Lysosomal Hydrolases in Calf Thyroid

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1. Two acid phosphatases (β -glycerophosphatase and phenylphosphatase), acid β -glucuronidase and cathepsin were demonstrated in the 0.25 M-sucrose homogenates from whole calf thyroid tissue and from isolated calf thyroid cells. 2. The main kinetic characters of these enzymes were studied. 3. All these acid hydrolases are partially sedimentable and display a latency that is unmasked by treatment with Triton X-100 and on dilution in hypo-osmotic media. It is concluded that these acid hydrolases belong to the lysosomes.

It is generally agreed that thyroid secretion depends on the hydrolysis of thyroglobulin. Proteolytic activity has indeed been observed in thyroid extracts; it is most marked at an acid pH and resembles that of the cathepsin found in other tissues (de Robertis, 1941; McQuillan & Trikojus, 1953; McQuillan, Stanley & Trikojus, 1954; Alpers, Petermann & Rall, 1956; Litonjua, 1960, 1961; McQuillan, Mathews & Trikojus, 1961; Beckers & De Visscher, 1961, 1962; Herveg, Beckers & De Visscher, 1963).

Recent morphological investigations have suggested a possible mechanism for the initiation of thyroglobulin hydrolysis, as it occurs in response to thyroid-stimulating hormone. Indications have been obtained that colloid is taken up from the follicle lumen by pinocytosis and that the vacuoles formed in this manner subsequently fuse with acid-phosphatase-positive granules believed to be of lysosomal nature (Nadler, Sarkar & Leblond, 1962; Novikoff & Vorbrodt, 1963; Nadler, Young, Leblond & Mitmaker, 1964; Wollman, Spicer & Burstone, 1964; Stein & Gross, 1964; Wetzel, Spicer & Wollman, 1965). It has been suggested that this process leads to the proteolytic degradation of thyroglobulin and to the release of hormonally active iodinated amino acids.

This interpretation is in line with the known function of lysosomes in other tissues (de Duve, 1959, 1963; Novikoff, 1961; de Duve & Wattiaux, 1966). However, it implies that the thyroid gland contains lysosomes comparable with those of liver and other organs, especially with respect to their catheptic activity. Preliminary investigations from this Laboratory have indeed demonstrated the existence of lysosome-like particles in calf thyroid (Herveg, Beckers, Jacques & De Visscher, 1965). The present paper provides a more complete account of the results obtained to date on the

subject. In addition, it shows the great advantage for such studies of first dispersing the thyroid cells with collagenase before homogenization.

MATERIALS AND METHODS

Tissue preparations

Fresh calf thyroid glands were obtained from the local slaughterhouse and brought to the laboratory on ice. The glands were dissected free of surrounding tissues and minced. Two types of tissue preparations were made. In the first one, the glands were homogenized with a mechanically driven homogenizer of the Potter & Elvehjem (1936) type, made of a smooth-walled glass tube and a Teflon pestle (manufactured by A. H. Thomas Co., Philadelphia, Pa., U.S.A.). Complete homogenization of the thyroids was obtained by three up-and-down strokes of the pestle rotating at about 1000 rev./min. This preparation is designated 'homogenate of whole tissue'.

In the second type of preparations the minced tissue was suspended in Eagle's solution (Eagle, 1955) containing 10% (v/v) of calf serum and buffered at pH 7.4 with NaHCO_3 (4 ml./g. of tissue). Satisfactory cell release was obtained by exposing the tissue for 30 min. at 37° to bacterial collagenase (Calbiochem, Los Angeles, Calif., U.S.A.) in the amount of 80 $\mu\text{g./g.}$ of tissue.

Microscopic examinations performed on the cell suspension obtained after incubation of the tissue with collagenase revealed the presence of rounded cells with well-defined nuclei and cytoplasm as well as broken cells. No follicular structure could be recognized after the treatment with collagenase. After incubation the resulting suspension was filtered through cheese-cloth and centrifuged for 10 min. at 700 rev./min. in an International refrigerated centrifuge (rotor no. 259). The cells were washed twice with a medium free of collagenase, resuspended in 0.25 M-sucrose and homogenized as described for the tissue homogenate to give the 'homogenate of isolated cells'. Both homogenates were prepared within 2 hr. of collection of the glands.

