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Subcellular Structure of Bovine Thyroid Gland

A STUDY ON BOVINE THYROID MEMBRANES BY BUOYANT-DENSITY-GRADIENT CENTRIFUGATION IN A B-XIV ZONAL ROTOR

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1. A combined mitochondrial and light mitochondrial fraction and a microsomal fraction were isolated from bovine thyroid gland and fractionated further in a B-XIV zonal rotor. A density gradient ranging from 20 to 50% (w/w) sucrose was used. The rotor was operated for 3h at 45000rev./min. All manipulations were performed at 4°C and at pH7.4. 2. Membranous material was recovered in two zones: zone I, containing microsomal material derived from both smooth endoplasmic reticulum and plasma membranes and probably also from other smooth membranes; zone II, containing material from rough endoplasmic reticulum. 3. Increasing the pH of the medium up to 8.6, or the addition of Mg^{2+} to the medium resulted in the formation of a single zone at intermediate densities (aggregation of membranes?). An analogous effect was obtained after treatment with $Pb(NO_3)_2$. 4. In the presence of heparin (50i.u./ml) the bulk of the membranes was found in zone I. This was due to the release of ribosomes from the rough endoplasmic reticulum.

After differential pelleting of thyroid tissue the bulk of plasma-membrane markers and endoplasmic-reticulum-membrane markers are recovered in the M+L fraction* and only a small portion is found in the P fraction. The present work was performed to characterize the M+L and P fractions and their subfractions in terms of chemical and enzymic composition.

For separating subcellular particles differential- and density-gradient-centrifugation techniques are widely used. The separations are often incomplete, since different particles may show considerable overlap in their centrifugal properties, and are hampered by the small capacities of commercially available high-speed centrifuge rotors and by difficulties in maintaining gradient stability during acceleration and deceleration. The development of the zonal rotor system has largely overcome these problems. In this paper we report our results on the buoyant-density-gradient centrifugation of bovine thyroid M+L and P fractions in a B-XIV zonal rotor.

* Abbreviations: M+L fraction, combined M and L fractions; N fraction, nuclear fraction; P fraction, microsomal fraction; S fraction, supernatant; L fraction, light mitochondrial fraction; M fraction, mitochondrial fraction; ATPase, Na^++K^+-stimulated ouabain-inhibited adenosine triphosphatase.

Materials and Methods

Biological materials and tissue preparation

Adult bovine thyroid glands were used throughout the study. Fresh thyroid glands were obtained from the local slaughterhouse and brought to the laboratory on ice as soon as possible. Further manipulations were performed in the cold-room (+4°C). The glands (10–40g) were trimmed free of surrounding tissue. Thyroid tissue (100g) was then cut into small blocks with scissors (for differential pelleting) or minced in a meat mill (for gradient studies). Subsequently the tissue preparations were washed with iso-osmotic sucrose solution (0.25M-sucrose–5mM-Tris–HCl buffer, pH7.4).

Preparation of subcellular particles

To obtain the distribution patterns for a series of markers, small blocks of thyroid tissue were homogenized in an all-glass home-made hand homogenizer and filtered through a double layer of cheesecloth. The filtrate was further fractionated (differential pelleting: N, M, L, P and S fractions) as described (Dierick & Hilderson, 1967).

For membrane studies (isopycnic-gradient centrifugations), thyroid tissue was prepared in a two-step procedure: (1) 100g of minced tissue was treated in a

Virtis (Virtis Co. Inc., Research equipment, Gardiner, N.Y., U.S.A.) homogenizer (300ml of iso-osmotic sucrose medium, pH7.4, at 11000rev./min for 2×1min) and (2) the resulting suspension was then homogenized in a Potter–Elvehjem homogenizer (Teflon pestle; 3000rev./min; five strokes). This homogenate was centrifuged at 1000*g* for 3min to remove blood cells, connective tissue and cell debris. The supernatant was then centrifuged (77300*g*, 15min) yielding a M+L fraction. Subsequently the P fraction was isolated from the resulting supernatant by centrifugation (104000*g*, 60min).

