Biochem. J. (1979) **184**, 527–538
Printed in Great Britain

Evidence for the Rapid Direct Control both *in vivo* and *in vitro* of the Efficiency of Oxidative Phosphorylation by 3,5,3'-Tri-iodo-L-thyronine in Rats

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(*Received* 9 *April* 1979)

1. Examination of the distribution of L-tri-iodothyronine among rat liver tissue fractions after its intravenous injection into thyroidectomized rats focused attention on mitochondria at very short times after administration. By 15 min this fraction contained 18.5% of the tissue pool; however, the content had decreased sharply by 60 min and even further over the next 3 h. By contrast, the content in all other fractions was constant or increased over 4 h. About 60% of tissue hormone was bound to soluble protein. 2. Mitochondria isolated from thyroidectomized rats showed P/O ratios that were about 50% of those found in normal controls, with both succinate and pyruvate plus malate as substrates. There was no evidence of uncoupling; the respiratory-control ratio was about 6. 3. Mitochondria isolated 15 min after injection of tri-iodothyronine into thyroidectomized rats showed P/O ratios and respiratory-control ratios that were indistinguishable from those obtained in mitochondria from euthyroid animals. The oxidation rate was, however, not restored. 4. Incubation of homogenates of livers taken from thyroidectomized animals injected with L-tri-iodothyronine before isolation of the mitochondria restored the P/O ratio to normal; by contrast, direct addition of hormone to isolated mitochondria had no effect. The role of extramitochondrial factors in rapid tri-iodothyronine action is discussed. 5. Possible mechanisms by which tri-iodothyronine might rapidly alter phosphorylation efficiency are considered: it is concluded that control of adenine nucleotide translocase is unlikely to be involved. 6. The amounts of adenine nucleotides in liver were measured both after thyroidectomy and 15 min after intravenous tri-iodothyronine administration to thyroidectomized animals. The concentrations found are consistent with a decreased phosphorylation efficiency in thyroidectomized animals. Tri-iodothyronine injection resulted in very significant changes in the amounts of ATP, ADP and AMP, and in the [ATP]/[ADP] ratio, consonant with those expected from an increased efficiency of ADP phosphorylation. This suggests that the changes seen in isolated mitochondria may indeed reflect a rapid response of liver *in vivo* to tri-iodothyronine.

In recent years there has been widespread recognition that much of the early work showing an effect of thyroxine on uncoupling oxidative phosphorylation was unphysiological (see Werner & Nauman, 1968). During the last 10 years or so the vast enthusiasm generated for molecular genetics has focused attention on the processes of DNA transcription and RNA translation, and a reasonable case for a nuclear site of action for thyroid hormones has been made (see Tata, 1970). More recently, work has centred on attempts to locate and isolate receptor sites for thyroxine and tri-iodothyronine in target tissues, and there is some evidence that these hormones bind in a specific manner to all cellular fractions (Tata, 1975), though perhaps the most complete evidence exists for nuclear sites (for a review see Oppenheimer *et al.*, 1976). However, there is controversy about how relevant all these binding sites are to the physiological action of the hormone (cf. Tata, 1975; Oppenheimer *et al.*, 1976; Sterling, 1976).

Some very rapid effects of thyroxine on protein synthesis have been observed *in vitro*. Sokoloff and his co-workers have reported a stimulation of incorporation of either labelled amino acid or aminoacyl-tRNA into protein 5–7 min after thyroxine addition to a rat liver or reticulocyte cell-free system (Sokoloff & Kaufman, 1961; Sokoloff *et al.*, 1963, 1968; Krause & Sokoloff, 1967; Sokoloff & Roberts, 1974). These workers report that the presence of mitochondria and oxidizable substrate are obligatorily required for thyroxine stimulation of microsomal protein syn-

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thesis and that the mitochondria cannot be replaced by an ATP-regenerating system or the provision of GTP. Although by present-day standards their cell-free system is crude and slow, the work appears to demonstrate a rapid direct effect of thyroxine on mitochondria.

A direct rapid effect of thyroxine on mitochondrial protein synthesis and oxidation has also been reported (Buchanan & Tapley, 1966; Buchanan *et al.*, 1970, 1971). The presence of 50 μM-thyroxine caused rat liver mitochondria to double their amino acid incorporation in 8 min, although the State-2 O_2 uptake also increased, the P/O ratio (succinate) fell from 1.5 to 1.1 and the respiratory-control ratio went from 3.5 to 1.9.

These findings have been largely ignored perhaps because of the general disenchantment with the mitochondrial model for thyroid-hormone action, and the conclusion that mitochondrial response owes as much to the properties of the individual mitochondrial preparations as to the level of hormone or prior thyroid status of the animal.

The long-standing speculation as to whether thyroxine during its peripheral metabolism gives rise to tri-iodothyronine was revived by the demonstration by Braverman *et al.* (1970) that athyreotic humans given highly purified thyroxine as replacement therapy had substantial amounts of tri-iodothyronine in their plasma. The same workers showed that more than 40% of the tri-iodothyronine in the plasma of normal humans came from thyroxine (Sterling *et al.*, 1970). Using whole-body homogenates of rat, Schwartz *et al.* (1971) have presented evidence that about 20% of thyroxine could be converted by extra-thyroidal tissues to tri-iodothyronine. As tri-iodothyronine has been estimated to be from 3 to 5 times more active metabolically than thyroxine in rat (Gross & Pitt-Rivers, 1953), they concluded that from 50 to 85% of the metabolic potency of thyroxine could be due to its conversion to tri-iodothyronine. Surks *et al.* (1973), using radioimmunoassay to measure tri-iodothyronine produced from thyroxine during replacement therapy to humans without endogenous thyroid function, estimated that about 42% of the thyroxine pool was converted to tri-iodothyronine per day. They calculate that this would supply sufficient tri-iodothyronine to account for the biological activity of thyroid hormone entirely. Thus the balance of evidence currently suggests that the major form of thyroid hormone active in target cells is tri-iodothyronine.

