HISTOLOGIC INVESTIGATION OF C CELLS IN FETAL CALF THYROIDS

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INTRODUCTION

In a monograph by Vappu Kossila, 1967, entitled "On the Weight and Basic Structural Components of the Thyroid in Dairy Cattle", no mention was made of the C cell content of thyroids from bovine fetuses. In view of the completeness of her study with regard to follicular epithelium and colloid content of thyroids from bovine fetuses, an additional study concentrating on the C cells would help make our present knowledge of the fetal bovhe thyroid more complete. This enumeration of C cells at various stages of development had many limitations which the researcher recognized. Although the enumeration is quantitative in nature the qualitative aspects of this research will be stressed. This study does not include the over-all distribution of the C cells throughout the thyroid gland nor has any attempt been made (nor could be, with any degree of accuracy) to quantitate the C cell content for the entire gland. The materials, methods and results are presented on their own merits as a rather thorough but yet preliminary type approach which may provide information for future investigation of the numeration of C cells and their distribution.

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REVIEW OF THE LITERATURE

Anatomical Development of the Thyroid Gland

The thyroid gland is the first gland to be differentiated in the embryo (Arey 1965). The major part of the thyroid gland, the thyroid tissue proper, is derived from endoderm as an evagination of the foregut (Bloom and Fawcet 1962). In the fetal calf this ventral evagination of the foregut occurs at 22-26 days gestation (Kingsbury 1935). With the rupture of the thyroglossal duct in the 8.5 mm. embryo the thyroid remains as a short solid plate or cord to cells (Anderson 1922). The principle epithelial components of the thyroid gland are the follicular cells which are derived from the outpouching on the ventral floor of the foregut. The follicular cells are thought to contribute to the synthesis of thyroglobulin and thyroxine. Monoiodotyrosine, diiodotyrosine, triiodothyronine and tetraiodothyronine are bound to the thyroglobulin in the lumen of the follicle. Triiodothyronine and tetraiodothyronine (thyroxine) are the major endocrine secretions of the thyroid gland (Ganong 1965).

A second source of tissue, not often included in a discussion of the formation of the thyroid gland, contributes some parenchyma to the gland which does not necessarily have thyroxine producing ability. This omission is not surprising as publications prior to 1963 dealing with the thyroid gland are primarily involved with the discussion of the origin of cells known to

produce secretions typical of the follicular epithelium.

The derivation of the entire cell population of the thyroid involves the caudal migration of the evaginated foregut after the 8.5 mm. embryo stage plus the medial migration of tissue originating from the pharyngeal pouches.

Developmental Relationships of the Ultimobranchial Body

In cattle as in humans four pharyngeal pouches develop (Kingsbury 1935). Figure I, redrawn after Goss (1961), illustrates the relationships of the pharyngeal pouches. Of primary interest regarding the development of the thyroid gland is the caudal pharyngeal pouch of branchial endoderm; in the calf, this endodermal pouch does not reach the ectoderm as do those pouches originating more cephalad. This last pouch in the 8.5 mm. calf embryo has a short cranial and a long caudal diverticulum (Anderson 1922). In the 11 mm. calf embryo this caudal diverticulum is seen and may be considered the same as the diverticulum of the last pharyngeal pouch described by Kingsbury (1935) Part of the thymus is derived from the last pharyngeal pouch, its origin being cephalad and lateral to the site of origin of the ultimobranchial body, also the caudal diverticulum of the third pharyngeal pouch contributes greatly to the formation of the thymus.

Parathyroid III arises from the cranial portion of the third pharyngeal pouch. In the adult it is often termed the inferior parathyroid.

Species variations exist in the ultimate location of the parathyroids. Some variations may exist within the same strain of the same species. Using the rat as as example: parathyroid IV may be surrounded by thyroid tissue thereby constituting an internal parathyroid or it may be adjacent to the thyroid and be termed as a "superior parathyroid" (Carvalheira and Pearse 1968). An internal parathyroid is generally the fate of parathyroid IV in the goat (Foster et al. 1964), sheep (Copp and Henze 1964, according to Hirsch and Munson 1969), rabbit (Carvalheira and Pearse 1967), horse (personal observation 1972), and human (Solcia et al. 1970). Carvalheira and Pearse (1967) reported finding cells in the internal parathyroid of sheep and rabbits which originated from the ultimobranchial body. This is not surprising as illustrated in Figure I both parathyroid IV and the ultimobranchial body arise from the same pharyngeal pouch.

Rogers (1927) found no fourth pharyngeal pouch in the white rat; the ultimobranchial body reportedly appeared prior to any trace of the fourth aortic arch. Kingsbury (1935) cites the rat as an example of a species having only three pharyngeal pouches with the ultimobranchial body originating from the third. However, Carvalheira and Pearse (1968) cite the ultimobranchial body of the rat as being derived from the fourth pouch. A variety of pharyngeal count assignments may have been used by various researchers. This variation in counting methods

may explain the discrepancy in the number of pouches cited for the white rat. The discrepancy could have arisen from a difference in the age of the embryo used for determination. The trend in thinking, regardless of the pharyngeal pouch number assigned, is that the ultimobranchial body of mammals arises from the caudal pharyngeal pouch (Kingsbury 1935).

Ultimobranchial body contribution to the thyroid gland

In the class Mammalia, with the exception of Echidna, the ultimobranchial bodies eventually become embedded to varying degrees in the thyroid. Mauer (according to Rogers 1927) reported the similarity of Echidna with the class Aves in which the ultimobranchial bodies never become embedded in the thyroid. Instead, they differentiate into a gland, which, while differing from the thyroid in structure has follicles containing colloid (Rogers 1927).

Ultimobranchial tissue differs histologically from tissue of the thyroid proper. The nuclei of the epithelial cells constituting the principle thyroid cells are of varying sizes and shapes, quite vesicular, and contain numerous small bits of peripherally placed chromatin. By comparison the nuclei of the cells of the ultimobranchial body are smaller, more spherical, more uniform in size, less vesicular and contain two or three centrally located nucleoli (Anderson 1922). The cytoplasm is scanty, resulting in more closely packed nuclei, and the nuclei

are markedly chromatic (Kingsbury 1935). Due to the previously described differences, the tissue of the ultimobranchial bodies may be distinguished from tissue of the thyroid proper even where adjacent (unincluded) and/or surrounded by (included) tissue of the thyroid proper (Anderson 1922; Kingsbury 1935; Jubb 1959).

Unincluded ultimobranchial tissues are defined as those portions of ultimobranchial tissue located adjacent to thyroid tissue of mid-pharyngeal origin. Included portions of ultimobranchial tissue are those portions which are surrounded on all borders by thyroid tissue of mid pharyngeal origin. Included ultimobranchial tissue may also be described as fused tissue.

Well-included portions of ultimobranchial tissue could not be distinguished from tissue of other origin in dog thyroids studied by Godwin (1937). This is interpreted to mean that no tissue with heterochromatic nuclei and scanty cytoplasm was found surrounded on all borders by typical epithelial follicles and/or thyroid cords. According to Godwin (1937), Badertscher (1918) and Rogers (1927) likewise found it impossible to distinguish fused ultimobranchial material from epithelial follicles and/or thyroid cords.

The ultimobranchial bodies come into contact with the lateral surfaces of the evagination of the foregut. A close association of the thyroid and caudal pharyngeal pouch was observed to occur in the 18-19 mm. calf (Kingsbury 1935). The incorporation of the ultimobranchial

body with the thyroid of the calf was considered by Kingsbury (1935) to take place in embryos of 19 to 62 mm. crown-rump length. The lateral side of the thyroid first contacted the ultimobranchial body and its growth progressively enveloped the body of the caudal pharyngeal complex.(Kingsbury 1935). Through changes in position associated with growth and migration, the derivatives of the caudal pharyngeal complex first are situated in or near the cephalic end of the lateral portions of the thyroid while in later stages, calf embryos 30 mm. and greater, they are found well down within the tissue of the thyroid. After thyroid cells have formed cords but before the thyroid cords form vesicles and before secretion of colloid is evident, the lateral portions of the thyroid of the 50 mm. calf embryo contained characteristic dark patches of cells, the cords of the ultimobranchial body (Anderson 1922). In pig embryos, Badertscher (1918) reported deeply stained nuclei of ultimobranchial origin to be most numerous in stages from 20 to 30 mm. crown rump length, with an active process of cell cord formation in those individuals 25-30 mm. crown rump length. The deeply stained nuclei diminish in number in developmental stages beyond 30 mm. and finally could no longer be distinguished (Badertscher 1918).

Anderson (1922) described the joining of the separately derived tissues as the ultimobranchial cords intermingling with those of the thyroid proper. Kingsbury (1935) phrased this incorporation as ..."thyroid cords

investing the ultimobranchial body intimately." This may only be a difference in the authors phraseology rather than a disagreement. According to Badertscher (1918) an ingrowth of vascular tissue and of structural elements of the median thyroid (evagination of the foregut) into the ultimobranchial body, plus the continued growth and branching of the epithelial buds found on the surface of the ultimobranchial tissue itself, results in the formation of cords of ultimobranchial cells.

Whether the thyroid grows into the ultimobranchial body or the ultimobranchial body grows into the thyroid, the greatest growth in bulk occurs in the thyroid tissue proper; this differential growth rate results in the ultimobranchial body becoming relatively smaller and smaller.

At the stage of first intermingling and for some stages thereafter the unincluded ultimobranchial tissue is evident in the calf. Appearing as thick branching cords of darkly staining cells first at the dorsolateral sides of the thyroid and around the blood vessels at the hilus (Kingsbury 1935). These cords of cells branch but are not fused with the parenchyma at the 50 mm. stage (calf) (Anderson 1922).

In the dog, fusion of the ultimobranchial body with thyroid tissue is present in embryos of 12.5 to 17 mm. in length (Godwin 1937). Godwin (1937) declared, "No limit can be drawn between the ultimobranchial and thyroid at points of fusion." In dog embryos 18 to 28 mm. the

majority of the ultimobranchial tissue loses its distinctiveness and becomes intimately intermingled with the thyroid tissue proper; the ultimobranchial tissue becomes progressively larger, more vascular and looser in arrangement. The ultimobranchial tissue was detectable by its growth character and by the occasional persistence of the original lumen of the complex.

Fusion of the ultimobranchial bodies with the thyroid proper occurs in the 20 mm. cat. It occupies the most lateral portion of the thyroid gland and is entirely reticulated in the 35 mm. cat (Mason 1931). At day 16 of gestation the ultimobranchial bodies of the rat begin to fuse with the thyroid proper (Carvalheira and Pearse 1968). The ultimobranchial bodies of the pig first fuse along the ventro-lateral border of the thyroid tissue proper at 19 to 27 mm. crown-rump length stage of development. The extent of their fusion to the thyroid gland is variable (Badertscher 1918).

The idea that the ultimobranchial bodies become the lateral thyroids (the tissue lateral to the isthmus) (Rogers 1927) has been disputed. "Some believe that the final fate of the ultimobranchial bodies is degeneration; others describe an actual conversion ('induction') of ultimobranchial into thyroid tissue, apparently through the dominating influence of a thyroid environment on a plastic, implanted tissue. Only to this degree at best can the ultimobranchial bodies qualify as 'lateral thyroid' primordia." (Arey 1965). This long standing dispute

is focused on the question of whether or not the ultimobranchial tissue gives rise to typical follicular cells. Kingsbury (1935) found no evidence for the origin of true thyroid parenchyma from any part of the fourth pharyngeal pouch in the calf, although he did state, "Where thyroid and ultimobranchial parenchymas are in regions of intimate association a decision as to whether the vesicle is of thyroidal or ultimobranchial origin is difficult to ascertain." In his study of the fate of the ultimobranchial bodies in the cat, Mason (1931) concluded that at no time during the development is there any indication that the cells of the ultimobranchial body transform into follicles or cords of thyroid tissue. The cells of the ultimobranchial body form cords which arrange into ducts which later become cysts in the thyroid of the cat.(Mason 1931).

The formation of cysts by ultimobranchial tissue in the thyroid glands of pig embryos rarely occurs; degeneration was not thought to be the fate of the ultimobranchial tissue. Badertscher (1918) reasoned ..."If the darkly staining nuclei of the ultimobranchial tissue were degenerating cells representing a general degeneration of the ultimobranchial tissue, large numbers of degenerated nuclei should be found in later developmental stages, but a contrary condition is the case." The darkly stained nuclei gradually decrease in number while the ultimobranchial bodies continue their growth (Badertscher 1918). Badertscher's view was that the cell cords of

ultimobranchial origin transformed into thyroid follicles. Nothing was found to indicate disintegration of the ultimobranchial tissue of the dog (Godwin 1937). VanDyke (1945) stated, "Kingsbury created a premise for considering the ultimobranchial body as relatively indifferent tissue which may be influenced in its further development by variable factors of environment."

Both Godwin (1937) and Kingsbury (1935) working on the dog and calf respectively, believed that the ultimobranchial body is not endowed with thyroid forming potencies but that it may form tissue indistinguishable from thyroid tissue by induction. Areas of ultimobranchial tissue which do not become included into the thyroid tissue show neither the growth character nor the appearance of follicular cells, whereas those incorporated (included) areas are indistinguishable from follicular cells. The cytoplasm of the cells of the unincluded ultimobranchial tissue is clear and there are signs of some degeneration. Numerous large light staining cells with a large nucleus possessing a distinct chromatin network represented ultimobranchial remnants in fetal tissue (Kingsbury 1935). In early postnatal life in cattle ultimobranchial tissue is still detectable at the cranial aspect of the thyroid and is encompassed to a greater or lesser degree by the parathyroid derived from pouch IV. Residues of the ultimobranchial persist within the thyroids of cattle throughout life. (Jubb 1959). As described by Jubb (1959), the early post natal calf possesses ultimobranchial tissue

in tortuous cell cord configurations with irregular lumina lined by simple columnar or transitional epithelium; this epithelium is in intimate contact with connective tissue and is surrounded by small vesicles of follicular formations derived from the epithelium of the thyroid proper. The ultimobranchial derived vesicles contain a lightly basophilic colloid or were devoid of detectable secretion. The ultimobranchial ducts and their derived vesicles were considered by Jubb (1959) to be transformed into typical follicular epithelium. The congenitally hyperplastic thyroids were used as a model to study the appearance of ultimobranchial tissue by Jubb (1959). Such thyroids presented ultimobranchial tissue relatively isolated from thyroid tissue. This relatively isolated ultimobranchial tissue had follicles which were indistinguishable from normal thyroid follicles except by the more hyperchromatic appearance of the cells' nuclei. These follicles showed a greater irregularity of size and contour and were generally void of colloid. Some follicles, although void of colloid, did contain cellular debris and in some instances scanty amounts of colloid plus cellular debries were seen in the follicle lumens.

Kingsbury (1935) and Jubb (1959) were in agreement as to the description of the appearance of the ultimobranchial tissue as it is found in the thyroid gland. Kingsbury did believe it was easily distinguishable, while Jubb believed the differentiation was more subtle and therefore not as obvious. Kingsbury (1935) described the

cytoplasm of the cells in the unincluded ultimobranchial material to be clear and he detected some cellular degeneration. He proposed that the numerous large cells with distinctly chromatic nuclei having a grayish granular or light staining cytoplasm resulted from ultimobranchial tissue. Cells believed to be of ultimobranchial origin very similar to the large cells, with chromatic nuclei, having a grayish granular cytoplasm were observed and called 'gray cells' regardless of their position. Godwin (1937) theorized, that many, perhaps all, gray cells arise from the ultimobranchial body. He described their formation from the ultimobranchial tissue as being rather indistinguishable from follicular cells at their early stage. The gray cells formed follicles with a cavity which became a mixed follicle consisting of gray cells, ordinary thyroid cells, and cells representing stages of transition from gray cell to follicular cell. It is interesting to note Godwin's (1937) observation that where a follicle is formed at the edge of a gray cell mass, the cells on the side of the follicle toward the thyroid tissue tend to transform into ordinary thyroid follicle cells first. Nonidez's (1931) observations of postnatal dog thyroids led him to the conclusion that follicular cells gave rise to parafollicular cells.

