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ABSTRACT

The seasonal variations in thyroid activity in Rana Catesbeiana are described, using several indices of thyroid activity, e.g., cell height, colloid stain affinity, thyroidal iodine, and circulating hormone iodine. The study was carried out between November, 1961, and November, 1962. Additional data was obtained in February and March, 1963. When frogs were available in the summer, the thyroids and serum were collected from freshly captured animals. In the winter the animals were kept under simulated hibernation conditions.

New evidence is offered to support existing theory of seasonal variations in thyroid activity which is based on morphometric observations. Two principle seasonal phases of thyroid activity in Rana catesbeiana were observed; in one phase, hormone storage predominated, and in the other phase, hormone secretion was predominate. The seasonal cycle of thyroid activities is as follows: (1) In the Fall the thyroid accumulates both organic and inorganic iodine, (2) during the winter hibernation period, the accumulation of organic iodine continues and it can be assumed to be hormonal from the colloid stain affinity, (3) just prior to emergence from hibernation, the thyroid appears to be replete with hormone, (4) after emergence from hibernation, the stored hormone in the gland is gradually depleted as the accent shifts to hormone secretion.

Evidence is offered to support the theory that a more direct relationship exists between thyroid activity and the general activity of the animals, rather than the environmental conditions.

## STATEMENT OF THE PROBLEM

Over fifty years have passed since Gudernatsch (18), in 1912, observed one of the better known functions of the thyroid hormone - its role in amphibian metamorphosis. Research on this one aspect of thyroid activity in amphibians continues, greatly increasing our understanding of the effect of thyroid hormones on metabolism and growth. However, the role of the thyroid hormone in the adult amphibian has not been as thoroughly investigated. Previous to the 1940's, investigation into the thyroid activity in lower vertebrates was confined, mainly, to a morphological study, and to the effect of exogenous thyroid treatment on various physiological functions. In the 1940's, however, greater use of antithyroid agents and, in the 1950's, the increased use of radioactive iodine intensified research into other parameters of the thyroid function in adult amphibians.

Poikilothermous animals are subject to seasonal variations in their living pattern. In general it is accepted that the ambient temperature influences the overall physiological activity of the animal. Following the emergence from hibernation there is a period of breeding activity, and it has been assumed that there is a concomitant increase in thyroid activity at this time. By the same token, it has been assumed that there is a decrease in thyroid activity with the onset of colder conditions and hibernation. However, the evidence of this has been largely

circumstantial and based upon the work of naturalists who have employed indirect methods of investigation. The variations between species, as reported by these authors, indicates that the role of the thyroid in poikilothermous animals requires closer scrutiny. It is the purpose of this study, then, to subject the thyroid gland and its activities (i.e., the synthesis, storage, and secretion of hormone) of one species of these animals to histochemical and biochemical analysis at various times of the year. It is our plan to establish the seasonal pattern of thyroid activity in Rana catesbeiana as a basis for future studies on the relationship of the environmental factors to this activity, and the role of the thyroid in the general physiological activity of this and other poikilothermous animals.

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This discussion will attempt to evaluate some of the criteria and the methods used in the investigation of thyroid function. Only descriptions of methods used directly upon the thyroid and phenomena immediately associated with the gland function will be considered here. Other methods and criteria have been used which are associated with the function of thyroid hormones and the pituitary thyroid stimulating hormone - TSH.

The criteria used by various authors in assessing the activity of the thyroid gland may be separated into four main categories:

(1) The first, and least reliable, criterium is the gross morphology of the gland.

(2) The second, micro-morphology involves histological, cytological, and histochemical observations.

(3) The third method of study is the chemical analysis of hormones and hormonal iodine, either in the gland or in the circulation.

(4) The fourth category involves the use of radioisotopes of iodine and the technique of replacement of circulating thyroxine.

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phenomena closely associated with thyroid activity, e.g., oxygen consumption.

1. The Gross Morphological Changes

There are three morphological characteristics of the thyroid often used by authors to assess thyroid activity; the size, the weight, and the colour of the gland (31,37,38 ). Changes in size and weight of the thyroid gland very often accompany physiological changes within organisms. In 1936, Cottle and Carlson (4 ) examined the turnover of thyroid hormone in cold-exposed rats. These authors found that for a short period following cold exposure the gross morphological changes correspond to changes in other parameters which give a more direct measure of thyroid activity. However, in time this correspondence disappears. It is also important to consider the factors that may initiate changes in thyroid size and weight, namely, increased or decreased vascularisation of the gland, hyperplasia, and changes in the colloid content of the follicles. Obviously, each factor alone, or in combination, may contribute to the changes in gross morphology. A change of colour in the gland in the fresh state can be attributed to a fluctuation in the vascular supply.

None of these gross morphological characteristics give an adequate estimation of thyroid activity in themselves, either alone or in relation to each other, but rather fall in the category of circumstantial evidence. How-

ever, they may be valuable when taken in concert with the micro-morphology of the gland and/or the chemical analysis of the gland.

2. The Micro-morphological Changes

The activities of the thyroid gland are four-fold: iodine trapping, the synthesis of hormone, hormone storage in protein form in the colloid and proteolytic release of the hormones from the colloid followed by their cellular reabsorption and secretion. Since these activities are controlled by enzyme systems, they will never be found in a static state. Therefore, at all times the four activities will go on, but at varying rates according to the physiological requirements of the organism.

As DeRobertis ( 8) defines the various states of thyroid activity, it is assumed that when the gland is least active, the fixed colloid is dense and is deeply stained by basophilic dyes; the walls of the follicle appear distended by the colloid substance, and the follicular epithelium is described as low. In this state, since the gland is synthesizing hormone at a faster rate than it is being released, the proteolysis of the colloid and the reabsorption of the hormone by the cells may be assumed to be very slight. As the gland becomes more active, the colloid stains less intensely, and there appears to be an infolding of the walls of the follicle, the cells increase in height and the nuclei assume a less basal position. In

this condition the proteolytic activity in the colloid is increasing and, as a consequence, reabsorption of hormone into the follicular cells increases. Finally, in the most active glands one observes an increase in the number of follicles, an increase in the epithelial cell height (with nuclei about midway between the base and the apex of the cell), a decrease in the size of the follicular lumen, and an increased vascularization of the entire gland. Further, there is a change in the staining property of the colloid which now appears less basophilic. This may be interpreted to mean that hormone is being secreted faster than it is synthesized and stored. Should these conditions of hormone demand by the organism persist, it may lead to a depletion of follicular colloid. Subsequently, colloid droplets begin to appear at the apex of the cell, indicating that the rate of synthesis exceeds reabsorption of hormone, and storage will again occur.

The colloid has been shown by various staining techniques to be basophilic (5). It has been observed, frequently, that the peripheral follicles appear larger, their cells more cuboidal, and that the colloid is more basophilic than is that of the central follicles (34). Although the colloid appears to be homogenous, the staining reaction need not be uniform in all areas of the colloid in the same follicle. It has also been noted that vacuoles frequently appear in the colloid of active glands (2, 3, 50). It is generally accepted that these vacuoles in the periphery of

the colloid are artifacts (6) due to fixation and due to the degree of liquefaction of colloid substance prior to fixation in active glands. DeRobertis found he could eliminate these fixation artifacts if he employed the freeze-drying technique. The supposition that these vacuoles represented a chromophobic colloid of physiological significance then had to be abandoned.

The fact that within a single follicle there are areas of differing affinity for basophilic dyes could not be adequately explained by varying densities of the colloid and, thus, it appeared that two substances might be present. LeBlond (25) has demonstrated that the colloid showing variation in the intensity of the staining reaction with basophilic dyes also, in autoradiographs, shows a variation in the incorporation of radioiodine. The acidophilic colloid, twenty-four hours after injection of I-131, contains more radioiodine than the basophilic colloid. By "in-vitro" experiments LeBlond has shown that trypsin can change the stain affinity of a formerly acidophilic colloid to basophilia. He attributes basophilia to the presence of proteolytic enzymes in the colloid. Desmarais and LeBlanc (11), in 1961, established a staining technique by modifying the Mallory stain. Using this technique, they found two types of colour pattern. In many follicles there appeared a yellow core of colloid surrounded by a blue ring, while in others a mosaic of blue and yellow was found. Since the active colloid is basophilic, one would expect it to be the

blue staining colloid, and this was substantiated by the use of I-131 and the preparation of autoradiographs. In all cases, the loci of radioactive material were located in the blue colloid. On this basis, the authors considered the possibility that two components do exist, one containing hormone (blue staining) and one without hormone (yellow staining). Both components are Periodic-acid-Schiff positive which, possibly, indicates a common polysaccharide stroma accompanying these two colloid components. While histological and cytological observations reveal phenomena associated with thyroid activities, they do not reveal anything about the actual chemical composition of the gland. Histochemical studies in the thyroid are rare, revealing enzyme activities only. Because of the unique structure of the gland, attention must be focused on both intra-cellular and intra-follicular material. It is natural that much of the chemical study should be centered upon the colloid substance itself. There are two views on the nature of the colloid as it appears in the droplets within the epithelial cells. DeRobertis and co-workers (7) favour the theory that these droplets represent the cellular synthesis of colloid material. DeRobertis describes the process of the extrusion of colloid material from the cells into the follicle in much the same manner as mucus is excreted into the lumen of the intestine by goblet cells. Other authors, notably LeBlond and co-workers, and Wollman and co-workers (57), suggest that these droplets represent reabsorption of the follicle

colloid by the cells. However, both theories agree that the droplets represent hormone containing colloid material.

3. The Chemical Analysis of Hormone

Derrien and co-workers (10) established that thyroglobulin represents approximately seventy percent of the crude aqueous extract of minced thyroid glands and accounts for the major part of the organically-bound iodine in the colloid. There have been other iodoproteins described in the colloid and the cells, but their physiological importance has not been elucidated. One, according to Shulman, et al, (43) is more soluble than thyroglobulin in aqueous solution, it reacts as an albumin in its electrophoretic mobility and possesses the same sedimentation properties. This protein is found in both colloid and epithelium in normal glands, but is more prevalent in neoplastic glands. A second, insoluble iodoprotein called a particulate protein by Rall and Hobbins (39) has been found associated with subcellular elements. Although thyroglobulin is described by all authors as a homogeneous protein, Rall and co-workers have also shown that thyroglobulin is readily and reversibly denatured by changes in pH. Below a pH of 8.5 the protein corresponds to a homogeneous one, with a molecular weight of 660,000. However, as one increases the pH to 9.5 there is a progressive denaturation halving the protein molecule. This is very evident at ph 9.5, when the new smaller molecule is in greatest abundance. At a higher pH a

quartering of the molecule takes place, and above pH 11.5 the protein is completely and irreversibly denatured. It is, then, necessary to keep the pH constant and near neutrality to ensure the consistency of results when studying the protein moiety of the colloid.

The chemical composition of thyroglobulin has been studied by Derrien and co-workers (9 ). They have identified, quantitatively and qualitatively, fifteen amino acid constituents of the protein. Interestingly, they show that only the noniodinated amino acid composition is constant, and the degree of iodine varies. Thyroxine accounts for only a fraction of one percent of the total protein iodine, while iodo-tyrosines, tryptophene and cystein each account for approximately ten times as much. To obtain this purified thyroglobulin, Derrien washed minced thyroids in physiological saline, then by alternate precipitation and washing with varying concentrations of ammonium sulphate, he extracted the purified protein. The importance of mincing the glands is that the rupture of the cell membranes is minimized, hence, the resulting extract can be identified as coming from the follicular colloid. However, the complicated washing and precipitation routine is rather a harsh treatment of the protein material, initiating a great loss and probably increasing the degree of denaturation.

The quantitative analysis of the hormone in the colloid can be related to morphological characteristics, gross morphology and cytology to give an indication of the

rate of hormone synthesis and storage. However, for an accurate assessment of the secretory activity of the gland, one must also have a measure of the circulating hormone, or better, a measurement of the fluctuations in circulating hormone along with an assessment of the metabolism and excretion of the hormone.

