p$		
Control	26.9 ± 1.2	194 ± 7	1.13 ± 0.12	0.94 ± 0.11	131 ± 2	43 ± 2	174 ± 1	105 ± 3	40 ± 4	146 ± 2	234 ± 12	1.22 ± 0.04
Hyperthyroidy	$42.4 \pm 1.4^{*}$	$441 \pm 7^{*}$	1.16 ± 0.11	1.09 ± 0.08	$89 \pm 3^{*}$	48 ± 5	$136 \pm 2^{*}$	$79 \pm 2^{*}$	46 ± 3	$126 \pm 3^{*}$	$390 \pm 16^{*}$	$0.89 \pm 0.04^{*}$
Hypothyroidy	$19.6 \pm 0.6^{*\ddagger}$	$117 \pm 3^{*\ddagger}$	1.08 ± 0.11	1.04 ± 0.12	$126 \pm 5^{\ddagger}$	$53 \pm 2^{*}$	$179 \pm 2^{*\dagger}$	$105 \pm 1^{\dagger}$	$51 \pm 2^{*}$	$156 \pm 2^{*\dagger}$	$184 \pm 14^{*\dagger}$	$1.60 \pm 0.06^{*\dagger}$

Note. Rat liver mitochondria (4 mg/mL) were suspended in the following medium: 125 mM KCl, 1 mM EGTA, 20 mM Tris–HCl (pH 7.2; 37°C), 5 mM Tris–Pi, supplemented with 5 mM Tris–succinate, 0.5 mM Tris–malate plus 1.25 μM rotenone and oligomycin 1.25 μg/mg protein (State 4). State 3 respiration was obtained after the addition of 1 mM ADP in absence of oligomycin and then ATP production (JATP) and ATP/O was measured. Protonmotive force measurements were performed in the same medium with succinate as substrate supplemented by [^{3}H]-TPMP$^+$ for $\Delta\Psi$ determinations, by [^{14}C]-DMO for ΔpH determinations, or by ^3H_2O and [^{14}C]-mannitol for matrix volume determinations. Each determination was performed in duplicate from at least four different rats in each group. Results are mean $\pm$ SEM. $p < 0.05$: *vs. control; ‡vs. hyperthyroidy.

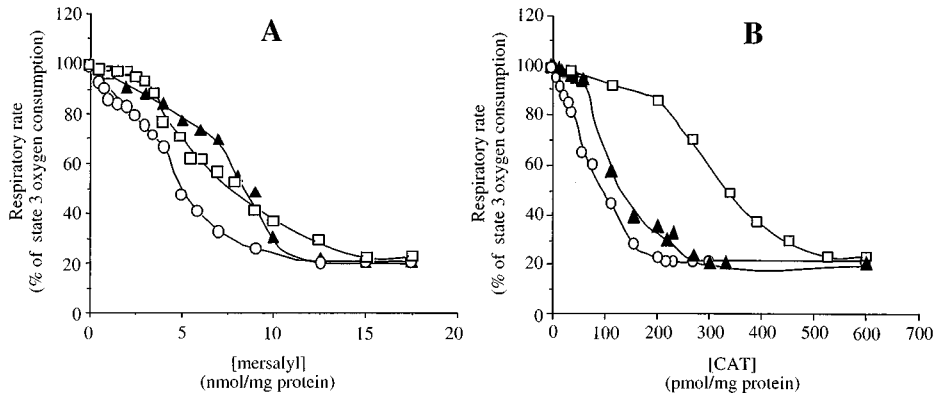

Fig. 4. *Determination of flux control coefficient of phosphate carrier and adenine nucleotide carrier on State 3 oxygen consumption of liver mitochondria isolated from control, hypothyroid, and hyperthyroid rats.* Rat liver mitochondria (1 mg/mL) were suspended in the medium described in Fig. 3 supplemented with 5 mM Tris–succinate, 0.5 mM Tris–malate, 5 mM Tris–Pi, 1.25 μM rotenone, and 1 mM ADP. Respiratory rate, expressed as percentage of State 3 oxygen consumption, was inhibited by increasing addition of (A) mersalyl or (B) carboxyatractyloside (CAT). One typical experiment out of four is shown. Control (▲), hypothyroid (○), and hyperthyroid (□).

