THESIS

AN EVALUATION OF A BIOASSAY METHOD FOR THYROID STIMULATING HORMONE AND ITS APPLICATION TO *CITELLUS RICHARDSONI*

Submitted by

William A. deGraw

In partial fulfillment of the requirements

for the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

May, 1965

COLORADO STATE UNIVERSITY

May 10 1965

WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY WILLIAM A. DEGRAW

ENTITLED AN EVALUATION OF A BIOASSAY METHOD FOR THYROID STIMULATING HORMONE AND ITS APPLICATION TO CITELLUS RICHARDSONI

BE ACCEPTED AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

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An *in vitro* bioassay for thyroid stimulating hormone (TSH) based upon the TSH-stimulated release of I^{131} was evaluated. Significant error was not introduced by the method of pipetting aliquots of incubation medium in order to estimate I^{131} activity. Incubation times for uptake and release agree with those suggested by Bottari's group.

While per cent uptake and absolute release of I^{131} were significantly related to the weight of tissue incubated, per cent release was not, and weighing of tissue before incubation was not necessary. It was found that storage of TSH in plasma at -10° C did not preserve TSH activity, thus fresh samples were used. U.S.P. reference standard TSH was quantitatively recovered from horse serum, rat plasma, and richardson ground squirrel plasma, with no apparent inhibition and with the linear range of the log-dose response lying between 5--200 ImU/100 ml.

Assay results are reported for male Holtzman strain rats. *C. richardsoni* plasma from summer and winter squirrels and one spring squirrel was assayed with the following results which support indirect findings of other investigators: summer, 34 ImU/100 ml; winter, 4 ImU/100 ml; spring, 116 ImU/100 ml.

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May, 1965

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CHAPTER I

INTRODUCTION

The ability of certain mammals to hibernate represents a complex adaptation for survival under adverse environmental conditions. The exact mechanism by which hibernators adapt to seasonal environmental stresses is not yet well understood. It is known, however, that prior to and during hibernation the anterior pituitary and other endocrine glands involute.

The precise role of endocrine involvement is not agreed upon, nor has the total significance of the hypophyseal-pituitary axis been directly established. Most studies, indirect in nature, of the endocrine glands indicate pituitary hypofunction during hibernation. However, few direct measures of pituitary activity have been attempted, and no evaluation of circulating thyroid stimulating hormone (TSH) in hibernators is known.

Nearly 100 methods have been reported for the bioassay of TSH. Of these techniques only a few possess the sensitivity necessary to quantitatively detect levels of this hormone in body fluids. Furthermore, many of the methods are difficult and time consuming. Some early qualitative and quantitative assays of blood and urine revealed TSH only in certain pathological states such as myxedema and Grave's disease. The quantitative determination of TSH in plasma of euthyroid individuals has been achieved by only a few methods. Often the TSH activity of the blood and urine must be concentrated prior to its assay due to the presence of inhibitors.

Delimitations

The first objective of this project was to review the available bioassay methods and select a sensitive and convenient technique. The usefulness of the method chosen for the detection of TSH in plasma was to be evaluated. The final goal was the estimation of circulating TSH in the ground squirrel *Citellus richardsoni*, including a study of seasonal variations, if possible.

CHAPTER II

REVIEW OF LITERATURE

Endocrine activity in hibernators

The thyroid gland shows a seasonal pattern of activity in hibernators. Prior to hibernation the thyroid involutes and reactivation may occur before arousal in the spring (65, 52). Neither the thyroid nor any other single endocrine gland is thought to be the controller of hibernation, but its variable activity with respect to hibernation does indicate involvement.

Associated with general endocrine involution in hibernators is decreased metabolic rate, activity, and food intake. The involution occurs at a definite time and is not correlated with environmental temperature change (52). Citellus richardsoni adults were not seen above ground after late July or early August, while pups disappeared later, August 30 (40). The same species, observed in this laboratory, decreased their food and water intake during September. These general changes are reversed in the spring at specific times, depending upon the species. Richardson's ground squirrel males were first observed above ground on the first of April, and the females beginning on the seventh of April (40).

Deane and Lyman (23) compared the responses of rats and hamsters to cold. The observed signs of a typical stress response in rats including enlargement of the zona fasiculata and lipid

depletion in the zona reticularis of the adrenal gland. Increased activity of the thyroid gland typified by increased epithelial cell height and colloid depletion was observed.

On the other hand, adrenal glands of hamsters exposed to cold showed an increase in zona glomerulosa thickness and augmentation of lipid in the zona reticularis. Thyroid activity was not significantly altered as a result of cold exposure (23).

The arctic ground squirrel, Spermophilus undulatus, has been studied in a similar manner by Mayer and Bernick (47). Thyroid activity in warm and active S. undulatus was characterized by round, oval, or polygonal follicles of varying sizes, containing abundant well-staining colloid, with some vacuoles and intracellular thyroglobulin present.

In arctic ground squirrels hibernating for at least one week, striking histological evidence of inactivity was observed. Thyroid follicles were distended, containing deeply staining vacuole-free colloid, squamous epithelium, and no apparent intracellular thyroglobulin. Within 24 hours after arousal, the thyroid appeared active with reduced follicular size, cuboidal epithelium and intracellular thyroglobulin.

The adrenal gland, which is sudanophilic in every layer in the non-hibernating arctic ground squirrel, showed lipid depletion in all layers during hibernation; the greatest loss occurred in the zona glomerulosa. The adrenals were essentially normal 24 hours post-arousal, with highest lipid content in the zona glomerulosa (47).

I^{131} uptake by the thyroid of hibernating ground squirrels was found to be negligible by Vidovic and Popovic (65). However, 4 hours after arousal, the I^{131} content of the thyroid was equivalent to that of non-hibernating squirrels or rats.

Foster, Foster, and Meyer (28) as well as Kayser, Petrovic, and Weryha (42) have observed the effects of administering thyroid hormone and TSH to animals in hibernation. In either case, hibernation ceased or the frequency decreased following treatment.

Suomalainen (62) measured per cent epithelium and per cent colloid in the thyroids of active and hibernating hedgehogs, and found thyroid activity greatest in the spring and lowest during hibernation.

In hibernators, then, thyroid activity does not increase in response to cold exposure due to apparent insensitivity of the hypophysis to cold. In fact, hamsters exposed to 5° C without bedding material maintained normal body temperature without evidence of thyroid or adrenal hypertrophy. This endocrine insensitivity is considered typical of hibernators, as a factor permissive to, but not the cause of hibernation by Deane and Lyman (23).

Mayer and Bernick (47) presented histological evidence of hypophyseal involution. They observed acidophils, basophils, and chromophobes in the hypophysis of the non-hibernating arctic ground squirrel. In those animals sacrificed during hibernation only chromophobes were observed. If the absence of staining in basophils is accepted as evidence of inactivity, then decreased thyroid activity during hibernation is explained.

Suomalainen (61), on the basis of histological findings in the hedgehog (Erinaceus europaeus), argued that the general adaptation syndrome occurred in this hibernator, and claimed pituitary hyperactivity during hibernation. Using chrome-alum-hematoxylin-phloxin and aldehyde-fuchsin stains, the seasonal changes in granulation of hypophyseal neurosecretory cells and the estimated volume of cell nuclei of the supraoptic nucleus were studied.

Nuclear volume was smallest in early summer, but steadily increased during the pre-hibernation period to a maximum during hibernation. Hypophyseal secretory cells contained few secretory granules during early summer, the axons being poorly stained. Granules were observed in the terminal Herring bodies, however, and Suomalainen suggested minimal neurosecretion in summer. In the fall accumulation of secretory granules was observed both in the neurosecretory cells and intracellular regions, and enlargement of the neurosecretory axons was also evident. During hibernation neurosecretory material was present as fine granules which were most evident in distal regions of the supraoptico-hypophyseal tract. From this, as well as capillary enlargement, Suomalainen concluded that intense neurosecretion occurs during hibernation (61).

With respect to TSH and thyroid activity in hibernators, histological evidence strongly supports the hypothesis of pituitary hypofunction during hibernation in most hibernators. However, low iodide uptake (65), responses to TSH and thyroid hormone, and removal of endocrine glands (28,42) represent indirect

evidence, only. In view of inherent uncertainties involved in such demonstrations, one might test the hypothesis by application of an objective biological assay for TSH in a hibernator.

