APPEARANCE AND FUNCTION OF ENDOGENOUS PEROXIDASE IN FETAL RAT THYROID

JUDY M. STRUM, JANICE WICKEN, JOHN R. STAN BURY, and MORRIS J. KARNOVSKY

From the Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115, and the Unit of Experimental Medicine, Department of Nutrition and Food Service, Massachusetts Institute of Technology, Cambridge, Massachusetts 02137

ABSTRACT

Iodination within the thyroid follicle is intimately associated with a thyroid peroxidase. In order to locate the in vivo site of iodination, the initial cytochemical appearance of this enzyme has been determined in fetal rat thyroid and its presence correlated with the onset of iodinated thyroglobulin synthesis. Peroxidase first appears in follicular cells during the 18th day of gestation. It is seen first in the perinuclear cisternae, the cisternae of the endoplasmic reticulum, and within the inner few Golgi lamellae. These organelles presumably represent sites of peroxidase synthesis. During the 19th and 20th days of gestation, there is a tremendous increase in peroxidase activity. In addition to the stained sites described, there are now many peroxidase-positive apical vesicles in the follicular cells. Newly forming follicles stain most conspicuously for peroxidase, the reaction product being heavily concentrated at the external surfaces of apical microvilli and in the adjacent colloid. Iodinated thyroglobulin becomes biochemically detectable in thyroids during the 19th day of gestation and increases greatly during the 20th day. The parallel rise in peroxidase staining that just precedes, and overlaps, the rise in iodinated thyroglobulin, suggests that apical vesicles and the apical cell membrane are the major sites of iodination within the thyroid follicle.

INTRODUCTION

The rat thyroid gland does not begin to function until late in fetal life (8, 9). During the 17th day of gestation thyroglobulin, or a precursor with similar immunochemical specificity, has been identified in rat thyroid by fluorescence microscopy (8). This material increases in amount from the 17th to the 20th day of gestation, and the iodide concentration of the gland increases exponentially during this time. Such evidence suggests the onset of thyroid hormone synthesis.

The process of synthesizing the biologically active thyroid hormones triiodothyronine (T₃) and thyroxine (T₄) includes the following steps (26). Iodide is actively transported from the circulation into the follicular cell. In the cell it becomes oxidized to an ionic species that, in turn, iodinates the tyrosyl residues of thyroglobulin to yield, first, monoiodotyrosine (MIT) and then diiodotyrosine (DIT). The coupling of these iodotyrosines results in the formation of T₃ and T₄. It is thought that the oxidation of iodide to a state in which it can iodinate tyrosyl residues is catalyzed by a thyroid peroxidase (26). Therefore, until this enzyme is present in developing follicular cells it is assumed that the iodination of thyroglobulin (and hormone synthesis) does not occur.

A cytochemical technique has made it possible to localize endogenous thyroid peroxidase at the fine structural level (31). This method was...
applied to fetal rat thyroids in order to determine when and where peroxidase first appears in differentiating follicular cells, and to establish whether or not its presence is correlated with the initial formation of iodinated thyroglobulin.

**MATERIAL AND METHODS**

**Fine Structure and Cytochemistry**

38 pregnant Sprague-Dawley rats, ranging in gestation from the 15th through the 20th day, were used in this study. (The 1st day of gestation was determined by the presence of a vaginal plug.) For electron microscopy and cytochemistry, 82 embryos from 15 rats were visualized in a dissecting microscope and their thyroid glands were carefully removed. These included five embryos in the 15th day of gestation, six in the 17th day, 25 in the 18th day, 37 in the 19th day, and nine in the 20th day. The thyroids were cut into tiny pieces and fixed at room temperature for 40 min in either 3% or 5% strength paraformaldehyde-glutaraldehyde fixative (14), then transferred to 0.2 M sodium cacodylate buffer, pH 7.4, and stored overnight in the refrigerator. For fine structural study, the tissue was postfixed either in Millonig’s osmium tetroxide-containing fixative (20) or in 2% OsO₄ buffered to pH 7.4 with 0.1 M cacodylate buffer (2), before being dehydrated in ethanol and embedded in Epon (18). For the cytochemical demonstration of endogenous peroxidase activity, thyroid tissue was incubated before postosmication in 0.05 M Tris-HCl buffer, pH 7.6, containing 0.05% 3,3’-diaminobenzidine tetrachloride (DAB) (Sigma Chemical Co., St. Louis, Mo.) and 0.0025% H₂O₂ (31).

