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Co-ordination between Membrane Phospholipid Synthesis and Accelerated Biosynthesis of Cytoplasmic Ribonucleic Acid and Protein

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1. The rate of synthesis of membrane phospholipid was studied in rat liver and seminal vesicles by following the incorporation of [^{32}P]orthophosphate, [^{14}C]choline and [^{14}C]glycerol. Particular emphasis was laid on the endoplasmic reticulum, which was fractionated into smooth microsomal membranes, heavy rough membranes, light rough membranes and free polyribosomes. 2. Phospholipid labelling patterns suggested a heterogeneity in the synthesis and turnover of the different lipid moieties of smooth and rough endoplasmic membranes. The major phospholipids, phosphatidylcholine and phosphatidylethanolamine, were labelled relatively rapidly with ^{32}P over a short period of time whereas incorporation of radioisotope into the minor phospholipids, sphingomyelin, lysolecithin and phosphatidylinositol proceeded slowly but over a longer period of time. 3. The incorporation of orotic acid into RNA and labelled amino acids into protein of the four submicrosomal fractions was also studied. 4. Rapid growth of the liver was induced by the administration of growth hormone and tri-iodothyronine to hypophysectomized and thyroidectomized rats and by partial hepatectomy. Growth of seminal vesicles of castrated rats was stimulated with testosterone propionate. 5. The rate of labelling of membrane phospholipids was enhanced in all major subcellular particulate fractions (nuclear, mitochondrial and microsomal) during induced growth. However, it was in the rough endoplasmic reticulum that the accumulation of phospholipids, RNA and protein was most marked. The effect of hormone administration was also to accelerate preferentially the labelling with ^{32}P of sphingomyelin relative to that of phosphatidylcholine or phosphatidylethanolamine. 6. Time-course analyses showed that, in all four growth systems studied, the enhancement of the rate of membrane phospholipid synthesis coincided with the rather abrupt increase in the synthesis of RNA and protein of the rough endoplasmic reticulum. Growth hormone and tri-iodothyronine administered to hypophysectomized rats had additive effects in all the biosynthetic processes. The latent period of action of each hormone was maintained so that two waves of proliferation of endoplasmic reticulum occurred if the hormones were administered simultaneously. 7. It is concluded that there is some mechanism in the cell that tightly co-ordinates the formation of membranes, especially those of the endoplasmic reticulum, when an increased demand is made for protein synthesis.

The acceleration of protein synthesis at the onset of rapid growth is accompanied in a variety of systems by the appearance of additional ribosomes and polyribosomes in the cytoplasm. This feature is particularly noticeable during regeneration of the liver after partial hepatectomy and after hormone-induced growth and development (Tata, 1967*a*, 1968*a*; Teng & Hamilton, 1967; Tsukada, Takako, Doi & Lieberman, 1968; Fujii & Villee, 1969). It had earlier been observed that hormone-induced acceleration of growth in rat liver or induction of metamorphosis in tadpole liver resulted in a higher proportion of total ribosomes (and polyribosomes) being recovered in the rough endoplasmic reticulum (Tata & Widnell, 1966; Tata, 1967*b*; Tata & Williams-Ashman, 1967). In liver, and to a smaller extent in other tissues, a large part of protein synthesized *in vivo* occurs on membrane-attached polyribosomes (Henshaw, Bojarski & Hiatt, 1963; Campbell, Cooper & Hicks, 1964; Campbell, 1965; Hendler, 1968) and the proliferation of both the smooth and rough endoplasmic reticulum is known to be a characteristic feature of growing cells (Dallner, Siekevitz & Palade, 1966; Pollak &

Ward, 1967; Siekevitz, Palade, Dallner, Ohad & Omura, 1967; Tata, 1967*b*). It was therefore decided to study the rate of accumulation of membrane constituents as a function of time either after acceleration of the rate of growth by hormones or after partial hepatectomy.

In the present paper the rates of labelling *in vivo* of nascent protein of microsomal fractions are correlated with both accumulation of newly synthesized cytoplasmic RNA and newly synthesized membrane-associated phospholipids. These studies were carried out on liver and seminal vesicles of hypophysectomized, thyroidectomized and castrated rats after growth induction by growth hormone, thyroid hormone and testosterone respectively. The additive effects of a combination of these hormones on the above processes were also studied and compared with the situation in regenerating rat liver. It is concluded that there is some mechanism in the cell that tightly co-ordinates the formation of membranes, especially those of the endoplasmic reticulum, when an increased demand is made for protein synthesis.

Some of this work has been reported in preliminary communications (Tata, 1967*c*, 1968*a*) and similar studies on bullfrog-tadpole liver during induced metamorphosis have also been published in full (Tata, 1967*b*).

EXPERIMENTAL

Animals and treatment. All experiments were performed with male Sprague–Dawley rats reared at the National Institute for Medical Research, Mill Hill. The procedures used for thyroidectomy, hypophysectomy and castration and hormonal treatment of hormone-deprived animals were the same as those described previously (Tata & Widnell, 1966; Widnell & Tata, 1966). Two-thirds hepatectomy was performed by the procedure of Higgins & Anderson (1931).

Subcellular fractionation. Nuclei, mitochondria, microsomes and cell sap were separated from liver homogenates by methods previously described (Widnell & Tata, 1964; Tata & Widnell, 1966). The subfractionation of microsomes into the rough and smooth membrane components was achieved by centrifuging through a discontinuous sucrose gradient (Tata & Williams-Ashman, 1967; Tata, 1969). The recovery of RNA in unfractionated microsomes relative to total RNA in the homogenate was 86±7% for liver and 71±9% in the seminal vesicles from normal rats. It was not possible to attain high recoveries of rat liver submicrosomal fractions because the material between the discontinuous sucrose gradient interfaces was not collected. Nevertheless 60–70% of microsomal phospholipid, protein and RNA was recovered in the fractions termed 'smooth membranes', 'light rough membranes', 'heavy rough membranes' and 'free polyribosomes' (see Tata, 1969).

Radioisotopic labelling of phospholipid, RNA and protein in vivo. Phospholipids were usually labelled by intraperitoneal injection of 50–100 μCi of carrier-free [^{32}P]orthophosphate but [^{14}C]choline and [^{14}C]glycerol were also used. The labelled phospholipids were extracted from particulate subcellular fractions with chloroform–methanol (2:1, v/v) after they had been first precipitated and washed three times with 0.4M-$HClO_4$. Four chloroform–methanol extracts were pooled (two extractions were carried out at 20°C and two at 45°C) and washed twice with 0.1M-HCl before they were evaporated to dryness. The individual phospholipids were separated and characterized by t.l.c. on silica gel with chloroform–methanol–water–acetic acid (25:50:2:4, by vol.) as the solvent system. The distribution of radioactivity in individual phospholipids was determined either by eluting the individual chromatogram spots, by double-beam microdensitometric (Joyce–Loebl) scanning of ^{32}P radioautograms or by direct radioactivity scanning with a Panax t.l.c. scanner. Radioautograms of t.l.c. plates were prepared with Ilford No-Screen X-ray films.

RNA was often labelled with ^{32}P at the same time as phospholipids. In some experiments cytoplasmic RNA was separately labelled by injecting 10–20 μCi of [^{14}C]-orotic acid or 40–80 μCi of [^{3}H]orotic acid. Extraction of RNA was carried out as described by Tata & Widnell (1966).

