The Action of Thyroid Hormones at the Cell Level

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A number of conflicting mechanisms have been proposed in recent years to explain one or more of the multiple biological actions of thyroid hormones, such as the stimulation of growth, acceleration of metamorphosis, the regulation of basal metabolic rate and effects on protein, lipid and electrolyte metabolism (see Pitt-Rivers & Tata, 1959). Although there is no evidence to show that the effect on basal metabolic rate is more fundamental than are the other actions of the hormones, the majority of the mechanisms proposed attempt to explain the calorigenic action of thyroid hormones, especially since the discovery that administration of large doses of thyroxine or direct addition *in vitro* leads to uncoupling of oxidative phosphorylation in liver mitochondria (Lardy & Feldott, 1951; Martius & Hess, 1951; Niemeyer, Crane, Kennedy & Lipmann, 1951). Other studies have also indicated some form of direct action of thyroid hormones on mitochondria or mitochondrial permeability as seen by swelling and contraction (Tapley, Cooper & Lehninger, 1955; Beyer, Löw & Ernster, 1956; Emmelot & Bos, 1958; Lehninger, Ray & Schneider, 1959), and interaction with dehydrogenases or their prosthetic metal groups (Wolff & Wolff, 1957). The literature on the action of thyroid hormones at the mitochondrial or submitochondrial level has been reviewed (Pitt-Rivers & Tata, 1959; Lehninger, 1960*a*; Bronk, 1960*a*; Tepperman & Tepperman, 1960; Lindberg, Löw, Conover & Ernster, 1961; Smith & Hoijer, 1962). However, there is increasing evidence to indicate that thyroid hormones can influence processes not directly dependent on mitochondrial function, such as protein and nitrogen metabolism, glycolysis and lipid synthesis and breakdown [F. Lipmann & C. Dutoit (see Dutoit, 1952); Glock & McLean, 1955*a*, 1956; Phillips & Langdon, 1956; Fletcher & Myant, 1960, 1961; Smith, 1960; Finamore & Frieden, 1960; Paik & Cohen, 1960; McGuire & Tomkins, 1959; Sokoloff & Kaufman, 1959, 1961; Stein & Gross, 1962; see Pitt-Rivers & Tata, 1959]. Further, the recent findings that many hormones, such as insulin, corticosteroids, testosterone, oestrogens and the growth hormone, have a marked effect on protein biosynthesis (see Kochakian & Dolphin, 1955; Korner & Manchester, 1960; Korner, 1961; Hultin, Decken, Arrhenius & Morgan, 1961*a*; Mueller, Gorski & Aizawa, 1961; Krahl, 1961; Manchester & Young, 1961; Leon, Arrhenius & Hultin, 1962) focus attention on the influence of thyroid hormones on this important cellular function. Such effects become especially important in correlating the effects of thyroid hormones at the cell level with their multiple physiological actions.

So far no biochemical mechanism can completely account for the multiple physiological actions of thyroid hormones. The principal shortcomings of earlier studies are: (1) The necessity of administering toxic doses of thyroid hormones to observe changes at the subcellular level. Uncoupling of oxidative phosphorylation in mitochondria or their swelling can usually only be produced with doses that are 50–500 times the amount of thyroxine necessary for maximal stimulation of basal metabolic rate. Further, the anabolic effect of thyroid hormones at low doses is reversed at higher doses. (2) Only rarely has the physiological status, such as basal metabolic rate, circulating lipids, rate of growth etc., been recorded for animals from which the tissues are removed for enzyme determinations. (3) The specificity of the relationship between chemical structure and biological activity is lost in experiments *in vitro*, as biologically inactive or only feebly active analogues of L-thyroxine, such as D-thyroxine and 3,5-di-iodo-L-thyronine, exhibit the same activity *in vitro* as the biologically active substance. Studies *in vitro* also do not account for the 'latent' period, which is so characteristic of action *in vivo*, especially as the time for hormones to enter into tissues appears to be comparatively short (see Tata, 1960). Further, isolated subcellular particles exhibit an intense thyroxine-binding *in vitro* which may be misinterpreted, since endogenous thyroid hormone is not exclusively concentrated in any given subcellular fraction (Tata, Ernster & Suranyi, 1962*b*; see Tata, 1962). Morphological structure, distribution of the hormones and metabolic patterns vary for different tissues but most biochemical studies on the action of thyroid hormone deal exclusively with liver. Thyroid hormones are known to affect many tissues and, in considering the effect on basal metabolic

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rate, changes in oxidative metabolism of skeletal muscle are at least as important as those in liver. (4) The majority of earlier studies have described the effects of 'chronic' hyperthyroidism or thyrotoxicosis so that the primary, or chronologically earlier, effects at the cell level are confused with the secondary or subsequent effects. No systematic studies in mammals have been reported which permit a distinction to be made between the primary and secondary effects at the cell level. The work of E. Frieden and P. P. Cohen and their collaborators (Herner & Frieden, 1960; Finamore & Frieden, 1960; Paik & Cohen, 1960; Metzenberg, Marshall, Paik & Cohen, 1961; see Frieden, 1961) on the sequential changes occurring during thyroxine-stimulated anuran metamorphosis has already demonstrated the importance of such a distinction.

In undertaking the studies reported below, we considered it essential to record in a co-ordinated manner as many major cellular processes sensitive to thyroid hormone as possible in evaluating the modes of action already suggested or before proposing a new hypothesis. The aim of this paper is to report changes in a large number of subcellular processes and constituents caused by the administration, deprivation and replacement of thyroid hormones, under conditions that overcome most of the objections raised above. These changes are also correlated with the basal metabolic rate and, in some cases, the rate of growth of the animals was recorded. Both 'chronic' and 'acute' effects of thyroxine and tri-iodothyronine were studied. The results indicate that, at the present state of knowledge, it is more important to consider simultaneously the activities of the major subcellular fractions than direct interactions with individual cell constituents to understand the multiplicity of the biological actions of thyroid hormones. They also indicate that a common, as yet unknown, fundamental action at the cell may underlie the diverse effects observed in the whole body.

A preliminary account of some of our findings has been published (Tata, Ernster & Lindberg, 1962*a*).

EXPERIMENTAL

Materials

All chemicals, enzymes (except carbamate kinase) and coenzymes were obtained commercially. Isocitric acid was prepared from the lactone according to Krebs & Eggleston (1944). Phosphoenolpyruvate was recrystallized before use (Baer, 1952) and converted into the potassium salt by treatment with HCl and K_2SO_4 added in slight excess. Carbamate kinase was prepared from *Streptococcus faecalis* and purified according to Mokrasch, Caravaca & Grisolia (1960).

All isotopically labelled substances were obtained from The Radiochemical Centre, Amersham, Bucks. The specific radioactivities of labelled amino acids were: L-[^{14}C]valine, 6·5 mC/m-mole; L-[^{14}C]leucine, 3·0 and 6·5 mC/m-mole; L-[^{14}C]isoleucine, 6·5 mC/m-mole.

Methods

Animals, treatment and measurement of basal metabolic rate. Male Wistar rats, 3–6 months old and weighing 150–220 g. at the time of killing, were used in all experiments. A meat- and fish-free cereal diet, supplemented with brewer's yeast, was used and, unless otherwise stated, they were throughout fed and watered *ad libitum*. Total thyroidectomy was performed by the administration to young rats (60–70 g. body wt.) of three doses of 50–60 μC of carrier-free ^{131}I at intervals of 1 week, the last dose being at least 6 weeks before the experiments. At the end of 6 weeks, the basal metabolic rate had dropped 20–30% below normal and the rate of body growth by about 15%. Thyroidectomized rats in which the fall in basal metabolic rate was less than 20% were eliminated and periodic determinations of basal metabolic rate were made before the experiments to ensure that it had stabilized at the lower values. Chronic hyperthyroidism was produced by repeated subcutaneous injection into normal rats of 25–35 μg. of L-thyroxine every fourth day or 15–25 μg. of 3,5,3'-tri-iodo-L-thyronine every third day for a minimum of 3 and 2 weeks respectively. The basal metabolic rate in treated animals increased 45–75% above normal and could be maintained at this for 2–3 months without any visible signs of toxicity, and with stimulation of growth.

For experiments on the acute effects of thyroid hormones a single injection of 15–30 μg. of tri-iodothyronine was given to thyroidectomized rats weighing 140–180 g. On an average, the basal metabolic rate was measured for every rat once or twice a week and a last determination made within 2–3 hr. before killing.

Basal metabolic rate was determined in a closed-circuit apparatus of the type described by Maclagan & Sheahan (1950). After an initial equilibration period of 5–10 min., consumption of O_2 by the rat was measured over a period of 20–30 min. when a linear relationship was observed. Each value of basal metabolic rate in this paper is an average for groups of three animals and is expressed as ml. of O_2 consumed/hr./g. body wt.

Preparation of subcellular fractions. Rats were killed by decapitation and tissue samples were pooled from two to four rats (usually three) of the same group. Liver mitochondria were isolated from homogenates in 0·25 M-sucrose (1 g. of tissue/10 ml. of medium) as described by Ernster & Löw (1955). Skeletal-muscle mitochondria were obtained from hind-leg and back muscle. The pooled tissue was first minced with scissors and squeezed once through the disk C of a Lepine tissue press (A. H. Thomas and Co. Philadelphia, U.S.A.) before homogenization (Potter–Elvehjem homogenizer with a Teflon pestle) in the ratio of 1 g. of tissue: 8 ml. of medium of the following final composition: 0·1 M-KCl, 0·05 M-tris buffer, pH 7·4, 1 mM-ATP, 5 mM-$MgSO_4$ and 1 mM-EDTA (Chappell & Perry, 1954). The mitochondrial pellet, isolated according to Azzone, Eeg-Olofsson, Ernster, Luft & Szabolcsi (1961), was suspended and washed twice in 0·25 M-sucrose. For studies other than on the incorporation of amino acid into protein, liver and muscle microsomes and cell sap (microsome-free supernatants) were obtained by centrifuging the mitochondria-free homogenate at 105 000*g* for 60 min. in rotor no. 40 of

a Spinco model L ultracentrifuge. Mitochondria and microsomes were finally suspended in 0·25 M-sucrose in the ratio of particles from 1 g. of liver or 5 g. of muscle/ml. (20–30 mg. of protein/ml.). The usual protein content of cell sap was: 8–10 mg./ml. (liver) and 6–9 mg./ml. (muscle).

A submitochondrial-membrane fraction sedimenting after centrifuging at 105 000*g* for 60 min. was obtained by treating liver mitochondria with 0·3% sodium deoxycholate (Watson & Siekevitz, 1956).

In the amino acid-incorporation experiments, livers were minced and washed in a medium containing 0·25 M-sucrose, 75 mM-KCl, 0·01 M-$MgCl_2$ and 35 mM-tris–HCl buffer, pH 7·8, and subsequently homogenized in the same medium (1 g. of liver/2·5 ml. of medium) in an ice-chilled all-glass homogenizer. The homogenate was centrifuged for 6 min. at 14 000*g* and the mitochondrial supernatant was siphoned off, the lipid layer on top of the supernatant being left. Microsomes and 'cell sap' were obtained by centrifuging the mitochondrial supernatant for 60 min. at 105 000*g*. The clear supernatant constituting the cell sap was siphoned off. After repeated washing of the pellet in the homogenizing medium, the microsomes were resuspended in the medium with a hand-operated all-glass homogenizer. The microsomal suspension contained 10–15 mg. of protein and 2–3 mg. of RNA/ml. and the cell sap contained 17–23 mg. of protein/ml.

