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The Subcellular Distribution of ^{32}P-Labelled Phospholipids, ^{32}P-Labelled Ribonucleic Acid and ^{125}I-Labelled Iodoprotein in Pig Thyroid Slices

EFFECT *IN VITRO* OF THYROTROPHIC HORMONE AND DIBUTYRYL-3′,5′-(CYCLIC)-ADENOSINE MONOPHOSPHATE

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1. The incorporation *in vitro* of [^{32}P]phosphate into phospholipids and RNA and of [^{125}I]iodide into protein-bound iodine by pig thyroid slices incubated for up to 6hr. was studied. The subcellular distribution of the labelled products formed after incubation with radioactive precursor in the nuclear, mitochondrial, smooth-microsomal, rough-microsomal and cell-sap fractions was also studied. 2. Pig thyroid slices actively took up [^{32}P]phosphate from the medium during 6hr. of incubation; the rate of incorporation of ^{32}P into phospholipids was two to five times that into RNA. 3. The uptake of [^{125}I]iodide by the slices from the medium was rapid for 4hr. of incubation, 6–10% of the label being incorporated into iodoprotein. 4. Much of the ^{32}P-labelled phospholipid accumulated in mitochondria and microsomes, whereas the nuclear fraction contained most of the ^{32}P-labelled RNA. After 2hr. of incubation most of the ^{32}P-labelled cytoplasmic RNA accumulated in the rough-microsomal fraction. The major site of localization of protein-bound ^{125}I was the smooth-microsomal fraction, and gradually increasing amounts appeared in the soluble cytoplasm fraction, suggesting a vectorial discharge of [^{125}I]iodoprotein (presumably thyroglobulin) from smooth vesicles into the colloid. 5. The addition of 0·1–0·4 unit of thyrotrophic hormone/ml. of incubation medium markedly enhanced the accumulation of ^{32}P-labelled phospholipids in the microsomal fractions and to a much smaller extent that of ^{32}P-labelled RNA without any increase in the total uptake of the label. Almost simultaneously the hormone increased the uptake of [^{125}I]iodide by the slices and enhanced the accumulation of protein-bound ^{125}I in the smooth-microsomal fraction. 6. As a function of time of incubation, thyrotrophic hormone had a biphasic effect on [^{125}I]iodide uptake and protein-bound ^{125}I formation, the stimulatory effect being reversed after 4hr. of incubation. 7. 6-*N*-2′-*O*-Dibutyryl-3′,5′-(cyclic)-AMP, but not 3′,5′-(cyclic)-AMP or 5′-AMP, mimicked the action of thyrotrophic hormone on iodine uptake as well as on iodination of protein. On the other hand, the mimicry by 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP of the stimulatory effect of thyrotrophic hormone on the formation of labelled thyroid phospholipids and RNA was only an apparent one resulting from an enhanced uptake of [^{32}P]phosphate. 8. It is concluded that thyrotrophic hormone causes a co-ordinated increase in the formation or accumulation of phospholipids, RNA and iodoprotein associated with the endoplasmic reticulum, and that 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP mimics the more rapid effects of thyrotrophic hormone on transport and metabolic functions of thyroid cells, but does not influence their slower biosynthetic responses to the hormone.

TSH† provokes numerous biochemical responses in its target cells that apparently seem to be unconnected with its major physiological action of regulating the formation and release of thyroid hormones (see Rall, Robbins & Lewallen, 1964). Important among such biochemical actions of TSH are the rapid effects on glucose metabolism in the thyroid (Dumont, 1964) and the relatively slower but very pronounced stimulation of phospholipid synthesis (Freinkel, 1964). Recently we observed

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† Abbreviations: TSH, thyrotrophic hormone; PB^{125}I, protein-bound ^{125}I (^{125}I that is incorporated into protein with a covalent linkage); c-AMP, 3′,5′-(cyclic)-AMP; DB-c-AMP, 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP.

that the acceleration of guinea-pig thyroid phospholipid synthesis by TSH *in vivo* coincided with increased rates both of synthesis of RNA and of ^{125}I-labelling of thyroglobulin (Kerkof & Tata, 1967). This observation seemed to conform to the co-ordinated accumulation of RNA and membrane phospholipids at the time of onset of additional protein synthesis after the administration of other growth and developmental hormones (Tata, 1967*a*, 1968). We therefore decided to extend our studies on TSH action to the intracellular distribution of thyroid phospholipid, RNA and newly formed iodoprotein. Such an analysis, however, presented numerous obstacles if studies were to be carried out *in vivo*, such as the small size of the thyroid gland in common laboratory animals, the relatively low rate of uptake by the gland of radioactive precursors of phospholipids and RNA and the large variability of precursor pool sizes. On the other hand, TSH can exert its varied physiological actions *in vitro* including those on phospholipid synthesis, provided that cellular integrity is maintained. Preliminary experiments on the subcellular distribution of ^{32}P-labelled phospholipids, ^{32}P-labelled RNA and ^{125}I-labelled iodoprotein revealed that investigations with isolated pig thyroid slices would indeed be more promising.

During this work, reports from Pastan's laboratory appeared describing the mimicry by DB-c-AMP of a variety of actions of TSH (including that on phospholipid metabolism) in thyroid slices and cell suspensions (Pastan, 1966; Pastan & Wollman, 1967; Pastan & Macchia, 1967). TSH was also found to activate thyroid adenyl cyclase when added to cell suspensions (Pastan & Katzen, 1967). It was therefore suggested that c-AMP might be an intracellular mediator of TSH action, in line with Sutherland's generalization for hormone action (Sutherland, Butcher, Robison & Hardman, 1967). These developments therefore led us to examine whether the mimicry of TSH by the cyclic nucleotide would apply to the tissue responses that we have studied.