In some experiments the homogenates were centrifuged for 30 min. at 40000 rev./min. and 0° ($3 \times 10^6 \text{g.-min.}$) in rotor no. 40 of the Spinco model L-50 preparative ultra-

centrifuge. The pellets were resuspended gently in ice-cold 0.25 M-sucrose with the help of a homogenizer operated at reduced speed. Both supernatant and particulate fractions were made up to the original volume of the homogenates.

Enzyme assays and analytical procedures

Most of the techniques for the enzyme assays are based on those used for liver tissue by de Duve and co-workers (Appelmans & de Duve, 1955; de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). The enzymic tests were all carried out at 37°. Free activities were measured in the presence of 0.25 M-sucrose, and total activities in the presence of 0.1% Triton X-100 (Wattiaux & de Duve, 1956). Activities are expressed in units/g. of tissue, 1 unit referring to the decomposition of 1 μ mole of substrate/min. under the conditions of the assay. According to the activity of each enzyme preparation, the volume of the incubation mixture was adapted to give the best sensitivity. Results substantiating the choice of methods are presented below.

Acid phosphatase. The homogenate was incubated for 30 min. in 0.1 M-sodium acetate-acetic acid buffer, pH 5.0, containing either β -glycerophosphate (50 mM) or phenyl phosphate (50 mM). The reaction was stopped by adding 5 vol. of 8% (w/v) trichloroacetic acid. After filtration, P_i was determined on the filtrate according to the method of Fiske & Subbarow (1925). An appropriate blank in which substrate was added after stopping the enzymic reaction was always run in parallel to the test and the result subtracted from the observed values.

β -Glucuronidase. Incubation was carried out for 30 min. in 0.1 M-sodium acetate-acetic acid buffer, pH 5.0, containing phenolphthalein glucuronide (1.25 mM). The reaction was stopped by adding 3 vol. of a solution containing glycine (133 mM), NaCl (67 mM) and Na₂CO₃ (83 mM), pH 10.7. The mixture was clarified by filtration and its phenolphthalein content determined by colorimetry. Enzyme and substrate blanks were run separately.

Cathepsin. Haemoglobin was used as substrate, generally after prior denaturation. It was present in the final incubation mixture at a concentration of 2% (w/v), together with enough acetic acid to bring the pH to the desired value and with 0.1 M-sodium acetate-acetic acid buffer, pH 3.6 or 5.0. The reaction was stopped by adding 2 vol. of ice-cold 5% (w/v) trichloroacetic acid. The mixture was filtered in the cold room. The products of the reaction were measured on the filtrate with the Folin-Ciocalteu reagent or by the protein method of Lowry, Rosebrough, Farr & Randall (1951), with tyrosine as standard. Blanks were run by incubating the complete mixture for 2 min. In general, the assays were performed at pH 3.6, except when free activities were measured. In the latter case, both the assay for free activity and that for total activity were carried out at pH 5.0.

Materials

The disodium salts of β -glycerophosphate and of phenyl phosphate were obtained from E. Merck A.-G. (Darmstadt, Germany) and from U.C.B. (Brussels, Belgium) respectively. Phenolphthalein glucuronide was from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Eagle's solution was prepared from packages of GBI powder medium MEM (General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A.).

Haemoglobin was prepared from ox erythrocytes, washed five or six times at 4° with 0.15 M-NaCl. After haemolysis and centrifugation, the preparation was filtered on absorbent cotton wool. Haemoglobin was then brought to pH 3.6, incubated for 20 min. at 37° and dialysed against distilled water until the pH value was 5.

RESULTS

Total enzymic activities

Effect of pH. As illustrated in Fig. 1, all four measured activities exhibited optima in the acid range; little or no activity was observed in the alkaline region.

Effect of enzyme concentration. Under the conditions adopted for the assays, the activities were essentially proportional to the amount of homogenate added, except for acid phosphatase with β -glycerophosphate as substrate, where deviation from linearity occurred with excess of enzyme (Fig. 2). This effect was not observed with phenyl phosphate.

Effect of substrate concentration. The following K_m values were obtained in experiments carried out with different substrate concentrations, under otherwise standard conditions: 15 mM for acid phosphatase (with β -glycerophosphate); 7.5 mM for acid phosphatase (with phenyl phosphate); 0.25 mM for β -glucuronidase; 0.036 mM for cathepsin at pH 3.6. The results obtained for acid phosphatase and β -glucuronidase are illustrated below (see Fig. 8).

