Ribonucleic Acid Synthesis during the Early Action of Thyroid Hormones

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1. The effect on RNA synthesis in rat liver of thyroidectomy and the administration of thyroid hormone, especially during its physiological latent period, was studied by determining: (a) the activity of DNA-dependent RNA polymerase in isolated nuclei; (b) the rate of synthesis of nuclear and cytoplasmic RNA *in vivo*; (c) polyribosomal sedimentation profiles; (d) the response of microsomes and ribonucleoprotein particles to polyuridylic acid; (e) the effect of inhibitors of RNA and protein synthesis on the biological activity of hormones. 2. The DNA-dependent RNA-polymerase activity of isolated rat-liver nuclei was lowered by thyroidectomy and stimulated by the administration of tri-iodo-L-thyronine or L-thyroxine (2–25 $\mu\text{g.}/100\text{g. body wt.}$) to both normal and thyroidectomized rats. In thyroidectomized rats, the activity of the Mg^{2+} -activated RNA-polymerase reaction (for which the product is mainly ribosomal type of RNA) was stimulated at 10–12 hr. after a single injection of tri-iodothyronine, reaching a peak value of 60–90% stimulation at 45 hr. after hormone administration. The Mn^{2+} /ammonium sulphate-activated RNA-polymerase reaction (for which the RNA product is more DNA-like) was not affected for 24 hr. after hormone administration but stimulated by 30–40% at 45 hr. The response of both RNA-polymerase reactions to the hormone *in vivo* paralleled the physiological response but the enzyme was not stimulated by the addition *in vitro* of the hormone to isolated nuclei. 3. Within 3–4 hr. after tri-iodothyronine administration to thyroidectomized rats, the specific activity of rapidly labelled nuclear RNA, after a 10 min. pulse of $[6\text{-}^{14}\text{C}]$ -orotic acid, was 30–40% greater than the control values, the stimulation reaching 100 and 200% at 11 and 16 hr. respectively after hormone administration. Longer exposures to $[6\text{-}^{14}\text{C}]$ -orotic acid and $[^{32}\text{P}]$ -phosphate showed that the hormone accelerated the synthesis of mitochondrial, microsomal (or ribosomal) and soluble RNA. The greater part of the labelled nuclear RNA was of the ribosomal type. The hormone-induced increases in the incorporation of radioactive precursors into RNA were not preceded, but followed, by enhanced uptake of the precursor. There was no change, per g. of liver, of DNA, nuclear RNA or soluble RNA, but there was a 40–60% increase in the amount of ribosomal RNA between 35 and 45 hr. after a single injection of tri-iodothyronine to thyroidectomized rats. 4. Coinciding with the increase in ribosomal RNA after hormone administration was an increase in the average size and amount of polyribosomes. The newly formed ribonucleoprotein particles, or messenger RNA attached to them, or both, were more firmly bound to microsomal membranes after hormone treatment. 5. Polyuridylic acid caused a bigger stimulation of incorporation of $[^{14}\text{C}]$ -phenylalanine by ribonucleoprotein particles, but not by microsomes, from thyroidectomized rats as compared with preparations from normal animals. The response of ribonucleoprotein particles to polyuridylic acid was lowered after tri-iodothyronine treatment of thyroidectomized rats. 6. Actinomycin D, 5-fluorouracil, puromycin and cycloheximide caused a 70–100% inhibition of the stimulatory effect of L-thyroxine and tri-iodo-L-thyronine on basal metabolic rate and growth rate in both normal and thyroidectomized animals. Administration of actinomycin D also abolished the stimulation of RNA polymerase by tri-iodothyronine. 7. It is concluded that regulation of nuclear and ribosomal RNA synthesis is an essential step leading to the biological action of thyroid hormones and that the formation of new ribosomes is an important aspect of the control of cytoplasmic protein synthesis by these hormones.

A single injection of small amounts of thyroid hormone causes, after a latent period of about 30 hr., a stimulation of the incorporation of amino acids into protein by microsomes and mitochondria (Tata *et al.* 1963; Roodyn, Freeman & Tata, 1965). Regulation of cytoplasmic protein synthesis is thought to be the basis for the growth-promoting and metabolic actions of L-thyroxine and 3,3',5-tri-iodo-L-thyronine (see Tata, 1964a). The importance of all types of RNA in the control of protein synthesis, the observation that the stimulatory effects of thyroid hormones *in vivo* were accompanied by changes in the cytoplasmic RNA concentrations and the latent period of nearly 30 hr. necessary for this effect to occur (Tata *et al.* 1963; Roodyn *et al.* 1965) prompted us to examine the possible influence these hormones may exert on the ability of the cell nucleus to synthesize RNA. At the time we initiated the studies reported below, there was no information on the effects of thyroid hormones on nucleic acid synthesis. Since then several reports have emphasized the importance of RNA synthesis in the action of other growth-promoting and developmental hormones (Korner, 1964; Williams-Ashman, Liao, Hancock, Jurkowitz & Silverman, 1964; Mueller, 1965; Sekeris, 1965).

We describe in the present paper the regulation of RNA synthesis by thyroid hormones, especially during the early period of their physiological action. This has been investigated from four different angles: (a) the activity of DNA-dependent RNA polymerase (EC 2.7.7.6) in isolated nuclei after thyroidectomy has been followed as a function of time after thyroid hormone administration; (b) the rate of incorporation of radioactive precursors into nuclear and cytoplasmic RNA *in vivo* has been followed under the same conditions; (c) polyribosomal sedimentation profiles and the response of microsomes and ribosomes to synthetic messenger RNA that accompany the changes in cytoplasmic protein synthesis induced by the hormone *in vivo* have been determined; (d) the effect of inhibitors of RNA and protein synthesis on the two major biological actions of thyroid hormones in mammals, namely growth promotion and regulation of B.M.R.,* were measured. We conclude from these studies that the regulation of RNA synthesis in the nucleus and the formation of new ribosomes are important features of the early action of thyroid hormones. Some of the results have already been reported briefly (Widnell & Tata, 1963; Tata, 1963, 1964b).

* Abbreviations: B.M.R., basal metabolic rate; s-RNA, 'soluble' RNA; m-RNA, messenger RNA; RNP, ribonucleoprotein; polyU, polyuridylic acid.

METHODS AND MATERIALS

Animals and treatment. Male albino rats (Wistar) were used, with no restrictions on their food intake throughout the experiments. Thyroidectomy was performed on young animals (45–55 g.) by a combination of surgery and radioiodine administration. Thyroidectomized rats were used about 4 weeks after the operation by which time their B.M.R. and body weight were about 20 and 30% respectively below the values in unoperated controls. Periodic checks were made on B.M.R. and growth rate as described by Tata *et al.* (1963). The usual dose of 3,3',5-tri-iodo-L-thyronine administered as a single subcutaneous injection was about 20 µg./100 g. body wt. Radioactive substances and inhibitors of RNA and protein synthesis were dissolved in 0.15 M-NaCl and injected intraperitoneally.

Isolation of subcellular fractions. All procedures were performed at 0–4°. For studies on isolated nuclei and RNA synthesis *in vivo*, livers from three rats were pooled and homogenized in 0.32 M-sucrose containing MgCl₂ (3 mM). Nuclei were isolated as described by Widnell & Tata (1964a) and 55–70% of the total DNA in the homogenate was recovered in the nuclear fraction. Mitochondria, microsomes (or ribosomes) and the cell sap were separated by differential centrifuging (Tata *et al.* 1963) from the nuclei-free homogenate after a preliminary additional centrifuging at 700 g for 10 min. For studies on the incorporation of amino acids into protein and distribution of polyribosomes, microsomes and ribosomes were isolated from livers homogenized in a medium containing sucrose (0.25 M), KCl (75 mM), MgCl₂ (10 mM) and tris-HCl buffer, pH 7.6 (35 mM), as described by Tata *et al.* (1963).

Distribution of polyribosomes. Ribosomes from 2 g. of liver were obtained from the mitochondria-free supernatant by treatment with 0.25–1.0% (w/v) sodium deoxycholate and were suspended in 1 ml. of a medium containing KCl (50 mM), MgCl₂ (1 mM) and tris-HCl buffer, pH 7.6 (20 mM). These were then layered over 26 ml. of 15–30% (w/v) discontinuous sucrose density gradient (Charlwood, 1963) with a cushion of 3 ml. of 60% (w/v) sucrose at the bottom of the Spinco SW25.1 rotor tube. After centrifuging at 22000 rev./min. for 110–140 min. at 0–4°, 1 ml. fractions were removed by siphoning from the bottom of the tube and extinctions at 260 mµ and 280 mµ and radioactivity were determined in the fractions (*E*₂₆₀/1.0 corresponds to 50 µg. of RNA). The pellet that had sedimented through the sucrose gradient was suspended in 3 ml. of 1.0% sodium deoxycholate and corrections were made for protein content from the *E*₂₆₀/*E*₂₈₀ ratio. The RNA, in the remainder of each fraction after samples had been removed for extinction measurement, was precipitated with 0.4 N-HClO₄, and washed with 70% (v/v) ethanol before ¹⁴C determination.