In this paper we describe the effect of TSH *in vitro* on the incorporation of ^{32}P into phospholipid and RNA and of ^{125}I into iodoprotein by pig thyroid slices and on the subcellular distribution of these constituents. It is shown that the labelling of all three constituents is almost simultaneously accelerated by TSH and that the major site of accumulation of additional RNA is the rough endoplasmic reticulum, whereas that of additional labelled phospholipid and iodoprotein is the smooth endoplasmic reticulum. DB-c-AMP, but not c-AMP, simulated the action of TSH on the uptake of ^{125}I and its incorporation into protein, and partially mimicked that on incorporation of ^{32}P into RNA and phospholipid. The mimicry of TSH action by DB-c-AMP with regard to labelling of phospholipid and RNA was doubtful, as the cyclic nucleotide increased the uptake of ^{32}P but did not influence the rate of synthesis as does TSH.

EXPERIMENTAL

Tissue preparation. Fresh pig thyroid glands were obtained from the abattoirs of T. Wall and Sons Ltd., London N.W.10. They were chilled in ice immediately and all manipulations were carried out in a cold-room at 2° thereafter. After the glands had been trimmed of fat and connective tissue, slices, cut manually, of uniform thickness (0·3–0·4mm.) were pooled and washed in the incubation medium. The slices were then weighed on a torsion balance and equal portions were distributed in 25ml. Erlenmeyer flasks to which 7·5ml. of Krebs–Ringer tris medium was added/0·5g. of slices.

Incubation. The incubation medium was modified Krebs–Ringer 50mM-tris–HCl buffer, pH7·4 (Freinkel, 1958). In experiments with ^{32}P, 0·6–1·1μc of [^{32}P]orthophosphate was added with 0·2μmole of non-radioactive orthophosphate (added as Krebs–Ringer phosphate buffer, pH7·4; Freinkel, 1958)/ml. of medium. In experiments in which the incorporation of ^{125}I into iodoprotein was studied, 0·3–0·5μc of [^{125}I]iodide was added/ml. of Krebs–Ringer tris medium. The radioactive precursors and TSH (where used) were usually added to the chilled slices just before incubation. Incubation was carried out at 37° with gentle shaking (30–40cyc./min.) in lightly stoppered flasks under atmospheric air. The medium was oxygenated only for incubations lasting longer than 4hr. Incubation was terminated by placing the flasks in an ice bath with the addition of 4μmoles of phosphate/ml. (for ^{32}P-incorporation experiments) or 0·54μmole of propylthiouracil/ml. (to stop further incorporation of ^{125}I into protein). For the extraction of total tissue RNA and phospholipid, the chilled slices were finely chopped with scissors, washed three times with 5ml. of Krebs–Ringer tris medium and homogenized in the same medium in a tight-fitting Potter–Elvehjem tissue homogenizer (8·6ml. of medium/g. of tissue). For each set of incubations, an identical sample was maintained in ice for the same period of incubation. The incorporation of label at 0° was usually minimal and was deducted from the values obtained at 37°. Unless otherwise stated, each value is the average of duplicate extractions or determinations.

Subcellular fractionation. In some experiments the chilled tissue, after incubation with radioactive precursor, was fractionated into its major subcellular fractions in the following way. The slices were washed twice with Krebs–Ringer phosphate medium (for experiments with ^{32}P) or Krebs–Ringer tris medium containing 2mM-propylthiouracil (for experiments with ^{125}I). They were then finely chopped and washed a further two or three times with phosphate-free Krebs–Ringer tris medium in experiments with ^{32}P, and with 0·15M-NaCl–tris medium containing 2mM-propylthiouracil for experiments with ^{125}I. After two more washes with 0·35M-sucrose, the tissue was homogenized in 3 vol. of medium of composition 0·35M-sucrose–25mM-KCl–5mM-$MgCl_2$–50mM-tris buffer, pH7·6, with a Teflon–glass Potter–Elvehjem homogenizer. A small portion of the homogenate was kept for chemical analysis

and determination of radioactivity, and the remainder was processed by differential centrifugation into the following fractions. A crude pellet of nuclei, debris and connective tissue, sedimented by centrifugation at 600*g* for 10min., and a pellet of mitochondria, sedimented by centrifugation at 6000*g* for 10min., from the nuclei-free supernatant were first separated. The post-mitochondrial supernatant was then layered over 1·3M-sucrose–10mM-$MgCl_2$ medium and was fractionated into smooth microsomal membranes, rough microsomal membranes and cell sap by the method described by Tata & Williams-Ashman (1967). Although this method described the subfractionation of rat liver microsomes, the distribution of RNA, protein and phospholipid and electron-microscopic examination of the fractions obtained in the present work showed that it was also applicable to pig thyroid microsomes. The particulate fractions were suspended in a small volume of 0·15M-NaCl or Krebs–Ringer tris medium before extraction of RNA, phospholipid and protein.

Extraction of ^{32}P*-labelled phospholipid,* ^{32}P*-labelled RNA and* ^{125}I*-labelled iodoprotein.* ^{32}P-labelled phospholipid and RNA were extracted from the homogenate and the various subcellular fractions by the method of Tata (1967*b*). The acid-soluble fraction obtained after the initial treatment and washing of the sample with $HClO_4$ was kept for determination of ^{32}P and phosphorus content. The incorporation of ^{32}P into individual phospholipids was checked by t.l.c., and that into the ribonucleotides by column chromatography of an alkaline hydrolysate of RNA (Tata, 1967*b*). The PB^{125}I content was determined by precipitating the iodoprotein with 20% (w/v) trichloroacetic acid at 0°, followed by washing the precipitate with 10% (w/v) trichloroacetic acid and determining the radioactivity in the precipitate.

Chemical analysis. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin as the standard. RNA was determined by the orcinol procedure (Ceriotti, 1955) or by a slight modification of the spectrophotometric method of Fleck & Munro (1962). Phosphorus was determined by the method of Ames & Dubin (1960), phospholipid content being calculated as 25× phosphorus content.

Determination of radioactivity. ^{32}P was measured in a Nuclear–Chicago thin-window gas-flow counter with an efficiency of about 40%, and ^{125}I was measured in a Packard Auto Gamma spectrometer with an efficiency of about 10%.

Materials. All chemicals were of analytical grade and were obtained commercially. Organic solvents were freshly distilled and all incubation and homogenization media were sterilized before use. TSH (Thytropar) and crystalline bovine serum albumin were purchased from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex. AMP, DB-c-AMP and c-AMP were obtained from C. F. Boehringer und Sohne G.m.b.H., Mannheim, Germany. Carrier-free [^{32}P]orthophosphate and [^{125}I]iodide were purchased from The Radiochemical Centre, Amersham, Bucks.