A direct approach was taken by Denyes and Horwood (24) in which quantitative measurement of circulating steroids was reported in active and hibernating hamsters.

The seasonal variations in 5-hydroxytryptamine content of various regions of the brain including the hypothalmus were studied by Uuspaa (64). However, no reports of quantitative determination of TSH in a hibernator were found.

Recognition of a pituitary-thyroid relationship

In 1851 Niepce observed enlargement of the pituitary in goitrous cretins, and is believed to be the first to recognize a relationship between the pituitary and thyroid glands (49). Renon and Delille demonstrated histologic changes in the thyroid glands of rabbits after injection of pituitary extracts in 1908, which is considered to be one of the first qualitative assays for TSH (49).

Allen (5) proposed the existence of an active substance of anterior pituitary origin which caused growth of the thyroid gland in 1920. His conclusion was based upon a series of experiments involving the implantation of adult Rana pipiens pituitary lobes in hypophysectomized tadpoles of the same species. By careful

dissection of the pituitary and implanting anterior and posterior lobes separately into hypophysectomized tadpoles with and without thyroid glands, he observed the effects of a pigment-producing factor (MSH), growth-producing factor, and a thyroid-stimulating factor. While Allen made no attempt to isolate or name the hormones involved, he recognized the wide-spread effects of the pituitary. P. E. Smith is credited with the discovery of TSH (59). In experiments with hypophysectomized tadpoles it was shown that administration of bovine pituitary affected the reactivation of the thyroid gland.

Early TSH bioassay

The first quantitative bioassay for TSH was devised by Junkmann and Schoeller in 1932 (41). The method was based on histological changes in the thyroids of guinea pigs injected with pituitary extracts. Measurement of extract potency was in terms of the Junkmann-Schoeller unit (J.S.U.) which was defined as "that amount of thyrotrophin extract required to produce definite signs of histological stimulation in one out of two guinea pigs after three daily injections" (41).

The Heyl-Lacquer method and other histological assays which evolved from the original Junkmann-Schoeller technique tended to be highly subjective and laborious, requiring several days to complete (16, 39).

Using the guinea pig, the limit of sensitivity was lowered by deRobertis (56) to 0.0025 J.S.U. per ml, or approximately 0.02 U.S.P. units per ml. The number of colloid droplets in a theoretical cell 100 microns in diameter was calculated as an index of TSH activity. TSH was detected with this method in acetone extracts of hemolyzed blood from euthyroid individuals.

Guinea pigs and day-old chicks were primarily used in TSH assays based on hormone-stimulated histological changes. Adams and Beeman (1), comparing guinea pigs and chicks, indicated a preference for chicks in such methods. Their thyroids were found to be more uniform histologically and they responded to a smaller quantity of test material. Rats have been used, but with little success in the quantitative determination of TSH using histological changes in the thyroid as the test response (38).

A rather long-term effect of TSH is stimulation of increased thyroid gland size, and consequently its weight. Assays based on the thyroid weight response were developed using the day-old chick, mainly because these animals have very low levels of endogenous TSH (29, 58). The method of Smelser (58) involved the subcutaneous injection of TSH or test substance twice daily for 5 days. The wet-weight of the thyroid was related to the dose level of TSH, but the sensitivity of the method was not great. TSH activity was detected in pituitary extracts, but the assay gave no indication of TSH activity in blood or urine. Methods utilizing chicks were probably the most useful of this type in the 1940's, since the weight response in guinea pigs was much

less sensitive (9). These methods were useful only in the assay of highly potent pituitary extracts.

A very sensitive assay was reported by D'Angelo in 1942 (20). Fasted, "stasis" tadpoles (R. pipiens) were injected with small volumes (0.05 ml) of test substance or TSH on 5 alternate days. Observation of thyroid stimulation, including hypertrophy, hyperplasia, colloid depletion, increased epithelial cell height, decreased body weight, and increased hind limb length were the basis of D'Angelo's tadpole unit of TSH. The tadpole unit was defined as the minimal amount of TSH which would result in a loss of body weight 50 per cent greater than controls (water injected) accompanied by thyroid activation after injections of the material on 5 alternate days (20).

This assay, based on a response in the tadpole first recognized by Allen (5) 22 years earlier, was 60--100 times more sensitive than any contemporary method, allowing the detection of TSH in some euthyroid serum samples (16, 21).

Very few bioassays had as their basis TSH induced alteration of metabolic rate. The response, particularly in vivo, is highly variable. Galli-Mainini reported the successful use of an in vitro method based upon the metabolic changes following incubation of thyroid tissue with TSH or thyroxine. Measuring oxygen consumption of guinea pig tissue using the Warburg apparatus he demonstrated that TSH stimulation produced an increase in Q O_2, while thyroxine depressed oxygen consumption. A composite assay was designed

based on this antagonistic effect of TSH and thyroxine, and was applied to several clinical states (30, 31).

An inverse relationship between phosphorus and iodine in the thyroid was first observed by Oswald in 1899. Borell and Holmgren later reported that TSH-stimulated uptake of phosphorus was useful in the determination of TSH (11).

Measurement of TSH-related changes in iodine (I^{127}) in thyroid tissue was unsuccessfully applied as a bioassay due to the lack of sensitivity and difficulty of chemically determining iodine (17).

TSH units

The numerous TSH units set forth by early assayists made comparison and standardization of various techniques difficult. Few attempts were made to equate the various "animal units" and the situation remained "chaotic" until the time of Albert's review in 1949 (4). This situation existed in spite of the fact that a reference standard had been established in 1938 (16).

Albert was critical also of the great number of modifications, pretreatments, and special diets described in TSH assays. Another disturbing feature of TSH bioassays, as Albert reviewed, was the ever present seasonal, species, and strain variations which plagued investigators (8). The result was a list of TSH units, many of which were "freely floating variables" depending on the above listed factors (4).

The establishment of an international TSH unit by a committee of the World Health Organization (50), and the expression of potency in terms of reference standard has greatly improved the situation.

Methods before 1949

Methods available up to 1949 were not suited for the bioassay of TSH in plasma and urine because of insensitivity to the very low circulating levels of TSH. The hormone was not often detected using untreated plasma or urine, and if at all, only after concentration of TSH from large volumes of blood or urine. Results were often inconsistent in repeated assays of the same sample. Separation and concentration of TSH activity in plasma and urine was generally inefficient. Using acetone precipitation, the most common method, only about 30 per cent of the TSH activity could be recovered (4).

Representative modern methods

The assays described above are mainly of historical interest, since their sensitivities have been surpassed by more recent methods. Improvement of histological methods and particularly the introduction of radioisotopes (I^{131} and P^{32}) made possible new simplicity, convenience, sensitivity, and objectivity. In reviewing these more recent methods emphasis will be on those assays which are useful in the determination of TSH in blood.

The further improvement of D'Angelo's "stasis" tadpole method placed it among modern assays (19, 20), even though its basis was TSH-stimulated increase in acinar cell height. A linear log-dose response curve was obtained over the range 0.1--30 milliunits of TSH. This highly sensitive method has been useful in the evaluation of pathological states (21) and the elucidation of pituitary-thyroid relationships (22).

The thyroidal uptake of P^{32}, first studied by Borell and Holmgren (11), was made the basis of a sensitive assay in the day-old chick by Greenspan, Kriss, Moses, and Lew (35). The 24 hour uptake of P^{32} *in vivo* quantitatively measured TSH between 0.02 and 0.05 U.S.P. units per ml, and was not affected by FSH, prolactin, ACTH, vasopressin, histamine, epinephrine, or thyroxine.

Bakke and Lawrence investigated *in vitro* radioiodide uptake and release by surviving beef thyroid slices, and found that there was also a weight increase associated with TSH stimulation (7). This latter response was made the basis of a bioassay technique which stands alone as to type (6). The increase in wet-weight of thyroid slices was linear over the range 0.08--17 milliunits of TSH per ml of plasma. Due to the presence of a TSH-inhibitor in plasma, TSH activity was separated from inhibitor substance by using an ion exchange resin column (37). The experimental design allowed analysis by the method of Bliss (10), and possessed high precision. The weight response is interesting in that it is not

altered by treatment with propylthiouracil or thiocyanate. The response is blocked, however, by cyanide or dinitrophenol (6).

