tractions whereas cholecystokinin does not. The reduced gastric emptying associated with duodenal acidification may thus be the consequence of release of secretin.

1. Johnson, L. P. and Magee, D. F., Surg. Gynecol. Obstet. **121**, 557 (1965).

2. Chey, W. Y., Lorber, S. H., Kusakcioglu, O., and Hendricks, J., (Abstr.), Federation Proc. **26**, 383 (1967).

3. Johnson, L. P., Brown, J. C., and Magee, D. F., Gut **7**, 52 (1966).

4. Lorber, S. H., *in* "The Stomach: Including Related Areas in the Esophagus and Duodenum" (C. M. Thompson, D. Berkowitz, and E. Polish, eds.), p. 152. Grune and Stratton, New York (1967).

5. Cummins, A. J., Schapiro, H., and Merker, P. C., Am. Surg. **32**, 187 (1966).

6. Brown, J. C., Gastroenterology **52**, 225 (1967).

7. Allen, G. L., Poole, E. W., and Code, C. F., Am. J. Physiol. **207**, 906 (1964).

8. Papasova, M. P., Nagai, T., and Prosser, C. L., Am. J. Physiol. **214**, 694 (1968).

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Localization of Calcium in the Thyroids of Rats* (33721)

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The thyroid gland contains relatively high concentrations of calcium. This has been reported as a mean value of 35 mg/100 g of wet weight in rats (1), and in humans as a mean of about 34 (2), 39 (3), and 52[1] (4) mg/100 g of wet weight. With the exception of the aorta, one value cited (3) was the highest of the 27 nonsupportive tissues reported (cartilaginous tissues such as larynx and trachea were higher, as are, of course, bones and teeth). In 1965 Kaellis and Goldsmith (1) found that thyroidal calcium concentrations in rats declined with thiourea treatment and rose with either inanition or the use of a high calcium–low iodine diet. This was part of a study of thyroidal citrate concentrations; and they proposed that the thyroid captures calcium along with citrate (which is also present in high concentration), and that this calcium was subsequently bound to thyroglobulin in such a manner as to inhibit the production of thyroxine. The present study was undertaken to determine the sites of localization of calcium in the thyroid by histochemical methods and to observe changes occurring in animals rendered hypercalcemic.

* Experiment performed under a grant from the National Research Council of Canada.

[1] Calculated, assuming 75% water.

TABLE I. Treatment of Rats.

Group	No. of rats	Treatment
I	11	Regular rat chow and water *ad libitum*
II	6	As in Gr. I with 2% $CaCO_3$ (w/w) added to the chow
III	6	Low calcium diet and water *ad libitum*, i.p. injections of dihydrotachysterol o.d., 5 days, 2.5 mg/kg of body wt.

Procedure. A total of 23 male CFN rats was used. This strain and sex were chosen to permit application of previously-reported results (1). The body weight of the rats averaged about 250 g at the time of death. During the 5 days of observation while the rats were under either control or experimental conditions, they all gained weight with the exception of a single animal (in Group II), the weight of which stayed very nearly the same. The animals were divided into groups as shown in Table I.

For all the experimental and for six of the controls the glyoxal bis(2-hydroxyanil) (GBHA) method of Kashiwa and Sigman (5), slightly modified, was used because of its sensitivity and discreteness in localization.

For the other five controls, the alizarin red S method of Dahl (6) was used as a check. This latter method has approximately the same sensitivity as the former, but its originator does not claim as much discreteness in localization.

At the end of each period the rats were anesthetized deeply with ether, blood was removed from the heart, and the serum was separated by centrifugation. The sera were subjected to calcium analyses by atomic absorption spectrophotometry. When the GBHA method was used, the larynx and portions of the trachea to which the thyroid was attached were cleaned free of esophagus and muscle. The tissue remaining was plunged into liquid nitrogen for 30 sec and allowed to thaw on a glass slide resting on solid carbon dioxide. The tracheal end was grasped with a forceps and a razor blade was used to cut sections of no more than 0.2 mm in thickness through the thyroid and the supporting cartilage. These were immediately placed in a vial containing 2 ml of a 3.4% solution in 75% ethanol in which 100 mg of GBHA had been dispersed just prior to use. The slices remained in the vial for 3 hr after which they were dehydrated in ethanol, cleared in *p*-xylene, infiltrated and embedded in paraffin. They were sectioned at 7μ. The sections were floated on a 1% NaOH solution at 40° and lifted on to albuminized slides where they were allowed to dry overnight at 37°. The sections were then dehydrated with ethanol, deparaffinized in *p*-xylene, brought through absolute ethanol to a saturated solution of sodium carbonate and potassium cyanide in 95% ethanol (to remove metals other than calcium which complex with GBHA), dehydrated, counterstained lightly with fast green FCF, dehydrated, cleared and mounted in Clearmount (E. Gurr, Ltd.) to avoid discoloration with time.

