Subcellular Structure of Bovine Thyroid Gland

THE LOCALIZATION OF THE PEROXIDASE ACTIVITY IN BOVINE THYROID

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1. After differential pelleting of bovine thyroid tissue the highest relative specific activities for plasma membrane markers are found in the L fraction whereas those for peroxidase activities (*p*-phenylenediamine, guaiacol and 3,3'-diaminobenzidine tetrachloride peroxidases) are found in the M fraction. 2. When M+L fractions were subjected to buoyant-density equilibration in a HS zonal rotor all peroxidases show different profiles. The guaiacol peroxidase activity always follows the distribution of glucose 6-phosphatase. 3. When a S_b fraction is subjected to Sepharose 2B chromatography three major peaks are obtained. The first, eluted at the void volume, consists of membranous material and contains most of the guaiacol peroxidase activity. Most of the protein (probably thyroglobulin) is eluted with the second peak. Solubilized enzymes are recovered in the third peak. 4. *p*-Phenylenediamine peroxidase activity penetrates into the gel on polyacrylamide-gel electrophoresis, whereas guaiacol peroxidase activity remains at the sample zone. 5. DEAE-Sephadex A-50 chromatography resolves the peroxidase activities into two peaks, displaying different relative amounts of the different enzymic activities in each peak. 6. The peroxidase activities may be due to the presence of different proteins. A localization of guaiacol peroxidase in rough-endoplasmic-reticulum membranes (or in membranes related to them) seems very likely.

It is generally believed that the iodination of thyroglobulin in thyroid is mediated by peroxidase. In most cytochemical studies peroxidase activity is measured by using benzidine (Armstrong *et al.*, 1975) or 3,3'-diaminobenzidine tetrachloride (Strum & Karnovsky, 1970) as co-substrate. In radioautographic studies peroxidase is detected by incorporation of radioactive iodide (Edwards & Morrison, 1976). In biochemical studies both guaiacol (Hosoya & Morrison, 1967) and *p*-phenylenediamine (Armstrong *et al.*, 1975) are used as co-substrate. Peroxidase activity can also be measured biochemically by iodination of tyrosine (Morrison, 1973) or mono-iodotyrosine (Neary *et al.*, 1973). Depending on the type of study, conflicting results are obtained for the subcellular localization of this enzyme activity. Cytochemical studies suggest a localization at the microvilli near the follicle lumen (Strum & Karnovsky, 1970) or in the colloid surrounding the microvilli (Novikoff *et al.*, 1974). In radioautographic studies the label is found either within the cells

(Edwards & Morrison, 1976) or in the follicle lumen (Strum & Karnovsky, 1970), depending on the perfusion technique used. Hosoya and co-workers (Hosoya *et al.*, 1973; Hosoya & Matsukawa, 1975; Hosoya *et al.*, 1971), using biochemical methods, claim that the iodination of thyroglobulin must take place in rough-endoplasmic-reticulum membranes. In the present paper we describe the distribution of peroxidase activity after differential pelleting and buoyant-density-gradient centrifugation of a M+L fraction in a HS zonal rotor. Also electrophoresis and column chromatography were applied.

Materials and Methods

Biological materials and tissue preparations

Biological materials were obtained and tissue preparation was carried out as described previously (Hilderson *et al.*, 1975).

Subcellular fractionation

Differential pelleting. Subcellular fractionation of bovine thyroid as described by Dierick & Hilderson (1967) resulted in a quantitative isolation of five subcellular fractions (N, M, L, P and S).

Abbreviations used: N fraction, nuclear fraction; M fraction, mitochondrial fraction; L fraction, light mitochondrial fraction; P fraction, microsomal fraction; S fraction, supernatant; M+L fraction, combined M and L fractions.

Subfractionation of the S fraction. The S fraction was centrifuged overnight (125000g, 16h). In this way four subfractions could be separated and collected through aspiration: S_a, a sediment at the bottom of the tube; S_b, a viscous red fraction located immediately above the sediment; S_c, a yellow fraction overlaying the previous one; S_d, the top fraction, a clear colourless supernatant.