Mitochondrial respiration and oxidative phosphorylation. Mitochondrial respiration was measured both by the Warburg manometric technique and polarographically with a stationary Pt electrode and a rotating cuvette (Chance & Williams, 1955). For the manometric method, mitochondria from 200 mg. of liver or 1 g. of muscle (4–5 mg. of protein) were incubated at 30° in a final volume of 2 ml. of a medium containing 10 μmoles of substrate, 100 μmoles of KCl, 100 μmoles of sucrose, 50 μmoles of tris buffer, pH 7·4, 50 μmoles of phosphate buffer, pH 7·5, 16 μmoles of $MgCl_2$ and 4 mg. of bovine serum albumin (Armour Laboratories). The medium was supplemented with 0·4 mg. of cytochrome *c* (Sigma Chemical Co.) for muscle mitochondria and the phosphate-acceptor system for all manometric measurements consisted of a catalytic amount of ATP (2 μmoles), 60 μmoles of glucose and 0·75 mg. of yeast hexokinase (Sigma Chemical Co., type IV). The final composition of the medium for polarographic determinations was the same as given above except that the final volume was 1 ml. and 5 μmoles of ADP were added as the phosphate acceptor. The respiratory quotient, q_{O_2}, is expressed as μg.atoms of O consumed/hr./mg. of protein.

Phosphorylation was measured from the amount of inorganic phosphate esterified, as determined by the ^{32}P-distribution method (Lindberg & Ernster, 1955). The P:O ratio was calculated by dividing inorganic phosphate esterified (μmoles) in 25 min. by O_2 consumed (μg. atoms) in 20 min. (with extrapolation for 5 min. of equilibration) as measured by the manometric procedure. Respiratory control index was measured both by the manometric and polarographic techniques and expressed as the ratio of O_2 consumed in the absence of phosphate acceptor to O_2 consumed in the presence of the acceptor.

Mitochondrial adenosine triphosphatase was determined in the presence and absence of 0·1 mM-2,4-dinitrophenol and 1 mM-Mg^{2+} ions with mitochondria from 50 mg. of liver or 200 mg. of muscle, as described by Siekevitz, Löw, Ernster & Lindberg (1958). The procedure of Löw, Siekevitz, Ernster & Lindberg (1958) was used to measure the capacity of liver mitochondria to catalyse an exchange of ^{32}P between inorganic phosphate and the terminal phosphate group of ATP (inorganic phosphate–ATP exchange). It was expressed as μmoles of phosphate exchanged/mg. of mitochondrial protein, according to the method of calculation of Boyer, Luchsinger & Falcone (1956).

Succinoxidase and cytochrome oxidase in submitochondrial particles. Succinoxidase activity in liver-submitochondrial preparations was estimated polarographically, an amount of the membrane fraction equivalent to 500 mg. of liver in 1 ml. of standard medium of the same composition as that used for whole mitochondria being used. The microspectrophotometric method of Cooperstein & Lazarow (1951) was adopted for measuring cytochrome-oxidase activity in membranes derived from 2–4 mg. of liver (5–10 μg. of protein/cuvette). Both activities in submitochondrial preparations from normal animals gave values comparable with those originally reported by Siekevitz & Watson (1956).

Mitochondrial stability in vitro. The stability of liver mitochondria *in vitro* was judged from the degree of swelling induced by inorganic phosphate, high concentrations of thyroxine (0·01–0·05 mM), 3 μM-sodium oleate and hypo-osmoticity (20 mM-tris, pH 7·4). ATP-induced contraction of mitochondria swollen by the above-named agents was also determined. The methods, which were based on those described by Lehninger and his colleagues (Lehninger, 1959; Lehninger *et al.* 1959; Wojtczak & Lehninger, 1961), essentially consisted in observing the changes in E_{520}, over a period 20–60 min., of a suspension of mitochondria from 50 mg. of liver/3 ml. (initial E between 0·4 and 0·6).

Amino acid incorporation into proteins by cell-free particles. The incubation mixture contained, in 1 ml., 0·7 ml. of the liver-mitochondrial supernatant or 0·6 ml. of cell sap combined with 0·1 or 0·2 ml. of the microsomal suspension, 0·08 μmole of the ^{14}C-labelled amino acid (L-valine, L-leucine or a mixture of equal parts of L-isoleucine and L-valine), 1 μmole of ATP, 10 μmoles of phosphoenolpyruvate and 20 μg. of pyruvate kinase. The final concentration of K^+ ions was 0·1 M. In some experiments, the ATP-generating system consisted of 15 μmoles of carbomoyl phosphate and 25 μg. of carbamate kinase instead of phosphoenolpyruvate and pyruvate kinase; there is no essential difference between these two systems under our experimental conditions (Hultin, Leon & Cerasi, 1961*b*). After 10 min. at 35°, the incubation was stopped by the addition of trichloroacetic acid to a final concentration of 5% and an excess of non-radioactive amino acid was added for isotope dilution of the non-incorporated labelled amino acid. Where microsomes were recombined with the cell sap, the samples were adjusted to equal protein content to avoid differences in isotope dilution of proteins (Hultin, Arrhenius, Löw & Magee, 1960). Protein precipitates were repeatedly extracted with hot 5% trichloroacetic acid in the presence of excess of unlabelled amino acid and subsequently with ethanol and ether, as described elsewhere (Hultin *et al.* 1960). The dry protein powders were plated on planchets and the radioactivity was measured with a thin end-window Geiger–Müller tube or an end-window gas-flow counter (Tracerlab). Specific radioactivity is expressed as counts/min./mg. of protein at infinite thinness.

Microsomal enzymes. The assay system for glucose 6-phosphatase consisted of 20 μmoles of glucose 6-phosphate, 1 μmole of EDTA and microsomes from 50 mg. of liver (in 0·5 ml. of 0·25 M-sucrose) in a final volume of 1 ml. After incubation at 30° for 20 min. the inorganic phosphate liberated was measured and the activity expressed as inorganic phosphate liberated/10 min./mg. of protein. NADH- and NADPH-cytochrome *c*-reductase activities were determined by following the reduction of cytochrome *c* at 550 mμ in a Beckman DK-2 recording spectrophotometer and expressed as ΔE_{550}/min./mg. of protein. The reaction mixture contained 166 μmoles of phosphate buffer, pH 7·5, 2 μm-moles of cytochrome *c*, 50 μm-moles of KCN in a final volume of 3·0 ml., and to this 100 μm-moles of NADH or NADPH were added.

Enzymes of liver-cell and muscle-cell sap. Specific activities of isocitric dehydrogenase and glucose 6-phosphate dehydrogenase in liver cells were determined from the rate of reduction of NADP observed spectrophotometrically by the increase in E_{340} (see Glock & McLean, 1953). Each cuvette contained, in a final volume of 3·0 ml.: 166 μmoles of phosphate buffer, pH 7·5, 2·5 μmoles of isocitrate or 10 μmoles of glucose 6-phosphate, 1·4 μmoles of NADP, 1·5 μmoles of $MnCl_2$ (for isocitric dehydrogenase) or 16 μ-moles of $MgCl_2$ (for glucose 6-phosphate dehydrogenase) and 0·1–0·3 ml. of cell sap (0·7–3·5 mg. of protein). Lactic dehydrogenase was measured in liver-cell and muscle-cell sap by recording the rate of decrease of E_{340} during the oxidation of NADH with pyruvate as substrate. The assay mixture contained, in a final volume of 3 ml.: 166 μmoles of phosphate buffer, pH 7·5, 10 μmoles of pyruvate, 0·3 μmole of NADH, 0·005 ml. of liver-cell or muscle-cell sap (3–10 μg. of protein). Changes in *E* were recorded with a Beckman DK-2 recording spectrophotometer and the results expressed as ΔE_{340}/min./mg. of protein.

Creatine-phosphokinase (creatine phosphate transphosphorylase) activity in muscle-cell sap was estimated as described by Luft, Ikkos, Palmieri, Ernster & Afzelius (1962) for human muscle. A known amount of creatine phosphate was incubated at 30° in the presence of cell sap, a catalytic amount of ADP and an excess of glucose and yeast hexokinase and the amount of creatine phosphate remaining was estimated from the acid-labile phosphate. Results are expressed as μmoles of creatine phosphate disappearing/20 min./mg. of protein.

Carboxyl activation of amino acids by liver-cell sap was estimated from the rate of [^{32}P]pyrophosphate–ATP exchange in the cell sap, dialysed overnight against the homogenizing medium containing 0·1 mM-glutathione, according to a modification (Hultin & Decken, 1958) of the method of Hoagland, Keller & Zamecnik (1956).

Chemical determinations. Except for amino acid-incorporation studies, protein was determined by the biuret method of Gornall, Bardawill & David (1949), particulate suspensions being cleared in 0·3–0·5% sodium deoxycholate. This method gives higher values in the presence of haem compounds (Wharton & Griffiths, 1962). The method of Lowry, Rosebrough, Farr & Randall (1951) was used for protein determination in experiments on amino acid incorporation.

Total tissue RNA and DNA in liver, kidney and skeletal muscle were measured by the colour reaction with orcinol and indole respectively (Ceriotti, 1955). The RNA content of microsomes and RNP particles was measured by a slight modification (Arrhenius & Hultin, 1962) of the method of Ogur & Rosen (1950).

Liver glycogen was measured according to Kemp & Kits van Heijningen (1954) and expressed as mg. of glycogen/g. dry wt. of liver.

The relative contents of haems and cytochromes in liver mitochondria and microsomes were estimated from difference spectra of the reduced (sodium dithionite) and oxidized constituents (Ernster, Siekevitz & Palade, 1962) in the presence of 0·3% sodium deoxycholate and KCN, with a Beckman DK2 recording spectrophotometer.

RESULTS

Effect of thyroidectomy and 'chronic' and 'acute' administration of thyroid hormone on basal metabolic rate and growth rate. Basal metabolic rate was markedly increased within 2–3 weeks of chronic treatment with thyroxine and then remained at that rate; in some experiments, tri-iodothyronine, used instead of thyroxine, gave qualitatively and quantitatively similar results. Since only young rats were used in our work, both thyroidectomy and treatment with thyroid hormone markedly affected the rate of growth, as illustrated in Table 1, after 14 weeks of treatment. The stimulation of growth in the thyroxine-treated animals suggests an absence of toxic effects since higher doses (200–500 μg./day/rat), commonly used by other workers, cause a loss of body weight.

The effect of single administration of 20–30 μg. of tri-iodo-L-thyronine to thyroidectomized rats was studied in a second series of experiments. The acute change in the basal metabolic rate after a single injection is shown in Fig. 1, for six experiments. There was a latent period of about 20–30 hr. after injection during which time no appreciable increase in the basal metabolic rate occurred. In two cases where animals were studied over a

Table 1. *Basal metabolic rate and rate of growth of normal, thyroidectomized and 'chronically' thyroxine-treated rats*

Radiothyroidectomy was completed 14 weeks before the measurement of basal metabolic rate. For the rats made 'chronically hypermetabolic', 20 μg. of L-thyroxine was injected every fourth day for 13 weeks and the basal metabolic rate determined 4 days after the last injection. Each figure represents the mean ± S.E. for a group of six rats. Rats were of approximately the same age and weight before treatment was started. Readings below were taken just before they were killed.