Assay of RNA polymerase. The two RNA-polymerase reactions of isolated rat-liver nuclei were determined by a slight modification of the procedure described by Widnell & Tata (1964b). The incubation medium for the Mg²⁺-activated reaction, carried out in the absence of (NH₄)₂SO₄, contained, in 0.5 ml.: 50 µmoles of tris-HCl buffer, pH 8.5; 2.5 µmoles of MgCl₂; 10 µmoles of cysteine; 3 µmoles of NaF; 0.3 µmole each GTP, CTP and UTP; 0.015 µmole of non-radioactive ATP; 0.005 µmole of [¹⁴C]ATP; 0.1 ml. of nuclear suspension containing about 0.3 mg. of DNA. Incubation was for 15 min. in air at 37°, after which time the reaction was stopped by the addition of 5 ml. of 0.5 N-HClO₄ at 0°.

The incubation medium for the Mn^{2+} -activated RNA-polymerase reaction, carried out in the presence of $(NH_4)_2SO_4$, contained, in 0.5 ml.: 50 μ moles of tris-HCl buffer, pH 7.5; 2 μ moles of $MnCl_2$; 0.05 ml. of a solution of $(NH_4)_2SO_4$, saturated at room temperature and adjusted to pH 7.5 with aq. NH_3 ; 0.3 μ mole each of GTP, CTP and UTP; 0.015 μ mole of non-radioactive ATP; 0.1 ml. of nuclear suspension. After preincubation for 15 min. at 37°, the reaction rate was followed by the addition of 0.005 μ mole of $[^{14}C]$ ATP, and the reaction was stopped, after a further 45 min. of incubation, by the addition of 5 ml. of 0.5 N-HClO₄ at 0°.

The RNA product of both reactions was extracted, and the radioactivity determined, as described by Widnell & Tata (1964a). Unless otherwise stated, each value presented for the specific activity of RNA polymerase represents the mean of either triplicate determinations at one concentration of nuclei in the incubation medium, or duplicate determinations at two concentrations; these values normally agreed to within 5%.

Other enzymic determinations. NAD pyrophosphorylase was determined as described by Widnell & Tata (1964a). ATP was determined enzymically by the method of Roodyn & Suttie (1961).

Nuclear RNA and cytoplasmic RNA labelled in vivo. Rats were killed at different time-intervals after the injection of 4–8 μ C of $[^{14}C]$ orotic acid or 60–80 μ C of carrier-free $[^{32}P]$ -orthophosphate and their livers were homogenized and subjected to subcellular fractionation. Nuclei, mitochondria and microsomes (or ribosomes) were suspended in an all-glass Potter-Elvehjem homogenizer as 2 g. equivalent of liver/ml. of the initial homogenizing medium. With microsomes, sodium deoxycholate was added to the suspending medium at a final concentration of 0.4%. RNA from the 1.0–2.5 ml. suspensions of the different particles and 12 ml. of cell sap (105 000 g supernatant) was precipitated with

0.4 N-HClO₄ at 0°. The precipitate was washed three times with 0.4 N-HClO₄ and then twice with ether-ethanol (3:1, v/v) and once with ether alone before two extractions of the RNA with hot 2 M-NaCl (90° for 60 and 30 min. for the first and second extractions respectively). One sample of the hot-NaCl extract was used for chemical determination of RNA; 200 μ g. of carrier yeast RNA was added to the rest, and the RNA precipitated with 66% (v/v) ethanol and hydrolysed in 0.5 ml. of 0.4 N-KOH or -NaOH at 35° for 18 hr. The hydrolysate was neutralized with HClO₄ or HCl, any precipitate removed by centrifuging and the DNA precipitated by the addition of 2 vol. of ethanol. The ribonucleotides in the ethanol supernatant were concentrated for the determination of radioactivity and chromatography.

Incorporation of L- $[^{14}C]$ phenylalanine into protein and the effect of polyU. The incubation mixture contained, in a final volume of 0.6 ml.: 3 μ moles of KCl; 14 μ moles of tris-HCl buffer, pH 7.6; 4.2 μ moles of $MgCl_2$; 0.09 μ mole of each of 19 amino acids except for phenylalanine; 6.0 μ moles of GSH; 2.5 μ moles of phosphoenolpyruvate; 0.25 μ mole of ATP; 0.0625 μ mole of GTP; 25 μ g. of pyruvate kinase; L- $[^{14}C]$ phenylalanine. The amino acid mixture was made up of: L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glycine, L-glutamine, L-glutamic acid, L-histidine, L-hydroxyproline, L-isoleucine, DL-leucine, L-lysine, L-methionine, L-proline, L-serine, L-tryptophan, L-tyrosine and L-valine. The reaction was stopped by the addition of 5 μ moles of non-radioactive phenylalanine in 0.2 ml. of N-NaOH; the incubation was continued for another 5 min. to hydrolyse the remaining s-RNA, after which protein was precipitated with 2.0 ml. of 10% (w/v) trichloroacetic acid at 0°. The precipitate was washed three times with 3 ml. of cold 5% (w/v) trichloroacetic acid, twice with the hot acid and twice with water on Oxoid membrane filters (Oxo Ltd., London, E.C.4). After drying, the radioactivity in the

Table 1. *Effect of thyroidectomy, and of thyroid hormone administration in vivo to thyroidectomized and normal rats, on the specific activities of RNA polymerase and NAD phosphorylase in isolated rat-liver nuclei*

Experimental details are given in the text. The specific activity of the Mg^{2+} -activated RNA-polymerase reaction is expressed as μ moles of $[^{14}C]$ ATP incorporated into RNA/15 min./mg. of DNA, and that of the $Mn^{2+}/(NH_4)_2SO_4$ -activated RNA-polymerase reaction as μ moles of $[^{14}C]$ ATP incorporated into RNA/45 min./mg. of DNA. The specific activity of NAD pyrophosphorylase is expressed in m μ moles of NAD synthesized/20 min./mg. of DNA.

Animals	Specific activity of RNA polymerase		Specific activity of NAD pyrophos- phorylase
	Mg^{2+} -activated	$Mn^{2+}/(NH_4)_2SO_4$ - activated	
Thyroidectomized	665	2205	492
Thyroidectomized, treated with 25 μ g. of tri-iodothyronine 24 hr. before death	1076	2325	475
Thyroidectomized, treated with 25 μ g. of tri-iodothyronine 45 hr. before death	1085	3225	508
Normal, unoperated	1135	3275	502
Normal unoperated, treated with 5 μ g. of tri-iodothyronine/day for 8 days before death	1445		

protein was directly determined on the filters. For comparing the response of microsomes and ribonucleoprotein particles to polyU, the usual time of incubation at 37° was 30 and 60 min. (Campbell, Cooper & Hicks, 1964).

Determination of nearest-neighbour frequencies and base composition of RNA. For the determination of nearest-neighbour frequencies of the four nucleotides of the hydrolysed product of RNA polymerase, the nucleotides were separated by the ion-exchange column-chromatographic method of Katz & Comb (1963) and specific radioactivity was measured in the eluates. For the determination of base composition of ^{32}P -labelled RNA formed *in vivo*, the nucleotides extracted into ethanol from the RNA hydrolysate were separated by paper chromatography by the method of Lane (1963). The relative distribution was estimated from the density of silver grains deposited on a radioautograph (Ilford, No-Screen X-ray film), by using a Joyce-Loebl double-beam densitometer.

Measurement of radioactivity. ^{14}C was generally measured in the Packard Tri-Carb scintillator spectrometer by depositing the RNA on a cylinder of filter paper (Widnell & Tata, 1964a) (counting efficiency 66%, background 20 counts/min.). The scintillator fluid contained 0.5% of 2,5-diphenyloxazole and 0.05% of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in redistilled toluene. In studies on the incorporation of [^{14}C]phenylalanine into protein, the radioactivity was measured on the membrane filter in a Nuclear-Chicago thin-window gas-flow counter (efficiency about 20%, background 2 ± 0.5 counts/min.). ^{32}P was also measured with an end-window Geiger-Müller counter (efficiency about 40%).

