The Stimulation by Treatment *in vivo* with Tri-iodothyronine of Amino Acid Incorporation into Protein by Isolated Rat-Liver Mitochondria

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1. Normal and thyroidectomized rats were treated with near-physiological doses of tri-iodothyronine. Liver mitochondria were isolated and incubated with radioactive amino acids. In normal rats tri-iodothyronine caused only a slight stimulation of incorporation into mitochondrial protein, but in thyroidectomized animals the incorporation was doubled. 2. There was a lag period of about 36 hr. after injection and the maximum effect was observed after 2 days. 3. Direct addition of tri-iodothyronine to the incubation medium had no effect on mitochondrial incorporation. 4. The incorporation was not due to bacterial, nuclear, lysosomal or microsomal contamination and the labelled particles had sedimentation properties identical with those of mitochondria, as followed by suitable enzyme markers. 5. Thyroid hormone treatment did not cause any marked alterations in the pattern of labelling of submitochondrial fractions and in all cases the most radioactive protein was in an insoluble lipid-rich fraction. The amino acid compositions of the total mitochondrial protein and the more radioactive lipoprotein were also unaltered. 6. Increases in the content of RNA and various cytochromes per mg. of mitochondrial protein were observed after treatment with tri-iodothyronine. These occurred slightly later than the stimulation of amino acid incorporation. 7. No uncoupling of oxidative phosphorylation was observed and the ATP production per mg. of mitochondrial protein increased. 8. It was concluded that tri-iodothyronine stimulated amino acid incorporation into mitochondrial protein and that the result is consistent with the view that treatment with thyroid hormone results in an enhanced selective synthesis of mitochondrial respiratory units.

In a recent study of the action of thyroid hormones, Tata *et al.* (1963) showed that, if thyroidectomized rats were given a single physiological dose of tri-iodothyronine, a two- to three-fold stimulation of amino acid incorporation into the protein of isolated microsomal fractions took place. The stimulation was not immediate and reached its maximum about 2 days after the hormone was injected. At the same time the activity of several mitochondrial enzymes was raised and there was an increase in the overall capacity of the mitochondria to carry out both oxidation and phosphorylation. There was no evidence for uncoupling of oxidative phosphorylation or loosening of respiratory control with the doses of thyroid hormone used. These results suggested that the

increase in respiratory activity in mitochondria observed after treatment with thyroid hormone was not due to a direct interaction with the enzymes involved in oxidative phosphorylation but rather to stimulation in the rate of synthesis of mitochondrial respiratory proteins.

While these experiments were in progress, the incorporation of radioactive amino acids into the protein of isolated rat-liver mitochondria was being studied (Reis, Coote & Work, 1959; Roodyn, Reis & Work, 1961a; Roodyn, Suttie & Work, 1962; Roodyn, 1962). It therefore became possible to determine whether treatment with tri-iodothyronine stimulated amino acid incorporation into protein in isolated mitochondria, as well as in isolated microsomal fractions. Experiments similar to those of Tata *et al.* (1963) were carried out, and a doubling of incorporation was indeed observed in the mitochondrial fraction, as reported briefly by Freeman, Roodyn & Tata (1963). The effect is now described in more detail, together with

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evidence that the subcellular particles that are stimulated have sedimentation properties identical with those of rat-liver mitochondria.

METHODS AND MATERIALS

Animals and treatment with thyroid hormone. Male albino (Wistar) or hooded (Mill Hill) rats were used, with no apparent difference in response. All animals were fed *ad libitum* before the experiments. Thyroidectomy was carried out on rats weighing 50–60 g. by a combination of surgery and treatment with radioactive iodine. 3,3',5-Triiodo-L-thyronine was injected subcutaneously at various times before the experiment. The doses used were in the range 20–26 $\mu\text{g}/100$ g. body wt. The animals were weighed and the basal metabolic rate was determined periodically and before killing as described by Tata *et al.* (1963).

Isolation of subcellular fractions. Rats were killed by a blow on the head, usually followed by decapitation. The livers were rapidly removed and washed with ice-cold 0.15 M-NaCl, and the homogenate was fractionated as follows.

Nuclear and whole cell fraction was isolated as described in Roodyn *et al.* (1961a).

Mitochondria were generally isolated in 0.3 M-sucrose–2 mM-EDTA–0.03 M-nicotinamide, pH 7.2, as described by Roodyn *et al.* (1961a) except that the five successive sedimentations were carried out at 8000 *g* for 10 min. each.

Submitochondrial fractions were prepared as follows (Roodyn, 1962). Mitochondria were incubated with radioactive amino acid and then sedimented at 10 000 *g* for 10 min. The supernatant was taken as the 'freely soluble fraction' (see Table 6). The sediment, suspended in sucrose–EDTA–nicotinamide medium at a concentration of 7.0 mg. of protein/ml., was treated with a final concentration of 0.30% (w/v) Triton X-100 and centrifuged at 105 000 *g* for 60 min. The supernatant was taken as the 'detergent-soluble fraction'. The sediment was resuspended in sucrose–EDTA–nicotinamide medium at a concentration of 2 mg. of protein/ml. and treated at 0° for 15 hr. with 0.45% Triton X-100. The suspension was then centrifuged again at 105 000 *g* for 60 min. to give a 'lipid-rich extract' and an 'RNA-rich sediment'.

Microsomal and soluble fractions were prepared by two methods. In the first the sucrose–EDTA–nicotinamide medium was used. The supernatant after removal of the mitochondria was centrifuged at 105 000 *g* for 60 min.; the sediment was washed gently by swirling medium over it, and was taken as the microsomal fraction. The supernatant was taken as the 'soluble fraction' (or 'cell sap'). In the second method the microsomal and soluble fractions were prepared in 0.25 M-sucrose–0.075 M-KCl–0.01 M-MgCl₂–0.035 M-tris-HCl buffer, pH 7.8, as described in Tata *et al.* (1963).

Gradient differential centrifuging. Mitochondria were suspended in 2 ml. of sucrose–EDTA–nicotinamide medium and layered over 22 ml. of a linear gradient between 0.3 M-sucrose and 0.9 M-sucrose. All solutions contained 2 mM-EDTA and 0.03 M-nicotinamide. The tubes (8.90 cm. $\times$ 2.54 cm. diam.) were centrifuged for 20 min. at 3000 rev./min. (1700 *g*_{av}) in a swing-out head of the MSE Major refrigerated centrifuge (*R*_{max}. 21.0 cm., *R*_{min}. 13.5 cm.). After the centrifuging the bottom of the tube was pierced with a pin and the contents were allowed to drip through.

The average volume of each drop was calculated from the total number of drops and the volume of fluid.