In order to determine the presence of peroxidase activity, 3-µ-thick sections of incubated thyroid tissue were cut. They were examined in a Zeiss Ultraphot II light microscope, and representative ones were photographed on Kodak Panatomic-X film. Thin sections were then cut on an LKB microtome. The sections were either left unstained, stained lightly with lead citrate (38), or double-stained with uranyl acetate (39) and lead before being examined in a Philips 200 electron microscope.

**Determination of Iodinated Thyroglobulin**

For the determination of iodinated thyroglobulin, rats in the 17th, 18th, 19th, and 20th days of gestation were used. 24 hr before the removal of the fetal thyroids, the pregnant rats were injected intraperitoneally with 15 µCi of Na¹²⁵I (New England Nuclear Corp., Boston, Mass.). Thyroid glands from 34 embryos in the 17th day of gestation, 44 in the 18th day, 29 in the 19th day, and 46 in the 20th day of gestation were removed. Glands from separate ages were pooled in 2 ml of 0.26 M sucrose (buffered to pH 7.4 with 0.1 M Tris-HCl buffer), and homogenized at 4°C. The homogenates were centrifuged for 60 min at 105,000 g in a Beckman L2-65 ultracentrifuge with a Spinco Type 50 Ti rotor head (Beckman Instruments). The supernatants were removed and dialyzed at 4°C against 0.01 M phosphate buffer, pH 7.4, for 3 hr (in order to eliminate the sucrose) before being layered on 5-20% sucrose gradients. The sucrose gradients were centrifuged at 65,000 g for 40 hr in a Spinco SW 25.3 rotor head. Human thyroglobulin standards were treated identically in order to permit comparison of their 19S peak with those in the fetal thyroid samples. The sucrose gradient fractions were read at 280 µm in a Beckman DC spectrophotometer and graphed with a Gilford automatic absorbance recorder (Gilford Instrument Company, Oberlin, Ohio). The fractions were then collected and counted for ¹²⁵I activity in a Nuclear-Chicago Automatic Gamma well counter, Model 1085 (Nuclear-Chicago Corporation, Des Plaines, Ill.). Protein was determined by a modification of the method of Lowry et al. (17)."

**Observations**

**Cytochemical Observations**

Light microscope observations of sections from fetal rat thyroids removed during the 17th day of gestation do not show cytochemical evidence of endogenous peroxidase activity (Fig. 1). At this stage of maturation, the cells are still arranged in epithelial sheets, although an occasional cluster of cells may be observed. Observations of thyroid glands from the 18th day of gestation reveal the first cytochemical appearance of endogenous peroxidase within the cells. This earliest detectable sign of the enzyme is easily overlooked in 3-µ-thick sections of incubated tissue (Fig. 2). (The intense staining in red blood cells is due to the peroxidatic effect of hemoglobin, Fig. 2.) Careful scrutiny of the sections is necessary in order to distinguish some cells which display a faint cytoplasmic staining. On the basis of light microscopy alone, however, one would have difficulty being certain that thyroids in the 18th day of gestation possess peroxidase activity. At this stage of development, although we do not have immunological proof of thyroglobulin, nor do we have proof of the presence of iodinated amino acids, what we are determining is the appearance of a protein at the position on the sucrose gradient where thyroglobulin runs, and which is iodinated with ¹²⁵I.
opment, most of the cells are clustered together in groups, although follicles have not yet formed.

By electron microscopy, peroxidase activity is still not evident within the cells of thyroids removed from embryos during the 17th day of gestation (Fig. 5). The cells lie clustered together and are attached to one another through prominent junctions. Their cytoplasm is filled with ribosomes, and occasionally a rare profile of rough endoplasmic reticulum can be seen. An occasional cell exhibits what appears to be the beginning of a Golgi region, but membranes are very scarce at this stage of development, and ribosomes (scattered free or organized into polyribosome aggregates) predominate in the cytoplasm. The cells are actively dividing, and their prominent nucleoli are commonly observed.