In contrast to Godwin's (1937) estimate that perhaps 1/3 to 1/2 of the thyroid was indirectly derived from the ultimobranchial bodies, Rogers (1927) estimated that the ultimobranchial body constitutes only a very small part

of the thyroid gland. He too thought cords at the site of the ultimobranchial body are transformed into colloid-containing follicles, morphologically indistinguishable from thyroid follicles.

Rather conclusive evidence that the C cells in rat thyroids are derived from the ultimobranchial body was offered by Carvalheira and Pearse (1968) Cytochemical studies of the embryonic thyroid gland originating only from the ventral evagination of the foregut showed no C cells at any stage. On day twelve of gestation, C cells were observed in the ventral part of the caudal pharyngeal pouch. By day fourteen of gestation the caudal pharyngeal pouch separated into dorsal and ventral out-pocketings. Up to day sixteen no C cells were found in the thyroid gland proper. From day sixteen thereafter C cells were found in the thyroid gland of the rat. Incorporation of the ultimobranchial body coincided with the finding of C cells in the thyroid tissue proper (Carvalheira and Pearse 1968).

C cells have been identified in the thyroid glands of chickens, a species in which the ultimobranchial body never mixes with the thyroid tissue (Solcia and Sampietro 1968). This is suggestive evidence that these cells may also develope independently from the ultimobranchial body.

Under experimental conditions the number of C cells has been demonstrated to increase by treatment with somatotropic hormone of hypophysectomy. Sarkar and Isler (1963) concluded that in the rat, C cells must arise from

follicular cells as the mitotic rate of C cells was not great enough to account for the increase in the number of C cells which were observed. Since the number of follicular cells and the diameter of the follicles remained constant and only the number of C cells increased, the concept of modulated follicular cells was suggested.

Using electron micrographs of rat thyroids, Calvert and Isler (1970) saw some cell types which revealed characteristics of both C cells and follicular cells. Some follicular cells containing a few granules the size of those found in C cells were observed. A few cells resembling light cells possessed an unusually well developed ergastoplasm with moderately distended cisternae which is more characteristic of follicular cells than C cells. The observation of these two forms of possible intermediate cell types offered suggestive evidence that follicular cells may change into C cells.

The concept that the ultimobranchial body is transformed into follicular as well as light cells is still being considered by some researchers. Although the concept that C cells are eventually transformed into follicular tissue is not the current general view (Hirsch and Munson 1969) more research is needed to clarify the relationship between definite follicular cells, definite C cells and the "intermediate" cell type observable in thyroids of many species.

History of C cells
various terms applied

Several terms have been applied at various times by the many researchers on the specific cell type under histologic investigation for this thesis. The designation C cell is currently accepted by most morphologists and has been the predominant term used throughout this discussion except in instances of direct quotations or if doubt existed that the cell described by a particular author is indeed identical.

Baber (1876) is generally credited as the first to describe cells in the thyroid that were different from typical follicular cells. He found distinct groups of "parenchymous cells" in the thyroid glands of dogs which he described as markedly different from follicular cells. Bensley (1914) called them "ovoid cells." There is considerable doubt the "mitochondria-rich" cells discussed by Seecof may be the same as C cells and according to Pearse (1968) they probably should be regarded as phospholipid-rich rather than mitochondria-rich, but nonetheless the counterparts of the parenchymatous cells of Baber. The term "mitochondria rich" cell is out of favor. (Hirsch and Munson 1969). Lietz (1971) stated, "The term is incorrect, as the motochondria-rich cells of the thyroid gland (oncocytes or Askanazy-cells) are not identical with C cells.

Nonidez (1931) called them parafollicular cells. He published a succeeding article entitled "The

'Parenchymatous' cells of Baber, the 'Protoplasmareichen Zellen' of Huerthle, and the 'Parafollicular' cells of the Mammalian Thyroid." He concluded these terms had been applied to the same cell type (Nonidez 1933).

Godwin (1937) believed the cells he termed 'gray cells' to be the same as the macrothyrocytes of Zechel and the parafollicular cells of Nonidez. Feyriter wrote of clear cells (Lietz 1971). Many years later the term light cell was introduced, referring specifically to the lack of PAS positive colloid droplets after the administration of thyroid stimulating hormone (Young and Leblond 1963).

Following the discovery of calcitonin, by Copp et al. in 1962, the thyroid gland was shown by Hirsch et al. in 1963, to be the site of production. It was suggested by Pearse (1966) that calcitonin was produced by particular cells within the thyroid gland which were not typical follicular cells. Since the parafollicular cells had not been attributed with a function they were investigated as a possible source of calcitonin. The term C cell was introduced by Pearse in 1965 (Pearse 1966). Its usage was suggested to indicate the common physiological function of cells which produce calcitonin, the majority of which are located in the thyroid gland in mammals. Calcitonin producing cells are found in smaller numbers in the thymus and parathyroid IV and are also termed C cells.

The wide variety of terms used to name what is

believed to be a single cell type is not surprising. The early investigators often did not learn about similar works or publications of similar research until some time after they had applied their personalized terminology and their paper had been published.

Parafollicular cell was the most widely adopted term used after its introduction by Nonidez (1931). However, it was criticized, and rightly so, due to its misleading description of the location of these cells in the thyroid gland. The term implies these cells are located by the follicle. In the species studied many cells are in an interfollicular cell position rather than exclusively in a parafollicular position. Some researchers used the term epifollicular (Idelman 1962). Studies by electron microscopy show the C cell of the rat to be located within the follicular basement membrane (Wissig 1962). If this holds true for other species, parafollicular is truly a misnomer.

Variations in appearance due to the staining technique, species of the subject, or age of the subject make the use of a morphologically based term unacceptable but enhances the acceptance of Pearse's apellation.

Function of C cells

The function of the C cells has been under rather intensive and fruitful investigation during the past decade. In the past, C cells were considered by some to be exhausted or degenerated follicular cells and by others to be promitotic follicular cells. More recent studies indicated that they are metabolically active cells having an enzyme pattern quite different from that of the principle cells (Bloom and Fawcett 1962).

Takagi (1922) observed that some cells in the thyroids of neonatal puppies contained numerous mitochondria but were void of granules and vacuoles. In 2 month-old puppies very few cells were filled with mitochondria while those containing granules, vacuoles and few mitochondria prevail. On the basis of his observation Takagi (1922) concluded that the large interfollicular cells were of glandular nature and that the secretion they produced was contained within the numerous vacuoles that occupied their cytoplasm.

Nonidez considered parafollicular cells functionally different from the follicular cells. He wrote that their granules appeared to disappear slowly in the cytoplasm, which suggested to him that a secretion was produced by these cells and that it found its way into the blood stream or into the lymphatics (Nonidez 1932).

Even as recent as 1962, the C cells were considered by some to represent a state of cyclic evolution of the principle cells. Their functional role was still obscure.

Kroon (1958) concluded from his histochemical studies that the C cell probably produced a mucoprotein precursor of thyroglobulin. Initial histochemical studies provided information on the nature of the secretion, and several authors implicated their function with colloid production, although none were explicit on the cells exact role or possible mechanism involved. Based upon the alloxan-Schiff reaction, a-amino acids were demonstrated. The reaction of Millon Pollister, by the Landing Hass method, showed the presence of aromatic amino acids. Sulfhydryl proteins were also demonstrated in these cells. Idleman (1962) concluded from his findings, 'les glucides et les protides sont vraisemblablement associes en une glycoproteine precurseur probably de la thyroglobuline."

Copp et al. (1962) perfused the thyroid-parathyroid complex in dogs with hypercalcemic blood and found it to exert a hypocalcemic effect. The hypocalcemic hormone was attributed to the parathyroid, and was called calcitonin. Hirsch et al.(1963) demonstrated a hypocalcemic hormone from thyroid extracts made from rat thyroids. They called it thyrocalcitonin it indicate its origin as the thyroid gland. Thus possible confusion with the hypocalcemic hormone, calcitonin, produced by the ultimobranchial body of lower classes was avoided. The differences and similarities of hypocalcemic hormones of various origins are still being investigated.

Foster et al. (1964), using the goat, showed that hypercalcemic perfusion of a parathyroid gland (with a blood

supply separate from the thyroid) had no significant change in the blood level calcium. Perfusion of the thyroid gland alone resulted in the plasma calcium lowering effect. Foster thus confirmed the origin of thyrocalcitonin from mammalian thyroid glands by gland perfusion studies in the goat. Care (1965) confirmed the origin of thyrocalcitonin to be the thyroid gland by gland perfusion studies in the pig. The parathyroid gland was not responsible for the production of a hypocalcemic hormone. The shared blood supply of the thyroid gland and the parathyroid gland of the dog resulted in both being perfused with hypercalcemic blood in the experiments of Copp et al. (1962). The hypocalcemic hormone produced was actually produced by the thyroid gland of the dogs and not the parathyroid gland.

Refuting the notion that the C cells were perhaps a transitory mitotic stage of the principle cells of the thyroid, Esper and Isler (1965) measured the DNA content of the C cells of the rat thyroid. The DNA content was the same as that of follicular cells. Therefore, the hypothesis that C cells are premitotic (tetraploid) cells on the verge of dividing was convincingly rejected.

Cytochemical studies by Pearse (1966) revealed more evidence linking thyroid C cells to the production of thyrocalcitonin. Discussion of the cytochemistry of the C cells will be included with the presentation of staining reactions.

Staining Reactions

There seem to exist some species and perhaps age differences with regard to the morphology and staining characteristics of the C cells. Nonidez (1931) found a difference in the affinity of the granules of the C cells for colloidal silver, the neonatal pup showing a lesser affinity than those of a few weeks of age. He (1932) stated ..." the young parafollicular cell appears less deeply stained than the other elements in the epithelium." He also noticed that the affinity of the granules of the C cells varied, being most marked in the dog. "The length of the impregnation also differs according to whether the animal belongs to the Carnivora or the Herbivora." It was suggested that the younger the animal from which the tissue was obtained, the greater the percentage of the silver-nitrate solution that must be used for successful impregnation of the material. C cells are much less differentiated in young rats than in adult rats when PAS is the stain (Stux et al. 1961).

Lietz (1971) stated that the existence of C cells could not be exhibited morphologically in rat and human thyroids by techniques available as late as 1959. In seemingly contradiction, he stated in another portion of his paper that the cytoplasm of C cells in all species appears coarse and granular due to a greater affinity for silver salts than the follicular epithelium, but in the very next sentence, "the selectivity of these techniques is not satisfactory in all species."

Several stains originally cited for their excellence in demonstrating pancreatic islet cells have been tried successfully to demonstrate thyroid C cells. One of these methods was divised by Manocchio (1964) for the demonstration of the alpha pancreatic cells. Velicky (1971) used the method of Manocchio to demonstrate thyroid C cells in sheep, rabbit and cat. Solcia et al. (1968) further modified the method of Manocchio and was able to demonstrate C cells in human thyroids. The similarity in methods consists of pretreatment of the adfixed tissue section. The pretreatment served to hydrolyze the tissue. Then stains such as toluidine blue, azure A or pseudoisocyanin revealed metachromatic C cells. Although obscure as to the mechanism, hydrolysis "unmasks" acid groups which are somewhat blocked in fixed tissues. "Groups binding basic dyes may be side-chain carboxyl groups of proteins" (Solcia et al. 1968). Following hydrolysis the carbodiimide reaction for carboxylic groups showed no evidence of a significant increase of the carboxyl groups. Nor could calcitonin be demonstrated after acid hydrolysis with the performic acid-pseudoisocyanin method. Thus Lietz (1971) suggested that calcitonin itself is extracted during hydrolysis. Since the fixatives, which seemed best for the "unmasked metachromasia" reaction, contained picric acid which binds to the tissue and is not completely removed during the dehydration and embedding procedure, it may be important in the staining reaction. There is no agreement as to the mechanism whereby hydrolysis

allows the demonstration of thyroid C cells by toluidine blue. It has been further suggested that the unmasking of staining properties by acid hydrolysis of APUD series cells (Amine and Precursor Uptake and Decarboxylation) is related to the random coil conformation of the protein precursors of polypeptide hormones (DeGrandi 1970). Pearse (1968) classified the C cell as a member of the APUD series. This series has been characterized as peptide-hormone secreting cells exhibiting common cytochemical traits.

In a comparison of various fixatives applied to thyroid tissue and the respective effect upon staining properties the choice of fixative was found to be a very important consideration. When subsequently hydrolyzed by 0.1N HCl and stained with a 0.01% toluidine blue in a 0.02 McIlvain buffer pH 5, Solcia et al. (1968) found that tissue fixed in 95% ethanol gave negative results for pancreatic A and D islet cells and thyroid C cells. Whereas tissue fixed in Bouin's fluid fixative with 1% acetic acid gave the best results for staining of C cells after acid hydrolysis with 0.01% toluidine blue pH 5 McIlvains buffer. Solcia et al.(1968) did not report attempts at fixing in ethanol and then refixing in Bouin's after the tissue was adfixed and deparaffinized.

The silver staining method of Grimelius was originally a pancreatic islet stain. The method was slightly modified by DeGrandi (1970) and applied to thyroid tissue. The C cells were argyrophilic. Variations were noted

in staining which were accounted for by the choice of fixative and the degree of autolysis. Formalin and glutaraldehyde fixed tissue did not stain or stained poorly. Marginal areas of freshly fixed tissue were negative in response to the stain. Small blocks of freshly fixed tissue had negative staining reactions. Autolytic tissues were positive in marginal areas as well as the deeper areas. DeGrandi (1970) proposed that autolysis seems to umask some chemical groups necessary to the argyrophilic reaction. However, acid hydrolysis did not furnish chemical argyrophilic groups nor did autolysis provide chemical groups responsible for " masked metachromasia" (DeGrandi 1970). The best fixative for the Grimelius method was Bouin's fluid fixative. Picric acid fixatives such as Bouin's were considered to yield very good results, being better than formalin fixatives which were better than glutaraldehyde fixed tissue (Solcia et al. 1970). The chemical groups responsible for the argyrophil reaction may be groups either of the polypeptide hormone such as SH groups or other substances accompanying calcitonin such as monoamines (DeGrandi 1970).

Fresh, quick frozen, cryostat prepared tissues may be subjected to various cytochemical studies. Among these are immunofluorescent localization technique based on antiserum. This technique was used by Bussolati (According to Hirsch and Munson 1969) to identify the C cell as the site of storage of thyrocalcitonin in the dog and pig.

By utilizing 5-HTP as a marker, Carvalheiro and Pearse (1968) identified the ultimobranchial origin of C cells in rats. C cells of most species have a high activity of cytochemically demonstrable esterases (Lietz 1971).

Frequency & Distribution

The distribution of C cells within the thyroid gland appears to be a species characteristic. The cow and calf have their greatest concentration of C cells in the periphery of the gland (Idelman 1962). In the rat, guinea-pig, and dog they are scattered in nearly all parts of the gland (Solcia and Sampietro 1968). Dogs have a greater concentration in the upper poles (Teitelbaum et al. 1970) In rabbit, horse, monkey and man they are usually grouped in the posterio-medial areas of the gland (Solcia and Sampietro 1968).

A through investigation of the rat thyroid by Stux et al. (1961) showed C cells "essentially, although not exclusively in the follicles making up the core of the thyroid gland." No C cells were found in the isthmus. The highest proportion were found halfway between the cranial and caudal poles. The average ration of light cells to follicular cells throughout the entire thyroid was calculated to be 0.7%. In the center of the thyroid the proportion was 1-2% with the data of some experiments averaging 5%. Rodents have more C cells than primates when comparing body weight to total amount of thyroid tissue (Lietz 1971).

C cells constitute 1-2% of the weight of hog thyroid (Copp et al. 1967). Lietz, (1971) referring to works of several authors stated the relative amount of C cells seems to be age-dependent in the rat.

Stux, et al. (1961) established that administration of thyrotropic hormone did not alter the number of C cells but did make them more conspicuous, especially in young (8 + day old) rats. Investigation of a frequency-age relationship employing administration of thyrotropic hormone indicated that the proportion of light cells to follicular cells did not vary between the ages of 8 days and 15 months. In newborn rats the C cells could not be counted even after large doses (200mu/gm. body wt.) of thyrotropic hormone (Stux, et al. 1961).