Rats	Basal metabolic rate (ml. of O_2/hr./g.)	Increase in body wt. during the last 6 weeks (% of initial body wt.)
Normal	0·65 ± 0·05	48·5 ± 7·5
Thyroidectomized	0·46 ± 0·05	36·0 ± 5·5
Treated with thyroxine	0·88 ± 0·09	70·6 ± 8·0

long period of time, the peak stimulation occurred at about 70 hr. after injection. It was not possible in acute experiments to record accurately changes in body weight accompanying those in the basal metabolic rate, but in Expt. 1 a weight gain of 8–10% over that of the uninjected controls was noted in the tri-iodothyronine-treated animals 9 days after the single injection. All acute effects on cellular activities to be described below will be referred to Expts. 1–6 of Fig. 1.

Mitochondrial function and stability

Respiration, phosphorylation and respiratory control. In Table 2 are summarized the respiratory quotient, P:O ratio and respiratory control index of liver and muscle mitochondria obtained from the groups of normal hypo- and hyper-metabolic rats of Table 1. For both tissues and all the substrates used, mitochondrial q_{O_2} was a function of the basal metabolic rate of the animal. The relative increase or decrease in q_{O_2} varied according to the substrate used and the tissue from which the mitochondria were obtained. On the other hand, P:O ratios and respiratory control index were not altered by a change in the basal metabolic rate produced by thyroidectomy or administration of small doses of

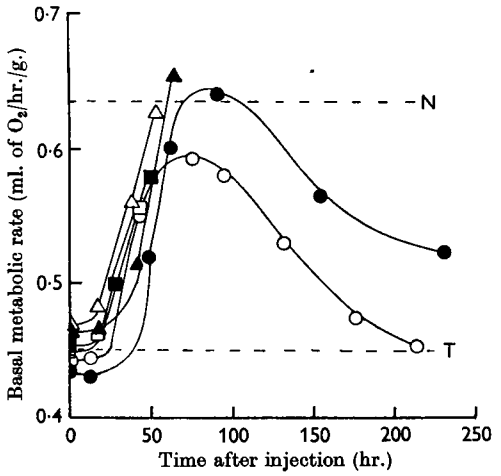

Fig. 1. Acute effect of a single injection of tri-iodo-L-thyronine on the basal metabolic rate of thyroidectomized rats. Tri-iodothyronine was injected at time 0 and changes in basal metabolic rate, as a function of time, are plotted for six different experiments, with an average of three rats for each point. Subsequent results of changes in cellular function will be referred to the experiment numbers given here. Broken lines indicate the average basal metabolic rate of untreated normal (N) and thyroidectomized (T) rats, measured under identical conditions. Doses of tri-iodothyronine: ○, Expt. 1, 26 μg.; ●, Expt. 2, 30 μg.; △, Expt. 3, 18 μg.; ▲, Expt. 4, 25 μg.; □, Expt. 5, 30 μg.; ■, Expt. 6, 27 μg.

Table 2. *Oxidative phosphorylation and q_{O_2} in liver and skeletal muscle mitochondria in normal, hypo- and hyper-metabolic rats*

Mitochondria were obtained from the groups of rats indicated in Table 1. q_{O_2} and P:O ratio were calculated from manometric experiments only; respiratory control index was determined both manometrically (ATP–glucose–hexokinase as phosphate acceptor) and polarographically (ADP as phosphate acceptor). Other details are as described in the Experimental section.

Rats	Substrate	q_{O_2} (μg.atoms of O/hr./mg. of protein)		P:O		Respiratory control index			
						Manometric		Polarographic	
		Liver	Muscle	Liver	Muscle	Liver	Muscle	Liver	Muscle
Normal	Glutamate	7·73	10·21	2·54	2·48	0·116	0·077	0·138	0·112
	Pyruvate + malate*	7·18	12·34	2·76	2·58	0·125	0·131	—	0·211
	Succinate†	7·72	10·80	1·48	0·88	—	—	—	—
	Glycerol 1-phosphate	0·72	3·86	—	0·99	—	—	—	—
Hypometabolic (thyroidectomized)	Glutamate	6·07	6·81	2·47	2·62	0·126	0·086	0·189	0·107
	Pyruvate + malate*	5·53	7·85	2·79	2·51	0·112	0·115	—	0·106
	Succinate†	6·05	9·00	1·69	1·04	—	—	—	—
	Glycerol 1-phosphate	0·34	2·25	—	0·92	—	—	—	—
Hypermetabolic (treated with thyroxine)	Glutamate	8·85	14·08	2·54	2·21	0·088	0·081	0·100	0·110
	Pyruvate + malate*	8·39	18·30	2·79	2·56	0·121	0·097	—	0·160
	Succinate†	8·36	12·31	1·58	0·92	—	—	—	—
	Glycerol 1-phosphate	2·62	8·42	—	0·95	—	—	—	—

* 5 μmoles of each substrate.

† 4 μmoles of Amytal added.

thyroxine. Thus phosphorylation was stimulated or depressed to the same extent as the respiratory activity of the mitochondria, without the P:O ratio or even the degree of tightness of coupling of these activities being lowered.

The increased overall respiratory activity of mitochondria from hypermetabolic rats was also discernible when the concentration of the phosphate acceptor was rate-limiting. In a separate experiment in which the basal metabolic rate was stimulated by repeated injections of tri-iodo-L-thyronine, mitochondrial q_{O_2} (glutamate) was substantially higher than the untreated controls at every hexokinase concentration (Fig. 2). The possibility that an early direct action of thyroid hormone on oxidative phosphorylation was masked by an adaptation of the mitochondria to a different rate of respiration was tested by studying mitochondrial function and stability *in vitro*, in groups of thyroidectomized rats killed at different time-intervals after a single administration of 15–30 μg. of tri-iodo-L-thyronine. The changes of basal metabolic rate in these animals have already been summarized in Fig. 1. Fig. 3 shows the changes in the respiration, measured manometrically with different substrates, of liver and muscle mitochondria at different stages of changes in the basal metabolic rate after a single injection of tri-iodothyronine. An increase in mitochondrial q_{O_2} was observed both in the presence and absence of the phosphate acceptor (ATP–hexokinase–glucose). The ratio of

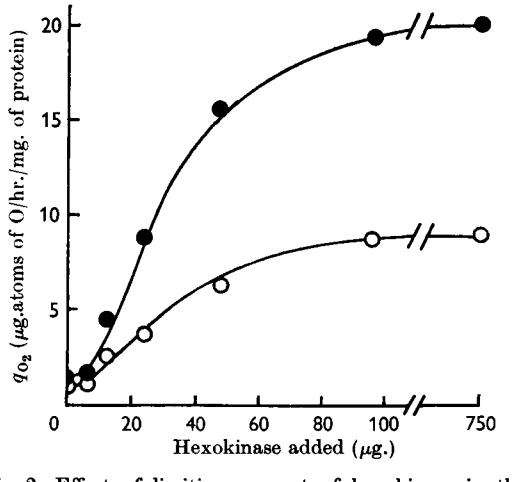

Fig. 2. Effect of limiting amount of hexokinase in the phosphate-acceptor system (hexokinase, glucose, ATP) on q_{O_2} of liver mitochondria from normal (○) and hypermetabolic (●) rats. Tri-iodothyronine (25 μg.) was injected every third day for 24 days to increase the basal metabolic rate and rats were killed 4 days after the last injection; basal metabolic rate values are given in Table 6. q_{O_2} was determined by the manometric procedure with glutamate as the substrate.

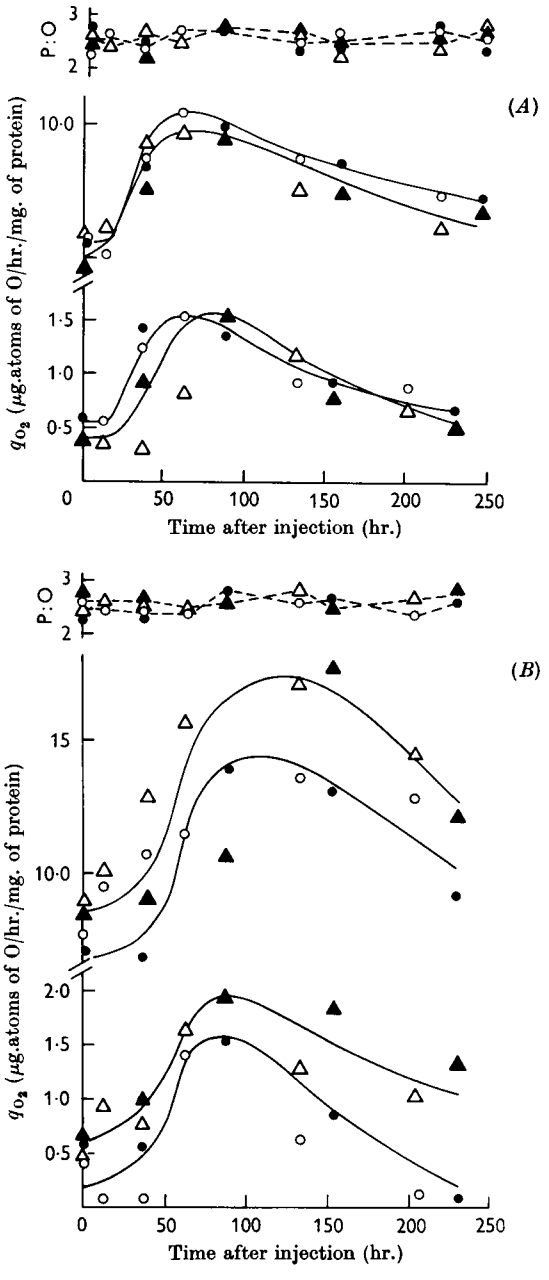

Fig. 3. Effect of a single injection of 25 μg. of tri-iodothyronine to thyroidectomized rats on q_{O_2} and P:O ratio of (*A*) mitochondria liver and (*B*) muscle mitochondria, as observed from two separate experiments (Expts. 1 and 2, Fig. 1). q_{O_2} and P:O ratio were determined manometrically with glutamate (○, ●) and pyruvate + malate (△, ▲) as substrates. The upper sets of q_{O_2} curves were obtained with the phosphate-acceptor system present and the lower set in its absence. Broken lines represent the respective P:O ratios. Open symbols are for values from Expt. 1 and filled symbols for those from Expt. 2. For other details, see text.

Table 3. *Effect of a single injection of tri-iodothyronine into thyroidectomized rats on the q_{O_2} of liver mitochondria measured with different substrates separately and in various combinations*

All animals were killed 48 hr. after injection of 18 μg. of tri-iodothyronine to one-half of the thyroidectomized rats. At this period the basal metabolic rate of the treated animals was 24% above that of the controls. Mitochondrial q_{O_2} was determined manometrically with 10 μmoles of each substrate (except for 5 μmoles each of pyruvate and malate).