Newly synthesized or nascent protein formed *in vivo* was labelled by a 5–10 min pulse of 4–8 μCi of a mixture of ^{14}C-labelled amino acids administered intraperitoneally for liver, and a 15 min pulse of 15 μCi for seminal vesicles. The radioactive protein was released from microsomal preparations with 1% (w/v) sodium deoxycholate and precipitated with 5% (w/v) trichloroacetic acid. The precipitate was filtered on Oxoid (Oxo Ltd., London E.C.4, U.K.) membrane filters and washed twice with 5% trichloroacetic acid at 5°C and twice at 80°C and then with water. The dried filters were used for radioactivity measurements and protein was chemically determined separately on a small portion of the original preparation. In experiments dealing with the effects of hormones, corrections were applied for (1) differences in weights of the organs from animals at different stages of growth stimulation by hormones or during liver regeneration and (2) the possible effect of hormones or partial hepatectomy on the uptake of isotope (see Bucher & Swaffield, 1969; Tata, 1968*a*). This was done by multiplying the specific radioactivity obtained by the factor:

$$\frac{\text{Uptake in control (c.p.m./g of tissue)} \times \text{wt. of organ in experiment (g)}}{\text{Uptake in experimental (c.p.m./g of tissue)} \times \text{wt. of control organ (g)}}$$

'Uptake' was taken as the total radioactivity recovered in the acid-soluble and acid-insoluble fractions.

Determination of radioactivity. ^{14}C and ^{3}H in solution were measured in a Packard Tri-Carb scintillation spectrometer in 10 ml of a fluid comprising 0.4% 2,5-diphenyloxazole, 0.01% 1,4-bis-(4-methyl-2-phenyloxazol-2-yl)benzene and 18% nuclear-grade naphthalene in redistilled dioxan. The counting efficiencies were 66% and 40% respectively. ^{32}P and ^{14}C-labelled proteins on

membrane filters were measured in a Nuclear-Chicago thin-window gas-flow counter with efficiencies of about 40% and 20% respectively (background 1.5±0.3 c.p.m.).

Chemical determinations. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) as standard. RNA was determined either spectrophotometrically by the method of Fleck & Munro (1962) or colorimetrically by the method of Ceriotti (1955) with yeast RNA (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) as standard. RNA and phospholipid phosphorus were measured by the Ames & Dubin (1960) modification of the method of Chen, Toribara & Warner (1956). The amount of phospholipid was taken to be 25 times the phosphorus value. DNA was determined by the diphenylamine method of Burton (1956).

Materials. All chemicals and reagents were of analytical grade and purchased from Sigma (London) Chemical Co., London S.W.6, U.K. or Boehringer. Phospholipid markers were obtained from Sigma (London) or Applied Science Laboratories Inc., State College, Pa., U.S.A. 3,3',5-Tri-iodo-L-thyronine was a gift from Glaxo Ltd., Greenford, Middx., U.K., human growth hormone (W.H.O. International Standard, 2 i.u./mg) was obtained from Biological Standards Division, National Institute for Medical Research, and testosterone propionate was purchased from Paines and Byrne Ltd., Greenford, Middx., U.K. Carrier-free [^{32}P]phosphate, [6-^{14}C]orotic acid (6.8mCi/mmol), [5-^{3}H]orotic acid (234mCi–13.4Ci/mmol), [U-^{14}C]glycerol (14.3mCi/mmol) and U-^{14}C-labelled amino acid mixture (640mCi/mg) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Any contaminating radioactive nucleotides were removed from the labelled amino acid mixture by ion-exchange chromatography. All organic solvents were freshly redistilled before use.

RESULTS

The kinetics of labelling of nuclear and cytoplasmic RNA in normal, thyroidectomized and hypophysectomized animals under our laboratory conditions have been described (Tata & Widnell, 1966; Tata, 1967*a*). The following is a brief description of the time-course of formation of membrane phospholipids and recovery of nascent protein in microsomal fractions in normal animals.

Labelling of membrane phospholipids. Fig. 1 shows the rate of labelling with [^{14}C]choline of the phospholipid of particulate fractions (nuclei, mitochondria, unfractionated microsomes) of normal rat liver. The incorporation rate was rapid and nearly linear for about 1.5h after administration of the labelled compound; the microsomal fraction had the highest specific radioactivity, which is compatible with the fact that it is virtually the sole site of phosphatidylcholine (lecithin) synthesis (McMurray & Dawson, 1969). Since the present study deals with co-ordination of membrane proliferation with protein and RNA synthesis *in vivo* the major

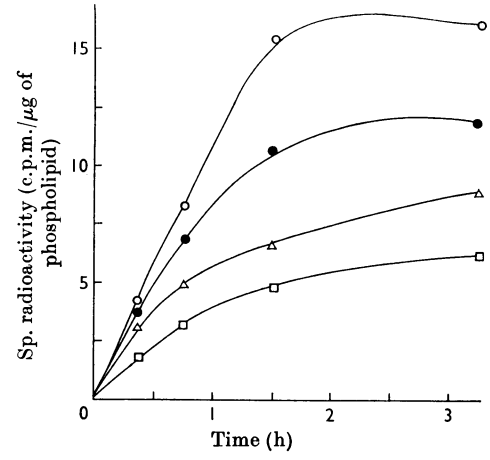

Fig. 1. Time-course of incorporation of [^{14}C]choline into phospholipids of particulate fractions of normal rat liver. [^{14}C]Choline chloride (7.5μCi) was injected into each of a group of three rats (140–150g). The rats were killed at the times indicated and nuclei (□), mitochondria (△), smooth microsomes (○) and rough microsomes (●) were isolated from homogenates of pooled livers by the methods described in the text. The phospholipids were extracted from the membrane fractions and their specific radioactivities determined. Each point is the mean of three determinations.

emphasis is laid on the formation of microsomal phospholipids. Fig. 2 shows the rate of incorporation of ^{32}P into phospholipids and RNA of smooth and rough microsomal fractions of rats.

The specific radioactivity of phospholipids in the smooth membranes at short labelling periods was found to be lower than in the rough membranes, but this pattern was reversed after 2–3h. The most likely reason for the higher specific radioactivity of RNA in smooth membranes is that they do not contain the large amount of unlabelled rRNA found in the rough membrane fractions, although a special class of microsomal membrane RNA has been postulated (Shapot & Pitot, 1966; Rodionova & Shapot, 1966). The rate of labelling of all phospholipids with ^{32}P did not follow the overall pattern of Fig. 2. In general, in the smooth and rough membranes, the major phospholipids, phosphatidylcholine and phosphatidylethanolamine, were not further labelled after 3h, whereas the specific radioactivity of minor phospholipids, such as sphingomyelin and 1-acylglycerophosphorylcholine (lysolecithin), continued to increase up to 24h. This effect is illustrated for phosphatidylcholine and sphingomyelin in Fig. 3. The ratio of ^{32}P incorporated into phosphatidylcholine to that into sphingomyelin decreased from 10–20 at 2h to 3–5 at 24h (see curves with broken lines in Fig. 3).