Bouin fixed tissue, which had undergone acid hydrolysis followed by toluidine blue stain, showed a change in the number of C cells with age. Thyroid glands of newborn guinea pigs and rabbits showed fewer C cells with less numerous secretory granules as compared to those of adult animals (Solcia and Sampietro 1968). An increase in the number of demonstrable C cells with age was indicated in the dog by Nonidez (1931, 1932) and in the rabbit by Raymond (1932). They had utilized the Cajal silver impregnation method.

Although the C cells of the rat thyroid are of ultimobranchial origin (Carvalheira and Pearse 1968), no relationship was found between identifiable ultimobranchial tissue surrounded by thyroid tissue and the

distribution of the C cells within the gland.

The C cells of the rat (Wissig 1962), the rabbit (Velicky 1971), and the dog (Teitelbaum et al. 1970), are confined within the follicular basement membrane and are separated from the lumen of the follicle by a follicular cell cytoplasm layer. The C cells do not come into contact with the colloid. This can only be demonstrated by the high resolution of the electron microscope.

INVESTIGATION

Materials and Methods

Tissue

Tissues used in this research were obtained by Dr. Wayne Oxender with the assistance of Dr. E. Paul Reineke. Financial support was from a grant awarded to Dr. Harold Hafs.

Dr. Oxender's research group interrupted the pregnancies of two-year-old Holstein heifers by Caesarean sections. Fetuses at three stages of gestation were obtained. Fetal age ranges were 88-95 days, 170-184 days and 260-270 days with 15, 14, and 11 thyroids from each respective age range. The sex of each fetus was recorded and the removed thyroids were weighed. Appendices I, II, and III list the data for each specimen collected. Tabulation of the data in Appendices I, II, and III was originally presented by Hernandez (1971) and is reproduced here slightly modified for ease of reference. Additional data has been included which was not in the original form.

Tissues from fetuses in the 1st trimester will be referred to as 90 day fetuses. Tissues from the 2nd trimester fetuses will be referred to as 170 day fetuses. Those specimens from the third trimester calves will be referred to as being from 270 day fetuses.

Each specimen was assigned an accession number. Appendices I, II, and III correlate the specimen numbers, with the actual age of the fetus. Thyroid tissue was

also obtained from adult cows at the time of slaughter. The cows were dams of some of the above fetuses.

All tissues were fixed in a mixture of acetic acid, formalin, ethanol, and tap water. (150 ml. tap water, 75 ml. of 95% ethanol, 25 ml. formalin, 40%, and 5 ml. glacial acetic acid.)

After a minimum of 25 hr. and a maximum of 48 hr. fixation, the tissue was cut into blocks. The small lateral lobes (one-half of the thyroid) from the 90 day fetuses were divided into two parts. One part was returned to the fixative and saved while the other part was dehydrated with tetrahydrofuran (Fisher Laboratories) and embedded in Paraplast (Sherewood Medical Industries). The details of dehydration and infiltration procedures have been elaborated upon in Appendix IV. Sections of appropriate size, approximately the same thickness but of larger diameters were cut from the fixed thyroids of the second and third trimesters and adults. These were processed in a manner identical to the 90 day specimens. Left over tissue was returned to the fixative.

Staining

A variety of stains were tried on the thyroid tissue described above in order to compare their relative abilities to differentiate the C cells. It was desired to select a stain which would facilitate enumeration of C cells and to gain general information as to the staining reaction(s) of the C cells of bovine thyroids.

The Cajal silver block staining technique was applied

to small blocks of fixed tissues. The procedure is described in Appendix V. This was the same technique which was so successfully applied by Nonidez (1931) to dog thyroids. Tissues from the 2nd and 3rd trimesters and adults were used. Four day impregnation and five day impregnation times with reduction times of 3 and 20 hr. were tried.

According to Kameda (1968) Davenport's silver impregnation method was applied as described in Appendix VI to 5u sections of randomly selected sections from randomly selected tissues of each age group. Since Kameda had recommended that Carnoy or formalin fixed tissues should be refixed in Bouin's fluid either for 12-24 hr. at 37°C or for 2 hr. at 50°C and then rinsed in tap water for 1 hr., a refixation of bovine tissue in Bouin's fluid was undertaken. Deparaffinized, adfixed tissues were subjected to the refixing technique. Then the silver impregnation method of Davenport was applied.

Rat thyroids which had undergone the same fixation, dehydration and infiltration process were also stained with Davenport's silver stain according to Kameda (1968). This was to test for possible species difference in the expected argyrophilia of C cells.

A comparison of various fixatives applied to thyroid tissue and their respective effects upon staining properties (when subsequently hydrolyzed with a 0.2 N HCl solution for 3 hr. at 60°C and stained with a 0.01% toluidine blue and a 0.02 M McIlvain buffer, pH 5.6) was

reported by Solcia et al. (1968). He found that tissue fixed in 95% ethanol gave negative results for thyroid C cells. In comparison, tissue fixed in Bouin's fluid with 1% acetic acid gave the best results for staining of C cells after acid hydrolysis.

Deparaffinized sections were hydrolyzed in 0.2N HCl solution at 60°C for 2, 3, 5, 7, and 25 hr., then stained with a 0.1% toluidine blue at pH 5 (formulae are given in Appendix XI) for 7 minutes. Adjacent sections were stained with 0.1% toluidine blue without prior hydrolysis. Bovine uterine tissue processed like the thyroid tissue was treated with hot HCl for 6 hr. and stained with toluidine blue. Adjacent sections were stained in the same manner omitting the hydrolysis step.

Tissues were sectioned at 7u for the application of routine stains. Each specimen was routinely stained with Harris Haematoxylin (Harleco) and Eosin. The technique and formulae are described in appendix VII.

One section from each specimen was stained with periodic acid Schiff and Harris Haematoxylin (Harleco). Appendix VIII contains the details of the technique and the formulae.

Mallory's trichrome stain was applied to a few randomly selected sections from randomly selected specimens, at least one from each of the age groups. Details of the technique and formulae are give in Appendix IX.

A silver nitrate method of staining developed by Grimelius (DeGrandi 1970) and slightly modified by

DeGrandi was tried. The technique and formulae are presented in Appendix X. The tissue was sectioned at a thickness of 5u.

Staining Results

The Cajal silver block staining technique yielded negative results for the impregnation times and reduction times tried for all three fetal age groups and the group of adult tissues.

The results of Davenport's silver impregnation method were so poor as to be considered unsuitable for future enumeration studies of the C cells. The silver granules were distributed evenly in all cells, and evenly in the colloid. Results of each repetition were negative. The procedure was lengthy and required much silver nitrate.

The rat thyroids which had undergone the same fixation, dehydration, and infiltration process showed very few cells (2 or 3 per cross section of an entire lobe) to be argyrophilic. It was assumed that the fixative was interferring with the staining reaction as rat tissue had been successfully stained for C cells by this method by Kameda (1968).

Thyroid tissue refixed in Bouin's fixative demonstrated no noticable improvement in selective argyrophilia. Refixation was deemed unsuccessful for the purpose of demonstration of argyrophilic C cells.

Thyroid tissues subjected to hot acid hydrolysis and stained with toluidine blue demonstrated metachromatic cells; these cells were demonstrated for each period of

hydrolysis. Tissue sections hydrolyzed for 7 hr. were better differentiated for metachromatic cells than those hydrolyzed for shorter periods of time due to a less intense blue staining of the follicular cells. Of the time periods tested those sections hydrolyzed for 25 hr. produced the best differentiated metachromatic cells.

In such treated tissue the granules of the metachromatic cells were a bright red-purple of a slightly lesser intensity than for the shorter periods of hydrolysis. These slides had the faintest blue stained follicular cells for the hydrolysis times tested. The follicular cells were such a faint blue that they were nearly colorless. These were the best differentiated for the purposes of locating, enumerating and photographing as evidenced in Figure II. Metachromatic cells were in areas of connective tissue as well as adjacent to the follicular cells. Figure II shows them in an area of connective tissue. They were well separated from each other. None were seen in apposition to another similarly stained cell. A metachromatic cell adjacent to follicular cells is shown in Figure III. No metachromatic cells were seen in the follicular lumens nor were any seen in close proximity to the colloid.

The sizes of the metachromatic cells were variable. They ranged from 4.6-11.5u in diameter and from 11.5-23u in length. Their appearance was that of a cell with two long processes extending in opposite directions from the midportion of the cell. There was a greater

consistency in the measurements of the diameters than in lengths of the metachromatic cells.

Staining with toluidine blue without pretreatment of the tissue stained mast cells metachromatically. Bovine uterine tissue so treated demonstrated red-purple metachromatic mast cells. Bovine uterine tissue pretreated with hot HCl for 6 hr. and stained with toluidine blue also revealed metachromatic mast cells. Hydrolysis did not destroy the stainability of mast cells in uterine tissue. Although difficult to locate due to poor contrast with the follicular cells, the location and number of metachromatic cells in nonhydrolyzed thyroid sections stained with toluidine blue was determined. This data corresponded with the number and location of the metachromatic cells in the hydrolyzed sections. Therefore, it was concluded that the fixative used for the thyroid tissue did not allow demonstration of C cells by the technique described in Appendix XI.

The C cells observability (degree of differentiation) and appearance varied with respect to the stain applied to the tissue. Haematoxylin and eosin, PAS, and Mallory's trichrome stains stained the cytoplasm of the follicular cells darker than the cytoplasm of the C cells. Such was the appearance of the "ovoid" cells of Bensley (1914) as he used the phosphotungstic-haematoxylin method of Mallory. Thyroid tissue stained with either of the three routine stains demonstrated only relatively few cells with a clear staining cytoplasm and a larger size. Due

to the description of the appearance of the C cells discussed in the literature review, those cells with clear staining cytoplasm were considered C cells. Contrary to Idleman (1962) and in agreement with Velicky (1971) the cytoplasm of the C cell was PAS negative in all three trimesters of fetal life and in the adults examined. Figures IV, V, and VI show C cells as distinct appearing cells. Only those with the clearest, best differentiated cytoplasm were photographed. The appearances of C cells in thyroids stained with Mallory's trichrome stain are shown in Figures VII, IIX, and IX. Cells with an intermediate morphology between C cells and follicular cells were seen in the thyroid tissue of all age groups studied.

The number of clear cells appearing at any age was extremely low. Rarely were C cells differentiated by routine stains in 90 day old specimens. The 170 day old fetuses seemed to show more well differentiated C cells than the other fetal age groups.

The results of the silver nitrate method of Grimelius were very encouraging. It was well suited to the bovine thyroid tissue fixed as described. Figures XI, XII, and XIV show C cells to be clearly differentiated from follicular cells. The C cells were highly argyrophilic. Figures X and XI reveal a vast difference in the differentiation of C cells with H&E and the Grimelius silver method. The tissue sections in Figures X and XII were of the same general area of the same thyroid. The distance between the sections approximately 14u. Figure X shows no cells with a clear

staining cytoplasm in the tissue stained with H&E. Figure XII shows thyroid tissue stained by the Grimelius method to have numerous argyrophilic cells. These silver stained preparations were ideally suited for enumeration of the C cells.

Methods of
Enumeration Studies

Tissue stained with toluidine blue after acid hydrolysis was examined for the number of metachromatic cells. The metachromatic cells were enumerated for each age group. The following procedure was followed for this enumeration. An ocular grating with a calibrated area of 0.0132 sq. mm. was used. The number of metachromatic cells which appeared in 100 fields at 450X magnification was the basis of comparison. The total area of tissue examined from each specimen was 1.32 sq mm. The fields were selected at random by advancing the slide in a predetermined random pattern. The ocular containing the grating was rotated one-quarter turn before each ensuing count of a new field in keeping with the random field selection.

Using silver stained preparations an enumeration of the argyrophilic C cells was accomplished. The following procedure was followed for this enumeration. An ocular grating with a calibrated area of 0.0132 sq. mm. was used. Argyrophilic cells appearing in 100 randomly selected fields were counted for each specimen studied. The total area of tissue examined from each specimen was 1.32 sq. mm. The magnification was 450X. The fields were selected at random by advancing the slide in a predetermined pattern. The ocular containing the grating was rotated one-quarter turn before each ensuing count of a new field.

Determination of Volume Ratios

The volume ratios were determined on the basic assumption that point ratios equal volume ratios. P p = V v if so many points out of all points hit on one kind of tissue, this tissue occupies the same percentage of space in the organ (Elias and Pauly 1969).

Volume percentages were determined for the follicular epithelium, follicular lumens and stroma. Included with stroma were, in addition to connective tissue, blood vessels and artifacts.

At a magnification of 450X a calibrated grating with 36 equidistant points located 23 u apart was used. The total area of the grating was 0.0132 $mm.^2$ Twenty-five randomly chosen fields were examined for each tissue specimen studied. At each point of intersection of the grating the type of underlying organ component was determined and recorded. Since each field had 36 points of intersection, 25 fields had a total of 900 points of intersection.

When Grimelius silver stained tissue was examined it was possible to clearly differentiate the argyrophilic C cells from the follicular cells. For those tissues volume percentages were determined for four classes of tissue components: follicular epithelium, lumens, C cells, and stroma which again included, in addition to the connective tissue, blood vessels and artifacts.

Methods of
Data Interpretation

Recalling that the standard total area of enumeration was 1.32 mm.^{2} and believing that certain component volume percentage changes would occur corresponding to changes in age a method was devised to evaluate the raw data into meaningful information. It was realized that for a uniform area of enumeration an expected increase in the percent volume of the lumena would have certain possible effects. An increase in follicular luminal volume in effect excluded C cells from an increasing area of the enumeration field. The effect of differential growth could have the following interpretations. For a constant area the number of C cells would seem to be less if the total number of C cells remained constant with age. If the number of C cells in the gland increased the number counted in a given area could theoretically remain constant. Higher counts in uniform area fields with specimens of increasing age could only be due to an increase in total C cell population. The actual increase would be greater than indicated in the raw data because the increased area occupied by the follicular lumens decreases the percentage of the total area which may be occupied by C cells.

To obtain a more meaningful appreciation of the increase in the number of C cells observed, the number of C cells was equalized on the basis of a constant area which could accomodate C cells. The equalized C cell

count (ECc) equaled the number of C cells per 1.32 mm.^{2} (N) divided by the percent volume of accomodating tissue (A). The percent volume of accomodating tissue is defined as the total volume minus the percent volume of the lumens.

$$ECc = \frac{N}{A}$$

To evaluate the raw data, equalized cell counts were established in the predescribed manner for each age group. The equalized cell counts of the 170 and 270 day old fetuses and adults were then compared to the equalized value of the 90 day old fetuses to establish the ratio of change in cell numbers. The 90 day old fetus counts were converted to a unit of one. The changes in the other age groups were evaluated in relation to the enumeration of the equalized cell count of the 90 day old fetuses.

Observation of
Ultimobranchial Tissue

The slides were examined for the possible presence of ultimobranchial tissue. Identification was based on the descriptions by Kingsbury (1935), Anderson (1922), and Jubb (1959). They described ultimobranchial tissue in calf thyroid as characterized by nuclei more heterochromatic than nuclei of follicular cells.

Ultimobranchial tissue seen in routine stained tissue sections was compared with silver stained tissue sections when such adjacent or very close sections were available.

Photographs were used as an aid to study ultimobranchial tissue and the argyrophilic C cells.

Enumeration Results

The metachromatic cells were enumerated for each age group. Appendix XII lists the raw data for the metachromatic cell enumeration. For the 90, 170, and 270-day-old specimens the respective metachromatic cell number ranges for 100 fields were: 0-15, 2-17, and 9-46. The average number of metachromatic cells per 100 fields for the 90, 170, and 270-day-old specimens were respectively: 3.5, 8.1, and 19 cells. The number of metachromatic cells increased with age. A numerical comparison was made between the number of metachromatic cells and the number or argyrophilic (Grimelius stain, see Appendix IX) cells seen in tissue sections which were either serially adjacent or not more than 21 u apart. Appendix XII lists the data for the comparison of the number of metachromatic cells per 100 fields with the number of argyrophilic cells per 100 fields.