Electron microscope observations verify the presence of thyroid peroxidase during the 18th day of gestation (Figs. 6, 7). Early in the 18th day, only an occasional cell displays the reaction product, and so unless unstained sections are examined it is easy to overlook this earliest cytochemical evidence of the enzyme. The perinuclear cisternae and the cisternae of the endoplasmic reticulum are sites at which the staining is first evident (Fig. 6). Although only a few profiles of rough endoplasmic reticulum exist in the cells at this stage of development, they all appear to contain the dark cytochemical reaction product. A number of small vesicular inclusions lying along the peripheral borders of the cells also contain the reaction product (Fig. 7). Although the origin of these inclusions cannot always be ascertained, many of them have been identified as cross-sectioned profiles of the rough endoplasmic reticulum. The Golgi apparatus is present and in fortuitous sections its inner few lamellae stain for peroxidase (Fig. 7). In most cells cytoplasmic ribosomes do not stain for peroxidase, but sometimes in cells that have just divided the ribosomes are darker and appear to be stained. Occasionally, the outer border of lipid droplets (Fig. 6) and the membranes of mitochondria (Fig. 9) are also positive. Microvilli exist along the cell surfaces and often intervene to separate the attached cells. Occasionally, just outside the microvilli, colloid-like material is seen, but neither it nor the membranes of the microvilli stain for peroxidase activity. These areas in which colloid-like material exists presumably represent the beginning of follicle formation. They are thought to become continuous with the deep extracellular spaces that invaginate into the apical regions of cells in 18-day fetal rat thyroids. As maturation continues, the extracellular colloid-containing regions will expand as more colloid is secreted by the cells. When a single layer of follicular cells envelops

Figures 1-4 are unstained 3-μ thick sections of rat thyroid glands that have been incubated for the cytochemical localization of endogenous thyroid peroxidase. The dark reaction product represents sites of peroxidase activity.

Figure 1  17th day of gestation. The cells do not yet show cytochemical evidence of peroxidase. Intercellular spaces (arrow) mark the boundaries between cells which are attached to one another in epithelial sheets. A single red blood cell stains darkly owing to the peroxidatic effect of its contained hemoglobin. X 40.

Figure 2  18th day of gestation. The cells have clustered into groups, but follicles have not yet formed. At this stage peroxidase activity is first detectable, as evidenced by the dark reaction product outlining nuclei (N) and staining the cytoplasm in a few of the cells (arrow). Red blood cells are intensely stained. X 40.

Figure 3  19th day of gestation. An increase in peroxidase activity is clearly evident in this section. The dark reaction product is heavily concentrated at the cell-colloid interface of newly formed follicles (arrows). The cytoplasm of follicular cells also stains for peroxidase activity, outlining the unstained nuclei (N) X 40.

Figure 4  Perfused adult thyroid gland. Endogenous thyroid peroxidase activity is seen within follicular cells. Although microvilli are not resolvable at this magnification, the cytochemical reaction product is concentrated along the apical cell borders (arrows) and often appears as a dark line. Follicular cell nuclei (N) and parafollicular cells (P) do not stain for peroxidase. X 45.
FIGURE 5 Electron micrograph of a rat thyroid gland in the 17th day of gestation that has been incubated for thyroid peroxidase activity. The cells do not yet display cytochemical evidence of peroxidase. They lie clustered together in sheets, and, although wide extra-cellular spaces separate them (ES), they are attached to one another through prominent cell junctions (J). Clusters of ribosomes fill the cytoplasm of these cells, and peripheral microvilli (MV) project from them. A few scanty profiles of endoplasmic reticulum (ER) are just beginning to make their appearance. Section stained lightly with lead citrate. X 12,400.

the colloid-like material, a typical follicle will be formed. The cells lining a mature follicle are polarized so that their apical microvilli project into the colloid and their basal surfaces lie oriented towards the parafollicular capillaries.