yield and respiratory flux was well shown when ATP/O ratio was plotted against the oxygen consumption rate (Fig. 5(B)). Indeed according to thyroid status, the differences in yield were marked: at any rate of oxygen uptake ATP/O was twofold lower or twofold higher according to thyroid status, that is, from T_3-treated or PTU-treated rats, respectively.

Since oxidative phosphorylation also depends on ATPase, the effect of thyroid status on this enzyme activity was determined. We found a significant increase in hyperthyroid state (796 ± 32 vs. 413 ± 66 nmol Pi/min/mg protein for the control group, $n = 9$, $p < 0.05$), while it was not affected in hypothyroidism (403 ± 15) as compared to controls.

DISCUSSION

Thyroid status affects energy metabolism in both intact liver cells and isolated mitochondria. Although the changes induced respectively by hyper- or by hypothyroidism appear to be opposed concerning some

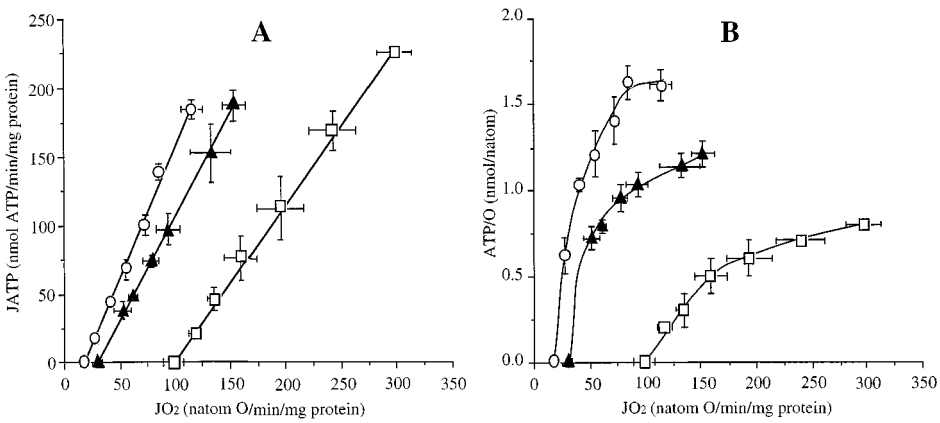

Fig. 5. *Relationships between ATP/O and respiratory rate in isolated mitochondria from control, hypothyroid, and hyperthyroid rats.* Rat liver mitochondria (2 mg/mL) were suspended in the medium described in Fig. 3 supplemented with 5 mM Tris–succinate, 0.5 mM Tris–malate, 5 mM Tris–Pi, 1.25 μM rotenone, 20 mM glucose, 125 μM ATP, and 1 mM $MgCl_2$. Oxygen consumption and ATP synthesis were titrated by addition of increasing concentrations of hexokinase. Results are mean ± SEM ($n = 4$). Control (▲), hypothyroid (○), and hyperthyroid (□).