Substrate	Mitochondrial q_{O_2} (μg.atoms of O/hr./mg. of protein)	
	Untreated, thyroidectomized	48 hr. after tri-iodothyronine
Glutamate	6·75	9·05
Citrate	4·32	4·82
α-Oxoglutarate	5·90	9·32
β-Hydroxybutyrate	3·31	4·82
Pyruvate + malate	6·69	11·15
Succinate	5·87	8·78
Pyruvate + malate + β-hydroxybutyrate	8·12	14·50
Succinate + β-hydroxybutyrate	9·30	12·79
Glutamate + β-hydroxybutyrate	8·25	11·65
Citrate + β-hydroxybutyrate	7·00	9·26

'maximal' to 'basal' q_{O_2} remained unchanged throughout, indicating that at no stage after the administration of tri-iodothyronine was there a loosening or loss of respiratory control. Esterification of inorganic phosphate was measured for all samples and a change in the P:O ratio was not observed. Although the overall pattern of stimulation of mitochondrial oxidative phosphorylation paralleled that of the whole-body consumption of oxygen, the peak of increase in q_{O_2} of liver mitochondria was reached about 24 hr. before those of muscle mitochondrial q_{O_2} or basal metabolic rate (compare Figs. 1 and 3).

The above-mentioned changes in q_{O_2} were observed with all substrates tested, with the possible exception of citrate (see Table 3), regardless of whether they were oxidized at a relatively high rate (such as pyruvate + malate, glutamate, α-oxoglutarate and succinate) or at a relatively low rate (β-hydroxybutyrate). This stimulation was also observed with various combinations of these substrates. Hence it appears that the respiratory stimulation or depression (according to the thyroid status) reflects an altered capacity of both the respiratory chain and, at least in certain cases, of the individual substrate dehydrogenases.

Table 4. *Changes in succinoxidase and cytochrome oxidase in submitochondrial preparations from liver of normal and hypermetabolic rats*

Hypermetabolism was induced by the injection of 20 μg. of tri-iodo-L-thyronine every third day for 16 days to normal rats weighing 160–180 g. The deoxycholate membrane fractions sedimenting at 105 000*g* for 60 min. were prepared as described in the text. Succinoxidase activity was determined poloragraphically with particles from 50–100 mg. of liver/ml. of suspending medium containing 10 μmoles of succinate and 5 μmoles of ADP. Cytochrome oxidase was determined spectroscopically by the method of Cooperstein & Lazarow (1951).

Rats	Basal metabolic rate (ml. of O_2/hr./g.)	Mitochondrial q_{O_2} (succinate) (μg.atoms of O/hr./mg. of protein)	Submitochondrial particles: Succinoxidase (μg.atoms of O/hr./mg. of protein)	Submitochondrial particles: Cytochrome oxidase (μmoles of cytochrome *c* oxidized/hr./mg. of protein)
Normal	0·68	6·93	40·6	84·0
Hypermetabolic	0·85	8·85	78·5	150·0

Table 5. *Cytochrome-oxidase activity in liver submitochondrial particles (deoxycholate particles) prepared from thyroidectomized rats killed at different time-intervals after a single injection of 25 μg. of tri-iodothyronine*

The deoxycholate-membrane fraction obtained at 105 000*g* from liver mitochondria was prepared and cytochrome oxidase measured as described in the text (Expt. 4, Fig. 1).

Time after injection (hr.)	Change in basal metabolic rate after injection of tri-iodothyronine (%)	Mitochondrial q_{O_2} (glutamate) (μg.atoms of O/hr./mg. of protein)		Cytochrome oxidase in deoxycholate particles (μmoles of cytochrome *c* oxidized/hr./mg. of protein)	
		Control	Injected	Control	Injected
4	−4	6·1	6·4	78	96
16	+5	6·5	6·6	96	90
40	+14	5·9	8·0	90	168
64	+37	6·6	9·7	96	186

Table 6. *Catalysis of* [^{32}P]*phosphate–adenosine triphosphate exchange by liver mitochondria from normal rats treated with tri-iodothyronine and untreated normal rats*

Treatment with tri-iodothyronine consisted of injecting 25 μg. every third day for 24 days (animals were those used for hexokinase experiment; Fig. 2). q_{O_2} of liver mitochondria was determined manometrically.

Rats	Basal metabolic rate (ml. of O_2/hr./g.)	Mitochondrial q_{O_2} (glutamate) (μg.atoms of O/hr./mg. of protein)	P exchanged (μmoles/mg. of mitochondrial protein) At 3 min.	At 8 min.	At 13 min.
Normal	0·59	8·6	0·58	1·01	1·92
Treated with tri-iodothyronine	0·88	16·4	1·42	3·33	4·75

The fact that an increased q_{O_2} was found both in the presence and absence of phosphate acceptor (cf. Table 2 and Fig. 3) suggested that both the respiratory chain and phosphorylating system were stimulated. This could be demonstrated further by measuring respiration with non-phosphorylating submitochondrial preparations or, conversely, by determining the inorganic phosphate–ATP-exchange rate of the intact mitochondria. Table 4 shows that the stimulatory effect on mitochondrial activity of both the long-term and acute treatments with thyroid hormones was also manifested at the submitochondrial level. A chronic increase in the basal metabolic rate of 40 % was accompanied by a nearly twofold increase over normal values in the succinoxidase and cytochrome-oxidase activities prepared from liver mitochondria by deoxycholate treatment. A single injection of tri-iodothyronine was soon followed by increased activity in cytochrome oxidase in the deoxycholate membrane preparations centrifuged at 105 000*g* (Table 5). There was no change in activity 16 hr. after the hormone was administered but there was an 80 % increase after 40 hr. These results further suggested that administration of thyroid hormone may increase the activity of electron-transfer systems in mitochondria. As shown in Table 6, the inorganic phosphate–ATP-exchange rate was substantially increased in mitochondria from hyperthyroid rats. Assuming that the rate of inorganic phosphate–ATP exchange reflects the capacity of the phosphorylating system linked to the respiratory chain (see, however, Vignais, Vignais & Stanislas, 1961), these results mean that the stimulatory action of thyroid hormones also involves the phosphorylating system.

Liver-mitochondrial stability in vitro. The rate and extent of phosphate-induced swelling, and its ATP-induced reversal, in liver mitochondria from normal, thyroidectomized and 'chronically' thyroxine-treated rats are compared in Fig. 4. In the last-named group of rats a greater increase in metabolism (basal metabolic rate + 75–80 %) was produced, since preliminary experiments with the usual stimulation of basal metabolic rate revealed

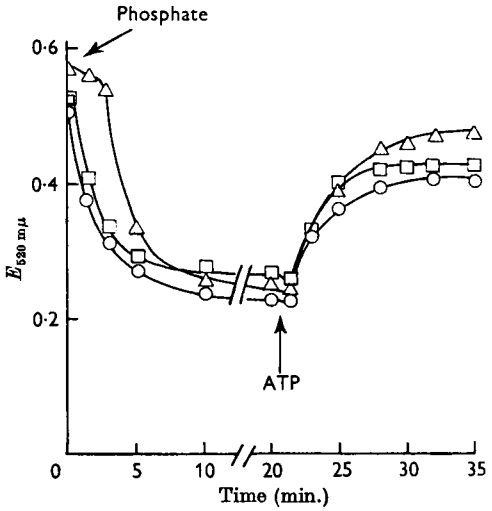

Fig. 4. Phosphate-induced swelling followed by ATP-induced contraction, *in vitro*, of liver mitochondria from normal (○), thyroidectomized (△) and 'chronically' hypermetabolic (□) rats. Hypermetabolism was induced by injecting normal rats with 35 μg. of L-thyroxine every fourth day for 22 days and the rats were killed 4 days after the last injection. See Table 7 for values of the basal metabolic rate. Swelling and contraction were measured by recording $E_{520\,m\mu}$. Each cuvette contained, in a final volume of 3 ml., mitochondria from the equivalent of 30 mg. of liver, 475 μmoles of KCl, 60 μmoles of tris–HCl buffer, pH 7·4, and 30 μmoles of phosphate as the swelling agent added at time ○. Contraction was induced (shown by vertical arrow) by the addition of 15 μmoles of ATP, 9 μmoles of $MgCl_2$ and 6 mg. of bovine serum albumin; t = 21·5°.

no differences. In spite of this, no significant changes were observed in the sensitivity of the three groups of mitochondria to phosphate, or to contraction with ATP, under conditions in which their respiratory and phosphorylative capacities were markedly altered. The same was true, as summarized in Table 7, when thyroxine, oleate or hypo-osmoticity were the swelling agents, both after chronic treatment and during the early phase of stimulation of basal metabolic rate after a single

injection of tri-iodothyronine. As figures normally varied by ± 10 %, the differences in the stability of mitochondria were not significant. In some cases, a short lag period was observed for the swelling of mitochondria from thyroidectomized rats (see Fig. 5), but this difference in stability from preparations from normal or hypermetabolic rats was not as marked as that reported by Tapley (1956).

Another expression of mitochondrial 'labilization' *in vitro* is the appearance of a high Mg^{2+} ion-stimulated adenosine-triphosphatase activity simultaneously with the disappearance of dinitrophenol-stimulated adenosine triphosphatase activity. The two activities were therefore measured in the same samples of mitochondria used for measurement of respiration and phosphorylation (Tables 2, 4, 5; Fig. 3). Differences exceeding ± 20–30 % could not be detected in liver or muscle mitochondria.

Effect of thyroid hormones on microsomal functions

Glucose 6-phosphatase and NADPH–cytochrome c reductase. Table 8 shows the increases, as a function of time after a single injection of tri-iodothyronine into thyroidectomized rats, in the specific activity of glucose 6-phosphatase and NADPH–cytochrome *c* reductase in liver microsomes isolated from the same homogenates as were used for mitochondrial studies (Fig. 3). A two- to three-fold stimulation of these enzyme activities was reached 60–88 hr. after injection of tri-iodothyronine and the increase and regression with time coincided with changes in the basal metabolic rate and liver-mitochondrial activity (see Figs. 2 and 4). The specific activities of glucose 6-phosphatase and NADPH–cytochrome *c* reductase in thyroidectomized-rat-liver preparations were 20–30 % lower than in normal preparations. In some experiments on the effect of 'chronic' administration of thyroid hormones the activities of these two microsomal enzymes were two to three times as high as normal when the basal metabolic rate was maintained at 30–40 % above normal over periods of 4–15 weeks.

Incorporation of amino acids into protein by cell-free liver systems. The effect of a single injection of tri-iodothyronine on the amino acid-incorporation activity of mitochondria-free supernatants of thyroidectomized-rat liver is summarized in Table 9. The amino acid-incorporation capacity remained practically unchanged 26 hr. after the administration of tri-iodothyronine but it increased markedly at 36 hr. and reached a peak at between 40 and 50 hr. It was followed by a gradual decrease, although at 219 hr. after injection the activity was still about 50 % above that of the controls.

Table 7. *Rates of swelling and contraction* in vitro *of liver mitochondria from normal and thyroidectomized rats after chronic or acute treatment with thyroid hormones*

Rats were made hypermetabolic by the administration of 35 μg. of thyroxine every fourth day for 24 days and killed 4 days after the last injection. For acute experiments, thyroidectomized rats were killed at various time-intervals after a single injection of 18 μg. of tri-iodothyronine (Expt. 3, Fig. 1). Rates of swelling and contraction were compared on the basis of time taken to reach half the maximal decrease in $E_{520\,m\mu}$ after the mitochondria were subjected to various swelling agents or half the maximal increase in $E_{520\,m\mu}$ after ATP was added. Other conditions were as described in the text.

