STUDIES ON THE HISTOCHEMICAL NATURE OF THE COLLOID OF THE THYROID GLAND OF THE MALE ALBINO RAT[1]

by

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ABSTRACT

A new staining technique has been developed for the thyroid gland of the rat which in addition to being an excellent histological stain is a histochemical method for the rapid identification of the colloid containing iodinated thyroglobulin from that which contains biologically inactive protein material.

Various indices such as incorporation of radioiodine 131, gland weight, epithelial height, and total number of follicles have been used to test the validity of the color reaction in the colloid. The comparison of these diverse indices of thyroid activity show that the blue colloid is directly related to the physiological activity of the gland.

As a result of our studies further insight has been gained into several aspects of thyroid physiology:

a) in the normal rat only about fifty percent of the gland contains active colloidal material;

b) complete incorporation of iodine 131 into the colloid requires longer than twenty-four hours in the normal rat;

c) information on the role of thiouracil raises the question of its action in iodine deficient rats;

d) evidence is offered for intracellular protein binding of iodine rather than the view that it is restricted to the follicular colloid;

e) additional evidence is presented that short exposure to cold brings about an immediate physiological and morphological response by the thyroid;

f) ascorbic acid has no apparent effect on the thyroid itself;

g) evidence favors the view that the pituitary gland is the rate-controlling principle in thyroid hormonogenesis;

h) the histochemical method may be of value in pathological diagnosis of thyroid slices and biopsies.

STATEMENT OF THE PROBLEM

For many years biologists have observed that the follicular colloid of the thyroid gland does not react homogeneously with tri-chrome stains such as the Mallory Connective Tissue Stain. Some have drawn attention to the fact that under different physiological conditions one or the other dye will dominate. In spite of this knowledge the general conclusion has been that these reactions are staining or fixation artefacts and cannot be interpreted as having any biological significance.

Having been faced with this problem several times and struck by the different staining pattern under different experimental conditions, serious doubt arose about the validity of dismissing these reactions as simple artefacts.

The present work, therefore, has two purposes:

1- The development of a histochemical method which would give a consistent color reaction with the follicular colloid.

2- To test the stain under known experimental conditions and, if valid, subsequently to use it in testing our hypothesis that the colloid of the thyroid gland is in fact heterogeneous. That is, that some follicles contain "active colloid" or iodinated thyroglobulin while others contain "inactive colloid" or the protein residue remaining in the follicle following the selective hydrolysis of the iodothyroglobulin by the proteolytic enzyme system.

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INTRODUCTION

The unique organization of the thyroid gland among the endocrines is undoubtedly respon sible for the extreme complexity of its physiological mechanisms. Although the impertant role of the thyroid in metabolism has been recognized since the classical observations of Magnus-Levy in 1895, in his study of Gulls Disease, today there still remain to be solved numerable fundamental problems related to the production, storage, secretion, and peripheral action of the thyroid hormones.

The thyroid gland differs fron all the other endocrine glands in that hormone storage has been developed to the highest degree. Other endocrines elaborate their secretion from the site of origin directly into the blood stream, but in the case of the thyroid the secretions are liberated into cup-like acini called follicles. Here the secretions remain for an indefinite period dependent upon the demands of the organisms for thyroid hormone. The initial secretion is thyroglobulin, a large molecular protein (630,000). Proteolysis takes place within the follicles to hydrolize the protein to sufficiently small constituents capable of being absorbed through the follicular cells into the blood stream. Briefly, then, the overall activity of the thyroid gland may be presented as follows.

There is a certain amount of iodine available in the body, which constitutes the "iodine pool". It consists of

the iodine stored in the tissues exclusive of the thyroid gland, which is available from the metabolized thyroid hormones or food absorption; it also includes that which is circulating in the blood stream.

The follicular cells of the thyroid gland take up iodide from the blood. The accummulated iodide is oxidized to iodine and combines with a glycoprotein secreted by the follicular cells to form thyroglobulin.

The thyroglobulin synthesized by the thyroid cells is stored in the lumen of each follicle where it is acted upon by a proteolytic enzyme system which hydrolizes it so that iodinated amino acids are relcased. The two biologically active ones are thyroxine and triiodothyronine. These compounds are sufficiently small to diffuse back through the cells of the follicles into the surrounding capillaries as the thyroid hormones which then act at the tissue level either as such or in a modified form.

Such a summary as the above might give the impression that the physiology of the thyroid gland is a clearly defined process when in fact the contrary is true. A survey of the more pertinent literature makes one acutely cognizant of this fact.

A. Sources of Iodine

1) Exogenous sources of iodine.

It is common knowledge that a deficiency of iodine results in simple colloid goitre. Historically, ashes of seaweed

and sponges were used in the treatment of goitre. Coindet (41) first used iodine therapeutically, after the element was discovered in 1812, incident to a search for gunpowder for Napoleon's armies; Chatin (40) in 1852, attempted to scientifically correlate iodine-deficient geographic areas with the incidence of goitre, but due to the inadequacy of his analytical procedures discrepancies arose which largely discredited his work. In the United States, however, Marine (142) established the value of iodine in goitre prevention in his classic studies on about four thousand school children in Akron, Ohio. Since that time the addition of iodine to food (usually as 1:10,000 of table salt) has reduced greatly the incidence of goitre in endemic districts. In spite of the overwhelming evidence at the present time to the contrary there are a few, notably Greenwald (87, 88), who contend that endemic goitre and iodine deficiency are unrelated.

This brief historical account establishes the necessity of a dietary source of iodine and points markedly to the necessity of supplementing the diet with iodine in those areas where the soil concentration of the element is deficient. However, endemic goitre still exists in areas where there is adequate iodine present, e.g., Derbyshire, England. In this case it is probably due to dietary factors since the introduction of iodized salt did not reduce the incidence of goitre (159). The goitrogens (sulfonamides, thiourea derivatives, and certain leafy vegetables) may conceivably be the causative factor.

Therefore, iodine deficiency may result from an absolute lack of the mineral in ingested food and water or from a relative insufficiency during periods of increased metabolic demand or from goitrogens occuring in the diet. In addition, it is probable that certain microorganisms in the digestive tract may reduce the iodine absorption from food as found by McCarrison and Madhara (145). Goitrogens, notaby the thiocyanates, inhibit the uptake of iodine in the thyroid gland. Presumably one or more of these substances is the active factor in foods such as raw cabbage, turnip, kohlrabi, and rutabaga which enhance the goitrogenic effect of an iodine-deficient diet. These vegetables are known to be high in cyanogen content which presumably converts to cyanides and cyanates. Lest it be concluded that only the cyanates inhibit iodine collection by the thyroid gland, it should be pointed out that perchlorate, chlorate, periodate, iodate, biiodate, and nitrate have the same effect. In fact perchlorates are ten times as potent as thiocyanates in discharging iodides already accummulated in the thyroid gland (241).

Not only then is the diet an essential source for iodine, but there are also naturally occurring foodstuffs which can inhibit the accumulation of this iodine by the thyroid gland.

b) Endogenous sources of iodine.

Although the principle source of iodide is dietary,

there is a limited amount made available from the de-iodinated hormones during metabolism. Radioactive hormones secreted by the thyroid are partly excreted in the feces and partly broken down in the body to liberate I^{131} int. the blood. The I^{131} derived from degraded hormones is partly excreted in the urine and saliva and partly recaptured by the thyroid. "Daily studies involving (a) measurements of the thyroid: renal sharing of I^{131} and the rate of excretion of I^{131} during the course of a release cure and (b) the distribution of I^{131} following injection of radio-thyroxine have shown that only about ten percent of the radio-activity lost from the thyroid in any one day is recovered as I^{131}" (110). The foregoing results pertain to the normal rabbit. These figures will vary depending upon the organism since the metabolic pathways differ. For example in the rat thyroxine is normally passed from the liver through the bile duct to the intestine from where iodine can be reabsorbed; however, in the case of man more of the thyroxine appears to re-enter the hepatic vein rather than pass via the intestine. In this case the destruction of the thyroxine appears to be largely a function of the hepatic cell (228). Regardless of the metabolic pathway the amount of iodine thus recovered by the body constitutes only a minor source of iodine for the thyroid gland.

B. Site of Iodine Trapping

The bulk of the data available on the site of thyroidal

iodide trapping has been obtained principally by applying autoradiographic techniques to radioactive sections of thyroid tissue (112). There are two methods of arranging the tissue slices: in one the thyroid tissue is fixed and all the iodine containing compounds soluble in water and organic solvents are eliminated. In the other the freezing-drying method is used which results in retention of all the radioactive iodinated products. Leblond and Gross (126) have shown that radioactive iodine was present in the colloid and epithelial cells one hour after injection of I^{131}. After twenty-four hours it was found in the colloid only, mainly in an organic form. Thus, iodide was taken up by the cells and was subsequently transferred to the colloid. Quantitative studies have shown that the small follicles which are generally located in the center of the gland are more active in the uptake of iodine than are the larger peripheral follicles (155). Therefore, for the entire thyroid of the rat iodine incorporation depends upon the total cell volume of its follicle agglomerates. The situation, however, appears to be the reverse in the shark (86). By following the formation of thyroglobulin by means of autoradiography at various time intervals after administration of I^{131}, it can be seen that after the first hour all the iodine was present in the colloid and there was none in the epithelial cells. The implication is that all the iodination reactions occur in the colloid. The results of Pitt-Rivers and Trotter are very

interesting (160). Administering 200 μc doses of I^{131} to rats previously treated with propylthiouracil, they found that the gland concentrates inorganic iodine in the colloid and perhaps in the epithelial cells. In contrast Wollman and Wodinsky (237), using mice in a study of the concentration problem, showed that organic iodine is concentrated in the colloid only, even in the remarkably short time of eleven seconds. This would indicate that iodination of thyroglobulin does take place in the colloid and is in agreement with Doniac, Howard and Pelc (62) who suggest that the iodination process normally occurs in the colloid and not in the cytoplasm of the epithelial cells. In contrast to the occasional rings found in normal rats by Gross and Leblond (94) these authors (63) have always found the iodine incorporated into colloid and never in the cells from five minutes onward. In fact these same authors found protein-bound iodine in the middle of the colloid of active follicles within two minutes of I^{131} administration and none in the cells, suggesting that the iodine simply diffuses through the cells but is bound in the colloid.

C. Mechanism of Iodine Trapping

From the foregoing review it would appear that there is some confusion over terms. Iodide trapping should not be confused with organic binding of iodine. The ability of the thyroid gland to concentrate iodine without forming diiodotyrosine

or thyroxine was recognized by Schachner, Franklin and Chaikoff (192) from in vitro studies in which azide and sulfanilamide were found to inhibit organic binding. Further studies by Franklin, Chaikoff and Lerner (78) showed that thiocyanate inhibited the capacity of the thyroid slices to concentrate iodide. Astwood and Bissell (16) demonstrated that administering thiouracil to normal rats depleted the thyroidal stores of organic iodine, and that the pituitary or thyrotrophin must be present for this to occur. Astwood (14) noted that under these circumstances the thyroid gland was able to concentrate promptly an amount of iodide which varied with the dose given, and Vander Laan and Bissell (223) found that iodine thus accumulated had almost entirely disappeared in twenty-four hours.

Vander Laan and Vander Laan (221) established the existence of a gradient between thyroid cells and serum (T/S ratio), the magnitude of iodide ion concentration being about 25:1. This gradient remained constant over a wide range of iodide levels. When thiouracil is chronically administered resulting in hyperplasia of the thyroid gland, thyroidal iodide accumulation rose to a level of approximately 250:1, while the size of the gland increased three to fourfold. Thiocyanate was shown to prevent not only the iodide ion accumulation, but also of effecting its discharge from the gland with the result that the T/S ratio is disrupted.

In human subjects Stanley and Astwood (205) found that

thyrotrophin administration brought about a marked increase in the capacity of the thyroid to trap iodide. Vander Laan and Greer (225) recognized that the pituitary was necessary for the maintenance of a high T/S ratio since hypophysectomy brought about a rapid fall from the high levels attained with chronic propylthiouracil treatment. It appeared from this study that thyrotrophin was required for the thyroid not only to bind iodine to protein and release the hormone, but also to concentrate iodide. These results appeared to be a contradiction of previous conclusions of Vander Laan and Vander Laan (227) which proposed that iodide concentration seemed to be independent of the hypophysis. The apparent differences were resolved by the important research of Halmi et al (103), showing that the increased capacity of the thyroid gland to accumulate iodide ion from serum in hypophysectomized animals receiving thyrotrophin was greatly increased about thirty-four hours after organic binding had been blocked with propylthiouracil. There was a sharp rise in the gradient after propylthiouracil administration even though the dose of thyrotrophin remained constant. Further, Halmi (99) established that in hypophysectomized rats the iodide ratio between thyroid cells and serum rose when the iodide content of the diet was reduced.

Vander Laan and Caplan (224) found the concentration of iodide to be partially independent of the pituitary which is in agreement with Halmi (99), and they found an inverse relationship

to exist between iodine stores in the thyroid gland and its capacity for concentrating iodide ion. These observations indicated that although thyrotrophin influenced the thyroid gland in its capacity for concentrating iodide ion, other factors within the gland were also important in its function.

Despite the foregoing evidence, the nature of the thyroid iodide concentrating mechanism has not been unequivocally established. Freinkel and Ingbar (79) have suggested that it involves "active transport". If such is the case, the term "thyroidal iodide pump" introduced by Vander Laan and Caplan (224) is justified (100). This would appear to be preferable since the term "iodide trap" ignores the fact that much of the thyroidal concentrated iodide is freely exchangeable.

Whether the transport of iodide into the thyroid is (25, 222) or is not (236) the sole rate-controlling mechanism in the formation of thyroid hormones, factors influencing this function can greatly effect thyroid hormone output and thereby play an important role in homeostasis (100).

The final mode of action of thyrotrophin on thyroidal iodine transport remains to be ascertained. Woolman and Scow (236) in analysing their own observations as well as those of Halmi (98) on the effect of graded doses of carrier iodide on the T/S ratio at various levels of thyrotrophic stimulation concluded that thyrotrophin does not act by increasing the <u>affinity</u> of the thyroidal iodide acceptor for its substrate.

Therefore, Halmi (100) suggested that a plausible explanation is that thyrotrophin augments the number of iodide acceptor sites. This is indicated though not proved by the observation that the thyroid's finite capacity for pumping iodide is expanded by thyrotrophin to the same extent as the T/S ratio for tracer doses of radioiodide is enhanced (97).

The following results obtained in hypophysectomized rats, quoted from Halmi (100), argue for the existence of an intrinsic mechanism which depresses the pump thus opposing the stimulating effect of thyrotropin: "(a) The responsiveness of the thyroidal iodide pump to exogenous TSH is enhanced by concurrent medication with PTU which depletes the thyroidal hormone stores (Halmi et al (103)); Vander Laan and Caplan (224). (b) The response of the pump to a standard dose of TSH is inversely proportioned to dietary iodine intake (Vander Laan and Caplan (224); Halmi (99). Such is not the case if PTU is also given (Halmi and Spirtos (102)). This shows that the effect is not due to the level of circulating iodide but rather to products arising through organification of iodide in the thyroid. In fact, if organic binding is blocked with PTU, the titres of serum iodide must rise exorbitantly (to over 100 μg per cent) before any diminution of the T/S becomes evident, Vander Laan and Vander Laan (227); Halmi (98). Moreover, this decline of the T/S is due to saturation and not to depression of the iodide pump, since the stable iodide concentration within the thyroid actually increases as the serum iodide titre rises, until the

capacity level is attained (Halmi (97)). (c) Both PTU and iodine intake effect the iodide pump in hypophysectomized rats not receiving TSH (Halmi (99), Halmi and Spirtos (101). This suggests that they do not merely modify the activity of TSH. (d) In our experience injected thyroxine did not diminish the iodide pump-stimulating effect of TSH (Halmi et al (103))."

All the above findings fit the hypothesis that the thyroidal iodide pump is directly inhibited by one or more organic iodine-containing compounds within the thyroid. Which of these iodinated compounds perform this task has yet to be determined.

There, are, however, two observations which appear to contradict the above hypothesis of pump regulations: first, in propylthiouracil treated rats given low doses of thyroxine or triodothyronine the T/S ratio is increased instead of decreased as it is with high doses; and secondly, in normal mice propylthiouracil, although producing goitre, lowers rather than elevates the T/S ratio (104, 105). Halmi (100) appears to reconcile these paradoxes by making the following assumptions: "(a) that activation of the iodide pump requires less TSH than maintenance or development of goitre, and (b) that the number of thyroidal iodide acceptor sites per thyroid cannot be enhanced beyond a limit."

Taurog, Tong and Chaikoff (212) studied I^{131} uptake and the chemical distribution of I^{131} in the thyroid gland and in the plasma of hypophysectomized rats. They found that total I^{131} uptake was reduced to only a few percent of control values and that the T/S ratios were greatly reduced. Previous iodine intake, however, had a marked influence on both values. The thyroglobulin that was formed contained only low percentages of I^{131} diiodotyrosine and a higher than normal value of I^{131} monoiodotyrosine. The plasma, likewise, was void of detectable I^{131} thyroxine. This striking reduction in all phases of thyroid iodine metabolism, under these conditions is interpreted as emphasizing the importance of the pituitary rather than autonomous control in the regulation of thyroid iodine metabolism.

In a follow-up to the above work TSH treated rats readily reversed the abnormal distribution of I^{131} which occurred in the thyroids of untreated hypophysectomized rats (211). They also concentrated iodine <u>in vitro</u> to a much greater extent. Evidence indicated that the effects of thyrotrophic hormone on both the thyroid iodide pump and on thyroid cellular growth were independent of any effect on iodine discharge. The authors suggested that the various steps in thyroid iodine metabolism were affected by thyrotrophic hormone independently, and further, that the effects of thyrotrophic hormone on iodine metabolism were probably secondary to its effect on some basic cellular process in the gland.