Chemical determinations. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin as standard. DNA and RNA were estimated by the methods of Burton (1956) and Ceriotti (1955) respectively, after extraction of the nucleic acids as described by Widnell & Tata (1964a), with calf-thymus DNA (Sigma) and yeast RNA (Boehringer) as standards. Phosphorus was measured by the method of Fiske & Subbarow (1925).

Materials. ATP, GTP, CTP, UTP, NAD and NMN were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.; yeast RNA, phosphoenolpyruvate, GSH, yeast alcohol dehydrogenase and pyruvate kinase were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; amino acids, nicotinamide and 5-fluorouracil were from Roche Products Ltd., Welwyn Garden City, Herts. Puro-mycin was obtained from Lederle Laboratories, Pearl River, N.Y., U.S.A., cycloheximide (Actidione) was from California Corp. for Biochemical Research, Lucerne, Switzerland, and actinomycin D was a gift from Merck, Sharpe and Dohme Co. Inc., Rahway, N.J., U.S.A. L-Thyroxine, 3,3',5-tri-iodo-L-thyronine and their derivatives were obtained from Glaxo Laboratories, Greenford, Middlesex; they were dissolved in 1% (v/v) propane-1,2-diol at pH 8.3–8.5. PolyU was purchased from Miles Chemical Co., Elkhart, Ind., U.S.A., and dissolved in water.

Carrier-free [^{32}P]phosphate, L-[^{14}C]phenylalanine (1.22 mc/m-mole) and [6- ^{14}C]orotic acid (6.8 mc/m-mole) were purchased from The Radiochemical Centre, Amersham, Bucks. [8- ^{14}C]ATP (11.2 mc/m-mole) was obtained from Schwarz Bio-Research Inc., Orangeburg, N.Y., U.S.A. [α - ^{32}P]ATP and [α - ^{32}P]GTP were synthesized as described by Nakamoto, Fox & Weiss (1964).

RESULTS

Effect of thyroid hormone administration in vivo on RNA-polymerase activity in vitro. Preliminary experiments (Widnell & Tata, 1963) showed that the specific activity of the Mg^{2+} -activated RNA-polymerase reaction of isolated nuclei was stimulated within 16 hr. after the administration of tri-iodo-L-thyronine to thyroidectomized rats. In a more detailed investigation (Table 1), it was found that the specific activities of both the Mg^{2+} - and Mn^{2+} /ammonium sulphate-activated RNA-polymerase reactions were depressed by thyroidectomy, and restored to normal 45 hr. after a single administration of tri-iodo-L-thyronine to thyroidectomized

Fig. 1. Time-course of the two RNA-polymerase reactions in liver nuclei isolated from thyroidectomized and tri-iodothyronine-treated thyroidectomized rats. The assay of the two RNA-polymerase reactions was as described in the text. (a) Mg^{2+} -activated RNA-polymerase reaction; (b) $\text{Mn}^{2+}/(\text{NH}_4)_2\text{SO}_4$ -activated RNA-polymerase reaction. ●, Nuclei isolated from thyroidectomized rats; ▲, nuclei isolated from thyroidectomized rats 45 hr. after an injection of 25 $\mu\text{g.}$ of tri-iodothyronine/100 g. body wt.

Table 2. *Characteristics of the two RNA-polymerase reactions of liver nuclei isolated from thyroidectomized and tri-iodothyronine-treated thyroidectomized rats*

Experimental details are given in the text. The deoxyribonuclease treatment was as described by Widnell & Tata (1966b). Tri-iodothyronine-treated rats received a single injection of 20 μ g. of the hormone 45 hr. before death.

Source of nuclei	Incubation medium	RNA-polymerase activity (μ moles of [14 C]ATP incorporated into RNA/mg. of DNA)	
		Mn $^{2+}$ /(NH $_4$) $_2$ SO $_4$ - activated	
		Mg $^{2+}$ -activated	
Thyroidectomized rats	Complete	684	2320
	GTP omitted	193	274
	CTP omitted	184	301
	UTP omitted	167	359
	GTP, CTP and UTP omitted	61	117
	Complete, + 25 μ g. of deoxyribonuclease	109	42
Tri-iodothyronine-treated thyroidectomized rats	Complete	1240	3540
	GTP omitted	379	336
	CTP omitted	342	352
	UTP omitted	296	397
	GTP, CTP and UTP omitted	78	139
	Complete, + 25 μ g. of deoxyribonuclease	187	97

rats. Table 1 also shows that the specific activity of the Mg $^{2+}$ -activated RNA-polymerase reaction was stimulated 24 hr. after tri-iodo-L-thyronine administration, at which time no change was detected in the specific activity of the Mn $^{2+}$ /ammonium sulphate-activated RNA-polymerase reaction. The nuclei of normal rats also responded to the administration of thyroid hormone but to a smaller extent than preparations from thyroidectomized rats. No change was observed, in any of the groups of experimental animals, in the specific activity of NAD pyrophosphorylase.

The major characteristics of the two RNA-polymerase reactions were not altered by thyroidectomy or by the administration of tri-iodo-L-thyronine to thyroidectomized rats. Fig. 1 shows that the incubation time-course of both RNA-polymerase reactions was the same in liver nuclei isolated from either untreated or tri-iodothyronine-treated thyroidectomized rats. In the Mn $^{2+}$ /ammonium sulphate-activated RNA-polymerase reaction, the radioactive precursor was added after 15 min. of incubation since by this time no more RNA was formed by the reaction taking place in the absence of the salt (see Widnell & Tata, 1964b). Table 2 shows that for both groups of animals, and for both RNA-polymerase reactions, incorporation of [14 C]ATP into RNA was dependent on DNA as well as on the presence of all four nucleoside triphosphates. The stimulation of RNA polymerase after tri-iodothyronine treatment could not be

Fig. 2. Stimulation of the two RNA-polymerase reactions of isolated liver nuclei after a single injection of 25 μ g. of tri-iodothyronine/100 g. body wt. to thyroidectomized rats. The experimental details were as described in the text. ●, ▲ and ■, Mg $^{2+}$ -activated RNA-polymerase reaction; ○ and △, Mn $^{2+}$ /(NH $_4$) $_2$ SO $_4$ -activated RNA-polymerase reaction. Different symbols denote the values obtained from separate experiments.

explained by a decrease in the size of the endogenous ATP pool of isolated nuclei, as the concentration of ATP in liver nuclei from thyroidectomized rats, which was found to be less than 1 μ mmole/mg. of DNA, was not sufficiently large to exert any effect on the specific activity of the enzyme. From this and the lack of effect on nuclear NAD pyrophos-

phorylase, it may be inferred that the effects of thyroidectomy and thyroid hormone replacement on the specific activity of RNA polymerase are unlikely to be due to some non-specific effect on nuclear metabolic activity, but that they represent a more selective action on RNA polymerase.

Analysis of the time-course of the hormonal stimulation revealed quite distinct responses of the two RNA-polymerase reactions to a single injection of tri-iodothyronine to thyroidectomized rats, as shown in Fig. 2. No change was detected in the specific activity of the Mg^{2+} -activated reaction 6 hr. after the single injection, but at 10 hr. a small but reproducible rise was observed. Peak stimulation was between 24 and 48 hr., after which time the specific activity gradually fell, returning to the control value by 140 hr. Essentially no change was observed in the specific activity of the Mn^{2+} /ammonium sulphate-activated reaction during the first 24 hr. after a single injection of tri-iodothyronine, but by 42 hr. the specific activity was stimulated to values found in nuclei isolated from normal animals; this stimulated level was maintained until at least 72 hr. after the injection. No change was observed in the protein/DNA or RNA/DNA ratio of any of the nuclear preparations described in Fig. 2.

The pattern of the dose response of both RNA-polymerase reactions to a single injection of tri-iodo-L-thyronine, which is shown in Fig. 3, was very similar to that found for whole body growth or increase in the weight of the liver (J. R. Tata & C. C. Widnell, unpublished work). Maximum stimulation of either reaction to give values close to those of the specific activity in the control unoperated animal was observed at a dose of about 2 $\mu g.$ of tri-iodo-L-thyronine/100 g. body wt. There was also a good correlation between the physio-

logical activity of structural analogues of thyroid hormones in the whole animal (see Pitt-Rivers & Tata, 1959) and their effects on RNA polymerase determined in isolated nuclei. Table 3 shows that the analogues tri-iodo-D-thyronine and 3,5-di-iodo-L-thyronine, which possess only a fraction of the biological activity of tri-iodo-L-thyronine, caused a slight stimulation of both RNA-polymerase reactions, but that the stimulation at high doses

Fig. 3. Effect of the dose of tri-iodothyronine administered to thyroidectomized rats on the stimulation of the two RNA-polymerase reactions of isolated liver nuclei. The animals were killed 45 hr. after hormone administration; other experimental details were as described in the text. ●, Mg^{2+} -activated RNA-polymerase reaction; ▲, $Mn^{2+}/(NH_4)_2SO_4$ -activated RNA-polymerase reaction.