RESULTS

Tissue composition. Table 1 summarizes the composition in terms of protein, RNA and phospholipid of fresh and incubated pig thyroid slices. After incubation for 4hr. at 0° some protein was lost, most of it into the medium. At 37° the loss of protein was considerably increased, presumably owing to the very strong proteolytic activity of the thyroid (see Rall *et al.* 1964).

Uptake and incorporation of ^{32}P*: effect of TSH.* During incubation at 37° pig thyroid slices actively took up [^{32}P]orthophosphate from the medium, as illustrated in Fig. 1. The rate of uptake of ^{32}P (as judged by the total radioactivity recovered in the homogenate) was rapid and linear for the first hour, after which the process continued at a lower rate for at least another 3hr. More than half of the ^{32}P taken up was recovered in the acid-soluble fraction, but its proportion fell with time of incubation (Fig. 1*a*), as a progressively increasing amount of acid-insoluble ^{32}P was recovered in the phospholipid and RNA fractions (Figs. 1*a* and 1*b*). (Less than 3% of acid-insoluble ^{32}P was recovered as DNA, and 5–10% as phosphoprotein, under any incubation condition.) The rate of incorporation of ^{32}P into phospholipid exceeded that into RNA, the phospholipid/RNA incorporation ratio for whole-tissue acid-insoluble ^{32}P being 1·5 after 1hr. of incubation and increasing to 2·5 at 3hr.

The addition of TSH to the incubation medium markedly enhanced the incorporation of ^{32}P into phospholipids and, to a smaller extent, that into RNA. The hormone did not appreciably alter the total uptake of ^{32}P, but lowered radioactivity of the acid-soluble fraction. This preferential channelling of ^{32}P-labelled precursors into phospholipids

Table 1. *Composition of pig thyroid slices in terms of protein, RNA and phospholipid, before and after incubation for 4hr. at 0° and 37°*

Values are the means ± S.D. of five separate determinations in duplicate. Phospholipid values were obtained by multiplying the lipid phosphorus content by 25. Details are given in the Experimental section.

	Content in thyroid (mg./g. wet wt.)		
Incubation	Protein	RNA	Phospholipid
None	144±16	1·81±0·06	1·57±0·13
4hr. at 0°	126±11	1·74±0·04	1·52±0·09
4hr. at 37°	113±12	1·56±0·07	1·48±0·12

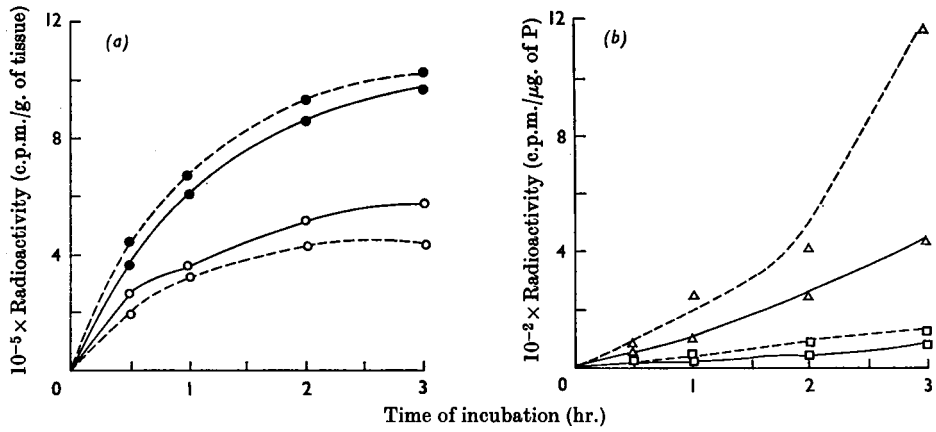

Fig. 1. Rate of (*a*) uptake of ^{32}P (measured by the total radioactivity recovered in the homogenate) (●) and ^{32}P recovered in the acid-soluble fraction (○) and (*b*) incorporation of ^{32}P into phospholipids (△) and RNA (□) of whole pig thyroid slices in the presence (----) and absence (——) of TSH. Thyroid slices (0·5g.) were incubated in 7·5ml. of medium and, where indicated, 0·28 unit of TSH was added/ml. At different times of incubation, the tissue was washed and homogenized; the ^{32}P content of the whole homogenate and of the acid-soluble fraction, and the specific radioactivities of phospholipid and RNA, were determined as described in the text.

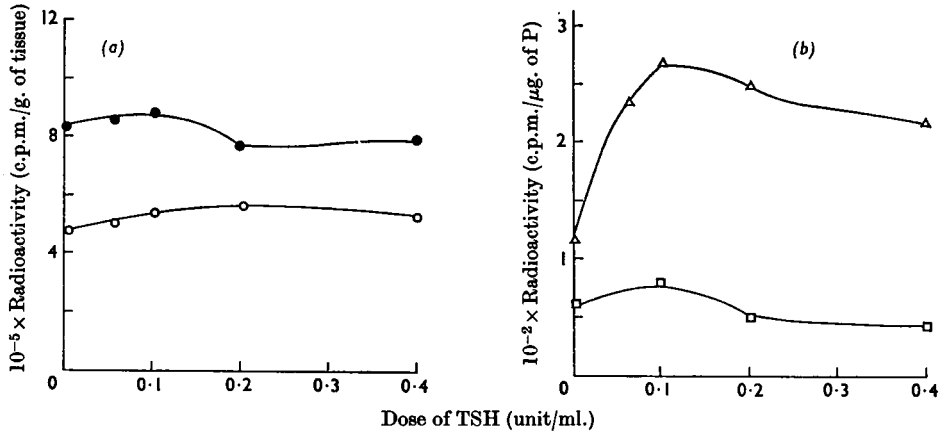

Fig. 2. Effect of dose of TSH added *in vitro* on the (*a*) uptake of ^{32}P (measured by the total radioactivity recovered in the homogenate) (●) and recovery of acid-soluble ^{32}P (○) and (*b*) specific radioactivity of phospholipid (△) and RNA (□) of whole thyroid slices. Pig thyroid slices (1·0g.) were incubated in 14·3ml. of medium for 3hr. and different amounts of TSH were added at the beginning of the incubation. For other details, see the text.