Metabolic and enzyme determinations. Basal metabolic rate was determined as described by Tata *et al.* (1963). Succinoxidase was determined by the method of Schneider & Potter (1943) except that AlCl₃ was omitted from the reaction mixture. Esterification of inorganic phosphate (ATP) was measured either in the normal amino acid-incorporation medium (Table 8) or in the medium of Greengard, Minnaert, Slater & Betel (1959) (Fig. 2) by using glucose and hexokinase, followed by glucose 6-phosphate dehydrogenase, as described by Roodyn & Suttie (1961). For the P:O ratios given in Fig. 2 oxygen uptake was measured manometrically. Malate dehydrogenase (EC 1.1.1.37) was determined at pH 9.9 as described in Roodyn *et al.* (1962). Glucose 6-phosphatase (EC 3.1.3.9) was determined as described in Roodyn *et al.* (1961a). Acid phosphatase (EC 3.1.3.2) was estimated by the method of Gianetto & de Duve (1955). The enzyme was activated by freezing and thawing several times before the assay.

Cytochromes were determined as follows: two cuvettes of 4 cm. light-path were each filled with 3 ml. of mitochondrial fraction, 1 ml. of m-potassium phosphate, pH 7.2, 2 ml. of 1% (w/v) Triton X-100 and 7 ml. of sucrose–EDTA–nicotinamide medium. The lysed suspension in one cuvette was reduced with 2 mg. of sodium dithionite and the difference spectrum between the reduced and non-reduced suspensions was read on a Unicam SP.700 automatic recording spectrophotometer. The amounts of cytochrome were calculated by the method of Estabrook & Holowsky (1961).

Incorporation of radioactive amino acids into protein. Mitochondria were incubated with radioactive amino acid at 30° as described in Roodyn *et al.* (1961a) with medium B and potassium succinate as an energy source. The medium had the following final composition: 0.1 M-sucrose, 0.04 M-KCl, 1.3 mM-EDTA, 0.02 M-nicotinamide, 0.01 M-potassium succinate, 0.016 M-potassium phosphate 4 mM-AMP, 0.5 mM-NAD, 8 mM-MgSO₄ and 50 μg . of synthetic amino acid mixture/ml. It was adjusted to pH 7.2 and contained 4–6 mg. of mitochondrial protein/ml. Antibiotics were not added to the medium.

In some experiments the mitochondria were incubated in 3–6 ml. of medium in 25 ml. conical flasks gassed with oxygen, as described by Roodyn *et al.* (1961a). However, during the course of this work, the effect of conditions of incubation was studied in more detail. It was found that the incorporation rate was greatly affected by the depth of fluid in the incubation vessel, the shape of the flask, the rate of oxygenation and the rate of shaking. The experiments suggested that, in the rather dense suspensions of mitochondria used (approximately 5 mg. of protein/ml.), when 3–6 ml. of fluid was used adequate oxygenation of the particles could only be achieved by gassing the flasks with oxygen and shaking them rapidly. However, if the depth of fluid in the incubation flask was greatly reduced, by incubating smaller volumes of suspension in flat-bottomed specimen bottles, rapid shaking and gassing with oxygen were no longer required. At the same time, the method of plating of radioactive protein described by Mans & Novelli (1961) was examined and found to be suitable for plating mitochondrial protein. The following method of incubation and radioassay based on these observations was then developed.

'Cylinder-plating' method of incubation and radioassay. A portion (0.4–0.6 ml.) of mitochondrial suspension was incubated in air at 30° in flat-bottomed specimen bottles (2.2 cm. diam. × 5 cm. long) at a shaking rate of 80 cycles/min. At suitable times, the incorporation was stopped by rapidly immersing the bottles in ice. A cylinder of Whatman no. 3 filter paper, rolled from a rectangle 9 cm. × 3 cm. and previously impregnated with a solution of non-radioactive amino acid (10 mg./ml.), was dropped into the bottle, so that as the suspension soaked into the paper any further increase in the radioactivity of the protein was effectively stopped. The inside of the bottle was wiped clean by the paper held in forceps and the cylinder was then dried in a stream of warm air. The various paper cylinders were then dropped into 5% (w/v) trichloroacetic acid containing 1 mg. of carrier amino acid/ml. After at least 60 min. at 0° in the solution the papers were washed with 5% trichloroacetic acid at room temperature and then treated with 5% trichloroacetic acid at 90° for 30 min., twice with ethanol-ether (1:1, v/v) for 30 min. at 40° and twice with anhydrous ether at room temperature for 15 min. The papers were dried in air and then placed in bottles filled with scintillation fluid (see below). There was little evidence for loss of protein from the paper rectangles during the washing procedure. In fact, it was difficult to remove the protein dried on to the paper, even by washing with 0.1 N-NaOH. Counts/min./mg. of protein were therefore calculated from protein N determinations on separate samples of the mitochondrial suspension and thus refer to the amount of protein added at the beginning of the experiment. Zero-time assays (usually between 5 and 15 counts/min.) were subtracted from all readings.

Incorporation into isolated microsomal fractions after incubation with radioactive amino acid, cell sap and energy source was studied as described in Tata *et al.* (1963).

Chemical determinations. Amino acid analyses were kindly carried out by Dr S. Jacobs at this Institute with a Spinco-Beckman Automatic Amino Acid Analyser. Protein from intact mitochondria, or submitochondrial fractions (Table 7), was hydrolysed in sealed tubes at 105° for 15 hr. in 6 N-HCl. The hydrolysate was filtered through Whatman no. 50 paper to remove humin and dried *in vacuo* over P₂O₅. Separate tryptophan determinations were not carried out. RNA and protein were determined as described in Roodyn *et al.* (1961a). DNA was determined by the method of Burton (1956), a sample of purified calf-thymus DNA being used as a standard.

Radioactive assay. Apart from when the cylinder-plating method was used radioactive protein was isolated by the method of Simkin & Work (1957). It was either counted at infinite thickness as described in Roodyn *et al.* (1961a) or, more usually, in a Packard Tri-Carb series 314E liquid-scintillation counter. The protein was dissolved in 98% (w/v) formic acid and a sample removed for protein determination by the micro-Kjeldahl method. (The formic acid was removed by gentle heating before digesting with conc. H₂SO₄ and catalyst.) A second sample was dried on Whatman no. 3 paper rectangles (9 cm. × 3 cm.). The rectangles were rolled into cylinders and dropped into counting bottles filled with scintillation fluid. This was dry AnalaR toluene containing 0.5% (w/v) of 2,5-diphenyloxazole and 0.5% of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene obtained from the Packard Instrument Co. Inc., La Grange, Ill., U.S.A. The orientation of the paper in the

bottle appeared to have no effect on the counting efficiency. The specific radioactivity of a sample of radioactive mitochondrial protein was determined by counting at infinite thickness with an end-window counter calibrated by a polymethacrylate standard as described in Roodyn *et al.* (1961a). A known weight was then dissolved in formic acid, dried on to paper and counted in the scintillation counter with a gate setting of 9–100, a gain of 13 and a high-voltage setting of 1050 v. It was found that 1 μμc gave 1.6 counts/min. The efficiency of counting radioactive protein was therefore about 70%, which agreed with the value obtained with the [¹⁴C]benzoic acid standard supplied by the manufacturers. The background was 30 counts/min.