The numbers of metachromatic cells were less than the number of argyrophilic cells in comparable sections in all age groups. Due to the much greater argyrophilic cell count than the metachromatic cell count in adjacent or nearby tissue sections and due to the variation in the locations of the greater concentrations it was concluded that the two cell types were not histochemically identical. Alcohol fixed tissue which was hydrolyzed and stained with toluidine blue did not differentiate C cells for Solcia *et al.* (1968)

It was suggested by the results of the staining investigation already discussed that the metachromatic cells may be mast cells. It was suggested by the above results and previously described examinations that the metachromatic cells were not C cells but were most likely mast cells.

The Grimelius silver stained tissues were used for enumeration of the C cells. Table I lists the average number of C cells for each age group for a constant area.

TABLE I* Average number argyrophilic cells per 1.32 mm.^{2}

Age	Average Number Cells
1st trimester	114
2nd trimester	236
3rd trimester	129
adults	278

* computed from raw data Appendix XIII.

In order to evaluate the meaningfulness of the variation in the number of C cells found in a uniform area at four age levels the differential change in components of the thyroid glands with age had to be considered.

Appendix XIV lists the raw data collected for computation of percent volume ratios. During the fetal period there was a gradual decrease in the proportion of epithelium. The volume occupied by follicular lumens greatly increased. The greatest increase of follicular lumen volume in fetuses was between the 1st and 2nd trimester. The third trimester luminal percent volumes

were only slightly greater than the second trimester luminal percent volumes, (Table II).

TABLE II Thyroid Gland Component Percentages

Age	Epithelium	Lumens	Stroma
1st trimester	61.4	2.9	35.5
2nd trimester	52.92	23.9	23.2
3rd trimester	56.77	26.1	17.1
adult	19.6	60.7	19.5

Routine stains did not differentiate C cells satisfactorily. Such stained sections will not accurately reflect the C cell component. Those C cells not clearly differentiated were counted as epithelial cells. Based on the data in Appendix XIV the percent volume of C cells which may be differentiated by routine stains is 0.22% of the entire thyroid gland for 2nd trimester thyroids. Also based on the data in Appendix XIV, the percent volume of argyrophilic C cells for 2nd trimester thyroids was 2.4 .

The changes in the volume of thyroid components were taken into consideration in evaluating the changes in C cell number of the various age groups examined. The following changes were observed with increasing age: the volume of stroma decreased but much less than the decrease in the percent volume of the epithelium. The percent volume of the follicular lumens greatly increased.

The percentage of tissue which could accomodate C cells decreased with an increase in age. This was due to the increase in the percent volume occupied by

follicular lumens. Since the C cells were never found in lumens of the follicles the increase in follicular luminal volumes in effect excluded C cells from an increasing percentage of the area of the total field enumeration area. Table III illustrates the magnitude of this effect.

TABLE III Percentage of Accomodating Tissue

Age	A%
1st trimester	97.1
2nd trimester	76.
3rd trimester	73.1
adult	39.9

Establishment of equalized C cell counts shown in Table IV shows a form of enumeration which may be used for comparison to establish the magnitude of change in the number of C cells with an increase in age.

TABLE IV Equalized C cell Counts

Age	ECc
1st trimester	119
2nd trimester	310
3rd trimester	178
adult	707

The increase in the number of argyrophilic cells in the thyroid glands of fetal bovines is not a uniform increase but is an overall increase. The increase between the 1st and 2nd trimester is offset by the decrease in C cell number between the 2nd and 3rd trimester.

Table V compares the change ratios of the various age groups based on the 90-day-old fetuses. The greatest fetal increase in the C cell number after the 1st trimester

is between the 90th and 170th day of gestation. Post-partum, a greater increase in the number of C cells (demonstrable with Grimelius silver stain) occurs. Second trimester fetal thyroids had the greatest number of demonstrable C cells of the three fetal age groups examined. A sharp decrease in the number of argyrophilic cells was noted between the 2nd and 3rd trimester.

TABLE V Ratio of Change of C cell Number Relative to the 90-day-old Fetus

Age	Ratio
170 day fetus	+ 2.6
270 day fetus	+ 1.41
adult	+ 6.

Summary

The fixative may limit the demonstration of C cells in bovine tissue of all ages. Stains such as H&E, PAS, and Mallory's trichrome were found to be poor for C cell investigation. Grimelius silver stain was very good for demonstrating argyrophilic C cells in fetal and adult bovine thyroid tissue. Tissue stained by this showed an overall increase in the number of argyrophilic staining cells with increase in fetal age. The greatest increase during fetal life occured between the 90th and the 170th day of gestation. Adult tissue showed six times as many argyrophilic cells as did tissue from 90-day-old fetuses.

Further Observations

The slides were examined for the possible presence of ultimobranchial tissue. Identification was based on the descriptions by Kingsbury (1935), Anderson (1922), and Jubb (1959). They described ultimobranchial tissue in calf thyroid as characterized by nuclei more heterochromatic than nuclei of follicular cells. Many routine stained sections had areas with clusters of cells having nuclei of the type previously described. Therefore such areas as shown in Figures IV and V were considered to be of ultimobranchial origin. The tissue of Figure IV was stained with H&E. Centrally located in Figure IV is an elongated cluster of cells with more heterochromatic nuclei and less cytoplasm than the follicular cells visible in the lower portion of Figure IV. Approximately four cells of the cluster were considered to be typical of H&E stained C cells. Figure V shows this cluster as it appeared in a section 19 u away, stained with Grimelius silver stain. The majority of the cells of the cluster were argyrophilic. It has been demonstrated that not all those cells which are argyrophilic are recognizable as C cells when stained with H&E.

Argyrophilic cells situated in an unquestionable interfollicular position were most frequently seen to be separated from the colloid by extensions of adjacent follicular cells. Several are seen in Figure XIII. A few argyrophilic cells appeared to abut upon the colloid. The use of the term 'appeared' was intended and is stressed.

Whether or not such appearing cells really contact the colloid cannot be determined by light microscopy.

To emphasize the need for the use of special stains tissues shown in Figures X and XII were compared. They were adjacent tissue sections, Stained with H&E the tissue shown in Figure X revealed no distinguishable C cells. The tissue shown in Figure XII was stained with Grimelius sliver stain. It shows many argyrophilic C cells.

On specimen showed transitional epithelium surrounding a lumen of an ultimobranchial cyst. Such was described by Jubb (1959). Figure XIX illustrates this feature. Ultimobranchial remnants and cysts were common in the 2nd and 3rd trimester fetal calves. These cysts were found in the connective tissue stroma. The most distinctive feature is the near absence of stainable material in the lumen of the cysts. Some contain scant amounts of PAS positive material. Trichrome stained tissues showed the material to be basophilic. Degenerating nuclei were seen frequently in these cysts. Figures XVII and XVIII show degenerating nuclei. Frequently as seen in Figures XVI and XVII C cells are seen in the outer portion of the cyst walls, However, this is not always observed. Figures XIX and XX show ultimobranchial cysts lacking C cells in their walls and without clusters of adjacent C cells. Several clusters of C cells were readily observable in (Figure XVIII) areas between the walls of the cysts.

The sections of adult tissue prepared for this study did not show ultimobranchial remnants. However, reference

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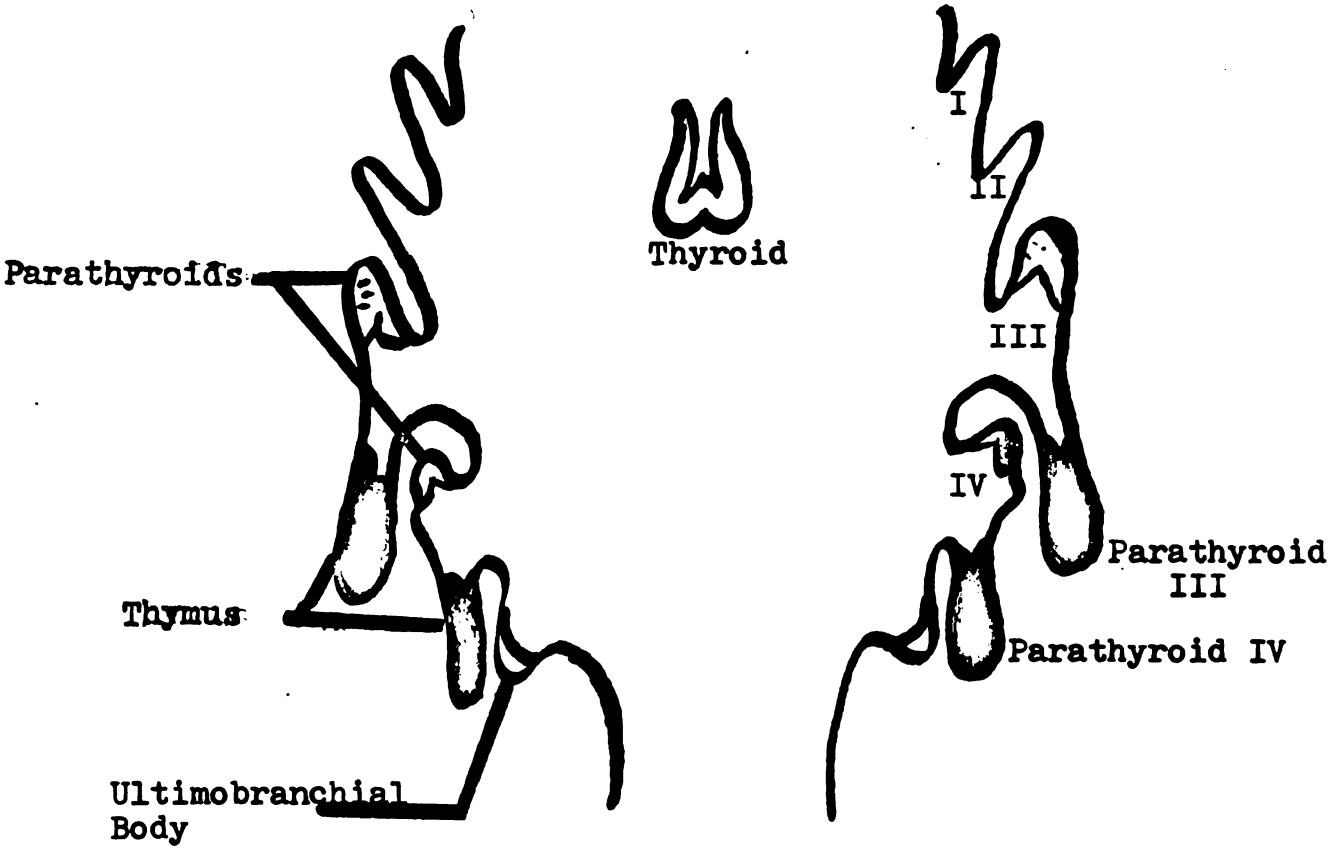
FIGURES

Figure I

Pharyngeal pouches and their derivatives.



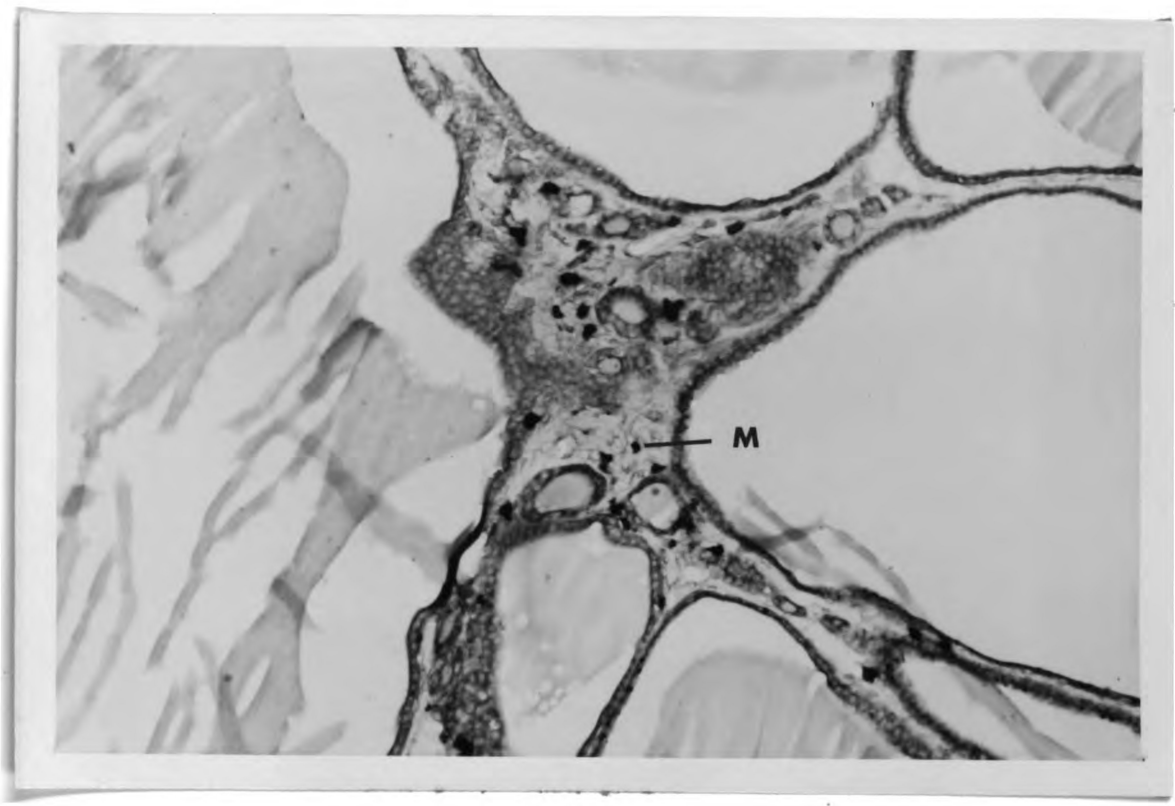
Drawn from Goss (1961).

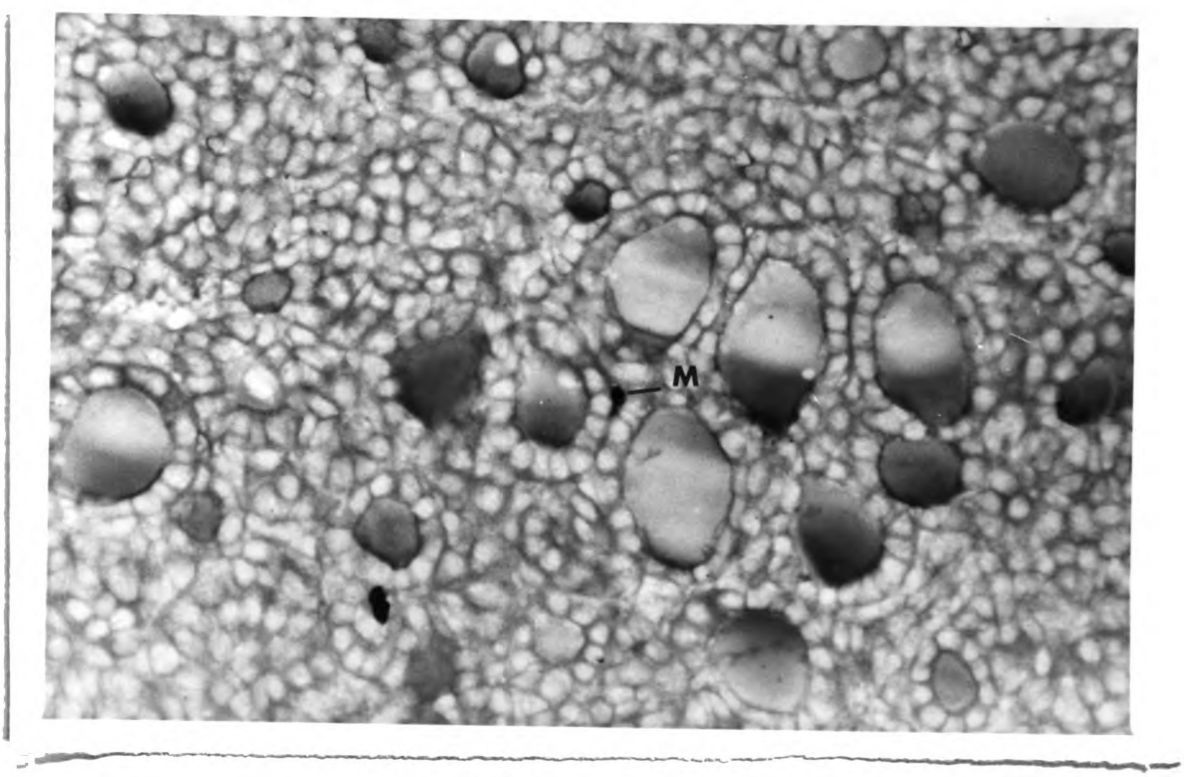
Adult thyroid, prolonged hydrolysis

Figure II Hydrolyzed and stained for 25 hr. and stained with toluidine blue. Adult (859-5)
M - metachromatic cells in area of connective tissue

Adult thyroid, moderate hydrolysis

Figure III Hydrolyzed 6 hr. and stained with toluidine blue. Adult thyroid, animal 859-5.
M - metachromatic stained cell adjacent to follicle.