Zonal centrifugation: standard procedure

Fractionation of the subcellular preparation was carried out in the B-XIV zonal rotor in a MSE Superspeed 65 centrifuge. The rotor was loaded at 2500rev./min by means of a variable-speed MSE gradient former [20–50% (w/w) sucrose in 5mM-Tris–HCl buffer, pH7.4] through the edge of the rotor. When the rotor was completely filled with the gradient, the sample (12ml) was introduced via the feed line to the centre, by using a hypodermic syringe as a small hand pump. The sample layer was then displaced with 100ml of an overlay solution [5% (w/w) sucrose] and finally centrifuged for 3h at 45000rev./min. At the end of the centrifugation period, the zonal rotor was unloaded at 2500rev./min. Fractions (20ml) were collected by displacement with 55% (w/w) sucrose solution. As a routine 36 fractions were collected.

Chemical analyses

Extraction and fractionation of phospholipids. Phospholipids were extracted, fractionated by two-dimensional t.l.c. and determined in the presence of silica gel as previously described (Hilderson *et al.*, 1974; Lagrou *et al.*, 1974*a*).

Cholesterol and phospholipids after zonal centrifugation. After centrifugation in the zonal rotor 5ml of each fraction was extracted as described by Bligh & Dyer (1959). Cholesterol and phospholipids were determined in the resulting underphase as described by Rouser *et al.* (1970).

Sialic acid. This was assayed as described by Lagrou *et al.* (1974*b*).

Protein. Portions (0.5ml) of the fractions were measured by an adaptation of the method of Lowry *et al.* (1951) with bovine serum albumin as a standard (Hilderson *et al.*, 1974).

Cytochrome b_5. This was determined as described by Omura & Sato (1964).

RNA. This was assayed in the fractions obtained after isopycnic-gradient centrifugation by both u.v. spectrometry and phosphorus determination. Where RNA is present, in the absence of DNA, the u.v. spectrum is maximal at 260nm. Concomitantly there is an increase of the phosphorus content.

Enzyme assays

Cytochrome *c* oxidase (EC 1.9.3.1) was determined as described by Cooperstein & Lazarow (1951). Acid and alkaline phenylphosphatase (EC 3.1.3.2 and EC 3.1.3.1) were determined by the method of Kind & King (1954) at pH4.6 and 10. β-Glucosidase (EC 3.2.1.21) was assayed as described by Patel & Tappel (1969*a*) with *p*-nitrophenyl-β-D-glucopyranoside as a substrate (pH 4 and 9). NADPH–cytochrome *c* reductase (EC 1.6.2.4) was measured as described by Masters *et al.* (1967) and Nordlie & Arion (1966). 5′-Nucleotidase (EC 3.1.3.5) and glucose 6-phosphatase (EC 3.1.3.9) were assayed as described by Morré (1974). ATPase (EC 3.6.1.3) was measured as described by Bonting *et al.* (1961).

Results

Differential pelleting

The distributions of different marker enzymes and components were investigated after isolating the N, M, L, P and S fractions (Table 1). Hilderson *et al.* (1971) found that thyroid mitochondria display a lower sedimentation coefficient than rat liver mitochondria and therefore higher centrifugal forces are necessary to sediment thyroid mitochondria (10min at 37000*g*).

In these circumstances, 5′-nucleotidase, ATPase and alkaline phosphatase, lipid-bound sialic acid and cholesterol all showed the highest relative specific activity in the L fraction. The bulk of cytochrome *c* reductase and of glucose 6-phosphatase are recovered in the M fraction. In rat liver both of these enzymes are endoplasmic reticulum markers. The distribution of β-glucosidase was different from the other markers. About 90% of the enzyme activity is recovered from the supernatant. In the M+L fraction (9% recovery) there is a latent enzyme activity ranging from 25 to 30% and a lag period of about 10–20min (Dierick & Hilderson, 1967).

Centrifugation of a M+L fraction in the B-XIV zonal rotor

A M+L fraction was subjected to zonal centrifugation (standard procedure). The distribution profiles of 5′-nucleotidase, cytochrome *c* reductase and glucose 6-phosphatase are shown in Fig. 1(*a*). Fig. 1(*b*) shows the distribution profiles of proteins, cholesterol and phospholipids as well as the slope of the gradient. Fig. 1(*c*) represents the profiles for acid phosphatase, β-glucosidase (pH4.0) and cytochrome oxidase. Peaks located at the far left end (top of the

Table 1. *Relative amounts (%) and relative specific activity of enzymes and constituents in the subcellular fractions obtained by differential pelleting*

All values are ±S.E.M. for the numbers of experiments given.

