Calorigenic effect of diiodothyronines in the rat

Antonia Lanni, Maria Moreno, Assunta Lombardi and Fernando Goglia*

Dipartimento di Fisiologia Generale ed Ambientale, Università degli Studi di Napoli 'Federico II', Via Mezzocannone 8, I-80134, Napoli, Italy

1. In hypothyroid rats, we determined the effects of administration of different doses of 3,3′,5-triiodo-L-thyronine (T_3), 3,3′-diiodo-L-thyronine (3,3′-T_2) and 3,5-diiodo-L-thyronine (3,5-T_2) ('T_2 isomers' refers specifically to these latter two isomers throughout this paper) on resting metabolism (RM) and on the oxidative capacity (measured as cytochrome oxidase activity) of tissues that are metabolically very active.

2. The T_2 isomers induced a dose-dependent calorigenic effect when injected I.P. into hypothyroid rats. The increase in RM was already evident at a dose of 2·5 μg (100 g body wt)$^{-1}$, and the greatest effect was observed at the highest dose, 10 μg (100 g body wt)$^{-1}$, when RM reached a value not significantly different from that of the euthyroid controls (1·92 $\pm$ 0·08 and 1·93 $\pm$ 0·13 (l O_2) kg^{-1} h^{-1} for 3,5-T_2 and 3,3′-T_2, respectively, *vs.* 2·1 $\pm$ 0·12 (l O_2) kg^{-1} h^{-1} for euthyroid controls). T_3 administration restored RM to normal euthyroid values, even at a dose of 2·5 μg (100 g body wt)$^{-1}$.

3. The effect of T_2 isomers on RM was paralleled by an increase in the oxidative capacity of tissues that are metabolically very active (liver, skeletal muscle, brown adipose tissue (BAT) and heart). The increases were between 33% (liver + 3,3′-T_2) and 63% (muscle + 3,3′-T_2). By contrast, T_3 induced its greatest effect on the liver, with a smaller effect on skeletal muscle, but no significant stimulation in heart and BAT, whatever the dose.

4. These results suggest that T_2 isomers might be mediators of the direct thyroid hormone regulation of energy metabolism.

Historically, the root of some modern approaches to the investigation of the mechanism of action of thyroid hormones can be traced to early observations of their calorigenic effects (Tata, Ernster & Lindberg, 1962). The control of metabolism by thyroid hormones, seen in many adult endothermic vertebrates, is an action that receives considerable attention, and yet the mechanism by which this control is exerted is still to be identified.

Thyroid hormones are especially active in liver, heart, brown adipose tissue (BAT), kidney and muscle. However, as a consequence of their complex mode of action, each tissue responds in a characteristic way and not all the effects of thyroid hormones are observed in all the responsive tissues. Moreover, there are problems linked to the wide variety of animal models used in investigating the actions of thyroid hormones. Apart from the differences due to the use of acute or chronic treatment, variability also arises from the different methods used to induce hypothyroidism, i.e. surgical or chemical thyroidectomy. Indeed, a clarification of the characteristics of deiodinase enzymes (Köhrle, 1994) has revealed that induction of hypothyroidism by surgical or chemical thyroidectomy results in very different animal models. Surgical thyroidectomy induces a fall in the serum level of thyroid hormones, but deiodinase activity is not inhibited. The situation with chemically induced thyroidectomy is far more complex and depends on the drug used. Thus when: (a) methimazole (MMI) is used, there is an inhibition of the synthesis of thyroid hormones, but not of deiodinase activity (Erickson, Cavalieri & Rosenberg, 1982; Leonard & Visser, 1986; Rondell, de Greef, Klootwijk & Visser, 1992); (b) propylthiouracil (PTU) is used, there is, as with MMI, a block of thyroidal hormone synthesis, via an inhibition of thyroid peroxidase activity, but unlike MMI, there is also a strong inhibition of type I 5′-D-deiodinase activity in liver and kidney (Oppenheimer, Schwartz & Surks, 1972; Silva, Leonard, Crantz & Larsen, 1982; Leonard & Visser, 1986); and (c) iopanoic acid (IOP) is used, there is no influence over thyroid hormone secretion despite fluctuations in iodine supply (Vagenakis, Downs, Burger, Braverman & Ingbar, 1973), the induced changes in circulating thyroid hormones being principally due to alterations in thyroid hormone metabolism in the periphery (Burgi, Wimpfheimer, Burger, Zaunbauer, Rösler & Lemarchaud-Béraud, 1976). The administration of IOP, in fact, inhibits all three known types of deiodinase enzymes;

* To whom correspondence should be addressed.

however, while its effect is strong on types II and III it is comparatively weak on type I (Burgi *et al.* 1976; Kaplan & Utiger, 1978; Obregon, Pascual, Mallol, Morreale de Escobar & Escobar del Rey, 1980; Köhrle, 1994; St Germain, 1994).