Table 3. Stimulation of the two RNA-polymerase reactions of isolated liver nuclei by thyroid hormone analogues administered in vivo

Experimental details for the assay of the two RNA-polymerase reactions are as given in the text. The specific activity of RNA polymerase is as expressed in Table 1. Compounds were injected as a single dose 45 hr. before killing the animals.

Rats	Compound	Amount injected ($\mu g.$)	Specific activity of RNA polymerase	
			Mg^{2+} -activated	$Mn^{2+}/(NH_4)_2SO_4$ - activated
Normal	—	—	960	3020
Thyroidectomized	—	—	704	2170
	3,3',5-Tri-iodo-L-thyronine	18	1255	3080
	3,3',5-Tri-iodo-L-thyronine	2	1105	2870
	3,3',5-Tri-iodo-D-thyronine	30	1040	2750
	3,5-Di-iodo-L-thyronine	150	1010	2650
	L-Thyroxine	30	1180	Not assayed

Table 4. *Effect of tri-iodo-L-thyronine treatment in vivo and in vitro on the Mg^{2+} -activated RNA-polymerase reaction of liver nuclei isolated from thyroidectomized rats*

Experimental details are given in the text. The specific activity of the Mg^{2+} -activated RNA-polymerase reaction is as expressed in Table 1.

Time after tri-iodo-L-thyronine treatment <i>in vivo</i> (hr.)	Tri-iodo-L-thyronine added to nuclei <i>in vitro</i> ($\mu g.$)	Specific activity of RNA polymerase
—	—	626
—	0.1	618
—	1.0	628
—	10.0	594
16	—	838
48	—	964

of the analogues was lower than that induced by a small dose of both the normally circulating hormones, tri-iodo-L-thyronine and L-thyroxine.

Table 4 shows that, under conditions in which treatment of thyroidectomized rats with tri-iodo-L-thyronine *in vivo* caused a stimulation of the Mg^{2+} -activated RNA-polymerase reaction, direct addition *in vitro* of tri-iodothyronine to nuclei isolated from the livers of thyroidectomized rats had no effect on the specific activity. No effect was observed at any concentration of tri-iodothyronine from 0.1 $\mu g.$ to 10 $\mu g.$ added to 0.5 ml. of the incubation mixture. This result, taken together with the finding (Fig. 2) that no change could be detected in the specific activity of this RNA-polymerase reaction until about 10 hr. after tri-iodo-L-thyronine administration, suggests that the hormonal stimulation of RNA polymerase *in vivo* does not stem from a direct hormone-enzyme interaction.

Effect of thyroid hormone on the synthesis and turnover of nuclear RNA and cytoplasmic RNA in vivo. [6- ^{14}C]Orotic acid was found to be more suitable than [^{32}P]phosphate as a precursor for studying the formation of rapidly labelled nuclear RNA in rat liver. Fig. 4 shows the time-course of incorporation of ^{14}C into RNA extracted from nuclei, mitochondria, microsomes and cell sap of livers of thyroidectomized rats after the injection of [^{14}C]orotic acid. This pattern is similar to that described for normal rat liver by other workers who have studied the incorporation of [^{14}C]orotic acid into RNA (Adams & Busch, 1963; Moulé & Delhumeau de Ongay, 1964). At early time-intervals after the administration of [^{14}C]orotic acid, over 75% of acid-insoluble ^{14}C was recovered as nuclear RNA, whereas at time-intervals exceeding 2 hr. an increasing fraction of radioactivity appeared in cytoplasmic RNA. This

Fig. 4. Time-course of incorporation of [^{14}C]orotic acid into nuclear and cytoplasmic RNA from livers of thyroidectomized rats. A 5.0 $\mu g.$ dose of [^{14}C]orotic acid was injected into each rat at the various time-intervals indicated before killing and the RNA extracted from the different cellular fractions as described in the text. ●, Nuclear RNA; △, mitochondrial RNA; ○, microsomal RNA; □, s-RNA.

pattern is consistent with the conclusion drawn from kinetic analysis in various mammalian and microbial systems that cytoplasmic RNA is formed in the nucleus (see Prescott, 1964).

Administration of tri-iodothyronine to thyroidectomized rats caused an early enhancement of the incorporation of [^{14}C]orotic acid into the rapidly labelled nuclear RNA (Table 5). The specific radioactivity of nuclear RNA, after a 10 min. pulse of labelled orotic acid, was over 3 times the control value in animals that had received a single injection of tri-iodothyronine 16 hr. before. No further stimulation was discernible if the animals were pretreated with the hormone for longer periods of time. The earliest significant increase (25% over the control values) in the specific activity of the rapidly labelled nuclear RNA was observed 3–4 hr. after the hormone administration, with a doubling of the specific activity at about 12 hr. A marked elevation of the total uptake of ^{14}C from [^{14}C]orotic acid (acid-soluble and acid-insoluble ^{14}C) was observed 16 hr. after tri-iodothyronine but not at earlier time-intervals, suggesting that the higher uptake of the precursor may reflect an adjustment to an accelerated rate of RNA synthesis.

In experiments in which the incorporation of [^{32}P]phosphate into RNA was studied after longer exposure to the isotope than with orotic acid, a similar increase after hormone treatment in the uptake of [^{32}P]phosphate by the liver did not accompany the enhanced incorporation of ^{32}P into RNA (Table 6). When rats were killed 10–16 hr.

Table 5. *Specific activities of rapidly labelled RNA in different cellular fractions and uptake of [¹⁴C]orotic acid in livers of thyroidectomized rats at different times after the injection of tri-iodothyronine*

A 5.0 μ C dose of [¹⁴C]orotic acid was injected into each rat 10 min. before killing. The dose of tri-iodothyronine was 18 μ g./100g. body wt., each rat weighing 140 ± 7 g. No change in B.M.R. was detected until 25 hr. after hormone injection but there was a 16% increase at 42 hr. Each value is the average of two separate experiments with variations within $\pm 10\%$. Corrections were made for variations in body wt. and amount of isotope injected.

Time after tri-iodo- thyronine (hr.)	Uptake of [¹⁴ C]orotic acid (counts/min./g. of liver)	Specific activity (counts/min./mg. of RNA)			
		Nuclear RNA	Mitochondrial RNA	Microsomal RNA	Cell-sap RNA
0.0	88650	14400	175	308	462
3.3	84000	18300	206	401	425
6.4	86300	21000	160	480	575
11.0	95350	27600	231	415	661
16.1	138900	45250	205	516	823
21.0	159500	43000	322	500	776
42.0	141100	40780	365	559	695

Table 6. *Stimulation of incorporation in vivo of [³²P]phosphate into liver nuclear RNA, ribosomal RNA and s-RNA of thyroidectomized rats after the administration of tri-iodothyronine*

The 75 μ C and 88 μ C doses of [³²P]phosphate were injected into each rat 9.8 and 16.2 hr. respectively before killing. Other conditions were as in Table 5 and the text. Mitochondrial RNA values were unreliable because of large amounts of ³²P-labelled nucleotides and proteins recovered in these particles.

Time after [³² P]phosphate (hr.)	Time between tri-iodothyro- nine and [³² P]phosphate (hr.)	³² P content (μ C/g. of liver)	Specific radioactivity ($m\mu$ C of ³² P/mg. atom of RNA P)		
			Nuclear RNA	Ribosomal RNA	s-RNA
9.8	0.0	1.72	8.35	3.10	9.20
	6.5	1.60	13.91	3.05	11.00
	12.0	1.91	23.46	3.43	9.95
	25.2	2.06	26.60	4.95	13.18
16.2	0.0	1.28	6.68	5.15	6.74
	6.5	1.33	9.15	6.35	10.22
	10.0	1.19	12.50	7.60	10.34
	16.2	1.70	19.39	8.42	11.31
	21.0	1.66	17.83	12.11	12.91
	30.5	1.49	14.20	12.00	10.05

Table 7. *Base composition of nuclear RNA and ribosomal RNA labelled with [³²P]phosphate in vivo in normal, thyroidectomized and tri-iodothyronine-treated thyroidectomized rats*

Rats were killed 16.2 hr. after receiving 88 μ C of [³²P]phosphate; 18 μ g. of tri-iodothyronine was injected into one group of thyroidectomized rats 46 hr. before killing. RNA from thyroidectomized rats was extracted from the same preparations used in experiments shown in Table 6. Each value is the percentage of the total ³²P in the recovered four nucleotides (92–96% recovery from RNA hydrolysates).