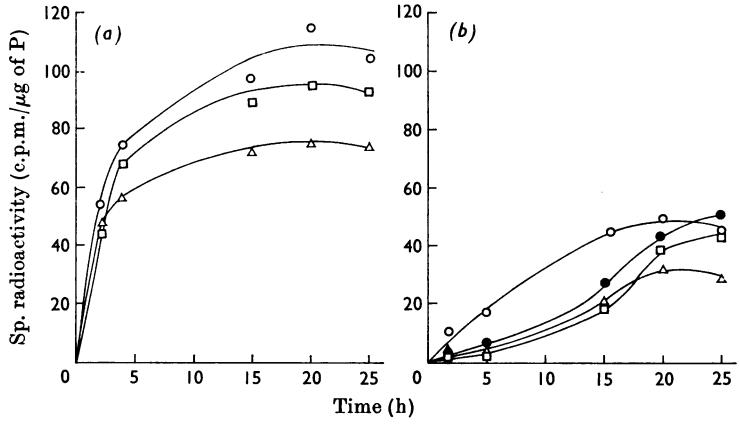

Fig. 2. Time-course of incorporation of [^{32}P]orthophosphate into phospholipids (*a*) and RNA (*b*) from submicrosomal fractions of normal rat liver. [^{32}P]Orthophosphate (85μCi) was injected into each of the groups of three rats (125–135g) and the microsomal subfractionation carried out on mitochondria-free supernatant from pooled livers as described elsewhere (Tata & Williams-Ashman, 1967; Tata, 1969). The results are expressed as specific radioactivity of phospholipid and RNA extracted from smooth microsomal membranes (○), heavy rough membranes (□), light rough membranes (△) and free polyribosomes (●). Each point is the average of two separate determinations.

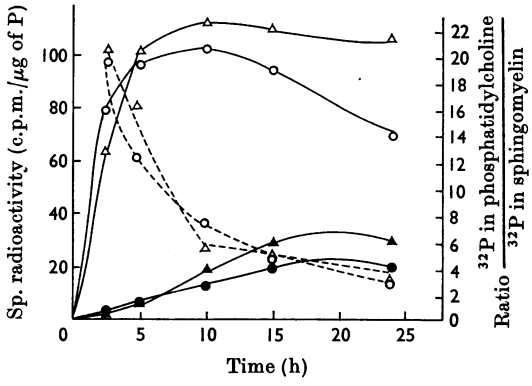

Fig. 3. Differential rates of incorporation of [^{32}P]orthophosphate into phosphatidylcholine (△ and ○) and sphingomyelin (▲ and ●) of smooth (△ and ▲) and rough (○ and ●) microsomal membranes. Groups of four normal rats (130–140g) were given 90μCi of [^{32}P]orthophosphate and killed at the times indicated. Smooth and rough microsomes were prepared from pooled livers and the individual phospholipids separated by t.l.c. of a chloroform–methanol extract as indicated in the text. Radioactivity and chemical phosphorus determinations were made in triplicate on each of the eluted phospholipids. ——, Specific radioactivity; ----, ratio of ^{32}P recovered in the two phospholipids.

Gurr, Prottey & Hawthorne (1965) also observed that the incorporation of ^{32}P into rat liver microsomes reached a maximum value between 3 and 6h after the administration of the radioisotope whereas incorporation into phosphatidylserine and phosphatidylinositol continued to increase up to 12–18h. Such differences in specific radioactivity, however, were not seen when [^{14}C]glycerol was used as a precursor in our experiments, in which case a rapid equilibrium was reached for all phospholipids. These effects reflect a complex heterogeneity of the type described in the synthesis and turnover of different phospholipid and protein constituents of the endoplasmic membranes (Omura, Siekevitz & Palade, 1967; Glaumann & Dallner, 1968; Arias, Doyle & Schimke, 1969).

In seminal vesicles, castration caused a marked fall in both the content of phospholipid and in the rate of its synthesis. Much of this decrease was due to the disappearance of a substantial amount of the smooth and rough endoplasmic reticulum, as already indicated by the ultrastructural studies of Szirmai & van der Linde (1964). The rate of labelling of phospholipids and RNA in unfractionated microsomes from seminal vesicles of castrated rats is shown in Fig. 4. Unlike that of liver microsomes (Fig. 2) the specific radioactivity of microsomal RNA phosphorus from seminal vesicles of castrated rats was higher than that in phospholipid at 24h after administration of [^{32}P]-orthophosphate. This difference is presumably because of a 5–10-fold decrease in the normal amount of cytoplasmic RNA recovered 8–10 days after castration.

Labelling of protein in vivo. Fig. 5 shows the

time-course of distribution of radioactive protein in the four microsomal subfractions of normal liver. At early times after injection of the radioactive amino acids much of the radioactivity was localized in the heavy rough-membrane fraction. This fraction is distinguished from other rough microsomal fractions by a high ratio of ribosomes per unit of membrane material to which they are attached (Tata, 1969). Since the specific radioactivity of labelled protein remained high for a substantial time after the administration of labelled amino acids this fraction represents the most active site of microsomal protein synthesis *in vivo*. This difference is accentuated if specific radioactivities are expressed per unit of rRNA since the RNA/protein ratio for heavy rough membranes is lower than that of free polyribosomes. With time, an increasing amount of the labelled protein appeared in the smooth endoplasmic membranes as radioactivity was lost from both the heavy and light rough membranes, which is in agreement with the current concepts of vectorial discharge of protein synthesis in secretory tissues (Palade, 1966; Redman, 1967). The free polyribosomes represented 10–15% of total rRNA in the cytoplasm and were less active than the membrane-attached ribosomes. This is not due to a loss of protein during fractionation, since virtually no radioactive protein appeared in the cell-sap fraction for 5–10 min after injection of the labelled amino acids, nor is the lower amino acid-incor-

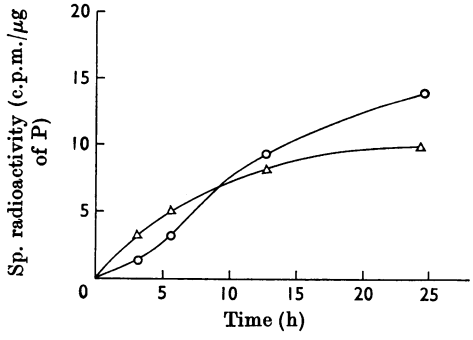

Fig. 4. Time-course of ^{32}P-labelling of total phospholipids and RNA of microsomes prepared from seminal vesicles of castrated rats. Groups of six rats (180–190 g) were castrated 11 days before the administration of 100 μCi of [^{32}P]orthophosphate/rat. Six pairs of seminal vesicles, weighing 30±4 mg/animal, were pooled and microsomes prepared as described in the text. RNA (○) and phospholipid (△) were extracted from the microsomes and each point gives the average value for triplicate determinations of specific radioactivity.

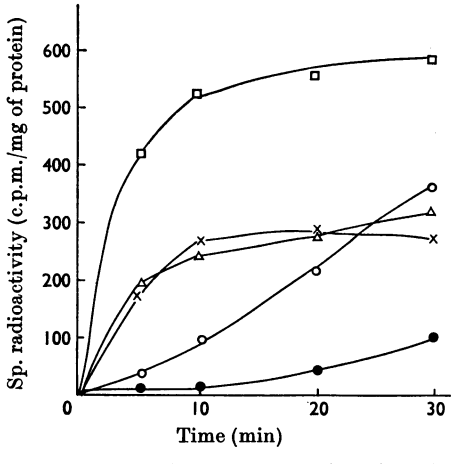

Fig. 5. Time-course of change in specific radioactivity of protein labelled *in vivo* and recovered in the different submicrosomal fractions and cell sap from normal rat liver. Protein was labelled by the intraperitoneal injection of 7.5 μCi of a mixture of ^{14}C-labelled amino acids/rat (125–130 g). Smooth membranes (○), heavy rough membranes (□), light rough membranes (△), free polyribosomes (×) and cell sap (●) were prepared from post-mitochondrial supernatant from three pooled livers. Each value is the average of three determinations of ^{14}C and protein/sample.