An assessment of the level of circulating hormone usually involves the measurement of the hormone iodine and not the thyronines, directly. Circulating hormone is found bound to pre-albumin, albumin, $\alpha$-globulin fractions of blood protein and as free amino acids. The $\alpha$-globulin fraction binds most of the hormones in circulation. The thyroid hormones utilize approximately one third of the binding capacity of the blood proteins (39). Under certain physiological conditions, such as pregnancy and certain pathological conditions, protein binding and its relationship to thyroid secretory activity is very important. An increase in blood protein concentration may increase the PBI level. Clinically, and in research laboratories, only PBI is studied. However, in 1950 Taurog (48) reopened the idea that free hormones exist in blood plasma as well as do protein conjugated hormones. The present theory (39) holds that there exists an equilibrium between the free and the bound hormone in circulation, but that the concentration of free hormone is insignificant.

The greater use of radioactive iodine (I-125 and I-131) in thyroid studies has greatly facilitated the

"in-vivo" and "in-vitro" investigation of thyroid activities. The uptake of radioiodine can be followed to assess the ability of the gland to trap iodine. Robertson and Falconer (40) found that the rate of secretion of thyroid hormone is correlated with the rate constant of the thyroid gland uptake and release of I-131. They also assert that the serum PBI and the rate constant for I-131 excretion are marginally correlated. The authors did not find, however, a correlation between maxima in PBI levels and increases in secretion of thyroid hormone. Similarly, the rate of release has been used to determine the secretion rate. In the light of Berrien's work, caution must be exercised as it is known that only a portion and, at that, not a constant portion of the entrapped iodine is converted to hormonal iodine. Also, when one is considering the rate of uptake and release of isotope, he must follow up with a chemical analysis of both the glandular and circulatory hormones to assess the degree of incorporation of iodine. This is necessary since the rates do not necessarily indicate release or retention of organified iodine. Robertson and Falconer (40) have shown, however, that the level of protein bound iodine in the serum and the rate constant for I-131 secretion can be marginally correlated with the rate of secretion of thyroid hormone. It is a question of correlation of rates of release and secretion, however, not absolute quantities. One gland may release radioiodine at a greater rate than another, but this does not indicate that the first

is producing more hormone - only that there is a greater turnover of iodine.

Studies involving the use of goitrogens and other indirect physiological measurements will be discussed in the following chapter dealing directly with the frog and other amphibians.

Part II The Physiology of the Amphibian Thyroid

Because of the scarcity of literature on thyroid function and physiology in Rana catesbeiana, reference is made to work on other frogs, toads, and amphibians in general. There have been only a few comprehensive studies of the annual variation in frog thyroid histology, although much work has been carried out during a single season. In Europe, Skowier (44) and Meisenheimer (27) reported observations on a complete annual cycle of thyroid activity in the species, Rana temporaria. This species has similar characteristics to Rana catesbeiana, namely, an extended period of development in the larval stage, and a mainly aquatic adult life. Skowier determined a low level of thyroid activity in winter, followed by a sharp increase in March and the level of activity remained high throughout the summer months, but in August began to decrease gradually until it reached the winter level in late October. Meisenheimer (27) found similar results, except that he reported a reduction in thyroid activity during the months of April and May, when it reached the winter level. Neither Skowier nor Meisenheimer found any correlation between the early spring peak of thyroid activity and the breeding season. Their theories are based, of course, on histological evidence. Both authors reported a flattening of the epithelial cells in winter and the follicles were distended with colloid, whereas in the month of March, the epithelium increased in height and the follicles flattened. This phase

of thyroid activity was extended into the summer and early fall months.

In opposition to Skowler and Meisenheimer, numerous authors, Wolf (56), Morgan and Stokes (33), and Holzapfel (20), report moderate activity from early January, with increasing activity as the breeding season nears. They report, also contrary to Skowler and Meisenheimer, a low summer activity. The above observations were made on Rana pipiens and Bufo americanus, amphibians that differ from Rana temporaria with respect to the degree of dependence on a water habitat in their adult life span. It may also be noted that these observations of the latter authors agree with observations made on a variety of urodels and reptiles that are nonhibernators; Evans and Hegre (13), Ikeda (22), Morgan and Fales (32), Miller and Robbins (30), Miller (29), Wolf (56). In hibernating urodels and reptiles, a pattern similar to that of Rana temporaria is found in the annual variation of the histological picture; Evans and Hegre (13) and Wilhoft (55). Only Tosia, et al, (49) determined the Rana pipiens thyroid cycle to be similar to that of Skowler's on Rana temporaria.

The extreme lack of chemical analysis of the amphibian thyroid is due, undoubtedly, to the size of the gland. Donoso and Trivellioni (12) have, however, studied the incorporation of I-131 in the toad thyroid. Chromatographic results of hydrolyzed glands revealed monoiodotyrosine, diiodotyrosine and thyroxine along with inorganic iodine. They found the

uptake of I-131 to be very low, and thyroxine accounted for only seventeen percent of the total organic iodine. No triiodothyronine was in evidence and they could not detect any thyroid products in the plasma. It is possible, however, that the chromatographic techniques were too crude to detect plasma hormones. Shellabarger and Brown (42) have also studied toad thyroids in the larvae and in the adults. These authors found that actively metamorphosing larvae produce thyroxine and triiodothyronine, the latter in trace amounts. There appears to be less thyroxine and no triiodothronine in tadpoles approaching the completion of metamorphosis, and no traces were found in the very early larvae. In adult toads these workers found that both iodothyronines and iodotyrosines were present. They suggested that the biosynthesis of thyroid hormones in amphibians is identical to that found in other higher vertebrates. They consider the physiological activity of thyroid hormones in the adult to be obscure. Apparently, they believe the only function of these hormones is as an activator in development and in heat production.

Serum studies on amphibians were, until recently, almost non-existent. The reason for this was a lack of a micro-technique for iodine analysis. Chromatographic techniques do not lend themselves to the detection of hormone in such small amounts as could be extracted from the plasma of a small animal. Donoso and Trivellioni (12) could not detect any trace of thyroid hormone in toad plasma.

In 1959, Wille and DeVisscher (55), pooling the plasma of several animals, obtained the following results in protein bound iodine determination on Rana temporaria. The PBI of the animals was undetectable in January, but between January and March there was a rapid increase reaching a maximum of 20 µg percent. This was followed by an immediate descent of PBI concentration to a low level of 1 µg percent in June. The early summer low level was followed by a second increase to approximately 5 µg percent, and this was maintained until December when there was a precipitous drop. It is interesting to note that this species breeds in the month of May. Wille and DeVisscher found that the variations in plasma PBI are more or less parallel to the sexual activity of the male. The authors, unfortunately, did not discuss how they maintained their animals, i.e., whether they were obtained from the natural environment the year round or had been kept under laboratory conditions.

Meisner (28) studied the plasma PBI and BEI of Rana pipiens in winter and found he could not determine the serum hormone levels in either the control or propylthiouracil treated animals. In November, he determined a PBI of 0.24 µg percent and a Butanol extractable iodine of 0.07 µg percent. From his experience, Meisner concluded that the thyroids of Rana pipiens have a very low activity during the winter season.

Radioiodine (I-131) combined with thiouracil or propylthiouracil has been used by several authors to determine the functional state of Anuran thyroids. Tosia and co-

workers (49) found that thiouracil, over a period of one to seven weeks, induces, in Rana pipiens, the same type of gross morphological and histological changes as are found in mammals. However, they found that after a longer treatment, up to eleven weeks, no manifestation of activation was evident. As a follow up in summer frogs, Talmage (47) found that the thyroids trapped and bound four to five percent of an injected dose of I-131. This I-131 uptake was cut to twenty percent of the control by daily injections of five mg of thiouracil. This would indicate that the thyroid glands of Rana pipiens are actively synthesizing hormone in both winter and summer seasons, but the gland appears to be more active in the winter season. Weisner's observations do not agree with this.

The work of Talmage (47) tends to lend support to the work of Mathews. Mathews (26) found Rana pipiens accumulated I-131 in the thyroid during the summer season, but at a slower rate than mammals, since the maximum uptake in the frog is not reached until forty-eight hours after injection. The author found that pre-chilling the animal to 3°C did not affect the initial rate of uptake, but did advance the time of maximum accumulation and the release of I-131. Talmage found that in summer frogs, environmental temperature and thiouracil treatment inhibited organification of iodine, while thiocyanate reduced I-131 uptake by thyroid tissues. This suggests that, as Warren (53) states, the thyroids of summer frogs exhibit both synthesis and release

of hormone.

The evidence to date indicates that amphibians, possibly, slowly build up a store of hormone during the winter hibernation, but that their thyroids are the most active in producing and secreting hormone during the summer months. The nature of the effect of thyroid hormones on amphibian physiology has not been reviewed here. Recently, two such reviews have been published by Corbman (17) and Gaiton and Ingbar (14). As yet, very little information is available on the subject, however, it is generally accepted that thyroxine has little calorgenic importance in these animals.

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Part III The Frog - Rana Catesbeiana

To North Americans, Rana catesbeiana means the common "bullfrog", receiving its name from the rather sonorous bull-like bellow of the male. It is the largest species of the genus, attaining a body length of approximately nine inches at full growth. Rana catesbeiana are abundant throughout eastern North America and have been successfully introduced into Japan and Italy. Bullfrogs possess teeth and are carnivorous, feeding on any small animal, even their own species. In turn, they are preyed upon by many aquatic and terrestrial animals, man included. This species is the principal source of the gourmet's delight, frog's legs. The larvae, on the other hand, are protected from predation by a secretion of their mucous glands that appears to be distasteful to most predators of frogs.

The colour of the species varies with the habitat from light yellow-green to a dark olive, always with dark blotches on the dorsal skin of the leg. The melanocytes producing these blotches are associated with iodine trapping (15). The belly varies in hue from light straw colour to deep yellow. The frog is a master at "freezing", it can flatten itself against a log or a leaf and blend in perfectly, leaping at the most dramatic moment into the water. The sexes are easily distinguished at any season by the size of the tympanum. Generally, in males the tympanum is twice the size of the eye orbit, while in the female it is equal to or smaller than the orbit.

Rana catesbeiana is the most aquatic species of this genus, rarely straying more than a few feet from the water's edge. Poorly developed lungs and a dependance on moist skin surface for respiration keeps this animal close to its breeding habitat. As is usual with aquatic amphibians, the bullfrog has an extended larval stage, requiring a year, or often two years, to complete metamorphosis.

Rana catesbeiana goes into hibernation in late September and does not emerge until early summer, May or June. This late emergence is thought to be timed to the availability of an adequate food supply. The mating season is extended throughout the summer months. Early spring breeding is not necessary since the larvae do not need to complete their metamorphosis before the next hibernation season (18,31,35).

## MATERIALS AND METHODS

### Source of Animals

The animals for this research project were obtained from two widely separated populations, Gatineau County, Province of Quebec, Canada, and Oshkosh, Wisconsin, United States. Frogs of both sexes were studied. At the beginning of the project, the sera and the thyroids were pooled without regard to sex, however, after June, 1962, all samples were segregated according to sex. The first group of frogs, represented by data from November, 1961, to May, 1962, were obtained from Oshkosh, Wisconsin. The second group of animals, represented by data from June, 1962 to November, 1962, were local frogs from the Gatineau area. The third and last group, February to March, 1963, were obtained from the Wisconsin supply house - Steinhibler. On November 3, 1962, we obtained a single Rana catesbeiana which was collected by a skin diver in Lac Bateille in the Gatineau area of Quebec.

Although the species abounds in Gatineau County during the winter months, it was not possible to obtain a continuous fresh supply. Therefore, a large number were kept in an aquarium under simulated hibernation conditions. In November, 1961, we obtained a large supply of Rana catesbeiana from Wisconsin. The frogs, on arrival were placed in two large horse drinking troughs covered with cardboard to keep out the light. The troughs had four inches of fine sand on the bottom and twenty-four inches of running

tap water. The temperature of the water was a constant 4°C ±0.5 at the outflow end of the trough (except for one short period of two days when the water was turned off). There was very little fungal infection after the first four weeks and until the water reached a temperature of 15°C±0.5 in March, 1962. At this time, the fungal infection known as "red-leg" appeared so frequently that one or two infected animals had to be removed each week. Because of the difficulty in feeding the frogs and keeping them disease free, we decided to place the remaining animals in a pond on the university property, where they could obtain sufficient food. A large cage, 10' x 5' x 5', was constructed and placed in the pond where the depth was four feet. In June, 1962, we received our fresh supply of local frogs. Half of the animals were sacrificed the day they were received, and the rest kept as described above until June 20, when, in a partially covered trough on the roof of the Biology building The supply continued, uninterrupted, until August 30, 1962. At this time a large number of these animals were being kept in the troughs. They were fed a mixture of homogenized beef liver and fox chow. This supply of frogs lasted until November 20, 1962. In February, 1963, a supply of frogs was received from Wisconsin, and these were kept in a refrigerator in a darkened vegetable crisper, fourteen inches square and six inches deep, which was filled with water at 4°C.