Buoyant-density-gradient centrifugation of an M+L fraction in an HS zonal rotor. To obtain the M+L fraction, thyroid tissue was subjected to a two-step procedure. First 100g of minced tissue was treated in a 1 litre Waring Commercial Blendor homogenizer (250ml of 0.25M-sucrose/5mm-Tris/HCl, pH7.4, at high speed for 30s). The resulting suspension was then homogenized in a Ten-Broeck hand-homogenizer (Teflon pestle, five strokes). This homogenate was centrifuged at 100g for 10min to remove blood cells, connective tissue and cell debris. The supernatant was centrifuged (73300g, 15min), yielding an M+L fraction. After two washings in the same medium further subfractionation was carried out in an HS zonal rotor (MSE 18 high-speed centrifuge). The rotor was loaded at 1500rev./min by means of a variable-speed MSE gradient former with 20–50% (w/w) sucrose in 5mm-Tris/HCl buffer, pH7.4 (unless stated otherwise), through the edge of the rotor. When the rotor was completely filled with gradient a 12ml sample was introduced through the feed line to the centre, by using a syringe. The sample layer was then displaced with 50ml of overlay solution [5% (w/w) sucrose] and finally centrifuged at 9000rev./min. At the end of the centrifugation period (usually 24h), the zonal rotor was unloaded at 1500rev./min. Fractions (20ml) were collected manually by displacement with 55% (w/w) sucrose solution. As a routine 36 fractions were collected. As the HS zonal rotor is transparent it is possible to collect, during the run and under visual control, a part of the gradient containing a given peak and to replace it by new gradient. When the denser section of the gradient was to be removed the zonal rotor was unloaded at 1500rev./min by displacement with water through the feed line of the rotor. Fractions were collected through the edge of the rotor. When enough gradient was pumped out (with visual control) new gradient was introduced through the edge of the rotor (light solution first).

Enzyme assays

Catalase (EC 1.11.1.6). This was determined by recording the decrease of the A_{240} (disappearance of the H₂O₂). The reaction mixture was prepared by adding 1ml of 3% (w/w) H₂O₂ to 50ml of 50mM-potassium phosphate buffer, pH7.25. Into a cuvette 3ml of this reaction mixture and 0.2ml of enzyme solution were introduced and the A_{240} was recorded

at 25°C during 5min against a blank solution (no H₂O₂ added).

Peroxidase activities (EC 1.11.1.7). These were followed by using different methods. *p*-Phenylenediamine peroxidase activity was assayed by a slight modification of the method of Armstrong *et al.* (1975). To 0.7ml of 0.15M-potassium phosphate buffer, pH7.4, 0.5ml of enzyme solution and 50μl of 3% (w/v) *p*-phenylenediamine were added. The reaction was started by addition of 50μl of 1mM-H₂O₂. The A_{485} was followed at 20°C against a blank solution (no H₂O₂ added). Guaiacol peroxidase activity was determined by a modification of the method of Hosoya & Morrison (1967). To 0.7ml of 0.1M-potassium phosphate buffer, pH7.4, 33mM with respect to guaiacol, 50μl of 0.09M-glucose and 50μl of glucose oxidase (1–2 units) were added. The reaction was started by the addition of 0.5ml of enzyme solution. The A_{470} was followed at 20°C against a blank solution (no glucose added). To measure 3,3'-diaminobenzidine tetrachloride peroxidase activity, to 0.7ml of 0.15M-potassium phosphate buffer, pH7.4, 0.5ml of enzyme solution and 50μl of 3% (w/w) 3,3'-diaminobenzidine tetrachloride were added. The reaction was started by addition of 50μl of 1mM-H₂O₂. The A_{475} was followed at 20°C against a blank solution (no H₂O₂ added). Iodination of tyrosine was followed by measuring the rate of production of moniodotyrosine at 290nm (Morrison, 1973). The conversion of moniodotyrosine into di-iodotyrosine was followed as described by Neary *et al.* (1973). To obtain reasonable recoveries (up to 50%) the peroxidase activities, being very labile, had to be measured as soon as possible (on the same day of the experiment). To stabilize the enzyme preparations KI (0.1mM) (Neary *et al.*, 1973) was added to all homogenates, fractions and eluents, resulting in improved recoveries throughout (up to 120%). For all peroxidases the amount of enzyme which gave a change of 0.001 A unit/s was defined as 1 munit.