It is now generally accepted that, although thyroxine (T_4) is the principal secretory product of the thyroid gland, its metabolic and developmental effects are all mediated by T_3, which is principally produced from T_4 by peripheral 5′-deiodination (Köhrle, Hesch & Leonard, 1991). However, no detailed studies have been performed on the possible effects of other circulating iodothyronines, such as the diiodothyronines. Recently, measurements made both *in vivo* and *in vitro* have shown that 3,3′-diiodo-L-thyronine (3,3′-T_2) and 3,5-diiodo-L-thyronine (3,5-T_2) have rapid and significant effects on the oxidative capacity of rat liver mitochondria (Horst, Rokos & Seitz, 1989; Lanni, Moreno, Cioffi & Goglia, 1992; O'Reilly & Murphy, 1992; Lanni, Moreno, Lombardi & Goglia, 1994*b*). (Throughout this paper, 'T_2 isomers' refers specifically to the 3,3′-T_2 and 3,5-T_2 isomers under investigation.) In addition, specific binding sites for the above diiodothyronines have recently been detected in rat liver mitochondria (Goglia, Lanni, Horst, Moreno & Thoma, 1994*b*; Lanni, Moreno, Horst, Lombardi & Goglia, 1994*a*). These results suggest that T_2 isomers might mediate the metabolic effects of thyroid hormones in the periphery, but so far there is no reported evidence that T_2 isomers have any calorigenic effect in animals or humans.

The above factors could help explain some of the conflicting information in the literature on the action of thyroid hormones. Furthermore, the same factors could have led to the masking of some effects actually attributable to iodothyronines, rather than to T_4 or T_3. Indeed, in situations in which deiodinase activities have not been inhibited, it is quite conceivable that some effects consequent upon T_4 or T_3 administration might be, at least in part, due to some of their deiodinated products, such as the diiodothyronines. We have now tested this possibility by investigating: (i) whether there is an effect of T_2 isomers on rat RM; and (ii) the involvement in such an effect of tissues that are metabolically very active (by measuring their oxidative capacity). The effect of comparable doses of T_3 has also been examined.

On the basis of previous considerations, we decided to use rats treated with PTU plus IOP, because this combined treatment produces animals with severe hypothyroidism and with a strong inhibition of all three known types of deiodinase enzymes. This gives rise to a better dose–response relationship and, at the same time, enables us to attribute the observed effects to the iodothyronines injected, rather than to any of their deiodinated products.

A preliminary version of these results has been published in abstract form (Goglia, Moreno, Lombardi & Lanni, 1995).

METHODS

Animals and treatments

Male Wistar rats (250–300 g) living in a temperature-controlled room at 28 °C were kept, one per cage, under an artificial lighting regime of 12 h light : 12 h darkness. A commercial mash (Mil-rats, Morini, S. Polo d'Enza, Italy) was available *ad libitum* and the animals had free access to water. All experiments were performed in accordance with local and national guidelines covering animal experiments. At the end of the experiment, the rats were anaesthetized by I.P. administration of chloral hydrate (40 mg (100 g body wt)$^{-1}$) and killed by decapitation. The rats had been made hypothyroid by administration of PTU (0·1% w/v in drinking water for 3 weeks (Lanni *et al.* 1992)) together with IOP (6 mg (100 g body wt)$^{-1}$ (Acheson & Burger, 1980)). To avoid undue stress, due to its long-lasting inhibitory effect, the IOP was injected I.P. once a week with the last dose being given 12 h before the animal was killed (St Germain, 1988). Each rat received, by a once-daily I.P. injection over a 3 week period, one of three different doses (2·5, 5 or 10 μg (100 g body wt)$^{-1}$) of T_3, 3,5-T_2 or 3,3′-T_2. In other words, a given rat would have received daily only one of the doses of only one of the agents. Saline was injected I.P. daily into hypothyroid control rats. In any given rat, the interval between injections was thus 24 h. The doses used in the present study were within the average range of the doses used in most studies on the effects of thyroid hormones on metabolism (for review see Towle, 1983).