Rats	Treatment	Basal metabolic rate (ml. of O_2/hr./g.)	Swelling agent	Time (min.) For half-maximal swelling	Time (min.) For half-maximal contraction
Normal	—	0·62	10 mM-Phosphate	1·9	3·1
			10 μM-Thyroxine	1·6	3·3
			3 μM-Oleate	4·6	1·0
			Hypo-osmoticity	<0·2	1·4
Normal	Thyroxine, chronic	1·08	10 mM-Phosphate	2·1	3·4
			10 μM-Thyroxine	2·4	4·6
			3 μM-Oleate	4·7	0·9
			Hypo-osmoticity	<0·2	1·5
Thyroidectomized	—	0·44	10 mM-Phosphate	4·0	4·0
			10 μM-Thyroxine	2·3	6·2
			3 μM-Oleate	5·1	1·2
			Hypo-osmoticity	<0·3	1·3
Thyroidectomized	Acute (39 hr. after tri-iodothyronine)	0·56	10 mM-Phosphate	2·2	4·0
			10 μM-Thyroxine	2·6	6·4
			Hypo-osmoticity	<0·3	1·0
Thyroidectomized	Acute (52 hr. after tri-iodothyronine)	0·60	10 mM-Phosphate	2·4	5·8
			10 μM-Thyroxine	2·0	5·8
			Hypo-osmoticity	<0·3	1·4

Table 8. *Glucose 6-phosphatase and NADPH–cytochrome c reductase in liver microsomes from thyroidectomized rats after a single injection of tri-iodothyronine*

Microsomes were isolated from mitochondria-free supernatants of the homogenates used for mitochondria in the experiments shown in Fig. 3. Values for normal rat-liver microsomes were: 0·98 ± 0·08 for glucose 6-phosphatase and 0·245 ± 0·022 for NADPH–cytochrome c reductase.

Time after injection of tri-iodothyronine (hr.)	Glucose 6-phosphatase (μmoles of inorganic phosphate released/20 min./mg. of protein)			NADPH–cytochrome c reductase (ΔE_{550} /min./mg. of protein)		
	Control	Treated with tri-iodothyronine	Increase after treatment (%)	Control	Treated with tri-iodothyronine	Increase after treatment (%)
12	0·81	0·78	0	0·185	0·213	18
36	0·76	1·35	84	0·227	0·396	75
60	0·84	2·51	199	0·192	0·425	121
88	0·78	2·72	245	0·208	0·410	97
159	0·86	1·20	39	0·170	0·306	80
230	0·78	1·12	44	0·196	0·285	45

Table 9. *Effect of a single administration of tri-iodothyronine on amino acid incorporation by liver mitochondria-free supernatants from fed thyroidectomized rats*

Thyroidectomized rats were killed at different time-intervals after the injection of 30 μg. of tri-iodothyronine. Incubation system: 0·7 ml. of mitochondria-free supernatant, 77 μm-moles of L-[^{14}C]valine (0·5 μc), 10 μmoles of phosphoenolpyruvate, 1 μmole of ATP and 20 μg. of pyruvate kinase in a final volume of 1 ml. Incubation was at 35° for 10 min. Amino acid-incorporation activity in injected rats is expressed as the percentage increase in counts/min./mg. of protein over the uninjected controls killed at every time-interval.

Time after injection of tri-iodothyronine (hr.)	Percentage increase over uninjected controls
26	13
36	86
40	92
50	104
78	85
219	66

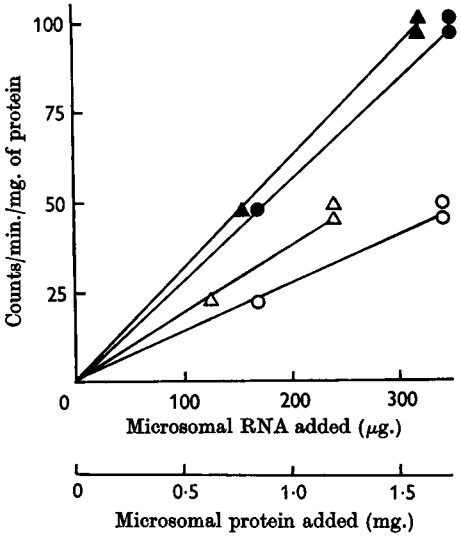

Fig. 5. Incorporation of amino acid into protein by liver microsomes from thyroidectomized rats, 50 hr. after a single injection of 27 μg. of tri-iodothyronine. Microsomal concentration is indicated as mg. of microsomal protein (○, ●) or μg. of microsomal RNA (△, ▲) added. Filled symbols: injected rats; open symbols: uninjected controls. Incubation system: 77 μm-moles of L-[^{14}C]valine (0·5 μc), 10 μmoles of phosphoenolpyruvate, 1 μmole of ATP, 20 μg. of pyruvate kinase, 0·6 ml. of cell sap from uninjected controls and microsomes as indicated, in a final volume of 1 ml. Incubation was at 35° for 10 min.

To evaluate to what extent this enhanced amino acid-incorporation capacity was due to an increased microsomal activity, microsomes from tri-iodothyronine-treated and untreated thyroidectomized rats were incubated with cell sap from either the untreated thyroidectomized rats or, in some experiments, from normal rats. In a typical experiment illustrated in Fig. 5, liver microsomes prepared from thyroidectomized rats 50 hr. after the administration of 30 μg. of tri-iodothyronine showed an increase in amino acid incorporation per unit of microsomal protein. It was comparable with the increase observed earlier in mitochondria-free supernatants (Table 9). The stimulation, although quite marked, was less pronounced when calculated on the basis of microsomal RNA.

When studied as a function of time after injection (Fig. 6), the amino acid-incorporation activity of isolated liver microsomes remained unchanged for at least 26 hr. after a single injection

of tri-iodothyronine into thyroidectomized rats. As with mitochondria-free supernatants (Table 9), there was a sharp and rather sudden rise in incorporation activity (when calculated per mg. of microsomal protein) at 36 hr. and reaching a peak value at about 40 hr., when the rate of incorporation was more than doubled. At 50 hr. after administration of tri-iodothyronine the incorporation capacity dropped sharply but remained at about 150% of the control value for more than 200 hr. Although qualitatively similar, the increase was once again less marked when expressed per unit of microsomal RNA. This difference can be explained by the increase in microsomal RNA:protein ratio accompanying the stimulation of amino acid incorporation. In considering the above-mentioned effects of a single replacement dose of thyroid hormone, it should be emphasized that both the amino acid-incorporation capacity and RNA:protein ratio for liver microsomes from thyroidectomized rats are one-half to one-third of values for preparations from normal animals (Table 10).

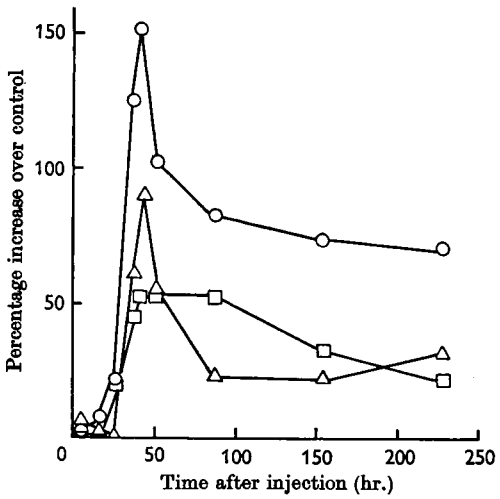

Fig. 6. Effect of tri-iodothyronine, as a function of time after a single injection into thyroidectomized rats, on amino acid-incorporation activity and RNA:protein ratio of liver microsomes. The stimulatory effect is expressed as percentage increase in microsomes from injected animals over those from uninjected controls killed at every time-interval. Values were averaged from data from Expts. 2–6 (Fig. 1). ○, Amino acid incorporation initially calculated as total counts/min./mg. of microsomal protein added; △, amino acid incorporation initially calculated as total counts/min./200 μg. of microsomal RNA added; □, microsomal RNA:protein ratio. Incubation was as in Fig. 5, except that 0·5 μc of L-[^{14}C]valine or L-[^{14}C]leucine or a mixture of equal parts of L-[^{14}C]leucine and L-[^{14}C]isoleucine (77 μmoles of amino acid) was used as the labelled amino acid.

The effects of feeding and starvation on 'chronically' hyperthyroid and normal rats were compared, since the nutritional status of the animals is known to influence the amino acid-incorporation activity of microsomes (Munro & Clark, 1960). Whether calculated on the basis of microsomal protein or RNA, the capacity of incorporation of L-[^{14}C]valine into protein was higher with microsomes from fed 'chronically' hyperthyroid rats than with those from fed normal controls (Table 11). Starvation for 24 hr. before the rats were killed, however, abolished or even reversed this relationship so that microsomes from hypermetabolic animals actually exhibited a slightly lower amino acid-incorporation rate than those from normal animals. The nutritional status of the animal at the time of killing therefore appears to condition the effect of the hormones on microsomal functions. There was no significant variation in the RNA:protein ratio accompanying these differences after starvation for 24 hr. in amino acid-incorporation capacity.

Effect of thyroid hormones on cell-sap enzymes

Activities of some enzymes known to be localized in the microsome-free supernatant were also measured for both the acute and chronic experi-

Table 10. *Effect of thyroidectomy on amino acid-incorporation capacity* in vitro, *liver-microsomal ribonucleic acid : protein ratio and liver-glycogen content of fed rats*

Animals were thyroidectomized 8 weeks before being killed. Incubation mixture contained 0·7 ml. of mitochondrial supernatant or 0·6 ml. of cell sap recombined with 0·1 or 0·2 ml. of microsomal suspension. Other conditions for amino acid incorporation were as described in Table 9.

	Incorporation of L-[^{14}C]valine into protein (total counts/min.)	
	Normal	Thyroidectomized
(*a*) Mitochondria-free supernatant	1150	660
(*b*) Microsomes + cell sap (per mg. of microsomal protein added)	264	75
(*c*) Microsomes + cell sap (per 200 μg. of microsomal RNA added)	231	87
Microsomal RNA/microsomal protein	0·23	0·12
Liver glycogen (mg./g. dry wt. of liver)	150	142

Table 11. *Influence of nutritional status on amino acid incorporation into protein by cell-free liver systems and microsomal ribonucleic acid:protein ratios in normal and 'chronically' hypermetabolic rats*

Hypermetabolism was induced by the administration of 20 μg. of thyroxine every fourth day for 35 days. Basal metabolic rate was 0·87 ml. of O_2/hr./g. for hyperthyroid rats and 0·63 ml. for normal controls. For starved rats, food was withdrawn 20 hr. before killing them; the others were fed *ad libitum*. The incubation system was as described in Table 2.

	Incorporation of L-[^{14}C]valine into protein (total counts/min.)			
	Normal		Hypermetabolic	
	Fed	Starved	Fed	Starved
(*a*) Mitochondria-free supernatant	1150	770	1430	604
(*b*) Microsomes + normal cell sap (calculated per mg. of microsomal protein added)	264	198	300	168
Microsomal RNA:microsomal protein	0·23	0·21	0·23	0·25

ments, starting with the same homogenates in which mitochondrial and microsomal activities were also measured and described earlier. In acute experiments (Table 12) the specific activities of lactic dehydrogenase and glucose 6-phosphate dehydrogenase did not appreciably alter during the first 2–3 days after the single injection of tri-iodothyronine into thyroidectomized rats, at which time the mitochondrial and microsomal activities from the same homogenates were considerably stimulated. The specific activities of these enzymes were still elevated or increasing on the sixth and ninth days after treatment, at a time when the basal metabolic rate, mitochondrial q_{O_2} and microsomal protein-synthesizing activity were progressively declining. Liver isocitric-dehydrogenase and muscle creatine-phosphokinase activities did not change within this long time interval. Activities of lactic dehydrogenase and glucose 6-phosphate dehydrogenase ranging from 40 to 80% above values in untreated normal rats were recorded for cell saps from rats treated 'chronically' with thyroxine.