The same response was restudied in vivo in the guinea pig by Gedda (32), but did not appear to be sensitive enough for an assay response.

The uptake of iodide (I^{131}) as a function of TSH stimulation has been studied as the basis of bioassay in tadpoles (18), rats (33, 34, 46, 51), mice (48, 55), and day-old chicks (53).

The use of rats requires either hypophysectomy (33) or pretreatment (51), since the normal activity of this species' thyroid is relatively high and insensitive to TSH stimulation. Pretreatment is preferred by most because of its convenience, and because assay results with pretreated animals are generally as good as those obtained with hypophysectomized animals. The sensitivity of uptake methods does not always approach that of other assay-types. The method of Overbeek (51) has a range of sensitivity between 20 and 200 milliunits and requires three days to complete, thus giving a time advantage over histological assays, but lacking the same order of sensitivity (19).

The method of Adams and Purves (2, 3) depends upon the TSH-stimulated release of isotopically labelled hormone from the thyroids of guinea pigs pretreated with thyroxine. The pretreatment period, which includes administration of thyroxine daily and 20 microcuries of I^{131} on the second day only, involves a total of 7 days. On the eighth day the I^{131} activity of the blood is

estimated before and 3 hours after TSH is administered intr-venously. The response of a given animal to various doses of standard is tested daily, since variations within individuals are small while animal-to-animal variations are quite large. The design allows determination of both a standard curve and a series of responses to unknown material in the same animal. Thus, the results of responses of a single animal on successive days to randomized treatments make possible analysis without animal-to-animal variation.

The range of sensitivity of this method lies between 0.5 and 3.0 milliunits of TSH per ml, the lowest detectable dose being 0.1 milliunit per ml.

McKenzie (48) has increased the sensitivity of the above method to include the range 0.05--3.2 milliunits of TSH per ml by using the mouse. Both assay designs (2, 3, 48) can be statistically analyzed. Both require pretreatment time, and the first requires considerable skill in handling guinea pigs. McKenzie's method would seem to involve less problem in this respect, and be more useful in terms of sensitivity.

In vitro bioassay

TSH-stimulated release of I^{131} *in vitro* was evaluated by Bakke and Lawrence (7) using beef thyroid, and by Bottari and Donovan (14) with guinea pig tissue. The assay based on the release response of guinea pig thyroid reported by Bottari, Donovan, and El Kabir (15) yields a linear log-dose relationship between

0.05 and 1.5 milliunits of TSH per ml, with 0.005 milliunits being the smallest detectable amount of TSH.

The method as reported appears to be quite simple, specific, precise, and requires only a single day to complete. However, uptake and release of I^{131} are estimated from the relative radioactivities of samples of the incubation medium taken before and after the addition of TSH, making the sampling process a critical step of the method.

In vitro methods have several advantages over *in vivo* techniques. The TSH material to be tested is "confined in a small volume with the target tissue;" the dilution which occurs in *in vivo* studies is no longer a factor. Rapid destruction or removal of TSH by other body tissues is avoided. Finally, thyroid tissue from a single animal can be used for a complete assay, including standard and unknown (13). The problem of animal-to-animal variability is not a major factor in such a design, since a standard dose-response curve is constructed in each assay.

The method of Bottari, *et al.* (15) is designed to allow complete regression analysis and estimation of the confidence interval on potency. It is also possible to check for significant deviation from parallelism between standard and unknown. As was noted, the method of McKenzie is designed to permit analysis, but unlike the *in vitro* method requires six animals at each dose level.

Bottari's *in vitro* technique is claimed to have the further advantage of permitting the use of untreated plasma, as opposed to

other methods which give best results after concentration of TSH activity or removal of inhibitory factors.

TSH inhibitor substances

The presence of an unidentified TSH inhibitor in human urine was demonstrated by Starr, et al. (60) and Lepp (45). Using several assay methods, including I^{131} release in day-old chicks, significant inhibitor activity was detected in the urine of hypothyroid patients. Since some of these patients were on thyroid therapy, l-thyroxine, d-thyroxine, triiodothyronine and triiodoacetic acid were also tested. However, none of these produced significant inhibition at normal circulating levels.

Greenspan and Lew (36) observed a flattening of the standard TSH curves using I^{131} release in the day-old chick due to an inhibitory substance in urine of normal and hypophysectomized dogs and man.

Heideman, Bakke, and Lawrence (37) experienced difficulty with a TSH inhibitor in human plasma using the in vitro beef thyroid weight response. The TSH-stimulated weight increase was not observed in response to human pituitary plus plasma, while human pituitary alone elicited the response. No activity was detected in whole plasma. A carboxylic ion exchange resin column was used to separate TSH and inhibitor. In 14 out of 15 trials 100 per cent recovery of TSH activity added to plasma was reported. This TSH fraction was free of inhibitor after elution.

Postel (53) used zone electrophoresis to successfully separate TSH and inhibitor, and McKenzie (48) employed starch block electrophoresis and Cohn fractionation to eliminate the problem of inhibitor interference in the assay of human serum. Using an I^{131} release assay in mice, TSH was detected chiefly in the gamma globulin fraction of both myxedematous and normal human serum, from which plasma concentration was calculated.

The *in vitro* I^{131} release bioassay devised by Bottari, *et al.* (15) has the advantage that treatment of plasma is not necessary. This is indeed an advantage in terms of time, equipment, and in the case of this study volume of plasma available. That treatment of plasma is not necessary is indicated indirectly by the results of El Kabir (25), Bottari (12, 13), and Bottari, *et al.* (15) in the rabbit and rat and by El Kabir, Doniach, and Turner-Warwick (26) in man.

Selection of a method

Of the modern methods, those of D'Angelo and Gordon (19), Adams and Purves (2, 3), Bakke, *et al.* (6), McKenzie (48), Kirkham (43), and Bottari, *et al.* (15) are most sensitive, all having a lower limit of sensitivity lying below 0.5 International milliunits of TSH per ml.

In addition to being one of the most sensitive, the method of Bottari, *et al.* (15) has the advantage of detecting TSH quantitatively in untreated plasma or serum. Bottari has not found TSH inhibition in his assay system, and reports TSH concentrations in

human blood which agree with previous reports by other assayists (13, 26, 49). The method has the additional advantage of convenience, since a determination can be completed in one day. This is in contrast to a similar *in vitro* technique with the same sensitivity proposed by Kirkham (43) which requires nearly two days for completion.

The method has disadvantages associated with tissue culture technique and with pipetting accuracy. The success of the method is dependent upon careful pipetting of aliquots of medium, since estimation of uptake and release of I^{131} is based entirely on changes in the radioactivity of the incubation medium. The method of Bottari, *et al.* (15) was chosen because of its sensitivity, simplicity, and because small volumes of untreated plasma can be tested for TSH activity quantitatively accurate results.

CHAPTER III

METHODS AND MATERIALS

Basis of method

The bioassay (15) is based upon the TSH-stimulated release of iodide (I^{131}) from guinea pig thyroid fragments *in vitro*. Chromatographic analysis of the incubation medium showed 88 per cent of the I^{131} released from the fragments after TSH addition to be iodide and that labelled thyroxine release was not detected (15).

Studying this phenomenon *in vitro*, Powell, Rahman, and Deiss (54) found the release of iodide by thyroid tissue to be "highly specific for TSH." While total iodide remained relatively constant in control slices, TSH (1 International milliunit per ml) caused iodide release without enhancing organification. A higher concentration of TSH first accelerated and later inhibited organification. Antithyroid agents which block organic binding did not alter the response. Enhancement of proteolysis was suggested as the origin of iodide. A release of iodide by the thyroid *in vivo* was observed by Rosenberg, Athans, and Behar (57).

Description of method

Male guinea pigs of mixed strain were obtained from a single supplier in Loveland, Colorado throughout the entire study. Prior to pretreatment these animals were caged in groups of six at temperatures between 70° and 78° F, and received food (Purina

guinea pig Chow) ad libitum. Body weights ranged from 250 to 350 grams at the time of use for bioassay.