Collecting Thyroid and Sera Samples

The animals were anesthetized by pithing the brain and nerve cord. To obtain the serum, the heart was carefully exposed and a blood sample drawn with a syringe and 24G needle. No anticoagulent was used, the blood was allowed to clot and then centrifuged at 2000 rpm for fifteen minutes in a clinical centrifuge. The sera were frozen immediately after centrifugation.

Some difficulty was experienced in obtaining blood samples from winter frogs, the heart beat being slow, the volume was small. The ventricle muscle did not relax sufficiently to allow it to expand to the same extent it did in the summer frog. Within the winter groups, the sera samples were pooled, each individual contributing a volume equal to the smallest volume. As mentioned above, the sera of the individual summer frogs were not pooled.

The thyroid gland is found attached to the hyoid apparatus in a groove formed by the thyroid-process, just posterior to the posteric-lateral process. It lies medial to the external jugular, beneath the procoracoid cartilage, the sternohyoideus and omohyoideus muscles, and just posterior to the origin of the hyoglossal muscle. Extraction of the gland must be accomplished under magnification and with fine forceps and needles. The thyroid lobes are very delicate in the winter when they are hypoemic. The glands lie flat against the thyroid apparatus, and are almost indistinguishable from the surrounding tissue. Careful

attention must be given to avoid tearing the gland when removing it. It was found best to grasp the anterior ligament connecting the gland to the cartilage and to tease the gland off the cartilage with a needle, cutting the posterior ligament with a pair of scissors. One lobe was placed immediately in Bouin's fixative and the other was frozen on dry ice. The frozen gland was dried in a freeze drying apparatus 10-100 Virtis Unitrap and kept in a deep freeze at -20° Centigrade until chemical analysis was undertaken.

Serial sections of the Bouin's fixed lobes were cut at five micra and stained according to the procedure described by DesMarais and LaMar (11). The colloid staining properties were measured in the following manner. The tissue section was divided horizontally into six areas by five equally spaced lines. With a micrometer passing along these lines, the blue and yellow stained colloid was measured and the results were interpreted as percent of the total colloid measured. The data from the five areas were averaged to give the percent of the total colloid staining with Aniline Blue.

The cell height and the nuclear diameters (measured perpendicular to the cell base) were measured in micra. Five follicles were chosen within each section and three cells were observed in each follicle. The follicles and cells were chosen in the following manner. Under low magnification, the section was centered in the field of vision, the follicle which was closest to the tip of the ocular hair pointer was

studied. This follicle would, of course, be a central one. Following this, the microscope stage was moved to the right until the edge of the section appeared, and again the follicle closest to the tip of the pointer was studied. The microscope stage was moved in this manner until all four major points of the compass had been scanned on the tissue section.

The chemical analysis of the thyroid gland was carried out after all the glands had been collected. The dried thyroids were weighed on a Sartorius balance and then homogenized in one c.c. of chilled (4°C) physiological saline. The saline solution was prepared with demineralized water to eliminate the possibility of introducing contamination by exogenous iodine. This was checked and the iodine content of the solution was found to be well within the range of the reagent blank. The homogenate was allowed to stand for fifteen minutes at 4°C to allow for the leaching out of the colloid. The homogenate was then centrifuged for fifteen minutes at 2000 RPM to remove cell debris. The extract was chemically analysed for iodine by the Mycal procedure for serum ( 2). Protein incorporated iodine, free thyronine iodine, and inorganic iodine were measured on each sample. The results were interpreted as micrograms of iodine per milligram of thyroid tissue.

The serum protein bound iodine (PBI) and butanol extractable iodine were also determined by the Mycal method ( 2), and the results were interpreted as micrograms of

iodine per hundred milliliters of blood.

The data collected in each parameter were expressed as a mean average value and plotted on a time series graph. The data was treated statistically with a simple analysis of variance, Snedecor's variance ratio test (45) was used with a one way classification (no sex difference) and a two way classification (46) taking into consideration sex differences. In both types of statistical treatment, a five percent level of confidence was the limit for significant differences. Those parameters which showed a significant difference between groups at the five percent level or better, were then studied by the Kramer modified Duncan range test for the between mean differences ($_{24}$).

## THE RESULTS

### A. Histological and Cytological Observations

#### 1. Cell Height

Our observations show that there is a seasonal cycle in regard to the variation in thyroid follicular epithelial cell height (Table I). There appear to be two seasonal peaks in cell height (Graph 1). Between November and January there is a minor increase from five microns to seven microns. Following this there is a period of rapidly decreasing cell height to a level of approximately five and one half microns, which is then maintained throughout the winter and early spring months. In the spring, the cell height increases rapidly to seven microns. This second high is followed by a gradual decrease in cell height throughout the summer and early fall months. The autumn low in cell height does not, however, reach that of the winter months. In late November, the cell height again increases.

A one-way analysis of variance (Table II), ignoring sex difference, gives a variance ratio ("F" value) which is greater than the one percent level of "F" found in Snedecor's Table for Variance ratio (45). When these results are analysed for sex and sex-time differences by the method described by Steel and Torrie (46), it is evident that the between group variation is not related to sex dimorphism (Table III).

Although the graphical (Graph 1) representation of

the data shows the nature of the trends in cell height variation, it was necessary to apply Kramer's modification of the Multiple Range (Fig. 2) Test to determine what time group means are statistically different. For this study we have chosen the five percent level of significance and this is uniformly applied in all our data for this test. The January, 1962, high (7.3μ) and the June, 1962, high (7.4μ) are statistically equivalent and are not significantly different from the February and March (1963) cell heights of 6.2 micra. The June, 1962, mean is not statistically different from the other summer group means, except the starved group of August 21, 1962. All the summer group means, except August 21, 1962, are statistically greater at the five percent level than the autumn, winter, and spring group means (except those mentioned above). There is no statistical difference at this level between the means of September to December and February to May.

The February and March, 1963, group means are statistically different at the five percent level from the February and March, 1962, group means.

The cell height observations in the single frog captured on November 3, 1962, can not be treated statistically with other groups, but the mean (5.3 micra) for this frog falls neatly into place between October 3 (5.5 micra) and the November 6 (5.0 micra) means.

Table I Epithelial Cell Heights in Micra

| (1) Date | (2) Replicates T | M | F | (3) Mean Cell Height | Date | Replicates T | M | F | Mean Cell Height |
|---|---|---|---|---|---|---|---|---|---|
| 11-12-61 | 3 | - | - | 5.9±0.87 | 31- 7-62 | 8 | 4 | 1 | 6.7±0.60 |
| 10- 1-62 | 4 | - | - | 7.3±0.3) | 9- 8-62 | 9 | 5 | 4 | 6.4±0.51 |
| 6- 2-62 | 4 | - | - | 5.2±0.2) | 21- 8-62*10 | 3 | 7 | | 5.8±0.6) |
| 26- 3-62 | 8 | - | - | 6.6±0.67 | 30- 8-62 | 10 | 5 | 5 | 6.6±0.71 |
| 4- 5-62 | 3 | - | - | 5.4±0.44 | 3-10-62 | 8 | 6 | 3 | 5.5±0.40 |
| 16- 5-62 | 3 | - | - | 5.7±0.66 | 3-11-62** | 1 | 1 | | 5.3 |
| 22- 6-62 | 6 | 5 | 1 | 7.6±0.28 | 6-11-62 | 3 | 1 | 2 | 5.0±0.20 |
| 28- 6-62* | 7 | 4 | 3 | 6.7±0.69 | 20-11-62 | 4 | 2 | 2 | 5.4±0.46 |
| 10- 7-62 | 5 | 4 | 1 | 8.8±0.81 | 18- 2-63 | 9 | 5 | 4 | 8.2±0.49 |
| 17- 7-62 | 9 | 5 | 4 | 6.4±0.4) | 14- 3-63 | 9 | 2 | 7 | 8.2±0.84 |
| 24- 7-62* | 10 | 7 | 3 | 6.3±0.49 | | | | | |

(1) Date refers to the day of autopsy, day-month-year.
(2) T, M, and F refer, respectively, to total, male and female replicates.
(3) Group means with standard error estimate.
* Summer, starved, frogs.
** Single frog captured at Lac Bataille.

Table II Analysis of Variance

| Source | Df | Sum of Squares | Variance Estimate | "F" Value | Theoretical "F" value |
|---|---|---|---|---|---|
| Total | 129 | 366.36 | | | 1% = 2.03 |
| Groups | 19 | 126.10 | 6.405 | | 5% = 1.66 |
| Error | 110 | 240.26 | 2.184 | 2.932 | |

Sm = 1.477

Table III The 8X2 Analysis of Variance

| Source | Df | Sum of Squares | Variance Estimate | "F" Value | Theoretical "F" value |
|---|---|---|---|---|---|
| A. Time | 9 | 75.046 | 8.338 | 6.249 | >1% |
| B. Sex | 1 | 0.282 | 0.282 | 0.211 | <5% |
| AxB | 9 | 9.248 | 1.027 | 0.770 | <5% |
| Error | 85 | 113.414 | 1.334 | | |

2. Nuclear Diameter

The nuclear diameters do not show as clear a seasonal pattern as do the cell heights (Table IV). As seen in the graphical representation (Graph I) there is a single peak of activity in spring (May) and the seasonal curve falls on both sides of this point. Statistical analysis (Table V) shows that there is a significant difference between the time groups, the "F" value is four and one half times greater than the Snedecor theoretical for one percent confidence limit. When treated for sex dimorphism (Table VI) there is no evidence of sex variation within any groups.

In the Multiple Range Test (Fig. 2) at the five percent level of significance, only the spring values are statistically different, in May the nuclear measurements are 5.3 and 5.4 micra. The mean nuclear diameter in February and March, 1963, are significantly different from those in February and March, 1962. There is no other clear separation of the group means according to seasons, as is seen in Table VIII.

The mean nuclear diameter observed in the single frog captured at Lac Bataille does not correspond to the value of October 3 and November 5 group of that same year. However, this difference would probably not be statistically significant at the five percent level.

Table IV Nuclear Measurements of Follicular Cells in Micra

| (1) Date | (2) Replicates T | M | F | (3) Nucleus Diameter | Date | Replicates T | M | F | Nucleus Diameter |
|---|---|---|---|---|---|---|---|---|---|
| 11-12-61 | 3 | - | - | 3.9±0.33 | 31- 7-62 | 5 | 4 | 1 | 3.8±0.50 |
| 16- 1-62 | 4 | - | - | 4.1±0.20 | 9- 8-62 | 9 | 5 | 4 | 3.7±0.20 |
| 6- 2-62 | 4 | - | - | 3.8±0.10 | 21- 8-62*10 | 3 | 7 | | 3.4±0.30 |
| 28- 3-62 | 8 | - | - | 3.8±0.67 | 30- 8-62 | 10 | 5 | 5 | 4.1±0.36 |
| 4- 5-62 | 3 | - | - | 5.3±0.40 | 3-10-62 | 9 | 6 | 3 | 3.4±0.14 |
| 16- 5-62 | 3 | - | - | 5.7±0.42 | 3-11-62** | 1 | 1 | | 4.0 |
| 22- 6-62 | 6 | 5 | 1 | 4.3±0.30 | 6-11-62 | 3 | 1 | 2 | 3.3±0. |
| 28- 6-62* | 7 | 4 | 3 | 3.8±0.20 | 20-11-62 | 4 | 2 | 2 | 3.8±0.25 |
| 10- 7-62 | 5 | 4 | 1 | 3.7±0.22 | 19- 2-63 | 9 | 5 | 4 | 4.4±0.38 |
| 17- 2-62 | 9 | 5 | 4 | 3.7±0.34 | 14- 3-63 | 9 | 2 | 7 | 4.5±0.20 |
| 24- 7-62* | 10 | 7 | 3 | 3.8±0.33 | | | | | |

(1) Date refers to the day of autopsy, day-month year.
(2) T, M, and F refer, respectively, to total, male, and female replicates.
(3) Group means with standard error estimates.
* Summer, starved, frogs.
** Single frog captured at Lac Bataille.