Enzyme or constituent	Fraction N		Fraction M		Fraction L		Fraction P		Fraction S		Yield (%)	No. of experiments
	% of total	Relative specific activity	% of total	Relative specific activity	% of total	Relative specific activity	% of total	Relative specific activity	% of total	Relative specific activity		
Proteins	3.5±0.6		7.1±0.6		2.4±0.2		2.6±0.4		84.6±1.0		101±4	15
Cholesterol	3.3±1.6	0.9±0.5	37.1±2.8	5.2±1.0	16.8±1.0	7.0±1.0	7.8±2.3	3.0±1.3	32.3±3.7	0.4±0.1	86±5	4
Phospholipids	3.5±1.4	1.0±0.5	43.1±3.5	6.1±1.1	13.6±1.4	5.7±1.4	9.2±1.3	3.5±1.1	30.9±2.7	0.4±0.1	109±5	4
Lipid-bound sialic acid	4.8±1.1	1.2±0.5	18.3±2.5	2.7±0.5	10.8±1.1	5.3±0.8	6.0±0.9	2.5±0.7	60.2±1.5	0.7±0.1	111±5	6
Acid phenylphosphatase	9.6±2.2	2.7±0.8	43.7±3.3	6.2±0.7	7.6±1.3	3.2±0.6	4.3±0.7	1.7±0.4	30.9±4.5	0.4±0.1	94±2	10
Alkaline phenylphosphatase	3.9±2.2	1.1±0.8	28.8±2.2	4.1±0.9	14.8±1.2	6.2±1.0	7.4±0.9	2.8±1.0	41.0±2.3	0.5±0.1	90±3	3
Cytochrome *c* oxidase	9.7±2.0	2.8±0.8	78.2±4.5	11.0±1.4	6.4±1.7	2.7±0.8	3.0±1.3	1.2±0.6	3.4±1.0	0.1±0.1	42±5	9
Cytochrome reductase	17.9±6.9	4.5±2.4	48.4±10.2	5.5±1.6	13.5±1.8	5.6±1.1	10.0±3.1	4.2±1.9	10.1±8.0	0.12±0.09	86±8	3
5′-Nucleotidase	14.9±2.7	1.9±1.0	22.4±8.8	3.6±1.8	18.0±13.2	9.0±7.4	4.0±0.4	2.3±1.0	42.1±17.6	0.5±0.2	68±6	2
Glucose 6-phosphatase	20.2±2.8	2.5±1.3	42.1±10.1	6.9±2.8	11.9±1.5	5.9±2.2	3.7±0.8	2.2±1.0	22.1±8.1	0.3±0.1	220±10	2
β-Glucosidase	0.5±0.4	0.14±0.12	5.9±1.3	0.8±0.2	2.3±0.2	1.0±0.2	2.1±0.2	0.8±0.3	89.4±1.8	1.1±0.1	116±20	3
ATPase	1.7±0.9	1.0±0.6	20.5±11.3	5.2±3.1	20.2±2.0	10.0±1.7	5.5±1.6	2.2±1.0	52.0±9.3	0.8±0.2	101±9	3

Fig. 1. *Zonal centrifugation of a M+L fraction: distribution profiles of some markers; zones I and II*

Experimental conditions: the standard procedure is described in the Materials and Methods section. (*a*) ○, 5′-Nucleotidase; ----, cytochrome *c* reductase; ——, glucose 6-phosphatase. (*b*) ○, Proteins; ----, cholesterol; ——, phospholipids; ●, slope of gradient. (*c*) ○, Acid phosphatase; ----, β-glucosidase (pH 4.0); ——, cytochrome oxidase.

gradient) of all profiles are always due to soluble material that does not penetrate into the gradient. Two distinct zones (I and II) can be observed in those profiles. Glucose 6-phosphatase and cytochrome *c* reductase are located in both zones. Glucose 6-phosphatase is enriched 15-fold in zone I over the original homogenate and ninefold in zone II. 5′-Nucleotidase coincides with ATPase and alkaline phosphatase activities (both not plotted): they are mainly recovered in zone I (specific activities 12 times higher than in the original homogenate). The phospholipid curve reflects the distribution of membranous material; it follows mainly that of glucose 6-phosphatase. The relative amount of sphingomyelin with respect to total phospholipids is 17±2.5% (S.E.M., three experiments) in zone I and 8.5±0.3% (three experiments) in zone II. The curve for cholesterol follows the distribution of 5′-nucleotidase in zone I, but tends to follow glucose 6-phosphatase distribution in zone II. The molar ratio of cholesterol/phospholipids decreases from left to right (0.37–0.19) of the gradient. Acid phosphatase is found over

