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Ultrastructural and Cytochemical Studies on the Thyroid Gland of Normal Metamorphosing Frogs (*Rana japonica* Guenther). II. Peroxidase Activity

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Summary. The peroxidase activity in the thyroid gland of developing frogs, *Rana japonica* Guenther, has been observed in the course of metamorphosis. The enzyme first appears in the vesicles near the Golgi complex or near the plasma membrane and sometimes in the cisternae of the inner parts of Golgi lamellae in the early premetamorphosis. As cytoplasmic organelles develop, the density of the reaction product and the number of positively reacted vesicles increase gradually. During climax metamorphosis, the peroxidase activity reaches its maximum. The reaction product is found in the numerous vesicles and multivesicular bodies at the apical cytoplasm, and many vesicles and the cisternae of a few inner lamellae of the Golgi complex. Figures showing reverse pinocytosis of the apical peroxidase positive vesicles into the follicular lumen are often seen.

Four tadpoles at stage XX and XXI were kept in water containing ^{125}I for 2 or 50 hrs, and sacrificed. After the technique for the cytochemical demonstration of endogeneous peroxidase activity, autoradiographic procedures were performed for simultaneous demonstration of peroxidase reaction and silver grains of ^{125}I were mostly localized on the colloid lumen and few were found on the peroxidase positive vesicles in the cytoplasm. It is suggested that the apical cell membrane region and peripheral colloid lumen are the major sites of iodination of thyroglobulin.

It is well known that the thyroid plays an essential role in amphibian metamorphosis. The fine structural changes of the amphibian thyroid during metamorphosis have been published by several authors (COLEMAN et al., 1968; NANBA, 1972a). The present author has also demonstrated the functional changes of the ultrastructural localization of the acid phosphatase activity in the developing frog thyroid in the previous paper (NANBA, 1972a). There were many droplets in the thyroid follicular cells at the climax metamorphosis that were positive for the acid phosphatase reaction. It is suggested that the thyroid hormone is actively released from the thyroid in this period. On the other hand, as to the ^{131}I uptake KAYE (1961) has found a 10-fold increase in prometamorphosis and a 40-fold increase near climax.

The thyroid peroxidase has been believed to be necessary for the iodination of thyroglobulin. In the present paper, the fine structural changes of the localization of peroxidase during metamorphosis is demonstrated and the site of iodination of thyroglobulin is discussed. In the preparations used in this study cytochemical demonstration for endogeneous peroxidase and ^{125}I autoradiography were applied simultaneously.

Materials and Methods

Thyroid glands were removed from *Rana japonica* Guenther at all the stages of

metamorphosis. The fresh glands were fixed in 2% glutaraldehyde solution buffered with Sodium cacodylate at pH 7.4 for 1.5 hrs and then rinsed in sodium cacodylate buffer (pH 7.4) for 12 hrs. The tissues were incubated for 40 min at room temperature in modified GRAHAM and KARNOVSKY's (1966) and ESNNER's (1969) medium. That is composed of 20 mg of 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) and 0.2 ml of 2% H_2O_2 in 10 ml of 0.2 M Tris-HCl buffer pH 8.6. Some blocks of the tissue were incubated in the medium without DAB or H_2O_2 . Following postfixation in OsO_4 , they were dehydrated in graded concentrations of alcohol and embedded in Epon epoxy resin; sections cut were left unstained or stained with Millonig's lead or, lead and uranyl acetate. Two tadpoles in the climax metamorphosis were bred in water containing propylthiouracil (0.1%) for 2 or 26 hrs and the peroxidase reaction technique was applied on the gland using the method mentioned above.

Four tadpoles at stage XX and XXI were kept in 50 ml of water containing 1.3 mCi of ^{125}I for either 2 or 50 hrs. Then the animals were sacrificed and sections were embedded following the procedures mentioned above for the cytochemical demonstration of endogenous peroxidase. Sections cut were stained with Millonig's lead and coated with Ilford L4 emulsion. After exposure for 3-5 weeks at 4°C, they were developed in Microdol X for 5 mins at 18°C or the phenidon developer (LETTYRE and PAWELETZ, 1966) for 1 min at 16°C.