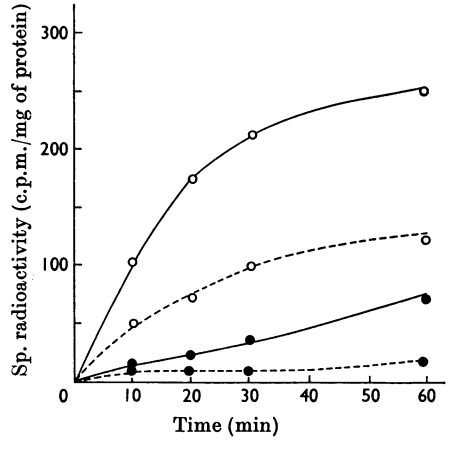

Fig. 6. Time-course of incorporation of ^{14}C-labelled amino acids into protein of microsomes (○) and cell sap (●) of seminal vesicles from control (——) and castrated (– – – –) rats. Rats were castrated or sham-operated (controls) 12 days before the experiment. Each rat was injected with 10.5 μCi of a mixture of ^{14}C-labelled amino acids and the microsomes and cell sap were prepared from seminal vesicles pooled from eight castrated rats (per point) or three sham-operated rats. The seminal vesicles weighed 11±4 mg/castrated rat and 61±9 mg/control rat. Each point is the mean of two separate specific-radioactivity determinations.

Table 1. *Effect of hormone deprivation and replacement on the recovery of protein, phospholipid and RNA in liver microsomal subfractions of young rats*

Rats of similar weight (125±15g) rather than similar age were used for these and other experiments. Rats were thyroidectomized at body wt. 60–70g and used 20–25 days later whereas hypophysectomies were carried out on rats weighing 90–100g, 9–13 days before the experiments. Treatment with tri-iodothyronine (T_3) consisted of two injections of 15 μg/100g body wt. at 37 and 82h before death and with human growth hormone (HGH) consisted of four injections of 75 μg/100g body wt. at 14, 26, 37 and 50h before death. The values for rough membrane fraction are derived from the sum of those obtained separately for 'light' and 'heavy' rough membranes and are about 15% lower than for total rough membranes.

Rats	Hormone given	Wt. of liver (g)	Microsomal fraction	Constituent recovered (mg/g of liver) Protein	Phospholipid	RNA
Normal	None	5.9±0.4	Total	20.6±2.3	6.6±1.1	4.5 ±0.2
			Smooth	7.9±1.5	3.0±0.6	0.15±0.06
			Rough	7.8±0.8	2.3±0.4	4.2 ±0.4
			Free polyribosomes	0.9±0.2	<0.02	0.5 ±0.1
Thyroidectomized	None	4.8±0.3	Total	17.5±1.5	6.4±1.3	3.4 ±0.2
			Smooth	7.6±0.8	3.2±0.3	0.10±0.05
			Rough	7.3±0.4	2.2±0.3	2.9 ±0.2
			Free polyribosomes	0.9±0.1	<0.02	0.6 ±0.2
Thyroidectomized	T_3	5.1±0.4	Total	18.8±2.0	7.0±1.4	4.2 ±0.3
			Smooth	7.8±1.2	3.4±1.6	0.21±0.06
			Rough	7.5±0.4	2.4±0.3	4.0 ±0.3
			Free polyribosomes	1.1±0.2	<0.03	0.7 ±0.1
Hypophysectomized	None	4.2±0.4	Total	14.2±1.3	5.4±1.0	2.9 ±0.3
			Smooth	6.3±1.4	2.1±0.3	0.10±0.002
			Rough	5.3±0.3	1.7±0.2	2.4 ±0.1
			Free polyribosomes	1.1±0.2	<0.01	0.4 ±0.1
Hypophysectomized	HGH	4.6±0.4	Total	1.6±0.9	6.4±0.5	3.8 ±0.4
			Smooth	6.2±1.0	2.0±0.2	0.08±0.01
			Rough	8.0±0.5	2.1±0.3	3.0 ±0.2
			Free polyribosomes	0.9±0.1	<0.02	0.3 ±0.04
Hypophysectomized	HGH+T_3	4.8±0.5	Total	21.2±2.8	6.2±0.5	4.4 ±0.3
			Smooth	8.3±0.6	2.8±0.4	0.12±0.02
			Rough	9.0±1.1	2.6±0.2	4.0 ±0.3
			Free polyribosomes	0.9±0.1	<0.02	0.5 ±0.05

porating activity of free polyribosomes due to a lower content of mRNA (Tata & Williams-Ashman, 1967). Other workers have, however, obtained a higher proportion of liver ribosomes as free polyribosomes and with a greater capacity to incorporate amino acids into protein (Bloemendal, Bont, De Vries & Benedetti, 1967; Blobel & Potter, 1967). The reason for this discrepancy may be because the rats used in these experiments were fed *ad libitum* whereas those used by the above workers were starved from 16–48h. I have consistently observed that starvation for even 16h causes a fall in the proportion of membrane-attached ribosomes as well as a disproportionately greater loss of amino acid-incorporation activity by this fraction.

Attempts to subfractionate microsomes from seminal vesicles were less successful than were those for liver. The results shown in Fig. 6 therefore represent the recovery of radioactive protein in total microsomes and cell sap. The rate of incorporation of amino acids into protein per unit of RNA or of protein was considerably lower in castrated than in normal rats. If the microsomal content is further taken into account (see Table 5) the rate of protein synthesis per organ is 10–20-fold lower in castrated than in normal rats.

Effect of hormone-induced growth of liver on the proliferation of endoplasmic reticulum. Table 1 summarizes the effect of experimental retardation and stimulation of growth on the amount of microsomal and submicrosomal constituents of liver. As expected, extirpation of the pituitary and the thyroid glands arrested the normal increase in liver weight of young rats. The rate of growth of the organ (in line with that of the whole animal) was found to be enhanced 2 days after repeated injections of human growth hormone or 3 days after injections of tri-iodothyronine. The effects of hormone deprivation and replacement were particularly pronounced on the amounts of protein, phospholipid and RNA recovered in all the microsomal subfractions. The hormone-induced accel-