by TSH confirms the work of Freinkel, Pastan and others, who showed, with [^{32}P]phosphate and other radioactive precursors ([^{14}C]glycerol, [^{14}C]inositol, [^{14}C]palmitic acid) that the hormone stimulates the net synthesis of phospholipids (Freinkel, 1958, 1964; Pastan & Macchia, 1967; Scott, Mills & Freinkel, 1968). A pattern of response to TSH similar to that described above was obtained at concentrations up to 0·4 unit/ml. (Fig 2); the optimum stimulation of ^{32}P incorporation into phospholipids and RNA was obtained with 0·1 unit/ml., and this dose was therefore adopted for most of the other experiments.

Subcellular distribution of ^{32}P*-labelled phospholipids and RNA.* The rate of incorporation of ^{32}P into mitochondrial phospholipids was 70–100% higher than that into microsomal lipids over a period of incubation of 6hr., so that two to three times as much ^{32}P-labelled phospholipid accumulated in the mitochondria as in microsomes at the

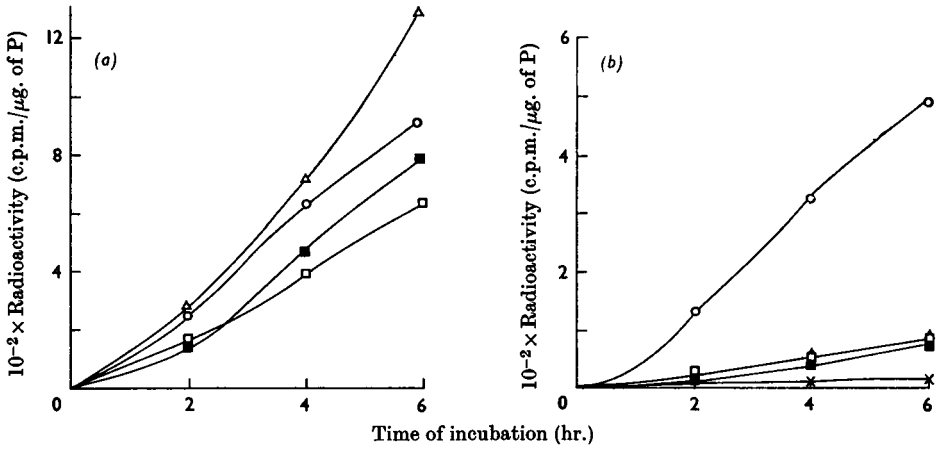

Fig. 3. Rate of incorporation of ^{32}P into (*a*) phospholipids and (*b*) RNA of different subcellular fractions of pig thyroid slices. Pig thyroid slices incubated for various times were homogenized, the subcellular fractions separated and the specific radioactivities of phospholipid and RNA determined as described in the Experimental section. ○, Nuclei; △, mitochondria; ■, rough microsomal membranes, □, smooth microsomal membranes; ×, cell sap.

Table 2. *Effect of TSH as a function of incubation time on the incorporation of* ^{32}P *into phospholipids recovered in particulate subcellular fractions of pig thyroid*

Pig thyroid slices were incubated with [^{32}P]phosphate for different times in the presence and absence of 0·1 unit of TSH/ml. before homogenization and subcellular fractionation. Details are given in the Experimental section.

		Sp. radioactivity of phospholipids (c.p.m./μg. of P)			
	Time of incubation (hr.) ...	2	3	4	6
Fraction	TSH				
Nuclei	−	158	236	274	465
	+	186	397	574	1091
Mitochondria	−	66	231	445	733
	+	135	489	809	695
Smooth membranes	−	83	111	218	240
	+	104	201	450	343
Rough membranes	−	32	92	154	250
	+	32	178	301	437

Table 3. *Effect of TSH on the incorporation of* ^{32}P *into RNA of different subcellular fractions of pig thyroid*

RNA was extracted from the same samples as in Table 2 after removal of phospholipids. Details are given in the Experimental section.

		Sp. radioactivity of RNA (c.p.m./μg. of P)		
	Time of incubation (hr.) ...	2	3	4
Fraction	TSH			
Nuclei	−	52	114	195
	+	48	152	326
Mitochondria	−	7	24	73
	+	6	35	64
Smooth membranes	−	2	11	68
	+	1	14	109
Rough membranes	−	1	7	30
	+	1	11	36
Cell sap	−	—	22	77
	+	—	18	162

end of 6hr. (Fig. 3*a*). The specific radioactivities of the ^{32}P-labelled phospholipids in rough-microsomal and smooth-microsomal fractions were virtually the same at all times. As expected, the major fraction of ^{32}P-labelled RNA was recovered in the nuclear fraction, which (except for a small amount in the cell sap) was virtually the only site of localization of labelled RNA for up to 2hr. of incubation (Fig. 3*b*). After this period, gradually increasing amounts of newly formed RNA were recovered from the cytoplasm, principally in the microsomal fraction. Chromatographic analysis of an alkali hydrolysate of RNA from both nuclear and cytoplasmic fractions led to the recovery of ^{32}P in all four nucleoside monophosphates.

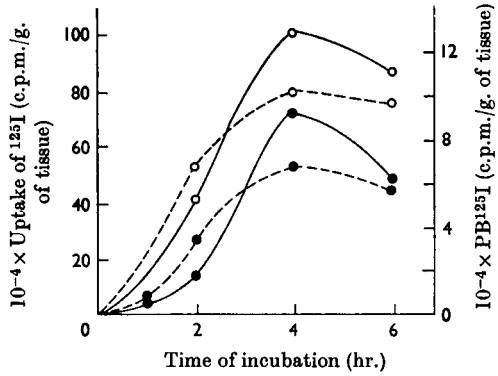

Fig. 4. Effect of TSH on the ^{125}I uptake (○) and $PB^{125}I$ content (●) in pig thyroid slices. A 5μc sample of [^{125}I]-iodide was used/7·5ml. of incubation medium containing 0·5g. of thyroid slices and 0·1 unit of TSH/ml.,' where used. ——, Control; – – – –, plus TSH.