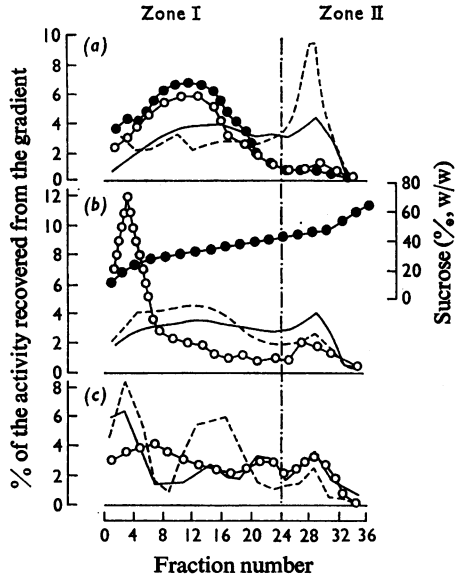

Fig. 2. *Zonal centrifugation of a P fraction: distribution profiles of some markers; zones I and II*

Experimental conditions: the standard procedure is described in the Materials and Methods section. (*a*) ○, 5′-Nucleotidase; ●, alkaline phosphatase; ----, cytochrome *c* reductase; ——, glucose 6-phosphatase. (*b*) ○, Proteins; ----, cholesterol; ——, phospholipids; ●, slope of gradient. (*c*) ○, Acid phosphatase; ----, β-glucosidase (pH4.0); ——, cytochrome oxidase.

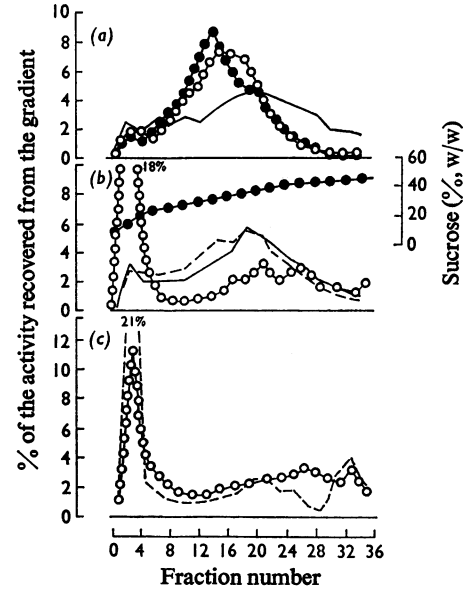

Fig. 3. *Zonal centrifugation of a M+L fraction, preincubated with digitonin: distribution profiles of some markers*

A sample (20ml) was preincubated for 10min at 0°C with 5mg of digitonin before loading the zonal rotor. Subsequent centrifugation was performed as described in the Materials and Methods section (standard procedure). (*a*) ○, 5′-Nucleotidase; ●, alkaline phosphatase; ——, glucose 6-phosphatase. (*b*) ○, Protein; ----, cholesterol; ——, phospholipids; ●, slope of gradient. (*c*) ○, Acid phosphatase; ----, β-glucosidase (pH4.0).

the whole gradient. The major part of cytochrome oxidase bands at a density of about 1.19. β-Glucosidase is recovered in several bands. A peak at the top of the gradient (Fig. 1*c*, left-hand side) had a pH optimum between 8.5 and 9.0 and a K_m of 2.3mmol/l. A band at density 1.19 (Fig. 1*c*, right-hand side) shows maximum activity at pH4.0 and has a K_m of 1.15mmol/l. However, in all bands both enzyme activities are present. Cytochrome b_5 can only be demonstrated in zone I. RNA is only found in zone II. The recoveries after zonal centrifugation are: proteins, 60±12% ($n=12$); acid phosphatase, 64±18% ($n=12$); 5′-nucleotidase, 42±8% ($n=12$); glucose 6-phosphatase, 64±14% ($n=12$); alkaline phosphatase, 68±20% ($n=5$); ATPase, 63±12% ($n=3$). The recoveries are lower than those reported in Table 1, this is chiefly caused by losses occurring during injection of the sample.