parameters, they are similar for others. Indeed, as compared to controls, hyperthyroidism is characterized by increased rate of respiration while the efficiency of oxidative phosphorylation (ATP/O) is decreased. Conversely, in hypothyroidism respiration is decreased while oxidative phosphorylation efficiency is increased. But simultaneously, both hyper- and hypothyroidism were responsible for a decrease in phosphate potentials in either cytosol or mitochondrial matrix when compared to the physiological euthyroid state. The finding of opposed changes in respiration between hyper- and hypothyroidism is in agreement with previous data from the literature (Bobyleva *et al.*, 1998; Gregory and Berry, 1991; Hafner and Brand, 1998; Harper and Brand, 1993; Verhoeven *et al.*, 1985), but the effects of thyroid hormones on oxidative phosphorylation efficiency (ATP/O) are much more controversial (Crespo-Armas and Mowbray, 1987; Hulbert *et al.*, 1976; Ida Chen and Hoch, 1977; Nishiki *et al.*, 1978). In the present work we have used two different approaches for assessing this efficiency. In State 3 mitochondria, both respiration and ATP synthesis occur at maximal rates in phosphorylating conditions, but with unphysiological redox and phosphate potentials due to high saturating concentrations of respiratory substrates and of ADP. Conversely when ATP/O is assessed by manipulating subsaturing concentrations of ADP with different amounts of hexokinase the mitochondrial phosphate potential is closer to physiological status but the rates of both respiration and ATP synthesis are much lower as compared to State 3. Nevertheless whatever the method used, when compared to euthyroid controls, hyperthyroidism is always responsible for a twofold decrease in ATP/O while hypothyroidism always increases this ratio to the same extent (see Table III and Fig. 5). This very clear finding of significant and opposed effects of hyper- and hypothyroidism on oxidative phosphorylation efficiency is the main result presented in this work.

The second striking finding is the effect of thyroid status on the regulation of the intrinsic coupling of the proton-pumps at the respiratory chain. Our results show that for a given overall thermodynamic driving force, hyperthyroidism increases respiration while hypothyroidism decreases it. On the one hand, the well-known effect of thyroid hormone on the activity of the mitochondrial bound glycerol 3-phosphate oxidase (EC 1.1.99.5), by transcriptional effect, could explain a decrease in respiratory chain efficiency (Lee and Lardy, 1965; Muller and Seitz, 1994). Indeed, the oxidation of glycerol 3-phosphate by this enzyme, results in the production of $FADH_2$, and the electron transfer to oxygen involves only two coupling sites. Therefore, the stimulation of this pathway results in decreased efficiency when compared to NADH pathway. But on the other hand, thyroid status affects simultaneously the mitochondrial cytochrome content. Particularly, cytochrome aa_3 is strongly affected since it decreases by 80% in hypothyroidism while it increases by 130% in hyperthyroidism. The effects of thyroid hormone on cytochrome content, and especially on cytochrome aa_3, have been already reported (Gregory and Berry, 1991; Horrum *et al.*, 1985; Nishiki *et al.*, 1978; Verhoeven *et al.*, 1985), although a demonstration of a specific effect of thyroid hormones on the transcription of cytochrome oxidase in the liver is still lacking. However, it must be noted that thyroid hormone activates cytochrome oxidase both directly (Lanni *et al.*, 1994a,b) and via an effect located at the cardiolipin surroundings by modifying the activity of the cardiolipin synthase (Hostetler, 1991; Paradies and Ruggiero, 1991). Whatever the actual mechanism, the effect of thyroid hormone on cytochrome oxidase is the major interest since this complex has been shown to be the location for slipping (Capitanio *et al.*, 1991; Papa *et al.*, 1991). This is confirmed by the data presented in Fig. 3, showing a decrease in the efficiency of the respiratory chain in isolated mitochondria incubated with the Site 2-respiratory substrate succinate. Hence we can conclude that by modulating cytochrome oxidase activity, thyroid hormones regulate the efficiency of the respiratory chain. Therefore, hyper- and hypothyroidism are responsible for opposed changes in respiratory chain efficiency.