In conclusion it would appear that the activity of the pump has a dual regulating mechanism: a stimulating effect of thyrotrophin, possibly through augmentation of thyroidal iodide acceptor sites, and a depression by intrathyroidal factors.

D. Organic Binding of Iodine

The question of the elaboration and secretion of the iodinated compounds which eventually yield the thyroid hormones has been approached by three methods: 1) cytological observations 2) investigations with radioiodine and 3) biochemical analysis.

In the cytological approach the function of the follicular cells is based on certain features of the cell structure which may serve as indicators of physiological activity.

The autoradiography techniques are mainly concerned with following the passage of the labeled I^{131} through the cells into the colloid and its rate of clearance from the gland.

The third approach, which has been extremely fruitful, consists mainly of identifying the iodinated constituents of thyroid extracts by paper chromatography techniques after incorporation of radioiodine.

1) Cytological evidence

Each follicular cell is a more or less pyramidal type of epithelical cell whose sides are in intimate contact with

adjacent cells, the apical face abuts the colloid of the lumen and the basal portion rests on a basement membrane which encloses the entire follicle. The cytoplasm which was investigated with the electron microscope (31, 51) contains mitochondria, Golgi apparatus, ergastoplasm, and colloid droplets. The apical border displays tiny cilia and terminal bars.

In the past the activity of the thyroid cells was based on the so-called "reversal theory" or some modification of it - that is, the cells alternately secrete towards the colloid and towards the outside of the follicle (54, 68, 72, 82, 196, 215, 233, 234). There were several indications of secretion in either direction: (a) the presence of droplets not only at the apex but also at the base of the follicular cells (24, 31). The great confusion over the nature and meaning of these droplets is apparent on reading earlier reviews on the histophysiology of the thyroid gland (19, 43, 145, 164) (b) the apparent change in the position of the Golgi apparatus between the apex and base of the cell (45): (c) Thomas (215) strongly contended that in any given thyroid there were several types of cells: flat, cuboidal, and tall, and that these were cytological evidence of a resting state, an active secretion toward the colloid and an active secretion towards the outside of the follicle. These changes he associated with the theory of reversal of secretory polarity. This work gained further

support when Ponse (164) reported cytological changes in the follicle under thyrotrophic hormone stimulation which had a sequence of colloid dissolution, excretion to the outside of the follicle and secretion of colloid into the follicle lumen. Further, each of these steps was associated with a definite cell size and shape corresponding to those described by Thomas. Finally, De Robertis (52, 54, 55) presented rather convincing evidence based on observations of thyroid tissue prepared by the freezing-drying method of Altmann-Gersh. Following injection of thyrotrophin there is first secretion towards the follicle, producing a kind of apocrine secretion. Afterwards apocrine secretion stops and release of the colloid through the cells is observed. "In some cases there is also an inversion of polarity" (52).

More recent work tends to cast doubt on the validity of the reversal theory. Nadler and Leblond (154) contend that thyroid hormone production is a continuous process going on at all times throughout the entire gland - the so-called "continuous activity" hypothesis.

It has also been contended by earlier works that changes in position of the Golgi apparatus and secretory droplets has no functional significance and may be simply due to mechanical factors (83, 157, 218). Nadler and Leblond (155) failed to find any significant change in the height of the follicular cells in 125g. rats. On the contrary, cells of every

follicle were found to be statistically identical. These authors go on to state that "the contention that a cycle similar to that observed after thyrotrophic stimulation occurs normally within any follicle presumes either that the circulating level of thyrotrophic hormone is fluctuating or that some follicles can be under stimulation while others are not. Evidence for either presumption is lacking".

2) Evidence from radioiodine studies

Much of the evidence for organic binding obtained from radioiodine studies has been reviewed in the section Mechanism of Iodine Trapping (page 7). In the process of organic binding the iodide first passes from the extracellular spaces through the thyroid cell. The uptake of the iodide ion appears to be directly related to the size of the thyroid cells (129). It seems that the epithelial cell may have an active role in concentrating iodide in the follicular colloid. This is substantiated by the work of Wollman and Zwilling (238) who found that in the chick embryo the development of the iodide concentrating power of the gland occurred when the epithelial tissue was fully formed but before any colloid was yet visible; moreover, the stage at which organic binding of radioiodine began coincided with the appearance of colloid. In contrast is the report of Trunnell and Wade (217) in their study of the chronology of the synthesis of iodinated compounds in the chick embryo. They found that monoiodotyrosine,

diiodotyrosine, or both can be formed prior to the advent of colloid production, but thyroxine does not appear until the first colloid droplets appear, indicating that organic binding can occur in the cell. This was also in apparent conflict with the work of Leblond and Gross (126) who at first thought that the reaction was localized to the apical region of the epithelium but subsequently revised their view after a study of profile radioautographs and stated that the "ring reaction" was due to a narrow ring of protein-bound radioiodine located within the boundary of the colloid and, in some cases, just at the colloid-cell interface (154). They interpreted their findings to mean that the epithelium secretes an oxidizing enzyme directly into the colloid where it comes in contact with the iodide present and causes its oxidation (50, 57, 84, 158). This implies that the cell secretes an uniodinated protein into the colloid which combines with iodine to yield ultimately thyroid hormones.

3) Evidence from biochemical analysis

Evidence for organic binding has also been obtained from the incorporation of iodine <u>in vitro</u>. Over ten years ago Taurog et al (210) showed that surviving thyroid slices were able to attract radioactive iodide in inorganic form. Roche and co-workers (179) observed the same phenomenon in normal human thyroid slices from a patient who died following an accident. More recently protein iodine has been obtained with

homogenates and mitochrondiia after incubation with I^{131} but copper and tyrosine had to be added (230). Fawcett and Kirkwood (70, 71) consider copper to be only a mechanical oxidizing agent to transform iodide to iodine. They believe that fixation on proteins is catalyzed by an enzyme "tyrosine iodinase" which transfers the iodine to the tyrosine. Wyngarten and Stanbury (240) suggest that the oxidation of iodide is chiefly enzymatic, enhanced by copper and dependent upon peroxide as the active oxidant. Recently, Taurog, Potter and Chaikoff (211) have obtained quite different results: copper and tyrosine were not necessary for organic binding in the presence of thyroid homogenates or of various separate cell fractions. Cell free preparations were able to change significant quantities of radio-active iodide to an organic form. Monoiodotyrosine was the chief constituent, but there were also traces of diiodotyrosine though no thyroxine. Harington and Pitt-Rivers (109) and Harington (108) have postulated that <u>in vitro</u> the oxidative coupling of diiodotyrosine to form thyroxine requires iodine as an oxidizing agent.

The finding of traces of diiodotyrosine by Taurog et al but no thyroxine is in accordance with the experiments of Fawcett and Kirkwood, but contrary to their claim, the monoiodotyrosine is a part of the protein molecule and has to be liberated by hydrolysis in order to be identified by chromatography (149). The latter work questions the validity

of the existence of "tyrosine iodinase" as well as the fact that studies on oxidative enzymes lead to the same results. Milk xanthine oxidase in the presence of iodide, casein, and xanthine under aerobic conditions resulted in iodination of the protein, principly as monoiodotyrosine and a limited production of diiodotyrosine (146, 184).

From the foregoing data the picture emerges that iodination of proteins probably occurs as two separate mechanisms: the first leading to the rapid synthesis of iodotyrosines, and the second, considerably slower, to the formation of the thyroid hormones.

E. Pituitary-Thyroid Relationships

Although it is common knowledge that a relationship exists between the adenohypophysis and the thyroid, and reference has been made to several aspects of this relationship in the preceding sections on thyroid physiology, it is a very complex problem and a review of the literature is prerequisite for further discussion.

The history of this relationship goes back at least as far as 1851. In that year Niêpce (156) described pituitary enlargement in goitrous cretins. Later in the nineteenth century Rogowitsh (188) and Stieda (207) among others, observed this syndrome following experimental thyroidectomy in animals. The impetus to research in this field, however, came from studies on amphibian metamorphosis. Adler (3) destroyed the

hypophysis of tadpoles by cautery and correlated their failure to metamorphose with their atrophic thyroids. Previously, Gudernatsch (97) had shown that anuran larvae metamorphosed precociously if fed mammalian thyroid substance.

It was not long before the implications of these seemingly unrelated phenomena were realized by investigators and intense research began. By the early twenties extirpation and transplantation studies made it clearly evident that growth and secretion of the amphibian thyroid were influenced by some hormonal factor (s) in the adenohypophysis (6, 7, 209). It was soon noted that the amphibian thyroid could be stimulated by mammalian hypophyseal substance (200, 203, 219), thus establishing what was to become the universality of the thyroid-stimulating principle, present with rare exception in the pituitary of all vertebrate animals (1, 4, 85).

Further impetus came from mammalian studies: Smith (198) described for the first time in mammals (rat) the involutionary and reparative changes occurring in the thyroid after hypophysectomy and replacement therapy; Loeb and Basset (134, 135) and Aron (10, 12) demonstrated the dramatic activating properties of extracts of beef pituitary on the guinea pig thyroid.

In the 1930's many unsuccessful attempts were made to isolate the thyroid-stimulating factor from the anterior pituitary, and it was referred to by various names until finally established as thyrotropin, thyrotrophin or thyroid-stimulating hormone (TSH).

The response of the thyroid to pituitary hormone and conversely the response of the pituitary to thyroid hormone were repeatedly studied. It soon became clear that the role of the thyroid was not a passive one. Excessive thyroid hormone resulted in histological manifestations of decreased activity (36, 205) which could be associated with deminished thyrotropin content of the pituitary (115, 124, 170). Further, histological activation of the thyroid with exogenous thyrotropin could be partially inhibited by concomitant administration of thyroid hormone (11, 133, 136, 137). "These findings and others of a more clinical nature led to the concept first promulgated by Aron et al (13), subscribed to by Salter (190), and more recently reiterated by Hoskins (117) that the thyroid and anterior pituitary exist in a state of reciprocal stimulation and inhibition thereby constituting one of the homeostatic mechanisms of the organism". (46)

The advent of radioiodine and chemical antithyroid agents as tools brought refinement to the investigation of pituitary-thyroid interplay. The excellent studies of Leblond et al (129) on the fixation of iodine, its reduction by hypophysectomy and augmentation by exogenous thyrotropin; confirmation and extention of these results by Morton et al (152, 153) made it possible to define rates of formation and release of thyroid hormone as a reflection of thyrotrophic hormone regulation. Working independently, the MacKenzies (139) and Astwood et al

(17) established that certain chemical agents induced functional hypothyroidism with thyroid hyperplasia. Thus it became possible to make a more critical study of the endogenous thyrotrophic mechanisms of the adenohypophysis under conditions of inhibited thyroid hormone formation with the target organ present. During this period a search for methods for the detection of thyrotropin were continuing, and several were reported which possessed the necessary sensitivity and precision to give quantitative estimation of the hormone in the circulation (47, 48, 56).

While the overall picture has been established, certain aspects of this relationship are quite obscure and even controversial (see iodine trapping, organic binding). At this point the review is restricted to the overall regulation of the thyroid by thyrotrophic hormone and the reciprocal action. Generally, the thyrotrophic hormone is considered as a single entity although periodically reports appear in the literature describing effects on the thyroid which can be interpreted as signifying more than one (26, 91, 92, 113). Also reported are the presence of thyroid-inhibiting factors (163, 199). Such results have usually gone unconfirmed (148, 165) or lend themselves equally well to more conservative interpretation. "One cannot be certain from the evidence at hand that thyrotrophic hormone acts to trigger a mechanism from which the thyroidal

processes unfold in a prescribed sequence with change in one contingent dependent upon previous change in another" (46).

Thyrotropin is considered as stimulating the thyroid processes directly and individually. Excessive circulating thyroid hormone is conceived as acting directly on the anterior pituitary to inhibit thyrotrophin production. Thyrotrophin content of the adenohypophysis decreases sharply after thyroid hormone administration (2, 114, 123, 148, 170), but the mechanism of action is completely unknown and this aspect of the interplay is in the highly conjectural phase. Radioactive thyroxine can significantly concentrated by the hypophysis of the rabbit, but in its posterior lobe (42, 43, 117)! In some species, selective concentration of labeled thyroxine in the pituitary does not occur at all (42, 93).

The role of the hypothalamus in the regulation of thyrotrophin activity is also a confusing issue. Complete stalk section leads to reduced thyroid function from which it has been inferred that thyrotrophic hormone production is under some measure of neural control (21, 32, 33, 110). It is argued whether these effects are due to neural control (32, 33) or to vascular insult (21, 89). Hypothalamic lesions in the rat have been found to abolish the thyroid hypertrophy which is a normal consequence of chronic thiouracil feeding (28, 46, 91), however, the T/S ratio undergoes the usual ten fold increase. The essential hypothalamic area involved in the above

responses seems to lie in the midline between the paraventricular nucleus and the median eminence (80). The same area appears to be involved in the dog (5). This casts serious doubt upon the concept of vascular damage since there is minimal damage to the portal system.

It must be emphasized that neither of the above procedures reduces thyroid function to hypophysectomy levels. For this reason D'Angelo (46) suggests that regulation of thyrotrophic tonus in the pituitary is accomplished by two distinct phases: a rapid one mediated through diencephalic centers highly sensitive to small fluctuations in thyroid hormone levels, and a more slowly aroused but sustained phase in which relatively wide excursions in levels of circulating hormone act on the adenohypophysis directly. In support of this speculation he points to the fact that changes in thyroid hormone levels (plasma protein-bound iodine) are too slow to account for the rapidity with which activation changes may be induced in the thyroid under certain conditions (5).

In spite of the confusion surrounding the physiological relationship between the pituitary and the thyroid gland very definite and convincing evidence has been obtained histochemically which has established the cells of the adenohypophysis responsible for thyrotrophin secretion in the rat, dog, man, and others. An excellent review of this problem has recently appeared in the literature (168). In the rat Purves and Griesbach (166, 167) have designated three types of cells

each containing a specific and chemically distinct glycoprotein which together constitute the group of cells known as basophils. These three types are classified according to function as the thyrotrophs, FSH gonadotrophs, and LH gonadotrophs. The thyrotrophs appear as glycoprotein granule-containing cells which are angular in outline; the gonadotrophs are round or oval.

Thus while we know, at least in some species, the source of thyrotrophin and also its primary effects on the thyroid, much remains to be uncovered regarding its locus of breakdown and its final metabolic fate. The effect at the cellular level of the thyroid is still unknown. It has yet to be established whether thyrotrophin stimulates the thyroid by stimulating its component processes at the same instant or by activating a single enzymatic mechanism which then sets off a sequential chain reaction.

F. Antithyroid Compounds and their Mechanism of Action.

As has been previously indicated the synthesis of thyroxine can be blocked or disturbed in more than one way depending upon which step in its synthesis one wishes to investigate. Thus the iodine concentrating mechanism can be disrupted by thiocyanate, perchlorate, and related compounds or by iodine itself. However, according to common usage of the term, as retained here, an antithyroid compound is one which

interferes with thyroxine and triiodothyronine synthesis without affecting directly other processes within the thyroid gland.

What happens when an antithyroid compound is given continuously to a normal animal in large doses can be seen by observing a young rat on thiouracil treatment. There is an almost immediate loss of iodine from the thyroid (16). From the onset of treatment no new hormones are formed and approximately half of the reserve store is used in one day and all is exhausted in five days (14). Subsequently, the amount of circulatory hormone decreases with a resultant compensatory enlargement of the thyroid gland which begins even before the gland is completely exhausted and continues at a steadily decreasing rate for several weeks. After about a month the thyroid enlargement just about keeps pace with body growth (14).

The iodine-depleted glands of animals on an iodine-deficient diet rapidly pick up iodine when potassium iodide is given. Thiouracil is unable to prevent the immediate uptake of iodide under these conditions (14). This iodide enters little if at all into the synthesis of thyroid hormone or, as is more commonly stated, is not protein bound (14). This is also the case <u>in vitro</u> (78).

While these studies pretty well established the site of action, they gave no insight into the mode of action of such compounds in inhibiting hormone synthesis. Since it was well established that iodine was the form in which iodine entered the

gland, the site of action of the antithyroid compounds lay somewhere along the chemical route between iodide ion and iodinated tyrosil radicals. An early observation that the goitrogenic effect of thiouracil was not prevented by the administration of diiodotyrosine suggested that the block was at a stage beyond iodinated tyrosine, and that the oxidative coupling of two diiodotyrosines to form thyroxine was inhibited (50). Iodination at this point alone should have permitted the accumulation of iodine bound to tyrosine, but such was not the case (17, 76, 78, 119). Furthermore, it was known that diiodotyrosine is rapidly degraded in the body and probably would not enter the thyroid to participate in hormone synthesis. Thus, it is unlikely that coupling of diiodotyrosine could be the only or even the chief locus of action (15). Currently, the weight of evidence favors the view that antithyroid compounds prevent the oxidation of iodide so that iodination of tyrosine cannot occur, and that this oxidation is enzymatic (15).

Inhibition of the cytochrome-cytochrome oxidase system of thyroid slices by the addition of azide, sulfide, cyanide, or carbon monoxide inhibited incorporation of radioiodine into iodotyrosine and thyroxine (193). However, sulfonamides and other antithyroid compounds inhibited conversion of iodide to organic iodine without affecting respiration which indicates that although an actively respiring cell is required for iodide

oxidation, the cytochrome system is not responsible (130). Tyrosinase, although inhibited by antithyroid compounds, is not known to effect the conversion of iodide to iodine, nor has it ever been found in the thyroid gland, and there is a poor correlation between antithyroid and antityrosinase activity of the compounds investigated (65). Similar considerations exclude catalysis by inorganic copper or iron as the normal mechanism (15). Most experimental data are in keeping with the theory that iodide oxidation by the thyroid is carried out by an enzyme with properties of a peroxidase and that antithyroid compounds act by interfering in some way with this process (15).