We report here investigations into some rapid effects of tri-iodothyronine on rat liver. Since tri-iodothyronine is extremely insoluble in aqueous medium, one problem seemed to be to answer how small physiological quantities of hormones could find their way to designated intracellular sites without saturating every hydrophobic structure in the cell. Thus the first objective was to examine the tissue distribution of tri-iodothyronine at short times after administration. This preliminary study focused attention very strongly on mitochondria, and the present paper describes some observations of a rapid effect of tri-iodothyronine both *in vivo* and *in vitro* on the efficiency of oxidative phosphorylation. Part of this study has already been presented in preliminary form (Palacios-Romero & Mowbray, 1977).

Experimental

Animal treatment

Male albino rats of an original Sprague–Dawley strain bred in this department were selected as soon as possible after weaning, supplied with GR3-EK diet (Dickinson and Sons, Ware, Middx., U.K.) and kept together in batches of 12–15 until they were in the weight range 130–150 g. They were then thyroidectomized under chloral hydrate anaesthesia and supplied additionally with water that contained 0.33 μM-calcium lactate to prevent hypercalcaemia (Gemmill, 1958; Gadaleta *et al.*, 1975). The animals were weighed each week and used after 6 weeks only when the weight was constant at 190–210 g. Normal or sham-operated animals of this age weighed 300–320 g.

Tri-iodothyronine administration

Except where noted, tri-iodothyronine was administered in 10 mM-sodium/potassium phosphate buffer, pH 7.4, via the tongue veins at a dose of 1 nmol/100 g body wt. to animals anaesthetized by prior intraperitoneal injection of 4% (w/v) choral hydrate (1 ml/100 g body wt.).

Tissue fractionation

The livers were perfused free of blood by injecting about 60 ml of 37°C Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) which contained only half the recommended concentrations of $CaCl_2$ and $MgSO_4$ (Greene & Power, 1931) and had been pre-equilibrated with O_2/CO_2 (19:1). The livers were then quickly removed to 5 vol. of ice-cold 0.25 M-sucrose/1 mM-$MgCl_2$/$NaHCO_3$ buffer, pH 7.4 (Johnston *et al.*, 1968), minced and homogenized in a Teflon/glass homogenizer (0.07 mm clearance). The homogenates were centrifuged in the 8 × 50 ml rotor of an MSE 18 centrifuge by accelerating until 6000 rev./min (5000*g*) and then switching off. The pellet gave a crude nuclear fraction; the supernatant was then centrifuged at 17000 rev./min (35000*g*) for 1 min to yield the mitochondrial pellet. The postmitochondrial supernatant was centrifuged at 40000 rev./min (105000*g*) for 1 h at 4°C in the 40 rotor of a Beckman L2-65B to give the microsomal pellet and the final supernatant.

All pellets were washed twice in 3ml of buffer and finally suspended in 0.4ml of ice-cold buffer/g original wet tissue wt. Cross-contamination of the fractions was estimated by measuring marker compounds, which were assumed to have a unique distribution (Allfrey, 1959). DNA can be assumed to be totally nuclear within experimental error and was determined by the method of Burton (1956). Cytochrome *b*, present also in the microsomal fraction but in negligible quantities, was determined by the differential spectroscopic method of Chance & Williams (1955) and used as a mitochondrial marker. The microsomal marker used was glucose 6-phosphatase (EC 3.1.3.9; Hers *et al.*, 1951). This enzyme was assayed by a continuous spectrophotometric technique in which the glucose produced from added glucose 6-phosphate was detected by using glucose oxidase. To 200μl of glucose oxidase (10 units/ml)/2,2'-azinodi-(3-ethylbenzthiazoline)-6'-sulphonate (1mg/ml) (Boehringer, Mannheim, West Germany) were added 680μl of 0.13M-KCl/2mM-$MgCl_2$/2mM-EGTA/5mM-Tris/HCl (pH7.2), 100μl of 0.1mM-glucose 6-phosphate and finally 10–70μl of extract. The reaction was followed spectrophotometrically at 420nm, and was linear with enzyme or fraction amount up to rates of 2 absorbance units/min. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) was assayed by the method of Shonk & Boxer (1964) and marked the cytoplasmic fraction (De Duve & Berthet, 1954).

Chromatography of [^{131}I]tri-iodothyronine

To 0.5ml of each tissue fraction suspension in a 2.5ml Eppendorf centrifuge tube was added 0.25ml of 0.2M-$HClO_4$. The tube was shaken, left at 4°C for 3–4h and then centrifuged if necessary. The solution was neutralized with NaOH, and samples were applied to a 10cm×30cm sheet of 3 MM Whatman paper and developed for 16h in the upper layer of butan-1-ol/dioxan/2M-NH_3 (4:1:5, by vol.; Schorn & Winkler, 1965). Spots were located under u.v. light, and 2cm segments were assayed for radioactivity.

Preparation of mitochondria for O_2-uptake measurement

Livers were quickly removed after cervical dislocation into cold 0.25M-sucrose/10mM-KCl/20mM-Tris/HCl buffer, pH7.2, minced, washed with buffer and homogenized in a Teflon/glass homogenizer at about 2°C. The homogenate was centrifuged in the SS-34 rotor of the RC2-B Sorvall SuperSpeed by accelerating up to 6000rev./min (about 3000$g_{av.}$) and then switching off. The supernatant from this spin was then subjected to 17000rev./min (about 24000$g_{av.}$) for 1min in the same rotor. The mitochondrial pellet was washed twice in the homogenization buffer and finally resuspended at about 50mg of mitochondrial protein/ml in the same buffer.

O_2-uptake measurement

This was carried out as described previously (Mowbray, 1974) in 0.13M-KCl/2mM-$MgCl_2$/2mM-EGTA/5mM-Tris/HCl/2% defatted (Chen, 1967) albumin (pH7.2). The electrode was calibrated by adding spectrophotometrically standardized NADH to a 3μg/ml solution of phenazine methosulphate in the chamber: 1mol of O_2 is reduced/mol of NADH added (Stanley, 1972).