Incubation of thyroid slices with TSH enhanced the rate of labelling of phospholipids in all particulate fractions (Table 2). To a smaller extent TSH increased the specific radioactivity of RNA recovered in the crude nuclear fraction, but more markedly accelerated the rate of accumulation of labelled RNA in the microsomal and cell-sap fractions (Table 3). Labelling of mitochondrial RNA was barely affected. From other experiments similar to those described in Tables 2 and 3 it was noticed that the onset of increased accumulation of newly formed microsomal RNA during incubation with TSH coincided with the increase in ^{32}P-labelled phospholipid recovered in the microsomes.

Incorporation of ^{125}I into iodoprotein, its subcellular distribution and the effect of TSH. The experiments on the incorporation of ^{125}I into iodoprotein were usually performed at the same time as those on the incorporation of ^{32}P into phospholipids and RNA. Fig. 4 shows that the uptake of ^{125}I from the medium and its organification and incorporation into thyroidal $PB^{125}I$ proceeded at a high rate during 4hr. of incubation in the absence of TSH. The fraction of total ^{125}I in the tissue that was incorporated into protein varied from 7% to 15%. Both these processes, especially that of $PB^{125}I$ formation, were stimulated by TSH during the first 2hr. of incubation. With longer time-intervals the hormone had the opposite effect, so that after 4hr. of incubation the values for both ^{125}I uptake and $PB^{125}I$ were lower with TSH than in the controls. The apparent reversal of TSH action is most likely to be due to the facts that with time TSH would increase the rate of breakdown of unlabelled thyroglobulin into iodo amino acids, and that the rapid deiodination of the latter would

Table 4. *Incorporation of ^{125}I into iodoprotein in thyroid cytoplasmic subcellular fractions and the effect of TSH* in vitro

Pig thyroid slices were incubated with [^{125}I]iodide without or with 0·1 unit of TSH/ml. for 1, 2, 4 and 6hr. before fractionation of the tissue. At 2hr. the uptake of ^{125}I and the fraction of the ^{125}I recovered in protein were: 415650 c.p.m./g. and 14% for the control, and 505325 c.p.m./g. and 17% with TSH, respectively. TSH was added with [^{125}I]iodide at the start of the incubation. When the amount of ^{125}I present in the initial pellet of nuclei and debris was taken into account, the recovery of radioactivity was 93–95%.

Fraction	Time of incubation (hr.) ... TSH	$PB^{125}I$/g. of slices (c.p.m.) 1	2	4	6
Mitochondria	–	2345	4590	12310	10720
	+	2516	6975	12270	11030
Rough membranes	–	310	506	2462	2070
	+	125	500	1438	1208
Smooth membranes	–	2641	15600	55950	47700
	+	4115	20400	36250	39500
Cell sap	–	875	3270	46300	8250
	+	1050	4040	30180	6900

effectively decrease the specific radioactivity of the [^{125}I]iodide picked up by the tissue (Rall *et al.* 1964). The decrease in both the ^{125}I uptake and PB^{125}I content of the control and TSH-treated slices after 4–6hr. of incubation is probably the result of loss of PB^{125}I from the slices as a result of tissue deterioration. Because of the decreased utilization of ^{125}I beyond 4hr. of incubation, many of the later studies were performed with only short periods of exposure to [^{125}I]iodide after incubation with the hormone.

The subcellular distribution of ^{125}I-labelled protein as a function of time, in the presence and absence of TSH, is summarized in Table 4. Although the rough membranes represent the major site of thyroglobulin synthesis (Ekholm & Strandberg, 1968), the major site of thyroglobulin iodination is different and is thought to be the smooth membrane (Seed & Goldberg, 1965; Nunez, Mauchamp, Jerusalemi & Roche, 1967). With prolonged incubation, an increasing amount of PB^{125}I became associated first with the smooth-membrane fraction, and then with the soluble fraction, which presumably represents the transport of freshly iodinated protein into the colloid for storage. After 6hr. of incubation there was a considerable decrease in the PB^{125}I content of the soluble fraction. The decrease in ^{125}I uptake and organification by the slices, noted above for the 6hr. incubation time, may be due primarily to loss of labelled protein from the soluble or cell-sap fraction. As mentioned above for ^{125}I uptake and

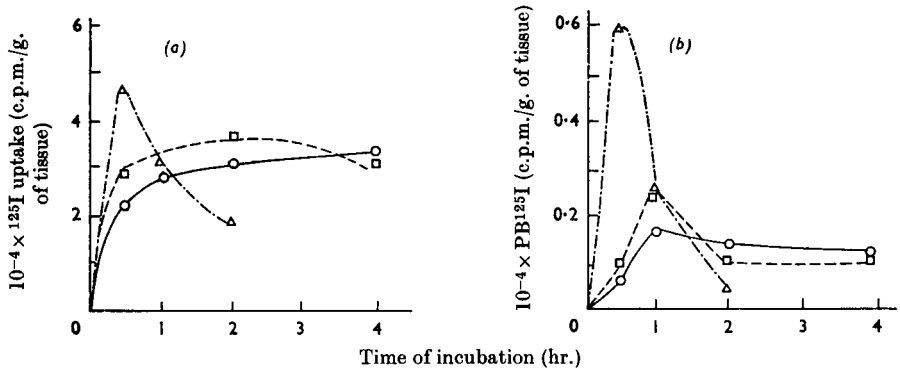

Fig. 5. Effect of TSH and DB-c-AMP on (*a*) the rate of uptake of ^{125}I and (*b*) the rate of incorporation of ^{125}I into iodoprotein. Thyroid slices (0·5g. in 7·5ml. of medium) were incubated, where indicated, with TSH or DB-c-AMP added at the start of the incubation, but [^{125}I]iodide was added in all instances 20min. before the incubation was terminated. ○, No additions; □, 0·12 unit of TSH/ml.; △, 0·2mg. of DB-c-AMP/ml.