Table V Analysis of Variance

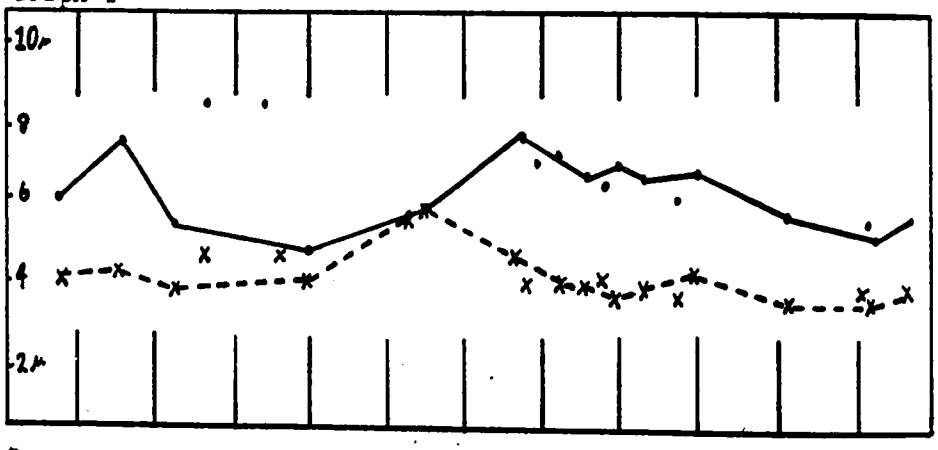
| Source | Df | Sum of Squares | Variance Estimate | "F" Value | Theoretical "F" value |
|---|---|---|---|---|---|
| Total | 129 | 39.77 | | | 1% - 2.03 |
| Groups | 19 | 24.71 | 1.235 | | 5% - 1.66 |
| Error | 110 | 15.06 | 0.136 | 9.080 | |

Sm - 0.369

Table VI The 8X2 Analysis of Variance

| Source | Df | Sum of Squares | Variance Estimate | "F" Value | Theoretical "F" value |
|---|---|---|---|---|---|
| A. Time | 9 | 11.355 | 1.261 | 4.486 | >1% |
| B. Sex | 1 | 0.018 | 0.018 | 0.065 | <5% |
| AXB | 9 | 2.795 | 0.310 | 1.104 | <5% |
| Error | 65 | 23.907 | 0.281 | | |

Graph I



Graph 1 Follicular Epithelium Measurements

●——● Cell height

x----x Nuclear diameter

The points through which the curves do not pass are the data observed on atypical animals, e.g., after interupted hibernation and prolonged captivity

3. The Colloid Histochemistry

The seasonal variation in the percentage of the colloid, which preferentially takes the Aniline-blue stain, is also well marked (Table VII, Graph II ). In the late spring, the maximum colloid affinity for this dye occurs in May, when one hundred percent of the colloid stains blue. This affinity falls steadily throughout the summer months, reaching a low of sixty-five percent during the late fall. This is followed by an increased Aniline-blue affinity in the mid winter to value of ninety percent.

The large "F" value for between group differences shows a highly significant variation between these group means (Table VIII). The 2X2 statistical analysis (Table IX) shows that this variation is not due to sex dimorphism. The Multiple Range Test shows that at the five percent level, the late winter means (February and March, 1962 and 1963) and the spring values (May and June) are not significantly different. The June, 1962, mean is not different from other summer means, nor the late winter means. The late summer value (August 30) and the autumn values are not different at the five percent level.

The February, 1963, frogs have a colloid affinity that is statistically different from the animals of the same period in 1962 (Fig. 3), while the March, 1963, mean percent blue colloid is statistically equivalent to that of the period March, 1962.

The starved summer frogs have mean values that are

statistically different from all others (Fig. 3). The early groups (June 28 and July 24, 1962) have mean values different (at five percent) from all other summer values and the August 21 mean value is significantly lower yet, indicating a trend toward increased Orange-G affinity in the colloid with a longer period of starvation and physical restraint in the aquarium tanks.

When the ratio of the follicular epithelial cell height and the colloid staining property (Table X) is plotted on the time axis (Graph III), the relationship of these two parameters is most distinct. The ratio is directly proportional to changes in cell height and inversely proportional to changes in the percentage of blue-stained colloid. It is evident that as cell height increases, the colloid loses its affinity for Aniline-blue, and conversely, as the epithelium becomes more cuboidal the percentage of the blue colloid increases.

Table VII Follicular Colloid Staining Property Expressed as Percentage of the Total Colloid Area Stained with Aniline-Blue

| (1) Date | (2) Replicates T | M | F | (3) % Blue Colloid | Date | Replicates T | M | F | % Blue Colloid |
|---|---|---|---|---|---|---|---|---|---|
| 11-12-61 | 3 | - | - | 78.4±6.4 | 31- 7-62 | 6 | 4 | 2 | 85.2±2.6 |
| 16- 1-62 | 3 | - | - | 76.0±2.7 | 9- 8-62 | 8 | 5 | 3 | 85.1±8.7 |
| 6- 2-62 | 4 | - | - | 97.3±8.6 | 21- 8-62* | 10 | 3 | 7 | 43.1±6.0 |
| 28- 3-62 | 8 | - | - | 92.2±5.7 | 30- 8-62 | 10 | 5 | 5 | 70.7±9.4 |
| 4- 5-62 | 3 | - | - | 100.0±0. | 3-10-62 | 9 | 6 | 3 | 85.7±5.9 |
| 16- 5-62 | 3 | - | - | 100.0±0. | 3-11-62** | 1 | 1 | | 66.7 |
| 22- 6-62 | 6 | 5 | 1 | 90.0±5.5 | 6-11-62 | 4 | 1 | 3 | 70.4±5.3 |
| 28- 6-62* | 8 | 5 | 3 | 53.8±7.0 | 20-11-62 | 4 | 2 | 2 | 89.4±7.9 |
| 10- 7-62 | 5 | 4 | 1 | 88.4±4.3 | 19- 2-63 | 9 | 5 | 4 | 84.4±9.3 |
| 17- 7-62 | 9 | 5 | 4 | 82.8±3.4 | 14- 3-63 | 9 | 2 | 7 | 89.4±4.2 |
| 24- 7-62* | 10 | 7 | 3 | 51.7±5.7 | | | | | |

(1) Date refers to the day of autopsy, day-month-year.
(2) T, M, and F refer, respectively, to total, male, and female replicates.
(3) Group means with standard error estimates.
* Summer, starved, frogs.
** Single frog captured at Lac Betaille.

Table VIII Analysis of Variance

| Source | Df | Sum of Squares | Variance Estimate | "F" Value | Theoretical "F" value |
|---|---|---|---|---|---|
| Total | 115 | 38,297.30 | | | 1% - 2.03 |
| Groups | 19 | 29,933.72 | 1,575.46 | | 5% - 1.66 |
| Error | 96 | 6,363.58 | 66.28 | 23.75 | |

Sm - 8.14

Table IX The RX2 Analysis of Variance

| Source | Df | Sum of Squares | Variance Estimate | "F" Value | Theoretical "F" value |
|---|---|---|---|---|---|
| A. Time | 9 | 15,429.64 | 1,714.40 | 4.075 | >1% |
| B. Sex | 1 | 438.50 | 438.50 | 1.042 | <5% |
| AXB | 9 | 578.26 | 64.25 | 0.153 | <5% |
| Error | 87 | 36,599.37 | 420.68 | | |

Table X Ratio of Percent Blue Colloid / Epithelial Cell Height

| Date | Ratio | Date | Ratio |
|---|---|---|---|
| 11-12-61 | 0.075 | 31- 7-62 | 0.079 |
| 10- 1-62 | 0.093 | 9- 8-62 | 0.075 |
| 6- 2-62 | 0.053 | 21- 8-62 | 0.134 |
| 28- 3-62 | 0.046 | 30- 8-62 | 0.093 |
| 4- 5-62 | 0.054 | 3-10-62 | 0.063 |
| 16- 5-62 | 0.057 | 3-11-62 | 0.079 |
| 22- 6-62 | 0.082 | 6-11-62 | 0.071 |
| 26- 6-62 | 0.124 | 20-11-62 | 0.078 |
| 10- 7-62 | 0.077 | 19- 2-63 | 0.128 |
| 17- 7-62 | 0.077 | 14- 3-63 | 0.091 |
| 24- 7-62 | 0.120 | | |

Table X Ratio of Percent Blue Colloid / Epithelial Cell Height

| Date | Ratio | Date | Ratio |
|---|---|---|---|
| 11-12-61 | 0.076 | 31- 7-62 | 0.079 |
| 18- 1-62 | 0.093 | 9- 8-62 | 0.075 |
| 8- 2-62 | 0.053 | 21- 8-62 | 0.134 |
| 28- 3-62 | 0.046 | 30- 8-62 | 0.093 |
| 4- 5-62 | 0.054 | 3-10-62 | 0.083 |
| 16- 5-62 | 0.057 | 3-11-62 | 0.079 |
| 22- 6-62 | 0.082 | 6-11-62 | 0.071 |
| 28- 6-62 | 0.124 | 20-11-62 | 0.078 |
| 10- 7-62 | 0.077 | 19- 2-63 | 0.128 |
| 17- 7-62 | 0.077 | 14- 3-63 | 0.091 |
| 24- 7-62 | 0.120 | | |

Graph II Seasonal Follicle Colloid Study

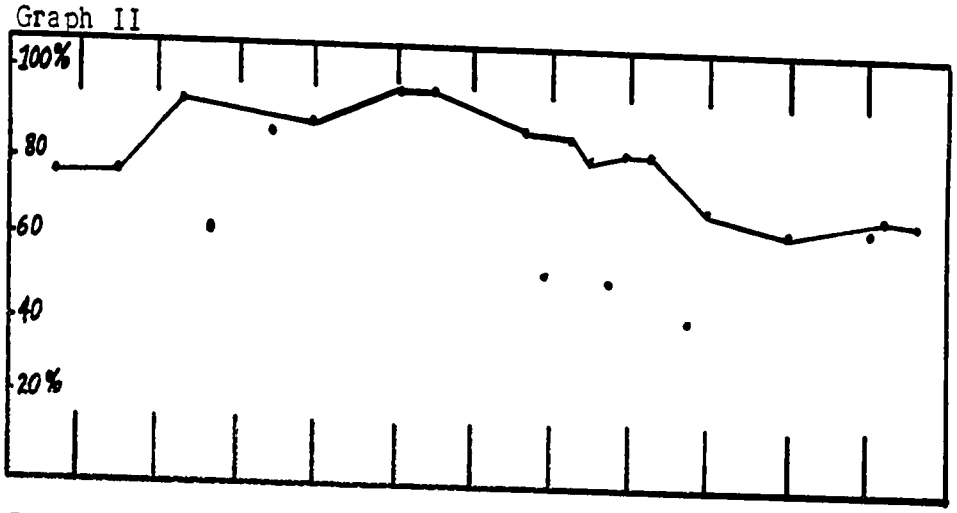
Percent Total Colloid Staining with Aniline-blue

The points through which the curves do not pass are the data observed on atypical animals, e.g., after interupted hibernation and prolonged captivity.