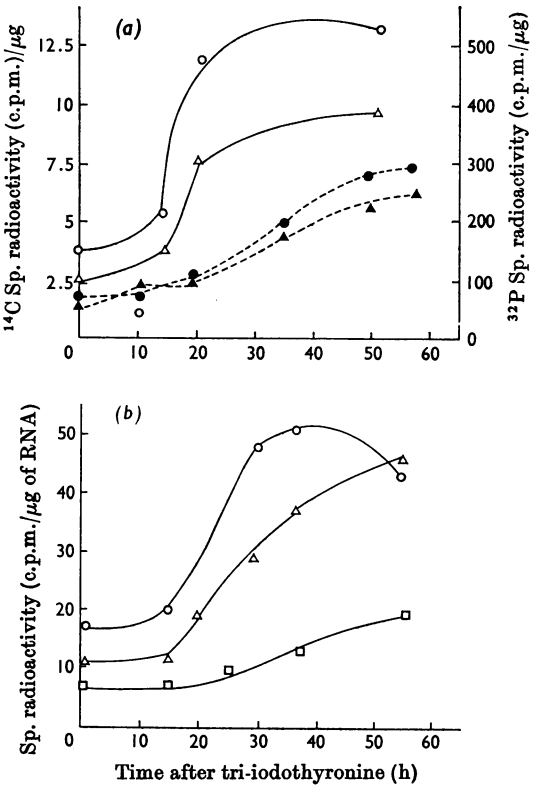

Fig. 7. Effect of tri-iodothyronine as a function of time after its injection on the formation of (*a*) phospholipid and (*b*) RNA in hepatic submicrosomal fractions of thyroidectomized rats. Thyroidectomy was performed 23 days before the experiment and a single injection of 16μg of tri-iodothyronine was given to groups of three animals weighing 120–135g each. The rats were killed at different times after the administration of hormone and labelled precursor; smooth membranes (○ and ●), total rough membranes (△ and ▲), and free polyribosomes (□) were isolated from the postmitochondrial supernatant. Each point is the average of two determinations. Corrections were applied for the effect of the hormones on uptake of radioactive precursors or labelling of acid-soluble components, as mentioned in the text. (*a*) Labelled phospholipid. One batch of animals was killed 1.5h after the injection of 8.5μCi of [^{14}C]choline chloride per rat (○, △). In another batch (●, ▲) the phospholipids were labelled by the administration of 70μCi of [^{32}P]orthophosphate 3h before death. (*b*) Labelling of RNA. [^{14}]Orotic acid (20μCi) was injected 6.8h before death. Time 0h represents thyroidectomized rats that did not receive the hormone.

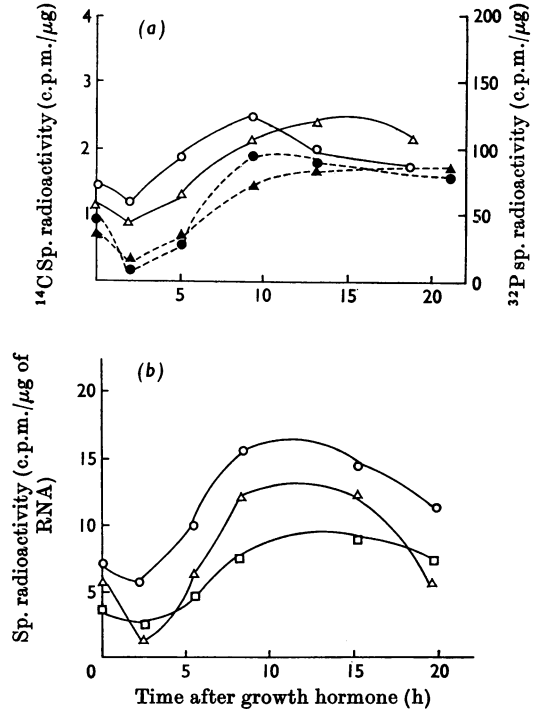

Fig. 8. Effect of human growth hormone as a function of time after its injection on the labelling of (*a*) phospholipid and (*b*) RNA in hepatic submicrosomal fractions of hypophysectomized rats. Rats were hypophysectomized 10 days before the experiment and weighed 105–115g at the time of death. A single injection of 50μg of human growth hormone was made at different times before death. Each point is the average of two determinations on material obtained from livers of three rats. (*a*) Phospholipids were labelled as in Fig. 7 with [^{14}C]choline and [^{32}P]orthophosphate, except that the animals were killed 30 and 45min respectively after administration of the two labelled compounds. (*b*) RNA was labelled with 35μCi of [^{14}C]orotic acid 1.8h before death. ○, ●, Smooth membranes; △, ▲, rough membranes; □, free polyribosomes. All other conditions were as given in Fig. 7. The early dip in specific radioactivity of cytoplasmic RNA and phospholipid has also been observed for nuclear RNA (Widnell & Tata, 1966).

eration of growth of the tissue was accompanied by a preferential proliferation of the smooth and rough endoplasmic reticulum, since there was a relatively much smaller change in the amounts of these constituents in the nuclei, mitochondria and cell sap (not shown in Table 1). The increased formation of microsomal constituents was most marked for the 'heavy rough membranes', which, as shown above, is the most active fraction in protein synthesis *in vivo* (see Fig. 5).

The manner in which microsomal protein, RNA and phospholipid accumulated on hormone-induced resumption of growth was next studied. Figs. 7 and 8 show the effects of tri-iodothyronine and growth hormone on the labelling of phospholipids

Table 2. *Relative rates of ^{32}P-labelling of individual phospholipids extracted from hepatic smooth and rough microsomal membranes of hypophysectomized rats with and without hormonal treatment*

Three groups of three hypophysectomized rats each were treated with 0.15M-NaCl (control), 15μg of tri-iodothyronine (T_3) or 50μg of human growth hormone (HGH) 22.2, 22.0 and 7.8h before death respectively. All animals received 88μCi of [^{32}P]orthophosphate intraperitoneally 3.0h before death and the extraction and analysis of phospholipids was carried out as described in the text. The values are averaged from six determinations in two separate experiments.

Microsomal fraction	Hormonal treatment	Specific radioactivity (c.p.m./μg of P)			Relative sp. radioactivity	
		Phosphatidylcholine	Phosphatidylethanolamine	Sphingomyelin	Phosphatidylcholine/sphingomyelin	Phosphatidylethanolamine/sphingomyelin
Smooth	None	855	670	129	7.1±1.2	5.5±0.7
	T_3	1396	985	395	3.4±0.8	2.6±0.6
	HGH	1272	1120	280	4.6±0.1	3.9±0.6
Rough	None	927	715	86	10.6±2.3	8.4±1.5
	T_3	1624	1008	330	5.3±2.0	3.0±1.1
	HGH	1317	639	181	7.1±1.7	3.3±0.8

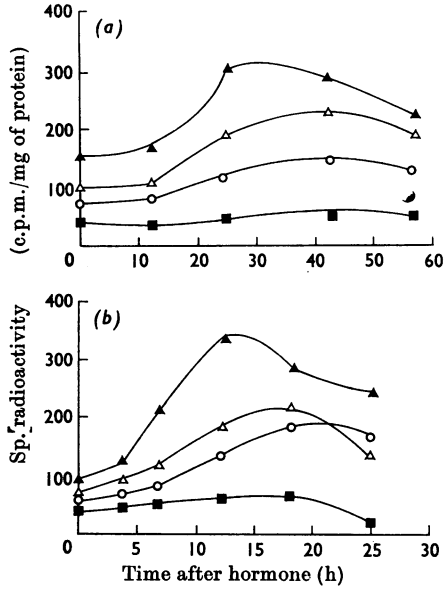

Fig. 9. Effect of injection of (*a*) tri-iodothyronine into thyroidectomized rats and (*b*) human growth hormone into hypophysectomized rats. The treatments were as described in Figs. 7 and 8. The postnuclear supernatants were fractionated into smooth membranes (○), heavy rough membranes (▲), light rough membranes (△) and free polyribosomes (■). The proteins were labelled by injection of 8.5μCi of a mixture of ^{14}C-labelled amino acid 10mins before death. Each point represents the average of three radioactivity measurements and duplicate protein determinations.

in microsomal subfractions with [^{32}P]orthophosphate and [^{14}C]choline and of RNA with [^{32}P]-orthophosphate in the same fractions. Although some of the increase in incorporation of label into phospholipids and RNA is likely to be due to enhanced turnover of these constituents, a significant part of the increased specific radioactivity must represent net synthesis, since increased amounts of these constituents accumulated after hormone administration. No significant qualitative changes were detected in the nature of the RNA or phospholipid after treatment. It was, however, noticeable that the relative rates of labelling with ^{32}P of the different phospholipids of both the rough and smooth microsomal membranes were altered (Table 2). As already indicated in Fig. 3 for normal rats, the major microsomal membrane phospholipids, phosphatidylcholine and phosphatidylethanolamine, are more extensively labelled with ^{32}P at 3h than the minor constituents, sphingomyelin, 1-acylglycerophosphorylcholine and phosphatidylinositol (the last two are not included in Table 2). The effect of administration of tri-iodothyronine and growth hormone, studied at a time when the stimulatory effect of the two hormones on overall phospholipid labelling is maximal (see Figs. 7 and 8) was to enhance preferentially the labelling of sphingomyelin relative to that of phosphatidylcholine and phosphatidylethanolamine. A 2–4-fold decrease in the ratio of ^{32}P incorporated into major and minor phospholipids would have occurred also in hormonally untreated animals but only after another 10–12h. Thus the effect of the hormone is to accelerate the normal process of a differential rate of synthesis and turnover of both smooth and rough microsomal membrane phospholipids.