Observations

Premetamorphosis

In the early premetamorphosis, peroxidase activity is faintly recognized in some follicular cells facing the primitive follicular lumen. Some vesicles in the Golgi complex and in the apical cytoplasm are positive for peroxidase reaction (Fig. 1a). Some of the cisternae of inner Golgi lamellae rarely reacted. During the latter part of this period, the cisternae of inner Golgi lamellae become positive in all the follicular cells, while the outer ones do not show any reaction product. Most of the small granules in the apical cytoplasm are negative for this reaction.

Prometamorphosis

Early in prometamorphosis, the rough endoplasmic reticulum and Golgi apparatus become well developed as those of the follicular cells of mammalia. The elements of rough-surfaced endoplasmic reticulum and the nuclear envelope never reacted for peroxidase. In the apical cytoplasm, a few vesicles reacted positively. The inner parts of Golgi lamellae and a few Golgi vesicles show deposition of reaction products (Fig. 1b). Although there are many moderately dense granules in the apical

Fig. 1. The peroxidase activity throughout metamorphosis. **a.** The first appearance of peroxidase activity at stage I (early premetamorphosis). The vesicles (arrows) near the Golgi apparatus and near the apical plasma membrane faintly react for peroxidase activity. $\times 30,000$. **b.** The peroxidase activity during prometamorphosis. The inner Golgi lamellae and a few apical vesicles (arrows) are reacted for peroxidase reaction. There are several granules negative for this reaction. $\times 18,500$. **c.** In the climax metamorphosis. The peroxidase reaction is the heaviest. Enlarged Golgi apparatus, many apical vesicles and the peripheral colloid lumen are reacted. However, the cisternae of r-ER are not reacted. The apical plasma membrane is uneven. $\times 11,000$. G Golgi apparatus, N nucleus, V apical vesicle, L colloid lumen, P pigment granule, M multivesicular body.

Fig. 2. a. The supra-nuclear region of a follicular cell at stage XXII (climax metamorphosis). Reaction products are seen over the Golgi lamellae, many Golgi vesicles, many apical vesicles and the peripheral colloid lumen close to the apical membrane. It seems that the positively reacted Golgi vesicles flow toward the apical plasma membrane. $\times 19,000$ **b.** At the apical plasma membrane, the reverse pinocytosis of peroxidase positive material is often observed (arrows). $\times 25,000$. *N* nucleus, *G* Golgi apparatus, *V* apical vesicle, *L* colloid lumen.

cytoplasm, they are negative for peroxidase reaction. As the stages proceed, there is an increase in the number of vesicles reacted positively in the apical cytoplasm. Some of these vesicles are close to the apical plasma membrane. The luminal colloid near the plasma membrane faintly reacts for peroxidase.

Climax metamorphosis

In this period, peripheral luminal colloid, many apical vesicles, multivesicular bodies and the Golgi apparatus heavily react for peroxidase (Fig. 1c). At stage XXII or XXIII, the peroxidase reaction of the metamorphosing frog thyroid shows tremendous activity. Even at the most active stage of peroxidase reaction, the elements

Fig. 3. a. The specimen on which peroxidase reaction and ^{125}I -autoradiography have been performed simultaneously. Reaction products for peroxidase are seen over the Golgi lamellae, a few Golgi vesicles and many apical vesicles. Most silver grains (dots) of ^{125}I autoradiography are located over the colloid lumen. $\times 10,500$. b. Peroxidase positive vesicles with grains of ^{125}I -autoradiography are scarcely seen. $\times 17,000$. N nucleus, G Golgi apparatus, V apical vesicle, L colloid lumen.

of rough-surfaced endoplasmic reticulum and the nuclear envelope do not generally react for peroxidase activity. This result is different from that in the rat thyroid reported by some authors (NAKAI and FUJITA, 1970; STRUM and KARNOVSKY, 1970; STRUM et al., 1971). Positively reacting apical vesicles are various in size. Small ones are same in size as the Golgi vesicles reacted positively. The large ones heterogeneously stained and about 1,500 Å in diameter might be vesicles being fused with several Golgi vesicles. Figures showing the reverse pinocytosis of the apical peroxidase positive vesicles into the follicular lumen are often seen (Fig. 2b). An enlarged Golgi complex is also positively reacted. Two or three Golgi lamellae, many Golgi lamellae, many Golgi vesicles and multivesicular bodies are positive. Many vesicles bubbling off from the Golgi lamellae also show the reaction product, while the apical moderately dense granules are negative for peroxidase reaction.