Other methods and materials. Bacterial counts were carried out by plating on blood-agar as described in Roodyn *et al.* (1961a). Radioactive amino acids were obtained from The Radiochemical Centre, Amersham, Bucks. The following were used: universally labelled L-[¹⁴C]valine (6.53 μc/μmole), uniformly labelled L-[¹⁴C]isoleucine (7.30 μc/μmole) and DL-[1-¹⁴C]leucine (7.25 μc/μmole). Purified hexokinase and glucose 6-phosphate dehydrogenase were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

RESULTS

Stimulation of mitochondrial amino acid incorporation by 3,3',5-tri-iodo-L-thyronine. Liver mitochondria isolated from thyroidectomized rats incorporated radioactive amino acid into protein less actively than mitochondria from normal rats. If the thyroidectomized rats had been treated 2 days previously with a single injection of tri-iodothyronine, however, the incorporation was approximately doubled and was then considerably greater than the normal (Table 1). A much smaller stimulatory effect was observed when normal animals were treated with the same amount of hormone (Fig. 1). Treatment with thyroid hormone stimulated both the initial rate of amino acid incorporation and the final level of radioactivity reached after incubation for 3 hr. The stimulation in incorporation is not observed immediately after treatment of thyroidectomized animals with tri-iodothyronine, but shows a lag period of about 36 hr. The incorporation activity then rises rapidly and reaches a maximum about 43–50 hr. after treatment (Fig. 2). At this time a 20% stimulation in basal metabolic rate was observed (Table 1). However, there was no detectable increase in liver weight (although 96 hr. after treatment with tri-iodothyronine a 10% increase in liver weight could be detected). Stimulation in microsomal incorporation was also observed at a similar time, in confirmation of the results of Tata *et al.* (1963).

Evidence that the effect is on mitochondria. Previous experiments had shown that it was unlikely that the incorporation of amino acid into protein observed under the conditions used in this

Table 1. *Effect of tri-iodothyronine on amino acid incorporation into protein of isolated liver mitochondria*

Results are given as percentages of values with thyroidectomized animals $\pm$ S.E.M. The numbers of experiments are given in parentheses. For the amino acid incorporation mitochondria were incubated under oxygen in conical flasks in the presence of $0.1 \mu\text{C}$ of [^{14}C]valine/ml.

State of animals used	Basal metabolic rate	RNA content of mitochondria	Amino acid incorporation
Thyroidectomized	100	100	100
Normal	124 ± 1.1 (3)	130 ± 3.7 (4)	126 ± 11.9 (5)
Thyroidectomized treated 43–48 hr. previously with tri-iodothyronine	120 ± 4.7 (7)	102 ± 1.9 (5)	201 ± 17.1 (10)
Absolute value with thyroidectomized animals	1.24 ml. of $\text{O}_2/\text{g.}/\text{hr.}$	15.8 $\mu\text{g.}/\text{mg.}$ of protein	35.2 $\mu\text{C}/\text{hr.}/\text{mg.}$ of protein

Fig. 1. Stimulation of amino acid incorporation into protein of isolated rat-liver mitochondria by 3,3',5-tri-iodo-L-thyronine. Expt. 1: ●, thyroidectomized rats; ○, thyroidectomized rats treated 2 days before with tri-iodothyronine; Δ, normal rats; ▲, normal rats treated 2 days before with tri-iodothyronine. Mitochondria were incubated with $0.1 \mu\text{C}$ of [^{14}C]valine/ml. under oxygen in conical flasks. Expt. 2: □, thyroidectomized rats; ■, thyroidectomized rats treated 2 days before with tri-iodothyronine. Mitochondria were incubated in air in specimen bottles with $0.1 \mu\text{C}$ of [^{14}C]valine/ml. and radioactive protein was assayed by the cylinder-plating method.

Fig. 2. Time-course of effect of tri-iodothyronine on amino acid incorporation and RNA content of mitochondrial and microsomal fractions. ●, Amino acid incorporation into protein of mitochondrial fraction; ○, amino acid incorporation into protein of microsomal fraction; ▲, RNA ($\mu\text{g.}/\text{mg.}$ of protein) in mitochondrial fraction; Δ, RNA ($\mu\text{g.}/\text{mg.}$ of protein) in microsomal fraction; □, basal metabolic rate; ■, P:O ratio during oxidation of succinate by mitochondria incubated in medium of Greengard *et al.* (1959). Values with untreated animals were: mitochondrial amino acid incorporation (carried out under oxygen in the presence of $0.1 \mu\text{C}$ of [^{14}C]valine/ml.), $48.7 \mu\text{C}/\text{mg.}$ of protein/hr.; microsomal incorporation (carried out in the presence of $0.48 \mu\text{C}$ of [^{14}C]valine/ml.), $279 \mu\text{C}/\text{mg.}$ of protein/10 min.; mitochondrial RNA, $17.6 \mu\text{g.}/\text{mg.}$ of protein; microsomal RNA, $196 \mu\text{g.}/\text{mg.}$ of protein; basal metabolic rate, 1.40 ml. of $\text{O}_2/\text{g.}/\text{hr.}$; P:O ratio, 2.00.

study was due to microsomal or bacterial contamination (Roodyn *et al.* 1961a). However, since treatment with thyroid hormone appeared to affect the activity of several subcellular fractions (Tata *et al.* 1963) it was important to establish that the incorporation was due to mitochondria and not to contamination.

Some experiments carried out with mitochondria from normal rats are relevant to this problem (Table 2). The incorporation was reduced by 30%

by isolation in the presence of 10 mM-EDTA (Expt. 1), a concentration which could seriously affect microsomal incorporation (see below). It is also inhibited by chloramphenicol (Expt. 2), which does not affect microsomal incorporation (Rendi, 1959; Mager, 1960). The incorporation is seriously affected by the osmolality of the isolation medium, being reduced in hypo- or hyper-osmotic media

Table 2. *Further studies on the amino acid-incorporating system of mitochondria from normal rats*

Mitochondria were incubated in air in specimen bottles in the presence of $0.1 \mu\text{C}$ of radioactive amino acid/ml. Radioactive protein was assayed by the cylinder-plating method.