Determination of total purine nucleotide

Rats were killed by cervical dislocation, and 2–3g of liver was removed and immediately frozen with Wollenberger tongs precooled in liquid N_2. The frozen tissue was homogenized and the purine nucleotide content assayed as described by Bates *et al.* (1978).

Radioactivity assay

^{131}I in the tissue fractions was measured by liquid-scintillation counting (Mowbray, 1975). Unbound tri-iodothyronine in the soluble fraction was removed by first adsorbing it on dried talc (1g/10ml of solution) at 4°C (Rosselin *et al.*, 1966). ^{131}I on chromatograms was assayed in a Panax Gamma 160γ-ray counter. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.) dried to constant weight as standard.

Materials

L-[3'-^{131}I]Tri-iodothyronine (specific radioactivity 30mCi/mg) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and, before injection, 1.8ml of 0.1M-sodium/potassium phosphate buffer, pH7.4, was added to 500μCi contained in 3.2ml of 5% propylene glycol solution. Glucose 6-phosphate, glucose 1-phosphate, disodium ATP, disodium $NADP^+$ and disodium NADH were obtained from Boehringer; 3,5,3'-tri-iodo-L-thyronine (free acid), rotenone and bovine serum albumin (fraction V) were from Sigma Chemical Co.; Charcoal Norit LSX was supplied by Hopkin and Williams, and talc by BDH. All other materials were of analytical quality.

Results and Discussion

Animals and hormone administration

Most of the experiments were performed on thyroidectomized animals. Thyroidectomy was carried out in order to eliminate the presence of endogenous tri-iodothyronine or thyroxine and thus

decrease competition for serum and cellular binding sites.

As one aim of the work was to investigate the effects of tri-iodothyronine at short times after administration, the route taken by hormone between injection and killing of the animal could be crucial. Some of the variations in lag times observed by different workers with the same experimental system (e.g. Tata *et al.*, 1963; Hoch, 1968) may be attributable to differences in the times taken by the hormone after administration to reach the target tissue. Intraperitoneal injections were considered unsuitable. Tri-iodothyronine has a very low solubility in aqueous solution at pH values less than 8 and the absorption of micro-crystals from the peritoneal cavity might be spread over a considerable time period. Moreover a certain degree of liver surface contamination has to be expected from this method of administration. This latter is particularly important when hormone with a high specific radioactivity is injected, since errors of considerable magnitude might be introduced if liver is chosen for examination. Intravenous injection via the clearly visible lingual veins appeared to provide a rapid controlled administration of hormone which, by allowing immediate access to plasma carrier proteins, was as near physiological as possible.

The physiological concentrations of tri-iodothyronine in rat are very low (approx. 1 pmol/ml of plasma; Heninger *et al.*, 1963). It seemed possible that injecting such small amounts might not reproduce the physiological amounts required by the cell, especially since most tri-iodothyronine is probably produced peripherally from thyroxine (see above); moreover, non-specific adsorption might obscure the data significantly. We thus decided to supply tri-iodothyronine at a concentration equal to about the sum of the concentrations of tri-iodothyronine and thyroxine in euthyroid animals. According to Reichlin *et al.* (1972), thyroxine administered at 2μg (approx. 2.5 nmol)/100g body wt. reduced the high thyrotropin concentrations found in hypothyroid rats to normal. In rat, a specific thyroxine-binding globulin is absent (Tanabe *et al.*, 1970), and both forms of thyroid hormone are carried largely by albumin, a circumstance that is generally held to account for the fact that, in this species, the half-lives of tri-iodothyronine and thyroxine are not too different (Van Middlesworth, 1974). It would appear therefore that, in the absence of thyroxine, sufficient extra binding sites exist to accommodate increased plasma tri-iodothyronine.

Distribution of injected tri-iodothyronine among liver tissue fractions

L-Tri-iodothyronine labelled with ^{131}I (30μCi/μg) was administered intravenously (1 nmol/100g body wt.) to rats that had been thyroidectomized 6 weeks previously. At given times after the injection the animals were killed the livers perfused free of blood, homogenized and tissue fractions separated by differential centrifugation. The radioactivity and the protein content in each fraction were measured. Fraction purity was assessed by use of marker enzymes, and corrections were made for cross-contamination. The radioactivity was extracted from three separate samples of each fraction and its identity investigated by chromatography. Unlabelled carrier tri-iodothyronine was always added to each sample. On occasion, some loss of recovery was found when the [^{131}I]tri-iodothyronine used for injection was investigated. This loss was never greater than 10% of applied radioactivity and was presumed to be the result of oxidation during chromatography. The major radioactive contaminant co-chromatographed with iodide ion. Thus recoveries of around 90% from the fractions (Table 1) are considered to imply that no other labelled compound was present in substantial quantities. This was so in all fractions except the soluble fraction, where the recoveries were significantly lower ($P<0.005$ versus microsomal fraction and nuclei; $P<0.025$ versus mitochondria) and increased quantities of iodide were found. Despite the location of deiodinases predominantly in the microsomal fraction (Stanbury, 1960; Wynn *et al.*, 1962; Nagakagua & Ruegamer, 1967), microsomal radioactivity was still largely identified with tri-iodothyronine (Table 1); presumably radioiodide is readily lost to the soluble fraction. It is also feasible that the deiodinase activity is very low in thyroidectomized animals and was not significantly induced by 4h after tri-iodothyronine injection, by which time the microsomal fraction had acquired around 20% of the tissue tri-iodothyronine (Table 1). A comparably large proportion (31%) of liver [^{131}I]-thyroxine was found in this fraction by Shimada (1963) 3h after injecting this compound into normal rats.