Graph III Seasonal Variations in the Ratio of

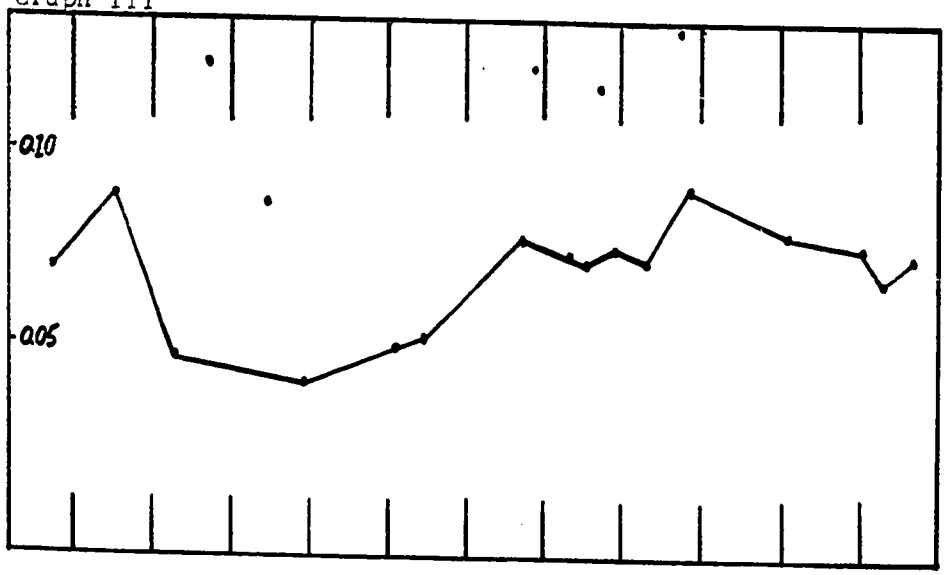
Percent Blue Colloid to Cell Height

Graph II




Dec. Jan. Feb. Mar. Apr. May June July Aug. Sep. Oct. Nov.

Graph III




Dec. Jan. Feb. Mar. Apr. May June July Aug. Sep. Oct. Nov.

MULTIPLE RANGE TEST, BETWEEN GROUP MEAN DIFFERENCES

Means underscored by the same unbroken line are not significantly different from each other, means not underscored by the same unbroken line are significantly different from each other.

Figure 1. Follicular Epithelial Cell Heights

4.6, 5.0,5.2,5.4,5.4,5.5,5.7,5.8,5.9, 6.3,6.4,6.4,6.5,6.7,6.7,6.8, .3,7.4, 8.2,8.2

Figure 2. Follicular Epithelial Nuclear Diameters

3.3, 3.4,3.4, 3.5,3.6,3.6,3.6,3.7,3.7,3.7,3.8,3.8,3.9,4.1,4.1,4.3,4.4 .5,5.3,5.4

Figure 3. Percent of Follicular Colloid Staining with Aniline-blue

43.1, 51.7,53.8,64.4,65.7,69.4,70.4,70.7, 78.0,78.4,82.8,85.1,85.2, .4,89.4,90.0,92.2,97.3,100.0

B. Chemical Analysis of the Thyroid Gland

1. Protein Incorporated Iodine

Our observations (Graph IV) indicate that it is during the colder season that the highest levels of protein incorporated iodine occur. Between October and early May, the mean values (Table XI) vary from 1.80 µg/mg to 2.85 µg/mg. Some low values were found, however, on May 4 the mean value was 1.9 µg iodine per milligram of tissue, and in one animal from Lac Bataille the average was 0.51 µg iodine per milligram of tissue.

The statistical analysis (Table XII) indicates a high level of significant difference between means, the variance ratio being greater than the one percent level. The RX2 analysis (Table XIII) shows that this difference is not due to any sex difference. The Multiple Range Test (Fig. 5) at the five percent level of significance, indicates that the summer frog thyroids contain significantly less protein incorporated iodine per milligram of tissue than the frogs taken in the colder seasons. The summer values range from 0.64 to 1.45 micrograms of iodine per milligram of tissue.

The February and March, 1963, observations indicate significantly less protein incorporated iodine in the frog thyroid than during the same period in 1962.

There is no significant difference in this respect between the thyroids of the summer starved group mean and the thyroids of the other summer group means.

The protein incorporated iodine level observed in the single frog from Lac Bataille is very much lower than even the lowest summer values.

2. Butanol Extractable Iodine

With the exception of the spring groups (May, 1962), the butanol extractable iodine values parallel the protein incorporated iodine values (Graph V Table XIV). There is not the same degree of variation, however, and as indicated in Table XV the variance ratio test indicates no significant differences between group means at the five percent level of confidence.

3. Inorganic Iodine in the Thyroid Gland

During the late summer and the autumn months (Graph IV) it is observed that the changes in inorganic iodine levels parallel the changes in protein incorporated iodine levels. When in late fall and early winter the latter level climbs, the inorganic iodine levels off to approximately 1.25 micrograms per milligram (Table XVI). Analysis of variance, one-way, indicates a significant difference between groups at the five percent level (Table XVII) and the $\chi^2$ statistics (Table XVIII) rule out any possible sex dimorphism in this parameter. The Multiple Range Test (Fig. 6) singles out the autumn and winter values to be generally higher (at five percent) than the spring and summer values.

In February, 1963, the inorganic iodine level was

observed to be significantly lower than it was in the same period in 1962 (Fig. 5). However, by March, 1963, the level had returned to the 1962 value. No effect of starvation and captivity was evident in regard to inorganic iodine concentration in the frog thyroid. As in protein incorporated iodine observations, the inorganic iodine concentration in the thyroid of the one frog captured on November 3 was much lower than the values observed for captive frogs in the same season.

Table XI Protein incorporated Iodine in the Thyroid Gland
( ug PBI/mg thyroid tissue)

| (1) Date | (2) Replicates T | M | F | (3) PBI | Date | Replicates T | M | F | PBI |
|---|---|---|---|---|---|---|---|---|---|
| 11-12-61 | - | - | - | --- | 31- 7-62 | 6 | 3 | 3 | 0.64±0.33 |
| 16- 1-62° | 4 | - | - | 1.81 | 9- 8-62 | 8 | 5 | 3 | 0.85±0.36 |
| 6- 2-62 | 4 | - | - | 2.24±1.01 | 21- 8-62* | 9 | 3 | 6 | 0.92±0.50 |
| 28- 3-62 | 5 | - | - | 2.36±0.50 | 30- 8-62 | 9 | 4 | 5 | 1.49±0.78 |
| 4- 5-62 | 3 | - | - | 1.61±0.64 | 3-10-62 | 7 | 5 | 2 | 2.89±1.07 |
| 16- 5-62 | 3 | - | - | 2.09±1.26 | 3-11-62** | 1 | 1 | | 0.51 |
| 22- 6-62 | 5 | - | - | 0.84±0.60 | 6-11-62 | 5 | 2 | 3 | 1.84±0.68 |
| 28- 6-62* | 9 | 5 | 4 | 1.12±0.71 | 20-11-62 | 5 | 3 | 2 | 2.70±0.96 |
| 10- 7-62 | 5 | 1 | 4 | 1.35±0.59 | 19- 2-63 | 9 | 5 | 4 | 1.85±0.63 |
| 17- 7-62 | 7 | 4 | 3 | 1.24±0.71 | 14- 3-63 | 10 | 3 | 7 | 1.80±0.63 |
| 24- 7-62* | 6 | 4 | 2 | 0.99±0.27 | | | | | |

(1) Date refers to the day of autopsy, day-month-year.
(2) T, M, and F refer respectively, to total, male, and female replicates.
(3) Group means with standard error estimates.
° Pooled sample not included in the statistical analysis.
* Summer, starved, frogs.
** Single frog captured at Lac Bataille.

Table XII Analysis of Variance

| Source | Df | Sum of Squares | Variance Estimate | "F" Value | Theoretical "F" value |
|---|---|---|---|---|---|
| Total | 114 | 118.887 | | | |
| Groups | 17 | 48.256 | 2.542 | | |
| Error | 97 | 70.08 | 0.728 | 3.491 | >1% |

Sm - 0.2699

Table XIII The 8X2 Analysis of Variance

| Source | Df | Sum of Squares | Variance Estimate | "F" Value | Theoretical "F" value |
|---|---|---|---|---|---|
| A. Time | 11 | 34.34 | 3.12 | 2.308 | >1% |
| B. Sex | 1 | 0.71 | 0.71 | 0.527 | <5% |
| AXB | 11 | 5.51 | 0.50 | 0.370 | <5% |
| Error | 71 | 96.05 | 1.35 | | |

Table XIV Butanol Extractable Iodine in the Thyroid Gland
(µg BEI/mg thyroid tissue )

| (1) Date | (2) Replicates T | M | F | (3) BEI | Date | Replicates T | M | F | BEI |
|---|---|---|---|---|---|---|---|---|---|
| 11-12-61 | - | - | - | --- | 31- 7-62 | 6 | 3 | 3 | 0.10±0.05 |
| 16- 1-62° | 4 | - | - | 0.05 | 9- 8-62 | 8 | 5 | 3 | 0.08±0.03 |
| 6- 2-62 | 4 | - | - | 0.19±0.044 | 21- 8-62* | 8 | 4 | 4 | 0.12±0.08 |
| 28- 3-62 | 5 | - | - | 0.15±0.070 | 30- 8-62 | 9 | 4 | 5 | 0.10±0. |
| 4- 5-62 | 3 | - | - | 0.11±0.0 | 3-10-62 | 7 | 5 | 2 | 0.25±0.04 |
| 16- 5-62 | 3 | - | - | 0.13±0.0 | 3-11-62** | 1 | 1 | | 0.08 |
| 22- 6-62 | 5 | 5 | - | 0.14±0.03 | 6-11-62 | 4 | 1 | 3 | 0.14±0.03 |
| 28- 6-62* | 9 | 5 | 4 | 0.12±0.07 | 20-11-62 | 5 | 3 | 2 | 0.17±0.03 |
| 10- 7-62 | 5 | 1 | 4 | 0.14±0.07 | 19- 2-63 | 9 | 5 | 4 | 0.22±0.06 |
| 17- 7-62 | 8 | 5 | 3 | 0.15±0.07 | 14- 3-63 | 10 | 3 | 7 | 0.17±0.03 |
| 24- 7-62* | 6 | 4 | 2 | 0.08±0.03 | | | | | |

(1) Date refers to the day of autopsy, day-month-year.
(2) T, M, and F refer, respectively, to total, male, and female replicates.
(3) Group means with standard error estimate.
° Pooled sample not included in the statistical analysis.
* Summer, starved, frogs.
** Single frog captured at Lac Bataille.

Table XV Analysis of Variance

| Source | Df | Sum of Squares | Variance Estimate | "F" Value | Theoretical "F" value |
|---|---|---|---|---|---|
| Total | 114 | 1.087 | | | |
| Groups | 17 | 0.255 | 0.013 | | |
| Error | 97 | 0.832 | 0.008 | 1.625 | <5% |

Sm - 1.131

Table XVI The Inorganic Iodine in the Thyroid Gland

| (1) Date | (2) Replicates T | M | F | (3) Iodide ug/mg | Date | Replicates T | M | F | Iodide ug/mg |
|---|---|---|---|---|---|---|---|---|---|
| 11-12-61 | - | - | - | --- | 31- 7-62 | 6 | 3 | 3 | 0,68±0,33 |
| 18- 1-62 | - | - | - | --- | 9- 8-62 | 8 | 5 | 3 | 0,73±0,45 |
| 6- 2-62 | 4 | - | - | 1,30 | 21- 8-62* | 9 | 3 | 6 | 0,76±0,14 |
| 28- 3-62 | 2 | - | - | 1,34±0,01 | 30- 8-62 | 9 | 4 | 5 | 0,55±0,33 |
| 4- 5-62 | 3 | - | - | 1,28±0,34 | 3-10-62 | 7 | 5 | 2 | 2,01±0-71 |
| 16- 5-62 | 3 | - | - | 1,04±0,40 | 3-11-62** | 1 | 1 | | 0,73 |
| 22- 6-62 | 5 | 5 | - | 0,85±0,20 | 6-11-62 | 5 | 2 | 3 | 2,04±1,11 |
| 28- 6-62* | 9 | 5 | 4 | 1,06±0,34 | 20-11-62 | 5 | 3 | 2 | 1,67±0,46 |
| 10- 7-62 | 5 | 1 | 4 | 1,25±0,45 | 19- 2-63 | 9 | 5 | 4 | 1,14±0,63 |
| 17- 7-62 | 7 | 4 | 3 | 0,81±0,20 | 14- 3-63 | 10 | 3 | 7 | 1,31±0,77 |
| 24- 7-62* | 6 | 4 | 2 | 0,99±0,37 | | | | | |

(1) Date refers to the day of autopsy, day-month-year.
(2) T, M, and F refer, respectively, to total, male, and female replicates.
(3) Group means with standard error estimate.
* Summer, starved frogs.
** Single frog captured at Lac Bataille.