Quantitative determinations. The results described above indicate the validity of the assay methods adopted for quantitative estimations. The values found for homogenates of whole tissue are given in Table 1. Table 2 shows the results obtained in assays performed with homogenates of whole tissue and on homogenates of isolated cells prepared from different batches of glands. It will be noted that the ratio of the activities observed on the two preparations is not of the same order of magnitude.

Latency of enzymes

Latency and sedimentability. According to the results presented in Table 3, homogenates of whole tissue contain 50–60% of the hydrolase activities in sedimentable form and about 40% in a form that does not react with added substrates under the conditions of the assays of free activity. Other experiments have demonstrated that the latent enzymes are entirely associated with sedimentable particles.

Of special interest are the results (Table 3) showing that a considerably higher proportion of the total acid-hydrolase activities are sedimentable and unreactive in homogenates prepared from

Fig. 1. Influence of pH on hydrolytic activities of homogenate of whole tissue: (a) acid phosphatase (with phenyl phosphate); (b) acid phosphatase (with β -glycerophosphate); (c) cathepsin; (d) β -glucuronidase. Incubations were performed in the following buffers at 0.1M concentration: glycine-HCl or glycine-NaOH ($\circ$); acetate-acetic acid ($\blacksquare$); HCl-sodium cacodylate ($\triangle$). Native haemoglobin was used in the cathepsin assays, denaturated haemoglobin being insoluble at its isoelectric point.

Fig. 2. Influence of enzyme concentration on enzyme activities (homogenate of whole tissue): (a) acid phosphatase (with phenyl phosphate); (b) acid phosphatase (with β -glycerophosphate); (c) cathepsin at pH 3.6 ($\bullet$) and at pH 5.0 ($\circ$); (d) β -glucuronidase.

suspensions of washed isolated cells. In contrast with the situation observed for homogenates of whole tissue, homogenates of isolated cells contain

more acid phosphatase than β -glucuronidase or cathepsin activity in the free form.

Effect of Triton X-100. When this detergent was

added in increasing concentration to the assay medium (containing 0.25 M-sucrose), the four activities were progressively released in an essentially parallel fashion in both types of preparations (Fig. 3). Complete release took place at a Triton

X-100 concentration of about 0.05%, which is half the detergent concentration used in the assays of total activity. No sign of inhibition of the enzymes was evident with detergent concentrations of up to 0.14%.

Effect of decreasing osmotic pressure. As shown in Fig. 4, temporary exposure of the preparations to a lowered sucrose concentration caused an irreversible increase in free activity. The magnitude of this effect was related to the degree of dilution achieved in a manner that was essentially similar for all four activities and in both types of homogenate.

Effect of freezing and thawing. In both preparations, the enzyme activities were all unmasked to a large extent by a single freezing-and-thawing cycle; a second cycle was sufficient to bring the activities to the same level as those observed in the presence of Triton X-100 (Fig. 5).

Effect of pH. Decreasing the pH at which the assays of free activity were run allowed increasing amounts of hydrolase activity to become manifest. As illustrated by Fig. 6, this effect was different for acid phosphatase and for β -glucuronidase. Experiments of this type were not run on cathepsin.

Table 1. *Total activities in homogenates of whole tissue*

With each homogenate of whole tissue the activities of all four enzymes were measured simultaneously. Denatured haemoglobin was used as substrate for the cathepsin assays. Results are expressed as means $\pm$ s.d. The numbers of individual determinations are given in parentheses.

Enzyme	Mean activity (units/g. of tissue)
Acid phosphatase	
With phenyl phosphate	4.84 $\pm$ 1.82 (4)
With β -glycerophosphate	0.928 $\pm$ 0.207 (4)
Cathepsin	
At pH 3.6	4.35 $\pm$ 1.13 (4)
At pH 5.0	0.57 $\pm$ 0.02 (4)
Acid β -glucuronidase	0.146 $\pm$ 0.039 (4)

Table 2. *Relative distribution of enzyme activities in homogenates of whole tissue and homogenates of isolated cells*

Results are expressed as means $\pm$ s.d., acid- β -glucuronidase activity being taken as unity. The numbers of individual determinations are given in parentheses.