The evidence favoring peroxidase as the enzyme responsible for the oxidation of iodide is based upon the following findings quoted from Astwood (15):1)"Peroxidase-positive granules can be stained in thyroid tissue sections; 2) this reaction is inhibited by thiouracil and thiourea; 3) peroxidases catalyze the oxidation of iodide by hydrogen peroxide; 4) all effective antithyroid compounds thus far investigated interfere with peroxidase reactions; and 5) thiourea can be oxidized by peroxidases as well as by living thyroid tissue."

Dempsey (49) was the first to describe fine blue granules near the nucleus of thyroid cells stained in frozen section with benzidine and hydrogen peroxide, and he found that a 1/10,000 solution of thiouracil inhibited the staining reaction. Numerous authors have confirmed these conclusions (57, 121, 229).

De Robertis and Grasso (57) described peroxidase activity in both the cells and the colloid in rat thyroids. Hydrogen peroxide, O- and p-phenylene-diamine, benzidine, guiacol, pyrogallol, and iodide were all oxidized. The reactions were inhibited, however, by 0.002 M thiourea and 0.01 M potassium cyanide but not by sulfanilamide at 0.01 M. When iodide was the substrate and a glycerine extract of thyroid was the enzyme, thicurea again inhibited whereas sulfathiozole prevented the appearance of iodine and a color change. The authors suggested that thiouracil inhibited peroxidase and the sulfanilamides prevented thyroid hormone synthesis by combining with the iodine formed.

The demonstration of peroxidase in thyroid tissue is doubly difficult. In the first place there are no specific tests for peroxidase, and secondly, the amount presumed to be necessary for the oxidation of iodide is very small. Manometric methods are unsuitable because the rate of iodide oxidation is too slow.

The rapid reduction of iodine by thiourea, thiouracil and related compounds suggested that a simple reduction might account for their antithyroid activity (36, 161). The failure of other reducing agents to be effective, including such thiols as cysteine, has been attributed to their ease of oxidation or to a failure to penetrate to the site of the enzyme within the thyroid cell (15). MacKenzie (138) suggested that antithyroid

compounds such as the sulfanilamides which reduce iodine at a very slow rate may inhibit the enzyme directly. Many of the polyphenols investigated by Arnott and Doniach (9) and Rosenberg (189) are strong reducing agents, but no correlation has been found between reducing power and antithyroid action; therefore, peroxidase inhibition was suggested (9).

With regard to the activity of the aromatic antithyroid compounds several researchers have suggested that the iodine formed by the action of a peroxidase is removed by forming a complex or compound with the antithyroid agent (57, 69, 160).

What is often called "direct" inhibition of peroxidase apparently may be either of a competitive or noncompetitive type. Compounds such as thiourea and thiouracil (169) or phenols, cresols, catecholquinol, gallic acid, β-naphthol, and pyrogallol (18, 67) can be readily oxidized by peroxidases and thus serve to divert the enzyme from another substrate; others such as resorcinol (67), sulfanilamide (132), and aniline (189), which are either not oxidizable or are so at extremely slow rates by peroxidases, can nevertheless inhibit thyroxine synthesis. These reults supported the contention that most classes of antithyroid compounds are either competitive substrates or inhibitors of peroxidase (189).

The iodide-oxidizing enzyme of the thyroid, like other oxidases, is apparently unable to act on other halogens

(197). At^{211} (astatine) accumulates to a limited extent (107) and resists washing out, but it leaves the gland more rapidly than radioiodine (106). According to Durbin and Hamilton (66), At^{-1} is very readily oxidized to At^{0} and then the iodide-oxidizing system oxidizes it to a higher valence state which makes organic binding impossible, accounting for its rapid loss from the gland. Thiouracil, by inhibiting the oxidation beyond At^{0}, permits At^{211} to accumulate within the gland.

The bulk of the available evidence thus points to the fact that the antithyroid compounds interfere with the conversion of iodide to iodine probably through destruction or inhibition of the enzyme peroxidase.

G. Specific Characteristics of Thyroglobulin

Salter et al (191) hypothesized that the protein of the colloid was a substrate able to bind free iodine under the influence of an oxidation enzyme. Derrien and co-worker (58) prepared pure thyroglobulins, giving on analysis different values for iodine, which led them to back the proposal of Salter. We have previously referred to the work of Nadler and Leblond (154) who came to the same conclusion following histological studies.

Research on the structure of thyroglobulin has often aimed at connecting the site of formation of the hormones or their secretion to specific characteristics of the protein.

Arginine has been known to be extremely stable in thyroglobulin (149); tyrosine and iodotyrosine are quite easily freed by pepsin digestion, which is not the case for most other proteins (56), and is a possible explanation for the liberation of iodotyrosines during thyroid proteolysis. The nature of the N terminal amino acids has been established: leucine, valine, glycine, serine, threonine, glutamic acid, alanine, tyrosine and also diiodotyrosine and thyroxine (182, 183); Michel (149) suggests that the presence of numerous N terminal amino acids might lead one to suppose the existence of several polypeptide chains and then the existence of a branched structure. This would fit the high molecular weight of thyroglobulin. At least two thyroidal proteins appear on electrophoretic patterns of rat thyroid extracts. One corresponds to thyroglobulin and the other is an uniodinated protein (232).

Another characteristic of thyroglobulin is its glycoprotein nature. The existence of a glycoprotein is based on histochemical studies (74). Galactose and mannose are known to be present in crude thyroid extracts (231); glucosamine is found in reasonably high concentration in purified thyroglobulin (116). Hooghwinkel et al (116) and Boas and Foley (27) have shown by electrophoresis that thyroglobulin is a glycoprotein, and Lacombe and Michel (125) have found galactose and mannose in addition to glucosamine; recently fucose has been detected (220). The glycoprotein nature of thyroglobulin could explain

the high viscosity and slow flow rate.

Of course, the biologically interesting aspect of thyroglobulin lies in the fact that it is an iodoprotein containing several iodinated amino acids, some being the thyroid hormones. The liberation of these iodinated amino acids follows enzymatic proteolysis of thyroglobulin. Two of these products, thyroxine and diiodotyrosine, have been known for a long time. In the last decade other iodinated amino acids have been identified largely by employment of I^{131} and chromatography techniques. The iodide trapped by the thyroid is oxidized and rapidly reacts with tyrosine and histidine to form monoiodotyrosine (73), diiodotyrosine and monoiodohistidine (175). These compounds are of interest only as precursors since they have no biological activity.

In addition to thyroxine, 3, 5, 3' triiodothyronine was shown to be present in thyroglobulin hydrolysates (95, 96, 176, 177). By perfecting their chromatography techniques Roche, Michel and Wolf established the presence of 3, 3', 5' triiodothyronine and 3, 3' diiodothyronine in the rat thyroid. These substances were shown to be present in rat thyroid from six to twenty-four hours after injection of I^{131} (186, 187). An excellent review of the various hypotheses concerning the mechanisms of thyroid hormonogenesis can be found in a paper by Michel (149).

H. Hormone Secretion

Thyroglobulin itself has no hormone properties. These appear only after the liberation of the iodothyronines. Thyroglobulin should be conceived as a biological reserve of the hormone (123).

Proteolysis takes place within the colloid resulting in the release of diffusible products of low molecular weights. The proteolytic system is of a catheptase nature and was first characterized by De Robertis in 1941 (53). The activity of these enzymes has since been tested in different substrates, and it was found that thyrotrophin activates catheptases *in vivo* (163) as well as *in vitro* (30). Further research indicated that the enzyme system behaves similarly to pepsin since it hydrolyzes the peptide linkage between two aromatic acids and its pH optimum is close to 3 (147). From thyroglobulin it liberates iodotyrosines, traces of monoiodohistidine, thyroxine, 3, 5, 3' triiodothyronine, and peptides (149). Thyroxine has also been identified by proteolysis of rat thyroglobulin (8). Although it has not been identified, it may be supposed that it is also liberated by the proteases since it has been distinguished among the free amino acids of rat thyroid originating from thyroid proteolysis (149).

Strong evidence exists also for a kind of physiological selection operating because of a deiodinating system (111, 181, 214, 216). In effect, diiodotyrosine is dehalogenated to

monoiodotyrosine which in turn releases its iodide to form tyrosine. In this way the thyroid metabolizes iodine with maximum efficiency.

Since the peripheral action of the thyroid hormones is not involved in the research undertaken no review of this aspect of thyroid physiology is given here.

EXPERIMENT I

INTRODUCTION:

Experiment I is actually a compendium of a great number of experiments which were performed in an attempt to find and refine a staining procedure which would permit a clear definition of the colloid material of the thyroid gland of the rat and at the same time give sharp histological and cytological definition. Different fixatives were used and the time of fixation varied. Many modifications of the staining solution were also tried. The pH values were checked in each staining combination to see if the pH of the solution would affect the histological picture. A protocol was kept of the time the tissues were left in each solution until the desired clarity was obtained. To recount the numerous trial runs on literally hundreds of slides would be repetitious and serve no useful purpose as far as the final results are concerned. Therefore, only the recipe of the stain and the procedure finally arrived at is recorded here.

PURPOSE:

The definition of a staining procedure which allows for the differentiation of the so-called "active" colloid from the so-called "inactive" colloid of the thyroid gland of the rat. By "active" colloid is meant that colloid which is believed to contain iodinated thyroglobulin; by "inactive" colloid

is meant the protein material residue of the thyroid follicle which it is felt does not contain the mother substance of the thyroid hormones.

MATERIALS AND METHODS:

Male albino rats of the Wistar strain weighing approximately two hundred grams were sacrificed in ether. The thyroid glands were removed in toto and immersed in Bouin's fixative for twenty-fours hours (not critical). Following fixation the tissue was dehydrated in ethanol, embedded in tissue mat and cut at 5 micra. Sections from the middle of the gland were mounted two each to albuminized slides and allowed to dry for twenty-four hours. The sections were then hydrated through ethanol to water.

After hydration the slides were placed into a staining solution of the following composition:

Phosphotunstic acid - 1 gram

Aniline blue-certified- C.I. 707 - 1 gram

Orange G.-certified- C.I. 27 - 0.5 gram

Distilled water - 100 ml.

The pH of this solution is 2.65

Staining time 8 minutes (optimal)

Wash in flowing tap water 5 seconds (critical)

95% ethanol I 30 seconds (critical)

95% ethanol II 30 seconds (critical)

100% ethanol 3-5 minutes

Xylene 5 minutes

RESULTS:

The nuclei of the follicular cells and connective tissue are yellow, the nucleolus a brilliant yellow; the cytoplasm of the follicular cells is greyish blue, but the colloid droplets within the cytoplasm give an intense blue reaction. The follicular colloid stains blue, yellow, or a mixture of the two colors. The reticular and collagenic fibers of the connective tissue stroma are dark blue; the vascular beds are a brilliant yellow. (Fig. 1, 2)

CONCLUSIONS:

From a purely histological viewpoint this is a good stain for the thyroid gland because it gives excellent histological and cytological definition without overstaining. In addition, as will be shown later, it affords a means of distinguishing between active colloidal substance (blue) and inactive protein residue (yellow).

DISCUSSION:

The histologist will immediately recognize that the stain used is a modification of the Mallory Connective Tissue Stain. Acid fuchsin has been eliminated for two reasons: 1) it gives a very intensive reaction which is difficult to control and therefore tends to obscure the cytological picture. The same observation was made for Azocarmine by De Robertis (52) in his modification of the Mallory Azan technique; 2) those structures having an affinity for acid fuchsin are stained by

Fig. 1
Normal gland (400x)

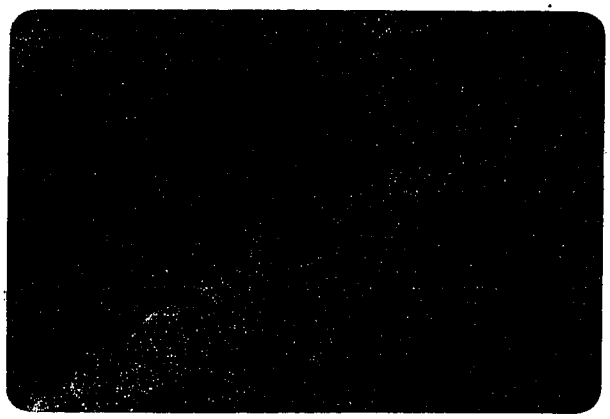

Fig. 2
Normal gland (1000x)
Note colloid droplets in cells

Orange G.

The fact that the colloid of some follicles stains blue while that of others stains yellow or a mixture of the two colors, would constitute an artefact according to De Robertis (55) since following freeze-drying he obtained homogenous blue staining colloid. However, as Leblond pointed out in the discussion following the paper by De Robertis such a contention should be modified. He feels if they are artefacts, they have a definite meaning since the color distribution varies considerably depending upon the physiological condition of the gland.

In the beginning we were inclined to agree with this latter view. However, further experimentation has brought out two points which are difficult to reconcile with either of the above statements; 1) the follicular colloid was almost one hundred percent blue after ten microcuries of I^{131} were given to rats that were on an iodine-deficient diet for from three to six weeks (see following experiments). Autoradiographs of parallel sections, also stained, showed all these blue follicles to be radioactive whereas the few yellow follicles were inactive; 2) animals administered thiouracil and thyroid powder for from two to four days prior to administration of radioiodine have yellow follicles to the extent of ninety percent and none of these are radioactive. Further, it has been observed that as the I^{131} uptake drops there is a corresponding decrease in the

number of blue follicles and a concomitant increase in the number of yellow follicles. Consequently, it is difficult to believe that the observed color reactions are artefacts; in fact it is more likely that there is a definite difference in the chemical composition of the two colloids, and that this difference, while not demonstrable in some techniques, is definitely brought out in this one.

The presence of more than one type of protein in the colloid is not a new concept in thyroid chemistry. Gersh (81) obtained evidence that the colloid contains at least two glycoproteins, one being responsible for most of the reacting groups and resembling purified thyroglobulin; the second may possibly act as a substrate for mucolytic activity. Likewise Wieme and Bekaert (232) have observed two proteins, one being thyroglobulin, and the other unidentified. It is likely, therefore, that the stain herein recorded is a histochemical method which distinguishes between iodothyroglobulin and at least one other protein in the colloid.

EXPERIMENT II

INTRODUCTION:

Since a staining procedure had been developed and an hypothesis formed that two different colloids could be detected in the thyroid gland of the rat, it became essential to design a series of experiments to check the validity of the hypothesis.

PURPOSE:

To establish whether a correlation exists between the staining characteristics and the physiological activity of the thyroid gland.

MATERIALS AND METHODS:

Sixty-one male albino rats of the Wistar strain weighing between eighty and one hundred grams were first divided into two groups: a normal control group (N) and an iodine-deficient group (D). The control group (12 animals) was reared on Masters Fox Breeder Cubes (Master Foods, Toronto Elevators, Ltd., containing 2.6 mg iodine per 100 grams) and water ad libitum under routine laboratory conditions. The remaining rats were fed with Remington's (172) iodide deficient diet (General Biochemicals, Inc., containing 15 μg.I/kg.) and distilled water ad libitum for three weeks at room temperature to bring them to a uniform low thyroglobulin level. This experimental group was then randomly divided into three sub-groups:

Group D: Remained at room temperature on the

Remington diet and were injected with 10 μc of carrier-free radioiodine twenty-four hours prior to sacrifice.

Group DC: Received the same treatment as group D but immediately after injection were placed in a cold room at 5°C, for twenty-four hours and then sacrificed.

Group DT: Remained on the Remington diet at room temperature, but three days before receiving radioiodine were given thyroid powder (USP) at a level of 0.8 gram per kilogram of diet and thiouracil as a 0.02% solution in the drinking water. These animals were sacrificed as the others, twenty-four hours after receiving 10 μc each of radioiodine.

A record of the animals weights was kept throughout the experiment.

All animals were sacrificed in ether, the thyroids removed in toto and fixed in Bouins. Alternately right and left lobes were prepared for microscopic examination as described in the previous experiment with the following modifications: Ten slides containing two sections each from the middle section of the gland of each animal were mounted from the same ribbon in serial fashion. Slides 1, 3, 5, 7, and 9 were used for

cytological examination, including follicle counts, measurement of the epithelial height by the standard optic micrometer technique, extent and distribution of cytoplasmic colloid droplets and histological notations of connective tissue infiltration and vascular changes. Slides 2, 4, 6, 8, and 10 were processed for autoradiography according to the technique of Belanger and Leblond (23). Immediately prior to staining the slides were individually counted for radioactivity on a "64 Scaler" (Tracer Lab) Geiger counter and corrected for background and percent decay of initial dose. The mean number of blue follicles present in each group was then plotted against the number of counts per minute: Statistical analysis was made employing the analysis of variance (202) and the differences between means assesed at the 1% or the 5% level of significance using the multiple range test of Duncan (65). Where unequal numbers of replications were encountered the extension of the multiple range tests by Kramer (122) was used to group the means.

RESULTS:

A. Histological observations

1. Normal Gland: (Fig. 3)

The most striking observation in the normal gland is that at any given time slightly less than fifty percent of the follicles are blue; the remainder are yellow or contain only a thin marginal ring of blue staining colloid.