Expt. no.	Radioactive amino acid used	Description of expt.	Amino acid incorporation ($\mu\text{C}/\text{mg. of protein/hr.}$)	Percentage of control value
1	Isoleucine	Mitochondria isolated in: (a) Normal medium (b) $0.3 \text{ M-sucrose}-0.03 \text{ M-nicotinamide}-10 \text{ mM-EDTA}$	99.7 69.3	69.5
2	Leucine	Mitochondria incubated in: (a) Normal medium (b) Chloramphenicol added ($1 \mu\text{mole}/\text{ml.}$)	75.5 8.0	10.6
3	Isoleucine	Mitochondria isolated in: (a) $0.05 \text{ M-sucrose}-0.03 \text{ M-nicotinamide}-2 \text{ mM-EDTA}$ (b) $0.20 \text{ M-sucrose}-0.03 \text{ M-nicotinamide}-2 \text{ mM-EDTA}$ (c) $0.30 \text{ M-sucrose}-0.03 \text{ M-nicotinamide}-2 \text{ mM-EDTA}$ (d) $0.50 \text{ M-sucrose}-0.03 \text{ M-nicotinamide}-2 \text{ mM-EDTA}$	18.5 50.9 60.9 38.7	30.3 83.6 100.0 63.5
4	Valine	Mitochondria incubated in: (a) Normal medium (b) Triton X-100 added (0.005% , w/v) (c) Triton X-100 added (0.05% , w/v)	49.5 22.8 0.0	46.0 0.0
5	Valine	Mitochondrial suspension: (a) Not treated (b) Homogenized for 3 min. in Waring Blendor	41.0 3.2	7.8
6	Valine	Mitochondria incubated in: (a) Normal medium (b) Tri-iodothyronine added ($0.001 \mu\text{M}$) (c) Tri-iodothyronine added ($0.1 \mu\text{M}$) (d) Tri-iodothyronine added ($10 \mu\text{M}$)	38.1 39.1 38.8 38.8	102.5 100.2 100.2

(Expt. 3). It is also sensitive to damage by excessive homogenization or by disruption with the neutral detergent Triton X-100 (Expts. 4 and 5). These properties are consistent with the properties of mitochondria. It may be noted that the direct addition of a range of concentrations of tri-iodothyronine (0.001 – $10 \mu\text{M}$) has no effect at all on incorporation (Expt. 6).

The effect was examined of incubating non-mitochondrial fractions in the medium normally used for studying incorporation with mitochondria. Nuclear, microsomal and soluble fractions, as well as the washings of the mitochondrial fraction, were isolated in sucrose-EDTA-nicotinamide medium and incubated with radioactive amino acid. In the absence of added mitochondria negligible incorporation was observed in all fractions, apart from the crude nuclear fraction which was heavily contaminated with mitochondria. (The nuclear fraction incorporated $21.8 \mu\text{C}/\text{mg. of protein/hr.}$ whereas the mitochondria alone incorporated $88.1 \mu\text{C}/\text{mg. of protein/hr.}$) When mitochondria were added as a source of energy (cf. Siekevitz, 1952) the nuclear fraction, mitochondrial washings and soluble

Fig. 3. Amino acid incorporation of mitochondria isolated at different centrifugal forces. ○, Total radioactivity in protein after incubation of fraction; Δ, total protein in fraction; ●, specific radioactivity of fraction. A rat-liver homogenate was centrifuged at $600 g$ for 15 min. and the supernatant centrifuged at increasing g values. Each pellet was washed a further three times by sedimenting at $20000 g$ for 10 min. and incubated in air with $0.1 \mu\text{C}$ of [^{14}C]isoleucine/ml. Cylinder plating was used for radio-assay of protein. $1 \text{ kg.-min.} = 10^{-3} \times [\text{centrifugal force (g)} \times \text{time of centrifuging (min.)}]$.

Table 3. *Deoxyribonucleic acid and bacterial content of mitochondrial fractions from rats treated with tri-iodothyronine*Mitochondria were incubated under oxygen in conical flasks in the presence of 0.1 μ C of [14 C]valine/ml.

Expt. no.	State of animals used	Time after tri-iodothyronine (hr.)	Amino acid incorporation (μ μ C/hr./mg. of protein)	DNA (μ g./mg. of protein)	10 ⁻³ $\times$ Bacterial colonies/ml. of suspension
1	Normal	—	28.9	25.4	—
	Normal treated with tri-iodothyronine	48	43.8	17.4	—
	Thyroidectomized	—	20.9	11.5	176.8
	Thyroidectomized treated with tri-iodothyronine	48	49.9	8.0	23.6
2	Thyroidectomized	—	45.0	7.1	1.0
	Thyroidectomized treated with tri-iodothyronine	22	55.0	4.3	5.5
		37	59.0	5.4	2.0
		47	91.0	5.9	1.2
		70	104.0	5.2	1.2
		95	64.8	10.7	1.8
3	Normal	—	22.7	1.2	—
	Thyroidectomized	—	18.1	2.1	
	Thyroidectomized treated with tri-iodothyronine	35	31.6	3.2	
		39	41.2	1.7	
		43	36.4	7.3	
		48	38.4	2.0	

Table 4. *Effect of tri-iodothyronine on incorporation in different media*

Microsomal fractions were isolated as described in the Methods and Materials section. The medium designed for mitochondrial incorporation was medium B (Roodyn *et al.* 1961a) and contained 0.1 μ C of [14 C]valine/ml. Incubation was in air in specimen bottles for 1 hr. and radioactive protein was assayed by the cylinder-plating method. Incubation in the medium designed for microsomal incorporation is described in the Methods and Materials section and was for 15 min. in the presence of 0.48 μ C of [14 C]valine/ml.

Expt. no.		Incorporation into protein ($\mu\mu\text{C}/\text{mg.}$)		$\frac{B}{A}$
		Thyroidectomized rats (<i>A</i>)	Thyroidectomized rats treated 2 days previously with tri-iodothyronine (<i>B</i>)	
	Incubation in medium designed for mitochondrial incorporation:			
1	Mitochondria alone	22.6	47.3	2.09
2	Mitochondria plus 17 $\mu\text{g.}$ of ribonuclease/ml.	20.2	58.8	
3	Mitochondria plus 0.5 mm-2,4-dinitrophenol	5.9	7.3	
	Incubation in medium designed for microsomal incorporation:			
4	Microsomal fraction isolated in sucrose-EDTA-nicotinamide	10.0	10.0	2.56
5	Microsomal fraction isolated in sucrose-KCl-MgCl ₂ -tris	353.0	903.0	
6	Microsomal fraction from Expt. 5 plus 17 $\mu\text{g.}$ of ribonuclease/ml.	0.0	0.0	
7	Mitochondrial fraction	0.0	0.1	

fraction showed little additional total incorporation, after correction for the activity due to the added mitochondria. The incorporation in the microsomal fraction in the first 30 min. was increased somewhat by adding mitochondria but by 60 min. little effect could be seen. If the increased activity observed in each fraction on adding mitochondria was corrected for mitochondrial activity and expressed per mg. of non-mitochondrial protein it is found that the mitochondrial fraction was the most active under the conditions of isolation and incubation used. This 'corrected' activity was as follows ($\mu\mu\text{C}/\text{mg. of protein/hr.}$): nuclear fraction 9.4, mitochondrial washings 22.9, microsomal fraction 14.3, soluble fraction 0.0 and the original mitochondrial fraction 88.1.

Attempts were made to separate 'heavy' and 'light' mitochondrial fractions to determine whether lysosomes contributed to the incorporation. Isolation of the mitochondria over a wide range of centrifugal force gave fractions of similar specific radioactivity. There was no indication that the less easily sedimented fractions, which would be enriched in lysosomes (Appelmans, Wattiaux & de Duve, 1955), had any greater activity (Fig. 3).