A sharp increase in endogenous thyroid peroxidase is observed during the 19th and 20th days of gestation (Figs. 3, 8, 9). At this time, the cells have become organized into follicles. In the light microscope the staining is seen heavily concentrated in the colloid of newly forming follicles, near the apical ends of follicular cells (Fig. 3). The product accumulates in such a way as to outline the periphery of the colloid (Fig. 3). Unstained follicular cell nuclei are also clearly outlined by the reaction product contained within their nuclear envelopes.

Sections of rat thyroids removed during the 19th day of gestation and observed in the electron microscope further emphasize the tremendous increase in peroxidase activity (Figs. 8, 9). The reaction product is heavily concentrated at the apical cell borders, dramatically outlining the microvilli that project into the newly synthesized colloid. The cytochemical reaction is so intense in these areas that intracellular sites staining for peroxidase are faint by comparison (Fig. 9). In addition to the concentration of reaction product along the external apical cell surfaces, peroxidase-stained apical vesicles now appear within the follicular cells. In thyroids removed during the 19th day of gestation such vesicles were essentially absent, but in 19- and 20-day fetal thyroids many...
FIGURE 6 Sections of thyroid glands in the 18th day of gestation show the cells clustered into groups, since this is just before the stage of follicle formation. Thyroid peroxidase begins to make its cytochemical appearance in rat thyroids at this stage of development. The dark reaction product is clearly demonstrated in the perinuclear cisternae (PC) and the endoplasmic reticulum (ER). The periphery of a lipid droplet (LD) also displays the cytochemical reaction product. Section stained lightly with lead citrate. × 9300.

Stained apical vesicles are seen in the follicular cells (Fig. 9). By a process of exocytosis, these vesicles apparently transfer their contents externally (Fig. 10), which could account (at least in part) for the intense cytochemical reaction product observed along the apical cell borders.

Intensely stained follicles, such as those in Figs. 8 and 9, also occur in 20-day fetal thyroids, but by this time most follicles have expanded so that the dark reaction product remains concentrated at the outer surfaces of the microvillous membranes and in the adjacent colloid (Fig. 11). This is also characteristic of the adult rat thyroid (31). However, in the adult gland the follicular cells themselves also stain heavily for peroxidase activity (Fig. 4).

Biochemical Observations

Sucrose gradients of homogenates from fetal rat thyroids removed during the 17th and 16th days of gestation showed no evidence of thyroglobulin and did not accumulate 125I. The accumulation of radioactive iodide first became evident during the
FIGURE 7  Section from a thyroid gland in the 18th day of gestation showing cellular sites of endogenous peroxidase activity. The perinuclear cisternae (PC), endoplasmic reticulum (ER), peripheral vesicles (PV), and an inner Golgi lamella (single arrow) contain the dark reaction product. Cisternae of the endoplasmic reticulum closely associated with a Golgi region stain markedly (double arrow). Well-developed Golgi areas (G) and a cross-sectioned centriole (C) are also evident. Section stained lightly with lead citrate. $\times 21,000$.

19th day of gestation. At this time, there were 170 cpm/mg protein, or a total of 209 counts in 29 glands. Of these counts, 52.7% were in the supernatant (19S region of the gradient) obtained by centrifuging the samples for 1 hr at 105,000g. There was a sharp increase in $^{131}$I counts in the 19S thyroglobulin region of homogenates from...
thyroids taken during the 20th day of gestation. During this stage there were 9096 cpm/mg protein, or 30,926 counts in 46 glands. Of these counts, 60.5% were contained in the supernatant remaining after the samples were centrifuged for 1 hr at 105,000 g.

DISCUSSION
Iodination of the tyrosyl residues in thyroglobulin is an essential step in the synthesis of thyroid hormones. After being actively transported into the follicular cell, iodide must be oxidized to a higher oxidation state before it is able to iodinate tyrosyl...
residues. In the thyroid gland, this is accomplished in the presence of \( \text{H}_2\text{O}_2 \) and is thought to be a peroxidase-mediated step (see Taurog and Howells for review of the literature, [33]). The exact ionic state of the oxidized iodinating intermediate is unknown, but it might be an iodinium ion \( (1^+) \) or a hypoiodite ion \( (10^-) \) (7). Although it has been proposed that an enzyme distinct from thyroid peroxidase might be required for iodinating the tyrosyl residues of thyroglobulin (29), more recent studies indicate that the site of oxidation is also probably the site of iodination, since the chemical activity of the oxidized iodide intermediate would be too great for its free existence in the tissue (37).