The greater number of colloid droplets appear to be associated with the mixed follicles, i.e., those with the marginal ring of blue colloid. While in general the larger peripheral follicles tend to be yellow this is not universally true. In some cases these contain blue colloid whereas the small central follicles are yellow. The appearance of vacuoles in the colloid was not as common as one might expect after reading earlier reviews (19, 44, 143, 164), but rather appeared in only a few preparations. They were never associated with the yellow colloid and were restricted to the larger peripheral blue follicles. The yellow colloid appears to be more brittle than the blue colloid since ridges and cracks are common. Both the blue and the yellow follicles give a strong positive Periodic Acid-Schiff reaction. (Fig. 4, 5)

In general after twenty-four hours most of the radioactivity was concentrated in the apical portion of the cell or at the periphery of the colloid. None the less its almost complete absence in the yellow colloid was evident. (Fig. 6)

In one slide we observed what would appear to be a reversal of polarity. While all adjacent follicles contained colloid droplets this one had none; the nuclei were in the apical portion of the cell, and autoradiography demonstrated an unusually high concentration of radioactivity in the basal portion of the cells rather than in the apical portion as true in most cases after twenty-four hours. (Fig. 6)

but ran as high as 160. The greater number of colloid droplets appears to be associated with the mixed follicles, i.e., those with the marginal ring of blue colloid. While in general the larger peripheral follicles tend to be yellow this is not universally true. In some cases these contain blue colloid whereas the small central follicles are yellow. The appearance of vacuoles in the colloid was not as common as one might expect after reading earlier reviews (19, 44, 143, 164), but rather appeared in only a few preparations. They were never associated with the yellow colloid and were restricted to the larger peripheral blue follicles. The yellow colloid appears to be more brittle than the blue colloid since ridges and cracks are common. Both the blue and the yellow follicles give a strong positive Periodic Acid-Schiff reaction. (Fig. 4, 5)

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Fig. 3 (100x) Normal gland.

Fig. 4 (100x) P.A.S. of normal gland. Positive regardless of color of colloid.

Fig. 5 (1000x) P.A.S. Note the fine granules in the apical portion of the cell as opposed to the larger colloid droplets.

Fig. 6 (400x) Camera lucida). After twenty-four hours most of the radioactivity is concentrated intracellularly, some in the blue colloid (light area); very little in the yellow colloid (dark area). Follicle on lower left side seems to exhibit a change in the polarity of the cells.

Fig. 3

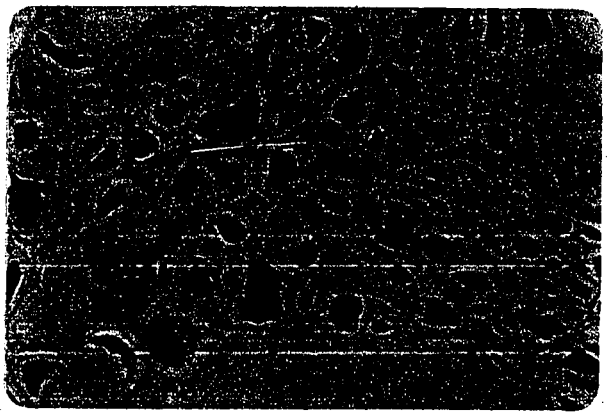


Fig. 4

Fig. 5

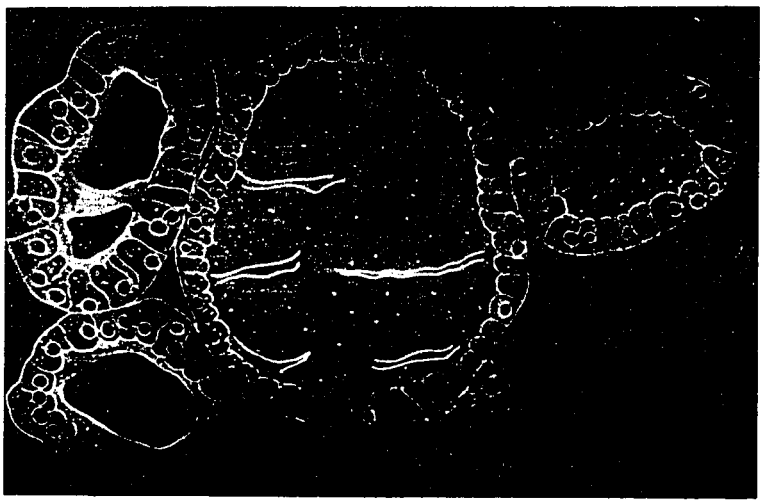


Fig. 6

2. Group D. (Fig. 7)

As was expected the glands of this group were very goitrogenous. There were numerous minute follicles and the amount of colloid, at one hundred magnification, appeared to the eye to be less than the diameter of a pin head. In three of the animals many of these small follicles were void of any discernable colloid. In general, however, the follicles did contain colloid and in the blue form. In some glands the blue follicles constituted ninety percent of the total. Examination of the autoradiogrphs of this group showed that almost all of the radioactivity was concentrated in the colloid, and that it was restricted to the blue or mixed follicles. (Fig. 8,9,10)

3. Group DC (Fig. 11)

There is considerable similarity in the histology of this group and Group D. The glands are goitrogenous but differ in that a greater number of follicles contain mixed colloid. The pattern of the colloid mixture tends to be more mosaic rather than the marginal blue ring configuration mentioned above. The appearance of the yellow colloid begins in the center of the follicle as a very dark core. There also appears to be an increased vascular supply to these cold stressed glands as the capillary beds are more easily discernable than they were in Groups N and D.

Fig. 7 (60x) Goitrogenous gland from an iodine-deficient rat 24 hours after I^{131} administration.

Fig. 8 (100x) Autoradiograph from an animal of same group as Fig. 7. All blue follicles are radioactive.

Fig. 9 (100x) As above but note single mixed follicle with radioactive blue area.

Fig. 10 (100x) As above but note two yellow-stained follicles in center.

Fig. 11 (100x) Goitrogenous gland from an iodine-deficient rat exposed to cold (5°C) for 24 hours after I^{131} administration.

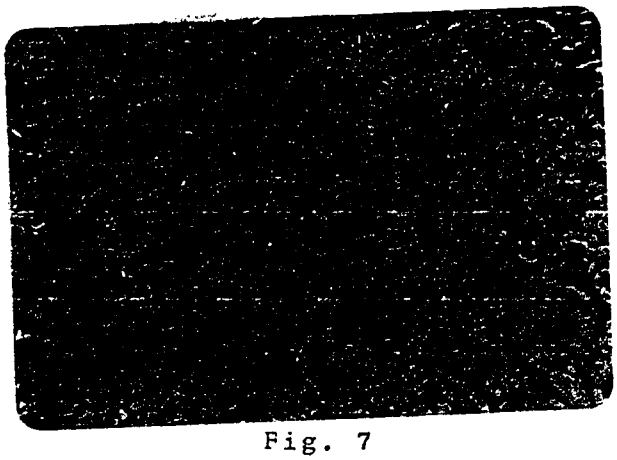


Fig. 7

Fig. 8

Fig. 9

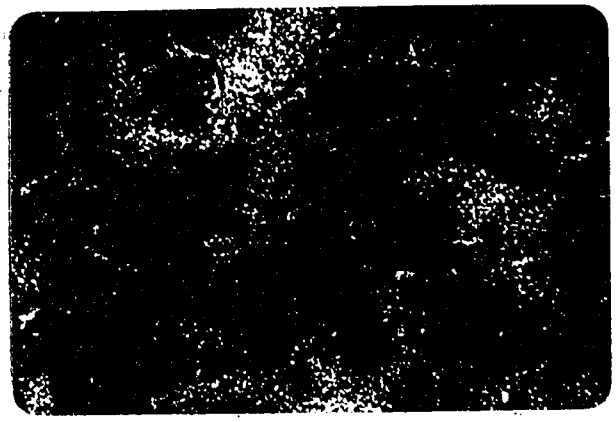


Fig. 10

Fig. 11

It is interesting to note that on one slide of this group mitosis was observed (Fig. 12) which is an exceedingly rare phenomenon in the thyroid (144).

Autoradiography studies established that after twenty-four hours most of the radioactivity was in the colloid. Since there were more mixed follicles, it was possible to establish a gradual change in concentration of radioactivity from very high in the blue colloid, to moderate and restricted to the blue portion in the area of mixed colloid, to very light or void in the yellow areas (Fig. 13, 14, 15, 16).

4. Group DT (Fig. 17, 18, 19, 20).

There are two very interesting histological features in the glands of this group: 1) the overwhelming number of yellow follicles, ranging from ninety to ninety-nine percent of all follicles present; 2) the reduction in the total number of follicles accompanied by considerable connective tissue infiltration. Colloid droplets were entirely absent from the cells. As in all other cases, however, the yellow colloid gave a strong Periodic Acid-Schiff reaction. In many follicles the nuclei of the squamous-like cells were pycnotic and the cytoplasm was scanty. Numerous follicles were in various stages of destruction and desquamated dying cells with pycnotic nuclei, fitting the description of the "colloid cells" of Langendorff, crowded the follicular cavity.

Autoradiography did not reveal any activity in the

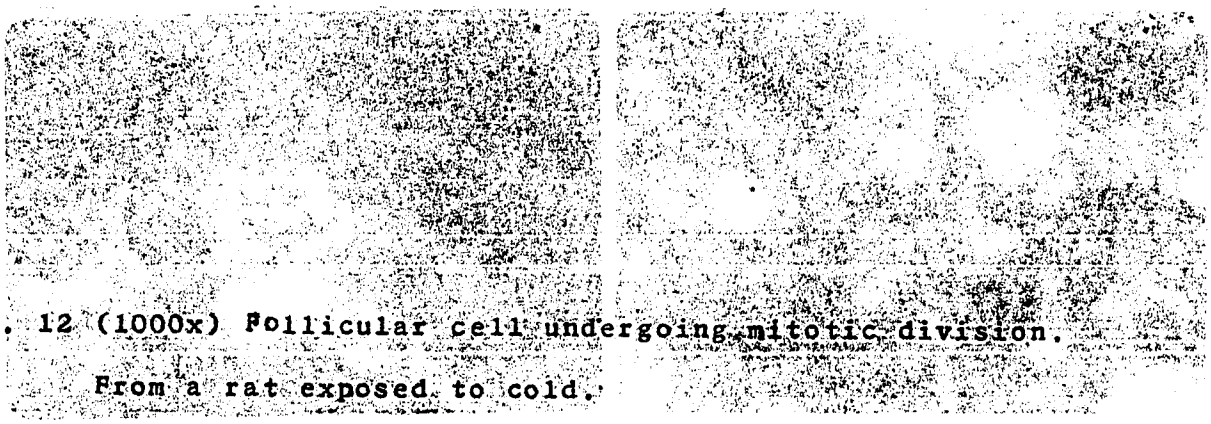


Fig. 12 (1000x) Follicular cell undergoing mitotic division. From a rat exposed to cold.

Fig. 13 (100x) Autoradiograph of the thyroid gland of a cold exposed rat. The yellow colloid shows little radioactivity.

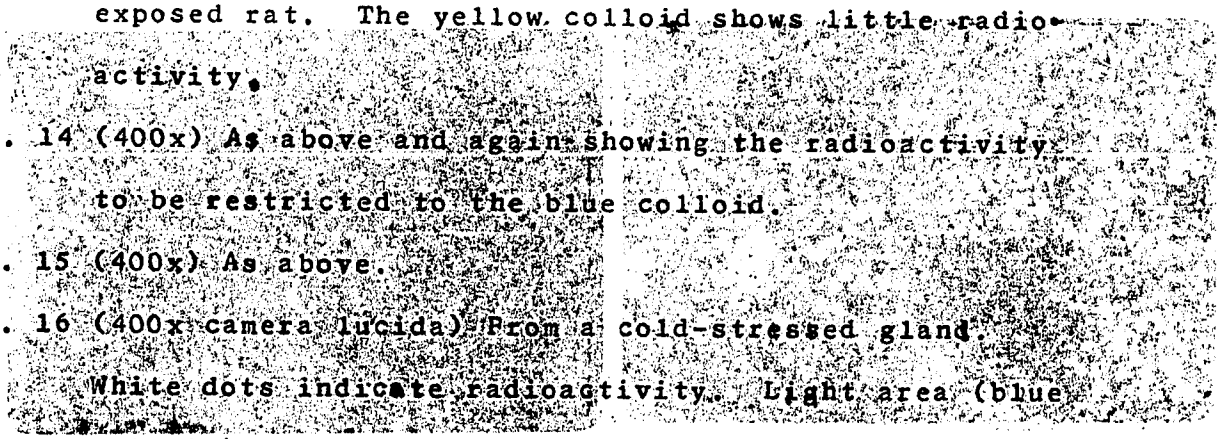


Fig. 14 (400x) As above and again showing the radioactivity to be restricted to the blue colloid.

Fig. 15 (400x) As above.

Fig. 16 (400x camera lucida) From a cold-stressed gland. White dots indicate radioactivity. Light area (blue colloid); dark area (yellow colloid). Between light and dark areas is mixed colloid. This drawing is offered because the photomicrographs do not show clearly the graded concentration of radioactivity in the three forms of colloid.

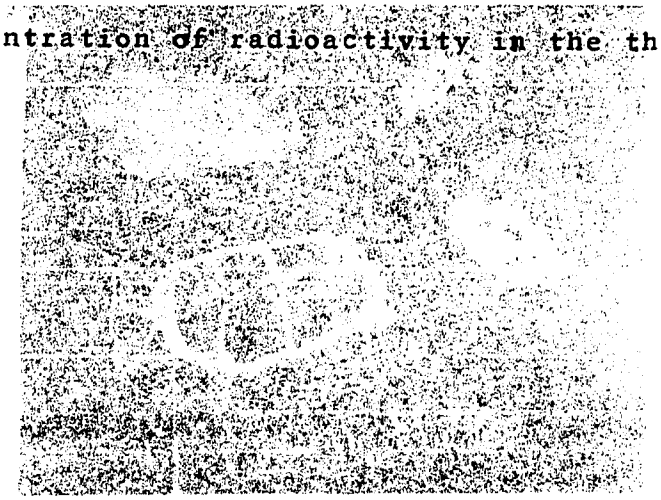


Fig. 12

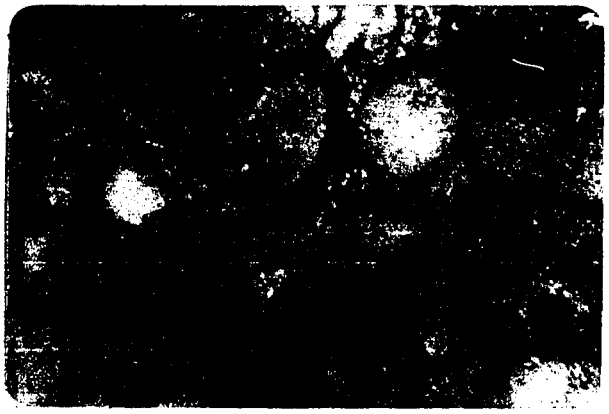


Fig. 13

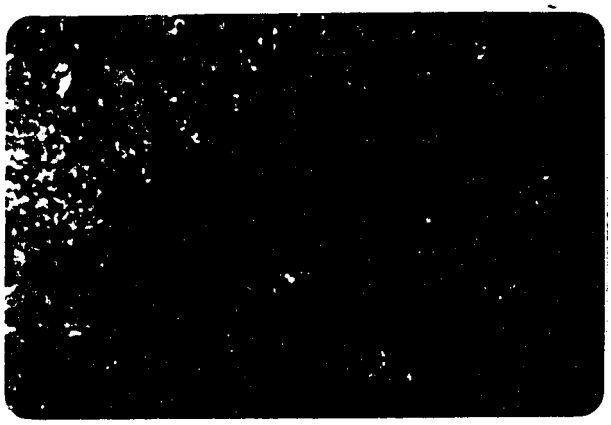


Fig. 14

Fig. 15

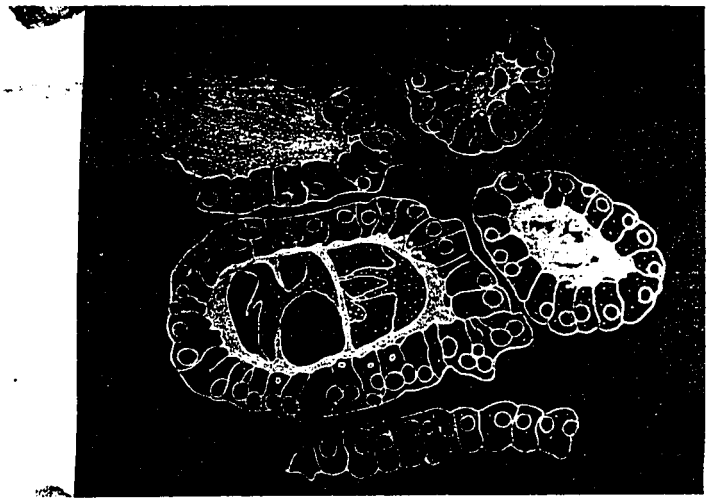


Fig. 16

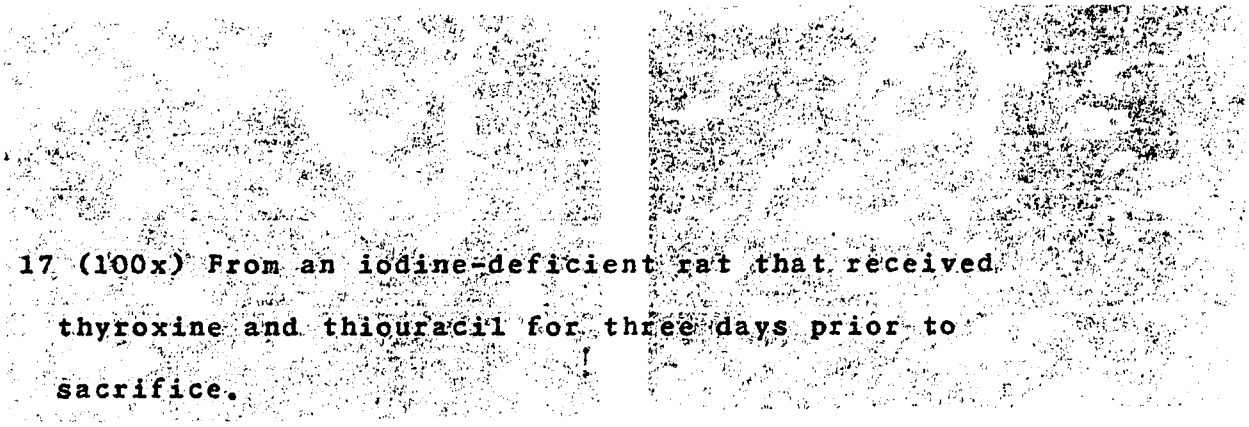


Fig. 17 (100x) From an iodine-deficient rat that received thyroxine and thiouracil for three days prior to sacrifice.