Marker enzymes

Monoamine oxidase (EC 1.4.3.4) (Mushahwar *et al.*, 1972; Wurtman & Axelrod, 1963) (outer mitochondrial membrane), cytochrome oxidase (EC 1.9.3.1) (Cooperstein & Lazarow, 1951) (inner mitochondrial membrane), acid phosphatase (EC 3.1.3.2) (Kind & King, 1954) (lysosomes), glucose 6-phosphatase (EC 3.1.3.9) (Morré, 1974), NADPH-cytochrome *c* reductase (EC 1.6.2.4) (Masters *et al.*, 1967) (endoplasmic reticulum), 5'-nucleotidase (EC 3.1.3.5) (Morré, 1974) and alkaline phosphatase (EC 3.1.3.1) (Hilderson *et al.*, 1975) (plasma membranes) and catalase (EC 1.11.1.6) (peroxisomes) were used as markers. P_i in glucose 6-phosphatase

assays was measured by the method of Rouser *et al.* (1970).

Chemical analyses

Extraction and fractionation of lipids. Lipids from the pooled fractions obtained by Sepharose 2B column chromatography of an S_0 fraction were extracted as described by Bligh & Dyer (1959). The lipids were further fractionated on a silicic acid column (Hilderson *et al.*, 1974). Cholesterol and lipid-bound P (total phospholipids) were determined as described by Rouser *et al.* (1970). Lipid-bound sialic acid was assayed as described by Lagrou *et al.* (1974). Sphingomyelin was measured by phosphorus determination after saponification of the phospholipid fraction with $HgCl_2$ as described by Abramson *et al.* (1965).

Protein. Portions (0.5 ml) 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.*, 1975).

RNA. This was determined by both u.v. spectrometry and phosphorus determination as described earlier (Hilderson *et al.*, 1975).

Extraction of peroxidase

Peroxidase activity was extracted from thyroid microsomal fraction with 0.05 M- Na_2CO_3 , pH 10.5, as described by Neary *et al.* (1973).

Polyacrylamide-gel electrophoresis

A sample (50 μ l, 50–100 μ g of protein) was layered on a 5% anionic gel (0.5 cm $\times$ 5.0 cm, cross-linking 2.5%) buffered with 0.1 M- Na_2CO_3 , pH 10.5, or 0.1 M-Tris/glycine, pH 8.3. A 4 mA current per gel was applied for 60 min at room temperature. Proteins were stained with 0.5% Amido Black dissolved in 7% (w/v) acetic acid. Separate gels were stained for peroxidase activity by immersing the gels in guaiacol, *p*-phenylenediamine or 3,3'-diaminobenzidine tetrachloride (incubation mixtures for enzyme assays, left overnight). Destaining was performed by immersion overnight in the same mixtures but in the absence of H_2O_2 and co-substrate.

DEAE-Sephadex A-50 column chromatography

A sample of the Na_2CO_3 extract (3 ml, 6 mg of protein/ml) was loaded on a DEAE-Sephadex A-50 column (2.5 cm $\times$ 12 cm) and eluted first with 50 ml of 0.01 M- Na_2CO_3 , pH 10.5, and then with 200 ml of a linear gradient of 0–0.6 M-NaCl in 0.01 M- Na_2CO_3 , pH 10.5. KI (0.1 mM) was added to the eluent to stabilize the enzyme preparation (Neary *et al.*, 1973).

Results

In a 1000g supernatant the peroxidase activities could be determined with guaiacol (0.00466 unit/mg of protein or 0.11 μ mol/min per mg of protein), 3,3'-diaminobenzidine tetrachloride (0.00030 unit/mg of protein), *p*-phenylenediamine (0.00283 unit/mg of protein). It was impossible to detect peroxidase activity with the moniodotyrosine and tyrosine iodination method. This could be due to interference of proteins (thyroglobulin ?).

Differential pelleting

The distributions of different markers and of peroxidase activities were investigated after isolation of the N, M, L, P, S_a , S_b , S_c and S_d fractions (Fig. 1). Thyroid mitochondria have a lower sedimentation coefficient than rat liver mitochondria (Dierick & Hilderson, 1967), necessitating higher centrifugal forces for sedimentation (37000g, 10 min). Under these conditions 5'-nucleotidase and alkaline phosphatase (plasma-membrane markers) show their highest relative specific activities in the L fraction. All other markers as well as the peroxidase activities are present at their highest relative specific activity in the M fraction. Therefore a localization of peroxidase in plasma membranes seems to be unlikely. To obtain a clear-cut conclusion an M+L fraction was further subjected to buoyant-density-gradient centrifugation in a HS zonal rotor.