M


M

Third trimester thyroid

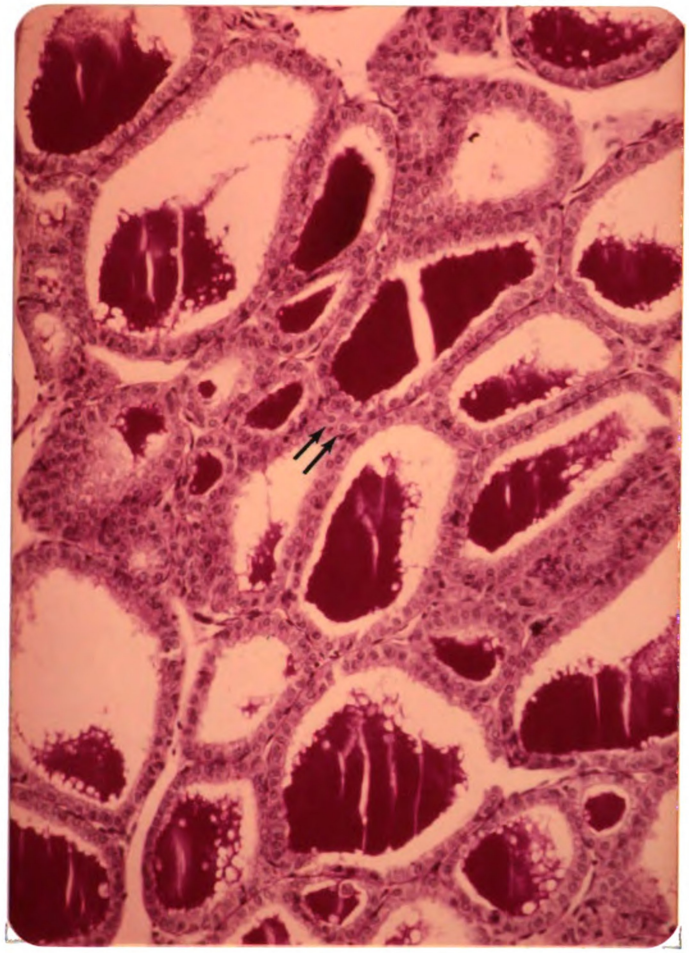
Figure IV Periodic acid Schiff and haematoxylin stained, 270 day old fetus, animal 817.

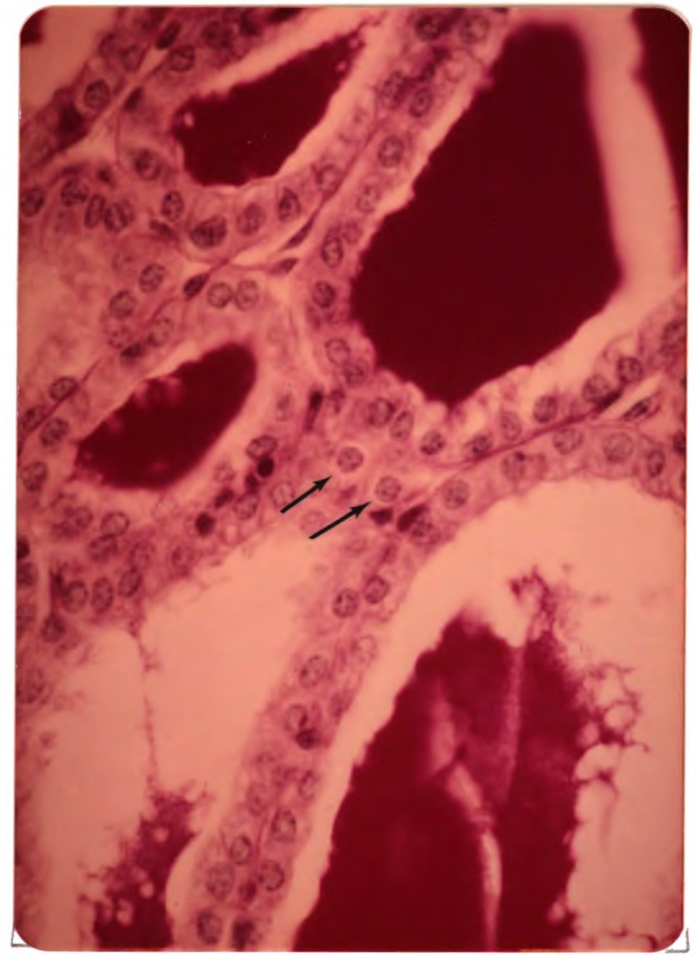
Arrows indicate C cells, distinguishable from the follicular cells by their lighter stained cytoplasm.

Third trimester thyroid

Figure V Periodic acid Schiff and haematoxylin stained, 270 day old fetus, animal 817.

Arrows indicate C cells. This is a higher magnification of the cells shown in Figure IV.





Third trimester thyroid

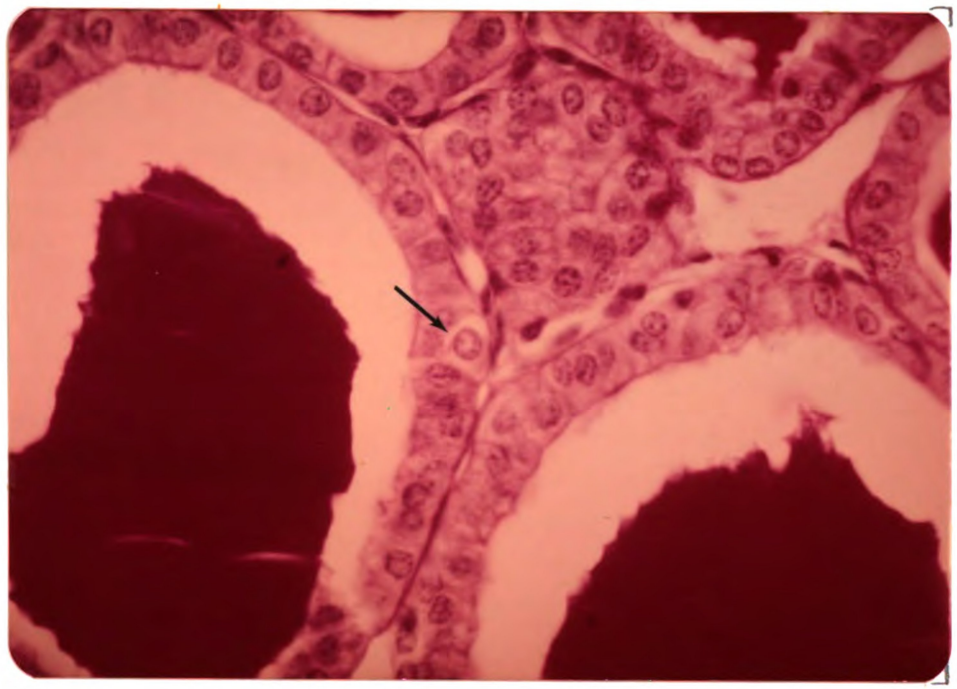
Figure VI Periodic acid Schiff and haematoxylin stained, 270 day old fetus, animal 817.

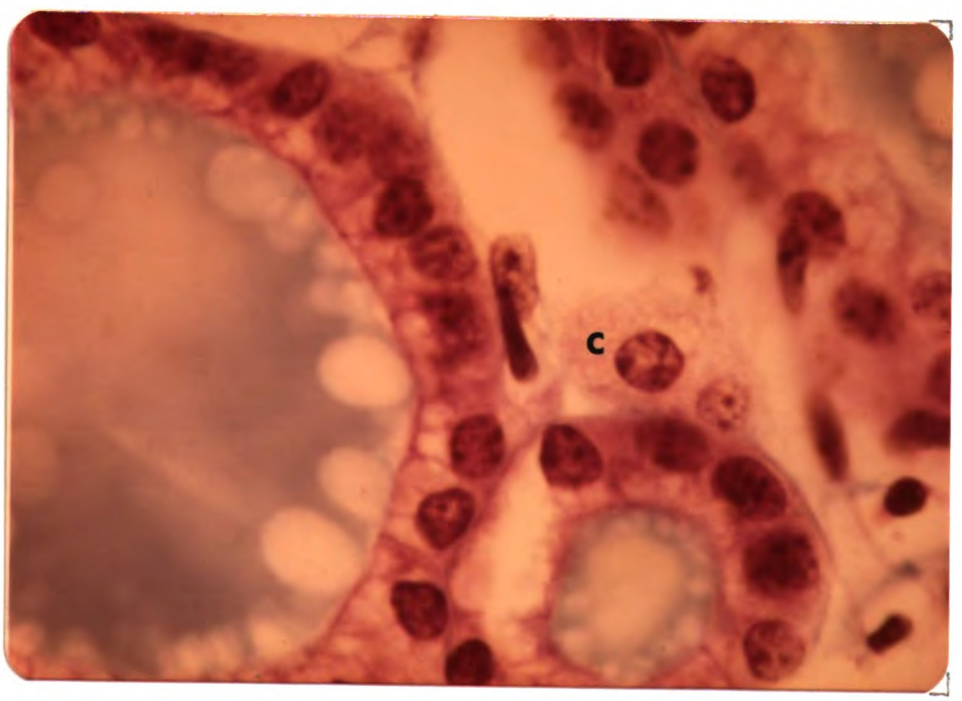
Arrow indicates C cell separated from colloid by extensions of follicular cells.

Second trimester thyroid

Figure VII Mallory's trichrome stained, 170 day old fetus, animal 841.

C - C cell




c

Second Trimester Thyroid

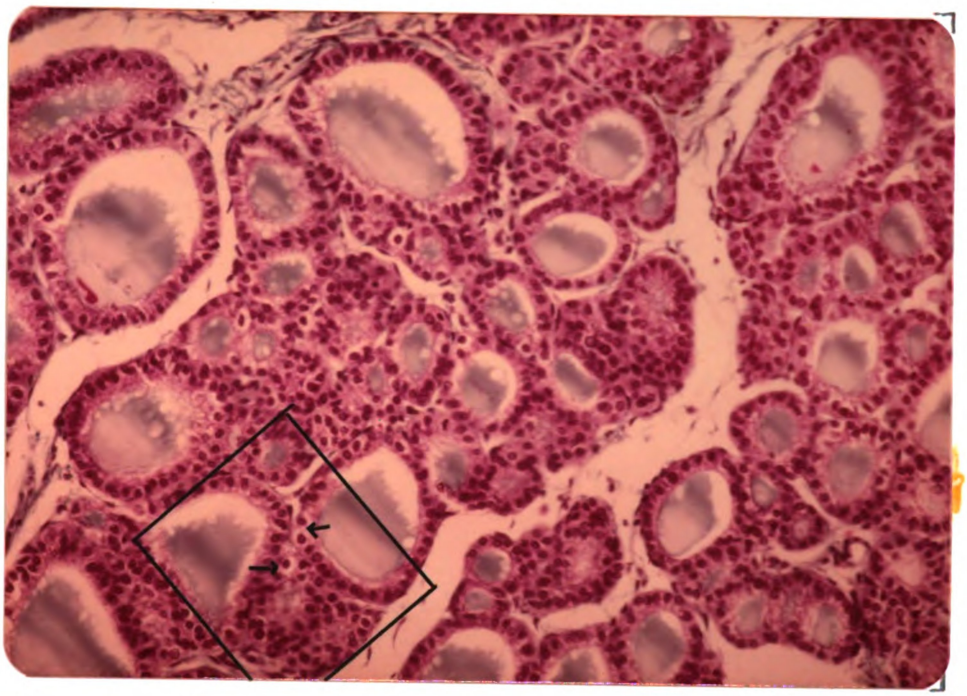
Figure VIII Mallory's trichrome stained, 170 day old fetus, animal 841

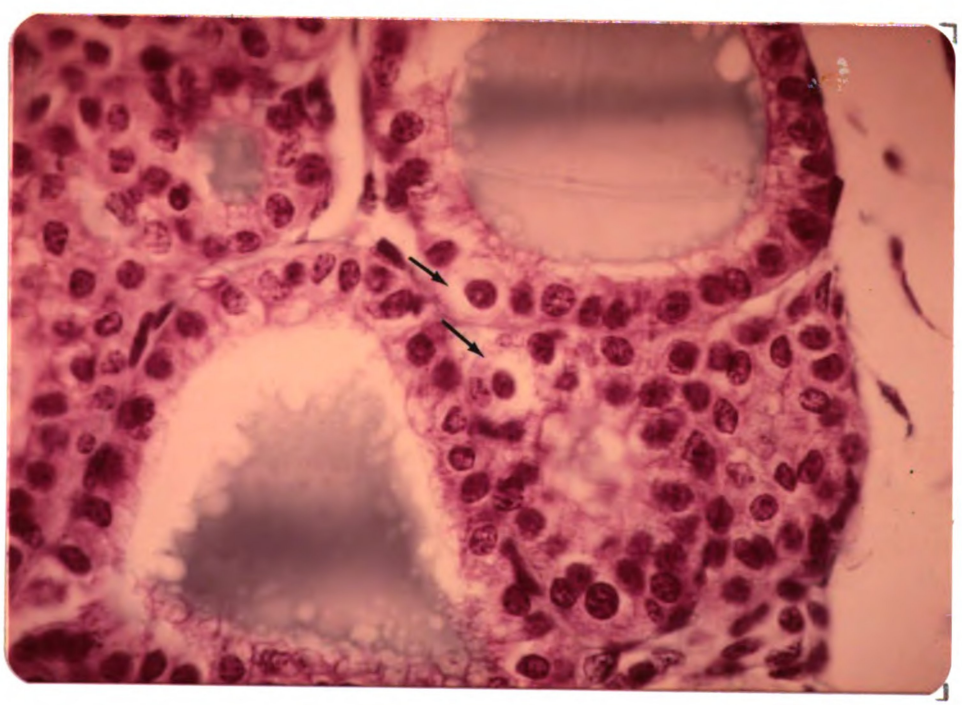
Arrows indicate C cells.

Second trimester thyroid

Figure IX Mallory's trichrome stained, 170 day old fetus, animal 841

Arrows indicate C cells. Higher magnification of the C cells within the area indicated by the rectangle in Figure VIII.





Routine stain

Figure X — H&E stained, second trimester, animal 825, 350X magnification.