During late stages of this period, many moderately dense droplets of various sizes which are negative for this reaction are accumulated in the cytoplasm. In addition, peroxidase-positive vesicles in the apical cytoplasm are reduced in number. The Golgi apparatus becomes smaller and the cisternae of one or two inner lamellae are positively stained. Golgi vesicles showing positive reaction are decreased in number. It is difficult to find vesicles bubbling off from the Golgi lamellae.

In the preparations on which both peroxidase reaction and ^{125}I autoradiography have been performed simultaneously, most silver grains for thyroglobulin bound ^{125}I are localized on the luminal colloid, while the silver grains of ^{125}I exist only on the 1.4% of all peroxidase positive vesicles (Fig. 3a, b).

The follicular cells that are incubated in a medium without H_2O_2 or DAB are negative from peroxidase reaction. In the thyroid glands of tadpoles that have been kept in water containing propylthiouracil for 26 hrs, the follicular cells are free of peroxidase activity.

Discussion

The thyroid hormone has been known to be necessary for amphibian metamorphosis from the results following several kinds of experiments, such as feeding experiment (GUDERNATSCH, 1912), thyroidectomy (ALLEN, 1916, 1938; HOSKINS, 1919; HOSKINS, 1922), and iodine deficiency (LYNN and BRAMBEL, 1935). The morphological changes in this organ during metamorphosis have been reported by several authors using the light microscope (ALLEN, 1938; D'ANGELE and CHARIPPER, 1939) as well as the electron microscope (COLEMAN et al., 1968; NANBA, 1972a). The present author attempted to clarify the following three things about the thyroid function of the metamorphosing frog: (1) the period and site of the first appearance of the peroxidase activity in the thyroid gland during metamorphosis, (2) the relationship between the peroxidase activity and thyroid function, and (3) the site of iodination of thyroglobulin in the frog thyroid.

Peroxidase has been believed necessary for the iodination of tyrosine contained in thyroglobulin (DEGROOT and DAVIS, 1962; KLEBANOFF et al., 1962; TAUROG and HOWELLS, 1966; HOSOYA and MORRISON, 1967). The ultrastructural technique for the demonstration of injected peroxidase has been devised by GRAHAM and KARNOVSKY (1966). This method also has enabled the localization at the fine structural level of endogeneous peroxidase of various cells such as leucocytes (YAMADA, 1966; BARTON

and FARQUHAR, 1967; 1970; ACKERMAN, 1968; COTRAN and LITT, 1969; MILLER and HERZOG, 1969), epithelial cells of the uterus (BRÖKELMANN, 1969), epithelium of the colon (VENKATACHALAM et al., 1970), the peritoneal macrophages (FAHIMI et al., 1970), Kupffer cells in the liver (FAHIMI, 1970), acinar cells of the parotid gland (HERZOG and MILLER, 1970), epithelial and glandular cells of the rat larynx (KATAOKA, 1971) and follicular cells of the thyroid gland of rats and adult frogs (NAKAI and FUJITA, 1970; STRUM and KARNOVSKY, 1970; STRUM et al., 1971; NANBA, 1972b).

In early premetamorphosis, a few apical and Golgi vesicles in some immature thyroid cells begin to show a faint peroxidase activity but those of most cells do not react for this reaction. KAYE (1961) has shown that the first concentration of ^{131}I uptake into the thyroid occurs stage 25 or the I stage of metamorphosis. The first appearance of peroxidase activity is coincident to this period. According to KAYE, the thyroidal ^{131}I uptake is relatively stable during stage II and stage XI (premetamorphosis), gradually rises during stage XI and stage XIV (early prometamorphosis), and reaches its maximum during stage XIV and stage XXII (late prometamorphosis and climax metamorphosis). The peroxidase activity in the thyroid cell seems to be parallel to ^{131}I -uptake into the thyroid gland throughout metamorphosis. In pre-metamorphosis the peroxidase positive materials are scanty in the thyroid gland, while the acid phosphatase activity is markedly high in this period (NANBA, 1972). Though the thyroid cell in this period has already had the ability to produce the peroxidase, the digestion of many pigment granules and yolk droplets might be needed more for differentiation of thyroid cells rather than synthesis of peroxidase. In the prometamorphosis, the elements of rough-surfaced endoplasmic reticulum, Golgi apparatus and apical vesicles are well developed.