Fig. 1 shows the tri-iodothyronine bound to the various liver fractions. The soluble fraction was treated with talc to remove any free hormone: about 30% was found to bind to the talc. Tri-iodothyronine appeared rapidly in all fractions, and in the case of nuclei and microsomal fraction showed an increasing trend but no substantial increase over the next 3h. By contrast, both the soluble fraction and the mitochondria showed some variation over the 4h period. The tri-iodothyronine content per mg of soluble protein ranged from 25 to 10 times that in any other fraction, and, although it showed a sharp fall between 15 and 16min, the proportion of hormone in this fraction remained unchanged. The principal reason for this apparent decrease in concentration is that the proportion of liver protein in the soluble fraction shows a dramatic increase between 15 and 60min after tri-iodothyronine injection. At 15min only

Table 1. *Distribution of tri-iodothyronine and total protein among the liver fractions*

The percentages of total tri-iodothyronine found in each fraction are quoted as means of findings for three animals at each time point. The values were calculated from the fraction radioactivity corrected for cross-contamination between fractions and from recovery of ^{131}I as [^{131}I]tri-iodothyronine (see the Experimental section). The average total tri-iodothyronine found was 929±26pmol ($n = 15$). The percentages of total radioactivity in each fraction co-chromatographing with authentic tri-iodothyronine and of fraction protein content are given as means±S.E.M. ($n = 15$). The protein content of the soluble fraction changed significantly between 15min and later times (see the text).

	Percentage of total tri-iodothyronine						
Fraction	15min	60min	120min	180min	240min	Percentage of total protein	Percentage recovery of ^{131}I as tri-iodothyronine
Nuclear	11.7	9.0	11.8	12.7	13.9	28.0±0.7	88.8±0.6
Mitochondrial	18.5	12.3	5.1	9.4	6.5	34.2±0.6	88.0±3
Microsomal	10.4	16.7	11.8	17.8	20.3	26.3±0.2	87.2±0.6
Soluble	59.4	62.0	71.3	60.1	59.3	11.7±0.3	84.0±1

6.8% (mean of three experiments) of the tissue protein was in the soluble fraction, whereas from 60min onwards the measured proportion was 12.9±0.3% ($n = 12$).

Although the early finding of most of the tri-iodothyronine in the soluble fraction was not unexpected, the predominance of this fraction over the whole 4h period was interesting. Of course, these data do not show whether the liver cytoplasmic tri-iodothyronine was turning over or static, and it is entirely possible that this apparently stable soluble-fraction pool results from a major role of liver in clearing plasma tri-iodothyronine (Gorman *et al.*, 1966). The finding of significant radioiodide in this fraction could support this. Oppenheimer & Surks (1974) have reviewed the large number of works dedicated to the quantitative aspects of thyroid-hormone distribution between the cell and the extracellular fluid, and the generally accepted view is that liver/plasma exchange of tri-iodothyronine is very rapid and apparently freely reversible.

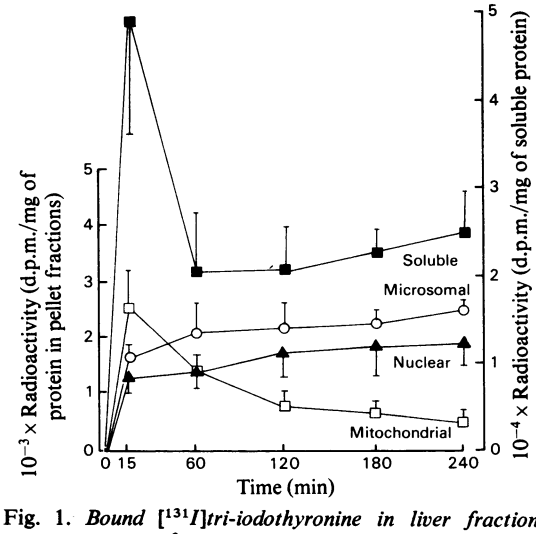

Fig. 1. *Bound [^{131}I]tri-iodothyronine in liver fractions after intravenous injection*

The values have been corrected for cross-contamination of tissue fractions and for recovery of radioactivity as [^{131}I]tri-iodothyronine (see the Experimental section; Table 1). The values are means from three experiments and the bars represent the range of values found. Free hormone was removed from the soluble fraction by adsorption on talc (see the text). The data for mitochondria (□), nuclei (▲) and the microsomal fraction (○) are plotted using the left-hand scale and that for the soluble fraction (■) using the right-hand scale.

Talc treatment shows that hormone in the soluble fraction was predominantly bound, presumably to protein. Care was taken to clear the liver of blood before homogenization, but it is possible that some plasma binding proteins in the interstitial spaces were poorly removed by perfusion. The existence of cytoplasmic binding proteins for thyroid hormones with properties apparently distinct from those in plasma has been reported in skeletal muscle (Tata, 1958, 1962), liver (Hamada *et al.*, 1970; Tata, 1975), kidney (Sterling *et al.*, 1974) and pituitary gland (Sufi *et al.*, 1973, 1975). On the other hand, Oppenheimer *et al.* (1976), using a titration technique, have claimed that no high-affinity limited-capacity sites, such as they have found for tri-iodothyronine in nuclei, exist in other liver cell fractions, although Tata (1975) has reported finding high-affinity stereospecific sites for tri-iodothyronine in all liver cell fractions, and Sterling (1976) has partially purified from rat liver and kidney mitochondria a thermostable lipoprotein with a very high affinity for this hormone. As tri-iodothyronine is extremely hydrophobic, the presence of hydrophilic carriers in cytoplasm would seem necessary in order to transport the very small quantities of hormone to specific intracellular sites. That interchange of tri-iodothyronine must occur between intracellular sites has been proposed by Oppenheimer *et al.* (1974) on the grounds that they could find no evidence that this

hormone is metabolized in nuclei. It seems not impossible that the high rate of binding to soluble proteins seen in this work may be related to an increased availability of binding sites in the cytosol of thyroidectomized animals, such as happens in the case of some steroid-hormone receptors when circulating hormone is low or absent (see Yamamoto & Alberts, 1976).

The most intriguing result is the observed time course of tri-iodothyronine binding to mitochondria (Fig. 1). By 15min this fraction had acquired more hormone per mg of protein than any other fraction except the soluble fraction and at this time had bound 18.5% of the tissue pool (Table 1), a greater proportion than nuclei, which are generally considered to be a major site of thyroid-hormone action, had acquired at any time in the 4h experiment. Rather surprisingly the mitochondrial tri-iodothyronine content decreased sharply by 60min and even further by 120min, so that after 2h only 5–10% of the tissue pool was in this fraction; and by 4h the mitochondrial content had fallen to 25% of the 15min concentration.