When the alizarin red S method was used, the thyroid glands and the supporting cartilage were sliced without first freezing. The slices were immersed for 36 hr in 95% ethanol, 12 hr in absolute ethanol, 4 hr in *p*-xylene, and then infiltrated with paraffin, embedded, and sectioned at 7 μ. The floated sections were taken up on nonalbuminized slides. The staining procedure was exactly as described by Dahl (6), except that fast green FCF was used as a counterstain.

TABLE II. Serum Calcium Levels.

Group	Mean (Ca) (mg/100 ml of serum)	SEM
I	11.05	0.13
II	12.10[a]	0.10
III	12.15[a]	0.09

[a] Significantly different from value for Gr. I at .001 level (Fisher *t* test).

Sections of three control rats were treated with either an alkaline Versene solution or a solution of equivalent alkalinity with no Versene and then subjected to the staining process.

Results. Analyses of the sera are indicated in Table II.

Examination of the GBHA-stained sections showed in all cases, experimental and control, a reddish-stippling in the follicular cells due to the formation of the colored calcium complex. In many sections, the nuclei had noticeably less stippling, and the immediate perinuclear area had a more dense concentration of granules. This was not correlated with any treatment the animals had received. The colloid was sometimes rather homogeneous, and sometimes had a terrazzo-like appearance with some stippling of the continuous phase of the colloid. This stippling had, however, a bluish cast and consisted of tiny globules rather than granules.

There was no evident change in the color intensity, concentration, or size of the granules or their location in any one of the experimental animals as compared with one another or with members of the control group (Figs. 1–3). The alizarin red S staining of control thyroids showed the same morphological and histochemical picture, although the contrast was somewhat heightened due, in part, to greater uptake of the counterstain. Control sections pretreated with Versene were negative for staining, while alkaline pretreated sections without exposure to Versene stained normally.

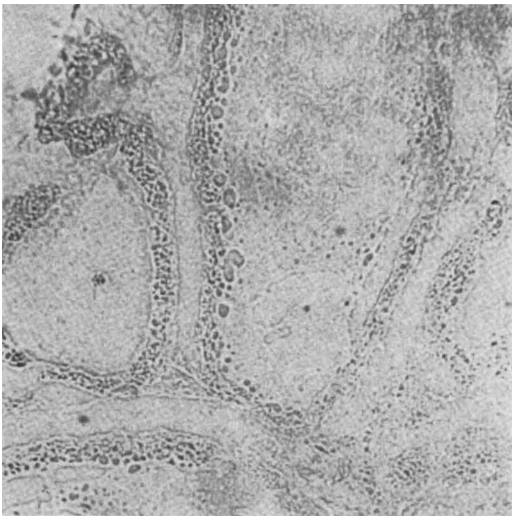

FIG. 1. Section of control thyroid stained with GBHA, L.P.

Discussion. Hueper (7) reported metastatic calcifications in the thyroids of dogs given such high doses of parathyroid extract that they died in coma. He observed two types of thyroid glands, *viz.*, one in which the normal architecture was maintained but in which calcium salts were precipitated in the colloid, and another which in appearance resembled parenchymatous goiter, but having an infiltration of calcium salts in the inerstitial connective tissue. Hass *et al.* (8) described the effects of very high doses of vitamin D on rabbit thyroids. The small arteries were usually severely affected, with atrophy of glandular epithelium and diminution of the amount of colloid. There were variable calcium deposits in the basement membranes and interfollicular stroma. These are, of course, results on animals *in extremis* and cannot be considered to be qualitatively comparable to those obtained in this study.

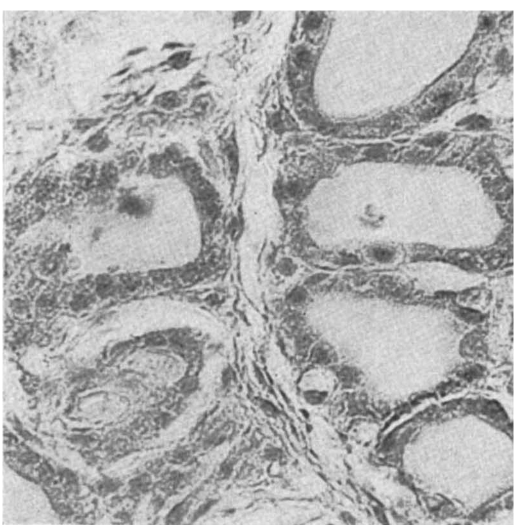

FIG. 2. Section of control thyroid stained with alizarin red S. L.P.