Centrifugation of an M+L fraction in an HS zonal rotor

Effect of centrifugation time. M+L fractions (100–300 mg of protein) were centrifuged in an HS zonal rotor during 5, 10 or 24 h. After 5 h of centrifugation the mitochondria had reached the zone of density 1.16 g/cm³, their isopycnic density being 1.18. The other markers had hardly penetrated into the gradient. After 10 h of centrifugation most markers were located in the vicinity of their isopycnic zones. Satisfactory resolution was obtained between the subcellular components (Fig. 2). After centrifugation for 24 h two distinct zones could be observed, as was the case in buoyant-density-gradient centrifugation in a B-XIV zonal rotor (Hilderson *et al.*, 1975), but good resolution was lost. Cytochrome oxidase and monoamine oxidase coincided and were recovered in the second zone. Catalase equilibrated at the same density as the mitochondria. *p*-Phenylenediamine, guaiacol and 3,3'-diaminobenzidine tetrachloride peroxidase activities displayed a double peak distribution (one peak in each zone). However, their profiles did not completely coincide. As the mitochondria migrated faster through the gradient than the other subcellular components, the denser section of the gradient was replaced after centrifugation for 5 h.

Fig. 1. Distribution patterns after differential pelleting of a 0.25M-sucrose homogenate

1, Nuclear fraction; 2, mitochondrial fraction; 3, light mitochondrial fraction; 4, microsomal fraction; 5, S_0 fraction; 6, S_0 fraction; 7, S_0 fraction; 8, S_0 fraction.

The remaining subcellular components were further centrifuged for an additional 19h. The results of this experiment are shown in Fig. 3. No good resolution was obtained between the maxima of catalase, *p*-phenylenediamine, guaiacol and 3,3'-diaminobenzidine tetrachloride peroxidase. However, the peroxidase activities did not show identical profiles. None of them coincided with the profiles of the plasma-membrane markers.

Effect of heparin. Better resolutions were obtained

when heparin (50i.u./ml) was added to both homogenization medium and gradient (Fig. 4). Glucose 6-phosphatase equilibrated at lower densities (around 1.11g/cm³). This shift in buoyant density towards lower values is due to the release of ribosomes from rough-endoplasmic-reticulum membranes (Hilderson *et al.*, 1975). The peak of guaiacol peroxidase shifted along with glucose 6-phosphatase, whereas *p*-phenylenediamine peroxidase was less affected by the heparin procedure. Catalase (1.169g/cm³) equilibra-

Fig. 2. Centrifugation of an M + L fraction in an HS zonal rotor

Experimental conditions: 9000 rev./min for 10h. (a) —, Protein; ●—●, A_{260} ; ○—○, A_{280} ; -.-.-, slope of gradient. (b) -.-.-, 5'-Nucleotidase; ●—●, alkaline phosphatase; ○, glucose 6-phosphatase; —, acid phosphatase; ▲, cytochrome oxidase. (c) —, Catalase; ●, guaiacol peroxidase; ○, *p*-phenylenediamine peroxidase.

ted between the density of the second peak of *p*-phenylene-diamine peroxidase (1.15 g/cm^3) and that of the cytochrome oxidase-monoamine oxidase peak (1.172 g/cm^3). From the results of these heparin experiments a localization of guaiacol peroxidase (1.12 g/cm^3) in plasma membranes (1.13 g/cm^3) or peroxisomes (1.169 g/cm^3) seems unlikely.

Effect of pyrophosphate. Sachs (1958) pointed out that pyrophosphate is able to release ribosomes from rough-endoplasmic-reticulum membranes. Thus an M+L fraction was treated overnight at 4°C with (a) 5mM-sodium pyrophosphate or (b) 50mM-sodium pyrophosphate (pH 7.4, in 5mM-Tris/HCl buffer) and subjected to buoyant-density-gradient centrifugation (Fig. 5, no pyrophosphate added to the gradient). In the presence of 50mM-pyrophosphate a first small band containing membranous material (identified by phase-contrast microscopy) was formed after 30 min of centrifugation at the edge of the rotor; after 3h a second band appeared at that position