Table 5. *Failure of c-AMP and 5'-AMP to mimic the effect of TSH and DB-c-AMP* in vitro *on the uptake and incorporation into protein of* ^{125}I *by pig thyroid slices*

Pig thyroid slices were incubated with the different substances indicated for the times indicated. [^{125}I]Iodide was added to each vessel only 20min. before the termination of the incubation. A 2·5μc sample of [^{125}I]iodide was added to 0·5±0·01g. of slices in 7·5ml. of incubation medium.

Substance added/ml.	Time of incubation (hr.)	^{125}I uptake (c.p.m./g.)	PB^{125}I (% of uptake)
None	0·5	22500	3·06
	1·0	28350	5·95
0·13 unit of TSH	0·5	29600	2·95
	1·0	20780	11·15
0·2mg. of DB-c-AMP	0·5	46400	12·62
	1·0	31240	8·25
1·0mg. of c-AMP	0·5	25500	6·45
	1·0	30600	6·50
1·0mg. of 5'-AMP	0·5	27745	5·51
	1·0	28910	5·97

incorporation into protein, the effect of the hormone on ^{125}I in smooth-membrane protein was a biphasic one, depending on the time for which the thyroid tissue was exposed to it.

Comparison of the effects of TSH and DB-c-AMP. Under our incubation conditions, DB-c-AMP qualitatively mimicked the action of TSH on the uptake and protein binding (or organification) of ^{125}I by pig thyroid slices (Fig. 5). There were, however, differences in the speed, magnitude and duration of response to the two substances. With TSH the action was manifested slowly but was longer-lasting, whereas the effect of the nucleotide was rapid but transient and gave a lower incorporation value after 2hr. of incubation. In these experiments [^{125}I]iodide was only added for the last 20min. of incubation and not from the beginning, and the magnitude of the effect of both TSH and the DB-c-AMP was consequently smaller than when the slices were exposed to [^{125}I]iodide for longer periods. The inactivity of c-AMP in isolated thyroid preparations (Table 5) was described by Pastan (1966) and is thought to be due to its susceptibility to breakdown by phosphodiesterases (Sutherland *et al.* 1967).

That the stimulation by DB-c-AMP of the rate of labelled iodoprotein formation was not merely a reflection of an enhanced uptake of ^{125}I from the medium is shown in Table 6. In this table the data of Fig. 5 are expressed as percentage increase or decrease in the radioactivity recovered as iodoprotein before and after correction for the change in total uptake of ^{125}I at the corresponding time of incubation with TSH or DB-c-AMP. Table 6 also

Table 6. *Biphasic changes produced by TSH and DB-c-AMP in the incorporation of* ^{125}I *into protein at different times of incubation*

The results are expressed as percentage changes from control values when the values were uncorrected or corrected for the increase or decrease caused by TSH and DB-c-AMP in the uptake of ^{125}I by thyroid slices. Other conditions were as given in Table 5 and Fig. 5.

		Change in PB^{125}I from control values (%)	
Substance added	Time of incubation (hr.)	Uncorrected for ^{125}I uptake	Corrected for ^{125}I uptake
TSH	0·5	+ 26	+ 6
	1·0	+ 38	+101
	2·0	− 37	− 63
DB-c-AMP	0·5	+755	+360
	1·0	+ 55	+ 48
	2·0	−205	−105

Table 7. *Effect of DB-c-AMP and TSH* in vitro *on the uptake of* ^{32}P *and its distribution in the acid-soluble, phospholipid and RNA fractions of thyroid slices* in vitro

Pig thyroid slices were incubated for the times indicated, with the [^{32}P]phosphate and the different substances, where indicated, added from the start of the incubation. A 8·4μc sample of [^{32}P]phosphate was added to each flask containing 0·5±0·01g. of slices in 7·5ml. of incubation medium.

				Sp. radioactivity (c.p.m./μg. of P)	
Substance added per ml.	Time of incubation (hr.)	^{32}P uptake (c.p.m./g.)	Acid-soluble ^{32}P (c.p.m./g.)	Phospholipid	RNA
None	1·0	882000	525	230	47
	2·0	1335000	650	435	135
	4·0	1887000	910	927	430
0·13 unit of TSH	1·0	940000	600	410	58
	2·0	1640000	650	1105	320
	4·0	2050000	690	2145	581
0·2mg. of DB-c-AMP	1·0	1465000	1150	240	103
	2·0	2045000	1475	612	225
	4·0	2180000	1290	1120	534
1·0mg. of c-AMP	1·0	950000	518	198	69
	2·0	1180000	725	365	118
	4·0	1900000	1030	870	369

shows that DB-c-AMP mimics the effect of TSH, with respect to both uptake of iodine and its incorporation into protein.

When the actions of TSH and DB-c-AMP on phosphorus metabolism of pig thyroid slices were compared, the mimicry by the nucleotide of hormonal action was less convincing. The experiment summarized in Table 7 showed that DB-c-AMP did cause a relatively modest (up to 50%) stimulation of incorporation of ^{32}P into phospholipid and RNA, compared with a 200–300% increase with TSH. Increased concentrations of the nucleotide failed to enhance the response of the tissue. As with the experiments with ^{125}I, unsubstituted c-AMP failed to alter the rate of incorporation of ^{32}P into phospholipid and RNA. That even the limited stimulation of ^{32}P incorporation induced by DB-c-AMP represented only an apparent mimicry of TSH action became clear when the effects on ^{32}P uptake and acid-soluble ^{32}P content were taken into account. From Table 7 (as well as Fig. 1), TSH had little or no effect on the amount of label entering into the tissue or present as ^{32}P in the acid-soluble pool; on the contrary, these parameters were affected by DB-c-AMP in every experiment to nearly the same extent as the increase in specific radioactivity of ^{32}P-labelled phospholipid and RNA.