On each of five days preceeding use for TSH assay guinea pigs received a subcutaneous injection of Tapazole (methimazole, Eli Lilly, Co.), 10 mg in a dose volume of 0.1 ml of physiological saline. On the day following the fifth injection of Tapazole, guinea pigs were sacrificed by a sharp blow at the base of the skull. An incision was made along the mid-ventral aspect of the neck, and the thyroid gland exposed by blunt dissection. The two lobes of the thyroid gland were quickly removed and placed in an inverted Petri dish containing cold Gey's solution.

The balanced salt solution used in the preparation of tissue and in the incubation medium was a modification of Gey's solution containing (in grams per liter): NaCl, 8.0; KCl, 0.38; $MgCl_2 \cdot 6H_2O$, 0.21; KH_2PO_4, 0.025; Na_2HPO_4, 0.120; glucose, 2.0; and $NaHCO_3$, 0.25 (Bottari, et al., 15).

Each thyroid lobe was trimmed of fat and connective tissue with care taken to keep the tissue moist during this procedure. Each lobe was then fragmented using small scissors. The diameter of fragments did not exceed 0.5 mm. The resulting tissue fragments were divided into 8 portions approximately equal in size.

Each of the 8 portions was blotted once on filter paper and placed in a 25 ml Erlenmeyer flask containing 1 ml of incubation medium. The incubation medium consisted of 70 per cent, by volume, Gey's solution, 30 per cent horse serum, and one drop of phenol red

indicator (1 per cent solution) per 50 ml of medium. Horse serum was prepared aseptically from blood collected from healthy animals in the Colorado State University Veterinary Clinic. Serum was frozen until use. Antibiotics were added such that the final concentrations were as follows: dihydrostreptomycin (sulfate), 0.25 mg per ml and penicillin G, 20 units per ml. The tissue was incubated for 8 to 10 hours in stoppered flasks, at pH 7.4--7.6, with a gas phase of 95 per cent oxygen, 5 per cent carbon dioxide. By using stoppered flasks, the pH was less variable than with open flasks under a gassing hood. Incubations were carried out at 25° C in a Dubnoff metabolic shaking apparatus, with the shaking rate set at 50 oscillations per minute. Three control flasks containing 1 ml of incubation medium, but no tissue, were incubated concurrently in order to calculate uptake of I^{131} by the tissue.

The initial incubation of guinea pig thyroid fragments for 8 to 10 hours allowed maximum I^{131} (iodide) uptake by the tissue. This period was sufficient to obtain a maximum uptake which was approximately 30 per cent in most cases as measured by decrease in the activity of the incubation medium. The activity of carrier-free NaI^{131} (obtained from Abbott Laboratories, Oak Radge) in the incubation medium was 0.5 microcuries per ml.

At the end of this incubation, 0.1 ml of NaI^{127} carrier solution, 6 micrograms per ml, was added to each flask containing tissue, and to three control flasks containing 1 ml of incubation medium, but no tissue. Carrier iodide serves to mask the effect

of iodide recirculation upon the assay index, TSH-stimulated iodide release. After the addition of carrier, additional incubation for one hour allowed equilibrium to be established.

One hour after the addition of carrier solution, 0.1 ml of either U.S.P. reference standard TSH or unknown plasma was added to each flask, including control flasks. Immediately after gentle shaking, one 0.20 ml aliquot of medium was taken to estimate uptake of I^{131} by comparison with analagous samples from control flasks. Reference standard TSH was obtained from U.S.P. Reference Standards, 46 Park Avenue, New York 16, New York. Both reference standard and unknown were prepared as serial dilutions in incubation medium which contained no I^{131}.

All pipetting was done with trap-type micropipettes with self-adjusting capillaries. They were quickly and thoroughly washed between samples successively with KI^{127} solution (1 g/l), distilled water, and acetone.

After removal of the first aliquot, flasks were returned to the Dubnoff apparatus for 1 hour and 45 minutes additional incubation with TSH or unknown. After this incubation period one 0.20 ml aliquot of medium was taken in order to estimate release of I^{131} by the thyroid fragments.

The radioactivity of the medium samples was estimated using a Nuclear-Chicago well scintilation counter and a Radiation Instrument Development Laboratories scaler. The aliquots of medium were prepared for counting by adding 2 ml of KI^{127} solution (1 g/l) to glass counting tubes.

Calculation of per cent release

The mean counts per minute of samples of the medium before and after TSH-stimulated I^{131} release and of control samples were employed to calculate per cent release.

Letting y_i equal mean counts per minute of a sample for a given flask before release, z_i equal mean counts per minute of a sample for the same flask after release, and $\bar{x}$ equal mean counts per minute of samples from control flasks, the calculation of uptake and release was as described below.

Since the total volume per flask after the addition of TSH or unknown was 1.2 ml (1 ml of incubation medium, 0.1 ml I^{127} solution, and 0.1 ml of TSH or unknown), total uptake of I^{131} was calculated by comparing radioactivity in each of the 0.20 ml aliquots, y_i, to the average of aliquots from the control flasks, $\bar{x}$, and correcting to total uptake in counts per minute per flask. In terms of the definitions above uptake, $U = 6\,\bar{x} - 6\,y_i$.

The next aliquot, taken after 1 hour and 45 minutes, was used to calculate release of I^{131}. This 0.20 ml sample was taken from the total volume remaining, 1.0 ml (1 ml + 0.1 ml + 0.1 ml - 0.20 ml). By comparison with the first estimate of activity, y_i, the total release in counts per minute per flask was calculated: $R = 5\,z_i - 5\,y_i$.

Per cent release was then calculated from the relation:

$$R_{\%} = \frac{5\,z_i - 5\,y_i}{6\,\bar{x} - 6\,y_i} \times 100 ,$$

which was linearly related to the logarithm of the dose of TSH.

Statistical analysis

Analysis of variance and factorial analysis were applied to 2 X 2 and 2 X 3 factorial bioassay experiments. The method of Bliss (10) was used.

There are certain assumptions and conditions associated with the factorial design. Both designs involve the use of a comparative standard curve in each assay. Thus the 2 X 2 was a comparison of two standard dose responses with two unknown dose responses. The 2 X 3 was a comparison of three standard and three unknown dose responses. The variance at each dose level must be of equal magnitude and there must be an equal number of replications at each dose level. In this study duplicate responses were observed at each dose level. While it is not necessary that the potency of the standard and unknown be matched perfectly, the log-dose interval must be equal in standard and unknown dilutions.

Equal log-dose interval and equal numbers allowed the use of factorial coefficients. In the 2 X 2 assay design, the null hypotheses of dose response, sample difference, and divergence from parallelism were tested. A valid assay required a significant dose response, or slope, and no significant divergence from parallelism. The 2 X 2 analysis can not indicate deviations from linearity, and could be used only in regions were linearity had been established.

Source and treatment of C. richardsoni

Richardson ground squirrels were collected at various intervals between June and August by W. A. House, using live-traps, on the Russell ranch near Tie Siding, Wyoming.

Blood was collected from active summer squirrels in a heparinized syringe by cardiac puncture under ether anesthesia. The blood was centrifuged immediately, and the plasma frozen. Some ground squirrel plasma was derived from captive squirrels in the same manner, but assayed immediately.

Squirrels trapped during the summer were maintained on laboratory diet (Purina Checkers) until January when they were placed in hibernation cages at between 0° and 5° C. Hibernating squirrels were decapitated and blood collected at that time. Plasma was stored by freezing.

CHAPTER IV

RESULTS AND DISCUSSION

Pipetting of aliquots

The greatest potential for error in this system was inconsistent pipetting of doses and aliquots, since the measurement of responses is determined from the radioactivity of samples of the incubation medium.

Micropipettes of constant bore gave less consistent volumes than micropipettes with self-adjusting capillary and trap. The latter type was used, and error in estimation of I^{131} activity was minimal when aliquots were transferred to a dry counting tube with no rinsing of the pipette. Two ml of carrier solution were later added before counting in the well scintillation counter. From a single sample of medium containing I^{131}, the activity, estimated from 5 aliquots prepared as described above, was 24,182 counts per minute with a standard deviation of 1.4 per cent. On the other hand, rinsing the micropipette with carrier and adding the rinse activity to the aliquot increased the standard deviation to over 2 per cent of the estimated activity.