containing very small membranous fragments. The third band, formed after 5h, consisted chiefly of mitochondria (identified by biochemical methods). During the centrifugation, profiles of all markers except those for mitochondria shifted to lower values. The peroxidases shifted along with the second peak of glucose 6-phosphatase to lower densities (1.13 g/cm^3). In the mitochondrial fractions (1.18 g/cm^3) obtained by replacement of the gradient after 5h cytochrome oxidase and monoamine oxidase did not coincide, the former having migrated further into the gradient. Therefore, the outer membrane of mitochondria was very probably separated from the inner membrane. Treatment with 5mM-pyrophosphate was less drastic. The two mitochondrial markers did coincide and the other markers migrated further into the gradient than in the presence of 50mM-pyrophosphate. The peroxidase activities equilibrated at the second half of the distribution profile of glucose 6-phosphatase (1.14 g/cm^3).

Effect of digitonin with heparin. In a previous paper (Hilderson *et al.*, 1975) it has been reported that the addition of digitonin to the sample results in a shift to higher densities of both plasma and endoplasmic-reticulum membranes. To eliminate possible aggregation the experiment was repeated in the presence of 50 i.u. of heparin/ml to both medium and gradient (3.5mg of digitonin/ml sample; 10min). In those conditions the plasma membranes and rough-endoplasmic-reticulum membranes shifted in opposite directions, the former to higher densities (from 1.13 to 1.14 g/cm^3), the latter to lower densities (from 1.19 to 1.125 g/cm^3). Guaiacol peroxidase shifted along with glucose 6-phosphatase (to density 1.12 g/cm^3). *p*-Phenylenediamine peroxidase on the other hand displayed a smaller shift to lower densities (1.13 g/cm^3) and showed a broader distribution than guaiacol peroxidase.

Subfractionation of a S_0 fraction on Sepharose 2B. Fraction S_0 (2ml, 125mg of protein/ml), made up with equilibration solution to 6ml, was loaded on a Sepharose 2B column ($35 \text{ cm} \times 2.5 \text{ cm}$ equilibrated with $0.14 \text{ M-NaCl}/10 \text{ mM-Tris/HCl}$ buffer, pH 7.4) and eluted with the same solution. Then 24 fractions of 6.5ml were collected. The distribution profiles of some markers and peroxidase activities are represented in Fig. 6. From this Figure it is apparent that most markers are eluted in two peaks. A first peak was eluted with the void volume (determined with Dextran Blue). The second peak emerged at 130ml. To investigate this phenomenon both peaks were pooled and the lipid content (cholesterol, total phospholipids, sphingomyelin, lipid-bound sialic acid) was determined (Table 1). The bulk of the lipids was recovered in the first peak, indicating that this peak probably consisted of membranous material. Therefore a large part of the enzymes present in the S fraction is membrane bound (mol.

Fig. 3. Biphasic centrifugation of an *M + L* fraction in an HS zonal rotor

Experimental conditions: after an initial phase (9000 rev./min, for 5 h) the denser part of the gradient was replaced (*d, e, f*) and the centrifugation was continued for an additional 19 h at 9000 rev./min (*a, b, c*). (*a*) and (*d*): —, protein; ●, A_{260} ; ○, A_{280} ; ----, slope of gradient. (*b*) and (*e*): —, acid phosphatase; ----, 5'-nucleotidase; ●, alkaline phosphatase; ○, glucose 6-phosphatase; ▲, cytochrome oxidase. (*c*) and (*f*): —, catalase; ○, *p*-phenylenediamine peroxidase; ●, guaiacol peroxidase; ■, 3,3'-diaminobenzidine tetrachloride peroxidase.

wt. $> 10^7$); 3% of the proteins was recovered in the first peak. The bulk of the proteins eluted between the major peaks of the markers and was shown by spectrometry to consist mainly of thyroglobulin (maximum at 280 nm, minimum at 256 nm, shoulder near 290 nm). It was also shown that the second peak of glucose 6-phosphatase activity was entirely due to the presence of acid phenylphosphatase (Hilderson *et al.*, 1976). The enzyme activity at the void volume was due to the presence of a true glucose 6-phosphatase (only 1.3% interference of phenylphosphatase). The profiles of the peroxidase activities did not coincide. Guaiacol peroxidase was chiefly recovered

in the first peak. The 3,3'-diaminobenzidine tetrachloride peroxidase and *p*-phenylenediamine peroxidase were present in both peaks in comparable amounts. Catalase was predominantly recovered from the second peak (enzyme solubilized from the peroxisomes during the fractionation).