The site within the thyroid follicle in which iodination occurs remains a subject of controversy. Radioautographic studies employing radioactive iodide have yielded conflicting results. Several investigators have reported that very soon after administering radioactive iodide to animals, the label (representing protein-bound iodine) appears concentrated around the periphery of the colloid (21, 30, 40); sections through such follicles often reveal a ring reaction, and this has been taken as evidence that iodination occurs extracellularly in the colloid, near the apical ends of the follicular cells. In contrast, other investigators have demonstrated the silver-grain label overlying the follicular cells (27), suggesting that iodination may occur within them. High-resolution electron microscope
**Figure 10** Apical portion of follicular cell from rat thyroid gland in the 20th day of gestation. Membrane-bounded apical vesicles (AV) contain peroxidase. Apical vesicles appear to release their contents into the follicular colloid (C) by a process of exocytosis (arrow). The external membranes of apical microvilli (M) stain for peroxidase. Section stained lightly with lead citrate. × 49,000.

**Figure 11** Section through a follicle from rat thyroid in the 20th day of gestation that has been incubated for peroxidase. The storage of more colloid (C) expands the follicles, but peroxidase activity remains concentrated (as a dark reaction product) near the apical microvilli of follicular cells (bracket). Stained lightly with lead citrate. × 23,000.

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radioautographs have repeatedly shown silver grains at the cell-colloid interface (19, 30), leading to the theory that the apical microvillous cell border is the site at which iodination takes place. In contrast, biochemical studies have consistently localized thyroid peroxidase activity in a particulate fraction of thyroid tissue (associated with mitochondria and microsomes) (1, 3, 4, 11, 12, 16), implicating an intracellular site of iodination. This correlates with experimental evidence that isolated follicular cells obtained by trypsinization are able to form iodide-labeled proteins (13, 25, 28, 34, 35). These labeled proteins are primarily iodothyronines, but small amounts of iodothyronines have also been detected (13, 35). Isolated thyroid cells grown in tissue culture in the presence of 131I have also been reported to form very small amounts of iodinated proteins, including T3 and T4 (13, 28). These findings indicate that the follicular cell is capable of incorporating radiiodide into organic combination, but the very small amounts of detectable T3 and T4 suggest that most enzymes necessary for the formation of these active hormones lie at the cell membrane or in the colloid itself. Trypsinization might easily damage enzymes of the cell membrane and could explain why silver grains (representing iodinated proteins) are not found underlying the plasmalemma in isolated follicular cells (34).

As a new approach to identifying the in vivo site of iodination within the thyroid follicle, we applied a cytochemical technique for thyroid peroxidase to adult rat thyroid (31). However, in the adult gland it was difficult to separate sites of synthesis from sites of iodination. For this reason, fetal thyroids were selected for the present study in order to pinpoint the time in development at which the differentiating cells first display evidence of thyroid peroxidase and become capable of iodinating thyroglobulin (in the process of hormone synthesis).

Our cytochemical observations of thyroid peroxidase activity suggest that its route of synthesis is similar to that of thyroglobulin (6, 10, 23, 31). The protein moiety of peroxidase is synthesized in the rough endoplasmic reticulum and, as we have observed, the cytochemical reaction product is characteristically found in the cisternae of this organelle. Peroxidase staining within only the inner few Golgi lamellae implies that the enzyme receives carbohydrate groupings at this site (24). Carbohydrate has been identified in bovine thyroid peroxidase (32). The presence of peroxidase in apical vesicles is commonly observed in differentiated follicular cells. Radioautographic evidence (10, 23) and biochemical-morphological findings (6) indicate that apical vesicles are associated with the transport of thyroglobulin to the follicular lumen. The present study suggests that a population of apical vesicles also seems to provide a route for the transfer of peroxidase to the outer cell membrane surface and/or into the colloid (by a process of exocytosis, see Fig. 10).