Table XVII Analysis of Variance

| Source | Df | Sum of Squares | Variance Estimate | "F" Value | Theoretical "F" value |
|---|---|---|---|---|---|
| Total | 110 | 49,17 | | | |
| Groups | 17 | 18,19 | 1,01 | | 5% |
| Error | 93 | 30,87 | 0,33 | 3,243 | |

Sm = 0,575

Table XVIII The RXS Analysis of Variance

| Source | Df | Sum of Squares | Variance Estimate | "F" Value | Theoretical "F" value |
|---|---|---|---|---|---|
| A. Time | 10 | 0.067 | 0.0067 | 2.046 | >1% |
| B. Sex | 1 | 0.0005 | 0.0005 | 0.149 | <5% |
| AXB | 10 | 0.026 | 0.0026 | 0.795 | <5% |
| Error | 71 | 0.231 | 0.0032 | | |

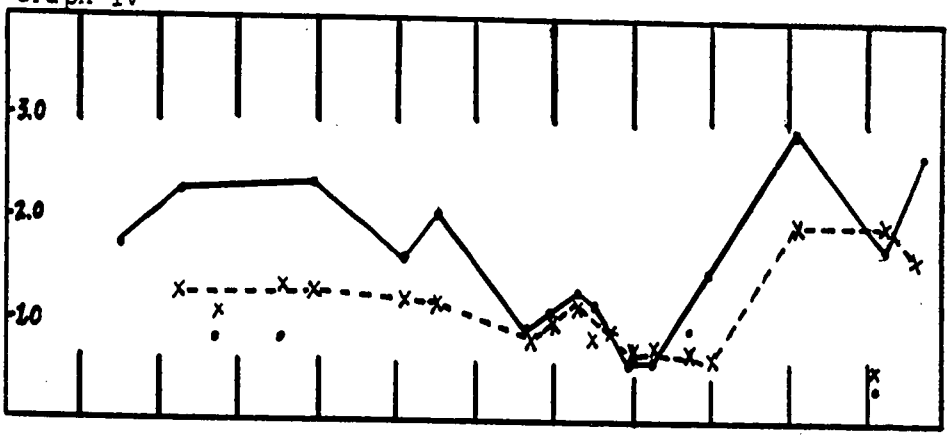
Graph IV Thyroid Iodine Components I
Expressed as Micrograms Iodine per Milligrams of Tissue (Dry weight)

●——● Protein incorporated iodine

x----x Inorganic iodine

The points through which the curves do not pass are the data observed on atypical animals, e.g., after interupted hibernation and prolonged captivity.

Graph IV

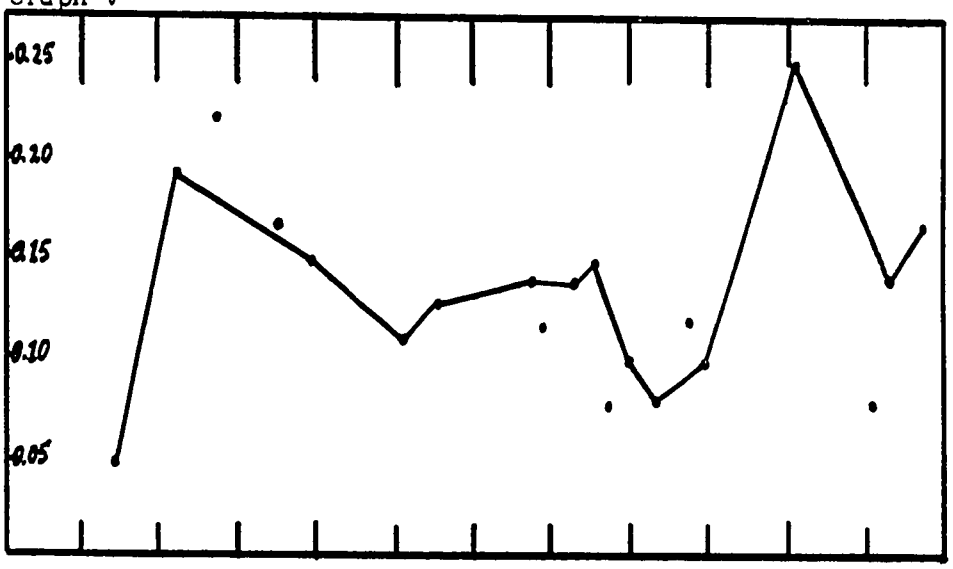


Graph V Thyroid Iodine Components II

Butenol Extractable Iodine Expressed as Micrograms of Iodine per Milligrams of Tissue (Dry weight)

The points through which the curves do not pass are the data observed on atypical animals, e.g., after interupted hibernation and prolonged captivity.

Graph V


0.25
0.20
0.15
0.10
0.05
Dec. Jan. Feb. Mar. Apr. May June July Aug. Sep. Oct. Nov.

C. Circulating Hormone Assay

The PBI observations as recorded in Table XIX and represented on Graph VI indicate that the late fall and winter PBI levels are greater than the summer and early fall values. Statistically, (Table XX) there is a significant difference (one percent) between group means which is not due to sex dimorphism (Table XXI). The February and March, 1963, values are statistically greater than all others except the October to November values (Fig. 6). While it is impossible to assess the statistical confidence of pooled serum means for the winter, 1962 groups, the winter, 1963, group means appear to fall into the same order of magnitude. The lower summer values, June 22, July 10, and August 8, 1962, are significantly (five percent) lower than the autumn values, but all other spring and summer values are statistically equivalent, and are not significantly different from the autumn values. There is no effect of starvation or captivity.

The butanol extractable iodine (Table XXII, Graph VI) shows that there is no real variation between seasonal groups with regard to actual hormonal iodine. The statistical treatment (Table XXIII) of these observations produces a variance ratio which is much below the accepted five percent level of significance.

Table XIX Serum Protein-Bound-Iodine
( ug Iodine percent cc. serum )

| (1) Date | (2) Replicates T | M | F | (3) PBI | Date | Replicates T | M | F | PBI |
|---|---|---|---|---|---|---|---|---|---|
| 11-12-61° | 3 | - | - | 3.9 | 11- 7-62 | 6 | 3 | 3 | 2.9±0.48 |
| 16- 1-62° | 2 | - | - | 10.9 | 9- 8-62 | 7 | 4 | 3 | 3.3±1.63 |
| 8- 2-62° | 4 | - | - | 4.0 | 21- 8-62* | 8 | 2 | 6 | 2.4±1.41 |
| 28- 3-62° | 6 | - | - | 5.0 | 30- 8-62 | 8 | 5 | 3 | 2.9±0.84 |
| 4- 5-62° | 3 | - | - | 3.5 | 3-10-62 | 7 | 4 | 3 | 3.8±0.59 |
| 16- 5-62° | 3 | - | - | 3.4 | 3-11-62** | 1 | 1 | | 3.2 |
| 22- 6-62 | 10 | 5 | 5 | 2.3±0.84 | 6-11-62 | 4 | 2 | 2 | 4.1±0.57 |
| 26- 6-62* | 10 | 5 | 5 | 3.2±0.73 | 20-11-62 | 3 | 2 | 1 | 4.3±1.23 |
| 10- 7-62 | 6 | 2 | 4 | 2.2±0.48 | 19- 2-63 | 8 | 5 | 3 | 4.6±1.09 |
| 17- 7-62 | 7 | 4 | 3 | 3.2±0.88 | 14- 3-63 | 10 | 3 | 7 | 4.5±1.12 |
| 24- 7-62* | 6 | 3 | 3 | 3.2±0.41 | | | | | |

(1) Date refers to the day of autopsy, day-month-year.
(2) T, M, and F refer, respectively, to total, male, and female replicates.
(3) Group means with standard error estimate.
° Pooled sample no included in the statistical analysis.
* Summer, starved frogs.
** Single frog captured at Lac Bataille.

Table XX Analysis of Variance

| Source | Df | Sum of Squares | Variance Estimate | "F" Value | Theoretical "F" value |
|---|---|---|---|---|---|
| Total | 99 | 240.8 | | | |
| Groups | 13 | 123.1 | 9.47 | | |
| Error | 86 | 117.7 | 1.37 | 6.905 | >1% |

Sm - 1.17

Table XXI The RK2 Analysis of Variance

| Source | Df | Sum of Squares | Variance Estimate | "F" Value | Theoretical "F" value |
|---|---|---|---|---|---|
| A. Time | 11 | 34.34 | 3.12 | 2.318 | >1% |
| B. Sex | 1 | 0.72 | 0.72 | 0.527 | <5% |
| AxB | 11 | 5.51 | 0.50 | 0.370 | <5% |
| Error | 71 | 96.05 | 1.35 | | |

Table XXI The 8X2 Analysis of Variance

| Source | Df | Sum of Squares | Variance Estimate | "F" Value | Theoretical "F" value |
|---|---|---|---|---|---|
| A, Time | 11 | 34.34 | 3.12 | 2.316 | >1% |
| B, Sex | 1 | 0.72 | 0.72 | 0.527 | <5% |
| AXB | 11 | 5.51 | 0.50 | 0.370 | <5% |
| Error | 71 | 96.05 | 1.35 | | |

Table XXII Serum Butanol Extractable Iodine
( ug percent cc. serum)

| (1) Date | (2) Replicates T | M | F | (3) BEI | Date | Replicates T | M | F | BEI |
|---|---|---|---|---|---|---|---|---|---|
| 11-12-61° | 3 | - | - | 5.4 | 31- 7-62 | 6 | 3 | 3 | 3.7±0.94 |
| 16- 1-62° | 2 | - | - | 3.9 | 9- 8-62 | 6 | 4 | 2 | 2.6±0.57 |
| 6- 2-62° | 4 | - | - | 4.7 | 21- 8-62* | 7 | 1 | 6 | 2.9±1.35 |
| 28- 3-62° | 6 | - | - | 3.4 | 30- 8-62 | 8 | 5 | 3 | 3.7±1.86 |
| 4- 5-62° | 3 | - | - | 3.5 | 3-10-62 | 4 | 2 | 2 | 3.8±0.98 |
| 16- 5-62° | 3 | - | - | 4.3 | 3-11-62** | 1 | 1 | | 5.2 |
| 22- 6-62 | 3 | 1 | 2 | 4.1±0.94 | 6-11-62 | 4 | 2 | 2 | 3.6±0.74 |
| 28- 6-62* | 9 | 5 | 4 | 2.9±1.10 | 20-11-62 | 3 | 2 | 1 | 4.8±0.68 |
| 10- 7-62 | 5 | 2 | 3 | 2.9±0.54 | 19- 2-63 | 9 | 6 | 3 | 3.5±0.60 |
| 17- 7-62 | 7 | 4 | 3 | 3.6±1.49 | 14- 3-63 | 10 | 3 | 7 | 3.1±0.75 |
| 24- 7-62* | 9 | 6 | 3 | 3.5±0.85 | | | | | |

(1) Date refers to the day of autopsy, day-month-year.
(2) T, M, and F refer, respectively, to total, male, and female replicates.
(3) Group means with standard error estimate.
° Pooled sample not included in the statistical analysis.
* Summer, starved, frogs.
** Single frog captured at Lac Baraille.