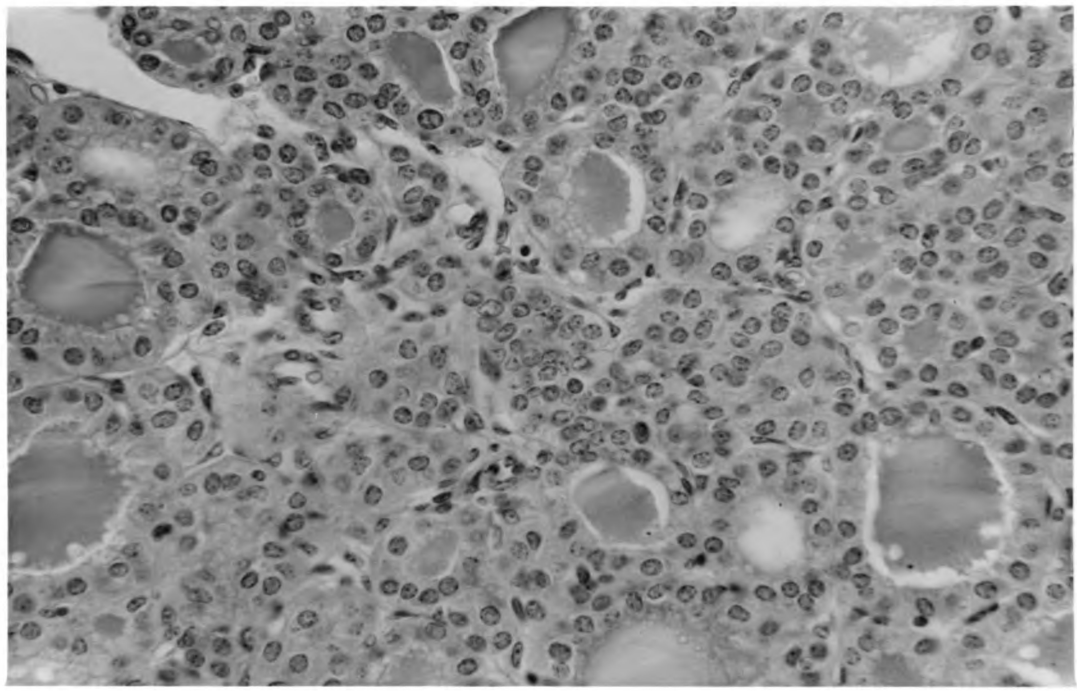
Area is from an adjacent tissue section to that of Figures XII and XIII.

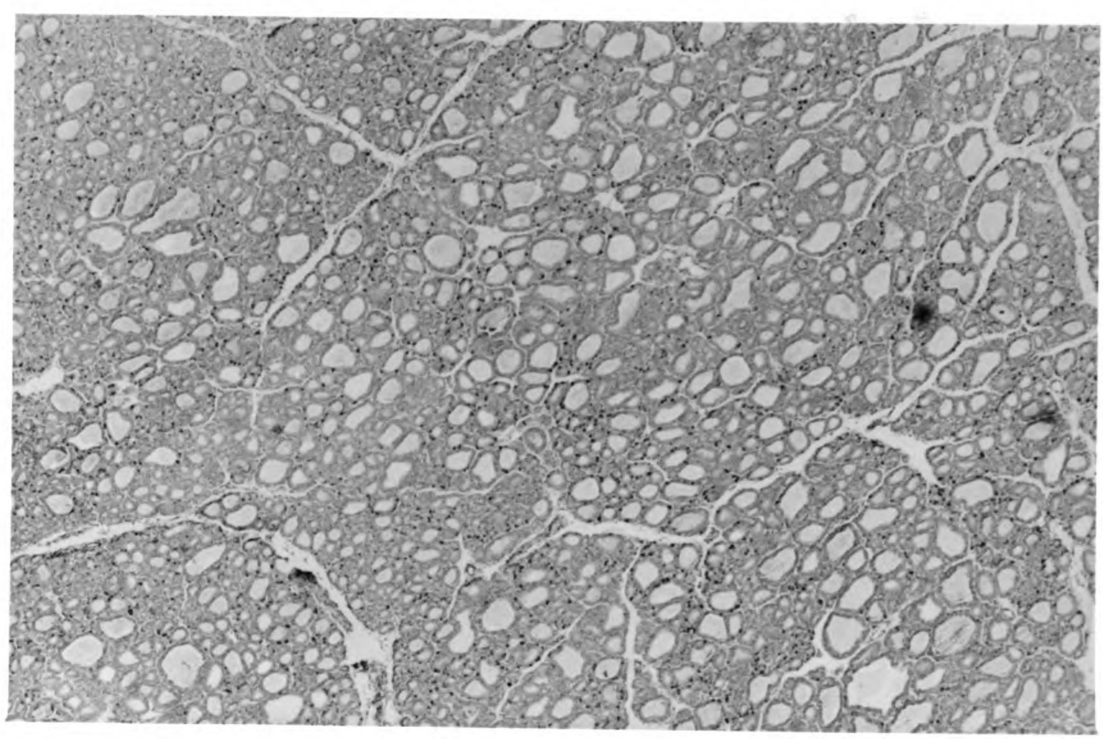
No C cells were differentiated by the H&E stain.

Specific stain

Figure XI — Grimelius silver stain, second trimester, animal 825, 35X magnification.

General distribution of C cells in section having a relatively large population of C cells. The C cells are those with the dark appearing granules in the cytoplasm. At this magnification the entire cell appears as a black dot as the granules are not resolved.





Specific stain

Figure XII Grimelius silver stain, second trimester, animal 825, 140X magnification.

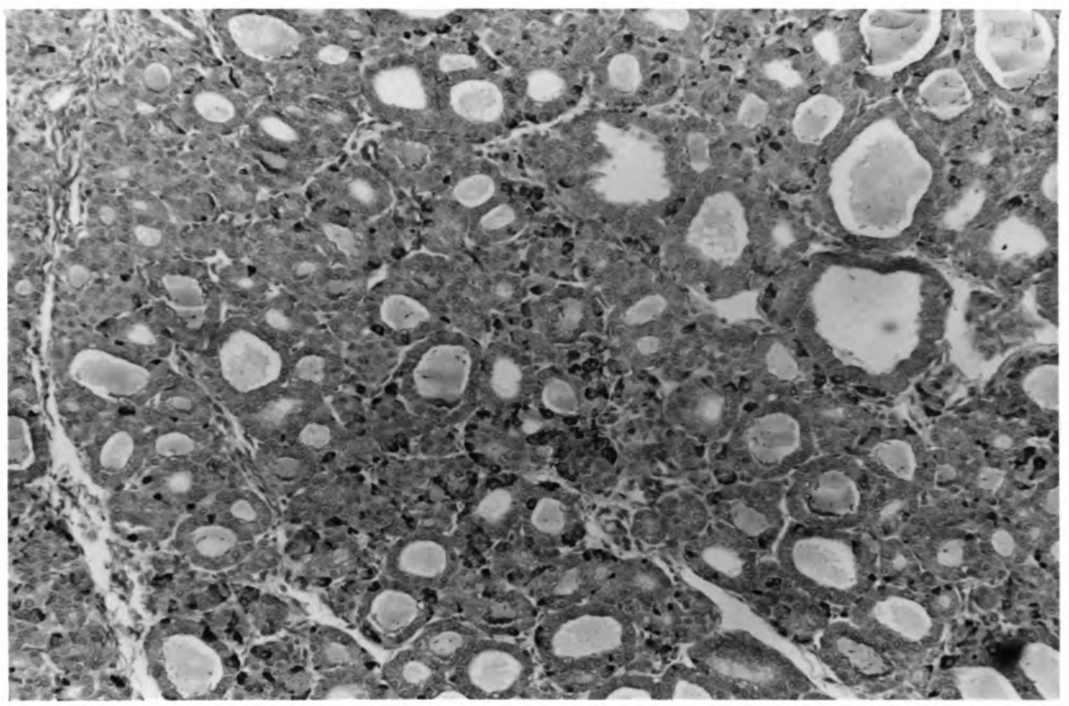
Numerous argyrophilic C cells visible with the Grimelius silver stain which differentiated them from the follicular cells. This is the same area of an adjacent section to that of Figure X.

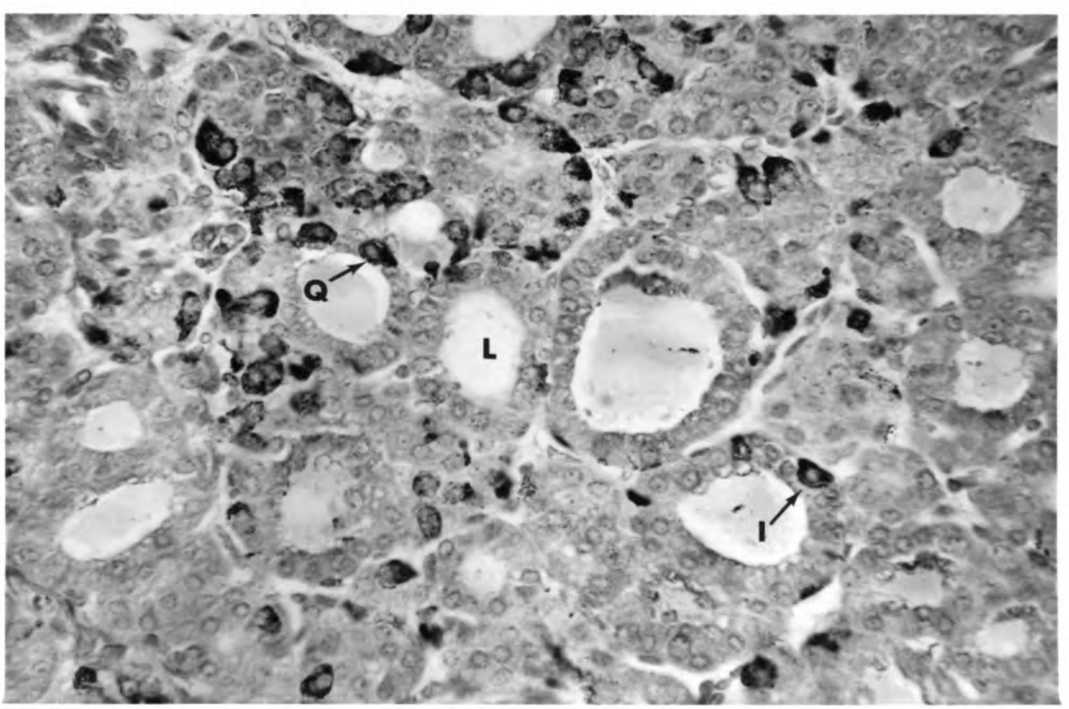
Specific stain

Figure XIII Grimelius silver stain, second trimester, animal 825, 350X magnification.

Higher magnification focused at upper two-thirds of center of Figure XII.

Q - C cell which appears very close to the colloid.
L - lumen of follicle containing colloid
I - C cell well separated from the colloid by adjacent follicular cells.




Q
L
I

Ultimobranchial tissue

Figure XIV H&E stained, second trimester, animal 825, 224X magnification.

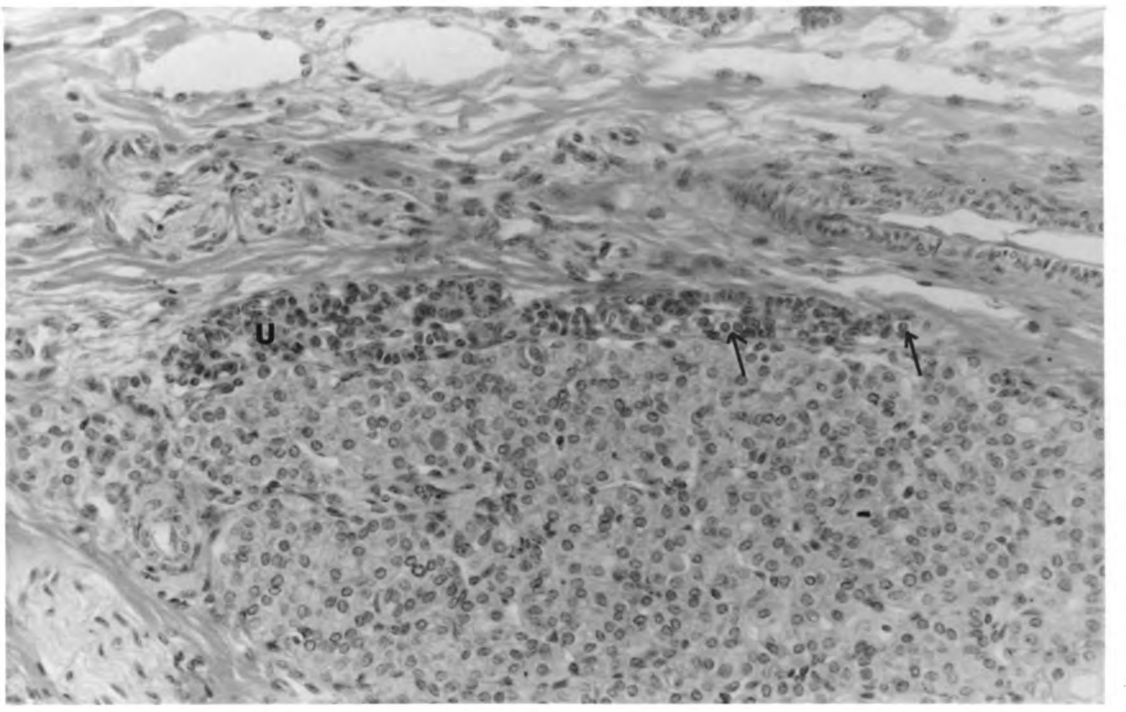
U - ultimobranchial tissue
Arrows indicate C cells

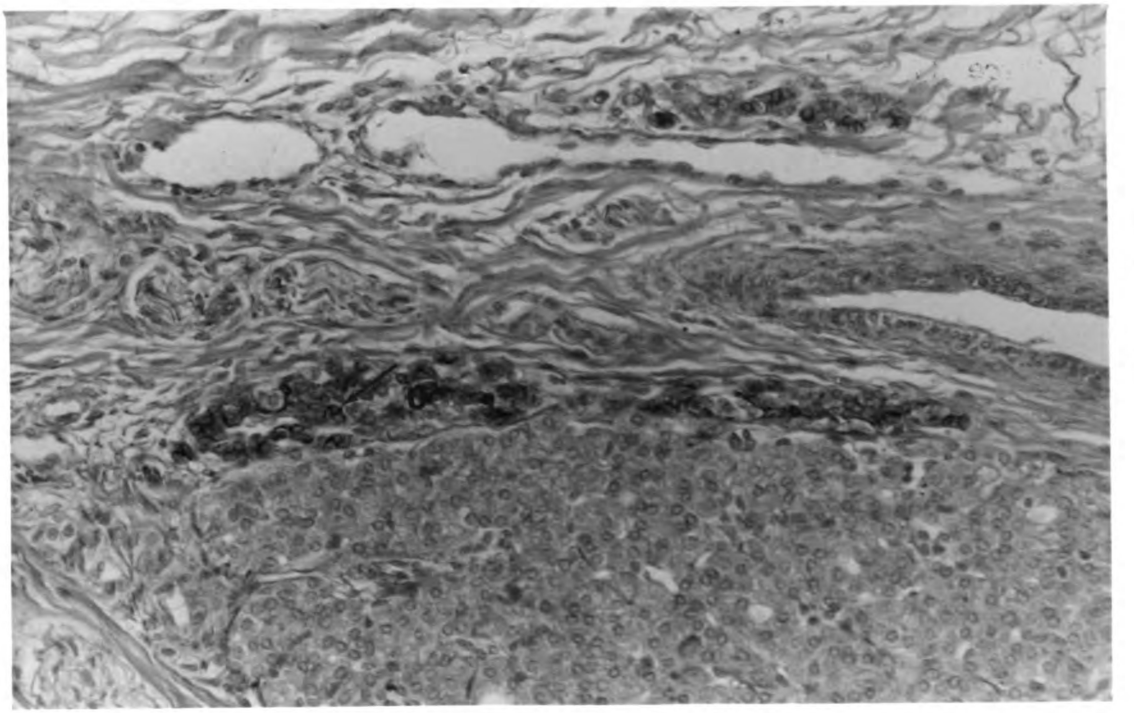
Ultimobranchial tissue

Figure XV Grimelius silver stained, second trimester, animal 825, 224X magnification

U - ultimobranchial tissue
Arrow indicates one of the many argyrophilic C cells.

Section is an adjacent serial section to that shown in Figure XIV.


U



Ultimobranchial cyst

Figure XVI H&E stained, third trimester, animal 840.

C - C cell differentiated by its light staining cytoplasm.