In view of the marked effect of thyroid hormones on amino acid incorporation into proteins by microsomes, the capacities of unfractionated cell saps for amino acid activation, as judged by the ATP–[^{32}P]pyrophosphate exchange, were also compared. Table 13 shows that there was little difference in the capacities of dialysed liver-cell saps from untreated or tri-iodothyronine-treated thyroid-ectomized rats to catalyse ATP–pyrophosphate exchange in the presence of a mixture of amino acids. These values should be considered in view of the facts that the activation of any specific amino acid was not measured and that variations in values obtained under these conditions would be quite high from one experiment to another.

Changes in concentration of cellular constituents

One of the most readily observed changes in the concentrations of cellular constituents measured during treatment with thyroid hormone was that of liver glycogen (Fig. 7). There was little difference between the concentrations of liver glycogen in thyroidectomized and normal rats but the administration of a single dose of 30 μg. of tri-iodothyronine to thyroidectomized animals caused a 50% decrease within a day. Decreases of about 25% were often observed within 16 hr. of administration, which thus preceded almost all the other actions we have observed.

Experiments on amino acid incorporation have shown that the microsomal RNA:protein ratio in thyroidectomized rats is almost half that in normal rats and that thyroid hormone increases this value (see Table 10, Fig. 6). The ratio total RNA:DNA in liver and muscle (but not kidney) was 20–30% lower in fed thyroidectomized rats than in fed normal rats; starvation for 24 hr. of both untreated and 'chronically' tri-iodothyronine-treated normal rats caused a similar drop in the total RNA:DNA ratio (Table 14). However, relatively little or no difference was observed in the total DNA and RNA content of many tissues either during acute or chronic alterations in the basal metabolic rate of normal or thyroidectomized rats (Table 14). Table 14 also shows little change in the water content of tissues under similar conditions. Protein determinations in various cellular constituents, performed regularly for expressing specific activities of different enzymes, revealed little difference in the amount of protein/g. of tissue in acute experiments, but the liver- and muscle-protein contents of 'chronically' hypermetabolic rats were 5–15% above those of normal rats of equal body weight.

DISCUSSION

In comparing our results with those of other workers the following differences in experimental procedure and design should be noted: (1) In our work, standardization and reproducibility of biological effects were achieved by frequently recording the basal metabolic rate, and, often, the rate

Table 12. *Changes in some enzyme activities in liver-cell and muscle-cell saps of thyroidectomized rats after a single injection of tri-iodothyronine*

Rats used in this experiment were from Expt. 2 (Fig. 1). The cell saps were therefore obtained from the homogenates from which mitochondria and microsomes were studied (Figs. 3, Table 8).

Enzyme	Tissue	Control (activity and range)	Percentage difference in activity over untreated controls after injection					
			12 hr.	36 hr.	60 hr.	88 hr.	159 hr.	230 hr.
Lactic dehydrogenase (ΔE_{340}/min./mg. of protein)	Liver	0·35±0·04	+ 5·5	− 8·8	+ 6·0	+41·9	+20·5	+21·5
	Muscle	1·32±0·21	0·0	0·0	+14·3	+22·0	+ 3·2	+ 6·6
Isocitric dehydrogenase (ΔE_{340}/min./mg. of protein)	Liver	0·55±0·05	+ 2·1	−21·7	+ 4·8	−28·0	−32·9	+ 1·1
	Muscle	0·06±0·005	− 2·0	−16·6	+21·0	+51·1	−13·6	+24·1
Glucose 6-phosphate dehydrogenase (ΔE_{340}/min./mg. of protein)	Liver	0·05±0·005	0·0	−16·9	− 2·4	+23·6	+53·2	+41·8
Creatine phosphokinase (μmoles of creatine phosphate broken down/10 min./mg. of protein)	Muscle	48·5 ±7·1	+11·6	−10·4	0·0	− 2·5	−13·6	0·0

of growth, for every animal. Cellular functions attributable to different subcellular fractions were correlated with changes in basal metabolic rate and examined, as often as possible, from the same tissue sample. (2) Changes at the cell level that we have studied as a function of time after a single replacement dose to thyroidectomized animals constitute a first systematic attempt to distinguish between the primary and secondary cellular actions of thyroid hormones. The use of a single replacement dose to thyroidectomized animals also provides greater specificity than chronic administration in interpreting the primary actions of thyroid hormones. (3) The doses of thyroid hormones used were selected on the basis of the minimum amount necessary to give clear-cut and measurable changes both at the cell and in the whole body. They were close to the physiological amounts, and the anabolic effect (rate of whole body growth) accompanying increased basal melabolic rate (see Table 1) was an indication of the absence of toxic effects, since high doses produce a marked catabolic effect (see Pitt-Rivers & Tata, 1959). Only a few recent reports describe the cellular effects of comparably low doses of thyroid hormones administered repeatedly (Fairhurst, Roberts & Smith, 1959; Smith, 1960; Fletcher & Myant, 1960, 1961; Stein & Gross, 1962), the majority of the earlier studies in effect interpreting the catabolic effects of severe hyperthyroidism or thyroid-hormone intoxication. All the biological actions of thyroid hormones can be elicited by minute amounts and some effects may even be reversed at higher doses (see Pitt-Rivers & Tata, 1959). (4) Skeletal muscle has been consistently used, in our studies, in addition to liver, in interpreting the calorigenic action of thyroid hormones. (5) We have avoided at this stage any comparison of the effects of thyroxine on cell constituents *in vitro* and those *in vivo*.

Action on mitochondrial function and structure

The salient feature of our findings on mitochondria is the failure to observe any change in the energy-coupling mechanism or mitochondrial structure in animals in which administration of thyroid hormones, in a single or in repeated doses, had markedly altered the basal metabolic rate. On the other hand, mitochondrial q_{O_2} was altered, the changes correlating well with those in the basal metabolic rate. This effect on mitochondrial respiration could not be localized at one given rate-limiting step of either the electron-transport or phosphorylation system but appeared to result from an 'overall' change in the capacities of the respiratory chain and phosphorylation. This conclusion is mainly drawn from the quantitatively similar changes in mitochondrial respiration with every substrate tried, and whether or not the

Table 13. *Effect of administration of tri-iodothyronine to thyroidectomized rats on the amino acid-dependent pyrophosphate–adenosine triphosphate exchange with liver-cell sap*

One group of thyroidectomized rats was injected with 25 μg. of tri-iodothyronine, 42 hr. before they were killed. ^{32}P exchange between [^{32}P]pyrophosphate and ATP was measured with varied amounts of dialysed cell sap and expressed as percentage of equilibrium value reached after 10 min. incubation at 35°. The incubation medium contained, in a final volume of 1·25 ml., 100 μmoles of tris–HCl buffer, pH 7·8, 4 μmoles of [^{32}P]pyrophosphate, 6 μmoles of ATP, 6 μmoles of $MgCl_2$, 60 μmoles of KF and, when added, 3 μmoles of each of the following amino acids: phenylalanine, valine, leucine, isoleucine, tryptophan, lysine, threonine, serine, alanine, histidine, arginine, glycine, glutamic acid, aspartic acid, tyrosine, methionine, cysteine and proline.

	Amount of cell sap (mg. of protein)	Percentage of equilibrium reached at 10 min.	
		Without amino acid mixture	With amino acid mixture
Untreated	0·88	2·8	28·5
	1·76	6·4	46·5
Treated with tri-iodothyronine	0·91	2·5	31·0
	1·81	3·5	58·0

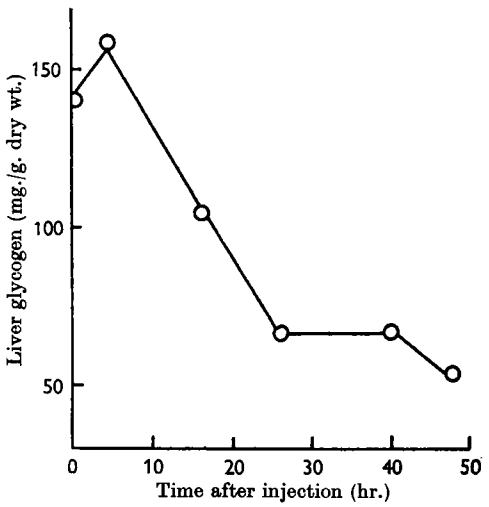

Fig. 7. Effect of a single injection of tri-iodothyronine on the concentration of glycogen in liver of fed thyroidectomized rats at different time-intervals after the injection. Values were averaged from Expts. 5 and 6 (Fig. 1), in which 27 μg. and 30 μg. respectively of tri-iodothyronine were used. Average glycogen content of normal rat liver is 140–160 mg./g. dry wt. of liver.

phosphate acceptor was limiting the respiration. The changes in non-phosphorylating electron transport, such as submitochondrial succinic oxidase and cytochrome oxidase, and the inorganic phosphate–ATP exchange (if it is a reflexion of phosphorylative capacity) also support this view.

Administration of thyroid hormone in amounts 10–100 times as great as we have used has been shown to increase mitochondrial respiration, but our failure to observe an uncoupling of oxidative phosphorylation or loosening of respiratory control is at variance with the interpretation offered in several reports in the past decade on the uncoupling action of thyroid hormones on mitochondria *in vivo* and *in vitro* (Lardy & Feldott, 1951; Martius & Hess, 1951, 1955; Niemeyer *et al.* 1951; Hoch & Lipmann, 1954; Maley & Lardy, 1955; see also Pitt-Rivers & Tata, 1959; Tepperman & Tepperman, 1960; Lindberg *et al.* 1961; Smith & Hoijer, 1962). The principal cause of this divergence is most likely to be the difference in the action of thyroid hormones at nearly physiological or toxic doses. With very low doses of thyroxine, administered daily for 6–16 days, Smith (1960) also failed to observe a lowering of the P:O ratio in liver mitochondria in the presence of phosphate acceptor. Both basal metabolic rate and mitochondrial respiratory quotient are depressed after thyroidectomy and stimulated by administration of thyroid hormone over a wide range of doses (Maley & Lardy, 1955; Martius, Bieling & Nitz-Litzow, 1955; Maley, 1957; Fairhurst *et al.* 1959; Tata *et al.* 1962*a*); on the other hand, whereas the P:O ratio is lowered only after the administration of high doses of thyroxine, it is unaffected after thyroidectomy and by low doses of the hormone. Thus it appears that mitochondrial respiration and phosphorylation on one hand, and the tightness of their coupling on the other, may respond differently according to the degree of hyperthyroidism or thyroxine intoxication. In earlier studies the effects on mitochondria *in vivo* and *in vitro* often have been compared and interpreted on the assumption that they are brought about by the same mechanism, in spite of many differences. For example, thyroid hormones fail to stimulate mitochondrial respiration *in vitro*, although the P:O ratio is lowered both *in vitro* and *in vivo* after high doses of the hormones [Maley & Lardy, 1953; Lardy & Maley, 1954; Klemperer, 1955; see Pitt-

Table 14. *Effect of chronic and acute treatment with tri-iodothyronine of normal and thyroidectomized rats on total tissue ribonucleic acid and deoxyribonucleic acid in liver, kidney and skeletal muscle*

For chronic treatment, 25 μg. of tri-iodothyronine was injected into normal rats every third day for 24 days; in acute experiments, thyroidectomized rats were given a single injection of 25 μg. of tri-iodothyronine. In chronic experiments, the effect of starvation for 24 hr. is also recorded. Values are averages from two experiments carried out separately with tissues from three rats pooled in each experiment.