The above experiments were with mitochondria from normal rats. Experiments with normal or thyroidectomized rats previously treated with thyroid hormone also showed that non-mitochondrial contamination did not contribute seriously to the incorporation. There was no evidence for an increase in either nuclear contamination (as measured by DNA) or bacterial contamination during stimulation by thyroid hormone (Table 3), and there was no correlation between amino acid incorporation and the content of DNA or bacteria in the various mitochondrial fractions studied.

Mitochondria were incubated in a medium normally used for microsomal incorporation, fractions from thyroidectomized rats treated 2 days before with tri-iodothyronine being used (Table 4). Negligible activity in the microsomal medium was observed (Expt. 7). It was shown above that the microsomal fraction isolated in sucrose-EDTA-nicotinamide medium incorporated poorly under conditions designed for mitochondrial incorporation. Microsomal fractions isolated in this medium also incorporate poorly even when incubated under conditions designed for studying microsomal incorporation (Table 4, Expt. 4) and have presumably been damaged by EDTA in the isolation medium. The most striking difference in the two systems, however, is in their response to ribonuclease, which completely abolished the microsomal incorporation (Allfrey, Daly & Mirsky, 1953) but did not affect the mitochondrial system (Table 4, Expts. 2 and 6). That the mitochondrial incorporation is still dependent on oxidative phosphorylation

after thyroid treatment is shown by the inhibition with 2,4-dinitrophenol (Expt. 3).

Positive correlation between mitochondria and incorporation was seen when the mitochondrial fraction incubated *in vitro* with radioactive amino acid was analysed by gradient differential centrifuging (see Anderson, 1955, for terminology). The radioactive protein sedimented very closely with the mitochondria, which were followed by malate

Fig. 4. Gradient differential centrifuging of mitochondria from thyroidectomized rats treated with tri-iodothyronine. □, Acid phosphatase; ■, glucose 6-phosphatase; ▲, malate dehydrogenase; ○, protein; ●, radioactivity in protein; △, succinoxidase. Thyroidectomized rats were treated with tri-iodothyronine and 48 hr. later liver mitochondria were isolated, incubated with $2 \mu\text{C}$ of DL- ^{14}C leucine in air for 30 min. and analysed on the sucrose gradient. C/C_{av} is the amount/ml. in the fraction (C) divided by the average amount/ml. in all fractions (C_{av}). Relative concentration is the amount/mg. of protein in the fraction divided by the amount/mg. of protein in the original mitochondrial fraction. C_{av} values/ml. were: acid phosphatase, $2.80 \mu\text{moles of phosphate/hr.}$; glucose 6-phosphatase, $1.02 \mu\text{moles of phosphate/15 min.}$; malate dehydrogenase, 0.95 unit ; protein, 1.08 mg. ; radioactivity, $70.5 \mu\mu\text{C}$; succinoxidase, $602 \mu\text{l. of O}_2/\text{hr.}$

dehydrogenase and the succinate-oxidase system as markers (Fig. 4a). Microsomal material, followed by glucose 6-phosphatase as a marker, and lysosomes, followed by acid phosphatase, sedimented in a different way from radioactive protein, and most of the activity remained above the mitochondrial peak. This difference and the close correlation between mitochondria and incorporation is shown more effectively in Fig. 4(b), when the results are expressed as relative concentrations (i.e. activity per mg. of protein in the fraction divided by activity per mg. of protein in the total mitochondrial fraction). Since total protein had a similar sedimentation pattern to the mitochondria and the radioactive protein, most of the protein in the original mitochondrial fraction was clearly due to mitochondria. The relative concentrations of the various mitochondrial markers and radioactive protein are near to 1.0 along the entire gradient. However, those of glucose 6-phosphatase and acid phosphatase range from 0.6 to 0.4.

Some separation between bacteria present in the mitochondrial fraction and radioactive protein was observed in an experiment in which only four fractions were taken from the gradient (Table 5), and there was also some indication of separation of DNA and radioactive protein.

Since the close correlation between mitochondria and radioactive protein along the gradient (Fig. 4) had been observed with mitochondria from the thyroidectomized rats given tri-iodothyronine 2 days previously, there is little doubt that the effect of thyroid hormone is on mitochondrial amino acid incorporation.

Studies on the mechanism of the stimulating effect of tri-iodothyronine. (a) *Effect on the endogenous pool of amino acids.* If treatment with thyroid hormone lowered the concentration of intramitochondrial amino acids the specific radioactivity of the incorporated amino acid might increase because of less dilution of added radioactive tracer. More

radioactive protein might then result without any increase in the absolute number of molecules of amino acid incorporated. An attempt was made to measure the total pool size of intramitochondrial amino acids by deproteinization and ninhydrin assay on the protein-free extract. However, the results were erratic and not reproducible. Such measurements are subject to several errors, not the least being that the pool with which the added amino acid equilibrates need not be identical with the total pool of 'free' or 'acid-soluble' amino acids. Also the pool will alter during incubation (Alberti & Bartley, 1963).

An attempt was made to overcome this difficulty by increasing the concentration of exogenous radioactive amino acid and so diminish the effect of dilution by any endogenous amino acid remaining after the repeated washing used during the isolation of the mitochondria. From the data on pool sizes of intramitochondrial amino acids given by Roodyn *et al.* (1961a) and Truman & Korner (1962) it was calculated that the concentrations of endogenous valine and leucine were not greatly different from the concentrations of amino acid usually added. A tenfold increase in concentration of exogenous amino acid had very little effect on the ratio of incorporation in tri-iodothyronine-treated animals to that in control animals. In one experiment this ratio was 1.45 when mitochondria were incubated in the presence of 0.034 μ mole of added valine/ml. and was 1.46 in the presence of 0.32 μ mole/ml. In a similar experiment with leucine, raising the concentration of added amino acid from 0.028 to 0.35 μ mole/ml. only changed the ratio from 2.99 to 3.04. This suggests that it is unlikely that the effect of thyroid hormone is due to changes in the intramitochondrial pool of amino acids.

(b) *Labelling of protein in submitochondrial fractions.* The distribution of radioactive protein in the major submitochondrial fractions of mitochondria from control and tri-iodothyronine-treated animals

Table 5. *Sedimentation of bacteria, deoxyribonucleic acid and radioactive protein in a sucrose-density gradient*

Mitochondria were incubated with 2 μ c of DL-[¹⁴C]leucine/ml. in air for 1 hr. They were sedimented for 10 min. at 8000 g and analysed by gradient differential centrifuging. (To avoid disturbing the small pellet at the bottom of the tube, fractions were collected from above with a bent-tip Pasteur pipette.)