At the end of the treatment, each rat was killed, the trunk blood collected, and the serum separated and stored at −20 °C for later measurement of T_3 concentration. Interscapular BAT, heart, gastrocnemius muscle and liver were dissected out, cleaned, immediately weighed (wet weight) and processed.

Energy expenditure

The day before a rat was to be killed, its resting metabolism was measured using open-circuit indirect calorimetry. The rat was placed in a respiration chamber (~32 × 20 × 19 cm), airflow was measured using an O_2-ECO mass flow controller (Columbus Instruments International Corporation, Columbus, OH, USA). A flow of fresh air (volume of air flowing into the cage, $V_{in} = 4$ l min^{-1}), containing 20·93% O_2 (X_{in}, the percentage of O_2 entering the cage) was pumped into the animal's cage through an input mass-flow controller, which regulates and measures the amount of air entering the cage. The air coming out of the cage went through a dryer, which absorbed water vapour and carbon dioxide before the air reached the oxygen sensor. The volume of the air flowing into the cage was always kept greater than the volume of air leaving the cage by this route (1 l min^{-1}). The excess air was let out into the atmosphere through a small outlet at the top of the cage. Thus, the total air entering the cage (V_{in}) equals the total amount of air leaving the cage (V_{out}) and the air composition does not vary except when an animal is in the cage. Then, the amount of oxygen changes because of its conversion to carbon dioxide by the animal (X_{out}, the percentage of oxygen in air leaving the cage). Since carbon dioxide and water vapour are completely absorbed from the air coming out of the cage, it is possible to define the rate of oxygen consumption ($\dot{V}_{O_2}$; expressed in l kg^{-1} h^{-1}) as:

$$\dot{V}_{O_2} = \frac{V_{in}(X_{in} - X_{out}) \times 60}{(1 - X_{out})(\text{kg body wt})}.$$

Since V_{in} and X_{in} are known, the equation gives the oxygen consumption when X_{out} is measured by the oxygen sensor.

The measurements for RM determinations (the lowest metabolic rate of a resting animal that is in a postabsorptive or fasting state and is not sleeping) were performed at 28 °C between 11.00 and 16.00 h, when the energy expenditure was at a lower level than in any other period of the day (see Fig. 1).

In order to be sure that our measurements of oxygen depletion would reflect resting metabolic activity and were not influenced by the movements of the rat around the cage, we decided upon the following procedure. We observed each rat over a 1 h period and took measurements whenever the rat was resting, but not asleep, in its cage. Such rest periods normally lasted about 4–5 min and, for that period, the readings were stable (whereas oscillations occurred when the animal was moving around). In this way, to calculate X_{out}, we took and averaged only the lowest values for oxygen depletion seen in non-sleeping rats.

Analytical procedures

Total T_3 levels were determined in 50 μl samples of serum using reagents and protocol supplied by Becton-Dickinson (Orangeburg, NJ, USA). The sensitivity of this T_3 assay was 0·10 nmol l^{-1}.

Cytochrome oxidase (COX) activity was determined polarographically at 25 °C, using a Clark oxygen electrode and a modification (Barrè, Bailly & Rouanet, 1987) of the procedure of Aulie & Grav (1979). This required 1·5 ml of reaction medium containing 30 μM cytochrome *c*, 4 μM rotenone, 0·5 mM dinitrophenol, 10 mM sodium malonate and 75 mM Hepes buffer, at pH 7·4. Samples of liver, skeletal muscle, BAT and heart were finely minced, diluted 1 : 10 (w/v) and homogenized in modified Chappel–Perry medium (mM): 1 ATP, 50 Hepes buffer adjusted to pH 7·4, 100 KCl, 5 $MgCl_2$, 1 EDTA and 5 EGTA. The homogenate was then diluted 1 : 2 (v/v) in the same medium with lubrol (100 mg (g tissue)$^{-1}$) in order to unmask the enzyme activity of the tissue. It was then left standing in ice for 30 min.

COX activity was measured as the difference between the rate of oxygen consumption observed after the addition of substrate (4 mM sodium ascorbate with 0·3 mM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine) and homogenate, and the rate of oxygen consumption observed after the addition of substrate alone, in order to take the auto-oxidation of ascorbate into account.

