PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 153, 383-387 (1976)

Calcitonin, Serotonin, and Parafollicular Cell Granules during the Hibernation Activity Cycle in the Bat (39551)

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The small, dense, smooth, membrane-enclosed granules of mammalian thyroid parafollicular cells have been recognized as storage granules containing calcitonin (1, 2). This peptide hormone inhibits bone resorption and thus lowers plasma calcium (3). A seasonal variation in the number and morphology of these granules in bats (4–8) and other hibernating mammals (9) suggests that secretory activity slows prior to hibernation, ceases during hibernation, and resumes during arousal.

Parafollicular cells of bats also store the monoamine serotonin (2, 10). Tritiated serotonin, synthesized from its tritiated precursor 5-hydroxytryptophan ([5-^{3}H]HTP), is localized in the small granules of the cell (11). The serotonin content of the bat thyroid varies in accordance with the morphologically defined seasonal secretory cycle of the parafollicular cell. It rises when the granules accumulate in early hibernation and gradually falls as granules are resorbed during mid and late hibernation.

These observations have led to the suggestion that the two hormones, calcitonin and serotonin, are stored in the same subcellular storage granules. If this hypothesis is correct, seasonal covariation of the thyroidal content of the two hormones is to be expected.

This report correlates thyroidal calcitonin, plasma calcium, thyroidal serotonin, and parafollicular cell morphology through the activity–hibernation cycle in the bat.

Materials and methods. Animals. Fully grown male and female bats of the species *Myotis lucifugus* were used. All animals were captured in their natural habitat. Active bats were captured in May, September, and October. Hibernating bats were collected in November, December, January, and February. The latter were placed gently, while in the cave where they were hibernating, into containers with ice, transported to the laboratory, and kept at 4° for at least 24 h before use. During this period, the animals were watched for signs of physical activity. Those exhibiting such activity were not used.

Sample preparation. At each seasonal time point, some animals were anesthetized by ether inhalation and blood was drawn by cardiac puncture. Blood from four to six bats was pooled for determination of plasma calcium. Other animals were decapitated and their thyroids were quickly excised and trimmed. Some glands were fixed in buffered 6.25% glutaraldehyde (pH 7.3) for 4 hr, washed, and processed for electron microscopy as described elsewhere (7). The remaining glands (between 40 and 60) were weighed and pooled into two separate samples (10 for serotonin analysis; the remainder for calcitonin). The pooled thyroids were quickly frozen by immersion in liquid nitrogen and were kept at −20° until processed.

Measurement of serotonin. The spectrofluorometric method of Snyder *et al.* (12) was used. Serotonin, extracted from an acid homogenate of the thyroids, was reacted in aqueous solution at neutral pH with ninhydrin. The excitation and emission spectra of the intensely fluorescent product were obtained at 380 and 490 nm, respectively, with an Aminco Bowman spectrofluorometer and were compared with those of serotonin-creatinine sulfate standards. Results were expressed as micrograms of serotonin per gram of thyroid wet weight.

Measurement of calcitonin. Pooled samples of 30 to 50 thyroids were processed for measurement of calcitonin at each seasonal time point by a rat bioassay method used previously in this laboratory (13). The glands were homogenized at 4° in 0.1 *N*

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HCl. The homogenate was centrifuged in the cold at 10,000 rpm for 20 min. The supernatant was collected, pH was adjusted to 4.0, and the protein content was estimated (14). Holzman albino male rats, averaging 100 g in weight, were fasted overnight and used for bioassay of hypocalcemic activity in the supernatant against the Medical Research Council (MRC) calcitonin standard B. The bioassay was designed as a two-dose factorial assay (15) with high and low points obtained for each homogenate. Blood was drawn by cardiac puncture 1 hr after injection of the supernatant and plasma calcium was determined by flame emission spectrophotometry (13). At least five rats were used for each point. Statistical evaluation was performed as described earlier (13, 15). Potency estimates were expressed as mean MRC milliunits per milligram of protein (MRC mU/mg) of the respective supernatant.

Bat plasma calcium was measured in pooled bat blood as described above by flame emission spectrophotometry and expressed in milliequivalents per liter (mequiv/liter). Significance of differences was tested by the Student's *t* distribution.