Gel electrophoresis of the peroxidases. The peroxidase activity was extracted from a P fraction with Na_2CO_3 as described in the Materials and Methods section. 3,3'-Diaminobenzidine tetrachloride, *p*-phenylenediamine and guaiacol peroxidase activity were detectable in the Na_2CO_3 extract. However, there was less 3,3'-diaminobenzidine tetrachloride

Fig. 4. Centrifugation of a heparin-treated *M+L* fraction in *na HS* zonal rotor

Experimental conditions: heparin (50 i.u./ml) was added to both medium and gradient. A 12 ml sample was injected and centrifuged during 23 h at 9000 rev./min. (a) —, Protein; ●—●, *A*₂₆₀; ○—○, *A*₂₈₀; ---, slope of gradient. (b) ---, 5'-Nucleotidase; ●—●, alkaline phosphatase; ○, glucose 6-phosphatase; ▲, cytochrome oxidase; ■, monoamine oxidase; —, acid phosphatase. (c) —, Catalase; ○, *p*-phenylenediamine peroxidase; ●, guaiacol peroxidase.

DEAE-Sephadex A-50 column chromatography. Na_2CO_3 extract (3 ml; 6 mg of protein/ml) was loaded on a DEAE-Sephadex A-50 column (2.5 cm $\times$ 12 cm) and eluted as described in the Materials and Methods section (Fig. 8). The recuperation for all peroxidase activities varied from 70 to 102%. All peroxidase activities displayed coinciding double-peak distributions. Part of all peroxidase activities did not adsorb on the column. The rest did elute with peak values at 0.3 M-NaCl. The percentage distribution for the individual peroxidase activities, however, was different in both peak fractions. In the first one the following series of decreasing peroxidase activities was found: 3,3'-diaminobenzidine tetrachloride, iodination of tyrosine, iodination of monoiodotyrosine, *p*-phenylenediamine, guaiacol. In the second peak fraction the decreasing series was the reverse: guaiacol, *p*-phenylenediamine, iodination of monoiodotyrosine, iodination of tyrosine, 3,3'-diaminobenzidine tetrachloride. This phenomenon can hardly be ascribed to the presence of one single enzyme protein. Catalase also displayed a double-peak distribution. However, the second peak fraction was eluted at 0.15 M-NaCl. This indicates that this enzyme does not have any peroxidase activity in those conditions.

Discussion

Generally one accepts that a peroxidase activity plays an essential role in the iodination process of thyroglobulin. Indeed, the physiological concentrations of iodine and iodide alone cannot account for the rapid iodination of thyroglobulin in the thyroid cells. Moreover, catalase inhibits the iodination through removal of endogenous H_2O_2 . Addition of exogenous H_2O_2 abolishes this effect (Tong, 1971). Furthermore, many peroxidases are capable of iodinating proteins (Pohl, 1976): horseradish peroxidase especially was studied intensively as a model for iodination of thyroglobulin (Morrison, 1973).

In the thyroid tissue the peroxidase activity is measured by different methods. In biochemical assays guaiacol peroxidase is preferentially used as the reaction catalysed seems to be a good model for the coupling reaction (Morrison, 1973). Aromatic phenols and amines have been frequently used for peroxidase determination in cytochemical studies. However, one must be cautious when interpreting the results of such experiments as haemoproteins, non-haem-iron proteins, copper-proteins etc. could also be able to convert these compounds (Morrison, 1973). Finally, one must keep in mind that it is not certain that the different co-substrates used in the different experiments are oxidized by the same enzyme. In this respect it is important to note that patients with Batten-Spielmeyer-Vogt disease are deficient for *p*-phenylenediamine peroxidase in both

peroxidase activity in this fraction; 50 μl of the Na_2CO_3 extract was subjected to polyacrylamide-gel electrophoresis. After electrophoresis at pH 10.5 the gel stained with Amido Black showed a major band and two minor components. The enzyme activity in the *p*-phenylenediamine-stained gel exactly corresponded with the major protein band. Guaiacol staining was only observed at the sample zone: this was also the case when horseradish peroxidase was subjected to an analogous procedure. No staining could be observed with 3,3'-diaminobenzidine tetrachloride. Similar results were obtained at pH 8.3; however, *p*-phenylenediamine peroxidase activity migrated further into the gel (Fig. 7).