Other workers have found that mitochondria can acquire a significant proportion of administered thyroid hormone. Lee & Williams (1954) found 40% of liver radioactivity in mitochondria prepared 5min after intravenous (carotid vein) injection of [^{131}I]-thyroxine to normal adult rats, and Tata *et al.* (1962) found 18% of liver thyroxine and 11% of liver tri-iodothyronine in the mitochondrial pellet 2 days after intraperitoneal injection.

The possibility that when tissue was homogenized the hydrophobic tri-iodothyronine might have redistributed among the fractions appears to be obviated by the findings of different distributions at different times. The distribution does not coincide with protein content (Table 1) nor does it appear related to lipid content, assuming this to be lower in the soluble than in the membrane fractions. The probability is then that the distribution may be specific. The presence of tri-iodothyronine in all cell fractions is not at variance with a proposal that all of these may be specific sites of hormone action. However, the rapid acquisition by mitochondria of hormone in considerable quantities and the subsequent loss suggests that an important primary action may be exerted in mitochondria. The reason for the decreasing concentration of tri-iodothyronine in mitochondria from the earliest time point, despite the apparent continued availability of hormone in the cytoplasm (logically the precursor pool), is unknown. However, the phenomenon may not be unlike the refractoriness that target tissues can exhibit to repeated or prolonged doses of hormone.

These findings together with the emphasis on the rapid response of mitochondria reported by other workers (see e.g. Sokoloff & Roberts, 1974; Sterling 1976) suggested that it was important to investigate whether effects of tri-iodothyronine could be observed by testing mitochondrial function a short time after hormone administration.

Effect of tri-iodothyronine on oxidative phosphorylation

Three groups of rats were used: normal, thyroidectomized and thyroidectomized–tri-iodothyronine-injected. The injected animals were killed 15min after injection, mitochondria were prepared and O_2 consumption was measured polarographically. The nomenclature of Chance & Hollunger (1963) has been used to follow the mitochondrial oxidative phosphorylation, and the O_2 consumption is expressed as μg-atoms of O/min per mg of protein. The rate of respiration in State 3 in the presence of ADP together with either succinate or malate plus pyruvate represents respectively oxidation of $FADH_2$ or of NADH by the electron-transport chain stimulated by the phosphate acceptor. State 4 represents the rate of respiration after the phosphate acceptor is phosphorylated. The respiratory-control ratio is the ratio of respiration rate during stimulation by ADP and P_i over that in the presence of ATP (rate in State 3/rate is State 4). The P/O ratio is the mol of P_i esterified/g-atom of O consumed, and represents the efficiency of phosphorylation.

The values for P/O ratios obtained by the Chance & Williams (1955) technique are shown in Table 2. No significant or progressive difference was detected in P/O ratios determined by repeated ADP additions to the sample. Normal rats exhibited a P/O ratio of about 1.9 when succinate was used as a substrate and 2.7 when malate plus pyruvate were added. Rotenone was added when the substrate was succinate in order to avoid the oxidation of endogenous NADH. Thyroidectomized rats showed P/O ratios with either succinate or malate that were significantly lower than those for normal controls ($P \ll 0.001$). By contrast, the P/O ratios found with mitochondria from thyroidectomized animals that had received an intravenous dose of tri-iodothyronine 15min before being killed were indistinguishable from normal.

Mitochondria from both euthyroid and thyroidectomized animals showed respiratory-control ratios of 5 or greater, showing them to be tightly coupled. In thyroidectomized animals the P/O ratio had dropped to 55% of normal with $FADH_2$ substrate and 52% of normal with NADH substrate. The reason for this decreased phosphorylation is unknown. It does not seem likely that there could be complete loss of coupling at a single phosphorylation site. Although such a mechanism either at site 2 or site 3 could explain the succinate results, the same mechanism would predict a decrease in efficiency to 66% of normal with NADH substrates. The observed decrease in this latter case was, however, to 52% of the euthyroid

Table 2. *Effects of thyroidectomy and tri-iodothyronine administration on oxidative phosphorylation*

For definition of respiratory-control ratio, P/O ratio and States 3 and 4, see the text. Where appropriate, tri-iodothyronine (2 μg/100 g body wt.) was administered intravenously 15 min before the rats were killed. In addition to 5 μmol of each substrate, the electrode chamber, thermostatically controlled at 37°C, contained about 0.5 mg of mitochondrial protein in 3 ml of 0.13 M-KCl/2 mM-$MgCl_2$/2 mM-EGTA/5 mM-Tris/HCl/2% defatted albumin buffer, pH 7.3. The P/O ratio was determined as the mean of three State 3–State 4 transitions induced by adding 0.25 or 0.5 μmol of ADP (Chance & Williams, 1955). Rotenone (12 μg) was additionally present when succinate was the substrate. The values are means ± S.E.M. for five different mitochondrial preparations.

			Oxidation rate (μg atoms of O_2/min per mg of protein)		
	Substrate	P/O ratio	State 3	State 4	Respiratory-control ratio
Normal	Succinate	1.87 ± 0.01	1.41 ± 0.05	0.28 ± 0.04	5.1
	Malate+pyruvate	2.69 ± 0.04	0.98 ± 0.02	0.19 ± 0.03	5.1
Thyroidectomized	Succinate	1.02 ± 0.03	1.11 ± 0.04	0.18 ± 0.02	6.2
	Malate+pyruvate	1.40 ± 0.03	0.75 ± 0.03	0.12 ± 0.02	6.0
Thyroidectomized–tri-iodothyronine-injected	Succinate	1.76 ± 0.03	1.18 ± 0.01	0.20 ± 0.04	5.8
	Malate+pyruvate	2.67 ± 0.01	0.78 ± 0.03	0.15 ± 0.03	5.2

value, suggesting that there was more likely an equal loss of efficiency at all three coupling sites.