Using the trap-type micropipette, samples of medium of low specific activity produced the results in table 1. Each of the samples from the same medium were counted three times.

Table 1: Analysis of error due to pipetting and counting

Sample	Replicate counts counts per minute		
1	1752	1746	1721
2	1694	1704	1721
3	1709	1752	1759
4	1745	1731	1740
5	1729	1712	1734

Analysis of variance showed that variations due to sample and to replicate counts were not greater than expected from random experimental error in either case. The F ratio for pipetting was 3.84 (NS), and that for counting 4.46 (NS).

In experiments involving thyroid fragments, removal of duplicate aliquots of the medium confirmed this precision of pipetting.

Uptake of I^{131}

Bottari and Donovan (14) suggested an 18 hour incubation of guinea pig thyroid tissue with I^{131} for optimal uptake. Later, El Kabir (25), a member of Bottari's research group, reported that an 8 to 10 hour incubation with I^{131} was sufficient. Since it was not possible to obtain guinea pigs of the same strain as those used by Bottari, and since incubation in flasks represents a modification of Bottari's roller-tube technique, the characteristic uptake of I^{131} was studied.

The basic method was followed in terms of tissue preparation and incubation conditions. Uptake was estimated by successive 0.1 ml aliquots of the incubation medium. I^{131} uptake was expressed as percentage of total I^{131} in the medium removed by the thyroid fragments. Figure 1 depicts a characteristic uptake curve for guinea pig thyroid fragments incubated at 25° C. Based on such results an 8 hour uptake incubation period was adopted.

The measured dose-dependent response, per cent release of I^{131}, involves estimation of both uptake and release. Bottari, et al. (15) reported that it was not necessary to distribute thyroid fragments equally on the basis of weight, since per cent release is a function of the active tissue present. Since this statement was not supported by published results, knowledge of the effect of amount of thyroid tissue on measured uptake and release was important in evaluating the method.

Therefore, the relationship of per cent uptake to tissue weight was studied. The regression of per cent uptake on tissue weight was significant, and is plotted in figure 2. Thus there exists a relationship such that if one were measuring uptake as the dose-dependent response, it would be necessary to control tissue weight in order to avoid introduction of an additional error due to amount of tissue present. However, this result alone does not oppose Bottari's suggestion that weight need not be equalized when per cent release is measured as the dose-dependent response. The nature of the release response must also be considered.

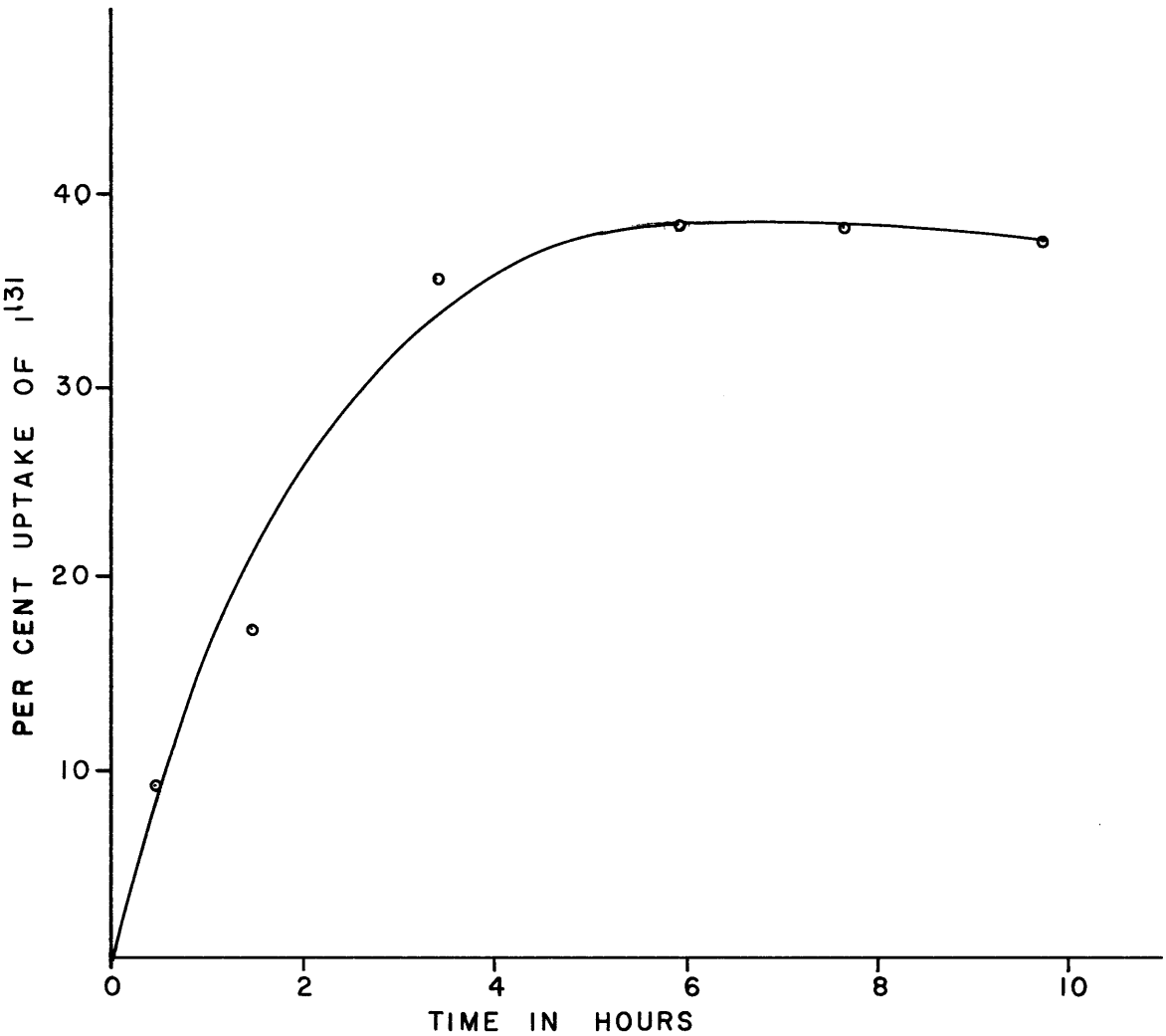

Figure 1: A typical I^{131} uptake curve for guinea pig thyroid fragments incubated at 25° C.