Fig. 5. Biphase centrifugation of a pyrophosphate-treated *M + L* fraction in an *HS* zonal rotor

Experimental conditions: An *M + L* fraction was treated overnight at 4°C with 50mM-sodium pyrophosphate (pH 7.4 in 5mM-Tris/HCl buffer). A 12 ml sample was injected and centrifuged as described in Fig. 3. (a) and (d): —, protein; ●—●, A_{260} ; ○—○, A_{280} ; —, slope of gradient. (b) and (e): —, 5'-nucleotidase; ○, glucose 6-phosphatase; —, acid phosphatase; ▲, cytochrome oxidase; ■, monoamine oxidase. (c) and (f): ●—●, guaiacol peroxidase; ○, *p*-phenylenediamine peroxidase; ■, 3,3'-diaminobenzidine tetrachloride peroxidase.

leucocytes and thyroid tissue, although their thyroid function appears to be normal. Leucocytes of these patients, however, show normal guaiacol peroxidase concentrations. Furthermore, in cytochemical studies peroxidase activity was reported to be deficient in the thyroid tissue, but not in leucocytes of some patients with thyroid hypofunction (Armstrong *et al.*, 1975; Clausen & Jensen, 1975). English setters with

neuronal ceroid-lipofuscinosis show deficiency for *p*-phenylenediamine peroxidase in leucocytes, but not for guaiacol peroxidase (Patel *et al.*, 1974). Therefore one can conclude that different peroxidases are likely to exist, even in thyroid tissue. It also appears possible that guaiacol peroxidase could be the enzyme involved in the iodination of thyroglobulin.

Fig. 6. Chromatography of an S_6 fraction on Sepharose 2B. For the experimental conditions see the text. (a) —, Catalase; ○, *p*-phenylenediamine peroxidase; ●—●, guaiacol peroxidase; ■, 3,3'-diaminobenzidine tetrachloride peroxidase. (b) —, Acid phosphatase; ○, glucose 6-phosphatase; ▲, cytochrome *c* reductase; ----, 5'-nucleotidase; ●—●, protein.

Fig. 7. Polyacrylamide-gel electrophoresis of an Na_2CO_3 extract of a P fraction. See the Materials and Methods section for details. (a) Proteins stained with Amido Black; (b) *p*-phenylenediamine peroxidase activity; (c) guaiacol peroxidase activity; (d) horseradish guaiacol peroxidase. Arrows indicate the positions of proteins and peroxidase activities.

Table 1. Sepharose 2B chromatography of an S_6 fraction

Compound	Distribution of lipids (%)	
	Peak A (void volume)	Peak B
Cholesterol	85	15
Total phospholipids	70	30
Alkaline stable phospholipids	70	30
Lipid-bound sialic acid	70	21

The results reported in the present paper are not in contradiction with this point of view. (1) During gel electrophoresis *p*-phenylenediamine peroxidase activity migrates into the gel, while guaiacol peroxidase activity remains in the sample zone. (2) The DEAE-Sephadex A-50 elution profiles of both activities are different. (3) Different Sepharose 2B elution profiles (S_6 fraction) are also obtained for guaiacol peroxidase and *p*-phenylenediamine peroxidase activities. (4) Buoyant-density-gradient centrifugation results in different distribution profiles, especially in the presence of heparin. (5) 3,3'-Diaminobenzidine tetrachloride activity is localized either in the

guaiacol peroxidase or in the *p*-phenylenediamine peroxidase activity zones. In the electrophoresis experiments 3,3'-diaminobenzidine tetrachloride peroxidase activity was not detectable. When reviewing the literature relating to the subcellular localization of peroxidase activities and iodination process conflicting results and interpretations become apparent. Strum & Karnovsky (1970), using a cytochemical method with 3,3'-diaminobenzidine tetrachloride as co-substrate, find peroxidase activity in perinuclear cisternae, endoplasmic reticulum cisternae, inner lamellae of the Golgi apparatus, vesicles at the apical side of the thyroid cell and microvilli near the follicle lumen. On the basis of these results they tend to localize the iodination process at the microvilli although they cannot preclude iodination in apical vesicles. However, Hosoya *et al.* (1973) using similar experiments localize iodination in the endoplasmic reticulum. Novikoff *et al.* (1974) claim that iodination is taking place in the colloid surrounding the microvilli. Conflicting results are also obtained when applying radioautography. Indeed, Strum & Karnovsky (1970) find labelling in the follicle lumen after 10s of incorporation. Croft & Pitt-Rivers (1970) on the other hand found the label inside the cells