Rats	Treatment	Time after single injection of tri-iodothyronine (hr.)	Basal metabolic rate (ml. of O_2/hr./g.)	Nutritional status	Tissue	Dry wt. of tissue (% of wet wt.)	RNA (μg./mg. dry wt. of tissue)	DNA (μg./mg. dry wt.)	RNA:DNA
Normal	—	—	0·67	Fed	Liver	26·3	69·9	13·9	5·03
					Kidney	18·2	30·9	30·7	1·01
					Muscle	21·4	11·3	4·3	2·62
				Starved	Liver	23·5	53·5	18·5	2·89
					Kidney	17·8	31·1	33·1	0·94
					Muscle	22·1	9·7	5·4	1·81
Normal	'Chronic'	—	0·79	Fed	Liver	26·6	74·3	15·3	4·86
					Kidney	19·6	29·2	28·7	1·02
					Muscle	21·7	11·0	4·1	2·68
				Starved	Liver	26·0	52·1	17·3	3·01
					Kidney	18·9	29·6	29·4	1·01
					Muscle	22·6	9·5	4·1	2·28
Thyroidectomized	—	—	0·47	Fed	Liver	25·5	61·6	18·8	3·28
					Kidney	17·6	33·0	32·8	1·01
					Muscle	21·9	8·3	4·1	2·02
Thyroidectomized	'Acute'	16	0·49	Fed	Liver	23·1	59·4	18·3	3·26
					Kidney	18·3	36·1	33·0	1·06
					Muscle	22·6	8·2	3·9	2·08
Thyroidectomized	'Acute'	25	0·52	Fed	Liver	—	58·6	19·8	2·96
					Kidney	17·5	28·8	31·7	0·91
					Muscle	20·9	6·3	3·1	2·06
Thyroidectomized	'Acute'	47	0·60	Fed	Liver	23·9	66·5	20·5	3·24
					Kidney	19·0	29·4	29·4	1·00
					Muscle	22·6	4·9	2·5	1·96

Rivers & Tata, 1959; Smith & Hoijer, 1962; Bronk's (1960*a*, *b*) demonstration of stimulation of mitochondrial respiration *in vitro* is exceptional in this respect]. Similarly, the inhibition of mitochondrial dehydrogenases by direct interaction is unlike the stimulatory effect *in vivo* at almost any dose (Maley & Lardy, 1953; Wolff & Ball, 1957; Barker, 1957; Wolff & Wolff, 1957; Lee, Takemori & Lardy, 1959; Wolff, 1962).

The degree of hyperthyroidism produced may account for our inability to detect alterations in mitochondrial stability, as seen by swelling and contraction *in vitro*. The discrepancy between our results and those of Tapley (1956) may be determined by whether or not the tightness of the energy-coupling mechanism of the mitochondria has been affected. Significant in this context are the findings that the swelling and contraction of liver mitochondria *in vitro* are linked to the state of respiratory-chain carriers and high-energy phosphate intermediates (Hunter & Ford, 1955; Lehninger *et al.* 1959; Lehninger, 1960*a*, *b*; Chappell & Greville, 1959). Muscle mitochondria do not swell or contract like liver mitochondria and it would be even more difficult to account for the identical effects we have observed on liver and muscle mitochondrial respiration on the basis of stability changes *in vitro*. However, our failure to detect differences in swelling and contraction by optical methods need not rule out the possibility that the biological action of thyroid hormones may involve more subtle effects on the fine structural organization of the mitochondrion or other cell organelles.

Microsomal glucose 6-phosphatase and NADPH–cytochrome c reductase

The chronological pattern of onset of stimulation, peak stimulation and decay in activity of these two enzymes, after a single injection of tri-iodothyronine into thyroidectomized rats (Table 8), was very similar to that of changes in the activity of mitochondria isolated from the same homogenate (Fig. 3*A*). In earlier studies, rats treated for several days with high doses of thyroxine (up to 0·5 mg./rat/day) also exhibited increased glucose 6-phosphatase activity in liver homogenates, mitochondria-free homogenates or isolated microsomes (Glock & McLean, 1955*a*; Maley, 1957; Harper & Young, 1959; Bargoni, Luzzati, Rinaudo, Rossini & Strumia, 1961), as well as increased NADPH–cytochrome *c*-reductase activity (Phillips & Langdon, 1956; Smith, 1960). Phillips & Langdon (1956) and Smith (1960) also found that NADH–cytochrome *c*-reductase activity in the same microsomal preparation did not increase. In a few measurements we also have failed to detect any stimulation of this enzyme activity. This dissociation may possibly be due to the ease of detecting changes in activities of enzymes that are present in relatively lower amounts (or more depressed after thyroidectomy) or due to the NADPH–cytochrome *c*-reductase values being more reproducible from one microsomal preparation to another, whereas those of NADH–cytochrome *c* reductase are not (Ernster *et al.* 1962). Glock & McLean (1955*b*) found that treatment with thyroxine reduced the total NAD^+ and NADH content of liver, although increasing the total $NADP^+$ (principally as NADPH) content. It has also been suggested elsewhere [on the basis of experiments on NADPH–cytochrome *c* reductase (Smith, 1960), increased participation of glycolytic pathway (Glock & McLean, 1956), stimulation of NADPH-dependent reduction of Δ^4-3-oxo steroids (McGuire & Tomkins, 1959) and inhibition of the transhydrogenase reaction (Ball & Cooper, 1957)] that thyroid hormones may act by regulating the amount of available NADPH. However, only the chronic effects of repeated doses of thyroid hormones were recorded in all the above-mentioned experiments so that the changes in the amounts of pyridine nucleotide may be a secondary rather than a primary consequence of action of thyroid hormone. Such a conclusion would be justified (*a*) by our findings in acute experiments that stimulation of microsomal NADPH–cytochrome *c* reductase did not precede that of mitochondrial respiration or amino acid incorporation by microsomes and (*b*) by the more delayed stimulation of enzymes that probably regulate the amounts of reduced pyridine nucleotides in the cytoplasm.

Incorporation of amino acids into proteins by microsomes

Thyroidectomy depresses the rate of protein formation or accumulation; small amounts of thyroid hormone accelerate it in the normal or thyroidectomized subject and accelerate protein breakdown when given in high doses or in thyrotoxicosis (Scow, 1954; Kochakian & Dolphin, 1955; Crispell, Parson & Hollifield, 1956; see Pitt-Rivers & Tata, 1959). Our results, which show that the capacity of amino acid incorporation into protein by cell-free systems was depressed in thyroidectomized rats and elevated after chronic or acute treatment with thyroid hormones (see Tables 9–11; Figs. 5 and 6), should be considered in the light of the growth-promoting action of thyroxine and tri-iodothyronine. Stein & Gross (1962) have also found that repeated injection of small amounts of tri-iodothyronine increased the rate of incorporation of amino acid by cell-free liver systems from thyroidectomized rats. In our acute experiments, a single administration of tri-iodothyronine to thyroidectomized rats was followed by a sharp

increase in amino acid-incorporation rate and microsomal RNA:protein ratio. In view of the stimulatory effect on amino acid incorporation even when expressed per unit of RNA, it appears that tri-iodothyronine may have caused an increase in the ribonucleoprotein particle population as well as enhancement of the overall activity of the new population. Tissières, Schlessinger & Gros (1960) have shown that only a part of the total amount of ribonucleoprotein particles are active and the possibility arises that thyroid hormones may in some way affect the ratio active : inactive ribonucleoprotein particles (see Leon *et al.* 1962).

At least part of the action of thyroid hormone on amino acid incorporation into protein can be localized at the microsome but we failed to detect any significant effect on the capacity of liver-cell sap to activate amino acids. Preliminary evidence, however, suggests that the cell sap from animals treated with thyroid hormone may influence the amino acid-incorporation capacity of microsomes from treated or untreated rat livers. The exact nature of this effect, which resembles effects observed with corticosteroid treatment (Leon *et al.* 1962), yet remains to be defined.

It is doubtful whether there is any relationship between the effect of thyroid hormones *in vitro* on a cell-free preparation (Sokoloff, Kaufman & Gelboin, 1961) and the mechanism by which amino acid incorporation is stimulated by relatively minute amounts of the hormone *in vivo*, especially as the immediate effect *in vitro* does not take into account the latent period *in vivo*. Further, Sokoloff & Kaufman (1961) have shown earlier that the biologically almost inactive analogue, D-thyroxine, failed to stimulate amino acid incorporation when administered to rats but was as active as L-thyroxine when tested *in vitro*; conversely, tri-iodo-L-thyronine, which is at least as active as L-thyroxine *in vivo*, showed a negligible effect *in vitro*.

Among the experimental conditions capable of modifying the response to thyroid hormones was the absence or reversal of the stimulatory effect of thyroid hormone in fed rats when the animals were starved (Table 11). Munro & Clark (1960) have shown that the metabolism of RNA in liver cells is controlled to some extent by the nutritional status and our observation of the fall in liver glycogen (Table 8) preceding the rise in microsomal RNA: protein ratio and microsomal amino acid-incorporation capacity, may be of some consequence. It is not possible from the present studies, whose principal aim was to study the cellular action of thyroid hormones in animals having free access to food, to explain the exact relationship between the regulatory roles of food intake (or other hormones such as insulin and cortisone) and thyroid hormones on biosynthetic mechanisms for protein.

Little work has so far been reported on the action of thyroid hormones on mechanisms of protein biosynthesis. Since the brief report by F. Lipmann & C. Dutoit (see Dutoit, 1952) that thyroxine treatment enhanced the uptake of alanine by liver slices, Sokoloff & Kaufman (1959, 1961) found that repeated administration to rats of 100 μg. of L-thyroxine daily increased the incorporation of L-[^{14}C]leucine into protein by cell-free preparations. However, there are major differences in the experimental conditions and interpretation of results of these workers from ours. First, the incorporation of [^{14}C]leucine during the first 10 min. period in the cell-free preparations of Sokoloff & Kaufman (1961) is about one-eighth to one-ninth of that in our mitochondria-free homogenates from untreated rats. This low activity may be partly due to starvation or to suboptimum concentrations of Mg^{2+} and K^+ ions during homogenization or incubation and a suboptimum pH (see Hultin *et al.* 1961*a*, *b*). Secondly, we have consistently shown all our effects directly with microsomes or in mitochondria-free preparations; Stein & Gross (1962) could also demonstrate a similar effect in the absence of mitochondria. In contrast with this, Sokoloff & Kaufman (1961) could only demonstrate a stimulation of amino acid incorporation in the presence of mitochondria and it was suggested that an interaction between mitochondria and thyroxine was a prerequisite. Among numerous possibilities, the requirement for mitochondria may be explained by the increased release of K^+ and Mg^{2+} ions caused by the severe hyperthyroidism or the high concentration of thyroxine *in vitro* (see Aebi & Abelin, 1953; Tapley, 1956). Further, the long duration of 60 min. for the linearity of amino acid incorporation in Sokoloff & Kaufman's work is contradictory to the relatively short active life-span in our cell-free systems (see Hultin *et al.* 1961*a*).