Rats	RNA	Distribution of ³² P (%)				(AMP + UMP) (GMP + CMP) ratio
		AMP	GMP	CMP	UMP	
Normal	Nuclear	20.5	29.3	28.7	20.0	0.70
	Ribosomal	19.2	32.9	29.4	18.0	0.60
Thyroidectomized	Nuclear	21.2	30.0	27.8	20.2	0.71
	Ribosomal	19.8	32.2	30.1	17.9	0.61
Thyroidectomized + tri- iodothyronine	Nuclear	21.4	30.2	27.0	20.2	0.72
	Ribosomal	20.5	31.4	30.0	17.6	0.62

after the administration of [^{32}P]phosphate, ribosomal RNA accounted for the bulk of the radioactive RNA. In these long-term labelling experiments, administration of thyroid hormone resulted in a 2–8-fold higher specific activity of both the nuclear RNA and cytoplasmic RNA. However, the hormone did not alter the base composition of the newly synthesized RNA. In Table 7 is summarized the effect of tri-iodothyronine on the distribution of ^{32}P in the four nucleotides of nuclear and ribosomal RNA in two groups of animals shown in Table 6. The distribution of labelled AMP, UMP, GMP and CMP in ribosomal RNA from thyroidectomized rats agrees well with the base composition of ribosomal or bulk RNA in rat liver reported by other workers (Hiatt, 1962; Lipshitz-Wiesner & Chargaff, 1963; Lieberman & Kane, 1965). Much of the ^{32}P -labelled RNA from the nucleus was of the ribosomal type, with a high guanosine + cytidine content, although the (AMP + UMP)/(GMP + CMP) ratio was consistently 10–15% higher than in RNA extracted from cytoplasmic ribosomes. In two experiments in which animals were killed at 30 and 90 min. after giving [^{32}P]phosphate, this ratio was considerably higher and therefore more DNA-like. No conclusions could, however, be drawn in this case as to the effect of the hormone on account of the high degree of quantitative variability from one group of control animals to another.

The absence of any effect of thyroidectomy and tri-iodothyronine administration on the base composition of RNA synthesized *in vivo* was paralleled by experiments *in vitro* where the nearest-neighbour frequency of the RNA products of the two RNA-polymerase reactions was measured (Table 8). It has already been demonstrated that it is the Mg^{2+} -activated, and not the Mn^{2+} /ammonium sulphate-activated, RNA polymerase in liver nuclei that is initially stimulated by thyroid hormone administration (see Fig. 1). In the presence of ammonium sulphate the product is more DNA-like; the functional significance of the different RNA products of the polymerase is discussed by Widnell & Tata (1966b).

Table 9 depicts the net accumulation of ribosomal RNA as a function of time after hormone treatment. The values for total liver RNA and ribosomal RNA, as well as the RNA/protein ratio in microsomes (see Table 11), were decreased 4 weeks after thyroidectomy to 50–60% of those found in normal rats. These values were restored to nearly normal levels at about 45 hr. after a single injection of tri-iodothyronine, which reproduces previous observations under identical conditions (Tata *et al.* 1963; Roodyn *et al.* 1965). Net accumulation of newly formed RNA in cytoplasmic ribosomes was not clearly evident for about 24 hr. after the hormone although the synthesis of rapidly labelled nuclear RNA was accelerated within 6 hr. of hormonal

Table 8. *Nearest-neighbour base frequency of the product of the two RNA-polymerase reactions of liver nuclei isolated from normal, thyroidectomized and tri-iodothyronine-treated thyroidectomized rats*

Polymerase reaction	Radioactive nucleotide	Source of nuclei	Percentage of ^{32}P counts/min. recovered				Percentage recovery of ^{32}P counts/min. from column
			In UMP	In GMP	In CMP	In AMP	
Mg^{2+} -activated	[α - ^{32}P]ATP	Normal rat	20	29	29	22	98
		Thyroidectomized rat	17	33	31	19	97
		Thyroidectomized rat, tri-iodothyronine-treated	18	33	30	19	102
	[α - ^{32}P]GTP	Normal rat	19	34	30	17	97
		Thyroidectomized rat	19	35	30	16	100
		Thyroidectomized rat, tri-iodothyronine-treated	18	34	28	20	95
$\text{Mn}^{2+}/(\text{NH}_4)_2\text{SO}_4$ -activated	[α - ^{32}P]ATP	Normal rat	26	24	20	30	96
		Thyroidectomized rat	25	25	23	27	99
		Thyroidectomized rat, tri-iodothyronine-treated	27	25	20	28	98
	[α - ^{32}P]GTP	Normal rat	34	27	10	29	101
		Thyroidectomized rat	37	24	9	30	94
		Thyroidectomized rat, tri-iodothyronine-treated	34	28	9	29	99

Table 9. *DNA, nuclear RNA, ribosomal RNA and s-RNA in livers of thyroidectomized rats at different times after a single injection of tri-iodothyronine*

Values were obtained from the liver preparations used for experiments in Tables 5 and 6.

Time after tri-iodothyronine (hr.)	DNA (mg./g. of liver)	Nuclear RNA (mg./mg. of DNA)	Ribosomal RNA (mg./mg. of DNA)	s-RNA (mg./mg. of DNA)
0.0	1.97	0.26	2.46	0.24
6.2	1.94	0.22	2.52	0.22
11.2	2.01	0.31	2.47	0.34
16	1.93	0.25	2.51	0.27
21	1.94	0.30	3.02	0.32
42.2	1.84	0.33	3.64	0.24
Normal, untreated	1.81	0.32	3.81	0.22

treatment. There was a small increase in the content of s-RNA but only a slight or no accumulation of RNA in the nucleus between 20 and 40 hr. after tri-iodothyronine administration. No change was recorded during this period in the DNA/liver, and a slight fall in the DNA/g. of liver at 40 hr., which is an indication of the hypertrophic action of the hormone. Insignificantly small amounts of [^{32}P]-phosphate and [^{14}C]orotic acid were incorporated into DNA in any of the experiments.

It may be concluded from the above studies on RNA synthesis *in vivo* that an early effect of thyroid hormone is to accelerate synthesis of RNA in the nucleus, consequently leading to the appearance of more newly formed RNA in cytoplasmic ribosomes or microsomes.

Effect of tri-iodothyronine in vivo on hepatic polyribosomal sedimentation profiles. Since polyribosomes are considered to be aggregates of ribosomes on m-RNA strands (Warner, Knopf & Rich, 1963; Noll, Staehelin & Wettstein, 1963; Munro, Jackson & Korner, 1964) analysis of polyribosomal profiles in sucrose-density-gradient centrifuging was used as an indication of the effect of the hormone on the concentration of m-RNA in the cytoplasm. Fig. 5 shows that the fraction of total liver ribosomal RNA recovered as polysomes was higher in thyroidectomized animals killed 42 hr. after a single injection of tri-iodothyronine. The increases in hormone-treated animals over the controls, as judged from the extinction at 260 m μ of fractions recovered in tubes 9–25, averaged 40–50%. At the same time, after hormone treatment, a higher amount of RNA sedimented through the 60% sucrose cushion as RNA associated with a lipid-rich fraction, presumably consisting of membranous structures. Sucrose-density-gradient centrifuging of ribosomes obtained by treating the mitochondria-free supernatant with different amounts of sodium deoxycholate showed that a higher detergent concentration was necessary to release the particles from the membranes after hormone administration.

Fig. 5. Polyribosomal sedimentation profiles of 0.4% sodium deoxycholate-treated mitochondria-free supernatant from thyroidectomized rat livers. A 1.5 ml. portion of the preparation was layered over discontinuous 15–30% sucrose density gradient with 3 ml. of 60% sucrose at the bottom of the centrifuge tube. Centrifuging was for 1.8 hr. at 22500 rev./min. in a Spinco SW25.1 rotor, and 1 ml. fractions were removed and their extinctions measured as described in the text. (a) Untreated thyroidectomized rats; (b) thyroidectomized rats 45 hr. after a single injection of 18 $\mu\text{g.}$ of tri-iodothyronine/100 g. body wt. P indicates the pellet; I and the broken line indicate the interface between the 30% and 60% sucrose layers. The arrow indicates the position of 78s single ribosomes.

Briefly, the effect of thyroid hormone administration led to the reversion of polyribosomal sedimentation profiles and of the association of the particles to membranous structures to those found in normal animals.