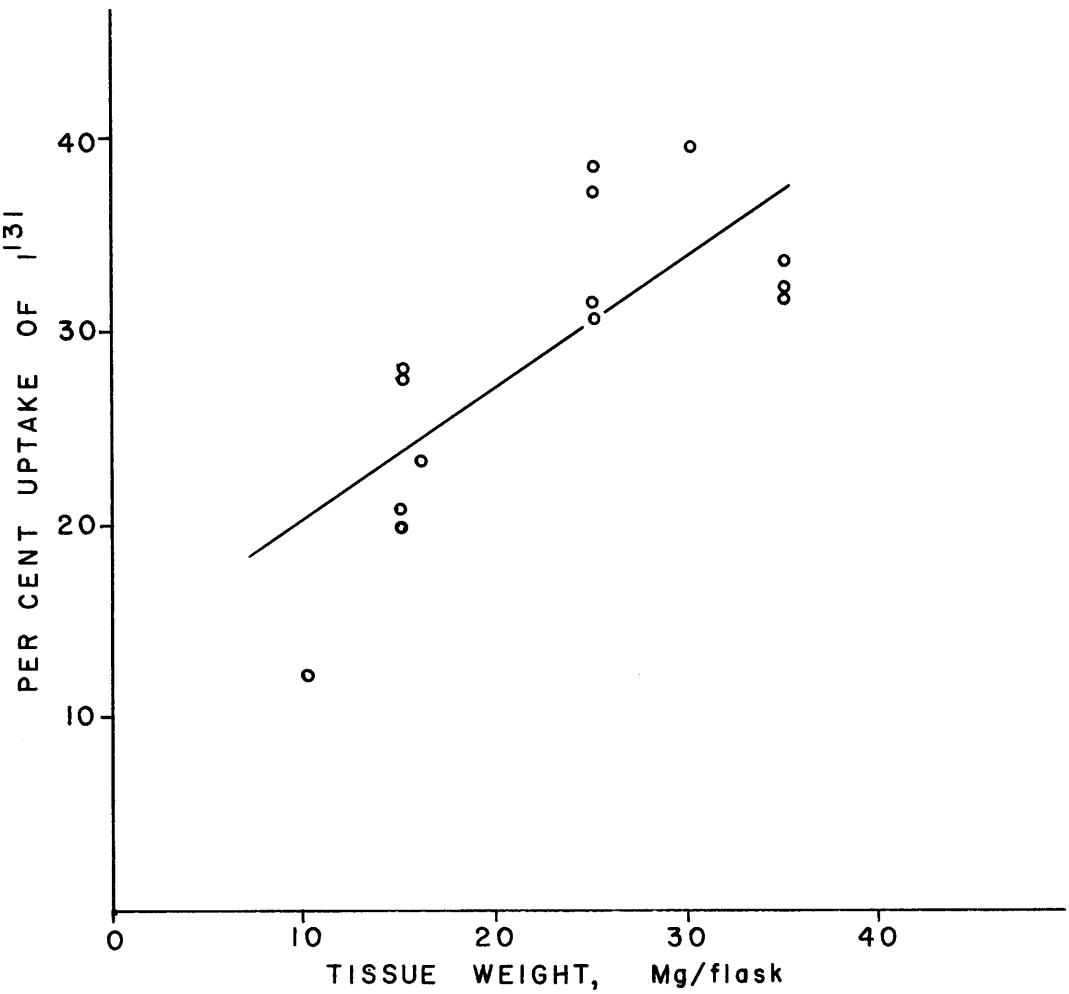

Figure 2: The relationship of per cent uptake of I^{131} to the amount of tissue incubated. The least squares equation of the line is Y=0.681 X+ 13.47. The F ratio for regression was significant (P less than 0.01), F_r=33.8.

Release of I^{131}

In order to explore the possibility that the per cent release of I^{131} stimulated by TSH was related to the amount of tissue present, thyroid tissue from two guinea pigs was randomized and quickly weighed before incubation. Release was stimulated by the addition of 80 International milliunits of reference standard TSH to each flask. Per cent release was calculated as previously described. Absolute release of I^{131} (counts per minute per flask) was found to increase significantly with increasing tissue weight. Per cent release data are plotted in figure 3. These were analyzed to determine the effect of tissue weight on the per cent release response. It was found that tissue weight per flask did not have a significant effect on per cent release of I^{131}.

Therefore, while the amount of tissue present significantly influenced per cent uptake and absolute release of I^{131}, TSH-stimulated release calculated as a function of uptake, per cent release, was not affected by tissue weight variations. As a result weighing of tissue fragments before incubation was not necessary, as suggested by Bottari, et al. (15).

In experiments involving a total of 80 flasks, thyroid tissue was divided into equal portions, and weighed after incubation in order to determine the actual variation in tissue weight per flask. The average standard deviation in this series was $\pm$ 2.2 mg per flask. Such variation would not be expected to cause significant variation in the assay response.

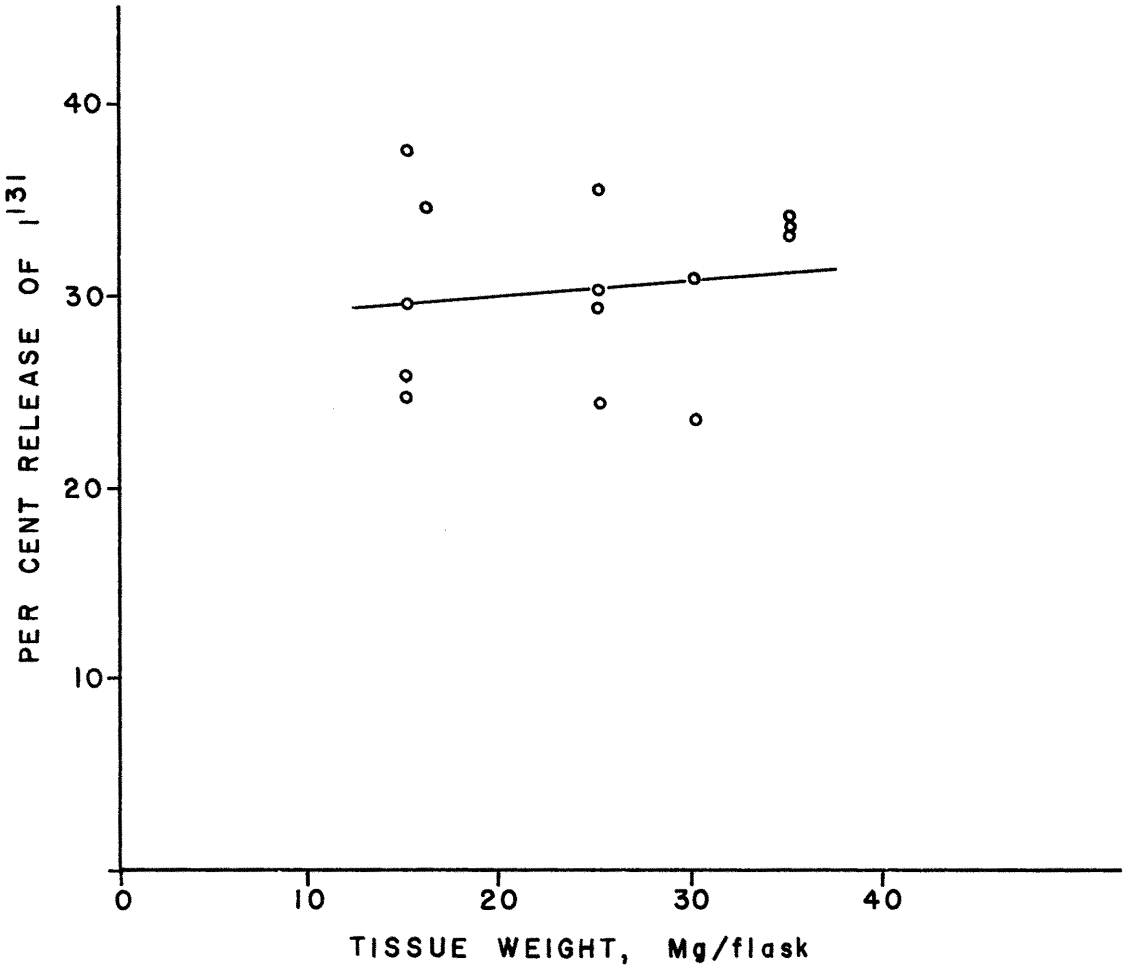

Figure 3: The relationship of per cent release of I^{131} to the amount of tissue incubated. The least squares equation for the line is $Y=0.083\ X + 28.48$. The F ratio for regression was not significant, $F_r=2.6$.

The relationship of per cent release to incubation time with TSH was studied and at all levels within the linear range of the response, maximum release was at approximately 1 hour and 45 minutes. This deviates very little from Bottari's incubation time of 2 hours (15). However, experience with the method has indicated that this time is critical, thus time between addition of TSH and successive sampling was held to the nearest minute.

Response to U.S.P. reference standard TSH

The response of tapazole-pretreated guinea pig thyroid fragments to serial dilutions of reference standard TSH was studied. As described under methods, incubation medium containing no I^{131} was used as a diluent. Figures 4 and 5 are examples of standard curves obtained. The lower limit of sensitivity was between 5 and 10 International milliunits per 100 ml, that is, the lowest dose of TSH which would produce a response significantly different from Gey's solution. The upper limit of linearity varied somewhat, but was usually at 200 International milliunits per 100 ml. An exception is illustrated in figure 5 where the relationship is linear from 15 to 405 International milliunits per 100 ml. Above the upper limit of linearity the response usually diminished, an effect similar to that reported by Powell, Rahman, and Deiss (54) with beef thyroid *in vitro*.