Table XXIII Analysis of Variance

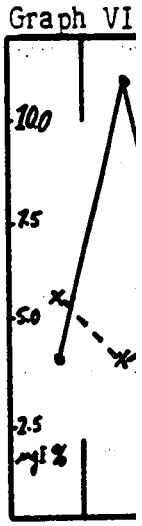
| Source | Df | Sum of Squares | Variance Estimate | "F" Value | Theoretical "F" value |
|---|---|---|---|---|---|
| Total | 89 | 127.8 | | | |
| Groups | 13 | 29.6 | 2.26 | | |
| Error | 76 | 98.2 | 1.29 | 1.76 | <5% |

Graph VI Seasonal Serum PBI and BEI Values
Expressed as Micrograms of Iodine Percent Serum

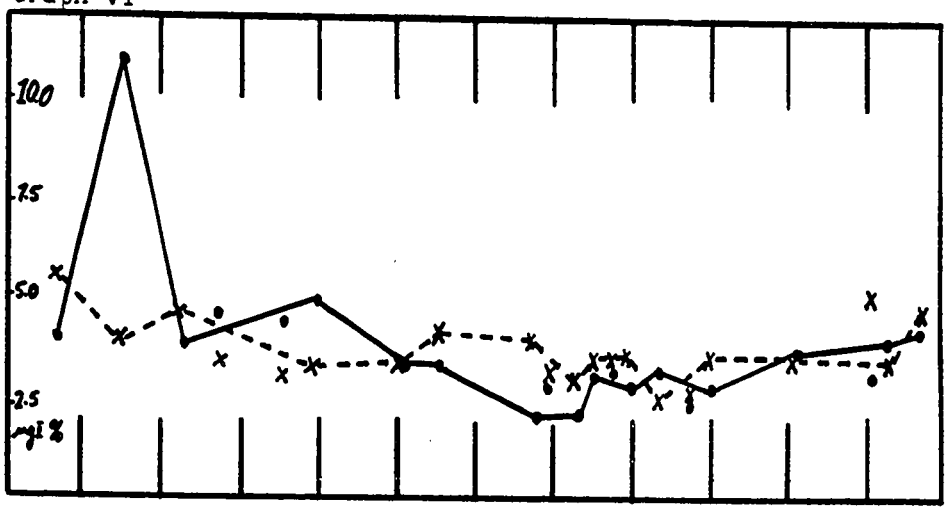
●——● Serum PBI

x----x Serum BEI

The points through which the curves do not pass are the data observed on atypical animals, e.g., after interupted hibernation and prolong captivity.



Graph VI




Dec. Jan. Feb. Mar. Apr. May June July Aug. Sep. Oct. Nov.

MULTIPLE RANGE TEST. BETWEEN GROUP MEAN D[illegible]FERENCES

Means underscored by the same unbroken line are [illegible]t significantly different from each other, means not underscored [illegible]y the same unbroken line are significantly different from e[illegible]h other.

Figure 4. Protein Incorporated Iodine in Thyroid

0.64,0.65,0.82,0.84,0.89,1.12,1.24,1.35,1.45,1.61,1.80,1.84,1.85,2.01 [illegible]2.24,2.36,2.70,2.80

Figure 5. Inorganic Iodine in the Thyroid Gland

2.04,2.01,1.67,1.34,1.31,1.30,1.25,1.23,1.14,1.08,1.04,0.99,0.85,0.81 [illegible]0.76,0.73,0.68,0.65

Figure 6. Serum Protein-Bound-Iodine

2.2,2.3,2.4,2.8,2.8,3.2,3.2,3.2,3.3,3.8,4.1,4.3,4.5,4.6

## GENERAL DISCUSSION

One of the interpretations of adaption in regard to physical changes in environment is that an animal makes some physical or physiological changes that enable it to survive. Essentially, acclimation refers to the special form of adjustments that animals undergo when subjected to a new environmental condition to which they have not before been subjected. For example, tame rats are adapted to a stable environment - small variation in temperature and light periods. When subjected to a sudden lowering of temperature, these animals undergo a period of acclimation during which time many of their physiological functions come into a state of imbalance while the animal attempts to maintain its normal body temperature. The function of this period of acclimation is to allow the animal to adjust its body functions to immediate and drastic changes in environmental conditions. In nature, however, environmental conditions are in a constant flux - there are diurnal changes of light and temperature which, in many climes, can be quite drastic. There are also seasonal changes, and within seasons there are variations in conditions. Animals which are exposed to these changes are accustomed to them and are said to be adapted to them. Due to repeated and gradual exposures, and due, also, to genetic adaptation, these animals have developed permanent cyclic physiological adjustments. If, however, the seasonal cycle is interupted or artificially reversed in a drastic manner,

then these animals will also attempt acclimation. Acclimation is, then a fighting effort to survive against adverse conditions.

In speaking of wide populations, one is not concerned about acclimation, but about the acclimatization process. There are many forms of adaption to seasonal temperature changes and the accompanying loss of food supply. Gross body changes, such as the increase of surface cover in the form of hair, feathers or other dermal derivatives and sub-dermal fat, generally occur in larger mammals and some birds. Smaller mammals turn to the formation of microcosm and all, but the true hibernators, increase food consumption. Among large herding animals there is a tendency to migrate. Few animals truly hibernate, however, but there are those which, for reasons of inadequate food supply or inadequate physiological mechanisms, simply give in to nature and allow their body temperature to fall and cease all normal activities that call for expenditure of energy. They fall into a state of stupor until favourable conditions are resumed.

Such an animal is the frog - this animal has not the facility to increase its body insulation, nor the facility to store a great deal of body fat of any description to tide it through lean times. Also, the frog lacks the ability to migrate or to create a useful microcosm. Some amphibians undergo complete hibernation, burrowing deep into favourable sub frost strata. Rana catesbeiana, like many of the species of fish, spends a period of complete or

semi-hibernation in deep water where there is no danger of frost or sub zero temperatures. The fact that this species chooses such conditions tends to indicate that it is probably not a complete hibernator.

## The Cyclic Nature of the Frog Thyroid Activity

### Gross Morphology

We have found that the thyroid gland in frogs is easily located during the warmer seasons on the year. Our observations have described this gland in winter frogs as inconspicuous and difficult to dissect from the animal. Skowier (44) described the thyroids of Rana pipiens as being smaller in the winter than during the breeding season, and rather pale in colour. Charipper, et al, (3) in agreement, described the vascularization of the gland as poor, but made no reference to size, except it was extremely variable. From the foregoing, it appears that the colour of the gland could be used in a limited way to assess the decrease of activity of the thyroid gland. This is most evident in glands that have been treated with goiterogens, a deepening of gland colour is due to hypermia. Similar results have been reported with rats on low-iodine diet.

### Thyroid Histology

Early studies in frog thyroid activity by Skowier (44) and Meisenheimer (27) involved cytological and histological measurements. In general, based on cell height and

semi-hibernation in deep water where there is no danger of frost or sub zero temperatures. The fact that this species chooses such conditions tends to indicate that it is probably not a complete hibernator.

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### Thyroid Histology

Early studies in frog thyroid activity by Skowler (44) and Meisenheimer (27) involved cytological and histological measurements. In general, based on cell height and

colloid observations, these authors found a low winter activity which was followed by a rapid increase in activity in the early spring. Up to this point, the authors agree, however Bowler reported a sustained high level of thyroid activity throughout the summer, until a decrease in late August and the activity finally reached a winter low in October. These observations would parallel the animal's seasonal peaks of activity and the seasonal temperature changes. Meisenheimer (27), however, found that the level of thyroid activity declined after the March-April peak and in May reached the winter level, only to be followed by a second increase in activity which culminated in a new summer peak in August and September. Following this, the thyroid slowed down until the winter low level of hormone production and secretion was reached. The curious fact about Meisenheimer's observations was that during the period of peak sexual activity, his animals would have a reduction in thyroid gland activity. Both authors found, however, that the summer period marked the height of thyroid activity which would correlate with the season of highest ambient temperatures and the greatest general and feeding activity. This was also the season of greatest body growth, however, in some areas Rana catesbeiana has been shown to exhibit body growth during the winter season (16).

Based on follicular epithelium cell height observations, we found there is a diphasic seasonal peak of activity. One short period of increased cell height occurred

during the December to January period, this was followed by a rapid lowering of epithelial height which leveled off between February and early April when it again increased. The cell height continued to increase during the spring months, reaching a summer maximum of activity in late June. This level of activity was then sustained through until September, when it again decreased to a second low level in early November. In general, our observations on epithelial activity agree with those of the previous authors, except for an early winter increase and an absence of an early spring decrease as found by Weisenheimer (27).

Among other species of amphibians, there was a great deal of variation in seasonal thyroid activity as observed through morphometric parameters. In the Urodele Triturus torosa, Miller and Robbins (30) found that the thyroid was least active during the summer period of estivation, then the thyroid is avascular with dense basophilic colloid and low follicular epithelium. These animals do not hibernate, but do spend the winter months in ponds. The thyroid activity increased in all animals during the fall migration to winter breeding ponds, as evidenced by increased vascularity, higher epithelial cells, and a more acidophilic colloid which contains many reabsorption vacuoles. In this species, the maximum thyroid activity occurred during the winter breeding season while the animal was aquatic. It is easily appreciated that during this period there would be great heat loss by the animal, yet there was very little

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feeding until early spring when thyroid activity decreased. Since this animal is a poikilotherm, it was considered that the thyroid did not increase its activity for caloric purposes. It must then be assumed that the hyperactivity of the thyroid gland is due to the frog's increased activity, both sexual and general. Miller and Robbins (30) suggested, however, that since only about one half of the colony are actually breeders, but all animals show morphological evidence of increased thyroid activity, then this activity must be associated with environmental conditions more than with sexual activity.

In contrast to the above observations, Morgan and Fales (32) and Uhlenhuth, et al, (51) in studying two species of Triturus found that increased thyroid activity by morphological standards corresponded to the periods of gametogenesis and spawning, that is spring and late summer, respectively. During other seasons, thyroid activity was moderately low.

Miller (29), in his study of the viviparous non hibernating lizard, Xantusia vigilis, found thyroid activity by morphological criteria was correlated with the various phases of its adult life history. The follicles were largest and the epithelium lowest during the dry fall months. During the winter months, the follicle size decreased and the epithelium increased. This, the author stated, was wholly related to the fact the animal was active and feeding during winter, the coldest months. The author found a

greater increase, however, during the spring and summer months, related to the gametogenesis and spawning periods. There was a sexual dichotomy in this species, however, in that the increase in the male animals preceded that of the females. In the latter, the increased thyroid activity was more related to sexual behaviour than to gametogenesis. Following the spawning season, there was a decrease in morphological evidence of thyroid secretion. In Rana pipiens, by radioiodine (I-131) conversion, Meisner (28) found that increased thyroid activity in late February was probably correlated with increased activity and spawning.

In all of the papers cited above, the authors found that in hibernating and non hibernating poikilotherms the period of increased activity corresponds to the period of increased thyroid hormone secretion, regardless of the season. In Rana catesbeiana, our observations support those of these authors. The major peak of epithelial activity was correlated with the period of increased activity and spawning. However, the highest cell heights were observed in the winter frogs which had been disturbed from hibernation. This indicates that seasonal temperatures are secondary factors in influencing thyroid activity. It is probable that in all animals the environmental conditions affect the animal's activity in a specific way, and that it is this species difference which determines the rate of thyroxine production, storage, and secretion.

The above mentioned authors describe the thyroid

follicular colloid as basophilic during the periods of estivation or hibernation. This basophillia decreases as animals become more active, the colloid then becomes more acidophilic. In Rana catesbeiana, during the colder months the follicular colloid exhibits a preference for Aniline-blue over Orange-G, and stains more intensely blue. This increased affinity for Aniline-blue represents an increased basophillia. During the Summer and Fall, this basophillia decreases until October when only sixty percent of the colloid stains blue.

In Rana catesbeiana reabsorption vacuoles were rarely noticed, only a few times in the summer of 1962 and again in the winter of 1963. This would indicate that these vacuoles are related to the condition of the colloid during periods of increased hormone secretion.

Uhlenhuth, et al, (51) studying the colloid and cell height in the salamander, Triturus torosus, in relation to thyroid secretory activity suggested that the absorption of intra-follicular colloid and the resulting secretion of hormone caused epithelial cells to be columnar, whereas apical excretion of the hormone into the colloid caused cells to decrease in height. These conclusions agree with our observations on the diphasic nature of the seasonal variation in cell heights and the changes in colloid staining properties.