Fig. 18 (100x) As above but better demonstrating the reduction in the number of follicles and connective tissue infiltration.

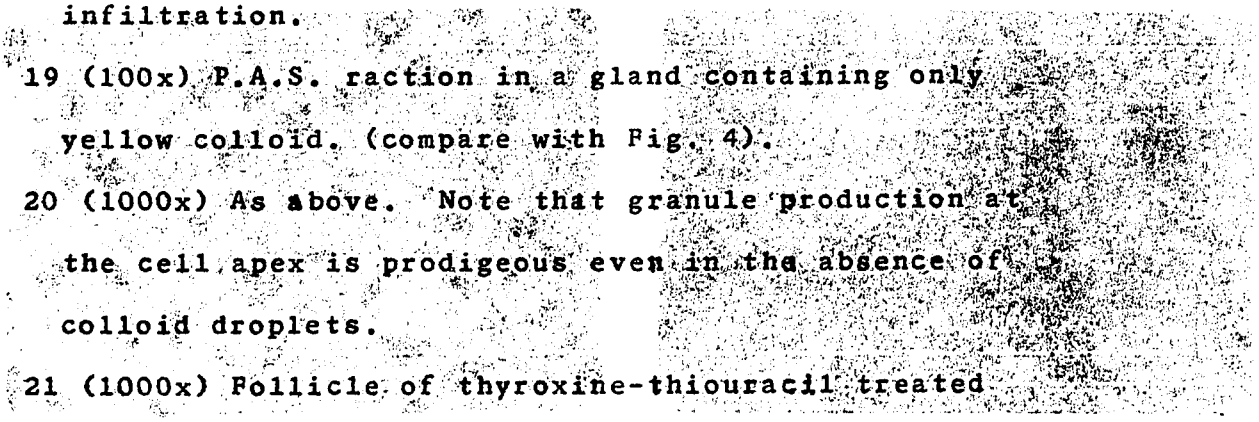


Fig. 19 (100x) P.A.S. raction in a gland containing only yellow colloid. (compare with Fig. 4).

Fig. 20 (1000x) As above. Note that granule production at the cell apex is prodigeous even in the absence of colloid droplets.

Fig. 21 (1000x) Follicle of thyroxine-thiouracil treated animal showing part of a yellow follicle in upper left corner and in the center a desquamating follicle containing colloid cells of Langendorf.

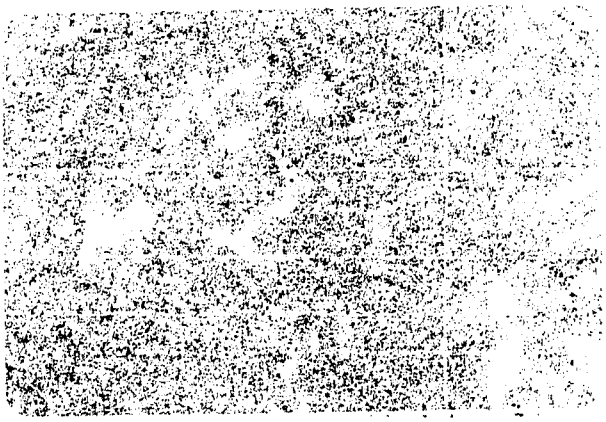


Fig. 17

Fig. 18

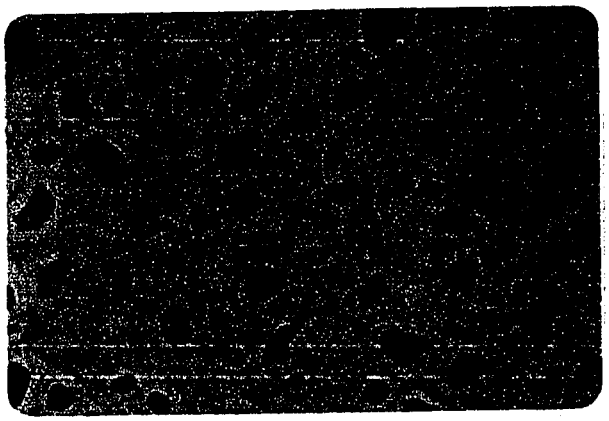


Fig. 19

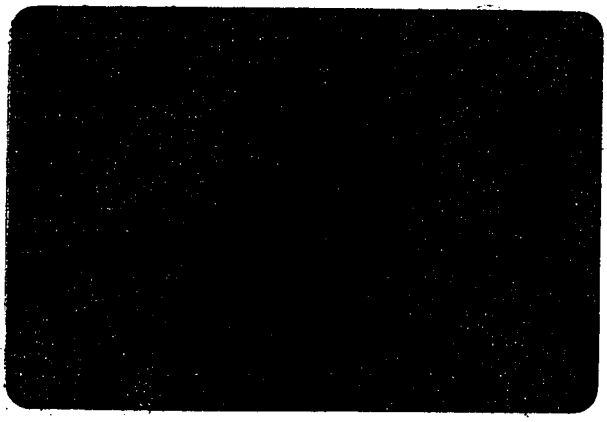


Fig. 20

Fig. 21

yellow colloid. When activity was apparent it was restricted to the cells. (Fig. 22)

B. Physical Measurements.

TABLE I

Group	N	CHE*	Activity**	Number blue follicles	Number yellow follicles	Total *** follicles
N	12	9.05	117.26(2.069)	147.8	225.9	373.7
D	17	15.34	4262.38(3.629)	356.0	163.6	519.6
DC	15	16.92	3349.26(3.525)	352.4	134.7	487.1
DT	15	6.99	6.49(0.813)	23.7	326.0	349.7

* cell height of epithelium in micra
** activity in counts per minute per two sections
*** mean from twenty mid-sagittal sections from the gland of each animal.
In parenthesis is the log of activity

1- Activity

Examination of Table I reveals that as the number of blue follicles increases there is also an increase in the uptake of I^{131}. This relationship is further emphasized when the number of blue follicles is plotted against the log of the activity (Fig. 23). Calculation of the curve by the method of least squares ($y = a + bx$) and determination of the correlation of regression results in $r = 0.984175$.

The Analysis of Variance (Table II) establishes that Groups D and DC while not differing between themselves are

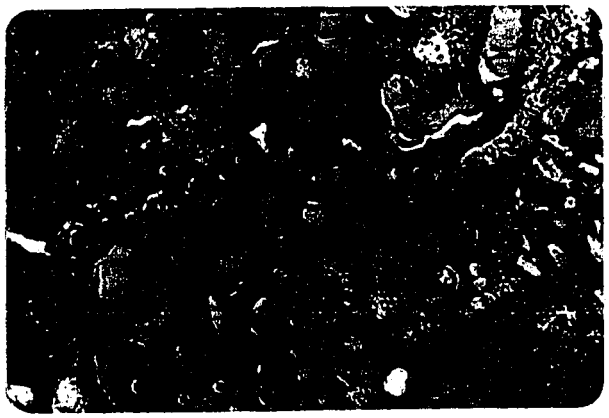


Fig. 22

Autoradiograph of the thyroid of a thyroxine-thiouracil treated rat showing radioactivity to be restricted to the follicular cells.

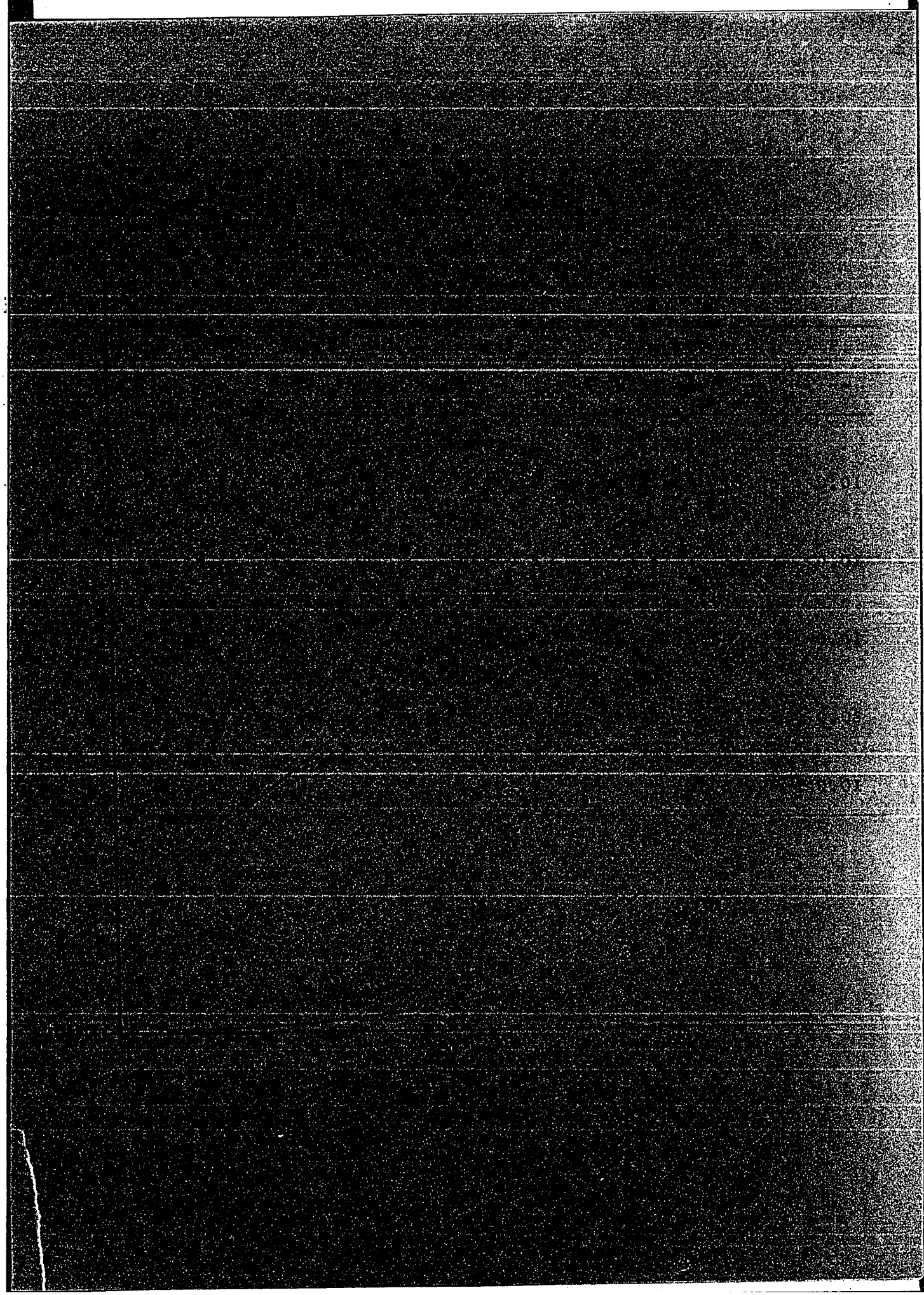


Table II

Source of Var.	Total	Groups	Ind. in gr.	F.	P.
d.f.	55	3	52	--	--
Activity $\sum$. sq.	301682896.1	215815458.6	85867437.5		
M. sq.	- - - -	71938486.2	1651296.8	43.56	<0.01
Blue fol. $\sum$. sq.	1915028.7	1213648.5	701380.2		
M. sq.	- - - -	404549.5	12752.4	31.72	<0.01
Yell.fol. $\sum$. sq.	1011o64.7	326378.7	685286.0		
M. sq.	- - - -	108792.9	13178.6	82.55	<0.01
Tot. fol. $\sum$. sq.	1715946.8	315924.0	1400022.8		
M. sq.	- - - -	105308.0	26923.5	3.91	<0.05
Cell H. $\sum$. sq.	1246.4	1025.1	221.2		
M. sq.	- - - -	341.7	4.0	84.95	<0.01

significantly different from Groups N and DT at the one percent level. Groups N and DT are statistically equivalent.

2- Blue Follicles

With the exception of the relationship of D to DC, all groups are significantly different between themselves at $P < 0.01$

3- Yellow Follicles

There is less correlation in this category. DT is significantly different from DC at $P < 0.01$ and D at $P < 0.05$. All other relationships are statistically equivalent.

4- Total Follicles.

Group D is significantly different from DT at $P < 0.01$ and N at $P < 0.05$. There is no difference between the means of D and DC. At $P < 0.05$ DC differs from DT but is equivalent statistically to the other groups. Groups N and DT are equivalent.

5- Cell height of Epithelium

With the exception of the differences between DC and D all the means are statistically significant. Further all are significant at $P < 0.01$ except for N and DT which are significant at $P < 0.05$.

DISCUSSION AND CONCLUSIONS:

Although many investigators have in the past (19, 24, 44, 45, 55, 143, 164) observed the phenomenon of color variations of the follicular colloid with a number of trichrome stains following chemical fixation of the thyroid

gland, to our knowledge no one has attempted, or if so apparently without success, to concretely relate it to a definite change in the nature of the colloid itself. As was previously stated (55) it has commonly been thought of as a fixation artifact which was interesting only because it followed a pattern associated with the physiological state of the animal. We have attempted to show that this phenomenon cannot be dismissed on such a simple supposition as an artefact but should probably be looked upon as an indicator of actual chemical change in the protein nature of the colloid. Several facts contained in the foregoing results and the degree of correlation between the different physical measurements all point directly to a relationship between the color of the colloid and the physiological state of the thyroid gland.

It is difficult to believe that the blue colloid does not contain the active iodinated amino acids of thyroglobulin when there is such exceptionally good correlation between Iodine 131 uptake and the increase in the number of blue follicles. A value for r of 0.9842 for biological data is an extraordinarily high correlation.

The fact that the animals comprising Groups D and DC incorporated the I^{131} to such a degree could be expected since they were starved for iodine and as a result the blood PBI was minimal and the TSH production of the pituitary probably maximal. Since such a condition results in hyperplasia, it follows that the number of iodine acceptor

sites (Halmi 98) would be tremendously increased in the follicular epithelium. It is interesting to note that in some individuals of this group ninety-nine percent of the follicles were blue and values in excess of eighty-five percent were common. Confirmation comes from the fact that autoradiographs show the iodine to be situated in the blue colloid only.

The opposite situation is equally remarkable. In Group DT which in addition to the iodine deficient diet received thyroid powder and thiouracil prior to iodine administration there was, as expected, practically no iodine incorporation; but the interesting fact is the reversal in the ratio of blue to yellow follicles. In this instance, with few exceptions, the thyroid glands of the individuals of the group were over ninety percent yellow. Since the animals were on an iodine deficient diet and received thyroid powder and thiouracil, we feel that it is safe to assume that there was very little if any thyroglobulin or iodinated amino acids in the thyroid gland of these rats. Therefore it appears feasible that what stains yellow in the colloid is protein residue after the hydrolysis of thyroglobulin. Since the P.A.S. reaction was possitive in this group as strongly as in the other groups, it is reasonable to assume that part of this residue is a glycoprotein. If the catepsin-like proteolytic enzyme acts very specifically, it is not inconceivable that the residual protein may also be of high molecular weight. Confirmation of this must await chemical analysis of the two colloid.

fractions by electrophoresis and chromatography.

The effect of thiouracil and thyroid powder on the number of follicles present in the thyroid gland is extremely interesting. If we assume that four days prior to sacrifice the thyroid glands of groups D, DC and DT were in the same physiological and morphological state it means that in this instance three days treatment with these compounds results in a thirty percent reduction in the number of follicles. So far as we know this observation has never been reported. Whether this is due to the combined action of the compounds or is primarily due to the action of one of them must be investigated. Although the differences in the uptake of radioiodine between groups D and DC and the difference between the number of blue follicles are obviously not statistically significant, it is felt that they are important in view of the following observations: 1) a difference in the distribution of the colloid in the mixed follicles and 2) a probable increase in vascularization in Group DC. Consideration of these points together with the fact that there was less iodine in the tissue at the time of sacrifice lead us to suggest that the difference may be due to the additional stress and that while as much iodine was incorporated by Group DC there was an even greater need and demand on the part of the animals and so more of the thyroglobulin was hydrolized to satisfy their peripheral demands.

While one follicle was observed that appeared to exhibit signs of a change in the polarity of the follicular cells we feel that, since it was the only instance in which this observation was made, it would be premature to express any opinion on the problem of reversal.

The autoradiography pattern for iodine is diffuse. For this reason cytological localization is difficult to determine precisely; however, it appears that intracellular iodination cannot be excluded entirely in the rat. While it may also occur at the colloid-cell interface (126), we suggest that there is also iodination of the colloid droplets within the cell; it is hoped that further experimentation will result in refinement of the technique in order to establish this fact conclusively.

The correlation between all the above factors, i.e., 1) increase in activity together with an increase in the number of blue follicles; 2) the decrease in activity coupled with the decrease in blue follicles and increase in the number of yellow follicles; 3) the concentration of radio-iodine in the blue colloid; 4) and the significant difference in the heights of the epithelium lead us to conclude that by the staining method described it is possible histochemically to differentiate the follicles containing iodinated thyroglobulin from those which do not and in the case of mixed follicles to estimate with reasonable accuracy the proportion of active colloidal substance.

EXPERIMENT III

INTRODUCTION

Since we felt a method was now available to determine the state of activity of the thyroid at any given time, we believed a study of the process of trapping, binding, secretion, hydrolysis and reabsorption of iodine and iodinated products could be undertaken.