Standard responses (figures 4 and 5) were comparable to those obtained by Bottari, Donovan, and El Kabir (15) with respect

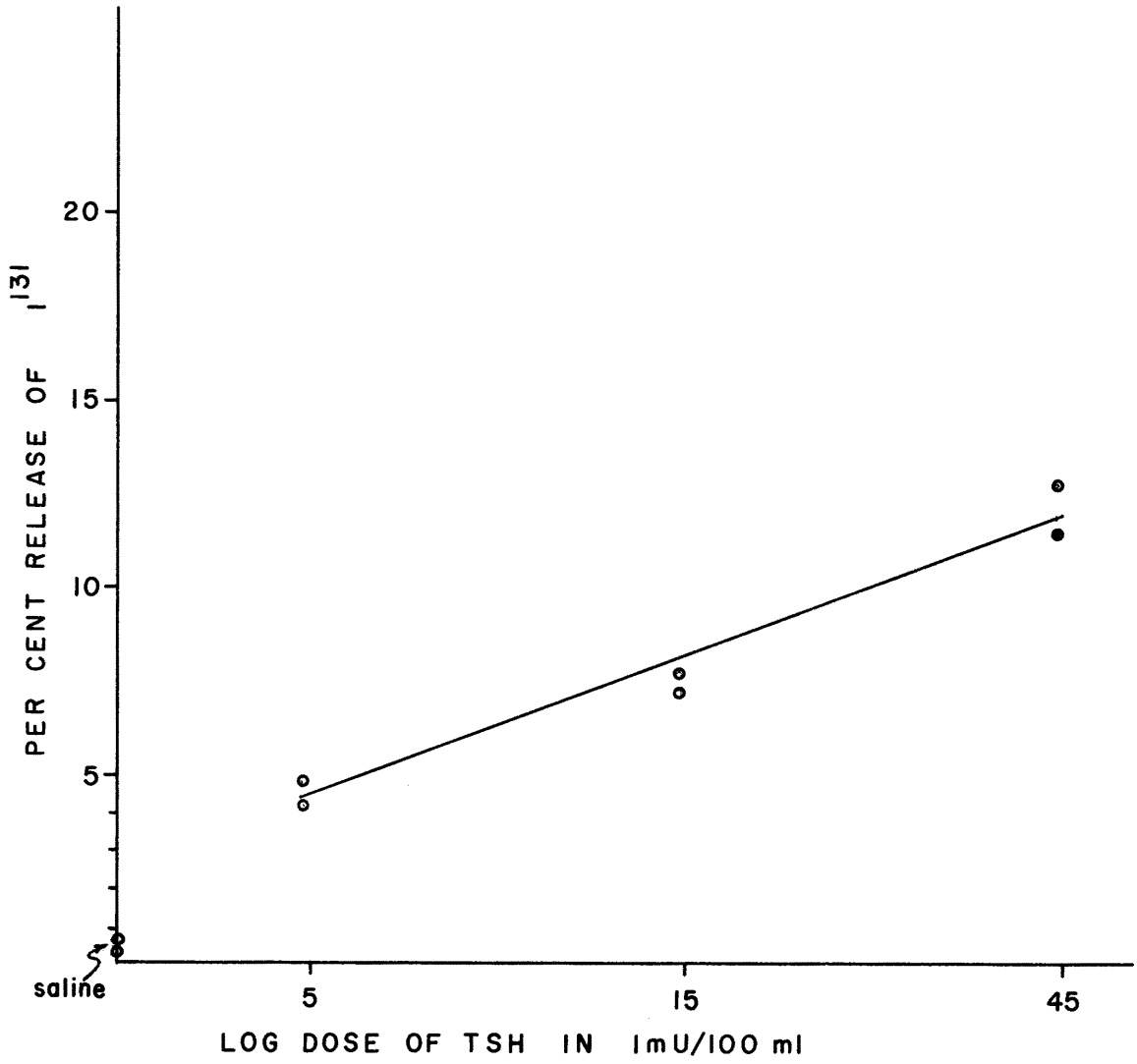

Figure 4: A typical dose-response curve for U.S.P. reference standard TSH. The least squares equation for the line is Y=7.757 X - 1.205. F_r=136 (S). A comparison of the responses to 5 ImU/100 ml of TSH and to saline is shown; the difference was significant, T=13.7 (P less than 0.01).

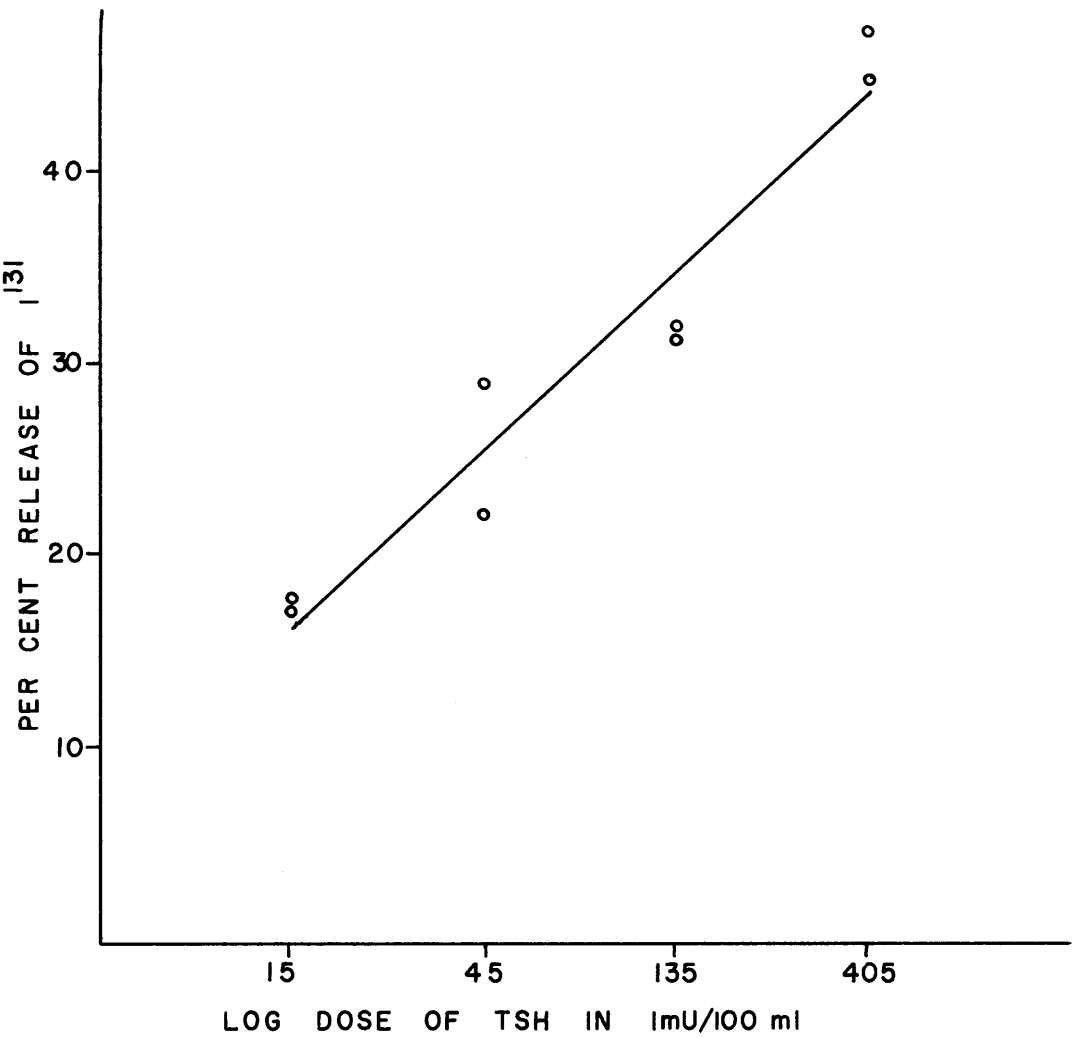

Figure 5: A dose-response curve for U.S.P. reference standard TSH. The least squares equation for the line is Y=19.227 X - 5.952. F_r=27.6 (S).

to range. However, the slope of the relationship was, in some assays, less than Bottari's group reports (15).