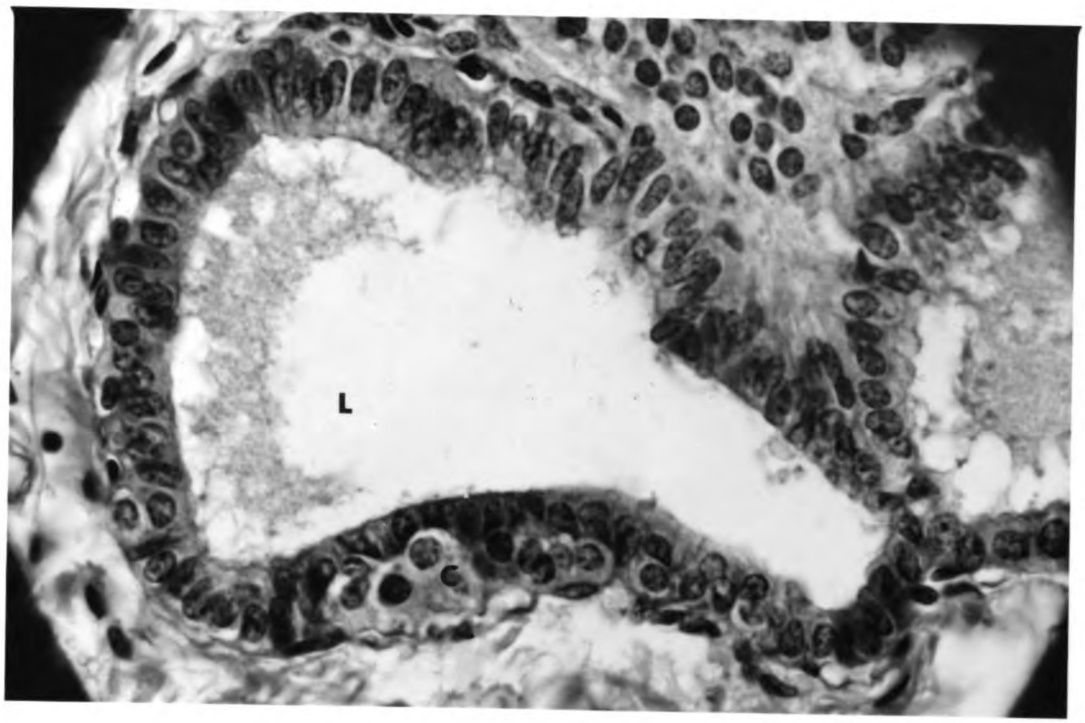
L - lumen of vesicle (cyst) containing a scant amount of stainable substance.

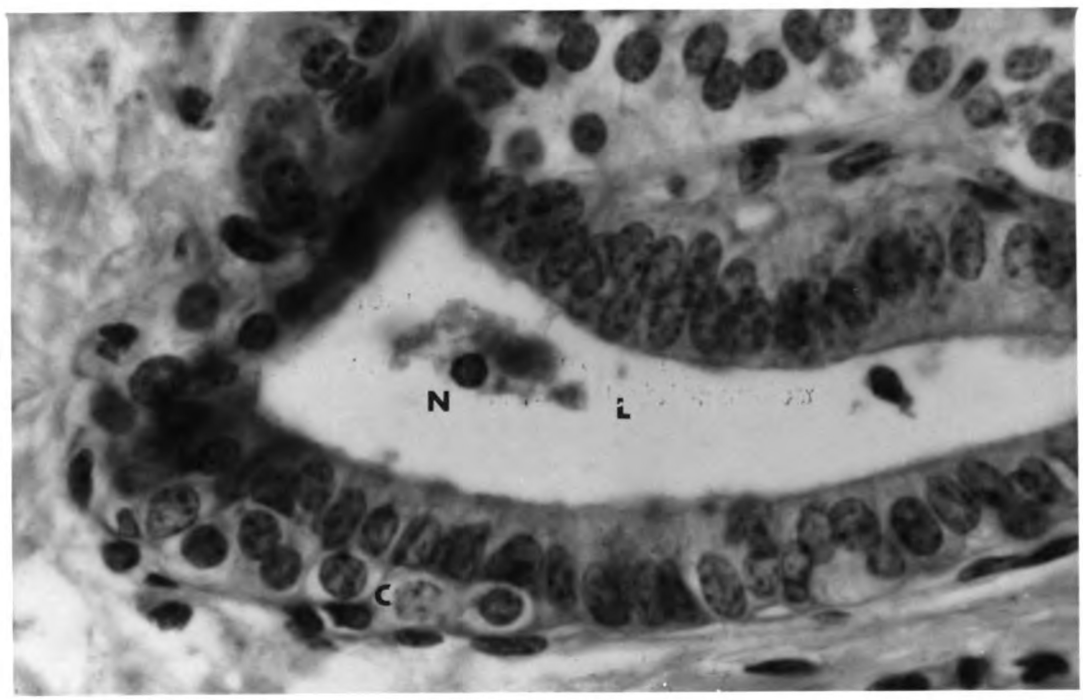
Ultimobranchial cyst

Figure XVII Period acid Schiff and haematoxylin stained, third trimester, animal 846.

C - C cell
N - unidentified nucleus of degenerating cell
L - lumen

Several C cells are seen clustered along the basal border of the epithelial cells constituting the major portion of the wall of this ultimobranchial cyst.


L
C


N
L
C

Ultimobranchial remnant

Figure XVIII PAS stained, third trimester, animal 846, 350X magnification

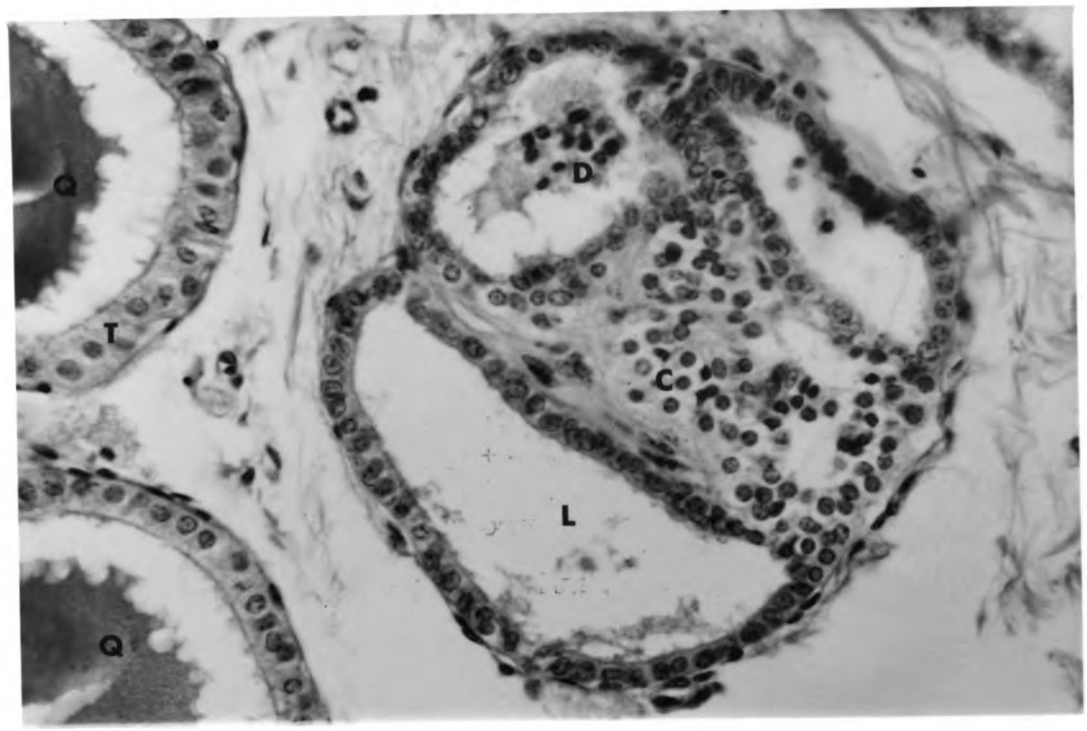
Ultimobranchial tissue showing cyst formation and clusters of C cells.

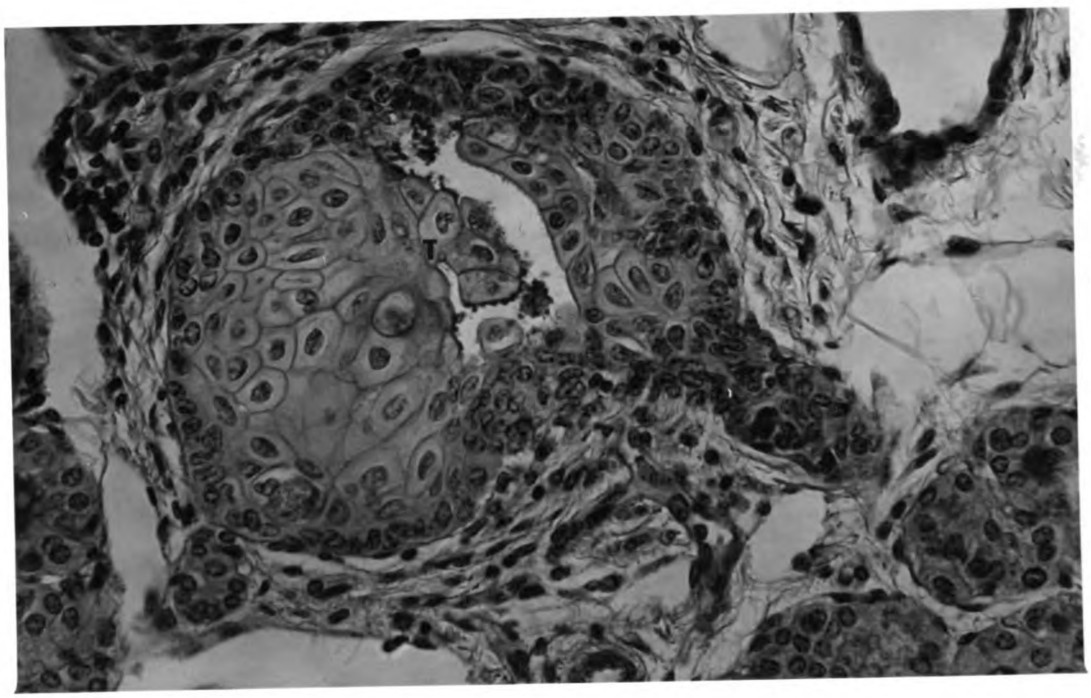
C - C cells
L - lumen of cyst, note scanty amount of material contained within
T - thyroid follicular cell in wall of typical thyroid follicle
Q - colloid
D - degenerating nuclei in lumen of cyst

Ultimobranchial cyst

Figure XIX PAS stained, third trimester, animal 828 350X magnification

T - transitional epithelium making up wall of ultimobranchial vesicle.


Q
T
D
C
L
Q


T

Ultimobranchial cysts

Figure XX H&E stained, third trimester, animal 828, 448X magnification

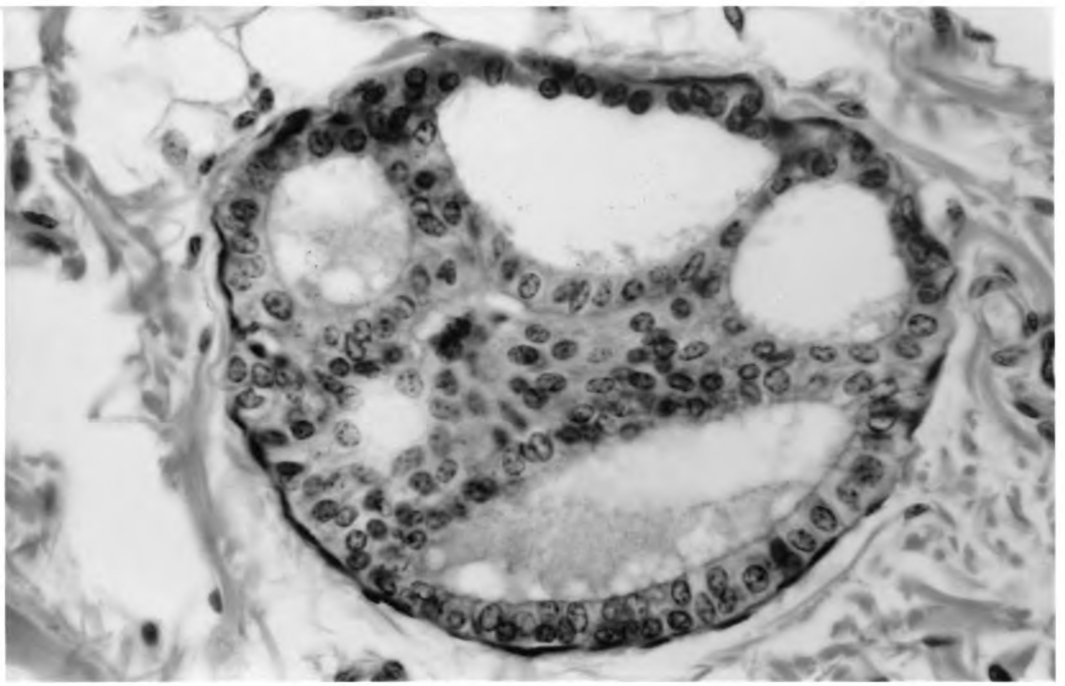
Ultimobranchial tissue showing cyst conformation. Lumen is void of typical colloid

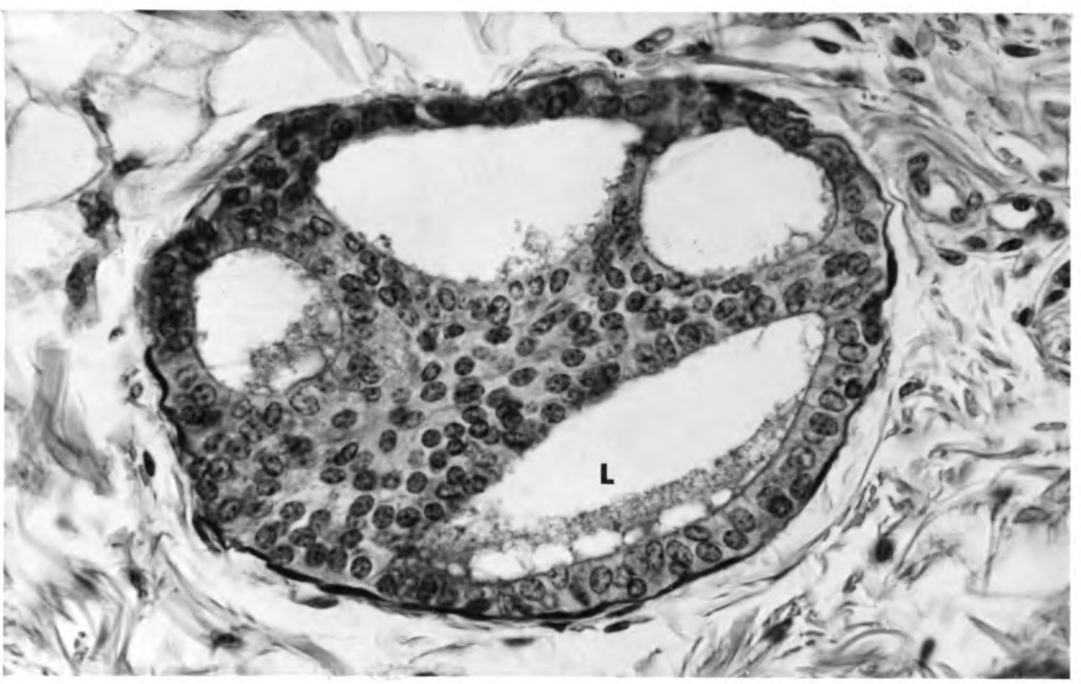
Ultimobranchial cysts

Figure XXI PAS stained, third trimester, animal 828, 448X magnification.

Same area as Figure XX from tissue section located 14 u from that of tissue in Figure XX.

L - lumen with small amount of PAS positive secretion.




L

Ultimobranchial cysts

Figure XXII Grimelius silver stained, third trimester, animal 828, 350X magnification.