The above hormone-induced changes in microsomal phospholipid synthesis coincided with the rather abrupt increase in the incorporation of amino acids into protein *in vivo*. Fig. 9 shows that

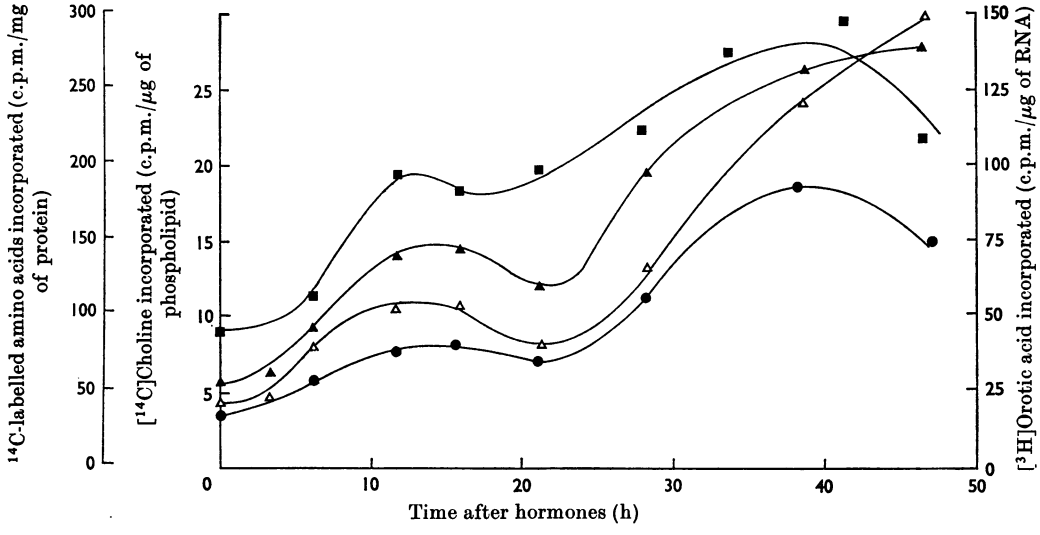

Fig. 10. Additive effects of tri-iodothyronine and growth hormone on the co-ordinated responses of labelling of hepatic microsomal phospholipids (△ and ▲), RNA (○) and protein (■) in hypophysectomized rats. The specific radioactivity of phospholipids was determined in both smooth (△) and rough (▲) microsomes after the administration of 10 μCi of [^{14}C]choline chloride 1 h before death. The specific radioactivities of RNA and protein are only shown for rough membranes, since this fraction contained the bulk of the radioactivity. RNA was labelled with 76 μCi of [^{3}H]orotic acid given 4.6 h before death and protein was labelled by injecting 8.8 μCi of a mixture of ^{14}C-labelled amino acids 10 min before death. Groups of three hypophysectomized rats (110–120 g) were killed at different times after the combined administration of 18 μg of tri-iodothyronine and 70 μg of human growth hormone. All other conditions were the same as in Figs. 7 and 8.

for both hormones recovery of 'rapidly labelled' or nascent protein in all four microsomal subfractions was enhanced coincidently with the accumulation of additional microsomal phospholipids and RNA, i.e. 15–20 h for tri-iodothyronine and 4–7 h for growth hormone. The most prominent effect of the hormone on protein synthesis *in vivo* was on the 'heavy rough membrane' fraction, whereas it was barely obvious with free polyribosomes. The effect on smooth membrane suggests that hormonal stimulus may also have accelerated the transfer of newly synthesized protein from the rough to smooth endoplasmic reticulum, assuming that a large part of hepatic nascent protein is destined for secretion.

Studies in which the additive effects of the two hormones were examined showed that the co-ordination between the enhancement of synthesis of microsomal constituents and protein-synthetic activity was related in magnitude and timing to the nature of the hormonal treatment. Fig. 10 summarizes the results of one type of experiment performed. The simultaneous administration of tri-iodothyronine and growth hormone produced two bursts of formation of microsomal RNA and phospholipids in the livers of hypophysectomized rats. (Only hypophysectomized rats were used in these experiments to standardize the conditions of the experiments. At 8–10 days after the operation hypophysectomized rats could also be considered 'functionally thyroidectomized' because of the loss of thyrotrophic hormone.) The timing of each burst corresponded to the different lag periods for the two hormones, i.e. 6–10 h for growth hormone (see Fig. 9) and 25–30 h for tri-iodothyronine (see Figs. 7 and 8). The pattern of incorporation of amino acids into protein *in vivo*, as judged from that recovered in the heavy rough microsomal fraction, followed that of the accumulation of newly formed microsomal phospholipid and RNA.

A variation of the experiment on additive hormone effects was also performed in which instead of simultaneous administration of the hormones the rats were killed at a time corresponding to the peak effects of each of the two hormones. The doses of growth hormone and tri-iodothyronine in both types of experiments were also such that they provoked a maximal response of the target tissue. Table 3 shows that under these conditions the effects of the hormones on proliferation of microsomal components and accelerated protein biosynthesis were superimposed. This additiveness may reflect a fairly tight coupling between a demand for additional protein synthesis and the formation or stabilization of the endoplasmic reticulum.

Table 3. *Additive effects of administration of tri-iodothyronine and growth hormone on the synthesis of phospholipid, RNA and protein associated with rough microsomal membranes of hypophysectomized rats*

Groups of three or four hypophysectomized rats (100–120 g) were killed at the same time but at different intervals after the administration of 22 μg of tri-iodothyronine (T_3) or 75 μg of human growth hormone (HGH) separately or in combination. Labelling and other conditions were as given in Fig. 10.

		Specific radioactivity			Amount (mg/g of liver)		
	Hormonal treatment	Phospholipid (c.p.m./ μg of P)	RNA (c.p.m./ μg)	Protein (c.p.m./ mg)	Phospholipid	RNA	Protein
Expt. 1	None	145	26	106	0.8±0.1	1.6±0.2	3.7±0.2
	5.2 h after HGH	167	34	151	0.7±0.1	1.6±0.2	3.9±0.1
	26.9 h after T_3	190	54	193	1.0±0.2	1.8±0.1	4.3±0.2
	HGH+T_3	206	64	228	1.1±0.1	2.2±0.3	4.6±0.3
Expt. 2	None	189	37	87	0.6±0.1	1.7±0.1	3.3±0.2
	9.5 h after HGH	415	60	159	0.8±0.2	2.0±0.2	3.6±0.4
	42.0 h after T_3	364	82	204	0.8±0.2	2.1±0.1	4.1±0.2
	HGH+T_3	529	99	316	1.0±0.1	2.5±0.3	5.1±0.5

Table 4. *Co-ordinated increase in formation of rough microsomal phospholipid and RNA after partial hepatectomy*

Each group consisted of four sham-operated or partially hepatectomized rats. They were given 52 μCi of [^{3}H]orotic acid or 65 μCi of [^{32}P]orthophosphate 3.2 and 2.0 h before death. The uptake of the label and acid-soluble fraction of radioactivity in the whole liver homogenate were measured to allow for the correction of specific radioactivities for increases in uptake of label after hepatectomy.