Fraction no.	Distance from meniscus (cm.)	Protein (mg./fraction)	Radioactive protein		Bacterial colonies		DNA	
			Total (μ c)	(μ c/mg. of protein)	Total per fraction	Per mg. of protein	Total (μ g.)	(μ g./mg. protein)
1	0-1.7	1.22	50.2	41.1	58	5	11.6	9.5
2	1.7-3.8	3.21	239.0	74.0	741	23	16.1	5.0
3	3.8-5.0	0.92	48.6	53.0	552	600	15.5	16.9
4	Pellet	1.60	65.1	40.8	1900	1180	33.6	21.0

was similar to that obtained by Roodyn (1962). No drastic alterations in the pattern of labelling occurred (Table 6) and in all cases the most radioactive protein was in the lipid-rich extract obtained after prolonged treatment with the detergent

Triton X-100. This extract contains normally insoluble proteins associated with the mitochondrial membrane (Roodyn, 1962).

(c) *Amino acid composition of labelled proteins.* There were no marked effects of treatment with

Table 6. *Effect of tri-iodothyronine on the radioactivity of proteins in submitochondrial fractions*

The basal metabolic rates of the three groups were *A* 1.68, *B* 1.36 and *C* 1.58 ml. of O₂/g./hr. Mitochondria were incubated under oxygen in conical flasks in the presence of 0.1 μ C of [¹⁴C]valine/ml. and then subfractionated with detergent as described in the Methods and Materials section. Values in parentheses are relative concentrations, i.e. radioactivity/mg. of protein in the subfraction divided by radioactivity/mg. of protein in the appropriate total mitochondrial fraction. (See Table 7 for amino acid composition of proteins in lipid-rich extracts.)

Fraction	Radioactivity in protein (μ C/mg. of protein/hr.)			$\frac{C}{\bar{B}}$
	Normal rats (<i>A</i>)	Thyroidectomized rats (<i>B</i>)	Thyroidectomized rats treated 2 days previously with tri-iodothyronine (<i>C</i>)	
Total mitochondrial fraction	23.6 (1.00)	20.5 (1.00)	43.4 (1.00)	2.11
'Freely soluble' fraction	0.0 (0.00)	3.1 (0.15)	4.7 (0.11)	1.51
'Detergent-soluble' fraction	15.4 (0.65)	13.4 (0.65)	28.5 (0.66)	2.13
Lipid-rich extract	92.8 (3.91)	60.9 (2.96)	118.0 (2.72)	1.94
RNA-rich sediment	47.4 (2.00)	42.5 (2.06)	71.1 (1.64)	1.67

Table 7. *Effect of treatment with tri-iodothyronine on the amino acid composition of protein in submitochondrial fractions*

See Table 6 for preparation of lipid-rich extract.

Amino acid	Percentage of total amino acids determined			
	Thyroidectomized animals		Thyroidectomized animals treated 2 days previously with tri-iodothyronine	
	Total mitochondrial protein	Protein in lipid-rich extract	Total mitochondrial protein	Protein in lipid-rich extract
Alanine	9.80	9.80	9.35	9.95
Arginine	4.56	4.38	5.20	4.92
Aspartic acid	10.35	9.98	10.05	8.58
Cystine	0.21	0.57	0.30	0.21
Glutamic acid	11.15	10.71	11.00	11.30
Glycine	9.15	9.57	8.83	9.38
Histidine	2.49	2.10	2.32	2.40
Isoleucine	5.16	5.15	4.96	5.01
Leucine	9.71	10.58	9.46	10.27
Lysine	7.18	6.49	7.57	6.92
Methionine	2.53	2.53	2.59	2.77
Phenylalanine	4.24	4.01	4.54	4.39
Proline	0.85	1.20	0.88	—
Serine	7.13	6.87	7.02	6.53
Threonine	6.06	6.35	5.93	6.18
Tyrosine	2.30	2.67	3.01	3.08
Valine	6.82	7.02	6.94	7.12
Amino acid incorporation (μ C/mg./hr.)	20.5	60.9	43.4	118.0

tri-iodothyronine on the amino acid composition of either the total mitochondrial protein or the radioactive lipoprotein fraction (Table 7). In particular, the amounts of valine and leucine were similar in all the fractions analysed. Differences in the radioactivity in the protein after thyroid hormone treatment and during fractionation with detergent were therefore not due to changes in the amount in the protein of the particular amino acid used as a radioactive tracer.

(d) *Effect of treatment with tri-iodothyronine on oxidative phosphorylation and respiratory enzymes.* The incorporation of radioactive amino acids into protein by isolated mitochondria is influenced by the rate of oxidative phosphorylation (Reis *et al.* 1959; Roodyn *et al.* 1961a). An enhanced rate of ATP production might therefore result in increased incorporation. Tata *et al.* (1963) showed that the mitochondrial q_{O_2} with many substrates is increased by thyroid treatment. In confirmation of this, the cytochrome content of the mitochondrial fraction was found to increase about 2 days after injection of tri-iodothyronine (Fig. 5). The

increase started at about the same time as the stimulation in incorporation, but was still proceeding 95 hr. after thyroid treatment and when the rate of amino acid incorporation had nearly returned to the initial value. Tata *et al.* (1963) could observe no uncoupling of oxidative phosphorylation after treatment with low doses of tri-iodothyronine. This was confirmed by following succinate oxidation in the medium of Greengard *et al.* (1959). There was little difference in the P:O ratios with mitochondria from control and treated animals (Fig. 2). If mitochondrial q_{O_2} is stimulated without uncoupling, increased ATP production would therefore be expected.

Accurate measurement of ATP production under the conditions used to study amino acid incorporation was unfortunately difficult. Negligible incorporation was obtained if the mitochondria were incubated with radioactive amino acid in the medium of Greengard *et al.* (1959), which was designed to measure oxidative phosphorylation. This was found to be partly due to the inhibitory effect on amino acid incorporation of sodium fluoride, added to inhibit adenosine triphosphatase. Also, glucose and hexokinase present in the medium lower the rate of incorporation (Roodyn, Reis & Work, 1961b) (Table 8). It was therefore not possible to add fluoride and the glucose-hexokinase 'trap' to the medium to reduce losses of ATP during incubation and still retain maximum incorporation. The ATP that had been formed during incubation in the presence of radioactive amino acid was therefore measured by stopping the reaction with 0.5 N-perchloric acid and then treating the neutralized protein-free extract with glucose, hexokinase and glucose 6-phosphate dehydrogenase. The ATP production per mg. of mitochondrial protein was greater in treated animals than in the controls (Table 8). Thyroid hormone therefore stimulated both energy production and amino acid incorporation.