There are at least two kinds of apical vesicles with regards to the peroxidase reaction: positive and negative. The negative ones are more numerous than the positive ones. Though it is difficult to distinguish them only by their fine structure, some of them must contain thyroglobulin. If the vesicles containing thyroglobulin are negative for this reaction, it is considered that the synthesis and release of thyroglobulin into the colloid lumen surpass those of peroxidase before the climax metamorphosis. Then thyroglobulin may be fully stored in the colloid lumen. At the climax metamorphosis, the peroxidase activity reaches its maximum. Apical vesicles are mostly positive for peroxidase reaction and many peroxidase positive vesicles seem to flow out of the large Golgi complex (Fig. 2a). At the apical plasma membrane, the figures showing reverse pinocytosis of peroxidase positive vesicles have often been encountered. The route of the transfer of peroxidase is similar to that of thyroglobulin reported by several authors (NADLER et al., 1964; WHUR et al., 1964; WHUR et al., 1969; HADDAD et al., 1971). Peroxidase and thyroglobulin may be respectively released into the colloid lumen from the follicular cell. The great secretion of thyroid hormone at the climax metamorphosis is related to the active synthesis of peroxidase, active uptake of iodine (KAYE, 1961), and active hydrolysis of re-absorbed colloid droplets by acid phosphatase (NANBA, 1972a). As soon as the peroxidase is released into the lumen by reverse pinocytosis, the iodination of thyroglobulin may occur in the peripheral colloid lumen close to the plasma membrane. This is also supported by the results of the preparation on which peroxidase reaction and ^{125}I autoradiography have been tried simultaneously. Most silver grains were

seen over the colloid lumen rather than on the peroxidase positive vesicles in the cytoplasm. On the basis of this finding the author concludes that the main site of iodination of thyroglobulin is the luminal colloid, especially in the peripheral region of the lumen as FUJITA (1972) emphasizes.

変態期の正常カエル (*Rana japonica* Guenther)

甲状腺の微細構造と超微組織化学

II. ペルオキシダーゼ活性

難 波 久 佳

ニホンアカガエルの変態期を通して、甲状腺のペルオキシダーゼ活性を電顕組織化学的に検索した。

1 変態前期の初期に、はじめてペルオキシダーゼ反応は陽性になり、反応産物は少数のゴルジ小胞、または細胞表層部の少数の小胞に弱く現われた。

2 細胞内小器官の発達に伴い、ペルオキシダーゼ反応はすべての濾胞上皮細胞の1～2層のゴルジ層板、少数のゴルジ小胞や細胞表層部の少数の小胞に陽性になった。変態最盛期に入るとペルオキシダーゼ活性は極めて強くなり、発達したゴルジ装置の1～2層の層板、層板から由来している多数のゴルジ小胞、細胞表層部の多数の小胞が強陽性を呈した。これらの小胞は多分逆パイノサイトーシスにより活発に濾胞腔内に放出されているであろうと思われる像がしばしば見られた。また濾胞腔の辺縁部も強陽性を呈した。甲状腺のペルオキシダーゼ活性の消長は、今まで報告されている甲状腺の機能の消長とよく平行している。

3 変態最盛期の甲状腺機能の非常に亢進した時に、濾胞腔辺縁部のペルオキシダーゼ反応が強陽性を呈すること、 ^{125}I のオートラジオグラフィを用いた報告により、サイログロブリンのヨード化の場合は濾胞腔辺縁部であることを強く暗示する。これをさらに裏づけるために、著者は同一切片試料上にペルオキシダーゼ反応と ^{125}I オートラジオグラフィを同時に行なった。銀粒子は細胞内のペルオキシダーゼ陽性場所にはほとんど見られず、大部分は濾胞腔内に集った。この結果より、サイログロブリンのヨード化の主な場は、濾胞腔とくに濾胞辺縁部であることが結論された。

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