Results. Calcitonin levels. A seasonal profile of bioassayable calcitonin in bat thyroid is shown in Fig. 1 The corresponding calcitonin levels are listed in Table I. Calcitonin levels in thyroid glands obtained late in hibernation (February) were similar to those observed in thyroids of active bats (May and September). The mean level of thyroidal calcitonin in active bats ranged between 30 and 43 MRC mU/mg. A marked increase in levels was observed in glands collected in early hibernation (November and December). The mean level observed during this peroid was two- to threefold that of active animals and ranged between 60 and 123 MRC mU/mg. In spite of a relative increase in the standard error estimate, indicating greater heterogeneity among pooled preparations, the difference between these values and those observed in late hibernation and during the active phase of the annual life cycle is clearly significant.

Plasma calcium levels. Pooled plasma calcium levels during the hibernation period revealed that relative hypocalcemia occurs in midhibernation (November; Fig. 1) with plasma calcium levels of 4.2 ± 0.2 mequiv/liter compared to 4.8 ± 0.2 mequiv/liter ($P < 0.01$) observed during the active phase of the annual life cycle. Hypocalcemia during hibernation in bats has also been observed by Riedesel (16).

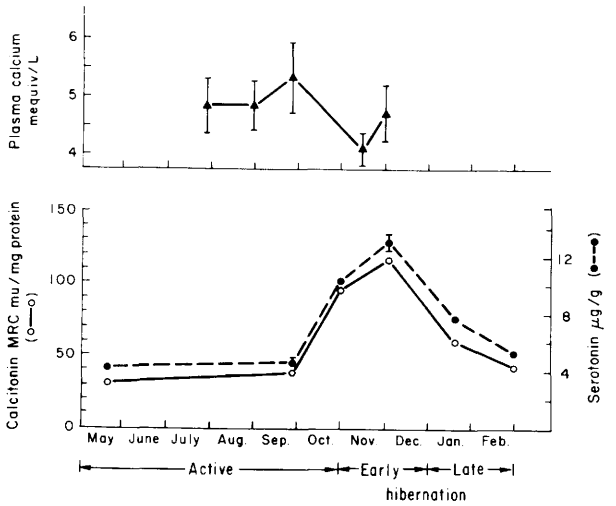

FIG. 1. A seasonal profile of bat thyroidal calcitonin, serotonin, and plasma calcium. (Upper panel): Plasma calcium in mequivalents per liter (mean ± SE). (Lower panel): Calcitonin potency estimates in bat thyroid extracts, expressed in mean MRC milliunits per milligram of protein. Open circles, solid line. Serotonin content expressed in micrograms per gram of thyroid wet weight. Filled circles, broken line. The bars at the September- and December-time points indicate the standard error.

TABLE I. CALCITONIN AND SEROTONIN CONTENT OF BAT THYROID.

Month	Calcitonin (MRC mU/mg)[a] Mean ± SE	Serotonin (μg/g)[b] Mean ± SE[c]
May	30 ± 4.50	4.05
September	42 ± 5.04	4.15 ± 0.1
November	93 ± 11.60	10.20
December	123 ± 24.6	12.68 ± 0.28
January	60 ± 8.40	4.67
February	40 ± 5.50	5.20

[a] Hypocalcemic activity expressed in MRC milliunits per milligram of protein of gland extract.

[b] Serotonin in micrograms per gram of gland wet weight.

[c] Multiple (three) determinations were performed at two seasonal time points: September and December. The mean and SE shown are for these time points.

Serotonin levels. The seasonal changes in thyroidal serotonin content in the bat are shown in Fig. 1 and the corresponding values are listed in Table I. Thyroidal serotonin in active bats ranged between 4 and 4.25 μg/g. In early hibernation, a threefold increase is observed with serotonin levels at almost 13 μg/g. This increase subsided by arousal time when serotonin content is back at prehibernation levels.