(e) *Effect of thyroid hormone on RNA in the mitochondrial fraction.* Treatment of thyroidectomized rats with tri-iodothyronine resulted in an increase in the RNA content of the mitochondrial fraction in several experiments (e.g. Figs. 2 and 5). However, this was only after the increase in amino acid incorporation into mitochondrial protein had occurred. As a result in several experiments doubling in incorporation was observed about 40–48 hr. after injection of tri-iodothyronine, without any increase in RNA in the mitochondrial fraction (Tables 1 and 9). It is seen in Table 9 and Fig. 2 that about 48 hr. after treatment with tri-iodothyronine, although there was no change in RNA in the mitochondrial fraction, the microsomal RNA had clearly increased, in agreement with the results of Tata *et al.* (1963). It is therefore

Fig. 5. Time-course of effect of tri-iodothyronine on amino acid incorporation and cytochrome content of mitochondrial fraction. ○, Cytochrome a ; △, cytochrome b ; □, cytochromes $c+c_1$; ▲, cytochrome a_3 ; ●, amino acid incorporation into mitochondrial protein; ■, RNA ($\mu\text{g./mg.}$ of protein). Thyroidectomized animals were treated with tri-iodothyronine and the results are given as percentages of the value with untreated animals. These were: cytochromes ($10^{10} \times \text{moles/mg. of protein}$) a , 4.32, b , 6.60, $c+c_1$, 5.32, and a_3 , 3.55; amino acid incorporation (after incubation in specimen bottles in air with $0.1 \mu\text{C}$ of [^{14}C]-valine/ml. and radioassay by the cylinder-plating method), $45.0 \mu\text{C/hr./mg. of protein}$; RNA, $23.6 \mu\text{g./mg. of protein}$.

Table 8. *Production of adenosine triphosphate during amino acid incorporation by rat-liver mitochondria*

Mitochondria were incubated in air for 30 min. in the presence of 0.1 μC of [^{14}C]valine/ml. ATP formed was estimated by conversion into glucose 6-phosphate, which was assayed with glucose 6-phosphate dehydrogenase. Where glucose and hexokinase were not present during the incubation, the suspension was deproteinized by precipitation with HClO_4 and the protein-free extract, after neutralizing, was then incubated with glucose plus hexokinase before addition of glucose 6-phosphate dehydrogenase.

State of animals used	ATP formed ($\mu\text{moles/mg. of protein}$)		Amino acid incorporation ($\mu\mu\text{C/mg. of protein}$)	
	Without glucose + hexokinase	With glucose + hexokinase	Without glucose + hexokinase	With glucose + hexokinase
Thyroidectomized (A)	0.58	2.84	37.6	6.1
Thyroidectomized treated 2 days previously with tri-iodothyronine (B)	0.94	4.28	51.8	13.3
Stimulation by tri-iodothyronine (B/A)	1.62	1.51	1.36	2.19

Table 9. *Effect of tri-iodothyronine on amino acid incorporation and content of ribonucleic acid of mitochondrial and microsomal fractions*

Mitochondrial incorporation was measured under oxygen in conical flasks in the presence of 0.1 μC of [^{14}C]valine/ml. Microsomal fraction was incubated with 0.48 μC of [^{14}C]valine/ml. as described in the Methods and Materials section. Basal metabolic rates and incorporation and RNA are expressed as % of value for untreated animals. These were: basal metabolic rate, 1.34 ml. of $\text{O}_2/\text{g.}/\text{hr.}$; mitochondrial incorporation, 18.1 $\mu\mu\text{C/mg. of protein/hr.}$; microsomal incorporation, 256 $\mu\mu\text{C/mg. of protein/15 min.}$; RNA content ($\mu\text{g./mg. of protein}$), mitochondrial fraction 19.1, microsomal fraction 142.

Time after injection of tri-iodothyronine (hr.)	Basal metabolic rate	Amino acid incorporation		RNA content	
		Mitochondrial fraction	Microsomal fraction	Mitochondrial fraction	Microsomal fraction
0	100	100	100	100	100
35	146	176	154	92	140
39	139	228	235	95	148
43	142	201	183	85	128
48	143	212	182	97	138

unlikely that the RNA in the mitochondrial fraction is due to microsomal contamination.

DISCUSSION

Although it still cannot be claimed that the synthesis of specific mitochondrial proteins has been observed *in vitro* under our conditions (cf. Roodyn *et al.* 1962) the results in this paper establish with more certainty that mitochondria can incorporate amino acids into protein. Also, it is reasonable to infer from its response to treatment of the whole animal with tri-iodothyronine that the incorporation system is related, at least in part, to the machinery of mitochondrial protein synthesis in the living cell.

Nature of the incorporating particle. The most likely forms of contamination are bacteria, nuclei

or nuclear fragments, lysosomes and microsomal material sedimenting with the mitochondrial fraction.

As regards bacteria, it is highly improbable that contaminant micro-organisms would respond in any way to the endocrine status of the rat. The lack of correlation between incorporation and bacterial contamination after thyroid hormone stimulation and the partial separation of bacteria and radioactive protein on sucrose gradients fully confirm the previous evidence (Roodyn *et al.* 1961a) for the unimportance of bacterial contamination.

Although some amino acid incorporation was observed with crude nuclear fractions this was most probably due to the heavy contamination of the fraction with mitochondria. The variable amounts of DNA in the mitochondrial fraction either arise from contamination with nuclei or nuclear frag-

ments or from a small amount of truly mitochondrial DNA (cf. Nass & Nass, 1963). However, the bulk of the DNA separates from radioactive protein on the gradient. Also, there is no obvious correlation between the amount of DNA in the mitochondrial fraction and the incorporation.

The mitochondrial fractions isolated in this work undoubtedly contain lysosomes since no precautions have been taken to exclude them. However, the identical activity of 'heavy' and 'light' mitochondrial fractions makes it most unlikely that lysosomes contribute significantly to the incorporation under the conditions used. The partial separation between acid phosphatase and radioactive protein on the sucrose gradient confirms this.

Since mitochondrial and microsomal amino acid incorporation increase at almost exactly the same time after thyroid hormone treatment, and the microsomal system is much more active, it is important to establish without doubt that microsomal contamination is not significant. Roodyn *et al.* (1961a) showed that repeated washing of the mitochondrial fraction greatly reduced the microsomal contamination without affecting the incorporation. The incorporation also differed from the microsomal system in being insensitive to ribonuclease and by showing no requirement for added cell sap or pH 5 enzymes. Further evidence has now been obtained. Microsomal fractions isolated in the sucrose-EDTA-nicotinamide medium normally used for isolating mitochondria incorporated poorly in media designed both for mitochondrial and microsomal incorporation. Thus any residual microsomal material contaminating the mitochondrial fraction is probably inactive. The insensitivity of mitochondrial incorporation to ribonuclease has been confirmed with control and tri-iodothyronine-treated animals as well as the lack of requirement for cell sap. Finally, microsomal material behaves differently from mitochondria on gradient centrifuging, with glucose 6-phosphatase used as a marker.