Enzyme	Activity relative to β -glucuronidase activity	
	Homogenate of whole tissue	Homogenate of isolated cells
Acid phosphatase		
With phenyl phosphate	33.15 $\pm$ 12.40 (4)	51.5 $\pm$ 8.3 (4)
With β -glycerophosphate	6.35 $\pm$ 1.41 (4)	22.7 $\pm$ 2.5 (4)
Cathepsin at pH 3.6	29.70 $\pm$ 7.73 (4)	40.5 $\pm$ 7.4 (4)
Acid β -glucuronidase	[1.00 $\pm$ 0.26 (4)]	[1.0 $\pm$ 0.20 (4)]

Table 3. *Latency and sedimentability of enzymes in homogenates of whole tissue and homogenates of isolated cells*

Free activities and sedimentable activities (30 min. assays) are expressed as percentages of the total activity elicited by Triton X-100 measured in the same type of homogenate. Results are expressed as means $\pm$ s.d. The numbers of determinations are given in parentheses.

	Free activity (% of total)		Sedimentable activity (% of total)	
	Homogenate of whole tissue	Homogenate of isolated cells	Homogenate of whole tissue	Homogenate of isolated cells
Acid phosphatase				
With phenyl phosphate	51.5 $\pm$ 5.0 (4)	26.6 $\pm$ 8.7 (5)	50.3 $\pm$ 7.5 (2)	70 (1)
With β -glycerophosphate	56.3 $\pm$ 11.1 (9)	28.5 $\pm$ 2.0 (4)	55.8 $\pm$ 8.7 (2)	74 (1)
Cathepsin at pH 5.0	51.6 $\pm$ 2.5 (5)	10.8 $\pm$ 6.2 (5)	50.8 $\pm$ 10.9 (5)	82 (1)
β -Glucuronidase	55.7 $\pm$ 3.6 (4)	16.4 $\pm$ 3.7 (7)	48.5 $\pm$ 6.2 (4)	87 (1)

Fig. 3. Influence of Triton X-100 concentration in the incubation medium on the free activities of acid phosphatase measured with phenyl phosphate (□) or β -glycerophosphate (○), cathepsin (Δ) and β -glucuronidase (●) of homogenate of isolated cells (a) and of homogenate of whole tissue (b). Results are expressed as percentages of the highest observed activities. Free activities were measured in 30 min. assays for both types of thyroid homogenates. With both preparations the detergent/tissue ratio was the same for each set of four assays.

Effect of incubation time. Fig. 7 shows the results of an experiment in which assays of free and total acid-phosphatase activity were run on a homogenate of whole tissue for increasing lengths of time, with β -glycerophosphate as substrate. The curvature, which has been attributed to P_i inhibition (Fig. 2), is manifest in both cases, but there is no indication that significant amounts of latent activity become unmasked during the incubation, even after 5 hr.

Effect of substrate concentration. Free and total activities of acid phosphatase (with β -glycerophosphate) and β -glucuronidase were measured on a homogenate of isolated cells with different concentrations of substrate. As shown in Fig. 8, the free and the total activities both obey Michaelis-Menten kinetics with identical K_m values.

Fig. 4. Influence of decreasing the osmotic pressure on the free activities of acid phosphatase measured with phenyl phosphate (□) or β -glycerophosphate (○), cathepsin (Δ) and β -glucuronidase (●) of homogenate of whole tissue (a) and homogenate of isolated cells (b). Samples of the homogenates were diluted with distilled water to give the various sucrose concentrations, kept for 5 min. at 0° and then made up to identical volumes by adding suitable amounts of sucrose and water to restore the sucrose concentration to its original value of 0.25 M. Assays of free and total activity were then performed on the treated preparations in 30 min. assays. Results are expressed as percentages of the corresponding total activities, which were unaffected by the treatment.

DISCUSSION

The results described in the present paper show that in thyroid tissue the acid hydrolases measured display the same characteristic properties of sedimentability and latency that they have been found to possess in similarly prepared homogenates of liver, kidney and many other organs. Although specific evidence of their association with lysosomes has not yet been obtained, this seems a fairly safe assumption in view of the numerous data that are now available on the presence of lysosomes in animal tissues.

Fig. 5. Influence of freezing at -80° and thawing at 4° on free activities of acid phosphatase measured with phenyl phosphate (□) or β -glycerophosphate (○), cathepsin (Δ) and β -glucuronidase (●) of homogenate of whole tissue (a) and homogenate of isolated cells (b). The values are expressed as percentages of the corresponding total activities.