Centrifugation of a P fraction in the B-XIV zonal rotor

Fig. 2 shows the distribution of different marker enzymes and constituents of a P fraction subjected to a similar density-gradient equilibration as for the M+L fraction. As in Fig. 1 two distinct zones can be observed. The distribution profiles for 5′-nucleotidase, alkaline phosphatase, glucose 6-phosphatase (Fig. 2*a*), cholesterol and phospholipids (Fig. 2*b*) are similar to those shown in Fig. 1. As with the M+L fraction, β-glucosidase (Fig. 2*c*) is recovered in several bands. However, a band at density 1.13 is appreciably larger than the band at density 1.19. The curve obtained for cytochrome oxidase activity (Fig. 2*c*) looks completely different from that for the M+L fraction.

Effect of digitonin

When rat liver microsomal preparations are exposed to low concentration of digitonin and then subjected to density equilibration, plasma-membrane markers (e.g. 5′-nucleotidase) selectively shift towards a region of higher density, whereas true endoplasmic reticulum markers (e.g. glucose 6-phosphatase and cytochrome *c* reductase) do not (Thinès-Sempoux, 1974). A similar digitonin treatment was applied to thyroid M+L fractions (Fig. 3). Comparison of Fig. 3 with Fig. 1 shows that the distribution profiles

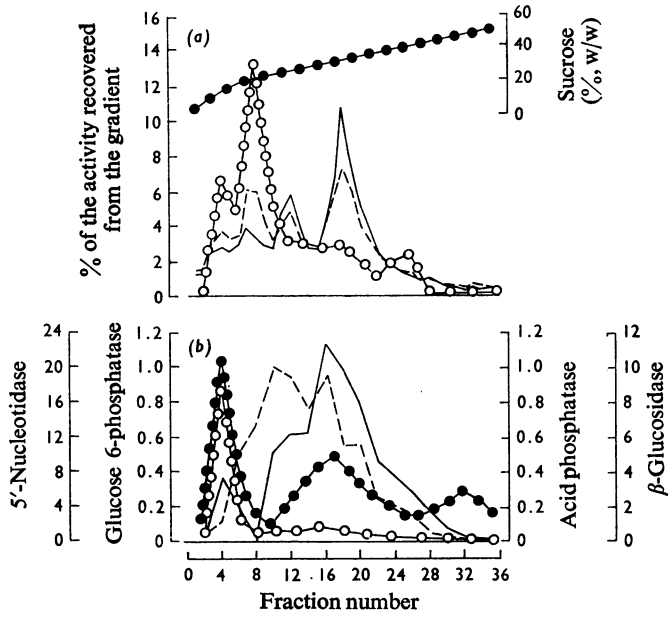

Fig. 4. *Zonal centrifugation of a P fraction treated with heparin: distribution profiles of some markers*

Heparin (50i.u./ml) was added to both homogenization medium and gradient. The P fraction was kept overnight in the cold-room (+4°C) before loading the zonal rotor. A 10ml sample was injected and centrifuged for 18h at 45000rev./min. (*a*) ○, Proteins; ●, slope of gradient; ----, E_{280}; ——, E_{260}. (*b*) Left-hand-side ordinates: 5'-nucleotidase and glucose 6-phosphatase, both expressed as phosphate released (nmol/min per ml). Right-hand-side ordinates: acid phosphatase, expressed as phosphate released (μmol/min per ml); β-glucosidase, expressed as % of activity recovered from the gradient. ○, Acid phosphatase; ●, β-glucosidase; ----, 5'-nucleotidase; ——, glucose 6-phosphatase.

of most markers have changed. 5'-Nucleotidase (Figs. 1*a*, 3*a*) shifts from a band with maximum density 1.13 to a band with maximum density 1.15. Alkaline phosphatase (Fig. 3*a*, not shown in Fig. 1) also shifts in the same direction (from density 1.13 to 1.14). The profile for glucose 6-phosphatase (Figs. 1*a*, 3*a*) has changed from a double-peak distribution (at densities 1.13 and 1.20) to a continuous distribution with broad maximum at density 1.16. The curve for phospholipids (Figs. 1*b*, 3*b*) has also acquired a maximum at density 1.16. The cholesterol profile (Figs. 1*b*, 3*b*) shows a shift of the maximum at density 1.13 to 1.15–1.16: the maximum at 1.20 has vanished. During the exposure to digitonin some acid phosphatase (Figs. 1*c*, 3*c*) and some proteins (Figs. 1*b*, 3*b*) were solubilized.