Even 6 weeks after thyroidectomy, the mitochondrial P/O ratio recovered within 15 min of a tri-iodothyronine injection, demonstrating a rapid control of phosphorylation efficiency in conjunction with the rapid acquisition of hormone by mitochondria. On the other hand, in State 3 the respiration rate was 79% ($FADH_2$) and 80% (NADH) of normal in thyroidectomized rats after tri-iodothyronine injection. This reduced capacity per mg of mitochondrial protein presumably reflects some adaptation to reduced activity and is not under rapid thyroid-hormone control. Chloral hydrate anaesthesia and sham injection of 10 mM-phosphate buffer had no detectable effect on liver mitochondria from normal or thyroidectomized rats (results not shown).

These findings are somewhat different from those of Hoch (1967), who reported that the high State-4 respiration and low respiratory-control ratios in mitochondria from livers of very young thyroidectomized rats were restored towards normal values 2 min after a very large dose of thyroxine (5.4 μg/g body wt.). However, he did find a decreased P/O ratio, which showed an increase over the following 3–4 h. By contrast, Heninger *et al.* (1970) found P/O ratios higher than normal with glutamate as substrate in liver mitochondria from thyroidectomized rats.

In much earlier work Tata *et al.* (1962, 1963) and Bronk & Bronk (1962) found the same P/O ratio with liver mitochondria prepared from either thyroidectomized or euthyroid animals. Possible reasons for this difference from our findings are that in these studies mitochondria were prepared in unbuffered sucrose and incubated at 30°C or less, which may account for the much lower oxidation rates reported. Further, the preparation of Bronk & Bronk (1962) is difficult to assess, since they found low P/O ratios even with normal animals (1.3–1.4 for succinate oxidation). Tata *et al.* (1963), unusually, used radioiodide administration to destroy the thyroid tissue. They found that the growth rate decreased by about 15% after 6 weeks, which, when compared with a decrease of more than 65% in this study (see also Bronk & Bronk, 1962), suggests that thyroidectomy may have been incomplete. Finally, in the light of the studies of Herd (1978), which suggest that tri-iodothyronine may regulate Ca^{2+} mobility in mitochondria, it is noteworthy that in these earlier studies no attempt was made to regulate Ca^{2+} concentration in the media.

Some evidence has been presented that mitochondrial exchange transport of ADP and ATP may be a major regulatory step in cellular energy production (Akerboom *et al.*, 1977). Thus one possible mechanism is that tri-iodothyronine might regulate ADP uptake by the mitochondria. In support of this, Babior *et al.* (1973) have found that, after intraperitoneal injection of a high dose of tri-iodothyronine (20 μg/100 g body wt.) to normal rats, an increase in the rate of ADP uptake by rat liver mitochondria isolated 3 days afterwards was observed. These workers say that the high dose led to no uncoupling. Further, Portnoy *et al.* (1973) found that ADP uptake in liver mitochondria from thyroidectomized rats was reduced compared with that in normal animals. Moreover, liver mitochondria, isolated from thyroidectomized rats 6 days after a physiological replacement dose of thyroxine (2 μg/g body wt.) was administered intraperitoneally, showed an increase in ADP uptake over that in untreated controls. Even though these findings were observed in liver mitochondria some considerable time after

hormone administration, it is possible that tri-iodothyronine could rapidly stimulate ADP uptake into mitochondria. Such an increased penetration rate would be expected to lead to an increased oxidation rate, however, rather than to an increase in P/O ratio in tightly coupled mitochondria. Just such an increased oxidation rate in proportion to increased ADP uptake was observed by Babior *et al.* (1973). By contrast, in this work no change in the oxidation rate was observed in response to tri-iodothyronine injection (Table 2). This by itself does not invalidate this mechanism, since it is perfectly possible that the increased oxidation rate is an adaptation which can be observed at much longer times than 15min after hormone administration and could account for the higher oxidation rate seen in mitochondria from normal animals (Table 2). Thus, admitting the possibility that ADP uptake could increase immediately and thus produce the 50% increase in P/O ratio observed, the fact that the oxidation rate is constant would imply that some other process using the high-energy intermediate in the absence of ADP has been inhibited. Such competition for high-energy intermediate has been observed in mitochondria, e.g. between ADP, Ca^{2+} transport and transhydrogenation (see Lehninger, 1962). If this were the case, however, there ought to be a greater degree of uncoupling in the absence of hormone, and this is contrary to our findings: in fact, these preparations show a high dependence on the presence of ADP for oxidation. Equally problematical would be a mechanism that involved impaired export of ATP in the absence of tri-iodothyronine. This would require an internal use of ATP (an ATPase) to lower the P/O ratio and would thus result in high State-4 respiration and a reduced respiratory-control ratio, which is again contrary to our findings (Table 2). Thus it seems doubtful whether the rapid alteration of adenine nucleotide translocation in mitochondria could provide a suitable mechanism to account for the observed results.

Although at the present state of knowledge of the mechanism of oxidative phosphorylation it may not be possible to suggest a molecular model to account for tri-iodothyronine control of phosphorylation efficiency, the general properties of such a model might be suggested. First, strict respiratory control by ADP is maintained and thus the lowered P/O ratio is unlike classical uncoupling. Second, energy conservation during oxidation appears to be reduced, since there is increased O_2 uptake/mol of ADP phosphorylated. Thus, in terms of the major hypotheses for oxidative phosphorylation, either the breakdown of a high-energy intermediate is catalysed by ADP, which fails to acquire a third phosphate in this process, or the protonmotive force is reduced. Interestingly, the uncoupling action of high thyroid-hormone concentrations might suggest that these molecules can act as proton ionophores at the mitochondrial membrane.

Addition of tri-iodothyronine to mitochondrial preparations in vitro

Since intravenous injection of tri-iodothyronine led to its rapid acquisition by mitochondria whose P/O ratio, impaired by thyroidectomy, was restored to normal within 15min, it was of obvious interest to investigate the effect of direct addition of hormone to preparations *in vitro*.