The results are expressed as mean values ± S.E.M. Statistical significance of differences between hypothyroid control animals and hypothyroid treated animals was determined using Student's *t* test. Values are considered significant when $P < 0·05$.

RESULTS

Hormone concentrations

The total T_3 levels were 0·92 ± 0·07 nmol l^{-1} in euthyroid control rats and 0·19 ± 0·02 nmol l^{-1} in hypothyroid (PTU + IOP-treated) rats. These values, showing an 80% decrease in T_3 serum levels in the hypothyroid rats, confirm the effectiveness of treatment with PTU plus IOP in inducing a severe hypothyroid status. For comparison, in rats treated with IOP alone, the T_3 level was 0·68 ± 0·05 nmol l^{-1}, only 20–30% lower than in the euthyroid controls. A similar percentage decrease has been reported by others using IOP alone (Burgi *et al.* 1976; Tuca, Giralt, Villarroya, Vinas, Mampel & Iglesias, 1994).

Effect of iodothyronines on the resting metabolism of hypothyroid rats

RM, which was considerably lower in hypothyroid rats than in euthyroid controls (1·47 ± 0·04 *vs.* 2·1 ± 0·12 (l O_2) kg^{-1} h^{-1}), was then significantly enhanced by the administration of either of the two diiodothyronines (Fig. 2) or T_3. In the

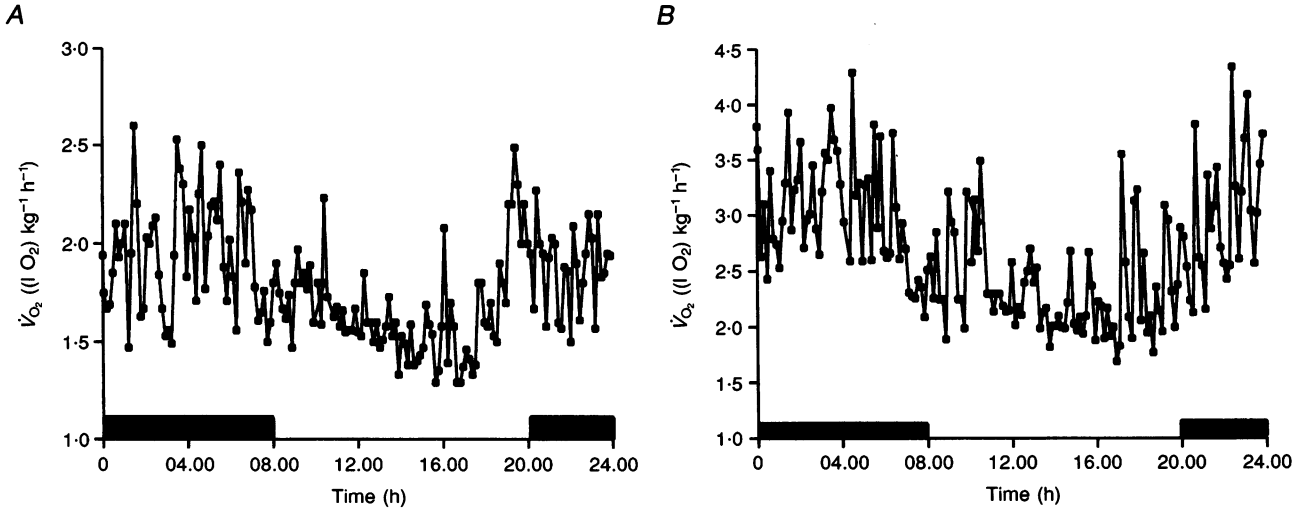

Figure 1. Representative patterns of energy expenditure during the day for a hypothyroid rat (*A*) and a euthyroid rat (*B*)

Throughout the day, at intervals of about 10 min and independent of the state of the animal, the oxygen consumption was regularly measured. Filled bars show hours of darkness. The figure shows that there is little or no difference in terms of daily pattern of energy expenditure between hypothyroid and euthyroid rats.

case of the two diiodothyronines, the increase in RM was already evident at a dose of 2·5 μg (100 g body wt)$^{-1}$ (RM increased to 1·77 ± 0·06 and 1·72 ± 0·04 (l O_2) kg^{-1} h^{-1} for 3,5-T_2 and 3,3′-T_2, respectively) and the greatest stimulation was observed at the highest dose used, 10 μg (100 g body wt)$^{-1}$ (RM increased to 1·92 ± 0·08 and 1·93 ± 0·13 (l O_2) kg^{-1} h^{-1} for 3,5-T_2 and 3,3′-T_2, respectively). These high values are not significantly different from that for the euthyroid controls.

