Fractionation of Iodinated Particles and Mitochondria from Thyroid by Zonal Centrifugation and a Study of their Heterogeneity

By RAYMOND MIQUELIS and CLAUDE SIMON

*Laboratoire d'Endocrinologie Cellulaire, Université de Provence, 3 place Victor Hugo,
13331 Marseille-Cédex 3, France*

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1. The subcellular particles of horse and rat thyroids were fractionated in a B XIV zonal rotor on a non-linear gradient of Ficoll after labelling with radioactive iodine *in vitro* (horse) or *in vivo* (rat). In the horse, the resulting fractions were analysed for radioactive iodine, protein and enzymes representative of certain subcellular particles. In the rat, iodine turnover and thyrotrophin stimulation were studied. 2. The population of iodinated particles could be subdivided into three main classes, characterized by differences in β -galactosidase and acid phosphatase content and position in the gradient. The presence of a fourth class of particles is suggested. 3. It is concluded that iodinated particles isolated from the thyroid are essentially secondary lysosomes. Their heterogeneity is established with respect to their position in the gradient, their content of acid hydrolases and their iodine turnover. 4. The iodine pools of these secondary lysosomes are increased by thyrotrophin without any change in their number. 5. Their functional significance is discussed. 6. The distribution of mitochondria as judged by succinate dehydrogenase was also studied. The succinate dehydrogenase was spread throughout the gradient with a maximum of activity (40%) in the upper layer of the gradient. Separation of mitochondria from lysosomes by this method was not successful.

The thyroid follicles contain in their luminal colloid large amounts of thyroglobulin in which the thyroid hormones are bound by peptide linkages. By pinocytosis, luminal colloid migrates into the follicular cells in the form of colloid droplets. It has been suggested that fusion of primary lysosomes and colloid droplets soon after the latter are formed results in the appearance of secondary lysosomes, which thus contain iodine (in the form of thyroglobulin) and typical lysosomal enzymes as well. Lysis of thyroglobulin occurs within these secondary lysosomes and is brought about by lysosomal acid hydrolases. Thyrotrophin enhances both pinocytosis and proteolysis of thyroglobulin with a subsequent increase in the release of thyroid hormones. All these biochemical and morphological aspects were reviewed by Wollman (1973).

The isolation and characterization of iodinated particles from the thyroid is thus of great interest for the elucidation of the process by which the thyroid hormones are secreted. In work on the rat (Simon *et al.*, 1971) it was shown that, after ultracentrifugation in a non-linear Ficoll gradient in a swinging-bucket rotor, the total population of iodinated particles was resolved into three peaks. They were characterized by iodine labelling and their particulate nature was demonstrated by Triton X-100 treatment. However, except for acid

phosphatase activity, the characterization of further enzymes was impossible, because the amount of material available in each tube was too small. The technique was thus adapted to a zonal rotor by using equivalent conditions with respect to the Ficoll gradient and the centrifugal force. In the present paper we report some of the characteristics of iodinated particles isolated from the thyroids of horse and rat. Separation of iodinated particles from mitochondria was also studied in the horse thyroid.

Materials and Methods

Reagents

Tris, CuSO₄, KCN and sucrose were obtained from E. Merck A.G., Darmstadt, Germany. Bovine serum albumin, *p*-nitrophenyl phosphate (disodium salt), *p*-nitrophenyl phosphate β -D-galactopyranoside and 2,6-dichlorophenol-indophenol (sodium salt) were purchased from Calbiochem, Los Angeles, Calif., U.S.A. Triton X-100 was from OSI, Paris, France, and Ficoll from Pharmacia Fine Chemicals, Uppsala, Sweden. Thyrotrophin 'Endo' was a gift from Laboratoires Endopancrine, Paris, France.

All other chemicals were of the best available analytical grade.

Biological materials

Rat. Wistar male rats, weighing about 300 g when used, were adapted to receive 50 μ g of iodine daily. A first group received 200 μ Ci of carrier-free 125 I and 1 i.u. of thyrotrophin by intraperitoneal injection 15 and 1 h before death respectively. A second group (control group) received in the same way 200 μ Ci of 131 I and 0.5 ml of the solvent of the thyrotrophin.