Fig. 6. (1) Effect of pH on the free activities of (a) acid phosphatase (with 50 mM- β -glycerophosphate) and (b) β -glucuronidase (with 1.25 mM-phenolphthalein glucuronide) of homogenate of isolated cells. Free and total activities were determined at various pH values in 0.2 M-sodium acetate-acetic acid buffers. (2) Effect of pH and substrates on the free activities of (c) acid phosphatase (with 50 mM- β -glycerophosphate in the presence of 1.25 mM-phenolphthalein glucuronide) and (d) β -glucuronidase (with 1.25 mM-phenolphthalein glucuronide in the presence of 50 mM- β -glycerophosphate). Free and total activities were determined at various pH values in 0.2 M-sodium acetate-acetic acid buffers.

On this assumption it would appear that calf-thyroid lysosomes resemble rat-liver particles closely in their sensitivity to various disruptive treatments. In particular, they show the same type of osmotic activation curve (Appelmans & de Duve, 1955; Gianetto & de Duve, 1955), and the same sensitivity to Triton X-100 (Wattiaux & de Duve, 1956) and to freezing and thawing when the freezing

process is carried out at sufficiently low temperature (Bendall & de Duve, 1960). These properties are best accounted for, as with the liver particles, by assuming that the enzymes are present within sac-like structures surrounded by a membrane impermeable to the substrates. Supporting this interpretation is the observation that the free activity of the enzymes is not increased by raising the

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Fig. 7. Influence of incubation time on free (O) and total (●) activities of acid phosphatase measured with β -glycerophosphate in homogenate of whole tissue.

substrate concentration. As pointed out by de Duve (1959, 1965) this property, which has also been observed with liver lysosomes (Appelmans & de Duve, 1955), indicates the lack of penetration of the substrate into the particles. Thyroid lysosomes differ from their hepatic homologues by a much greater resistance to incubation at pH 5 and 37°. In this respect they resemble more closely those of spleen and thymus (Rahman, 1964) and of bone (Vaes & Jacques, 1965; Vaes, 1965) and differ from the ox-thyroid lysosomes described by Balasubramaniam & Deiss (1965).

Homogenates prepared from suspensions of cells dispersed with collagenase contain a higher proportion of their hydrolases in latent and sedimentable form than do homogenates prepared from whole thyroid tissue. It is possible that the colloid contains relatively large amounts of soluble hydrolases that are removed by washing the cells, or the lysosomes may be better preserved in the cell than in the tissue homogenate. Thyroid tissue is very fibrous and its homogenization is undoubtedly associated with greater mechanical stresses than that of the dispersed cells. Greater damage to the lysosomes in the homogenate of whole tissue would therefore not be surprising.

In general, the results obtained support the hypothesis that the acid hydrolases investigated occur together in the same particles, as indicated by their closely parallel release under several different conditions. However, homogenates of isolated cells contain significantly more acid phosphatase than β -glucuronidase or cathepsin in the free form. This difference, which is not observed with homogenates of whole tissue, is open to several

Fig. 8. Influence of substrate concentration on free (O) and total (●) activities of (a) acid phosphatase measured with β -glycerophosphate and (b) β -glucuronidase. Incubation was carried out with (total activity) and without (free activity) Triton X-100. The ratio of the substrate concentration (s) to the velocity (v) is plotted against the substrate concentration. $-K_m$ is the intercept of the experimental straight line with the base line; the slope on the line represents $1/V_{max}$. (Dixon & Webb, 1964).

interpretations. Thyroid could, like liver (Neil & Horner, 1964), contain a soluble acid phosphatase in addition to the lysosomal enzyme, or the more fragile lysosomes could be richer in acid phosphatase than in the other enzymes, or again phosphatase present in soluble form in the suspension of isolated cells could be selectively retained by the cells through adsorption. Supporting the last-mentioned interpretation is the fact that the relative phosphatase content is somewhat higher in the homogenate of isolated cells than in the homogenate of whole tissue (Table 2). Another possible indication of heterogeneity is provided by the results of Fig. 6, which show a greater release of β -glucuronidase than of acid phosphatase under the influence of decreasing pH. This observation is not an artifact, since the two enzymes were assayed under identical conditions. In any case, some degree of enzymic heterogeneity cannot be considered a serious

objection against the hypothesis that the acid hydrolases are present together in a single population of lysosomes, since such heterogeneity is a common feature of these particles in other tissues as well.

In conclusion, the present results indicate strongly that lysosomes are present in thyroid cells. In particular, they support the interpretation that the acid-phosphatase-positive particles described in morphological studies are lysosomes and that they contain cathepsin, which could be involved in the hydrolysis of engulfed thyroglobulin.

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