However, since mitochondria deteriorate drastically (increased uncoupling and ATPase activity) even at 0–4°C after about 2h, it seemed possible that incubation at high temperatures might lead to a much more rapid decline. This possibility was investigated experimentally by incubating mitochondria at 37°C in buffer and removing samples every 3min for P/O ratio determination. These preliminary experiments showed that there was no significant alteration in the ratio for about 25min of incubation. Thereafter, however, the deterioration was extreme and very rapid. Thus incubation of mitochondria with tri-iodothyronine *in vitro* could be for a maximum of about 15min before polarographic measurement; at longer incubation times any hormone effects might

Table 3. *Comparison of the P/O ratio and respiratory-control ratio obtained with succinate as substrate from isolated mitochondria of thyroidectomized rats after addition of tri-iodothyronine in vitro*

For definition of parameters see the text and legend to Table 2. Enough tri-iodothyronine was added to the incubations to give a final concentration of 1 μM. Each value is the mean ± S.E.M. for six different mitochondrial preparations.

Preparation	P/O ratio	Oxidation rate (μg-atoms of O/min per mg of protein) State 3	State 4	Respiratory-control ratio
Isolated mitochondria after 15min incubation with tri-iodothyronine	1.17 ± 0.01	1.17 ± 0.02	0.19 ± 0.04	6.3
Isolated mitochondria from homogenate incubated for 15min with tri-iodothyronine	1.71 ± 0.01	1.22 ± 0.01	0.22 ± 0.01	5.5

well be obscured by the impaired performance of the organelles.

Mitochondria, prepared from thyroidectomized animals as before, were incubated at a concentration of about 10 mg of protein/ml for 15 min in the presence of 1 μM-tri-iodothyronine. Samples were then removed to an oxygen electrode chamber, and the P/O ratio and respiratory-control ratio determined. The values obtained (Table 3) showed P/O ratio and the slightly raised respiratory-control ratio characteristic of mitochondria from untreated thyroidectomized animals (Table 2).

Liver homogenate, prepared as usual (see the Experimental section), was made 1 μM in tri-iodothyronine and incubated at 37°C for 15 min. The mixture (about 3 ml) was then cooled in ice and immediately subjected to centrifugation at 2°C. The mitochondria were separated and washed as described above. The coupled respiratory parameters of these mitochondria were clearly comparable (Table 3) with those from euthyroid animals or from thyroidectomized animals previously injected with tri-iodothyronine (Table 2).

The lack of effect of tri-iodothyronine on isolated mitochondria might be explained by the absence of some factor (or factors) necessary to allow the hormone to interact with mitochondria, whereas its presence in homogenate permits this. It seems possible that this extramitochondrial factor (perhaps cytosol binding protein) is necessary to take the hormone to the correct sites rather than to metabolize it, since tri-iodothyronine is readily recovered from mitochondria (see above, Table 1). Rabinowitz & Swift (1971) and Gross (1971) have presented evidence that in thyroidectomized tri-iodothyronine-injected rats a new population of mitochondria is accumulated in the cell, whereas the previous population undergoes a rapid degradation. According to this view the physiological changes observed after thyroid-hormone administration are the result of the nature of the new mitochondrial population and not due to metabolic modification of the previous one. These conclusions are not supported by the observation of a rapid restoration of phosphorylation efficiency, although it seems quite possible that adaptation to an increased metabolic rate may result in changes at longer times to the average mitochondrial enzyme complement.

Total rat liver nucleotide pool

Mitochondrial studies *in vitro* have always been criticized as being excessively artificial, and it seemed pertinent to ask whether such an increase in phosphorylation efficiency actually took place in the intact cell. A method of approaching an answer to the question seemed to lie in measuring the tissue concentrations of purine nucleotides, since under conditions of decreased phosphorylation efficiency, muscle systems show a decrease in ATP and ADP and an increase in AMP and IMP concentrations (Tornheim & Lowenstein, 1975). Thus livers from thyroidectomized animals 15 min after intravenous injection of either phosphate buffer or buffered tri-iodothyronine were freeze-clamped in liquid-N_2-cooled tongs, homogenized and the purine nucleotide concentrations in the extract determined by high-pressure liquid chromatography. This chromatographic method yields in a single run the concentrations of all purine and pyrimidine nucleotides in the extract (Perrett, 1976). The concentrations of purine nucleotides found in livers from thyroidectomized rats and thyroidectomized–tri-iodothyronine-treated rats are shown in Table 4. At 15 min after tri-iodothyronine injection the amount of ATP in the tissue extract of thyroidectomized animals showed a dramatic 85% increase over that in untreated animals. The concentrations of ATP found in rat liver freeze-clamped *in situ* are around 15 nmol/mg of protein (Start & Newsholme, 1968). This compares very well with the value found after hormone administration and demonstrates that one effect of thyroidectomy has been to lower the normal tissue ATP concentrations. The concentrations of AMP and ADP found *in situ* in the same study (Start & Newsholme, 1968) were respectively about 1 and 5 nmol/mg of protein; these are a little lower than those measured in this study after tri-

Table 4. *Total liver nucleotide pool in thyroidectomized rats before and 15 min after tri-iodothyronine injection*
The values are means ± S.E.M. for seven preparations from thyroidectomized animals and eight preparations from thyroidectomized–tri-iodothyronine-injected rats (2 μg/100 g body wt). The mean [ATP]/[ADP] ratio was calculated from the ratios found in each individual sample. All values in which untreated versus treated are compared by Student's *t* test, except for those of IMP, GDP and GTP, are significantly different ($P<0.001$).

	Content (nmol/mg of protein)						[ATP]/[ADP] ratio	Total purine (nmol/mg of protein)
	AMP	IMP	ADP	GDP	ATP	GTP		
Thyroidectomized	4.1 ± 0.6	3.1 ± 0.5	6.4 ± 0.3	0.5*	8.1 ± 0.7	0.5*	1.34 ± 0.09	23 ± 2
Thyroidectomized + tri-iodothyronine	2.4 ± 0.1	2.6 ± 0.5	7.3 ± 0.3	1.2*	15.0 ± 1.0	1.5 ± 0.1	2.22 ± 0.04	30 ± 2

* These values are approximate; in several extracts the amount of material present was too low for reliable determination.

iodothyronine treatment. The AMP content is significantly higher in the untreated animals, whereas the ADP content is lower, which may reflect a response of the adenylate kinase equilibrium to the lowering of the ATP concentration.