Effect of (a) Mg^{2+}, (b) pH8.6 and (c) the combined use of $Pb(NO_3)_2$, glucose 6-phosphate and sonication

After the addition of Mg^{2+} to a P fraction a single peak is observed for all markers at a medium density of 1.135. At pH8.6 most markers of the P fraction equilibrate predominantly around density 1.13, although some glucose 6-phosphatase remains at about 1.19. The addition of $Pb(NO_3)_2$ (2mmol/l) together with glucose 6-phosphate (5mmol/l) to a P fraction, followed by sonication (Hinton *et al.*, 1970) also does not result in a selective shift of the position of membranes: all membranes equilibrate at about density 1.14.

Effect of heparin

To decrease cross-contamination between the different membranes of muscle cells caused by the aggregation of various subcellular particles, Haedon & Duggan (1970) decided to incorporate heparin at a concentration of 50i.u./ml in both homogenization medium and gradient. This resulted in a shift of buoyant density towards lower values. Similar experiments were performed on bovine thyroid tissue (P fraction) (Fig. 4). 5'-Nucleotidase equilibrates at about density 1.112. In the gradient only one glucose 6-phosphatase peak is observed (around density 1.14). The other glucose 6-phosphatase peak, normally occurring at about density 1.20 (Fig. 2), has disappeared. β-Glucosidase (pH4.0) and acid phosphatase equilibrate at almost identical densities (1.14). When comparing the E_{260} and E_{280} profiles of

untreated and heparin-treated P fractions it is obvious that two new peaks (Fig. 4*a*), with a RNA-like u.v. spectrum (maximum at 260nm, minimum at 240nm), appear in the heparin experiments. Coincident with these new peaks one also finds two additional phosphorus peaks. This indicates that these two peaks contain sedimentable RNA-like material (ribosomal subunits ?). From these results and from the disappearance of the second glucose 6-phosphatase peak (density 1.20), one may conclude that ribosomes have been released from the rough endoplasmic reticulum membranes. It is necessary to centrifuge for 18h to allow both RNA-like peaks to migrate halfway in the gradient. Indeed, when the standard procedure for zonal centrifugation is used (centrifugation time only 3h) the RNA-like material stays near the top of the gradient and there is no clear-cut formation of the RNA-like peaks. The distribution profiles of some marker enzymes are represented in Fig. 4(*b*). It is clear from those profiles that during this procedure most of the acid phosphatase and some glucose 6-phosphatase are solubilized. Moreover, the differences in profiles for 5′-nucleotidase and glucose 6-phosphatase suggested the possibility of a moderate resolution between plasma membranes (5′-nucleotidase) and endoplasmic reticulum membranes (glucose 6-phosphatase).

However, heparin treatment of a zone II preparation, collected after zonal centrifugation (standard procedure) of a M+L fraction, did not result in further separation of those membranes neither by buoyant-density sedimentation nor by flotation. Moreover, a profoundly damaging effect on these membranes (solubilization of marker enzymes, disappearance of their profiles) was noted.

Discussion

Hinton *et al.* (1970) pointed out that cytochrome b_5 in rat liver is largely confined to smooth endoplasmic reticulum. In bovine thyroid cytochrome b_5 was only demonstrated in the first part of the gradient (Figs. 1 and 2). Therefore one can conclude that smooth endoplasmic reticulum material is confined to this first part of the gradient (zone I) and that the rest of the gradient (zone II) is probably free of smooth endoplasmic reticulum material. Yamashita & Field (1970) and Wolff & Jones (1971) located 5′-nucleotidase and ATPase in thyroid plasma membranes. The bovine thyroid profiles for 5′-nucleotidase, ATPase and alkaline phosphatase (Figs. 1 and 2) were confined to zone I and run parallel to each other. Therefore zone I also contains plasma membranes. For alkaline phosphatase (Table 1) as well as for ATPase and 5′-nucleotidase (Lagrou *et al.*, 1974*b*) differential pelleting resulted in distribution patterns that are comparable with each other. This could indeed imply a joint localization. Concurring with this localization of plasma membranes in zone I is the experimental finding that the relative amount of sphingomyelin is higher in zone I than in zone II. This fits in with the statement made by Morré *et al.* (1974) that increasing amounts of sphingomyelin are present in the series: smooth endoplasmic reticulum < Golgi apparatus < secretion vesicles < plasma membranes.