Fig. 8. Chromatography on DEAE-Sephadex A-50 of an Na_2CO_3 extract of a P fraction

For experimental conditions see the Materials and Methods section. (a) —●—, Catalase; ●---●, iodination of monoiodotyrosine; ■—, iodination of tyrosine; —, slope of NaCl gradient. (b) ●---●, Guaiacol peroxidase; ○, *p*-phenylenediamine peroxidase; ■, 3,3'-diaminobenzidine tetrachloride peroxidase.

provided that the incorporation time does not exceed 55s. When fixation is delayed for 2min the label is predominantly found over the peripheral region of the follicle lumen. Therefore, iodide seems to be captured initially within the cells. However, it was not clear whether or not the binding protein is thyroglobulin. In agreement with these results Edwards & Morrison (1976) demonstrated that after prefixation of the tissue the label is localized within the cells and not in the follicle lumen. The bulk of the label was found at the level of the endoplasmic reticulum. Using biochemical methods Hosoya *et al.* (1971) suggest the localization of guaiacol peroxidase to be in the rough endoplasmic reticulum. From their experiments it is clear that the peroxidase cannot be localized in the plasma membranes, in the follicle lumen, the Golgi apparatus or the mitochondria. A clear-cut localization, however, could not be provided. Summarizing, one can say that iodination seems to be a very rapid process and that the conflicting results found in the literature could be due to the speed of this phenomenon.

From our experiments the following conclusions can be drawn. (1) The peroxidase activities are sedimentable. They concentrate predominantly in the M fraction. From the distribution patterns it can be concluded that a localization in plasma membranes is

unlikely. (2) Guaiacol peroxidase activity is almost completely membrane bound, as it is eluted with the void volume when performing chromatography on Sepharose 2B and is never observed at the far left end of the gradient during buoyant-density-gradient centrifugation. (3) The distribution profile of 3,3'-diaminobenzidine tetrachloride peroxidase activity is different from those of the other peroxidase activities (Fig. 3). Its elution profile on Sepharose 2B (Fig. 6) differs from the guaiacol profile. 3,3'-Diaminobenzidine tetrachloride peroxidase activity cannot be demonstrated when performing gel electrophoresis (Fig. 7) while *p*-phenylenediamine peroxidase and guaiacol peroxidase activities can be easily demonstrated in those conditions. (4) Guaiacol peroxidase and *p*-phenylenediamine peroxidase activities are probably attributable to separate proteins (Figs. 2, 3, 4, 6 and 7). (5) Guaiacol peroxidase activity is localized in rough-endoplasmic-reticulum membranes. This is substantiated by the distribution profiles in the zonal rotor, that always follow the distribution of glucose 6-phosphatase in the gradients. This is particularly clear-cut when comparing the buoyant densities in the absence and in the presence of heparin, digitonin or pyrophosphate (Figs. 3, 4 and 5). The guaiacol peroxidase peaks never coincide with the maxima for plasma-membrane markers. As guaiacol peroxidase always coincides with a part of the second peak of glucose 6-phosphatase one can say that this enzyme activity belongs to a specialized region of the rough endoplasmic reticulum or to membranes very closely related to them, e.g. apical vesicles (Strum & Karnovsky, 1970) or A granules (Novikoff *et al.*, 1974).

The question then arises where exactly the iodination process itself occurs. From our experiments rough-endoplasmic-reticulum membrane localization of the peroxidase activity involved is very likely. However, it is therefore not absolutely certain that the iodination does occur where the bulk of the guaiacol peroxidase is localized. Indeed, one could argue that the peroxidase, synthesized at the level of the rough-endoplasmic-reticulum membranes and stored there, is ultimately functioning in small amounts (not as yet detectable) elsewhere in the thyroid cell where H_2O_2 is provided. One could also argue that the distribution profiles recorded in our experiments are simply the distribution profiles of the so-called Novikoff A-granules, that do not behave differently from rough-endoplasmic-reticulum membranes. Finally, if, as suggested by Novikoff, iodination occurs in the colloid fluid, we want to stress that the peroxidase must remain membrane bound.

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