PURPOSE

To study thyroid activity from three to forty-eight hours after the injection of I^{131} in normal and iodine-deficient rats.

MATERIALS AND METHODS

Forty-one male albino rats of the Wistar strain weighing between fifty and eighty grams were divided into two groups: Group N, normal animals under routine laboratory conditions and Group D, animals which were placed on an iodine deficient diet and distilled water ad libitum for three weeks. At the end of this period both groups were subdivided on the basis of treatment.

Group N1. General control group receiving no treatment.

Groups N2 - N6. All animals received a 10μc subcutaneous injection of I^{131} and were sacrificed at 3, 6, 12, 24, and 48 hours respectively.

Group D. Served as a general control for the diet animals and received no further treatment.

Groups D_2 - D_6. All received a 10μc subcutaneous injection of I^{131} and were sacrificed at 3, 6, 12, 24 and 48 hours respectively.

Group D_7. Three days prior to sacrifice the members of this group were given 0.8 grams of thyroid powder (USP) per kilogram of diet and a 0.02% solution of thiouracil ad libitum. No isotope was given these animals.

The animals were sacrificed in ether and the thyroids removed in toto and fixed in Bouin's and prepared for study as described in the preceeding experiment.

RESULTS

A. Histological Observations

1) Normal Groups.

a) After three hours. (Group N_2)

Almost all the radioactivity is intracellular with only a small amount found in the peripheral area of the follicle. (fig.24)

b) After six hours. (Group N_3)

The situation after six hours is not remarkably changed from that seen after three hours. Most of the radioactive material is still intracellular although some ringing of the colloid is observed. (fig.25)

Fig. 24

Autoradiograph of a normal gland 3 hours after I^{131} administration. Radioactivity predominently intracellular.

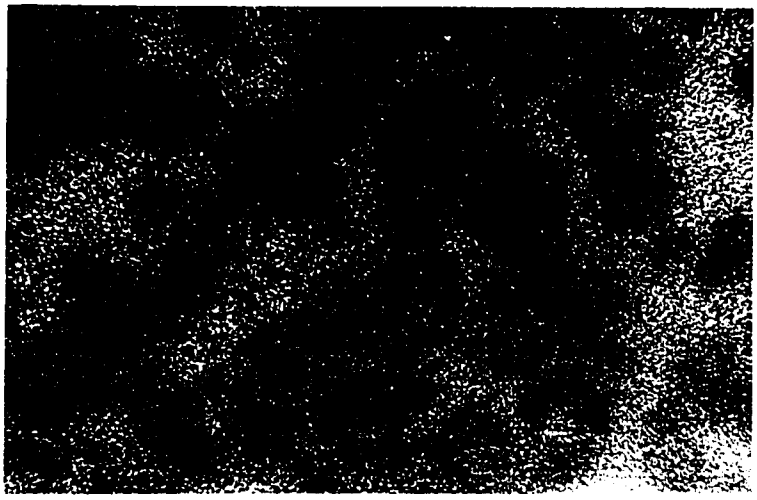


Fig. 25

As above but 6 hours after receiving I^{131}. Much activity still intracellular but some ringing of the colloid (100x).

c) After twelve hours. (Group N_4)

By twelve hours the radioactive compounds are definitely moving into the colloid. There is still a large amount of "activity" in the cells but the periphery of the colloid has now concentrated more of the radioactive iodine (fig. 26)

d) After twenty-four hours. (Group N_5)

At this time the radioactive material is diffusing throughout the colloid. The ring reaction is still the strongest, but there is still some radioactive iodine in the cells. (fig.27)

e) After forty-eight hours. (Group N_6)

Most of the radioactive material is by this time diffusing throughout the colloid and the picture is more homogeneous (fig.28)

2. Iodine Deficient Diet Groups

a) After three hours. (Group D_2)

Considerably more radioactive material has moved into the colloid within three hours than in its normal counterpart. The ring reaction is strong and there is some diffusion into the deeper colloid. Considerable radioactive material, however, still is located intracellularly. (fig.29)

b) After six hours. (Group D_3)

Almost all of the radioactive material is now located within the colloid and is concentrated on the periphery. Some diffusion toward the center has occurred. (fig. 30)

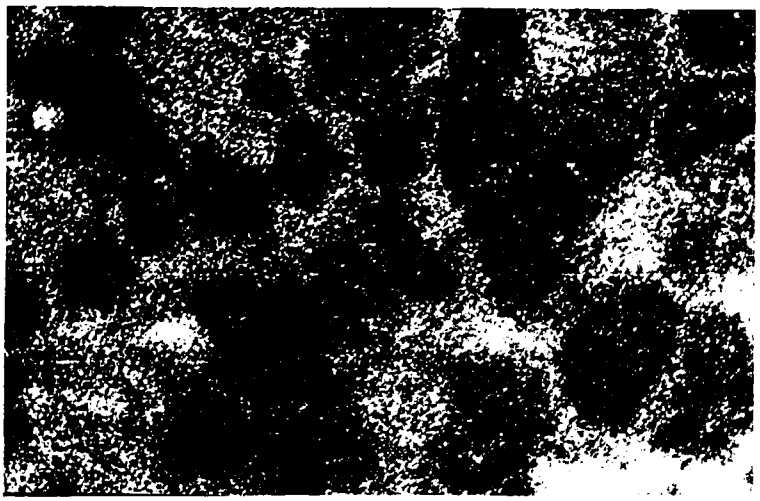


Fig. 26

Normal gland 12 hours after I^{131} administration. Some intracellular activity but colloid ring predominates (100x).

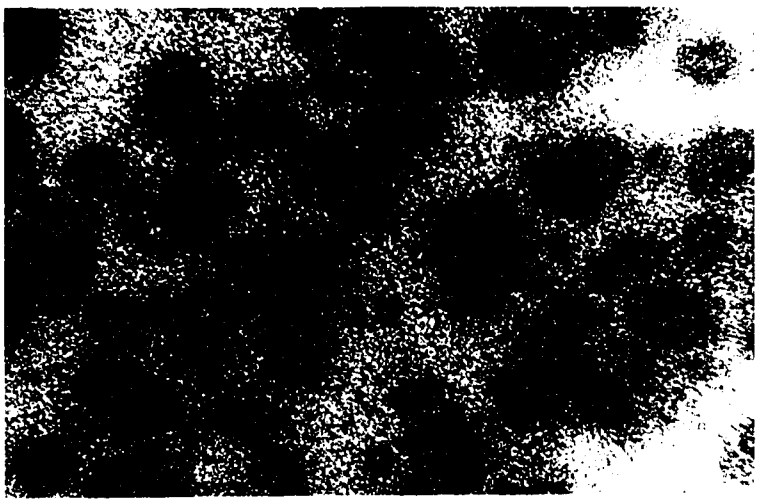


Fig. 27

As above but 24 hours after I^{131} administration. The ring reaction has reached its peak. Some radioactivity is still intracellular (100x).

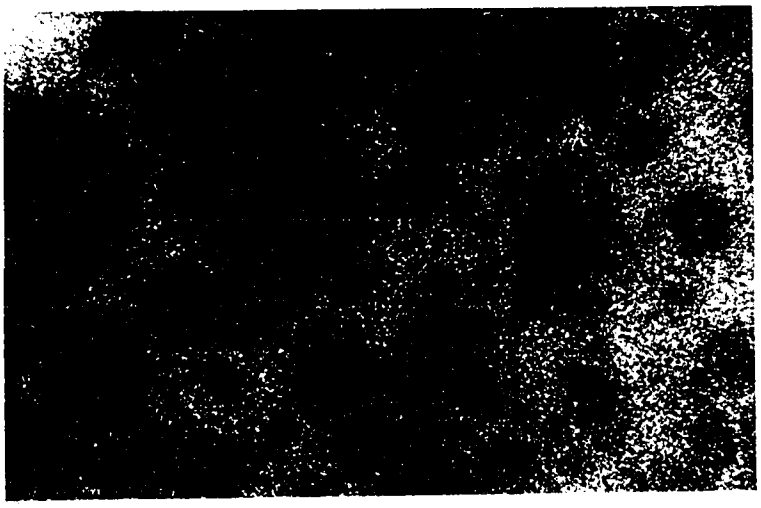


Fig. 28

Normal gland 48 hours after I^{131} administration. Radioactivity diffusing throughout colloid but evidence of the ring reaction still persists in some follicles (100x).

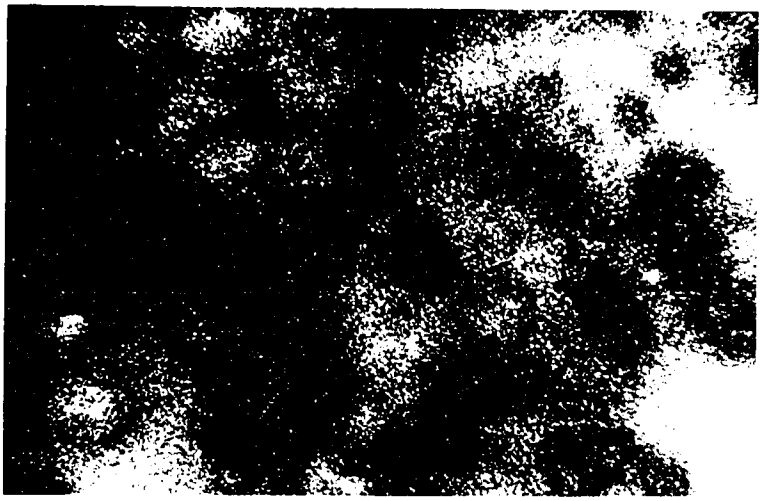


Fig. 29

Iodine-deficient rat thyroid 3 hours after I^{131} administration. Ring reaction already evident. Some activity is still intracellular. Compare with figure 27 (100x).

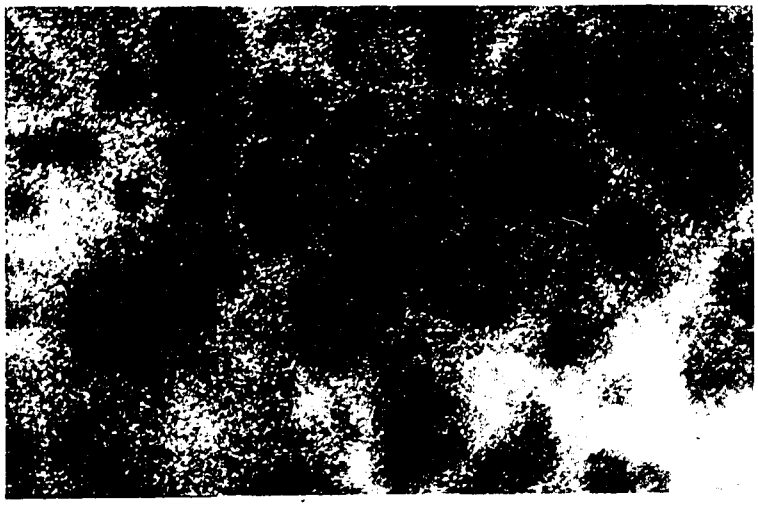


Fig. 30

Thyroid from an iodine-deficient rat 6 hours after I^{131} administration. I^{131} is diffusing toward center (100x).

c) After twelve hours. (Group D_4)

By this time the reaction is spreading from the periphery toward the center of the colloid. (fig. 31)

d) After twenty-four hours. (Group D_5)

Generally the picture is about the same as after twelve hours. There is a heavy concentration of I^{131} on the periphery, but there is a progressive diffusion to the interior of the colloid so that in some cases the colloid I^{131} is more uniformly distributed. (fig.32)

e) After forty-eight hours. (Group D_6)

The colloid is homogeneously impregnated with radioactive compounds, although in some cases a strong ring reaction still predominates. (fig.33)

In all cases, as the radioiodine diffuses into the colloid the blue color reaction occurs in the same area. (Fig. 34,35)

B. Physical Measurements (Table III)

Examination of the curve for normal animals (fig. 36) shows a small but steady increase in the number of counts per minute through twenty-four hours and then a drop in activity to a point below that of twelve hours.

It is obvious that all iodine-deficient diet groups show a significantly greater uptake of iodine^{131}. Examination of the uptake curve for these groups (fig,36) and comparison with the curve for controls show that, although the uptake is much greater in the iodine-deficient animals, the uptake

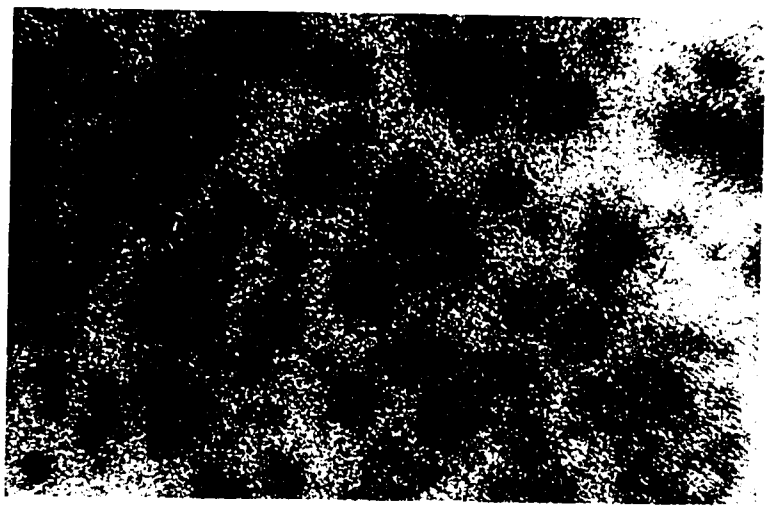


Fig. 31

Thyroid from an iodine deficient rat, 12 hours after I^{131} administration. Continued infiltration of colloid by iodine (100x).

Fig. 32

As above but 24 hours after I^{131} administration. Infiltration of colloid by iodine complete or nearly complete (100x).

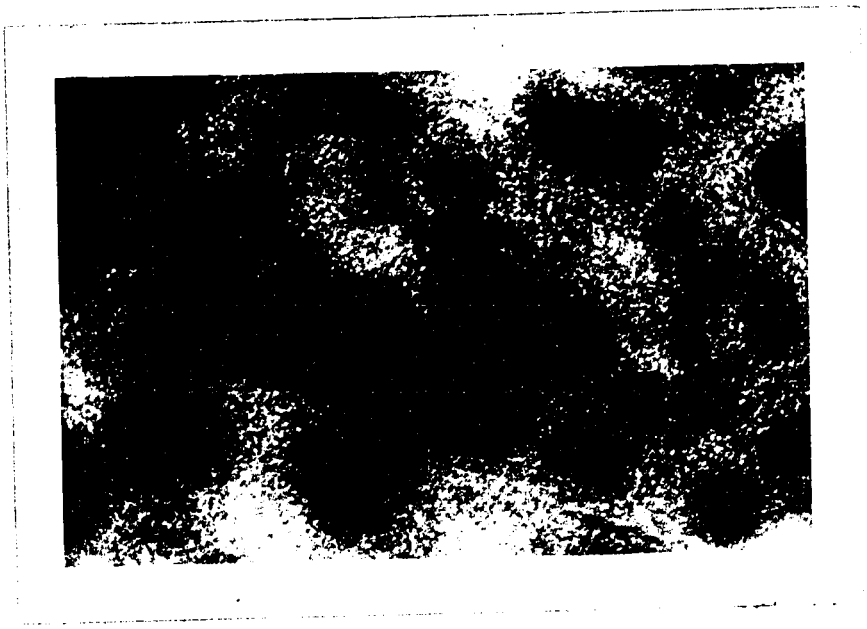


Fig. 33

Thyroid from an iodine defecient rat 48 hours after I^{131} administration. Infiltration of colloid by iodine complete.