Zone II, lacking cytochrome b_5, is the only part of the gradient where RNA is found. Therefore rough endoplasmic reticulum must be localized in zone II of the gradient. Cytochrome *c* reductase and glucose 6-phosphatase are found in both zones. As Lee *et al.* (1969) located cytochrome *c* reductase in rat liver endoplasmic reticulum and de Duve (1971) stated that rat liver glucose 6-phosphatase is also located in the endoplasmic reticulum, we may conclude from the cytochrome *c* reductase and glucose 6-phosphatase profiles (Figs. 1 and 2) that these enzymes qualify as endoplasmic reticulum markers in bovine thyroid. Glucose 6-phosphatase can indeed qualify as a marker for endoplasmic reticulum membranes in zonal profiles, as we were able to show that the hydrolysis of glucose 6-phosphate in bovine thyroid tissue is only partially due to an unspecific phosphatase: in whole tissue 40%, in zonal profiles of the P fractions never higher than 15% (H. J. Hilderson, M. de Wolf, A. Lagrou & W. Dierick, unpublished work). Further, phospholipid profiles generally follow the glucose 6-phosphatase profiles rather closely (Figs. 1, 2 and 3). Indeed, phospholipid profiles reflect the distribution of membranous material in the gradient. Cholesterol profiles run parallel to the 5′-nucleotidase and alkaline phosphatase profiles (plasma-membrane markers) in zone I. In zone II, however, there is a tendency to follow the glucose 6-phosphatase profile (endoplasmic-reticulum marker). This phenomenon could imply the presence of cholesterol in endoplasmic-reticulum membranes. Although this is still controversial for rat liver (Thinès-Sempoux, 1974; Glaumann *et. al.*, 1974), it is confirmed for bovine thyroid by: (1) the cholesterol profiles in all experiments reported in the present paper; (2) the relatively high cholesterol/phospholipid molar ratio in zone II (0.19); it is noteworthy in this respect that in purified bovine thyroid nuclei a cholesterol/phospholipid molar ratio of 0.088 was found (Hilderson *et al.*, 1974); (3) the shift of the position of plasma and endoplasmic reticulum membranes after digitonin treatment (Fig. 3). The cytochrome oxidase profile (Fig. 1) shows a peak at density 1.19. This peak is very probably due to the presence of intact mitochondria in zone II. Indeed, mitochondria obtained by a mild homogenization procedure also equilibrate at a similar density (range 1.17–1.19) (Hilderson & Dierick, 1973). The cytochrome oxidase profile also extends into zone I, indicating to what extent mitochondria were damaged during the experiment.

Rat liver β-glucosidase (EC 3.2.1.21) is associated with lysosomal membranes (Patel & Tappel, 1969*a*). In rat kidney, however, there is also a β-glucosidase activity in the supernatant differing in pH optimum (Patel & Tappel, 1969*b*). In bovine thyroid at least two different β-glucosidase activities are present. One of these activities has a lysosomal localization. This can be concluded from the existence of latent enzyme activity with a lag period for β-glucosidase in the M+L fraction (differential pelleting experiments). Zonal centrifugation of a M+L fraction (Fig. 1) shows β-glucosidase activity at density 1.19 (pH optimum 4.0) and activity at the top of the gradient (soluble enzyme, pH optimum 8.5–9.0). Both activities did show different K_m values. The activity localized deep in the gradient is presumably lysosomal (particulate nature, acid pH optimum). In Fig. 2 (P fraction) the peak at density 1.19 is appreciably smaller than in Fig. 1. However, there is a considerable β-glucosidase activity at density 1.13. This could be due to the presence at that density of damaged lysosomes and would imply that the lysosomal β-glucosidase is membrane bound. In Figs. 1 and 2 acid phosphatase, known to be a lysosomal enzyme (Hilderson *et al.*, 1970), is distributed throughout the whole gradient. There is no clear-cut explanation for this phenomenon. It could be due to one or several of the following possibilities: (*a*) heterogeneity of the lysosomal population; (*b*) the presence of lysosomal fragments; (*c*) non-specific adsorption of released enzyme to membranes.

Treatment of bovine thyroid tissue with heparin did not yield enriched fractions of different membrane structures (plasma membranes, rough and smooth endoplasmic reticulum) in different zones of the gradient. It did result in the release of ribosomes from the rough endoplasmic reticulum membranes and in partial solubilization of some enzymes and phospholipids. Therefore heparin has a rather damaging effect on bovine thyroid membranes. This was demonstrated when incubating a zone-II preparation (instead of a M+L fraction) with heparin. Neither the sedimentation nor the flotation experiment yielded any purification of endoplasmic reticulum membranes.

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