The site at which iodination occurs is not so obvious as the pathway of peroxidase synthesis. If an organelle within the follicular cell, such as the endoplasmic reticulum, were the primary site of iodination, one would perhaps expect to observe a noticeable increase in cytoplasmic staining as hormone synthesis begins. This is not observed in fetal rat thyroids when hormone synthesis commences during the 19th and 20th days of gestation. However, a marked cytochemical staining alteration does occur in fetal rat thyroids at this time. The surfaces of the apical microvilli and the colloid just adjacent to them now stain intensely for peroxidase activity. The dark reaction product is so concentrated at this site that all other cellular sites staining for the activity of the enzyme are pale by comparison (see Figs. 8 and 9). Even at the light microscope level, sections clearly reveal a heavy concentration of reaction product at the cell-colloid interface (Fig. 3). Correlated with this intense apical border staining are increased numbers of peroxidase-stained apical vesicles, which were rare in follicular cells from thyroids in the 18th day of gestation. Morphological evidence indicates that apical vesicles release their contents into the colloid (Fig. 10) and might well account for the presence of the dark reaction product in the colloid just next to the microvilli.

Our biochemical findings further substantiate these cytochemical observations. Thyroids in the 17th day of gestation showed no iodinated thyroglobulin, and the cells did not stain for peroxidase activity. In the 18th day of gestation, follicular cells began to show cellular sites of peroxidase staining, but their sparse microvilli and the scanty colloid-like material seen accumulating just outside of them showed no evidence of peroxidase activity. Moreover, iodinated thyroglobulin was not yet biochemically detectable in sucrose
gradients of thyroids removed at this stage of development.\(^2\) It would appear that peroxidase is being synthesized by follicular cells during this time, but it presumably is not yet functioning in the iodination reactions of thyroid hormone biosynthesis (at least not to an extent where our technique can measure it).

During the 19th day of gestation, iodinated thyroglobulin becomes biochemically detectable. This coincides with the sharp increase in peroxidase activity that is observed cytochemically along the apical cell borders. Apparently, the peroxidase is now functioning in hormone synthesis to catalyze reactions that result in the iodination of thyroglobulin. The intense apical cell border staining for peroxidase is sustained throughout the 20th day of gestation when biochemically there is a striking increase in iodinated thyroglobulin and when follicle formation is well underway. These observations indicate that a marked cytochemical increase in peroxidase activity just slightly precedes, but also overlaps, the rapid rise in iodinated thyroglobulin. This correlation could be coincidental, but biochemical investigations have demonstrated the ability of thyroid peroxidase to iodinate tyrosine in vitro (3, 4, 16, 29). For this reason, and because of the findings reported here, it is concluded that endogenous thyroid peroxidase functions in vivo to catalyze the oxidation reactions that result in the iodination of thyroglobulin.

The experimental results obtained by many investigators (5, 15, 22, 23, 36) indicate that the enzymes for the iodination of thyroglobulin exist in the colloid, rather than within the follicular cells. Our cytochemical observations do not support this theory completely, for the following reasons. If the enzymes for iodination occur within the colloid, one would expect peroxidase activity to be dispersed evenly throughout the colloid contained within the follicles, but this is not observed (see Fig. 3). Peroxidase activity is associated only with colloid just next to the external surfaces of apical microvilli. Therefore, it appears that the peroxidase is, for the most part, probably bound to the cell membrane or cell coat thus preventing its diffusion into the interior of the follicular colloid. The presence of peroxidase activity at the apical cell border can be accounted for by the process of exocytosis from apical vesicles. In fact, by exocytosis, the inner surface of the membrane of a peroxidase-positive apical vesicle would contribute to, and become a part of, the outer cell membrane surface. However, the exact time at which iodination occurs in this process cannot be ascertained in the present investigation. Apical vesicles have been shown to contain thyroglobulin in transport to the follicular lumen (10, 23), and because peroxidase activity is associated with apical vesicles it is possible that iodination might occur within them. However, from our observations that the greatest concentration of peroxidase reaction product is associated with the surfaces of apical microvilli and the cell border, it would appear that the apical cell membrane is the major site of iodination within the thyroid follicle.

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