As expected, the administration of T_3 to hypothyroid animals restored their RM to euthyroid values even at a dose of 2·5 μg (100 g body wt)$^{-1}$, the values being 2·2 ± 0·20, 2·3 ± 0·18 and 2·6 ± 0·20 (l O_2) kg^{-1} h^{-1} for T_3 at 2·5, 5 and 10 μg (100 g body wt)$^{-1}$, respectively (not shown).

In the literature, reported values for the resting metabolism of euthyroid rats vary over a considerable range, namely between 1·2 and 2·1 (l O_2) kg^{-1} h^{-1} (Nagasaka, Hirata, Sugano & Shibata, 1979; Liverini, Iossa & Barletta, 1994). The values obtained in our experiments are the same as the means reported by the latter group and close to those in other reports (Buser, Kopp, Gehr, Weibel & Hoppeler, 1982; Whitaker, Hussain, Hervey, Tobin & Rayfield, 1990; Himms-Hagen *et al.* 1994).

Effect of iodothyronines on COX activity in various tissues

To investigate the involvement of liver, BAT, skeletal muscle and heart in the calorigenic effect of diiodothyronines, we used an indirect method based on the determination of their COX activity, which has been shown to be an indicator of the metabolic potential of individual organs (Jansky, 1961; 1973). COX activity was significantly lower in each tissue from the hypothyroid animals than in the corresponding tissue from euthyroid ones. The values, expressed as nanograms of oxygen atoms (nAtom O) per minute per milligram protein were, for hypothyroid rats: 353 ± 30 for liver, 1442 ± 109 for BAT, 230 ± 13 for muscle and 1734 ± 168 for heart. The corresponding values for euthyroid rats were: 475 ± 30 for liver, 2420 ± 180 for BAT, 431 ± 30 for muscle and 2653 ± 188 for heart.

Administration of either 3,3′-T_2 or 3,5-T_2 to hypothyroid rats significantly stimulated the oxidative potential of each tissue in a dose-dependent manner, the maximal effects occurring at a dose of 10 μg (100 g body wt)$^{-1}$. In percentage terms, 3,3′-T_2 exerted its greatest stimulatory effect on muscle oxidative capacity (+63% at a dose of 10 μg (100 g body wt)$^{-1}$) which reached a value of 359 ± 10 (nAtom O) min^{-1} (mg protein)$^{-1}$. The tissue least responsive to 3,3′-T_2 was the liver where, at the same dose, stimulation caused a 33% increase, giving a value for maximal COX activity of 469 ± 13 (nAtom O) min^{-1} (mg protein)$^{-1}$.

By contrast, 3,5-T_2, at the highest dose used, 10 μg (100 g body wt)$^{-1}$, exerted its greatest stimulatory effect on oxidative capacity in BAT (+60%) and liver (+61%), where maximal values were 2307 ± 187 and 570 ± 53 (nAtom O) min^{-1} (mg protein)$^{-1}$, respectively. The tissue least responsive to 3,5-T_2 was the heart, though even there the stimulation was of the order of 40%.

On administration of T_3 (10 μg (100 g body wt)$^{-1}$), the greatest effect on oxidative capacity was observed in the liver (+74%), which reached a value for COX activity of 614 ± 50 (nAtom O) min^{-1} (mg protein)$^{-1}$, while in BAT and heart no significant stimulation was observed, whatever the dose.

Figure 3 summarizes these effects of iodothyronines on COX activity in the liver, skeletal muscle, BAT and heart of hypothyroid rats.

DISCUSSION

The effects of the T_2 isomers documented in this study were observed in rats treated with PTU and IOP together. In a previous study (Lanni *et al.* 1992) we reported that in rats treated with PTU alone, the administration of either of the T_2 isomers at a dose of 2·5 μg (100 g body wt)$^{-1}$ induced an insignificant increase in resting metabolism (+10%). This is smaller than the effects reported in the present study (+20%), suggesting that IOP treatment had the effect of