When kinetic study was desired, rats adapted to the same daily iodine intake were isotopically equilibrated for 60 days with 125 I (Simon, 1964) and received 200 μ Ci of carrier-free 131 I by intraperitoneal injection 15 h before death.

Horse. Fresh horse thyroid glands were obtained from the local slaughter-house and brought to the laboratory on ice within 30 min of death. The glands (10–40 g) were trimmed free of surrounding tissues, and 10–12 g of gland tissue was cut into small blocks (80–100 mg each) with razor blades. The whole blocks were incubated for 1 h at 37°C with shaking (100 cycles/min) in 20 ml of Krebs–Ringer phosphate buffer, pH 7.4 (De Luca & Cohen, 1964), containing 500 μ Ci of carrier-free 125 I. After the incubation, the blocks were washed twice in a buffer system, pH 7.0, containing 0.1 M-Tris-HCl and 0.15 M-sucrose. A 20% (w/v) homogenate was prepared in this medium which had been shown (Simon *et al.*, 1971) to retain the maximum integrity of the iodinated particles during preparation.

Preparation of subcellular particles

For both rat and horse, the bulk of the iodinated particles was prepared as described by Simon *et al.* (1971). Briefly, a 34000 g pellet was obtained and washed twice. The sample to be submitted to zonal centrifugation was obtained by resuspending the washed pellet.

Zonal centrifugation. Fractionation of the subcellular preparation was carried out in the B XIV rotor in an MSE SuperSpeed 65 ultracentrifuge. The rotor was loaded at 2500 rev./min by means of a variable-speed peristaltic pump (F. A. Hughes Hilo-flow). Various solutions of Ficoll, prepared in the 0.1 M-Tris-HCl buffer, pH 7.0, containing 0.15 M-sucrose, were successively pumped into the edge of the rotor, as follows (all concns. given as w/v): 0.5% Ficoll (40 ml), 1.5% (60 ml), 3% (70 ml), 10% (130 ml), 12% (130 ml) and 14% (cushion, 220 ml).

When the rotor was completely filled with gradient material, the sample 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 radially with 80 ml of an overlay solution (0.1 M-Tris-HCl buffer, pH 7.0, without sucrose) and finally centrifuged for 25 min at 100000 g_{av} .

At the end of the centrifugation period, the zonal rotor was unloaded at 2500 rev./min. The contents were collected in 6.5 ml fractions by displacement with a 14% Ficoll solution pumped to the rotor edge. As a routine 100 fractions were collected.

Collections of fractions for biochemical assays. In certain cases, a sample (0.2 ml) of each fraction was kept for determination of acid phosphatase activity throughout the gradient.

In other cases, the fractionation of the iodinated particles was first checked by rapid measurements of radioactivity. Thereafter the corresponding fractions were pooled by zones (see Fig. 1). Zones (A) (58.5 ml), (C) (58.5 ml) and (E) (78 ml) represented peaks 1, 2 and 3 respectively (Simon *et al.*, 1971). Zones (B) (58.5 ml) and (D) (58.5 ml) were between peaks 1 and 2 and peaks 2 and 3 respectively. For each zone sample, the viscosity was lowered by addition of 0.5 vol. of homogenization medium before centrifuging at 80000 g_{av} for 35 min in an MSE 59119 rotor. The resulting pellets were each resuspended in 0.5 ml of 0.1 M-sodium phosphate buffer, pH 7.4, and used for enzyme assays and protein determination.

Measurement of radioactivity

The two radioisotopes were obtained carrier-free from the Commissariat à l'Energie Atomique (C.E.N. Saclay). The 131 I or 125 I content of each fraction collected was measured with a two-channel Packard Autogamma spectrometer. In double-labelling experiments, interference of 131 I with the 125 I-counting rate was corrected. When isotopically equilibrated rats were used, the 127 I content of each fraction was calculated from its 125 I content as described by Simon (1964).