Thyroid Chemistry

The relatively small size of the amphibian thyroid gland, and its relative inaccessibility have, perhaps, discouraged many workers from undertaking a chemical study of the thyroid hormones. Two groups of workers were exceptions, Donoso and Trivelloni (12) worked on a South American toad and Shellaberger and Brown (42) on a North American toad. The former authors identified, by paper chromatography and I-131 incorporation, all the common iodinated amino acids, mono- and diiodotyrosine, thyroxine, and triiodothyronine in the thyroid gland. They have not, however, detected any hormones in the plasma. Shellaberger and Brown (42) found both hormones and iodinated tryosines in the adult toad gland. They failed to find any evidence of hormones in the plasma. Our observations indicated that in Rana catesbeiana there was a good deal of organic iodine in the thyroid gland. Generally, authors have found that in amphibians, the thyroid does not pick up radioiodine in large quantities, and that it is excreted from the body rapidly. Concentration of I-131 by other tissues, notably the ovary and the skin, have been reported. The ovarian tissue accumulated inorganic iodine (32), while the skin accumulated organic iodine (15). The melanocytes of the dorsal skin are accepted as I-131 binding sites. This is not surprising when one considers the fact that the integumentary system in amphibians is more highly active than in other animals, e.g., respiration, ion exchange. Volpert (32) found that the

ratio of skin iodide to plasma iodine was in the order of 5:1 in Rana temporaria. Gennaro and Clements (15) found "in-vitro" incorporation of I-131 into tyrosine molecules by the skin. Talmage, et al, (47) found that in summer frogs, the removal of radio-iodine from the blood stream followed the same pattern as in mammals, and that the maximum accumulation in the gland occurred forty-eight hours after injection. Treating these frogs to low temperature (3°C) for twenty-four hours prior to I-131 administration enhanced the uptake for a short period, however, after five hours the uptake decreased rapidly. Similarly, treating the frogs with PTU resulted in a short-lived increased I-131 uptake by the thyroid gland. Most authors reported less than ten percent I-131 accumulation in the thyroid gland, and as low as two percent after ninety-six hours.

DesMarais and LaHam (11) have shown that a high degree of positive correlation existed between the quantity of Analine-blue staining colloid and the incorporation of radioactive iodine in the colloid substance. This, they maintain, would be organically-bound iodine, since the histological treatment would wash out any free or non-protein-bound iodine. We had hoped to be able to characterize this iodine as incorporated thyronine by a direct total hormone assay on the thyroid colloidal substance. However, due to the smallness of the gland in the frog, we could not isolate the protein thyroglobulin by the usual methods. Similarly, attempts to hydrolyze single glands and chemically analyze

the hydrolisate by BEI method failed. There simply is not enough protein incorporated hormone in a single frog thyroid to allow for chemical determination. Failing this, we decided to analyse the crude saline homogenate for protein incorporated iodine (the regular PBI technique) and butanol extractable iodine.

The protein-incorporated iodine in this context included all the possible organic forms of iodine. Since the animals involved in the DesMarais-LaHam experiments were iodine deficient prior to receiving I-131, there is good reason to assume that the colloid radioactivity represented to a high degree - hormonal iodine. However, in our case we had no way of assessing the proportion of hormonal iodine in our protein incorporated iodine study. As Derrien, et al, [9,10] and many other authors have pointed out, there are large variations in the percent incorporation of thyroxine iodine in the total thyroid iodine among individuals of the same species. Normally, thyroxine iodine accounts for only a fraction of one percent of the total protein iodine.

The average butanol extractable iodine for the year was quite high, being 0.13 $\mu$g per mg of tissue. This represented about 0.20 $\mu$g of thyroxine, and was a surprizingly high value, constituting more than ten percent of the protein incorporated iodine values. It seemed unlikely that so much free hormone would be available in the thyroid. There was a high concentration of inorganic iodine in the thyroid and perhaps the alkaline wash was not efficient enough to remove all.

Circulating Hormone

In our studies, we have employed both the protein-bound-iodine assay and the butanol extractable iodine assay. Essentially, these two techniques were used to measure the same thing - the total hormonal iodine in circulation. The PBI method had one disadvantage, it measured all protein-bound-iodine, hormonal and non-hormonal. These results, then, can be affected by iodine contamination. If serum iodide levels were sufficiently high, a significant amount of inorganic iodine would be bound to the serum proteins and some would be trapped in the precipitated proteins as well. Butanol also extracts inorganic iodine, but by washing the extract with Blau's reagent, the iodide can be removed. There is some loss of thyroxine in the washing procedure, but with care this can be minimized.

We have found that the BEI and PBI values were in general agreement, except during the winter months when there was an elevated protein-bound-iodine value. The annual BEI average was 3.77 $\mu$g percent and for PBI it was 3.86 $\mu$g percent. From this we can conclude that there was no great loss of hormonal iodine in the BEI technique.

In the winter months, between December and March, PBI values were found to be very high, particularly in January, when a peak value of 10.8 $\mu$g percent was observed on the pooled samples. At this time, the BEI values were relatively normal with two high values in December and February. In Belgium, Wille and DeVisscher (55) made observations on

the seasonal changes in plasma protein-bound-iodine in Rana temporaria. They found very marked seasonal differences in protein iodine. There was a marked winter increase in PBI, reaching a maximum of over 20 $\mu$g percent in March, which was the period of spawning for this species. Following this, the PBI level declined until a low of approximately 3 $\mu$g percent was obtained in late May. This low was then followed by an increase to approximately 5 $\mu$g percent in August, and this level maintained, with only a slight decrease, until early December when the blood hormone level dropped off. Curiously, no thyroid hormone was detectable in the month of January.

DeVisscher's PBI values varied greatly from our PBI values, and our butanol extraction indicated that much of the winter PBI was actually inorganic iodine. It would be surprising to find that a hibernating animal would actively secrete thyroid hormone, only to store it in the circulation for use later in the spring spawning season. The thyroid gland would appear to be a more efficient and safer organ of storage. This was all the more puzzling due to the fact that the authors kept feeding their animals. Ordinarily, winter frogs cease to eat, or eat sparingly. However, assuming the thyroid was secreting hormone and the animal was digesting normally, one would expect these frogs would be excreting a large quantity of thyroid in their feces. With such a rapid increase in circulating hormone, the actual secretion rate of these animals would have to be great, indeed.

It is possible that those animals on laboratory diets received a large amount of dietary thyroxine or thyroid activators, and that the animals were, in effect, in a hypothyroid state as far as body tissue was concerned, and the hormones, exogenous or endogenous, simply remained blood protein-bound, while the winter liver was unable to excrete the excess. A second possibility is that the diet was high in iodine, and that it was this inorganic iodine which contaminated the blood protein. As mentioned above, our observations would tend to confirm the possibilitity that the authors were loading the circulation with iodine.

We suggest that the winter PBI peak, which in our animals was not as high, nor as sustained, as in Rana temporaria, could be a mechanism to conserve iodine for hormone production and prevent its excretion. It has been shown by Ardell and Thorson (1) that frogs dissipate water in the fall and early winter. The peripheral tissues and skin, which held a great deal of hormone and iodide would, then, tend to lose these and other metabolites into the plasma water. Therefore, we can postulate an increase in blood hormone and iodine level with a concomitant increase in feces and urinary excretion of thyroxine and iodide. We postulate this because it is usual for an euthyroid individual to maintain a normal level of circulating thyroid hormone by balancing secretion and excretion of this hormone. The low capacity of the amphibian thyroid to accumulate iodide, as evidenced by I-131 experiments, indicated some other

storage method was required to provide the necessary iodine for this extended period of hormone production.

Meisner (28) found that propyl-thiouracil had no effect on oxygen consumption in Rana pipiens in winter, and suggested that thyroxine was of no calorigenic importance in this animal. The use of radioactive iodine in amphibians and other poikilotherms facilitated the identification of hormones in blood, but has not led to new knowledge of hormone secretion by the gland. In general, however, the summer animal tended to accumulate more organically-bound radio-iodine in the blood than did the winter animal. If, indeed, the frog thyroid hormone does not have a calorigenic effect, then it would be all the more surprising to learn that animals with no defence against extremes of low temperatures should accumulate circulatory thyroxine during the Winter.

Hibernation Interupted and Starvation in Summer Frogs

In the frogs which were received in the winter of 1963, the follicular epithelial cells were greatly elevated over those of animals kept in hibernation the previous winter. The nuclei of these cells were also much larger in the 1963 groups than in the 1962 groups. In regard to the chemical analysis of the gland, we found that the PBI remained lower in the 1963 groups, however, the BEI which was elevated in February returned in March to values observed the previous year. While the February, 1963,

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inorganic iodine level in the thyroid was slightly lower than in 1962, it was not significant and by March had reached the normal value.

The colloid stain properties in these two groups showed a lower value in February, 1963, over the 1962 level, but by March it had returned to the level observed the previous year, while the cell height remained higher. This observation does not agree with the conclusions of Uhlenhuth and other, that excretion from the apical pole necessarily lowers the cell height. Perhaps cell height may not be so significant a measure of secretion, nor apical excretion of hormone. The serum hormone assays showed no difference between the two different annual groups. These frogs had been taken from their natural habitat and transported by air from Wisconsin. It is obvious that the stress due to this treatment would have a profound effect on the thyroid activity. Once the animals were placed in simulated natural conditions, most of the parameters returned to normal, with the exception of cell height and total PBI of the gland.

Starvation had no apparent effect on the cytology of the thyroid gland. However, there was a drastic change in the staining of the colloid substance. In the starved animals there was a dramatic lowering of follicular colloid affinity for Aniline-blue and a reciprocal increase for Orange-G. The iodine determinations did not show any evidence of effects of starvation. It remains to be seen, however, what relationship stain affinity has to hormone concen-

tration in the colloid. This can be determined only when sufficient yellow-staining colloid material can be extracted.

The excretion of thyroxine and thyroxine metabolites was not measured. The decrease in the active colloid was not accompanied by an increase in serum thyroid hormone. There are three possible explanations; increased excretion of thyroxine, increased utilization of thyroxine, or an accumulation of this hormone in peripheral tissues, e.g., melanocytes of the dorsal skin.

## CONCLUSIONS

1. There is a definite, diphasic seasonal cycle in regard to epithelial cell height. During January, there is a short period of "high" cells which is followed by a sharp decrease in cell height throughout the Winter. In early Spring, the cell height begins to increase again, reaching a maximum in June. This second "high" is followed by a more gradual decrease in cell height throughout the Summer, reaching an autumn low height in November which is followed by the early Winter increase in cell height.

2. The nuclei of the epithelial cells of the thyroid follicles do not show a seasonal pattern of morphological changes.

3. The follicular colloid stain affinity also follows a definite seasonal pattern. The staining property of the follicular colloid, regarding its affinity for Analine-blue or Orange-G, is a good index of thyroid secretory activity. Throughout the year, in normal animals, the colloid is predominately basophilic, however, it is more basophilic during the hibernation period than it is in the Summer. In abnormally hyperactive glands, the colloid is predominately acidophilic. The colloid stain affinity is inversely proportional to the cell height.

4. There is a higher concentration of protein incorporated iodine in the thyroid during the period of hibernation than in the Summer.

5. The period of greatest inorganic iodine concentration within the thyroid gland occurs just prior to hibernation. This iodine concentration is maintained at fairly constant levels during hibernation and during the mid Summer.

6. From the foregoing conclusions, it is possible to further conclude that during the winter months of hibernation the thyroid gland in Rana catesbiana is more active in producing and storing thyroxine, while in the Summer the emphasis shifts back to hormone production and secretion. Just prior to emergence from hibernation, the thyroid has its greatest concentration of hormone, and before entrance into hibernation the gland's hormone content is lowest. This conclusion is in agreement with the accepted theory of thyroid activity in other poikilothermous animals.

7. The winter serum PBI level is higher than the PBI level of the summer serum. However, the actual serum thyroid hormone concentration does not change from season to season. It is concluded that the elevation in PBI concentration over BEI represents endogenous inorganic iodine contamination. Since the thyroid hormone level in serum does not increase with increased thyroid secretory activity, one must assume that this hormone is either being utilized as it is being secreted, or it is excreted from the body in some manner.

8. Prolonged starvation and the restraint of captivity decreases the thyroid follicular colloid basophilia. Since no concomitant increase in serum hormone is evident, it is assumed that the secreted thyroxine is possibly excreted

directly, metabolized, or becomes fixed in some manner in the peripheral tissues.

9. Interruption of hibernation increases the epithelial cell height, decreases colloid basophillia, and lowers the concentration of protein incorporated iodine in the gland. This is concluded to indicate hormone release, which hormone does not remain in circulation. From the dramatic increase in cell height over normal animals, it is concluded that the seasonal pattern in thyroid activity is not directly influenced by environmental conditions, but rather by metabolic requirements in various states of activity.

10. There is no evidence of a sexual dimorphism in regard to the seasonal variation in thyroid gland activites.

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