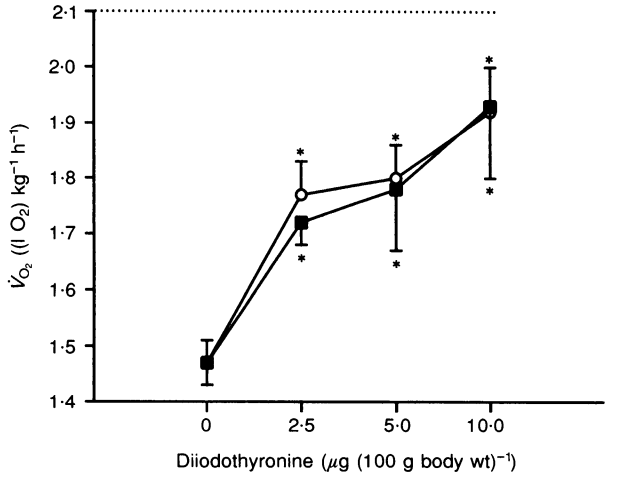

Figure 2. Effect of diiodothyronines on the resting metabolism of hypothyroid rats

The results are expressed as the means ± S.E.M. of 4 different experiments. * $P < 0·05$ for difference from corresponding value at dose 0. Dotted line represents euthyroid control value. ○, 3,5-T_2; ■, 3,3′-T_2.

improving the expression of the responses to the T_2 isomers. Unfortunately, to our knowledge no data are to be found in the literature on the metabolism of T_2 isomers or on the effects of IOP on their metabolic pathway. Thus, any attempt at an explanation for the improvement in the effect of T_2 isomers on RM observed with IOP must be, at best, tentative. The most likely explanation is that IOP preserves the diiodothyronines from deiodination, inhibiting both inner- and outer-ring deiodinase activities. However, it remains to be fully investigated as to what extent deiodination and conjugation of T_2 isomers are influenced by IOP before we can attempt to relate the metabolic clearance rate of T_2 isomers to any change in their effects.

All the tissues that are metabolically very active are sensitive to diiodothyronines. In the present study, their oxidative potentials were significantly stimulated by the administration of T_2 isomers, the increases ranging from 33% (liver + 3,3′-T_2) to 63% (muscle + 3,3′-T_2). The effect of T_2 isomers on resting metabolism mimicked to a large extent the effect of T_3 while, at the tissue level, some differences in the stimulation of oxidative capacity can be observed. Under our conditions, T_3 exerted its greatest effect on the liver and had little or no effect on the heart or BAT, while T_2 isomers were most active in muscle and BAT, with a smaller effect on the heart (about 40%). These results are not surprising, since previous reports have shown that, in hypothyroid animals, administration of T_3 was unable to stimulate the metabolic activity of BAT (Bianco & Silva, 1987) and that T_2 isomers, but not T_3, were able to stimulate *in vitro* the activity of isolated COX complexes from the bovine heart (Goglia, Lanni, Barth & Kadenbach, 1994*a*).

The mechanism of action of T_2 isomers, however, would seem to be different from that of T_3. The effects of T_3 are dependent on its effect on protein synthesis (Tata *et al.* 1962) and are mediated by binding to specific nuclear c-erb-A-related receptors (Oppenheimer, Schwartz, Mariash, Kinlaw, Wong & Freke, 1987). The T_2 isomers, on the other hand, may have mitochondria as their cellular target. The effects of T_2 isomers on metabolism are rapid and