Enzyme assays

Two acid hydrolase activities were assayed in the presence of 0.2% Triton X-100 to give total enzyme activities (Wattiaux & de Duve, 1956).

Acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2). Enzyme activity was assayed with *p*-nitrophenyl phosphate (disodium salt) as substrate. Samples were incubated with *p*-nitrophenyl phosphate in 0.2 ml of 0.2 M-sodium acetate buffer, pH 4.8, in a total volume of 0.4 ml. The reaction was stopped by addition of 2.6 ml of 0.2 M-disodium tetraborate buffer, pH 9.0, to the reaction mixture. *p*-Nitrophenol was measured at 400 nm in a DU2 Beckman Spectrophotometer. The unit of enzyme activity was given as μ mol of *p*-nitrophenol formed/ml of sample per/h.

To study the distribution of acid phosphatase activity throughout the gradient, 0.2 ml of each fraction collected was incubated for 1 h at 37°C.

To study the distribution of acid phosphatase activity between zones, portions (10 μ l) of samples (A), (B), (C), (D) and (E) were incubated for 30 min at 37°C.

β -D-Galactosidase (β -D-galactoside galactohydrolase, EC 3.2.1.23). Enzyme activity was assayed with *p*-nitrophenyl phosphate β -D-galactopyranoside as substrate. Portions (50 μ l) of samples (A), (B), (C), (D) and (E) were incubated for 1.5 h at 37°C with 5 mmol of substrate in 0.2 M-sodium acetate buffer, pH 4.8. The reaction was stopped and enzyme activity measured as described for acid phosphatase.

Succinate dehydrogenase (succinate oxidoreductase, EC 1.3.99.1). Portions (0.2 ml) of samples (A), (B), (C), (D) and (E) were assayed by the method of Green *et al.* (1955) and Bachmann *et al.* (1966). The unit of enzyme activity was given as μ mol of reduced 2,6-dichlorophenol-indophenol formed/min.

Protein. Portions (0.2 ml) of samples (A), (B), (C), (D) and (E) were measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Results and Discussion

Iodinated particles

Horse. As a preliminary control of the zonal centrifugation method, the distribution of acid phosphatase activity and of radioactive iodine (after labelling *in vitro*) was measured throughout the gradient.

(a) Distribution of radioactive iodine and acid phosphatase activity. Fig. 1 shows that acid phosphatase activity is distributed between three peaks. The radioactive iodine is distributed between

four peaks, three of them being superimposed on the three peaks of acid phosphatase activity. These results are in perfect agreement with those we have obtained in the rat with a conventional swinging-bucket rotor (Simon *et al.*, 1971). In the rat, it was demonstrated that the three peaks in which radioactive iodine is associated with acid phosphatase activity are particulate in nature and that the fourth peak was composed of iodinated molecules. Thus similar results are obtained with the horse thyroid in the zonal rotor and with the rat thyroid in the swinging-bucket rotor.

(b) Distribution of β -galactosidase and acid phosphatase activities. From each gradient, five zones of interest were selected in which both β -galactosidase and acid phosphatase activities were measured and their ratio was calculated. Table 1 shows average values of the activity ratio of β -galactosidase/acid phosphatase obtained from four gradients. For three gradients, the minimum value of the ratio was found to be associated with zone (D). In the four gradients, the ratio of the three peaks was decreasing from zone (A) (peak 1) to zone (E) (peak 3). It is concluded that the three classes of iodinated particles isolated from horse thyroid are heterogeneous with respect to their content of β -galactosidase and acid phosphatase. In addition, zone (D), lying between peaks 2 and 3, exhibits a ratio whose value is not intermediate between those of the peaks. From this, it is concluded that this zone does not result from an overlap of these two peaks. It is likely that a fourth class of iodinated particles is present in these gradients. It was also probably present in the rat after centrifugation in a swinging-bucket rotor but it is more readily visible after zonal centrifugation.