Fig. 34

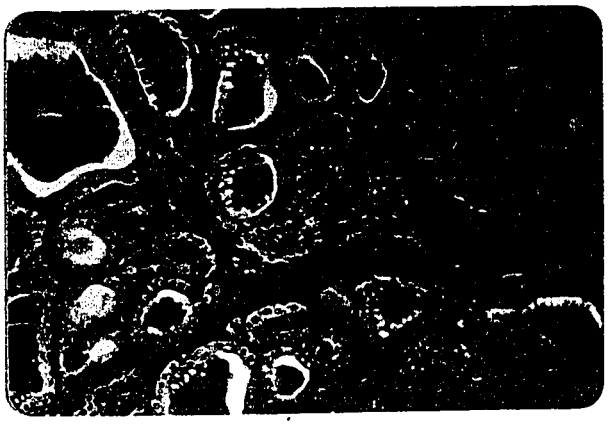
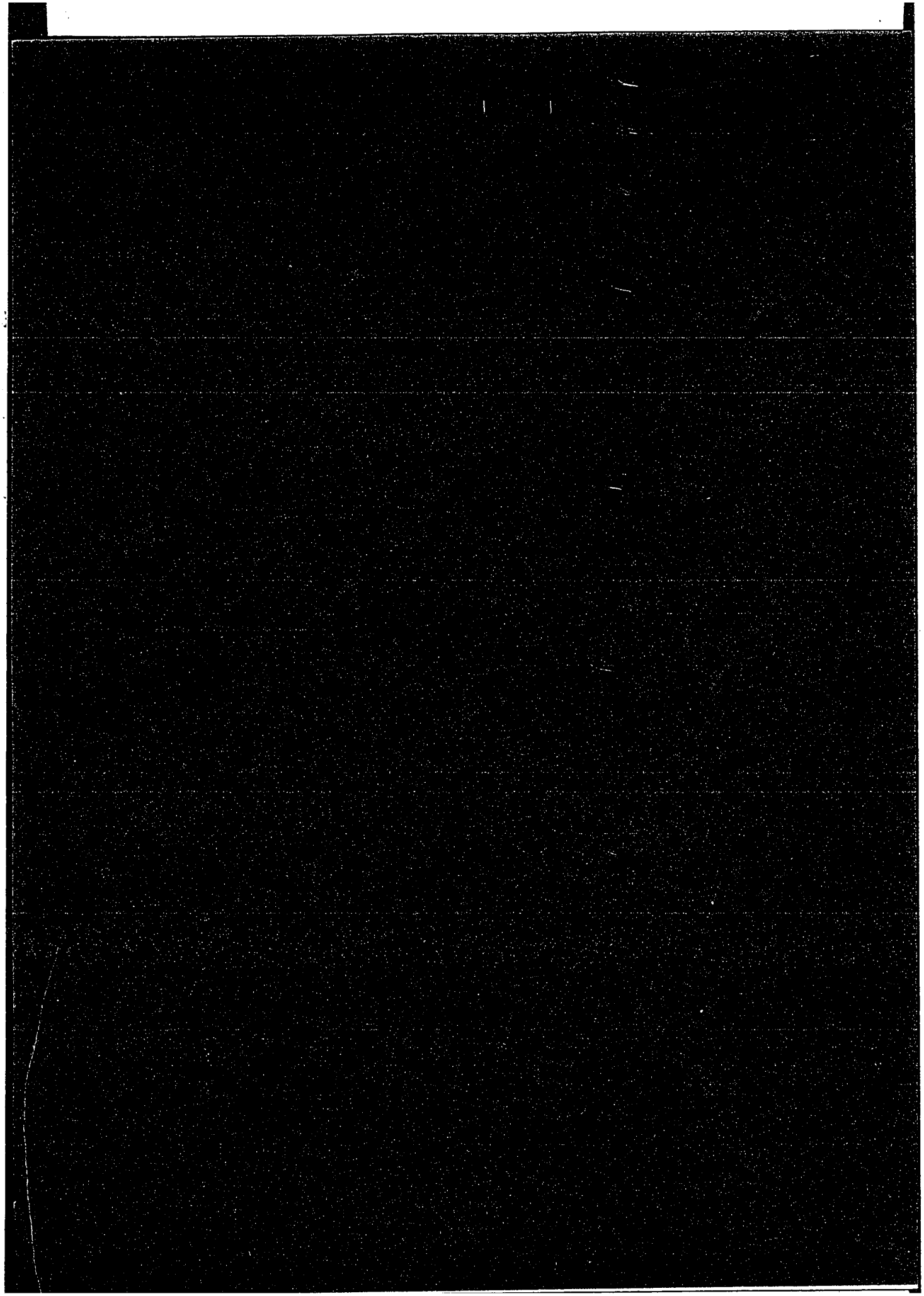
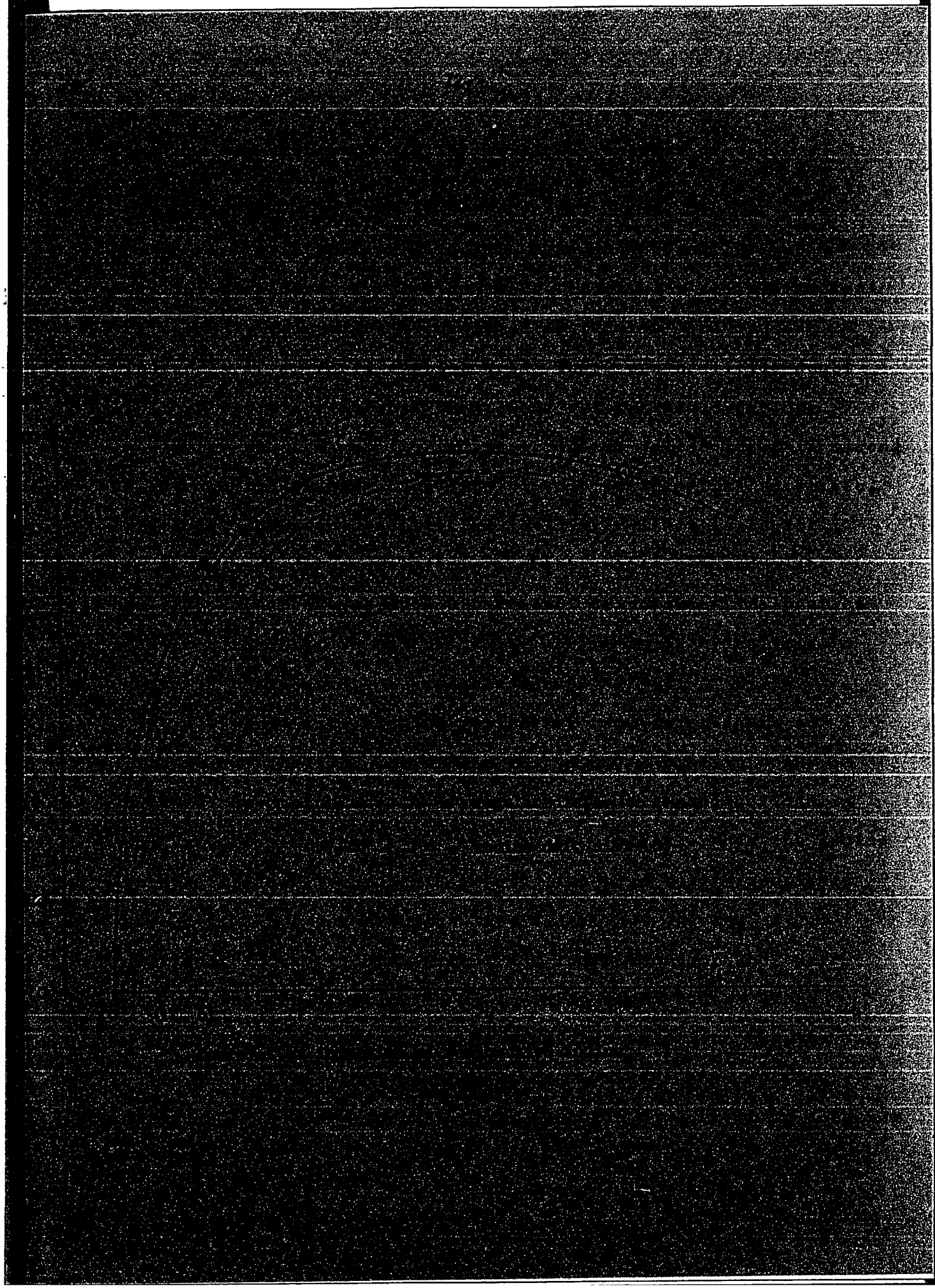


Fig. 35

Fig. 34 (100x) Thyroid from an iodine-deficient rat 6 hours after I^{131} administration. Note blue colloid surrounding yellow core (Compare with Fig. 30).

Fig. 35 (100x) As above but twenty-four hours after I^{131} administration. Note blue colloid (Compare with Fig. 32).





pattern appears to be similar in both treatment groups.

DISCUSSION AND CONCLUSIONS

Unlike the result of Leblond and Gross (127) we still find radioactive iodine in the follicular cells of normal rats twenty-four hours after injection of I^{131} and in some instances after forty-eight hours. There can be little doubt that it is in the organic form since the technique employed would have washed out the inorganic iodide unless there is strong electrostatic binding of iodine to some protein-like substrate, which has not been demonstrated yet. Even if it is in the form of monoiodotyrosine it is probably protein-bound since *in vitro* studies have shown that monoiodotyrosine is a part of the protein molecule and has to be liberated by hydrolysis in order to be identified by chromatography (149).

The fact that the peripheral ring reaction is so intense and that the colloid in this area is blue in color is further supporting evidence that the blue staining colloid is the biologically active material in the follicle. As the iodine spreads through the colloid we find the blue color associated with it. Since serum iodine levels were not checked, it is difficult to say to what extent the iodinated compounds were re-entering the circulation up to forty-eight hours. The morphological picture of a gradual disbursement through the colloid indicated that most of the radioiodine remained there. The drop in number of counts does indicate however, that some was

leaving but this is not statistically significant. Not all of the follicles captured radioiodine. We interpret this as meaning that these follicles were in a release phase of activity. This is not the case in iodine deficient rats. Incorporation of I^{131} is much more rapid and almost every follicle in the gland was radioactive and contained blue-staining colloid. Obviously, previous iodide intake is a limiting factor in the rate of colloid incorporation of radioiodine. Morphologically, it appears that by forty-eight hours it is entering the circulation; however, this is not established statistically. In some instances it undoubtedly entered before forty-eight hours since the autoradiographs showed the entire colloid of some follicles to be impregnated with I^{131} after six hours and in a few instances after three hours. These facts warrant serious consideration when one interprets results since the physiological state of the thyroid is going to determine the rate of incorporation of I^{131} and no definite time for this to occur can be forecast with complete accuracy.

EXPERIMENT IV

The effect of thiouracil alone and thiouracil plus thyroxine on the thyroid gland of the rat.

INTRODUCTION:

In previous experiments we observed that following treatment with thyroid powder and thiouracil there was a thirty percent reduction in the total number of follicles after three days.

PURPOSES:

To study the effect of the administration of thiouracil alone and in combination with thyroid powder over different time intervals.

MATERIALS AND METHODS:

Male albino rats of the Wistar strain weighing between 80 and 100 grams were fed the Remington iodine deficient diet and distilled water ad libitum for five weeks at room temperature. They were then divided into nine groups:

Group A: Control group which received no treatment other than the special diet.

Groups B, C, D and E: These were given thiouracil as a 0.02% solution in the drinking water for 1, 2, 3 and 4 days respectively prior to injection of I^{131}.

Groups F, G, H and I: In addition to the thiouracil these animals were given thyroid powder (USP) at a level of 0.8

gram per kilogram of diet for 1, 2, 3 and 4 days before being injected with I^{131}.

The animals were weighed once a week and at the time of autopsy. They were sacrificed with ether 24 hours after the injection of 10μc of I^{131}, carrier-free. The thyroids were removed in toto, weighed on a Roller-Smith balance and fixed in Bouin's. From the middle of a lobe (alternately right and left) 20 serial sections were selected and mounted on ten slides, two sections on each, and allowed to dry overnight. The slides were then individually counted for radioactivity on a "64 Scaler" (Tracer Lab.). Subsequently, they were stained according to the technique cutlined in the first experiment. Slides 1, 3, 5, 7 and 9 were then prepared for autoradiography while the others were cleared and covered for cytological examination, counting of the follicles, and measurement of the cell height of the epithelium using the optic micrometer technique.

RESULTS:

I. Thyroid Weight (tables IV, V, and VII)

Groups B, C, D and E which received thiouracil for periods of 1, 2, 3, and 4 days respectively prior to administration of I^{131} are not significantly different from control group A. Further, group F which received thiouracil and thyroid powder for one day is statistically equivalent to A, B, C, D and E. However, groups G, H and I given thiouracil and thyroid powder

Table IV

Group	N	Weight*	CHE**	Activity***	Number Blue foll.	Number Yel. foll.	Total n. Follicules
A	5	35.7	16.8	35528.6	1114.0	5.8	1119.8
B	5	34.2	14.2	8391.8	959.2	8.6	967.8
C	4	34.4	14.9	7658.0	814.2	2.2	816.4
D	5	36.0	13.9	13179.0	808.4	1.8	810.2
E	4	37.7	12.7	11830.0	753.7	2.7	756.4
F	5	33.0	11.6	91.6	553.8	543.4	1097.2
G	5	22.1	8.8	97.2	422.4	520.8	943.2
H	4	14.1	7.5	54.6	204.5	638.5	843.0
I	5	12.1	7.1	68.6	125.0	595.4	720.4

* Fresh weight in mgm. ** Cell Height of the Epithelium in micra.
*** Activity in counts per minutes per two sections.

Table V

Source of Var.	d.f.	Weight Σ. sq.	Weight M. sq.	CHE Σ. sq.	CHE M. sq.	Activity Σ. sq.	Activity M. sq.
Total	41	6741.48	—	519.83	—	327658998.8	—
Groups	8	4344.80	543.10	446.53	55.81	175496279.6	21937034.9
Ind. in groups	33	2396.68	72.63	73.30	2.22	142162719.2	4307961.1
F		7.48		25.13		5.09	
P		<0.01		<0.01		<0.01	

Table VI

Source of Var.	d.f.	Blue follicles Σ. sq.	Blue follicles M. sq.	Yellow follicles Σ. sq.	Yellow follicles M. sq.	Total follicles Σ. sq.	Total follicles M. sq.
Total	41	5586718.0	—	4411121.1	—	3150999.6	—
Groups	8	4306125.0	538265.6	3380034.5	422504.3	792711.2	99088.9
Ind. in groups	33	1279873.0	38784.6	737086.6	22335.9	2358288.4	71463.3
F		13.87		18.91		1.38	
P		<0.01		<0.01		>0.05	

Table VII

Ranges. p:	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Rp:	8.2	9.8	10.9	11.6	12.2	12.6	13.1	13.4
Results. Group:	K	H	G	F	B	A	D	E
Mean:	12.1	14.1	22.1	33.0	34.2	35.7	36.0	37.7

Table VIII

Ranges. p:	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Rp:	1.42	1.49	1.53	1.57	1.60	1.62	1.63	1.64
Results. Group:	I	H	G	F	E	D	B	C
Mean:	7.1	7.5	8.8	11.6	12.7	13.9	14.2	14.9

Table IX

Ranges. p:	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Rp:	2005.3	2102.2	2164.4	2212.8	2254.3	2281.9	2302.7	2318.7
Results. Group:	H	I	F	G	C	B	E	D
Mean:	54.6	60.6	91.6	97.2	7658.0	8391.8	11830.0	13174.0

Table X

Ranges. p:	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Rp:	190.4	199.6	205.5	210.1	214.0	216.6	218.6	220.2
Results. Group:	I	H	G	F	E	D	C	B
Mean:	125.0	204.5	422.4	553.8	753.7	808.4	814.2	914.2

Table XI

Ranges. p:	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Rp:	144.4	151.4	155.9	159.4	162.3	164.3	165.8	166.9
Results. Group:	D	C	E	A	B	G	F	I	H
Mean:	1.8	3.2	3.7	5.8	6.6	520.8	543.4	595.4	638.4

for 2, 3 and 4 days respectively are significantly different from all other groups though there are no significant differences between themselves.

2. Cell Height of the Epithelium (Tables IV, V, and VIII)

The cell height of the epithelium is significantly decreased in all experimental groups as compared with control group A. It is further and significantly reduced in groups G, H and I compared to groups B, C, D, E and F.

3. Thyroidal I^{131} (Tables IV, V, and IX)

The highest level of radioactivity is found in group A. It is significantly lower in thiouracil-treated groups D and E and still lower in similarly treated groups B and C, but groups F, G, H and I receiving both thiouracil and thyroid powder show the lowest and significant level of thyroid radioactivity.

4. Number of Blue Follicles. (Tables IV, VI, and X)

The thyroids from animals of group A have the greatest number of blue follicles. These are significantly less numerous in groups B, C, D and E. The reduction in number is more important in groups F and G and still more in groups H and I.

5. Number of Yellow Follicles. (Tables IV, VI, and XI)

The number of yellow follicles is low and statistically equivalent in control group A and in thiouracil-treated groups B, C, D and E. However, the addition of thyroid powder to thiouracil in groups F, G, H and I results in a significant increase in the number of yellow follicles present in the thyroid.

6. Total Number of Follicles. (Tables IV and VI)

Although there are no significant differences between any of the groups, there appears to be a trend toward a decrease in total follicles with increasing duration of treatment. In this respect thiouracil alone would seem to be as effective as the combination of thiouracil-thyroid powder.

DISCUSSION AND CONCLUSIONS:

Thiouracil, over a four day period, has little or no effect on the weight of the thyroid gland of an iodine-deficient rat. This would be expected since the plasma iodine level is probably already minimal. In a normal rat thiouracil administration results in an almost immediate loss of iodine from the thyroid so that no new hormonal secretion can be observed. (14). In our experiment the addition of thyroid powder to the thiouracil treatment reduces the weight of the thyroid three fold in four days. Again, this would be expected since an increase in the plasma PBI checks thyrotrophic hormone secretion.

Thiouracil alone, and more so in combination with thyroid powder administration, retards the uptake of I^{131}. The blocking effect of the thyroid powder may be expected for the very same reason given above. However, this effect of thiouracil was rather surprising since it is generally considered that thiouracil does not affect the trapping of iodine by the thyroid but only prevents the binding of iodine to the protein molecule thereby blocking the formation of iodinated thyroglobulin (14, 49, 239). Not only do our results cast some doubt on the question

of the effect of thiouracil on the trapping of iodine, but what iodine was trapped apparently was not prevented from becoming protein bound, since the follicular colloid contained most of the I^{131} after 24 hours. It may be that the effect of thiouracil is not the same in iodine-deficient rats as it is in normal rats, or that the amount of thiouracil taken by the animals was not sufficient to prevent protein-binding at this level but did in some way affect the pituitary-thyroid axis. Therefore, it seems to us that the whole question of the action of thiouracil should be subjected to further research. This has already been suggested but for other reasons by Roche and Michel (178).

Thiouracil has a limited effect on the height of the follicular cells, but its combination with thyroid powder decreases the cell height by more than 50%. The interpretation given above applies as well in this case.

From the above conclusions it would appear that we can no longer be certain that thiouracil does not affect the pituitary-thyroid axis. Not only did it block iodine uptake, but it also decreased the height of the epithelial cells. Although this was not statistically significant, the trend was quite noticeable.

The number of blue follicles decreased along with the weight, the height of the cells and the radioactivity of the gland. The correlation is so consistent that we feel there can be little doubt that a relationship exists between the number of

blue follicles and the physiological state of the thyroid. This was further substantiated by the fact that the radioactivity was invariably associated with the blue follicles.

Inversely, as the number of yellow follicles increased, there was a decrease in the number of blue follicles, the weight, the epithelial cell height, and the radioactivity of the gland. Moreover, examination of the autoradiographs failed to show any radioactivity associated with the yellow colloid.