There is a parallelism in the time-sequences of our results of acute experiments in thyroidectomized rats and the stimulation of amino acid incorporation by preparations of liver from the bull-frog tadpole during thyroid-hormone-induced metamorphosis (Paik & Cohen, 1960; see Frieden, 1961). It is also significant that thyroxine did not affect amino acid-activating enzymes in the tadpole (Degroot & Cohen, 1962). Metzenberg *et al.* (1961) demonstrated a new synthesis of carbamoyl phosphate synthetase and Herner & Frieden (1960, 1961) found that the increased activity of protein-synthetic mechanisms was reflected in a very marked alteration in the pattern of blood proteins and tissue enzymes, 3–5 days after treatment with thyroid hormone. As a function of time, the

thyroid-hormone-induced stimulation of amino acid incorporation in the bull-frog tadpole was preceded by marked alterations in nucleic acid metabolism (Finamore & Frieden, 1960).

Cell-sap enzymes

Several workers have shown that the activities of enzymes principally localized in the cell sap, particularly glycolytic enzymes, were either depressed after thyroidectomy or increased after repeated or long-term administration of large doses of thyroid hormones (Glock & McLean, 1955*a*, 1956; Huggins & Yao, 1959; Dow & Allen, 1961*a*, *b*; Bargoni *et al.* 1961; see Pitt-Rivers & Tata, 1959). Under our conditions thyroidectomy also resulted in a lower specific activity of lactic dehydrogenase in liver and muscle and glucose 6-phosphate dehydrogenase in liver, but the magnitude of stimulation of these enzymes after a single dose of tri-iodothyronine was inferior to that of mitochondrial and microsomal activities at the same time-interval. Also, significant changes in the cell-sap enzymes were only noticeable 2–3 days after the peak stimulation of the mitochondria and microsome-linked activities had occurred. This suggests that the changes observed in the 'chronic' type of experiments, by other workers, might have been secondary to action of thyroid hormones on mitochondrial respiration and phosphorylation and microsomal incorporation of amino acids into protein.

Changes in cellular constituents

Many thyroid-hormone-induced changes in the relative amounts of cellular constituents, such as pyridine nucleotides, ATP, nucleic acids, proteins, cytochromes, ubiquinone etc. have been recorded (Tissières, 1948; Glock & McLean, 1955*b*; Maley, 1957; Labouesse, Chatagner & Jollès-Bergeret, 1960; Edwin, Green, Diplock & Bunyan, 1960; Beyer, Noble & Hirschfeld, 1962; Fletcher & Myant, 1961; Sesso, 1962; see Pitt-Rivers & Tata, 1959). Whether or not they represent the cause or effect of changes in various cellular functions, or of other constituents, cannot be ascertained because of the 'chronic' treatment of animals In other experiments in our Laboratory, the increase in liver ubiquinone observed after chronic treatment with thyroid hormones could not be detected in acute experiments during the early phase of the stimulation of mitochondrial respiration and basal metabolic rate (Pedersen, Tata & Ernster, unpublished work). The decrease in liver glycogen that we have observed (Fig. 7) within 16 hr. after a single injection of tri-iodothyronine into thyroidectomized animals may be important because it precedes changes in other cellular constituents or activities. Fletcher & Myant (1961) have recently reported a dose relationship for the glycogen-lowering effect of repeated doses of thyroxine. The mechanism of this effect remains unknown but preliminary results indicated that it is independent of adrenaline since it is reproducible in thyroidectomized and adrenal-demedullated rats (Dr G. Bray, personal communication). The overall character of the increase in mitochondrial activities favours the idea that the action of thyroxine on mitochondria is more a question of alterations in the number of respiratory phosphorylating assemblies rather than a single rate-limiting enzyme. Further, the findings of Tissières (1948) and Drabkin (1950) of a large increase in the cytochrome *c* of liver in experimental hyperthyroidism is suggestive of an actual increase in the amounts of enzymic components rather than an activation of already existing assemblies. (Increases of 5–20 % in the amount of cytochromes *a* and *c* per total rat-liver mitochondrial protein and cytochrome b_5 in microsomes were also detected in both our acute and chronic experiments with much smaller doses.) The changes in microsomal RNA have already been discussed with reference to amino acid incorporation. Total RNA in liver, kidney and muscle was only slightly elevated or unaffected after thyroid treatment (Table 14). However, as Finamore & Frieden (1960) have pointed out, in tri-iodothyronine-induced metamorphosis in *Rana grylio*, a lack of marked change in total tissue RNA might conceal a rapid turnover. In contrast with tadpole metamorphosis, there was no decrease in DNA of rat tissue after treatment with thyroxine and tri-iodothyronine.

Action at the cell level and biological activity

Our 'mapping' of the various cellular activities in the absence, presence, replacement and excess of thyroid hormones should be considered in the light of the multiplicity of the biological actions of these hormones. Some of the actions on the whole body are likely to be more fundamental manifestations of biological activity than others, just as actions at the cell may be primary, secondary, tertiary and so on. Thus in view of our failure to detect an immediate or direct action on the efficiency of oxidative phosphorylation, one can ask to what extent the regulation of basal metabolic rate by thyroid hormones in itself represents a fundamental hormonal action. On the other hand, the almost simultaneous changes in a large number of cellular functions (mitochondrial as well as extramitochondrial) focus attention on the importance of the action of thyroid hormones in promoting growth and maturation as a fundamental action. It is becoming increasingly clear that many hormones with entirely different biological manifestations

(insulin, androgens, growth hormone, corticosteroids) can profoundly affect one or more mechanisms regulating protein biosynthesis, although differences in the modalities of the effects of individual hormones are apparent (Korner & Manchester, 1960; Korner, 1961; Krahl, 1961; Manchester & Young, 1961; Smith, Koepke & Franz, 1961; Leon *et al.* 1962). Does this then represent a common basis for the action of many hormones? At the present, it can only be speculated that whereas the effect on overall amino acid incorporation may only be a gross manifestation of their biological activity, the differences in their biological actions may stem from specific suppression or stimulation of different types of enzymic or non-enzymic proteins.

Although a larger number of cellular functions and constituents have to be studied before any categorical generalizations can be made, it appears from our results that enzymes or functions linked firmly to membranous subcellular structure are more markedly affected during the early phase of thyroid-hormone action than activities not dependent on structural integrity. Lee *et al.* (1959) found that administration of thyroid hormone markedly elevated the activity of glycerol 1-phosphate dehydrogenase that is firmly bound to mitochondria but was ineffective on the 'soluble' glycerophosphate dehydrogenase of the cell sap. The incorporation of acetate into cholesterol, which is linked to the membrane fraction of microsomes, has also been shown to be sensitive to small amounts of thyroid hormone (Fletcher & Myant, 1960). If a generalization could be made, then thyroid hormones might act primarily by either preferentially altering the relative rates of synthesis or turnover of the more tightly bound structural components or through a reorganization of structural elements so as to alter their efficiency. This interpretation of the cellular action of thyroid hormones bears on Hechter's idea that hormonal action may be translated by a participation in the structural organization of the cell (Hechter, 1955; Hechter & Lester, 1960), or Peters's (1956) suggestion of effects on the 'cytoskeleton'.

Our studies do not elucidate the mechanism of action of thyroid hormones; however, the method of chronologically mapping cellular activities after a single hormonal administration in thyroidectomized animals, initiated in these studies, should make this possible. The question then is to detect earlier and earlier changes in important cellular functions. It is essential that the changes in system(s) affected at the earliest time should be compatible with the sequential consequences. At this stage, model systems *in vitro* would be of value in proposing a new mode of action of thyroid hormones.

SUMMARY

1. Changes in a number of cellular activities and constituents localized in different subcellular fractions have been correlated with the effect on basal metabolic rate and growth of thyroidectomy and small doses of thyroid hormones, administered 'chronically' (15–35 μg. of tri-iodothyronine or of thyroxine every third or fourth day for 18–30 days) or 'acutely' (single injection of 15–30 μg. of tri-iodothyronine to thyroidectomized rats). In the acute experiments, measurements were made as a function of time after the single injection of the hormone to distinguish between the primary and secondary biochemical changes preceding, accompanying or following those in basal metabolic rate.

2. Thyroidectomy depressed, and both chronic and acute hormonal treatment stimulated, the respiratory quotient of liver and skeletal-muscle mitochondria with a variety of substrates in a fashion parallel to the changes in the basal metabolic rate. Maximum stimulation of q_{O_2} occurred between 45 and 60 hr. for liver mitochondria and between 60 and 70 hr. for muscle mitochondria, after a single injection of tri-iodothyronine into thyroidectomized rats.

3. Phosphorylation was not uncoupled from respiration at any stage before, during or after the acute or chronic changes in the basal metabolic rate or mitochondrial q_{O_2}.

4. The combined results of measuring mitochondrial respiration with various substrates in different combinations, succinoxidase and cytochrome oxidase in non-phosphorylating submitochondrial particles, inorganic phosphate–ATP exchange and cytochrome content, suggested that thyroid hormones affect the overall capacity of both the electron-transport chain and phosphorylation.

5. Changes in liver-mitochondrial respiration and phosphorylation were not preceded or accompanied by changes in stability *in vitro* as seen by swelling induced by phosphate, thyroxine, oleate or hypo-osmoticity or by ATP-induced contraction.

6. There was a marked stimulation of glucose 6-phosphatase and NADPH–cytochrome *c* reductase in liver microsomes accompanying, as a function of time after treatment, the stimulation of activity of mitochondria isolated from the same homogenates.

7. Amino acid incorporation into protein by cell-free liver systems was depressed to half the normal rate by thyroidectomy, and thyroid-hormone administration increased it two- to three-fold. In acute experiments, the maximal stimulation occurred 40–50 hr. after a single dose of tri-iodothyronine to thyroidectomized rats. The stimulation of amino acid incorporation into protein by

microsomes was greater when expressed per unit of microsomal protein than per unit of microsomal RNA as thyroid hormone administration increased the microsomal RNA:protein ratio.

8. The stimulatory effect of thyroid hormone on amino acid incorporation was not observed, or was even slightly reversed, in tissues from rats starved for 18–24 hr. before they were killed.

9. Thyroidectomy or administration of thyroxine or tri-iodothyronine did not alter the capacity of liver-cell sap (microsome-free supernatant) to activate amino acids as measured by the [^{32}P]pyrophosphate–ATP-exchange method.

10. Among the enzymes localized in the cell sap, the specific activities of liver and muscle creatine phosphokinase were unaffected by changes in the basal metabolic rate. Lactic dehydrogenase in liver-cell and muscle-cell sap and liver glucose 6-phosphate-dehydrogenase activities were increased by 20–50% in acute experiments. These increases did not occur until 60–90 hr. after tri-iodothyronine was given, reaching a peak between 90 and 160 hr., and therefore appear to be secondary to increases in basal metabolic rate and mitochondrial and microsomal activities.

11. Amounts of liver glycogen were unaffected by thyroidectomy but dropped by 50% within 24 hr. after a single injection of 30 μg. of tri-iodothyronine into non-starved animals.

12. Total tissue RNA and DNA and water content in liver, muscle and kidney were only slightly different after thyroidectomy and acute or chronic hypermetabolism.

13. The above results have been discussed with reference to some current mechanisms proposed for the action of thyroid hormones.

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