Fig. 1. Distribution of acid phosphatase activity and radioactive iodine after zonal centrifugation of a subcellular fraction of horse thyroid

For experimental details see the Materials and Methods section. Iodinated particles are distributed into five zones (A)–(E). The last peak of ^{125}I , which is near the centre of the rotor (right side), is composed of iodinated molecules. ●, Acid phosphatase activity; —, ^{125}I radioactivity content. The fraction size was 6.5 ml.

Table 1. *Distribution of acid phosphatase and β -galactosidase activities in a subcellular fraction of horse thyroid*

Experimental details were as given in Fig. 1. Individual values for each enzyme activity are given for four experiments. In each experiment, the activity ratio of β -galactosidase to acid phosphatase is then calculated.

Enzyme activity	Zone ... Expts.	(A)	(B)	(C)	(D)	(E)
Acid phosphatase activity ($\mu\text{mol/h}$ per ml of sample)	(1)	0.530	0.406	0.936	1.178	2.574
	(2)	0.886	0.927	1.183	0.983	2.699
	(3)	1.067	0.830	1.560	1.685	1.654
	(4)	1.585	0.715	1.841	1.186	3.058
β -Galactosidase activity ($\mu\text{mol/h}$ per ml of sample)	(1)	0.049	0.025	0.053	0.038	0.089
	(2)	0.036	0.031	0.035	0.032	0.067
	(3)	0.037	0.017	0.035	0.031	0.031
	(4)	0.064	0.016	0.045	0.015	0.056
$10^{-2} \times \text{Ratio} \left[\frac{\beta\text{-galactosidase activity}}{\text{acid phosphatase activity}} \right]$	(1)	9.24	6.16	5.66	3.22	3.46
	(2)	4.06	3.34	2.96	3.25	2.48
	(3)	3.47	2.05	2.24	1.84	1.87
	(4)	4.04	2.24	2.44	1.26	1.83

The finding by Bauer & Meyer (1965) of three categories of 'colloid droplets' in the mouse thyroid by electron radioautography is also noteworthy. These three categories were distinguished by their electron density and their radioactive iodine content.

Thus in the thyroid of rat, horse and mouse there exist at least three distinct classes of iodinated particles which are likely, as indicated by their β -galactosidase and acid phosphatase contents, to be secondary lysosomes. To date, the significance of this heterogeneity is unknown. It may be that they represent three different stages (ages) of the whole population of secondary lysosomes.

Rat. Because of the ease of both labelling *in vivo* and control of daily iodine intake in the rat, this species was used in our previous work with the conventional swinging-bucket rotor. After the zonal centrifugation technique had been developed with horse thyroid, our attention turned again to the rat. Experiments were performed to test the action of thyrotrophin on iodinated particles separated by the zonal method; in addition their iodine turnover was examined.

(a) Action of thyrotrophin. In rats treated with 1 i.u. of thyrotrophin for 1 h, the fractionation by zonal centrifugation of iodinated particles shows that: (1) the fractionation is as good as for the control; (2) the number of radioactive iodine peaks and their positions in the gradient are the same as for the control; (3) the radioactive iodine content of each peak is increased (Fig. 2).

In this experiment, thyrotrophin was injected 1 h before death, a time interval during which pinocytosis has been shown to be increased (Wetzel *et al.*, 1965). Thus the increase in radioactive content of the peaks is probably the result of increased pools of iodinated particles. Further, under thyrotrophin

Fig. 2. *Effect of thyrotrophin on the population of iodinated particles in the rat thyroid*

Whole populations of iodinated particles were prepared in the form of pellets from nine control rats and nine rats treated with 1 i.u. of thyrotrophin as described in the Materials and Methods section. Control pellet (^{131}I label) and thyrotrophin pellet (^{125}I label) were resuspended together and simultaneously centrifuged in the zonal rotor as in Fig. 1. The results are expressed as percentage of total radioactivity present in the homogenate (600 g supernatant). ●, Control rats; —, thyrotrophin-treated rats.

treatment, the same number of classes of iodinated particles with the same sedimentation properties is found. Such a result suggests that after treatment with thyrotrophin for 1 h the number and/or the iodine turnover of iodinated particles is increased but the type and number of classes of these particles are unchanged.