The above facts support our contention that the staining procedure described is a valid technique for estimating the activity of the thyroid.

EXPERIMENT V

PURPOSE:

To study the effect, if any, of ascorbic acid on the thyroid gland of iodine-deficient rats treated with thiouracil alone or in combination with thyroid powder.

MATERIALS AND METHODS: (Table XII)

Ninety male rats of the Wistar strain weighing between 80 and 100 grams were placed on iodine deficient diet for four weeks and then divided into eighteen groups:

Group A served as a general control and received no treatment.

Groups B, C, D, and E received thiouracil at a level of 0.02% in their drinking water for one, two, three and four days respectively.

Groups F, G, H, and I received in addition to thiouracil 0.8 grams per kilogram of diet of thyroid powder (USP) for one, two, three and four days respectively.

Group J, differed from Group A only in that the animals received a single intra-peritoneal injection of 75 mg. of vitamin C.

Groups K, L, M and N received the same treatment as groups B, C, D, and E except that they received ascorbate for one, two, three and four days respectively.

Groups O, P, Q and R were treated as groups F, G, H and I with the addition of ascorbic acid for one, two, three and four days respectively.

Table XII

Groups:	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
Thiouracil:		+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+
Thyroid:						+	+	+	+						+	+	+	+
As.Ac.										+	+	+	+	+	+	+	+	+
Days of Treatment	0	1	2	3	4	1	2	3	4	0	1	2	3	4	1	2	3	4

All animals received ten micro-curies of I^{131} twenty-four hours prior to sacrifice.

RESULTS: (Table XIII)

The results of this experiment proved to be a confirmation of the preceeding experiment.

Statistical analysis showed control groups A and J to be considerably different from all other groups regarding radio-activity. However, group for group there was no significant difference between those receiving vitamin C and those not receiving a vitamin supplement.

Regarding thyroid weight, the combination of thiouracil and thyroid powder brings about a progressive drop in weight. Again, however, vitamin C played no apparent role in any of these changes.

A statistical analysis of the number of follicles, the color of the colloid and the epithelial height was not made; however, observation of the sections showed that the same pattern appears as did in experiment four.

DISCUSSION AND CONCLUSIONS:

As has previously been stated by DesMarais (60) ascorbic acid does not have any effect on the thyroid itself, but in animals subjected to cold it appears to enhance the peripheral action of the thyroid hormones. This experiment indicates that supplements of vitamin C do not affect the histochemistry, physiology or morphology of the thyroid gland.

Table VIII

	Group	Number of Animals	Activity c.p.m.	Cell Height micra
Without ascorbate	A	5	13871.1 ± 1966.01	18.06 ± 0.81
	B	5	1553.0 ± 514.82	25.52 ± 2.03
	C	4	4245.2 ± 264.16	24.02 ± 0.28
	D	4	666.3 ± 227.14	17.05 ± 1.20
	E	4	1985.7 ± 1071.18	18.70 ± 1.39
	F	5	712.9 ± 296.47	15.96 ± 0.93
	G	3	464.9 ± 101.73	14.27 ± 1.07
	H	4	535.1 ± 296.31	14.85 ± 1.61
	I	5	183.5 ± 31.51	13.04 ± 1.06
With ascorbate	J	4	13487.9 ± 2227.63	18.77 ± 1.78
	K	3	1424.6 ± 262.20	19.80 ± 1.79
	L	5	5249.0 ± 1044.45	20.56 ± 1.53
	M	5	302.2 ± 84.67	21.68 ± 2.04
	N	5	1324.6 ± 292.40	22.40 ± 1.66
	O	5	1716.9 ± 737.45	17.36 ± 0.47
	P	5	429.4 ± 149.36	19.38 ± 0.79
	Q	4	284.1 ± 40.71	15.67 ± 1.02
	R	4	234.7 ± 65.00	11.25 ± 1.52

ADDENDUM

A piece of human thyroid gland was given to us by a pathologist for examination and was accompanied by the case history, microscopic description and diagnosis.

The patient, a forty-five year old female, was admitted for surgery following the clinical diagnosis of hyperthyroidism.

The following is quoted from the pathology report:

Microscopic Description: "Section of thyroid reveal a diffuse hyperplasia of acini characterized by multiple small acini containing only moderate amounts of colloid and lined by cuboidal epithelium. In many areas, the acini are actively secreting but the lumina are very small and almost devoid of colloid. There is moderate lymphatic infiltration and numerous germinal follicles are encountered in the fibrous septae."

Diagnosis: "Diffuse hyperplasia of thyroid."

We prepared the tissue according to the technique previously described.

While there are certain statements made in the foregoing description to which issue could be taken, in general we agree with it except for one important point. We found <u>every</u> follicle without exception to contain colloid and in what appears to us to be normal amounts. Most important, however, was that without a <u>single</u> exception the follicular colloid stained blue. The cells contained colloid droplets and the P.A.S. reaction was uniformly positive.

We concluded that not only was there diffuse hyperplasia but that the gland was producing thyroid hormones at a very high rate.

It would be desirable to examine more specimens of different thyroid pathologies and also to know what if any chemical therapy preceeded surgery.

Although it is perhaps risky to draw conclusions from one case, we believe that this is further confirmation of the hypothesis that the staining procedure developed is a means of assaying the physiology of the thyroid gland and of distinguishing the so called "active colloid" from the "inactive colloid."

It seems likely that this method could be applied with advantage in the pathological study of the thyroid, since a brief examination of the section would give the pathologist an easily recognizable diagnostic aid.

GENERAL DISCUSSION

The principal purpose of this research was to develop a histochemical method for the follicular colloid of the thyroid gland. During the course of this work, however, many interesting observations were made which bear directly on the physiology of the gland. Since some of them do not agree with those made by other researchers in this field certain questions must be raised.

There is, first of all, the question of the rate of incorporation of iodine into the colloid. Leblond and Gross (126) reported that after twenty-four hours radioiodine is found in the colloid only, mainly in the organic form. Our results with normal animals have clearly shown that even after forty-eight hours not all of the iodine is in the colloid. Previous iodine intake plays an important role in the rate of incorporation of organic-bound I^{131}, however, since in the case of iodine deficient rats most of the radioactive material is in the colloid within 24 hours. Further, while in general perhaps the smaller central follicles may be more active as the above authors contend (155), we have observed numerous cases where the autoradiograph indicated a higher rate of incorporation of radioiodine by the larger peripheral follicles than by

the smaller central ones, an observation also made by Wollman and Wodinsky (237). It appears to depend more on the nature of the colloid itself than on the position of the follicle. The question of where organic binding occurs and the rate of organic binding appears to us to be a more variable factor than those who would restrict it to the colloid only seem to believe. Since so many variations occur even within the same species, one cannot project what the situation is in any other so that it is quite possible that, for example, in the shark (86) iodination occurs entirely in the colloid.

Pitt-Rivers and Trotter (162) found inorganic iodine in the colloid of rats previously treated with propylthiouracil. While we found iodine in the cells and the colloid of thiouracil treated rats we strongly suspect that it was in the organic form. Our dosage level of thiouracil may have been inadequate to stop organic binding completely and may be the explanation for our results. However, the fact that Pitt-Rivers and Trotter administered such large doses of I^{131} (200 μc) lead us to suggest that the thyroid was literally flooded by iodine which could well upset the entire physiology of the gland; or, as Michel (149) suggested, in thiouracil treated rats, the iodination reaction in the colloid would not result in organic iodine compounds but more likely in the oxidation of the goitrogenic substance from which iodide would be derived. Whatever the explanation it appears to be true as Barker (20) stated:

"Radioautography obviously cannot reveal the intimate nature of the material comprising radioactive colloid". In like manner while the results of Woolman and Wodinsky (237) are extremely interesting in view of the fact that they found most or all of the radioiodine in the colloid after eleven seconds In C_3H mice, the dose (500-600 μc) via the external juggular route may be sufficient as Linsay et al (214) have pointed out to cause thyroid lesions of a very serious nature, so that damage to the follicles cannot be excluded in their work. They do point out, however, that their results do not preclude the possibility of intracellular iodination. From our observations, we favor the view that iodination can occur both in the cell and in the colloid.

Doniac, Howard, and Pelc (62) have referred to the occasional ring reaction found in normal rats by Gross and Leblond (94) as not observed by them (63). In contrast we have found the ring reaction to be an almost universal reaction in the normal rat through twenty-four hours. We are unable to account for the wide variations in the reports in the literature since in every experiment performed on over five hundred rats the ring reaction has been a consistent observation. One possible factor could be the level of iodine present at the time of administration of the I^{131}.

Our observations, perhaps, agree with those of Leblond and Nadler (154) in their contention that thyroid hormone production is a continuous process going on at all times throughout

the entire gland. Our interpretation may, however, be different. While all follicles appear to be active in the gland in some physiological phase or other, some are in active states of synthesis while others are in an active state of proteolysis. In other words it is felt that the effect of thyrotropin on the individual follicle depends upon the amount of "active colloid" in the follicle. If there is a high concentration of iodothyroglobulin the effect will be to bring about a release of thyroid hormones. On the other hand a follicle which has been depleted of hormone will enter into a state of protein synthesis. If this is what is meant by the "continuous activity hypothesis" then we certainly concur, but if it means that thyroglobulin synthesis is going on at all times in each follicle, then we cannot agree with this view. The presence of an intrathyroidal mechanism controlling the effect would be a reasonable assumption.

Our observations on the appearance of morphological changes in the thyroid due to a low iodine diet appear to conform more closely with those of Maloof et al (140) who found increased thyroid size in eleven days. As was pointed out, our earliest autopsies were made after the rats were on the Remington diet for three weeks, but the gland enlargement was such that it obviously had begun sometime earlier. This is in contrast to Chapman (39), fifty-five days; Leblond and Mann (128), seventy days; Money et al (151), three months, and Halmi (99) who found none after nineteen to twenty days. These

discrepencies may not be as discordent as they appear since the age and weight of the animals varied. Our rats were young (60-80 grams) at the time they were placed on diet. Experiments by others in our department working on vitamin A deficiency have shown that a difference in weight of ten grams at an early age results in weeks of delay in bringing about deficiency symptoms. This may also be true for iodine: the younger the rat, the more rapid the onset of goitre.

Taurog, Tong, and Chaikoff (212, 213) as well as many others earlier have again stressed the importance of the pituitary gland, rather than autonomous control, in the regulation of thyroid iodine metabolism. If we assume that the principal effect of the addition of thyroid powder to the diet of our rats was to increase the blood iodine level mainly as thyroxine, and that this in turn depressed thyrotropin secretion; then the reduction in total number of follicles by one third in three days, thyroid weight loss and the colloid color becoming over ninety percent yellow strongly suggest that the pituitary is the principal controlling mechanism not only in thyroid iodine physiology but of its morphology as well. This does not mean that there is not a measure of autonomous control as suggested by Halmi (100, 104) and Vander Laan (222, 226), but we agree with Taurog et al that this is secondary to the role of the pituitary.

There can be little doubt that the principal effect of the antithyroid drugs is to prevent the organic binding of

iodine. The mode of action of these drugs is not completely understood but is probably due to inhibition of peroxidase activity in the thyroid gland (15). Yet, as has been pointed out, we observed organic binding both intracellularly and within the colloid and have suggested that our dosage level and duration of treatment may have been inadequate. However, a second observation though not statistically significant indicated a trend toward reduction in the height of the epithelial cells over a period of one to four days. We are unable to offer an explanation for this phenomenon but suggest that there may be some secondary effect of this drug at least in iodine-deficient rats. This may be a direct action on the thyroid itself or may in some yet unexplainable way affect the pituitary thyroid axis. Though the two cases are not parallel it is interesting to note that Sellers and Schönbaum (195) have observed an enhancement of the goitrogenic action of propylthiouracil by small doses of thyroxine over a long period. The mechanism of this action is not known, but it indicates that the antithyroid compounds should be subjected to further investigation. It may be that if small doses of thyroxine enhance the goitrogenic effect of propylthiouracil, then the absence of any thyroid hormone may result in slight depression of this action and could account for the drop in the height of the follicular cells in our experiments.

Our observation that Iodine deficient rats exposed to cold for twenty-four hours after receiving radioiodine have

lower radio-activity and changes in the color pattern of the colloid agrees with what has previously been observed of the effect of cold on thyroid function. Seidell and Fenger (194) found that the thyroid glands of sheep, oxen, and pigs in North America have three times as much iodine during June to November as during December to May. Riddle (173) showed that the thyroid of the pigeon enlarged and became more active during the winter months. Ring (174) found that cold stimulated the thyroid in rats and caused a rise in the BMR. Numerous reports of histological changes after cold exposure appear in the literature (61, 114, 204). It has been repeatedly shown that moderate exposure of rats to cold stimulates I^{131} uptake by the thyroid (29, 127, 235) and increases its discharge from the gland (34, 35, 239). In this regard it has also been shown that short-term exposure to cold is more effective than prolonged exposure (above references and (120). The correlation between these facts and our results offer further confirmation that the histochemical stain developed by us is a barometer of the state of activity of the gland.

It is interesting to note that in thyroid glands which are known to be inactive in so far as hormone synthesis is concerned the production of P.A.S. positive granules continues. These granules are for the most part small compared to the colloid droplets one observes in the cells. While in general the colloid droplets are found anywhere in the apical half of the cell, the P.A.S. granules are restricted to a dense layer at the

apical tip of the cell. They can also be seen in great numbers at the edge of the colloid and at the colloid-cell interface. The nature of the secretion appears to be apocrine. This may constitute the glycoprotein moiety associated with thyroglobulin to which the intracellular colloid droplets bond wither physically or chemically. This substance may be partially responsible for the yellow reaction in the colloid with our stain. However, there are undoubtedly other factors. As mentioned, by electrophoresis, Gersh (81) and Wieme and Bekaert (232) have found two proteins present in the colloid of the rat, one being thyroglobulin the other an uniodinated protein. Derrien et al (58,59) in their researches on the preparation and properties of pure thyroglobulin have found three thyroglobulin fractions with constant solubility characteristics. Examination of these products has shown appreciable proportions of non-iodinated impurities and in certain cases denaturation products of thyroglobulin. The solubility characteristics of the thyroglobulin preparations are independent of their iodine content and iodinated amino acids. They conclude that thyroglobulin is probably a protein of practically constant structure and composition in non-iodinated amino acids but whose degree of iodination varies with different physiological conditions. They consider the three fractions probably represent different degrees of association of the thyroglobulin molecules. Even in different species (pigs, cattle, dogs) the thyroglobulins showed little differences in

composition (59). The thyroid protein of each species can contain varying amounts of iodine, but its content of tyrosine and other amino acids is always the same. In thyroglobulins of goitrous animals changes have been observed in the amino acid composition especially as regards the content of tyrosine and cystine.

These facts all point to the possibility that uniodinated thyroglobulin and iodinated thyroglobulin, the extent of iodination, and the degrees of association of the thyroglobulin molecules may influence the staining characteristics of the colloid. Together with the experimental results obtained by us we feel that the evidence indicates that we can distinguish between iodinated thyroglobulin and non-iodinated thyroglobulin, or as we have referred to it as the protein residue remaining in the colloid after proteolysis has split away the thyroid hormones.

Final proof must await the results of the chemical analysis of the two types of colloid which is actually under way.

CONCLUSIONS FROM THE FOREGOING EXPERIMENTS:

1. A staining technique has been developed for the thyroid gland which may be of value in the study of muscle cells and blood cells.

2. Most important it apparently differentiates between thyroid gland colloid containing the biologically active amino acids and that which does not.

3. The above observation indicates that in the normal art at any given time only about fifty percent of the follicles contain potentially biologically active colloidal matter.

4. Under conditions of iodine deficiency almost one hundred percent of the follicles became active immediately after iodine administration and the colloid is blue.

5. In contrast thiouracil-thyroid powder treated rats, which approach hypophysectomy conditions, are unable to produce thyroid hormones and over ninety percent of the follicles are yellow.

6. Since both types of colloid give a positive P.A.S. reaction, it is reasonable to assume that the glycoprotein remains in the colloid and serves as a substratum to which the thyroglobulin is intimately bound either physically or chemically.

7. The P.A.S. staining granules are produced independently of the colloid droplets and for the most part are smaller and are restricted to the apical tip of the cell. This production continues even in the absence of any apparent colloid droplet formation by the cell.

8. It has been found that the Remington iodine-deficient diet is capable of producing morphologically recognizable conditions of a state of goitre in three weeks and that this is advanced to such a degree as to be effective in even a shorter period.

9. Thiouracil would appear to affect more than the protein binding of iodine in the iodine-deficient rat although its role by other researchers has been restricted to this action. This fact is indicated morphologically and physiologically.

10. Contrary to most reports in the literature it is felt that evidence has been obtained for the fact that protein-binding of iodine can and does occur intracellularly. This does not imply that it does not occur as well in the colloid or at the colloid-cell interface.

11. It also appears that not all the iodine is located within the colloid after twenty-four hours in normal rats. It has been our experience that it is closer to forty-eight hours. In iodine deficient rats and rats exposed to cold, however, incorporation into the colloid occurs more rapidly and is usually, though not always, complete after twenty-four hours.

12. In one case a reversal of polarity appeared to occur; since this was the only obvious case it does not appear that this is the usual situation in the thyroid.

13. The regression of the hyperplastic gland by the addition of thyroid powder in three days favors the hypothesis that the pituitary is the main pace-maker in thyroid iodine metabolism.

14. Short exposure to cold has been known to bring about the more rapid release of hormones from the thyroid gland. Our results though not statiscally significant support these findings.

15. Short term ascorbic acid supplementation does not appear to affect either the physiology or morphology of the thyroid gland.

16. It is suggested that the histochemical method developed may be of practical aid to the pathologist in his diagnosis of thyroid disorders.

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