Morphology of parafollicular cell secretory granules. The morphological appearance of parafollicular cell secretory granules observed in the active phase, pre- or early hibernation phase, hibernation, and arousal phases are depicted in Fig. 2a–d. During the active phase of the annual cycle (May through early September), only small granules with a diameter between 0.1 and 0.2 μm were observed (Fig. 2a). They are usually spherical, contain a solid dense core, and are bounded by a smooth membrane. During pre- or early hibernation (September through late October), large dense intracisternal granules are found in addition to the small dense secretory granules. These intracisternal granules are bounded by a membrane dotted with ribosomes and reach diameters up to 5 μm (Fig. 2b). Large intracisternal granules are absent later in hibernation. At this time, however, the small, dense, secretory granules lose their solid dense core and often contain membranes or myelin-like figures (Fig. 2c). The granules at this stage do not exhibit uptake of tritiated 5-hydroxytryptophan (11). At arousal, small, solid, dense granules are again found in parafollicular cells (Fig. 2d).

Discussion. A parallel seasonal variation in thyroidal calcitonin content and thyroidal serotonin content is reported in the present study. The thyroidal content of both hormones increases at the start of hibernation and decreases as hibernation proceeds. A rise in thyroid calcitonin during hibernation has also been found in the ground squirrel (17). The changes in hormonal content described above are closely related to the morphological changes in parafollicular cell secretory granules. Thus, in early hibernation these granules accumulate and large dense granules appear in the cisternae of the rough endoplasmic reticulum. These suggest a slowing of intracellular transport of secretory material from the rough endoplasmic reticulum to the Golgi apparatus. During midhibernation, autophagy of preformed granules is commonly observed. The number of granules decreases and the matrix of residual granules becomes less electron dense (4, 6, 8). By prearousal, new granules appear (6) and arousal is accompanied by signs of exocytosis of granular contents (6).

It seems likely that the increase in calcitonin content of the thyroid in early hibernation is due to slowed or inhibited release of the hormone from the parafollicular cells. Relative hypocalcemia, actually observed during hibernation (this report; 16) is undoubtedly one factor responsible for and contributing to this. It is reasonable to assume that the absence of a dietary source of calcium during hibernation could explain a relative hypocalcemia. In fact, evidence for increased parathyroid activity (18) and increased skeletal resorption to the point of skeletal demineralization in hibernating bats (19) indicates the extent of corrective mechanisms induced by the hypocalcemia. The inhibition of intracellular transport and release of calcitonin from parafollicular cells during hibernation, clearly facilitates the effect of these corrective mechanisms toward homeostasis of calcium during hibernation. Thus, we would postulate that parafollicular and parathyroid cells have reciprocal roles and act synergistically in the regulation of calcium metabolism during the seasonal life cycle of mammalian hibernators.