Fig. 3. Double labelling *in vivo* of iodinated particles in the rat thyroid

The whole population of iodinated particles was prepared from fifteen rats as described in the Materials and Methods section. The zonal centrifugation was as in Fig. 1. Rats, maintained in isotopic equilibrium with ^{125}I , were injected intraperitoneally with carrier-free ^{131}I 15h before death. The ^{125}I (—) and ^{131}I (●) contents of each fraction were measured (lower part of the figure). The ^{127}I content of each fraction was calculated from its ^{125}I content (Simon, 1964) and expressed in μg . The specific radioactivity of each fraction was calculated and is given in the upper part of the Figure.

Fig. 4. Distribution of succinate dehydrogenase activity and protein content in a subcellular fraction of horse thyroid

Determination of succinate dehydrogenase activity and protein content and preparation and centrifugation of subcellular fractions were as described in the Materials and Methods section. The five zones selected in each gradient after centrifugation were as in Fig. 1. The bars in the columns refer to standard deviation of the percentage distribution. □, Succinate dehydrogenase activity; ▨, protein content.

(b) Iodine turnover. Fig. 3 shows the distribution of iodine (^{127}I) and radioactive iodine (^{131}I) after labelling for 15h *in vivo*. With respect to their specific radioactivity (^{131}I % of dose injected/ μg of ^{127}I), the three peaks exhibit different labelling rates. In addition, zone (D) has a greater specific radioactivity than those of the three peaks.

This result is comparable with that observed in the horse for the enzyme contents. Further, we have shown that the specific radioactivity of zone (D) is always higher than the specific radioactivity of the three peaks from 0.5 to 24h after labelling *in vivo* (J. Dang, R. Miquelis, P. Bastiani & C. Simon, unpublished work).

These converging results are compatible with the existence of a fourth class of iodinated particles whose enzyme content is different from and iodine turnover is higher than that of the first three classes.

Mitochondria

Fig. 4 shows the distribution of succinate dehydrogenase activity and protein content, which is given as a percentage of the total recovered from the five zones (A)–(E). Specific activities for succinate dehydrogenase were not calculated because most of the proteins in the gradient are of lysosomal origin and are not uniformly distributed. It was observed that: (1) the distributions of succinate dehydrogenase and proteins are different; (2) about 40% of the succinate dehydrogenase activity is associated with zone (E) (peak 3); (3) the distributions of succinate dehydrogenase and acid phosphatase are different (Fig. 4 and Table 1).

With the gradient used, it is therefore possible to show heterogeneity of distribution of the thyroid mitochondria. This distribution is different from that of secondary lysosomes as judged from the distribution of succinate dehydrogenase and acid phosphatase respectively. Nevertheless our present method does not succeed in giving a complete separation of mitochondria from secondary lysosomes.

General discussion

We were able to separate the iodinated particles isolated from the thyroid of horse and rat into at least three classes. This result agrees with our work in the rat (Simon *et al.*, 1971). The quantities of particles obtained by our method, which involves zonal centrifugation in a non-linear Ficoll gradient, are adequate for biochemical studies.

In addition to their iodine content (which is controlled by thyrotrophin), these particles were shown

to contain β -galactosidase and acid phosphatase activities, which are known to be characteristic of lysosomes. From this, it is concluded that iodinated particles isolated from the thyroid are essentially secondary lysosomes. With respect to the same criteria, it is also suggested that a fourth class of secondary lysosomes exists. Nevertheless, it was poorly resolved from the two surrounding peaks of particles. Its apparently high iodine turnover is very noteworthy and further improvement in its purification is now required.

By our method, it was clearly established that secondary lysosomes of the thyroid are heterogeneous with respect to their acid hydrolase content. Such a heterogeneity suggests that they represent classes of secondary lysosomes of different ages which are engaged in the process of thyroglobulin degradation to prepare the hormonal secretion of the gland. Further elucidation of lysosomal heterogeneity is now required, particularly to determine any functional significance.

Separation of mitochondria from lysosomes was unsuccessful, but we were not directly faced with this problem in studying the iodine turnover of the thyroid particles.

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