The GBHA method has been described as demonstrating "ionic" calcium (9). Actually, in the opinion of the author, a more accurate term would be "loosely-bound" since the reagent competes under the local chemical conditions with whatever is binding the calcium, *e.g.*, protein or hydroxyapatite, and will, in fact, form the colored complex with calcium as long as the dissociation of the complex, under the staining conditions, is less than that of the calcium complex or compound in the tissues. Although the alizarin red S stain is aqueous and only very mildly alkaline, there was no apparent diffusion of the calcium as compared with the GBHA method, which is designed to suppress diffusion of calcium. This may be due to the relatively short time that sections are immersed in the alizarin-red S (2 min) or may indicate that the calcium is somehow bound

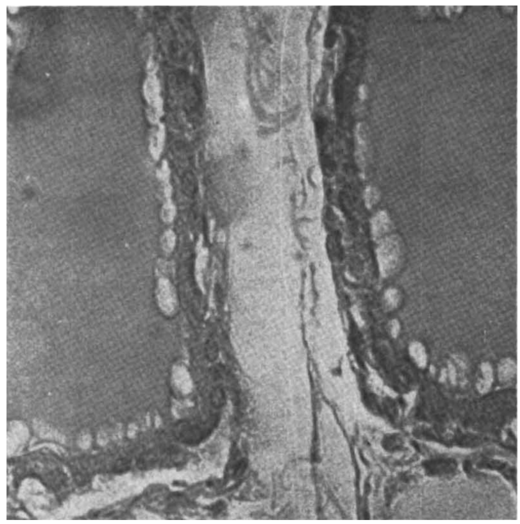

FIG. 3. Section of thyroid from Group III stained with GBHA, H.P.

and not readily diffusible as ionic calcium presumably would be. Furthermore, a very rapid formation of the colored complex with alizarin red S might "fix" the calcium in place.

What is perhaps most interesting is the observation that the thyroid, which has a high calcium concentration and a high total to cellular volume ratio, seems to have all of its calcium intraceullularly. Also interesting is the observation that the staining is relatively meager compared to what one would expect from chemical analysis of the thyroid; that it is apparently invariant with a hypercalcemic condition of several days' duration; and that the manner in which the hypercalcemia is induced, *i.e.*, either exogenously (by diet) or by leaching from the rat tissues seems to make no apparent difference.

The role of calcium in thyroid metabolism is not, of course, fully known. It seems to exert a direct inhibitory effect on the formation of thyroxine (10), and the gland must have some way of monitoring serum calcium levels if it is to release thyrocalcitonin in response to a rise.

Summary. Rat thyroid has a high concentration of calcium which is known to decline with thiourea treatment and increase with inanition or a high calcium-low iodine diet. The histochemical localization of this calcium in normo- and hyper calcemic states was studied. Use of glyoxal bis(2-hydroxyanil) and alizarin red S revealed localization in the follicular cells. Rats rendered hypercalcemic by increase in dietary calcium or by injections of dihydrotachysterol showed virtually identical structural and histochemical appearance of their thyroids as those of control animals.

1. Kaellis, E. and Goldsmith, E. D., Acta Endocrinol., Suppl. **48**, 95 (1965).

2. Cameron, A. T., Can. Med. Assoc. J. **16**, 753 (1926).

3. Tipton, I. H. and Cook, M. J., Health Phys. **9**, 103 (1963).

4. Butt, E. M., Nusbaum, R. E., Gilmour, T. C., DiDio, S. L., *in* "Metal Binding in Medicine" (M. J. Seven and L. A. Johnson, eds.), p. 43. Lippincott, Philadelphia, Pennsylvania (1960).

5. Kashiwa, H. K. and Sigman, M. D., Jr., J. Dental Res. **45**, 1796 (1966).

6. Dahl, L. K., Proc. Soc. Exptl. Biol. Med. **80**, 474 (1952).

7. Hueper, W., Arch. Pathol. Lab. Med. **3**, 14 (1927).

8. Hass, G. M., Trueheart, R. E., Taylor, C. B., Stumpe, M., Am. J. Pathol. **34**, 395 (1958).

9. Kashiwa, H. K. and Atkinson, W. B., J. Histochem. Cytochem. **11**, 258 (1963).

10. Taylor, S., J. Clin. Endocrinol. **14**, 1412 (1954).

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