Taken with the previous results of Roodyn *et al.* (1961a) and the experiments of other workers (e.g. McLean, Cohn, Brandt & Simpson, 1958; Truman & Korner, 1962; Kalf, 1963; Kroon, 1963), the above results show that it is most unlikely that microsomal contamination makes any serious contribution to mitochondrial incorporation under the conditions of isolation and incubation used. With the results on nuclear, bacterial and lysosomal contamination it now seems that the most likely sources of contamination have been eliminated. Mitochondria were measured by the markers malate dehydrogenase (Beaufay, Bendall, Baudhuin & de Duve, 1959a; Beaufay, Bendall, Baudhuin, Wattiaux & de Duve, 1959b; Roodyn *et al.* 1962) and the succinate-oxidase system

(Hogeboom, Claude & Hotchkiss, 1946). Radioactive protein and total protein closely followed these markers in sucrose gradients under conditions in which some separation had occurred between mitochondria, nuclei, lysosomes, microsomal material and bacteria. Thus a positive correlation was observed between the incorporation activity and mitochondria in a variety of fractions of different purity. Without confirmation by radioautography that the mitochondria have indeed incorporated radioactive amino acid, perhaps the most precise conclusion from the above results is that incorporation takes place *in vitro* into particles with sedimentation properties identical with those of mitochondria.

Mechanism of stimulation of mitochondrial incorporation by tri-iodothyronine. From the above results there is little doubt that the administration of small doses of tri-iodothyronine stimulates amino acid incorporation in isolated rat-liver mitochondria. Similar results have been obtained by Bronk (1963). Since there is a long lag-period of 36 hr. after injection before any effect appears, and since tri-iodothyronine added directly to the incubation medium has no effect on the incorporation, it is unlikely that the stimulation of amino acid incorporation is due to direct interaction between mitochondria and thyroid hormone. We are therefore concerned with different effects from the direct ones described by some other workers (Roche, Michel & Kamei, 1962; Sokoloff & Kaufmann, 1961; Sokoloff, Campbell, Francis & Klee, 1963). Our results also suggest that the stimulation of mitochondrial incorporation is not due to alterations in the intramitochondrial pool of amino acids or to changes in the amino acid composition of mitochondrial proteins.

Because of current concepts of the control of protein synthesis, it is important to consider the role of mitochondrial RNA, and also the possible existence of messenger RNA that controls the synthesis of mitochondrial proteins. Unfortunately, the role of RNA in the mitochondrial fraction in the incorporation process is far from clear at the present, mainly because the incorporation is not inhibited by ribonuclease. In the present experiments, although increase in the RNA content of the mitochondrial fraction did occur after treatment with thyroid hormone, this took place somewhat later than the stimulation of mitochondrial incorporation. As a result it was possible to obtain mitochondrial fractions with increased amino acid-incorporation activity but similar RNA contents (Table 9 and Fig. 2). It has been observed previously in many experiments with normal rats that there is no obvious relationship between the amount of RNA in the mitochondrial fraction and the rate of amino acid incorporation (Roodyn *et al.*

1961a). However, it is probable that the RNA in the mitochondrial fraction has some significance and is not due entirely to microsomal contamination. The low amounts present persist after repeated washing of the mitochondrial fraction (Roodyn *et al.* 1961a) and are closely associated with the most active site of incorporation in the mitochondrial membrane (Roodyn, 1962). Also, the difference in time before the RNA content of the mitochondrial and microsomal fractions increased after treatment with thyroid hormones (Table 9, Fig. 2) shows that RNA in the mitochondrial fraction cannot be due to microsomal contamination. As regards the action of thyroid hormones, the rate of turnover of mitochondrial RNA is probably more important than the absolute amount. Tata (1964a) has observed increased incorporation of radioactive orotic acid into RNA in the mitochondrial fraction as soon as 16 hr. after injection of tri-iodothyronine. This effect thus takes place before the stimulation of amino acid incorporation in the mitochondria. Another early effect that confirms that RNA synthesis is affected is the marked stimulation of DNA-dependent RNA polymerase on the nuclear fraction (Widnell & Tata, 1963). These results are consistent with the idea that a primary effect of the hormone is on nuclear mechanisms that regulate the synthesis of cytoplasmic proteins (see also Roodyn *et al.* 1961b).

The effect of thyroid hormone on energy production is also relevant to the problem. The experimental conditions in this work were similar to those used by Tata *et al.* (1963), in which the effect of thyroid hormone on energy production by liver and muscle mitochondria was studied. Thyroid hormones increased mitochondrial respiration and phosphorylation by selectively increasing the 'respiratory and phosphorylative assemblies' at the expense of other proteins. After mild, acute or chronic stimulation by thyroid hormones, mitochondria oxidized a variety of substrates (including glutamate, pyruvate, malate, succinate and glycerol 1-phosphate) at rates that were 50–500% higher than in the thyroidectomized controls. There was no loss in respiratory control or change in the P:O ratio. Increase in ATP production per mg. of mitochondrial protein, and absence of uncoupling, are also reported in this paper (Table 8 and Fig. 2). Other workers have observed increased q_o with no change in the P:O ratio when small doses of thyroid hormone are given (Fairhurst, Roberts & Smith, 1959; Fletcher, Myant & Tyler, 1962; Bronk, 1963). This mitochondrial response of increased respiration and phosphorylation is in contrast with the uncoupling of oxidative phosphorylation, the loss of respiratory control or the swelling of mitochondria that are observed when toxic amounts of thyroid hormones are administered

in vivo or when mitochondria are incubated *in vitro* with high concentrations of the hormones. The catabolic effects of such toxic doses may mask anabolic effects such as the increase in amounts of cytochrome *c*, cytochrome oxidase, succinoxidase and glycerol 1-phosphate oxidase observed by several workers (Tissières, 1946; McShan, Meyer & Erway, 1947; Drabkin, 1950; Smith & Williams-Ashman, 1951; Lee, Takemori & Lardy, 1959; see reviews by Barker, 1951; Pitt-Rivers & Tata, 1959; Hoch, 1962; Tata, 1964b). Gustaffson & Tata (1963, and unpublished work) have observed that liver and muscle mitochondria from rats given small doses of thyroxine or tri-iodothyronine were considerably enlarged, with increased packing of the cristae. There was no evidence of swelling. From the above evidence there is little doubt that mitochondria from rats treated with small doses of thyroid hormone have an enhanced capacity for carrying out oxidative phosphorylation.

It is not known whether increased production of respiratory energy is the cause or the effect of changes in mitochondrial protein synthesis. Amino acid incorporation into mitochondrial protein and the production of respiratory energy are closely associated (Roodyn *et al.* 1961a,b) and it is not yet known if the rate of production of ATP (or some other high-energy intermediate; Bronk, 1963) is rate-limiting during amino acid incorporation. It is possible that increased ATP production and amino acid incorporation may be two results of a common stimulatory effect on the machinery of the cell that controls the synthesis of mitochondrial proteins.

Action of thyroid hormones at the cellular level. The results given above are fully consistent with the conclusions drawn elsewhere that the primary site of action of thyroid hormones is close to nuclear mechanisms that regulate the synthesis of cytoplasmic proteins (Tata, 1963, 1964a; Widnell & Tata, 1963). They are also consistent with the view that the calorogenic action of thyroid hormones, observed at doses near to the physiological concentrations, results from a selective synthesis of mitochondrial respiratory units and not from uncoupling of oxidative phosphorylation or some other direct interaction with the mitochondrion. Thus the calorogenic action may be secondary to the more fundamental growth-promoting and developmental actions of thyroid hormones (see Tata, 1964b).

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