			Specific radioactivity		Amount recovered (mg/g of liver)	
Time after operation (h)	Operation	Wt. of liver (g)	^{3}H-labelled RNA (c.p.m./mg)	^{32}P-labelled phospholipid (c.p.m./ μg of P)	RNA	Phospholipid
4	Sham	6.8	12100	270	4.4	2.3
4	Hepatectomy	1.5	18480	339	4.1	2.1
9	Sham	6.9	11633	465	4.1	2.2
9	Hepatectomy	1.6	21070	808	4.3	2.4
18	Sham	6.7	10450	366	4.2	2.4
18	Hepatectomy	1.6	24600	543	4.9	2.8
24	Sham	7.0	9335	297	4.3	2.3
24	Hepatectomy	1.8	15870	413	4.9	2.8
45*	Sham	7.2	12640	275	4.2	2.1
45*	Hepatectomy	2.3	17300	392	4.8	2.6

* The animals were killed 6.5 and 5.5 h after [^{3}H]orotic acid and [^{32}P]orthophosphate administration respectively.

Co-ordinated synthesis of microsomal constituents in hypertrophy after partial hepatectomy. The early hypertrophic phase after hepatectomy is known to involve a massive synthesis of RNA and protein (Tsukada *et al.* 1968). Table 4 shows how accelerated synthesis of RNA and proteins in rough microsomes is correlated with the rate of microsomal phospholipid synthesis. Table 5 shows that proliferation of the rough endoplasmic reticulum was paralleled by an accelerated rate of synthesis of proteins in the same fraction. With longer time-intervals after ^{32}P administration, the differences between the specific radioactivities of phospholipid were less marked, as Gurr *et al.* (1965) observed for a 6 h labelling period and 30 h after hepatectomy. Although the results in Tables 4 and 5 refer only to rough endoplasmic reticulum, similar increases in rates of RNA and phospholipid synthesis were recorded for free polyribosomes and smooth membranes. An important feature in studying the

Table 5. *Increase in amino acid incorporation into rough microsomal protein after partial hepatectomy*

The experiment was performed in the same way as that in Table 4, except that 8min before death hepatectomized rats received 4μCi of a mixture of ^{14}C-labelled amino acids and sham-operated animals were given 10μCi. Different amounts of radioactivity were administered to the two groups of rats to allow for the different amounts of tissue and corrections were also applied for uptake.

Time after operation (h)	Operation	Wt. of liver (g)	Specific radioactivity (c.p.m./mg of protein)	Protein (mg/g of liver)
2	Sham	6.4	435	9.2
	Hepatectomy	1.6	398	8.8
4	Sham	6.6	490	8.9
	Hepatectomy	1.9	410	9.3
8	Sham	6.3	385	8.7
	Hepatectomy	1.7	588	9.1
16	Sham	6.3	361	8.8
	Hepatectomy	1.9	639	9.3
24	Sham	6.6	343	8.9
	Hepatectomy	2.0	605	9.6

hypertrophy induced by regenerating liver is that it is essential to apply corrections for the vastly greater uptake of label and consequent increase in precursor specific radioactivity that is found in hepatectomized rats (Bucher & Swaffield, 1969). Without these corrections the increases in specific radioactivity at early time-intervals after partial hepatectomy were highly exaggerated. Nevertheless the net increase in phospholipid and RNA (much of the latter being rRNA) with higher specific radioactivity suggested that the growth stimulus of hepatectomy simultaneously promoted the rate of formation of endoplasmic membranes and ribosomes and, possibly, slowed down their turnover.

Co-ordinated synthesis of microsomal RNA, protein and phospholipid in the seminal vesicles. In an earlier study (Tata, 1967c) a co-ordinated onset of increased accumulation of newly formed RNA and phospholipid was shown in the whole seminal vesicles of castrated rats after testosterone administration. It was also shown that the effect of the hormone was commensurate with the extent of hypertrophy produced in seminal vesicles and the liver. These studies were extended to subcellular fractions of the seminal vesicles and Fig. 11 illustrates the results obtained with whole microsomes. (These studies were restricted to whole microsomes from seminal vesicles as their subfractionation into smooth and rough microsomes was not satisfactory.) As with the hepatic systems described above, the onset of enhancement of incorporation of amino acids into 'nascent' protein recovered on microsomes after hormonal stimulation coincided with an increase in the accumulation of newly formed

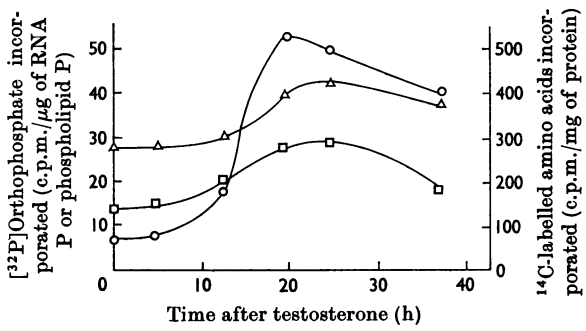

Fig. 11. Co-ordinated acceleration of formation of microsomal phospholipid (△), RNA (○) and protein (□) after a single administration of 250μg of testosterone propionate to castrated rats. Phospholipid and RNA were both labelled with 95μg of [^{32}P]orthophosphate administered 1.5 and 3.5h before death whereas newly synthesized protein was labelled with 15μCi of ^{14}C-labelled amino acids given 15min before death. Corrections were made for uptake of label in each determination. Six castrated rats (160–180g) were used for each time-interval. Seminal vesicles weighed 14±4mg in control rats (time 0) and 32±6mg at 35.5h after hormone injection.

microsomal RNA and phospholipids. The downward trend in the curves shown in Fig. 11 is not due to a biphasic biosynthetic pattern, but merely reflects a rapid accumulation of newly formed materials at the time the labelled precursor was administered. Table 6 shows that the rate of microsomal proliferation and growth of the seminal vesicles continued to rise at even 40h after a single injection of testosterone.

Table 6. *Effect of a single injection of testosterone propionate on the proliferation of microsomal constituents and growth of seminal vesicles in castrated rats*

All conditions of treatment were the same as those in Fig. 11. Each value is the mean of three determinations performed in duplicate on seminal vesicles pooled from three rats.