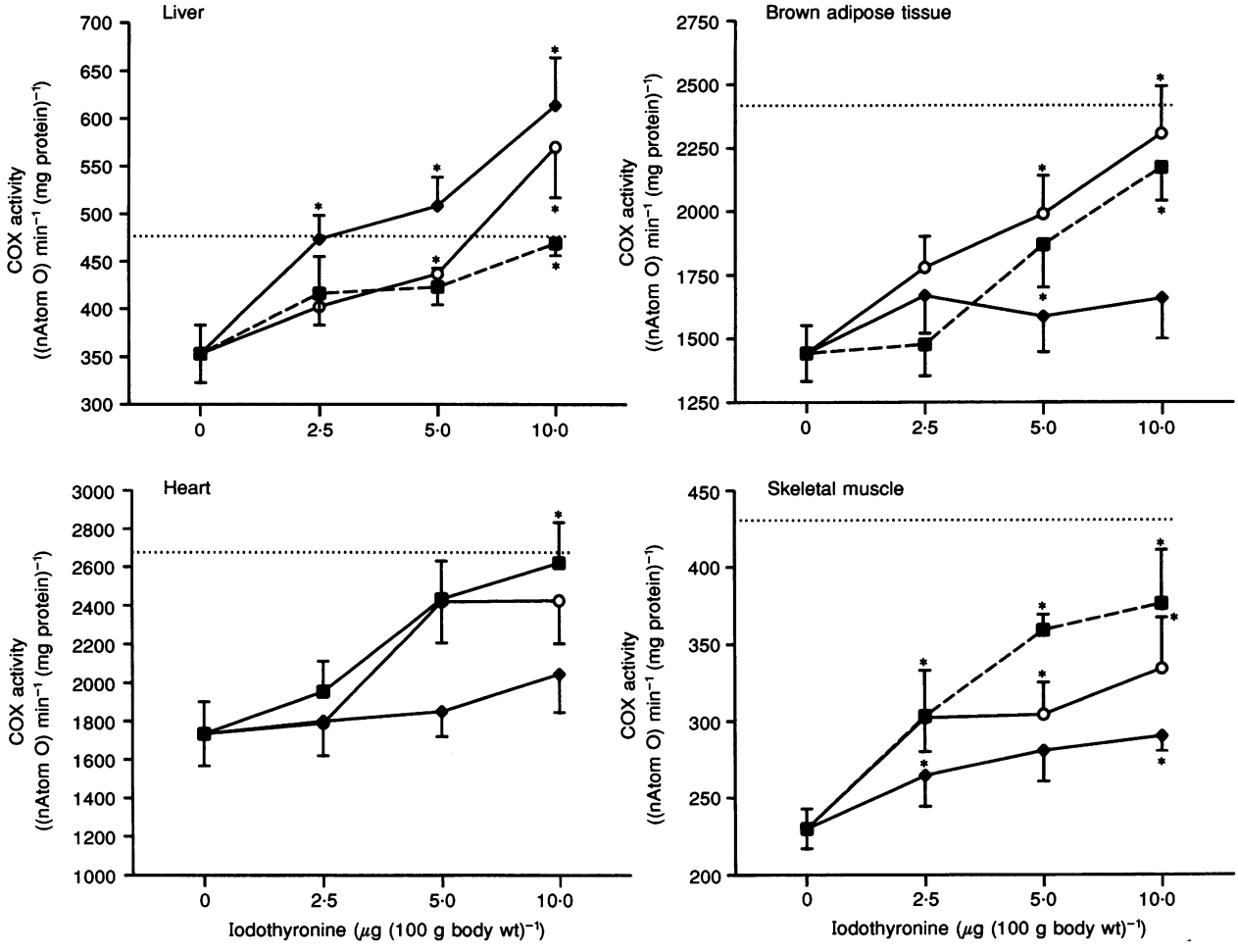

Figure 3. Effect of iodothyronines on COX activity of liver, heart, brown adipose tissue and skeletal muscle from hypothyroid rats

The results are expressed as the means ± S.E.M. of 4 different experiments. $*P < 0{\cdot}05$ for difference from corresponding value at dose 0. ○, 3,5-T_2; ■, 3,3′-T_2; ◆, T_3. Dotted line represents euthyroid control value.

independent of protein synthesis (Horst *et al.* 1989; O'Reilly & Murphy, 1992; Lanni *et al.* 1994*b*). In addition, the presence of specific binding sites for both diiodothyronines in rat liver mitochondria is consistent with the idea of a direct interaction with these organelles.

Although we cannot be sure as to the actual mechanism of action, we believe the results reported here provide new data which improve our understanding of how the individual iodothyronines might regulate metabolic rate. In addition, these results raise some doubts about the generally accepted idea that the various effects of thyroid hormones on different tissues are all mediated through T_3. Several studies have reported a stimulation of metabolism or mitochondrial activity by T_3 either *in vivo* or *in vitro* but, in most studies, large amounts of hormone were used (for review see Towle, 1983; Katyare, Bangur & Howland, 1994) and the deiodinase enzymes were not inhibited (Palacios-Romero & Mowbray, 1979; Katyare *et al.* 1994). Because of the large doses used, a pharmacological, rather than a physiological, effect may have been induced. Moreover, the failure to inhibit deiodinase enzymes means that deiodination of T_4 or T_3 to T_2 cannot be excluded.

In the light of previous considerations, the results and experimental approach reported here can also supply an explanation for the widely differing results obtained *in vitro* on the effects of T_4 and T_3 on mitochondria from hypothyroid animals in which hypothyroidism was induced by a variety of methods.

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