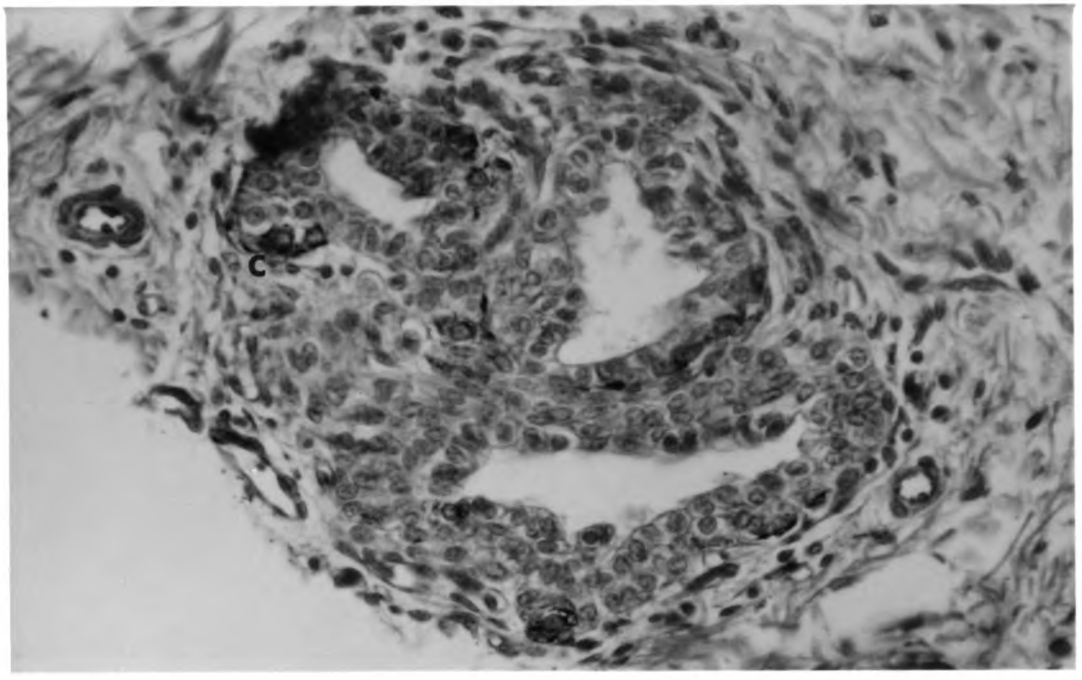
C - C cell

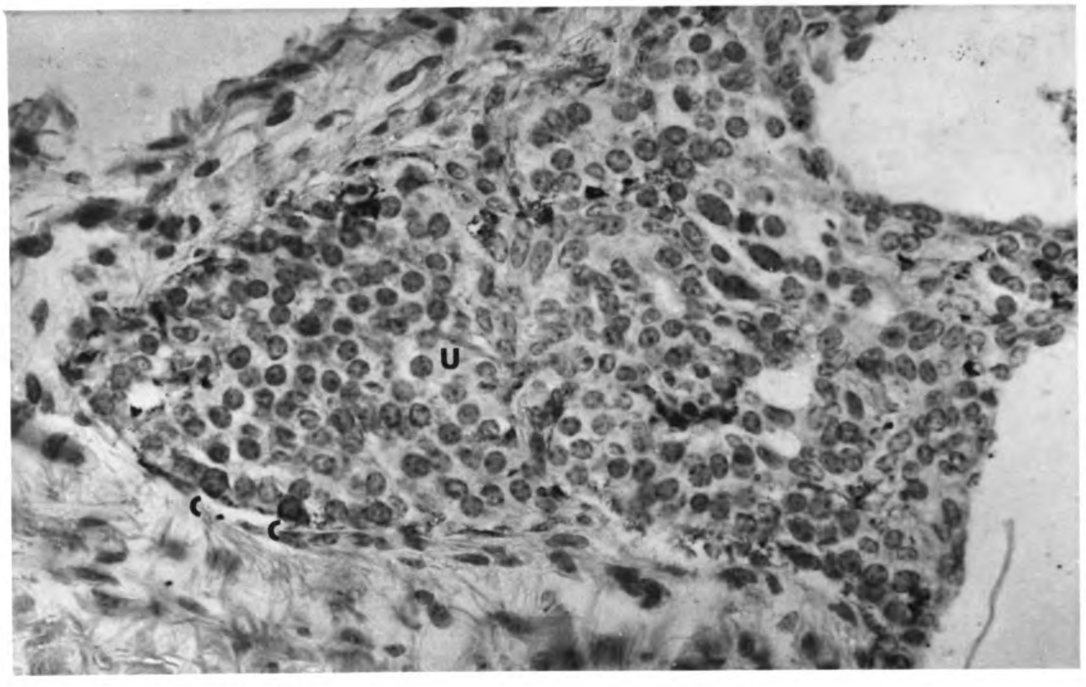
Ultimobranchial tissue

Figure XXIII Grimelius silver stained, third trimester, animal 846, 448X magnification

U - ultimobranchial cluster of cells with a few argyrophilic cells

C - C cells, argyrophilic


C


U
C
C

APPENDICES

APPENDIX I

FIRST TRIMESTER FETUSES

Anat. Dept Acc. No.	Cow and Fetus No.	Days of Gestat.	Fetus' Sex	Body Wt.Kg	Thyroid Wt. Tot. gm	Thyroid Wt. gm/Kg
826	543	91	F	0.22	0.076	0.345
827	554	91	M	0.28	0.125	0.446
823	545	91	F	0.24	0.086	0.358
830	548	89	M	0.40	0.132	0.330
832	552	88	M	0.33	0.125	0.383
834	553	88	F	0.34	0.135	0.400
848	570	90	F	0.425	0.153	0.360
849	571	91	F	0.52	0.216	0.416
850	572	90	F	0.44	0.172	0.390
852	573	90	F	0.22	0.071	0.323
853	574	91	F	0.475	0.190	0.400
854	575	93	M	0.665	0.246	0.435
855	576	93	F	0.345	0.112	0.325
861	577	95	M	1.05	0.381	0.363
860	578	92	F	0.64	0.215	0.336

Total No.	15
Male fetuses (M)	5
Female fetuses (F)	10

APPENDIX II

SECOND TRIMESTER FETUSES

Anat Dept Acc. No.	Cow and Fetus No.	Days of Gestat.	Fetus' Sex	Body Wt.Kg	Thyroid Wt. Tot. gm	Thyroid Wt. gm/Kg
821	540	184	M	4.5	1.33	0.296
822	541	183	F	5.9	2.27	0.385
820	542	179	M	6.30	1.49	0.237
824	546	178	F	6.72	2.65	0.394
825	547	177	F	7.44	2.46	0.330
829	551	178	F	6.10	2.13	0.349
833	554	180	M	5.92	1.98	0.334
835	555	179	M	7.14	1.76	0.246
838	556	180	M	6.68	1.58	0.237
839	557	180	F	6.02	1.94	0.322
840	558	180	M	6.14	1.83	0.298
841	559	180	M	7.90	2.35	0.297
844	561	180	M	4.64	1.697	0.366
851	567	180	F	6.20	2.160	0.348

Total No. 14

Male fetuses (M) 8

Female fetuses (F) 6

At the 2nd trimester the mean thy. gld. wt. (2.268 gm) was significantly higher (P 0.001) than the mean thy. gld. wt. (1.752 gm)

APPENDIX III

THIRD TRIMESTER FETUSES

Anat. Dept Acc. No.	Cow and Fetus No.	Days of Gestat.	Fetus' Sex	Body Wt.Kg	Thyroid Wt. Tot.gm	Thyroid Wt. gm/Kg
819	515	270	M	38.8	11.23	0.289
817	524	270	M	21.3	5.76	0.270
818	525	270	F	19.3	7.92	0.410
804	539*	270	M	23.94	80.50	0.269
831	549	264	M	33.1	9.21	0.278
828	550	264	F	18.6	5.02	0.270
842	563	265	F	37.65	13.31	0.354
843	560	265	F	29.94	6.206	0.207
845	562	262	M	16.78	5.366	0.320
846	564	265	F	20.41	5.788	0.284
847	566	265	M	29.48	7.964	0.270

Total No. 11

Male fetuses (M) 6

Female fetuses (F) 5

*Calf with goiter

APPENDIX IV

Infiltration

THF 1 hr.
THF 1 hr.
THF 1 hr.
THF and Paraplast 1:1 at 60°C 1 hr.
Paraplast 60°C 5 lb. vac. 1 hr.
Paraplast 60°C 5 lb. vac. 1 hr.
Embed in paraplast

APPENDIX V

Cajal Block Method

10 - 16 days 70% ethanol
24 hr. alcohol with chloral hydrate
4-5 days 1.5% aqueous $AgNO_3$ in dark at 38°C
reduce

Formulae

alcohol with chloral hydrate

50 ml. 95% ethanol
5 gm. chloral hydrate

reducing mixture

pyrogallol 2 gm.
formaldehyde 8 cc.
distilled H_2O 100 cc.

APPENDIX VI

Davenport's Silver Impregnation (Kameda 1968)

Deparaffinize
Refix Carnoy or formalin fixed tissue in Bouin's fluid
for either 12-24 hr. at 37°C or 2 hr. at 50°C,
rinse with tap water for 1 hr.
Dehydrate to 95% ethanol
Mordant 48 hr. at 25-28°C (in dark)
5 sec. 95% ethanol
60 sec. reducing agent (keep solution in motion)
1 min. 95% ethanol
1 min. 95% ethanol
1 min. 95% ethanol
10 sec. 100% ethanol
10 sec. 100% ethanol
5 min. xylene
5 min. xylene
mount

Mordant
10 gm. $AgNO_3$
10 ml. distilled H_2O
90 ml. 95% ethanol
0.1 ml. 1 N nitric acid

Reducing solution
5 gm. pyrogallol
5 ml. 40% formalin
100 ml. 95% ethanol

APPENDIX VII

Haematoxylin and Eosin Technique and Formulae

Deparaffinize in 2 changes xylene
100% ethanol 2 min.
95% ethanol 2 min.
80% ethanol 2 min.
70% ethanol 2 min.
50% ethanol 2 min.
distilled H_2O 2 min.
Harris Haematoxylin (Harleco) 2 min.
acid water (0.25% HCl) 10 sec.
running tap water 5 min.
Eosin 10 sec.
100% alcohol 10 sec.
100% alcohol 10 sec.
xylene 5 min.
xylene 5 min.
mount

Eosin

Eosin Y	3.1 gm.
Orange G	1.4 gm.
Erythrosin B	0.5 gm.
95% ethanol	500.0 ml.

APPENDIX VIII

Periodic Acid Schiff and Haematoxylin Technique and Formulae

Deparaffinize 2 changes xylene
100% ethanol 2 min.
95% ethanol 2 min.
Periodic Acid 5 min.
Distilled water 5 min.
Schiff's reagent 15-20 min.
Sodium metabisulfite 2 min.
Sodium metabisulfite 2 min.
Sodium metabisulfite 2 min.
Tap water 10 min.
Harris Haematoxylin (Harleco) 2 min.
Acid water (0.25% HCl) 10 sec.
Tap water 5 min.
95% ethanol 10 sec.
100% ethanol 10 sec.
100% ethanol 10 sec.
Xylene 5 min.
Xylene 5 min.
Mount

Lillie's "Cold Schiff" (Gridley 1957)

Distilled water	384 ml.
HCL	16 ml.
Sodium metabisulfite	7.6 gm.
Basic fuchsin	4. gm.

Stopper and shake at 2 to 3 min. intervals until mixture is clear brown to reddish brown. Add 1 gm. decolorizing carbon and shake for 2 min. Filter through fine filter paper. Filtrate should be clear and colorless (if not colorless filter again with charcoal). This should be kept refrigerated.

Periodic Acid
5 gm. periodic acid
100 ml. H_2O

APPENDIX IX

Mallory's trichrome (Crossman 1937)

Deparaffinize in 2 changes of xylene
100% ethanol 1 min.
100% ethanol 1 min.
95% ethanol 1 min.
80% ethanol 1 min.
50% ethanol 1 min.
Tap water, running, 5 min.
Groates Haematoxylin 8 min.
Distilled water 10 sec.
Tap water, running 5 min.
Distilled water 10 sec.
Acid fuchsin-Orange G 5 sec.
Distilled water 10 sec.
3% Phosphotungstic acid 4 hr.
Distilled water 10 sec.
Anilin blue 20 sec.
Distilled water 10 sec.
2% acetic acid 2 min.
Distilled water 10 sec.
100% ethanol 4 changes for 1 min. each.
Xylene 5 min.
Xylene 5 min.

Groates Haematoxylin

Distilled H_2O	100 ml.
H_2SO_4	1.6 ml.
Ferric ammonium sulfate	2 gm.
Ethanol 95%	100 ml.
Haematoxylin	1 gm.

Allow to ripen 5 hr. Lasts 8 weeks.

Acid Fuchsin-Orange G

Acid fuchsin	1. gm.
Orange G	0.4 gm.
Distilled H_2O	300. ml.
Thymol	0.2 gm.
Glacial acetic acid	3. ml.

APPENDIX X

Grimelius Silver Stain (DeGrandi 1970)

Deparaffinize, 2 changes xylene 5 min. each
Hydrate
0.05% $AgNO_3$ in pH 5 acetic acid sodium acetate buffer
incubate at 60°C for 3 hr.
1% hydroquinone and 5% sodium sulfite solution at 45°C 1 min.
Distilled water 10 sec.
Fix in 5% sodium thiosulfate for 2½ min.
Distilled water 10 sec.
Kernechtrot 2 min.
Distilled water 10 sec.
95% ethanol 10 sec.
100% ethanol 10 sec.
100 % ethanol 10 sec.
Xylene 5 min.
Xylene 5 min.
Mount

Kernechtrot (Nuclear fast red) (Gridley 1957)

Kernechtrot	0.1 gm.
Aluminum sulfate	5.0 gm.

Use heat to dissolve, cool, add thymol and allow to dissolve. Filter.

APPENDIX XI

Hot Acid Hydrolysis and Toluidine Blue Stain

Deparaffinize 2 changes xylene 5 min. each
3 - 5 - 25 hr. 0.1 N HCl at 65°C
rinse distilled H_2O
7 min. 0.1% toluidine blue pH 5.6 McIlvaine buffer
(modification of original 0.01% toluidine blue)
mount in distilled water
(Solcia and Sampietro 1968b)

McIlvain buffer (Pearse 1961)
103.0 ml. .2 M Na_2HPO_4
97.0 ml. .1 M citric acid

.2 M Na_2HPO_4 = 28.41 gm./1000 ml.

.1 M citric acid = 21 gm. citric acid/1000 ml.

APPENDIX XII

Comparison Metachromatic and Argyrophilic Cell Enumerations

Animal Number	Toluidine Blue Metachromatic	Silver Argyrophilic
848	2	96
827	3	85
849	1	140
852	3	147
853	3	35
854	0	45
855	2	8
860	15	206
820	12	458
821	17	113
822	8	294
829	9	133
839	2	89
840	8	186
841	6	19
851	3	132
817	9	184
818	11	72
819	9	93
847	46	95
859-5	24	210

APPENDIX XIII

Raw Data Argyrophilic C cell Enumeration

Age	Accession No.	No. C cells
First trimester	826	127
	827	85
	832	322
	848	96
	849	140
	852	147
	853	35
	854	45
	855	8
	860	206
	861	159
Second trimester	820	458
	821	113
	825	132
	829	133
	833	323
	835	238
	838	277
	839	89
	840	186
	841	19
	844	677
	851	132
Third trimester	817	184
	818	72
	819	93
	828	102
	831	215
	843	119
	845	171
	846	113
	847	95
Adult	859-3	192
	859-4	549
	859-5	210
	856	161

APPENDIX XIV

Thyroid Tissue Component Raw Data

Stain	Accession No.	Epithelium	Lumen	C cell	Stroma
PAS	826	553	15	1	331
PAS	823	615	21	0	264
PAS	827	470	20	0	410
PAS	823	553	14	3	235
Ag	861	595	56	14	235
Ag	852	467	32	52	349
PAS	820	511	172	3	214
Ag	822	450	254	13	183
H&E	825	516	189	1	194
Ag	844,	343	263	47	247
Ag	835	400	225	5	270
Ag	828	650	102	6	142
Ag	846	356	379	14	151
PAS	845	390	314	0	196
Ag	831	460	247	30	163
Ag	828	647	131	3	119
Ag	856	152	624	14	110
Ag	856	223	486	16	175
Ag	859	115	517	25	243
Ag	859-3	151	561	11	177

Included with stroma, in addition to connective tissue, were blood vessels, intercellular artifacts.

' only 864 points of intersection