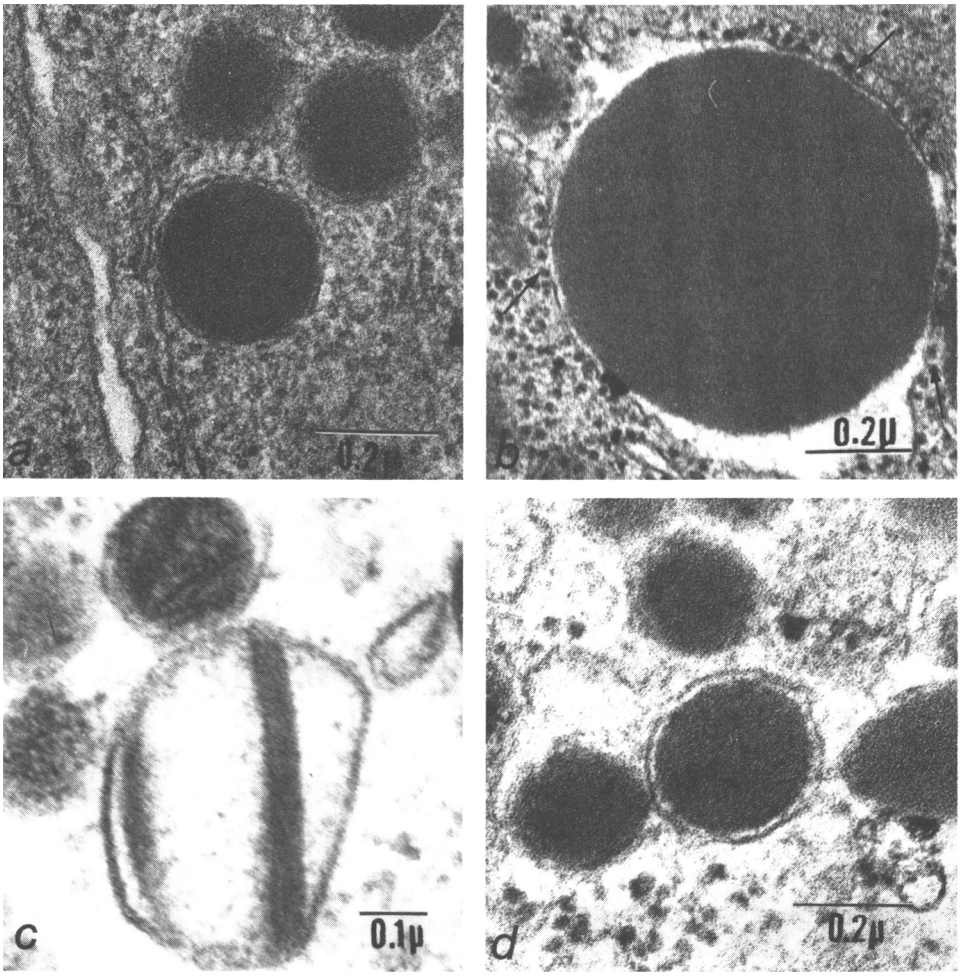

FIG. 2. A profile of seasonal ultrastructural changes in bat thyroid parafollicular cell granules. (a) Secretory granules during the active phase of the life cycle. Electron-dense core bounded by a smooth membrane. Magnification, × 80,000. (b) Large intracisternal granules seen during prehibernation and early hibernation. Arrows indicate the ribosome-studded limiting membrane. Magnification, × 76,000. (c) Resorbing secretory granules seen during mid- to late hibernation. Note gradual loss of electron-dense core. Magnification, × 90,000. (d) Secretory granules as seen at the arousal phase of the annual life cycle. Magnification × 80,000.

The present data, taken together with earlier observations (2, 10), support the hypothesis that serotonin and calcitonin are stored in the same parafollicular cell granules. The coincidence of storage would seem to ensure that both hormones would be released together by exocytosis. Serotonin release from parafollicular cells by calcium, the natural stimulus for calcitonin release, has recently been demonstrated (20).

Since serotonin is rapidly cleared from the circulation through uptake and inactivation by platelets, liver, and pulmonary endothelial cells (21), its role, once released from thyroid parafollicular cells, is likely to be a local one. Biogenic amines have been suggested as endogenous stimulators of thyroid follicular cells (22–24). Serotonin, specifically, has been implicated in the action of thyrotropin on follicular cells (25). Similarly, in bat thyroid, serotonin has been proposed to act as a local activator "messenger" between the parafollicular "storage" cells and the follicular cells (26). The latter are capable of serotonin uptake and inactivation (26). Thus, changes such as hypo- or

hypercalcemia might indirectly affect thyroid hormone secretion as well as calcitonin secretion.

Brain serotonin and the serotonergic neurons of the nuclei of the median raphe have been implicated in the control of hibernation (27). The presence of serotonin in the bat thyroid and the seasonal variation in its content suggest that thyroidal serotonin too may play an important role in the hibernation process. The definition of such a role remains to be determined.

Summary. A seasonal covariation in calcitonin and serotonin content of bat thyroid has been found. Whereas the mean level of thyroidal calcitonin in active bats ranged between 30 and 48 MRC mU/mg of protein, a two- to threefold increase in this level was observed prior to and during early hibernation. Associated with this increase, a relative hypocalcemia was observed. In late hibernation, thyroidal calcitonin level returned to the range observed in active bats. A parallel seasonal profile was found for bat thyroid serotonin content, which varied from a mean of 4.1 μg/g during the active phase of the annual life cycle to a peak close to 13 μg/g observed in early hibernation. The seasonal covariation of calcitonin and serotonin reflects ultrastructural changes observed in the secretory granules of thyroid parafollicular cells during the annual life cycle of the bat.

The authors wish to acknowledge the competent technical assistance rendered by Mary Ann Viloria and John Patrikes.

This work was supported in part by USPHS Grant No. AM11431 and by a grant from Ciba-Geigy Corporation, Summit, New Jersey.

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Received May 21, 1976. P.S.E.B.M. 1976, Vol. 153.