Table 10. *Specific radioactivity of RNA in the 0.4%-sodium deoxycholate-resistant membranes, polyribosomes and monomeric ribosomes from livers of thyroidectomized rats with or without treatment with tri-iodothyronine*

All thyroidectomized rats were given 9.6 μ C of [14 C]orotic acid 2.2 or 15.2 hr. before killing; 24 μ g. of tri-iodothyronine was injected 42.4 hr. before death to thyroidectomized rats weighing 160 ± 10 g. Treatment of mitochondria-free supernatant preparation and sucrose-density-gradient centrifugation were as in Fig. 5. 'Membranes' correspond to the resuspended pellet in Fig. 5, interface to the zone included in fractions 1-4, polysomes to fractions 9-25, monomeric ribosomes to fractions 27-29 and the 'top' to fractions 31-35. RNA was estimated from E_{280} after correction for protein content. The 14 C determinations were carried out on individual fractions after the addition of 0.2 mg. of carrier yeast RNA for precipitation.

Time after [14 C]orotic acid (hr.)	Time after tri-iodothyronine (hr.)	Specific activity of RNA (counts/min./ E_{280})				
		'Membranes'*	Interface	Polysomes	Monomers	'Top'
2.2	0.0	1250	—	2100	750	< 1000
	43.0	6700	—	3800	1200	< 2000
15.5	0.0	5600	7000	11000	7800	< 2000
	42.2	34500	14300	19800	17300	< 4500

* The radioactivity recovered in the membranes from control animals was 5-8% of the total radioactivity put on the density gradient whereas it was 14-17% for the hormone-injected animals.

Radioactive-labelling experiments showed that the hormone-induced changes in polyribosome distribution were accompanied by the appearance of newly formed RNA in both the monomeric 78s ribosomes as well as their aggregates. The following facts emerge from Table 10: (a) The specific radioactivity of RNA in all regions of the sucrose-density-gradient profile was higher after hormone administration, in agreement with the 32 P-labelling experiments (see Table 7). (b) The 4-6-fold increase in specific activity of the RNA associated with lipid-rich constituents in hormone-treated animals, compared with a 2-fold stimulation in that of free polysomes and monomers, suggests that a larger proportion of newly formed m-RNA and RNP particles are firmly associated with microsomal membranes in preparations from hormone-treated animals than from the uninjected controls. There is also twice as much membrane-bound RNA in the livers of tri-iodothyronine-treated rats. (c) The specific radioactivity of RNA in polysomes 16 hr. after administration of [14 C]orotic acid is only 10% higher than in the 78s monomers in both the treated and control animals. This suggests that an equilibrium has been achieved among the pre-existing and newly formed ribosomes and m-RNA, as suggested by Girard, Latham, Penman & Darnell (1965). There was also little variation (not exceeding $\pm 10\%$) in the specific radioactivity in polysomes of different sizes in both groups, which indicates an almost equal spacing of ribosomes along m-RNA strands of different length, in agreement with the conclusion of Marcus, Bretthauer, Halvorson & Bock (1965).

Fig. 6. Time-course of incorporation of [14 C]phenylalanine into trichloroacetic acid-soluble material by liver microsomes and RNP particles from thyroidectomized rats, in the presence and absence of polyU. ○ and ●, Microsomes; △ and ▲, RNP particles. ○ and △, PolyU absent; ● and ▲, incubation carried out in the presence of 100 μ g. of polyU/incubation tube.

Response of microsomes and ribosomes to synthetic m-RNA. The response of ribosomes to polyU and polyUG (mixed polymer of uridylic acid and guanylic acid) in the incorporation of phenylalanine and valine into protein has been used to evaluate

the effect of testosterone and growth hormone on the cytoplasmic m-RNA content of rat prostate (Liao & Williams-Ashman, 1962) and liver (Korner, 1964). We have applied the same principle to our studies on thyroid hormones. Since it has been reported that rat-liver microsomes and RNP particles may respond differently to polyU (Campbell *et al.* 1964; Kato, Loeb & Gelboin, 1965), both preparations were used under various conditions. In general, we were able to confirm the observations of Campbell *et al.* (1964) of the influence of polyU and Mg^{2+} concentrations on the incorporation of [^{14}C]phenylalanine into acid-insoluble material. However, the addition of polyU to both microsomes and RNP particles increased the duration of their ability to incorporate [^{14}C]phenylalanine (Fig. 6). The period of incubation used for comparing the stimulation by polyU of phenylalanine incorporation is therefore an important factor and the time-interval of 30 min. was found to be a convenient one when dealing with both preparations.

Table 11 summarizes the results of an experiment in which treatment of thyroidectomized rats with tri-iodothyronine moderately decreased the ability of polyU to stimulate [^{14}C]phenylalanine incorporation by RNP particles but not by microsomes. The values of 7.72- and 5.61-fold polyU-directed stimulation of phenylalanine incorporation by RNP particles from untreated and treated rats respectively were reproducible and the difference was significant. In agreement with previous studies on the regulation of protein synthesis by thyroid hormones (Tata *et al.* 1963; Roodyn *et al.* 1965),

thyroidectomy decreased the protein-synthesizing capacity of microsomes and ribosomes and tri-iodothyronine significantly increased it (by about 40%) about 40 hr. after its administration. Again, no hormonal effect on cytoplasmic protein synthesis could be observed at 22 hr. (It should be noted that the relative stimulation due to tri-iodothyronine was lower for the incorporation of [^{14}C]phenylalanine than that observed for [^{14}C]leucine, [^{14}C]isoleucine and [^{14}C]valine.)

RNP particles, whether obtained with 0.4% or 1% deoxycholate, showed a greater response to polyU than did unfractionated microsomes, as was also observed by Campbell *et al.* (1964). A further addition of 6 μ moles of magnesium chloride to the incubation medium markedly impaired in both preparations the extent of incorporation and decreased the polyU stimulation by 2-3-fold. The stimulation by polyU of RNP particles from thyroidectomized rats was decreased from 7.7-fold to 5.7-fold in particles from animals treated 42 hr. earlier with tri-iodothyronine, the latter value being close to that found in normal animals. No such differences could be detected with microsomes, which underlines the difficulty in 'titrating' endogenous m-RNA concentration with polyU in the presence of membranous structure. Further, during the 42 hr. after hormone administration, a marked increase had occurred in the cytoplasmic content of RNA, the microsomal RNA/protein ratio and firmer attachment of newly formed ribosomes to endoplasmic reticulum membranes (see Tables 9 and 10, Fig. 5 and the Discussion section).

Table 11. *Stimulation by polyU of the incorporation of [^{14}C]phenylalanine by liver microsomes and RNP particles from normal, thyroidectomized and tri-iodothyronine-treated thyroidectomized rats*

A 17 μ g. dose of tri-iodothyronine was injected/100 g. body wt. 42 hr. before killing. RNP particles were prepared by treating the mitochondria-free supernatant with 1% sodium deoxycholate. Each incubation vessel contained microsomes or RNP particles from 50 mg. of liver and cell sap from 40 mg. of liver; 125 μ g. of polyU was added wherever indicated. Other conditions are described in the text.

Rats	Preparation	RNA (mg./mg. of protein)	PolyU	Radioactivity in protein (counts/ min./mg.)	Stimulation by polyU
Thyroidectomized	Microsomes	0.13	—	171	5.65
			+	998	
	RNP particles	0.26	—	447	7.72
			+	3420	
Thyroidectomized + tri-iodothyronine	Microsomes	0.19	—	235	5.84
			+	1380	
	RNP particles	0.36	—	599	5.61
			+	3370	
Normal, untreated	Microsomes	0.27	—	365	5.51
			+	1908	
	RNP particles	0.46	—	969	5.43
			+	5232	

Table 12. *Effect of some inhibitors of RNA and protein synthesis on the stimulation of B.M.R. and growth rate of normal and thyroidectomized rats*

Tri-iodothyronine and the inhibitors were injected twice at an interval of 48 hr. The increase or decrease in the B.M.R. and body wt. 72 hr. after the first injection were expressed as percentage changes from values recorded 1-2 hr. before treatment was begun. Thyroidectomized rats weighed 150 ± 10 g. and normal rats of the same age were 235 ± 15 g. Each value is an average of two separate experiments with three rats/group. B.M.R. (ml. of O_2 /g./hr.) was 1.46 ± 0.10 and 1.05 ± 0.06 for normal and thyroidectomized rats respectively; growth rate (increase in body wt./72 hr.) was 17 ± 3 g. and 7 ± 3 g. for normal and thyroidectomized rats respectively. The dose of tri-iodothyronine was $24-27 \mu\text{g./100 g. body wt.}$ The doses of inhibitors/100 g. body wt. were $8 \mu\text{g.}$ of actinomycin D, 12 mg. of puromycin, 5 mg. of 5-fluorouracil and 10 mg. of cycloheximide (7 mg. for thyroidectomized rats).