Although the total ADP content only slightly but significantly increased, the ATP content and thus the ATP plus ADP content was much higher after tri-iodothyronine treatment. This increase in total ATP plus ADP is only partially accounted for by a decrease in AMP content. Examination of the total purine nucleotide content in liver before and after injection (Table 4) shows that the hormone injection increased the pool size by 30%. The explanation for this increase in purine pool size is not clear. Incubation of rat hepatocytes with adenosine has been found to lead to a considerable increase in tissue adenine nucleotides (Lund *et al.*, 1975). Thus it is possible that *in vivo* tri-iodothyronine altered plasma adenosine concentration or transport into liver. Alternatively, steady-state perfused rat hearts have been observed to show systematic variations with time in the total adenine nucleotide pool; these variations are of the order of 30% of the total tissue pool and appear to suggest that some form of rapid storage facility may exist in heart (Bates *et al.*, 1978). Should liver also possess such a sequestration ability, it may explain the rapid alteration in overall purine content in these experiments. Another alternative is a rapid synthesis of purine *de novo*. This pathway would be expected to be more active in conditions where the purine concentrations are low, since purine nucleotides inhibit the rate-controlling enzyme (see Blakely & Vitols, 1968). However, it may be that the low energy status of the thyroidectomized liver is the limiting factor, and this is altered by the increased phosphorylation potential after hormone addition.

The [ATP]/[ADP] ratio, which after thyroidectomy was lowered (see Start & Newsholme, 1968) to 1.3, showed a highly significant increase to 2.2 after tri-iodothyronine treatment (Table 4). This result is what would be expected from an increase in the efficiency of phosphorylation. It thus correlates well with the effects of tri-iodothyronine found with isolated mitochondria and supports the contention that the mechanism by which the cellular adenine nucleotide concentrations are altered is by a direct rapid effect of tri-iodothyronine on mitochondria. The actual [ATP]/[ADP] ratio found is somewhat lower than that measured *in situ* in liver from normal animals (3.2) and is much more closer to that obtained with starved animals (2.2–2.3; Start & Newsholme, 1968).

General discussion

These findings on the role of mitochondria in the mechanism of the effect of thyroid hormone at short times after intravenous administration of tri-iodothyronine single out this organelle as a primary target for rapid thyroid-hormone action.

Comparison of the effects of tri-iodothyronine found at short periods with those at the much longer times used by many other workers (see Ingbar & Woeber, 1974) suggests that thyroid hormone could act at two different levels: one responsible for the primary effects of the hormone, such as an immediate action on microsomal or mitochondrial protein synthesis or on changes in ATP or energy production, though not all of these may be independent or primary effects; a second level might be adaptative responses to the hormone. These could, of course, be dependent basically on the primary effects of the hormone and not necessarily be an independent co-ordinated direct action. For example, one could speculate that the delayed response in RNA polymerase activity observed by Tata and his co-workers (see Tata, 1970) is secondary to increased mitochondrial energy provision, which produces an upward growth shift. In this view tadpole metamorphosis is a normal embryological development system temporarily arrested by a limited energy output. This is a problem common to attempts to understand the mode of action of all fast-acting hormones: virtually all of these, and insulin must be the best known, lead to considerable adaptations in target cells. These adapations appear to involve at least as great a contribution from protein degradation as from protein synthesis (Goldberg & St. John, 1976). Since protein degradation is greater on denatured enzymes, a well used enzyme has a longer life. Thus many adaptations may easily result directly from potentiation of a particular set of enzymes without a direct effect of the hormone on the transcription or translation system. So in this case it is possible that the multiplicity of action of thyroid hormones could be the result of one unitary command; and perhaps the main command centre is located in the mitochondrion.

The observed relatively slow effects on transcription and translation, together with the reports of the existence of specific nuclear binding sites, all reminiscent of steroid-hormone action, nevertheless suggest that an independent nuclear role may exist for thyroid hormone. Looked at teleologically, biological advantage accrues from being able to respond rapidly to new conditions: thus a stimulus that exerts a direct effect and in addition promotes increased adaptation through simultaneous effects on protein synthesis or degradation seems likely to have conferred success in evolution. Furthermore, part of the argument has been that tri-iodothyronine is not randomly distributed in lipophilic areas in the cell, but must be transported to its site of action. If this is so, then the nuclear hormone and microsomal hormone (? deiodinases) would be expected to have as distinct functions as mitochondrial tri-iodothyronine.

The finding of a rapid and transient influx of tri-iodothyronine to mitochondria suggested that this hormone might have a short-term rapid effect. This led us to examine mitochondrial function *in vitro* 15min after a hormone dose. However, some other factor is required in addition to mitochondria since incubation of mitochondria and tri-iodothyronine *in vitro* was ineffective in restoring lowered phosphorylation efficiency. The identity of the non-mitochondrial component required for the tri-iodothyronine effect on mitochondrial P/O ratio could be a cytosol receptor. However, these experiments do not exclude the rapid stimulation of other processes whose products mediate the change in phosphorylation efficiency.

Many effects previously claimed for thyroxine on mitochondria have not survived critical appraisal. It therefore seemed important to try to find some general effect of tri-iodothyronine on intact cells, which would show a rapid response to a change in phosphorylation efficiency if this did indeed occur *in vivo*. Just such an effect was found when the tissue content of purine nucleotides was investigated. Thus this study appears to present a consistent model for at least one rapid effect of tri-iodothyronine on rat liver, which by its nature must exert a very significant effect on the metabolism of the whole body.

We are very indebted to Dr. David Perrett, Medical Unit, St. Bartholemew's Hospital, West Smithfield, London EC1A 7BE, U.K. for performing the nucleotide analysis by high-pressure liquid chromatography. R.P.-R. was supported by an award from Consejo de Desarrollo Cientifico y Humanistico, Venezuela.

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