The apparently similar effects of TSH and DB-c-AMP on ^{32}P incorporation can be further distinguished from each other if the changes in specific radioactivities of phospholipids and RNA are corrected for by the changes in ^{32}P uptake (specific radioactivity of acid-soluble phosphorus). An example of such a correction is given in Fig. 6, which shows that DB-c-AMP has no effect on the true rate of ^{32}P incorporation if subtractions are made for the increased entry of ^{32}P into the tissue. On the other hand, such a correction has no effect when applied to the stimulation by TSH. From other work (Freinkel, 1964; Scott *et al.* 1968; Pastan & Macchia, 1967; Kerkof & Tata, 1967) TSH is known to promote net synthesis of phospholipid and, to a smaller extent, that of RNA. It seems therefore that DB-c-AMP does not truly mimic this particular action of TSH, but only enhances the labelling of phosphorylated precursors in the acid-soluble pool.

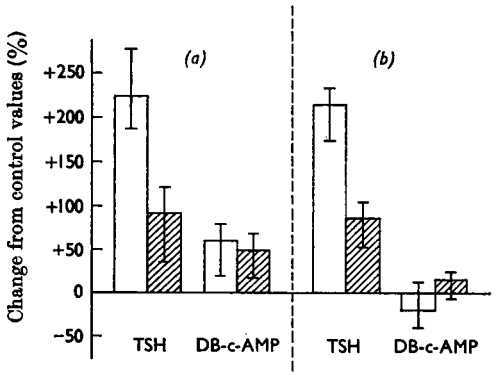

Fig. 6. Effect of DB-c-AMP and TSH on the incorporation of ^{32}P into pig thyroid-slice phospholipid and RNA after corrections are applied for the changes in ^{32}P uptake. (*a*) Values uncorrected for changes in uptake of ^{32}P caused by TSH or DB-c-AMP; (*b*) values corrected for changes in uptake. □, Specific radioactivity of phospholipids; ■, specific radioactivity of RNA. The results are expressed as the means of ten determinations from five separate experiments with S.D. shown as vertical bars. Details of the experiments were as given in Table 6 and the text.

DISCUSSION

Our results confirm some earlier findings and establish new facts about the effects of TSH on the metabolism and distribution of iodine and phosphorus in the thyroid. In general, pig thyroid slices seem to respond to TSH *in vitro* in much the same way as do thyroid slices and cell suspensions from ox, sheep and dog with regard to iodine uptake and protein-binding (Rall *et al.* 1964; Raghupathy, Kerkof & Chaikoff, 1965), phospholipid synthesis (Freinkel, 1958, 1964; Altman, Oka & Field, 1966; Pastan & Macchia, 1967) and RNA synthesis (Hall & Tubmen, 1965; Lecoq & Dumont, 1967). That the magnitude of some of our effects is lower than that reported elsewhere may be due to the fact that the pig thyroids we used may be relatively more stimulated by endogenous TSH than are glands in other species. Another variable may be the different periods of time elapsed between the death of the animal and the beginning of the experiment.

Our results of subcellular fractionation of thyroid tissue show that much of the labelled phospholipids, RNA and iodoprotein accumulates in the microsomal fraction. The effect of TSH was to enhance this accumulation and, with iodoprotein, to accelerate its breakdown after longer periods of incubation. It could be argued that the stimulated rate of phospholipid and RNA synthesis may be an important cellular response to an enhanced demand, created by TSH, for additional thyroglobulin. We have shown that in guinea-pig thyroid the administration of TSH *in vivo* is followed by a co-ordinated increase in the rate of synthesis of phospholipid, RNA and a thyroglobulin-like iodoprotein (Kerkof & Tata, 1967). Such a co-ordination has been demonstrated in the formation and function of the different cellular components for the synthesis and transport of proteins, and this in turn is associated

with the induction or acceleration of growth and development of target tissues by many hormones (Tata, 1967*a*, 1968). In the present studies we assumed that the main iodinated protein is thyroglobulin. It is, however, worth noting that the sites of synthesis and iodination of thyroglobulin in the microsomal fractions are thought to be distinct, i.e. the rough and the smooth endoplasmic reticulum respectively (Seed & Goldberg, 1965; Nunez *et al.* 1967; Ekholm & Strandberg, 1968). Other indirect results have further suggested that the stimulatory actions of TSH on the processes of RNA synthesis, thyroglobulin synthesis and its iodination are not directly linked to one another (Taurog & Thio, 1966; Halmi, Westra & Polly, 1966; Tong, 1967; Hall & Tubmen, 1968; Nataf, 1968).

Work from Palade's laboratory (see Palade, 1966) has established that the vectorial discharge of newly synthesized protein into the vesicles of smooth endoplasmic reticulum reflects the transport of proteins for export in cells with a predominantly secretory function. Our findings of the persistence of a large amount of [^{125}I]iodoprotein in the smooth-microsomal-membrane fraction is compatible with this mode of transport, as well as with the kinetic analysis of thyroid protein synthesis and secretion described by Ekholm & Strandberg (1968). However, the proportionally small amount of ^{125}I-labelled protein recovered in the cell sap at the earlier time-intervals suggests that thyroglobulin may be enclosed in vesicles until its discharge into the colloid. The relatively larger amounts of radioactivity that are recovered in the cell sap beyond 4hr. of incubation may represent this colloidal fraction. The dual effects of TSH on thyroglobulin iodination and breakdown, however, complicate a precise interpretation of our results of the hormonal effects on the kinetics of subcellular movement of iodoprotein.

No systematic attempt was made to characterize the additional thyroidal phospholipids and RNA synthesized under the influence of TSH. Freinkel and co-workers have, however, shown that TSH preferentially stimulates the synthesis of specific phospholipids, although it was not shown how these were distributed among the different cellular organelles (Scott, Jay & Freinkel, 1966; Scott *et al.* 1968). The significance of such a selective effect on phospholipid synthesis or accumulation may lie in the hormonal influence on membrane formation and structural reorganization of the target cells. It is also tempting to suggest that the additional RNA in the nucleus and cytoplasm is the messenger for thyroglobulin, but it should be realized that many of the acute effects of TSH on the synthesis and iodination of thyroglobulin could be elicited in the absence of synthesis of RNA or protein (Taurog & Thio, 1966; Halmi *et al.* 1966; Hall & Tubmen, 1968). Even if small amounts of messenger RNA were synthesized in response to TSH, our experience with other hormone-induced growth and developmental systems has shown that that the massive synthesis of ribosomal RNA in response to the hormone makes it very difficult to demonstrate the possible formation of small amounts of hormone-specific messenger RNA (Wyatt & Tata, 1968).