Treatment	Normal rats			Thyroidectomized rats		
	Δ B.M.R.	Δ Growth	Percentage inhibition*	Δ B.M.R.	Δ Growth	Percentage inhibition*
None	± 2.7	$+4.2$	—	± 1.5	$+ 5.4$	—
Tri-iodothyronine	$+16.5$	$+6.1$	—	$+35.3$	$+ 8.1$	—
Actinomycin D	-12.6	-9.2	—	-14.0	-13.0	—
Tri-iodothyronine + actinomycin D	-10.0	-7.7	85	-13.8	-14.0	100.0
Puromycin	-4.8	-6.3	—	$+ 1.0$	$- 7.7$	—
Tri-iodothyronine + puromycin	$+ 4.0$	-5.0	73	$+ 9.8$	$- 6.0$	69
5-Fluorouracil	-10.0	-8.8	—	-11.2	-13.2	—
Tri-iodothyronine + 5-fluorouracil	$- 7.0$	-8.4	79	$- 9.0$	-11.6	83
Cycloheximide	$- 2.0$	-1.5	—	0.0	$- 5.0$	—
Tri-iodothyronine + cycloheximide	0.0	-1.0	98	$- 4.8$	$- 4.8$	95

* Average of inhibition of increases in both B.M.R. and growth rate caused by thyroid hormone.

Effect of inhibitors of RNA and protein synthesis. In a previous report (Tata, 1963) it was shown that actinomycin D and puromycin were very effective in blocking the stimulatory effect of thyroid hormones on both the B.M.R. and growth rate. These studies have been extended to include two more inhibitors, 5-fluorouracil and cycloheximide, and the results are summarized in Table 12. All these inhibitors, which act at different steps of RNA and protein synthesis, were effective on normal as well as thyroidectomized rats treated with either thyroxine or tri-iodothyronine. The doses of inhibitors shown in Table 11 are those that gave the maximum inhibition of hormonal activity without producing a single fatality. Under these conditions, cycloheximide was the only substance that completely prevented the hormonal stimulation of B.M.R. and growth rate, presumably because it is a potent inhibitor of both protein and RNA synthesis (Wettstein, Noll & Penman, 1964; Fiala & Davis, 1965; Fukuhara, 1965). The relative timing of the administration of the inhibitor and the hormone was also an important factor in obtaining the antagonistic effects. For example, pretreatment of rats with the hormone 24 hr. before the inhibitor considerably decreased the inhibition; pretreatment with actinomycin by more than 2 days before the hormone caused the animals to be refractory to the antibiotic in long-term studies. It is for this reason that the simultaneous administration of the anti-

Table 13. *Effect of actinomycin D treatment in vivo on the stimulation of the Mg^{2+} -activated RNA-polymerase reaction of isolated liver nuclei by tri-iodothyronine*

Times of injection and doses of tri-iodothyronine and actinomycin D were as described in Table 12. The B.M.R. was determined 36 hr. after the second injection of the two substances and nuclei were isolated for assay of RNA polymerase immediately afterwards.

Treatment of experimental animal		Relative B.M.R.	Specific activity of RNA polymerase (μmoles of [^{14}C]ATP incorporated into RNA/mg. of DNA)
Tri-iodo- thyronine	Actinomycin D		
—	—	100	621
—	+	87	596
+	—	147	1030
+	+	92	653

biotic and the hormone in two doses spaced out by 48 hr. was adopted and the same procedure followed for other inhibitors for the sake of standardization.

Actinomycin D is well known to be a potent inhibitor of DNA-dependent RNA polymerase in bacterial and mammalian systems (Goldberg & Rabinowitz, 1962; Hurwitz, Furth, Malamy &

Alexander, 1962). In view of the above results it was important to see what effect actinomycin D had on the hormonal stimulation of nuclear RNA synthesis, described above, under conditions in which the antibiotic abolished the physiological effects of the hormone. Table 13 shows that the same procedure of treatment with the antibiotic that blocked the growth-potential effect of two doses of tri-iodothyronine (Table 12) also inhibited the rise in hepatic RNA-polymerase activity that was observed after the same hormone treatment without having any effect on the activity of RNA polymerase when administered to control animals.

DISCUSSION

The relatively early effects of thyroid hormone on RNA synthesis in the nucleus could be detected well within the latent period preceding the hormonal stimulation of cytoplasmic protein synthesis (Tata *et al.* 1963; Roodyn *et al.* 1965). Although many immediate biochemical effects of thyroid hormones have been described (see Pitt-Rivers & Tata, 1959; Tata, 1964a), the accelerated synthesis of rapidly labelled nuclear RNA and the higher RNA-polymerase activity are the earliest effects *in*

vivo under conditions in which both the B.M.R. and growth rate of most tissues would be stimulated.

In Fig. 7 we have summarized the sequential stimulations of RNA synthesis in the liver nucleus and cytoplasm as a result of a single injection of tri-iodothyronine into thyroidectomized rats. The first phenomenon observed was an increase in the specific activity of rapidly labelled nuclear RNA beginning at 3–4 hr. after hormone administration, and reaching a peak value of 300% increase at about 16 hr. Between these time-intervals a stimulation of Mg^{2+} -activated RNA-polymerase reaction was first recorded and this activity continued to rise until 42 hr. after tri-iodothyronine was given. When, however, the polymerase was assayed in the presence of ammonium sulphate and Mn^{2+} , no hormonal effect was discernible for 24 hr., after which only relatively small changes could be detected (45% as against 80% for the Mg^{2+} -activated system). A similar dissociation of the stimulations of RNA-polymerase activities in the presence and absence of ammonium sulphate has been observed, in our Laboratory and elsewhere, for growth hormone (Widnell & Tata, 1966a; Pegg & Korner, 1965) and oestrogen (Gorski, 1964; Hamilton, Widnell & Tata, 1965). A substantial rise in the amount of cytoplasmic ribosomal RNA and the microsomal RNA/protein ratio took place at about 24–30 hr. after the hormone was given, thus elevating the hepatic ribosomal RNA content by 50–70%. (The concentration of hepatic ribosomal RNA in young thyroidectomized rats is 40–50% below that found in normal animals and consequently a single injection of thyroid hormone to non-thyroidectomized rats fails to produce the same dramatic increase in RNA content.) There was only a slight change in s-RNA concentration, and previous work (Tata *et al.* 1963) had shown that thyroid hormones under similar conditions did not affect the activation of amino acids or the capacity of cell sap to support protein synthesis by microsomes. No significant differences were recorded in the content of nuclear RNA. In view of the sustained increase in the synthesis of nuclear RNA (Tables 5 and 6) and the net accumulation of RNA in the ribosomal fraction (Tables 6 and 9), it may be concluded that thyroid hormone administration accelerated nuclear RNA synthesis and its transport into the cytoplasm. (The fraction of RNA synthesized in the nucleus that is transported into the cytoplasm under different conditions is not known.) The close association between the increase in cytoplasmic RNA and the higher amino acid-incorporating activity of cytoplasmic particles is interesting, although other factors besides RNA synthesis could also stimulate protein synthesis.

Sokoloff, Francis & Campbell (1964) have reported that thyroid hormones do not stimulate RNA

Fig. 7. Summary of data on the sequential stimulations of liver RNA synthesis, determined according to different parameters, in thyroidectomized rats after a single injection of 15–22 $\mu g.$ of tri-iodothyronine/100 g. body wt. In most cases, two or more parameters were determined in groups of six rats for each point on the curves. ●, Incorporation *in vivo* of [^{14}C]orotic acid into rapidly labelled nuclear RNA; △, Mg^{2+} -activated RNA-polymerase reaction in isolated nuclei; ▲, $Mn^{2+}/(NH_4)_2SO_4$ -activated RNA-polymerase reaction in the same nuclei; □, mg. of ribosomal RNA/mg. of DNA; ■, incorporation of [^{32}P]phosphate into ribosomal RNA *in vivo* (9 hr. after [^{32}P]phosphate administration); ○, mg. of nuclear RNA/mg. of DNA; ○—○, incorporation of ^{14}C -labelled L-valine, L-leucine or L-phenylalanine into protein by microsomes [this curve is based on combined data from Tata *et al.* (1963) and Roodyn *et al.* (1965)]. See the Discussion section for explanation.

polymerase. This report was based on the observation that the addition of either nuclei or inhibitors of DNA-dependent RNA synthesis to a reconstituted rat-liver homogenate had no effect on the slight stimulation of amino acid incorporation that was induced by the addition of high concentrations of thyroxine *in vitro*. We have never observed any effect of the addition of thyroid hormone *in vitro* on the specific activity of RNA polymerase (see Table 4 and Widnell & Tata, 1963). In addition, the incorporation medium used by Sokoloff *et al.* (1964) contained neither CTP nor UTP, so that the incorporation of [¹⁴C]AMP into RNA that they observed was more likely to represent terminal addition of ribonucleotides than actual RNA synthesis. Further, for the stimulation of amino acid incorporation into protein in a cell-free system by the addition of nuclei to have any significance, it would be necessary to demonstrate that RNA synthesized in the nucleus did stimulate amino acid incorporation by the cytoplasmic ribosomes present in the system, and no such evidence was presented by these authors.