Whatever the actual mechanism of mitochondrial energy wastage related to thyroid status, that is, slipping of the redox proton-pump or proton-leak, its consequence on phosphate potential in intact cells would have been mimicked by using appropriate pharmacological uncoupling. When inducing such an artificial uncoupling by DNP, we found that this was not the case (Table I). For similar respiratory rate and $\Delta\Psi$, ATP/ADP ratios and phosphate potentials were very different. This led us to conclude that the phosphorylating part of the pathway, that is, ATP synthase *plus* phosphate and adenine nucleotide carriers, was also influenced by thyroid status. In the hyperthyroid state the significant increase in the ATPase activity was associated with functional increases in both ANC and Pi carrier. Indeed since we found a decrease in the flux control coefficient of both carriers simultaneously with an increase in inhibitor concentrations required for maximal inhibitors, we can conclude that both quantities and activities of these carriers were increased. Thus it appears that the changes related to hyperthyroid status permit an increase in the capacity of both ATP synthesis and exportation out of the matrix. These results are in agreement with data from the literature since it has been shown that thyroid hormone increases the transcription of ATP synthase (Izquierdo *et al.*, 1990; Nelson *et al.*, 1995) and of both transcription and translation of Pi carrier and ANC (Dummler *et al.*,

1996; Luciakova and Nelson, 1992). Moreover, thyroid hormone was also responsible for a direct activating effect on ANC (Sterling, 1991; Sterling and Brenner, 1995). The changes occurring in hypothyroid status were not exactly opposed to that observed in hyperthyroidism. Indeed as compared to controls, ATP synthase activity and flux control coefficient of Pi carrier were not affected, but simultaneously the control exerted by ANC as well as the amounts of both Pi carrier and ANC, as estimated from inhibitor concentrations required for maximal inhibitions, were decreased. Hence the lack of effect of hypothyroidism on ATPase activity and on the control of Pi import in the matrix led us to propose that hypothyroidism did not strongly affect the mitochondrial phosphorylation capacity. However, the increased flux control coefficient of ANC is in favor of an effect of limitation of ATP export toward the cytosol in hypothyroid status. These results are in agreement with the reported inhibitory effect of hypothyroidism on the transcription of ANC and Pi carrier (Dummler *et al.*, 1996; Luciakova and Nelson, 1992). Conversely, there is a discrepancy between the reported inhibitory effect of hypothyroidism on the transcription of ATP synthase (Izquierdo *et al.*, 1995; Nelson *et al.*, 1995) and the lack of effect on activity as reported here. This could be explained by posttranscriptional effects either on the enzyme or on the cardiolopin environment (Dabbeni-Sala *et al.*, 1981).

The different changes induced by thyroid hormone status, that is, either hyper- or hypothyroidism, occuring in the oxidative phosphorylation pathway are rather complex, but the resulting effect on energetic metabolism is quite clear. In hyperthyroidism the respiratory capacity is dramatically increased at the expense of the overall oxidative phosphorylation yield. Indeed the increased respiratory-chain slipping, probably linkend to the large increase in cytochrome oxidase activity, represents the cost of the increased respiratory capacity. The changes occuring at the level of nucleotide phosphorylation and transport are well integrated to these changes oriented toward a huge increase in ATP synthesis and utilization capacity. Indeed despite a twofold decrease in efficiency, the rate of ATP synthesis was actually increased in hyperthyroidism as compared to controls (see Fig. 5 and Table III). Nevertheless the decrease in the growth of these hyperthyroid animals indicated that the cost of such high rate of ATP synthesis was high.

Conversely in hypothyroidism, the threefold decrease in cytochrome oxidase activity permitted a large reduction in the energy wastage by lowering the slipping process occurring at this level. The resulting increase in efficiency is clearly shown by the twofold increase in the ATP/O ratio (see Fig. 5 and Table III). But this increase in oxidative phosphorylation yield occurs at the expense of a dramatic limitation in flux. Hence simultaneous to the increase in ATP/O ratio, the rate of ATP synthesis is actually decreased in hypothyroidism as compared to controls. Indeed, despite an increased efficiency the rate of animal growth was dramatically reduced in hypothyroidism, not only when compared to controls but also when compared to hyperthyroid rats.

As already pointed out in the literature, it appears that energy homeostasis relies on both rate and efficiency (Fitton *et al.*, 1994). The data presented in this work support such a view of a necessary compromise between these two parameters: the cost of a high rate is a decreased efficiency and conversely the consequence of high efficiency is a low rate. Thyroid hormone appears to exert a major role in this homeostatic regulation.

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