Time after testosterone (h)	Wt. of seminal vesicles (mg)	Microsomes recovered (mg/g of gland)		
		RNA	Phospholipid	Protein
0	12.0	0.42	0.65	3.0
6.2	13.5	0.45	0.68	2.8
12.5	16.8	0.54	0.81	3.1
18.0	20.5	0.73	0.95	3.4
20.5	25.3	0.89	1.18	4.1
39.5	31.6	1.03	1.23	5.3

DISCUSSION

Phospholipids of endoplasmic reticulum of rat liver and seminal vesicles are rapidly labelled with different radioactive precursors. The slight differences in the kinetics of labelling of the hepatic submicrosomal fractions (Fig. 2) made it difficult to draw a precursor–product relationship between the rough and smooth microsomal fractions as was done by Dallner *et al.* (1966). However, it was possible to show from the kinetics of labelling of individual phospholipids that there exists a heterogeneous, and possibly very complex, pattern of synthesis and turnover of different moieties of membrane lipids. In general, incorporation of ^{32}P revealed that the major microsomal phospholipids, phosphatidylethanolamine and phosphatidylcholine, were rapidly labelled, but further incorporation virtually ceased after 3h. The minor components, sphingomyelin and 1-acylglycerophosphorylcholine, were labelled relatively slowly, but the specific radioactivity continued to rise until 24h after the administration of the [^{32}P]orthophosphate. There was a small but reproducible difference in the ratio of ^{32}P incorporated into sphingomyelin to that into phosphatidylcholine of smooth membranes and rough membranes (see Fig. 3 and Table 2). No differential labelling was, however, detected with the glycerol moiety. Workers from other laboratories have also described such heterogeneity in the rate of accumulation or turnover of lipids of the endoplasmic reticulum (Ulsamer & Glenn, 1966; Glaumann & Dallner, 1968; Dallner & Ernster, 1968).

It was not intended to study the protein components of the endoplasmic-reticulum membrane. However, work in other laboratories on the different 'constitutive' membrane proteins such as glucose 6-phosphatase, NADPH–cytochrome *c* reductase, cytochrome b_5 and drug hydroxylases has shown that different membrane proteins turn over with different half-lives and may have a heterogeneous distribution (Omura *et al.* 1967; Orrenius & Ericsson, 1966; Dallner, Bergstrand & Nilsson, 1968; Arias *et al.* 1969). This has led to the concept of a mosaic model of endoplasmic-reticulum cell membranes in general in which the membrane is a dynamic entity with different functions located within small compartments (see Siekevitz *et al.* 1967). In these labelling studies it has been assumed that since both liver and the seminal vesicles are predominantly protein-secreting tissues the major part of the rapidly labelled protein would be destined for secretion. However, some of the incorporation must reflect synthesis of membrane proteins, and in future studies it may be possible to distinguish between these two classes of proteins in a rapidly growing tissue.

The most salient feature of our present studies on accelerated growth of the liver and seminal vesicles is the existence of some mechanism in the cell that allows for the co-ordinated enhancement of the assembly of ribosomes and membranes of the endoplasmic reticulum in response to a demand for more or different proteins. Such a co-ordination is manifested as a simultaneous onset of an enhanced rate of protein synthesis with the accelerated accumulation of newly formed ribosomes and membranes of the endoplasmic reticulum. Although only the phospholipid moiety was studied here, our attention had already been drawn to proliferation of membrane proteins by the absolute increase in three constitutive proteins, glucose 6-phosphatase, NADPH–cytochrome *c* reductase and cytochrome b_5, during thyroid hormone-induced growth of liver under very similar conditions (Tata *et al.* 1963). It was shown in a previous study (Tata, 1967*c*) that the pattern of hormone-induced changes in labelling of phospholipids is very similar in all subcellular particulate fractions. However, it is in the rough endoplasmic reticulum that a relatively rapid accumulation of membrane phospholipids occurs (Table 1). These two observations may reflect (*a*) that phospholipids destined

for all cell membranes are made at the same site and (*b*) that the mechanisms for both accelerated formation and retarded degradation may operate for membranes for which the demand is most acute. The question of membrane stability is an important one and ought to be studied along the lines laid down by the groups of Palade, Dallner and Schimke in studying drug-induced proliferation of the endoplasmic reticulum (Omura *et al.* 1967; Orrenius & Ericsson, 1966; Dallner *et al.* 1968; Arias *et al.* 1969). How the phospholipid and protein components of membranes are assembled in an orderly fashion to yield precise structures is not known. It may be that the initial response to a growth stimulus may be to accelerate the assembly or 'crystallization' of rigid structures from existing components, which in turn would trigger off the synthesis of additional membrane phospholipids and proteins.

The preferential accumulation of rough endoplasmic reticulum that, as this work has shown, is also the ribosomal fraction most active in amino acid incorporation *in vivo* underlines the importance in protein synthesis of the attachment of ribosomes to membranes (see also Henshaw *et al.* 1963; Campbell, 1965; Campbell, Lowe & Serck-Hanssen, 1967). A preferential effect of hormonal stimulation on membrane-bound relative to free ribosomes was also observed when amino acid incorporation was measured with isolated preparations, but the differential effect was much less noticeable than in those studies in which the labelling of proteins was carried out *in vivo* (Tata & Williams-Ashman, 1967). This observation suggests that disintegration of the cell structure may eliminate some factor that maintains a high degree of functional distinction between membrane-bound and free ribosomes. It has been estimated that efficiency of protein synthesis by cell-free preparations may be less than 1% of that *in vivo* (see Hendler, 1968). Until now the major emphasis on ribosome–membrane attachment in animal cells has been focused on the export of proteins in predominantly secretory cells (Palade, 1966; Siekevitz *et al.* 1967). However, studies in this laboratory on non-secretory tissues, such as the brain and muscle, have shown that the membrane-attached ribosomes play an important role in synthesizing proteins for intracellular destinations (Andrews & Tata, 1968; T. M. Andrews & J. R. Tata, unpublished work). One has to consider the possibility that some factor determining the efficiency of protein synthesis may be dependent on the interaction between the ribosome and the membrane (see Hendler, 1968; Mainwaring, 1969).

In this paper it has been established that in hepatic regeneration and in three systems of hormone-induced growth there is co-ordination between the formation of rough endoplasmic reticulum and increase in protein synthesis. A similar co-ordination was observed in thyrotrophin-induced synthesis of thyroglobulin both *in vitro* and *in vivo* (Kerkof & Tata, 1967, 1969) and in induced amphibian metamorphosis (Tata, 1967*b*). Perhaps a common fundamental mechanism may underlie the numerous observations of a marked acceleration of phospholipid synthesis anticipating or accompanying the initial burst of protein synthesis in a variety of situations of hormone-dependent or -independent growth and development as well as in immune response (Dallner *et al.* 1966; Pollak & Ward, 1967; Aizawa & Mueller, 1961; Fisher & Mueller, 1968; Ursprung & Schabtach, 1968; Nicholls, Follett & Evennett, 1968; Doeg, 1968; Freinkel, 1964; Tata, 1968*c*). What could be the meaning of an enhanced coupled formation of membranes and ribosomes during growth and development? It has already been suggested (Tata, 1968*a*,*b*) that such a coupling may reflect a topographical segregation of different populations of ribosomes, presumably differently pre-coded according to the developmental or environmental stimulus. The advantage of such a segregation in cells that have to adapt rapidly to such external stimuli as those regulating growth, development, detoxication etc. would be to synthesize preferentially proteins involved for the adaptation response with the minimum of perturbation of synthesis of those proteins that are not involved in the response to the stimulus. The additive effect of different stimuli on the proliferation of membrane-bound ribosomes (Fig. 10 and Table 3; see also Tata, 1968*a*,*b*) can be interpreted as a reflexion of such a process in which different types of proteins are preferentially made in response to different agents. Of course, such results do not constitute direct evidence for this idea. It can, however, be tested by determining whether or not new enzymes formed during development are initially localized in the region of newly formed rough endoplasmic reticulum, much in the same way as has been feasible for the ultrastructural localization of antibody in differentiating plasma cells (Leduc, Avrameas & Bouteille, 1968).

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