Thyroidectomy or thyroid hormone administration did not alter the base composition of ³²P-labelled RNA *in vivo* or the nearest-neighbour frequencies in RNA formed by the Mg²⁺-activated RNA-polymerase reaction (see Tables 7 and 8). In both cases the RNA synthesized was largely ribosomal in that it had a high guanosine + cytidine content, and a large part of rat-liver nuclear RNA is known to be ribosomal (Hiatt, 1962; Sporn & Dingman, 1963). Shorter ³²P-labelling periods than the ones reported above did give a more DNA-like RNA both in the nucleus and microsomes, as also noted by others (Hiatt, 1962; Munro *et al.* 1964; Trakatellis, Axelrod & Montjar, 1964), but the quantitative variations with short pulses of [³²P]-phosphate from one sample to another in the same group precluded any interpretation of hormonal effects.

A selective control of m-RNA synthesis by thyroid hormones could explain the specificity of their biological effects. Results of experiments on the response to polyU of the incorporation of [¹⁴C]phenylalanine by microsomes and RNP particles (Table 11) and the polyribosome sedimentation profiles (Fig. 5 and Table 12) provide indirect but functional evidence for the subnormal cytoplasmic m-RNA content in the livers of thyroidectomized rats, which can be elevated by a single injection of tri-iodothyronine. However, the lowering of the response to polyU of RNP particles from thyroidectomized rats after tri-iodothyronine administration was not as marked as with ribosomes from the prostate stimulated with testosterone and from the liver with growth hormone (Liao & Williams-Ashman, 1962; Korner, 1964). A possible

explanation for this difference may lie in the fact that the appearance of a large amount of new ribosomes, at the time after tri-iodothyronine administration at which polyU stimulation dropped from 7·7- to 5·5-fold, could lead to only slight increases in the overall m-RNA/ribosomes ratio in the cytoplasm.

The large increase in amino acid-incorporation activity per unit of microsomal RNA (Tata *et al.* 1963) suggests that the m-RNA or ribosomes or both synthesized after hormonal treatment is more active in protein synthesis than in the untreated animals. Since the sucrose-density-gradient analyses were limited to a few time-intervals after hormonal stimulation, it is not clear how the hormone would affect the stability and rates of entry and degradation of m-RNA and ribosomal RNA in the cytoplasm. Density-gradient analysis has also revealed a hormonal effect on the association between ribosomes or polysomes and microsomal membranes or some other lipid-rich component. The increase in RNA recovered as a pellet sedimenting through 60% sucrose in preparations from hormone-treated animals also meant that a higher detergent/RNA ratio was necessary to obtain the same degree of release of free particles. The specific-radioactivity distribution in the different gradient fractions suggests, further, a preferential attachment of newly synthesized RNA to the membrane-like fraction. A similar phenomenon has been observed in tadpole liver after thyroid hormone-induced metamorphosis (Tata, 1965). This disparity between 'old' and 'new' ribosomes does not suggest a constant exchange between free and bound ribosomes. With regard to the biological significance of the attachment of RNP particles to membranes of the endoplasmic reticulum, two recent studies have indicated that only those ribosomes attached to membranes represent the site of protein synthesis *in vivo* (Henshaw, Bojarski & Hiatt, 1963; Campbell *et al.* 1964).

That the four inhibitors of RNA and protein synthesis (actinomycin D, puromycin, 5-fluorouracil and cycloheximide) are powerful antagonists of thyroid hormone action in the intact animal enhances the biological implications of the present studies. Weiss & Sokoloff (1963) also reported the inhibition of the calorogenic action of thyroxine by puromycin but these workers interpreted this suppression as supporting evidence for a direct action of the hormone on the aminoacyl-s-RNA linkage to the polypeptide chain (Sokoloff, Kaufman, Campbell, Francis & Gelboin, 1963). Inhibition *in vivo* does not indicate the site of action of the hormone. Further, actinomycin D, which was initially used as an inhibitor of m-RNA synthesis *in vivo* (Reich & Goldberg, 1964), is an even more potent inhibitor of the synthesis of ribosomal RNA and conse-

quently of ribosomes (Perry, 1963; Franklin, 1963; Trakatellis *et al.* 1964). Similarly, puromycin and cycloheximide, which have largely been used for inhibiting cytoplasmic protein synthesis (see Darken, 1964), are also potent inhibitors of ribosomal RNA synthesis (Tamaoki & Mueller, 1963; Fiala & Davis, 1965; Fukuhara, 1965). Thus inhibitor studies in themselves only demonstrate that unimpaired RNA and protein synthesis is essential for the physiological activity of thyroid hormones.

The present results as a whole do not indicate the mechanism or site of action of thyroid hormones. However, stimulation of nuclear RNA synthesis is the earliest response to thyroid hormone under growth-promoting conditions and the sequential stimulation of cytoplasmic protein synthesis certainly indicates that control of RNA synthesis is one of the important initial steps in the cell leading to the diverse hormonal actions. Basically similar conclusions have been drawn for the developmental changes induced by thyroid hormones in amphibian metamorphosis (see Bennett & Frieden, 1962; Tata, 1965). A salient feature of these observations is that, although the synthesis of nuclear RNA is stimulated relatively early, the appearance of newly formed RNA in the cytoplasm is a slow process. The close association of increased protein-synthetic activity with the appearance of new ribosomes may perhaps reflect the transfer of 'pre-coded' ribosomes from the nucleus to the cytoplasm, as has been suggested for a bacterial system by Byrne, Levin, Bladen & Nirenberg (1964). Since the nucleolus appears to be the exclusive site of ribosomal RNA synthesis (see Prescott, 1964), it would be worth while to extend investigations on the possible regulation of nucleolar function by thyroid hormones. Whatever the site of action of thyroxine, the mechanisms of RNA transport and the specificity of ribosomal activity, our present results are compatible with the idea expressed elsewhere that thyroid hormones control not only growth and development but also B.M.R. and mitochondrial respiration via a selective control of protein synthesis (see Tata, 1964a; Tata *et al.* 1963; Lee & Lardy, 1965; Michels, Cason & Sokoloff, 1963; Roodyn *et al.* 1965; Gustafsson, Tata, Lindberg & Ernster, 1965).

Finally, almost all the effects of thyroid hormones on hepatic RNA metabolism described above have also been observed for a variety of growth and developmental hormones in their respective target tissues. Growth hormone, testosterone, oestrogen, adrenocorticotrophic hormone and ecdysone, soon after their administration, produce a 2-4-fold increase in the specific activity of rapidly labelled nuclear RNA (Talwar, Gupta & Gros, 1964; Korner, 1964; Wicks & Kenney, 1964; Bransome

& Chargaff, 1964; Mueller, 1965; Gorski & Nelson, 1965; Sekeris, 1965) and a stimulation of RNA polymerase. Not surprisingly, actinomycin D, puromycin and cycloheximide are potent inhibitors of the hormonal action *in vivo* (Mueller, 1965; Hamilton, 1964; Gorski & Axman, 1964; Ferguson, 1963; Sekeris & Karlson, 1964). It was also found with testosterone acting on the seminal vesicles (Wicks & Kenney, 1964) and oestrogen on the uterus (Greenman & Kenney, 1964; Moore & Hamilton, 1964) that the hormonal stimulation of cytoplasmic protein synthesis accompanied the appearance of new ribosomes, although acceleration of nuclear RNA synthesis occurred much earlier. The simplest way to explain how the synthesis of different constituents is regulated by different hormones is to assume a specific de-repression of genes by each of these hormones. Nevertheless, the question arises whether the similarity of the biochemical phenomena produced by these hormones reflects some common site or route for the control of RNA synthesis in higher organisms. We have attempted to answer this question in the following paper (Widnell & Tata, 1966a).

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