Sutherland has suggested that c-AMP is a universal intracellular 'second messenger' or mediator for the action of a number of mammalian hormones (Sutherland *et al.* 1967). The mimicry by the more stable derivative, DB-c-AMP, of so many of the actions of TSH has therefore generated much interest in the possibility of c-AMP being a mediator of hormone action in the thyroid. However, the nucleotide stimulates mostly those actions of TSH that are rapid and do not involve any biosynthetic processes, such as iodine uptake, iodination, lysosomal activation for breakdown of thyroglobulin, glucose oxidation and thyroxine release. It is for this reason that the claim by Pastan & Macchia (1967) of a net increase in phospholipid synthesis provoked by DB-c-AMP deserved to be carefully investigated. Our results show clearly that, whereas the TSH-like enhancement by DB-c-AMP of iodine uptake and iodination of protein may be a real one, the simulation of the effect of TSH on incorporation into phospholipids is more apparent than real. This discrepancy is most likely to be due to a stimulatory effect of DB-c-AMP on the penetration of [^{32}P]orthophosphate into thyroid cells, leading to a corresponding increase in specific radioactivity of the acid-soluble phosphorus pool. The distinction between effects of the cyclic nucleotide and of the hormone on phospholipid synthesis, as well as on RNA synthesis, became obvious when corrections were made for changes in ^{32}P uptake and specific radioactivity of the acid-soluble phosphorus fraction (see Fig. 6). Pastan & Macchia (1967) did not consider this factor of labelling of precursor pools, but their results could also be similarly explained. Further, the kinetics and magnitude of the effects of the two substances were dissimilar (Tables 5 and 7). This type of situation is not restricted to TSH and thyroid cells. In fact mimicry by c-AMP of the action of almost all the other hormones is virtually restricted to rapid and 'purely metabolic' hormonal actions (Sutherland *et al.* 1967). In the few instances in which biosynthetic functions of hormones have been claimed to be mimicked it still remains to be shown that c-AMP did in fact enhance the net synthesis of protein, RNA or phospholipid (see Tata, 1968). c-AMP indeed seems to be an important intracellular mediator in rapid hormonal effects on cellular permeability and modulation of enzyme activity, but is unlikely to exert a direct

influence on the slower biosynthetic responses to growth hormone and developmental hormones.

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REFERENCES

Altman, M., Oka, H. & Field, J. B. (1966). *Biochim. biophys. Acta*, **116**, 586.

Ames, B. N. & Dubin, D. T. (1960). *J. biol. Chem.* **235**, 769.

Ceriotti, G. (1955). *J. biol. Chem.* **214**, 59.

Dumont, J. E. (1964). *Bull. Soc. Chim. biol., Paris*, **46**, 1131.

Ekholm, R. & Strandberg, U. (1968). *J. Ultrastruct. Res.* **22**, 252.

Fleck, A. & Munro, H. N. (1962). *Biochim. biophys. Acta*, **55**, 571.

Freinkel, N. (1958). *Biochem. J.* **68**, 327.

Freinkel, N. (1964). In *The Thyroid Gland*, vol. 1, p. 131. Ed. by Pitt-Rivers, R. & Trotter, W. R. London: Butterworths Scientific Publications.

Hall, R. & Tubmen, J. (1965). *J. biol. Chem.* **200**, 3132.

Hall, R. & Tubmen, J. (1968). *J. biol. Chem.* **243**, 1598.

Halmi, N. S., Westra, J. P. & Polly, R. E. (1966). *Endocrinology*, **79**, 424.

Kerkof, P. R. & Tata, J. R. (1967). *Biochem. biophys. Res. Commun.* **28**, 111.

Lecocq, R. E. & Dumont, J. E. (1967). *Biochem. J.* **104**, 13c.

Lowry, D. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

Nataf, B. (1968). *Gen. comp. Endocrin.* **10**, 159.

Nunez, J., Mauchamp, J., Jerusalemi, A. & Roche, J. (1967). *Biochim. biophys. Acta*, **145**, 127.

Palade, G. E. (1966). *J. Amer. med. Ass.* **198**, 815.

Pastan, I. (1966). *Biochem. biophys. Res. Commun.* **25**, 14.

Pastan, I. & Katzen, R. (1967). *Biochem. biophys. Res. Commun.* **29**, 792.

Pastan, I. & Macchia, V. (1967). *J. biol. Chem.* **242**, 5757.

Pastan, I. & Woolman, S. H. (1967). *J. Cell Biol.* **35**, 262.

Raghupathy, E., Kerkof, P. R. & Chaikoff, I. L. (1965). *Biochim. biophys. Acta*, **92**, 118.

Rall, J. E., Robbins, J. & Lewallen, C. G. (1964). In *The Hormones*, vol. 5, p. 159. Ed. by Pincus, G., Thimann, K. V. & Astwood, E. B. New York: Academic Press Inc.

Scott, T. W., Jay, S. M. & Freinkel, N. (1966). *Endocrinology*, **79**, 591.

Scott, T. W., Mills, S. C. & Freinkel, N. (1968). *Biochem. J.* **109**, 325.

Seed, R. W. & Goldberg, I. H. (1965). *J. biol. Chem.* **240**, 764.

Sutherland, E. W., Butcher, R. W., Robison, G. A. & Hardman, J. G. (1967). In *Wirkungsmechanismen der Hormone*, p. 1. Ed. by Karlson, P. Berlin: Springer-Verlag.

Tata, J. R. (1967*a*). *Nature, Lond.*, **213**, 566.

Tata, J. R. (1967*b*). *Biochem. J.* **105**, 783.

Tata, J. R. (1968). *Nature, Lond.*, **219**, 331.

Tata, J. R. & Williams-Ashman, H. G. (1967). *Europ. J. Biochem.* **2**, 366.

Taurog, A. & Thio, D. T. (1966). *Endocrinology*, **78**, 103.

Tong, W. (1967). *Endocrinology*, **80**, 1101.

Wyatt, G. R. & Tata, J. R. (1968). *Biochem. J.* **109**, 253.