In order to determine whether or not quantitative detection of TSH was possible with various diluents, the standard response was compared simultaneously with the response to serial dilutions of reference standard TSH in horse serum, rat plasma, and Richardson ground squirrel plasma. Due to the necessity of comparing standard and test diluents in a single assay over a wide range of doses, tissue from two guinea pigs of approximately equal weight was distributed among the incubation flasks. The comparisons were made using the 2 X 3 factorial design.

The data plotted in figures 6 through 8 were analyzed and parallelism tested. Standard TSH in horse serum, rat plasma, and ground squirrel plasma produced a linear response in each case which did not differ from the standard response (TSH in incubation medium). Furthermore, the difference between standard and test samples was not significant at the 95 per cent level for horse serum and squirrel plasma, indicating a low level of endogenous TSH in horse serum and squirrel plasma. Although no statistically significant, some endogenous TSH was apparent in ground squirrel plasma (figure 6). Rat plasma showed significant endogenous TSH activity (figure 7).

These data demonstrated that TSH could be recovered quantitatively from rat and squirrel plasma. It appeared that the use of a diluent containing horse serum did not introduce additional TSH

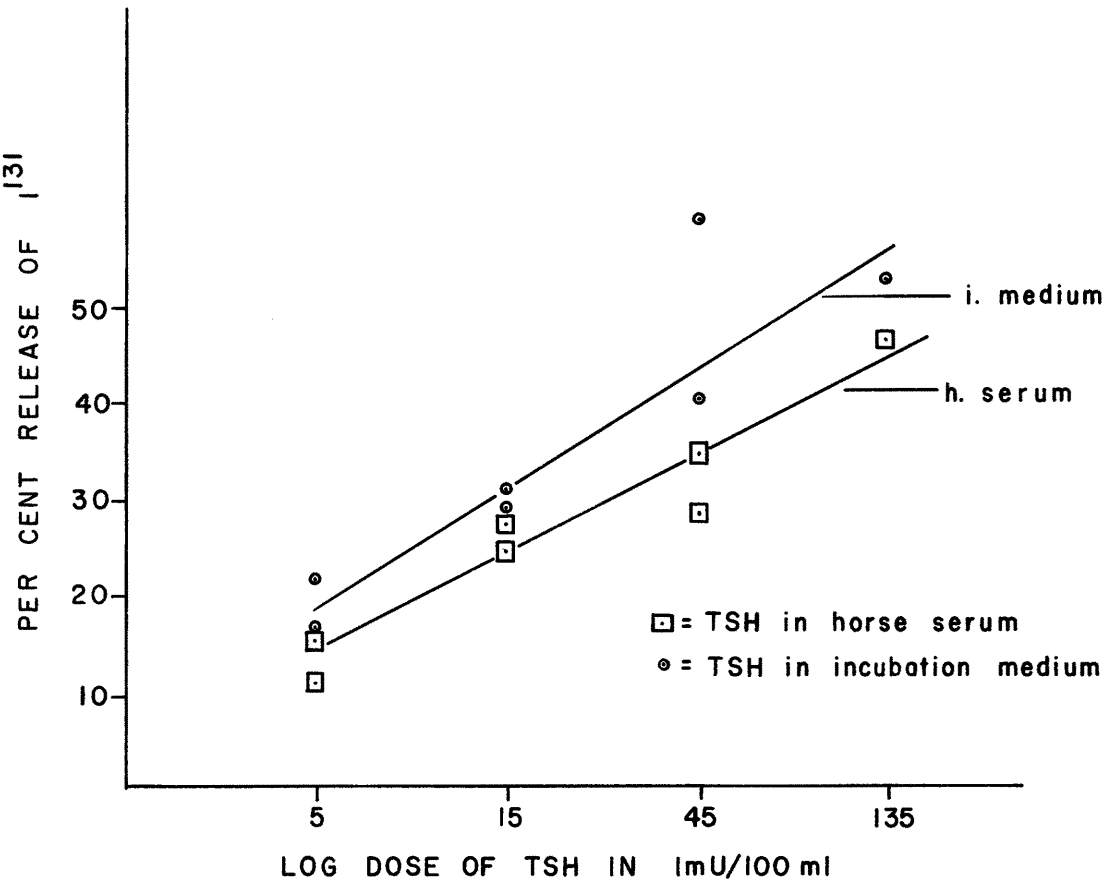

Figure 6: A comparison of dose-responses to U.S.P. reference standard TSH in incubation medium and horse serum. The least squares equations are: incubation medium, Y= 27.345 X - 0.496; horse serum, Y= 20.867 X + 0.45. Deviation from parallelism is not significant, F_p= 0.9.

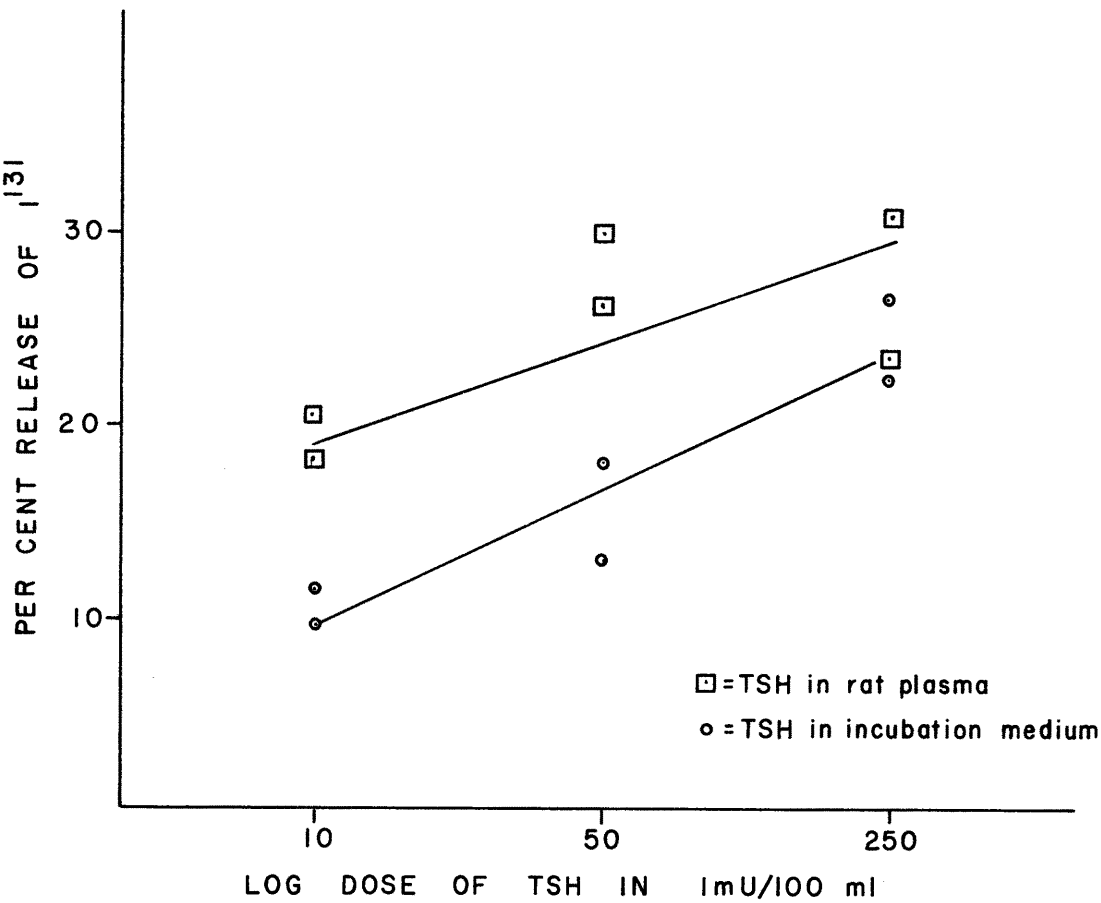

Figure 7: A comparison of dose-responses to U.S.P. reference standard TSH in incubation medium and rat plasma. The least squares equations are: incubation medium, Y=9.944 X - 0.0148; rat plasma, Y=7.074 X + 12.71. Deviation from parallelism is not significant, F_p=1.9.

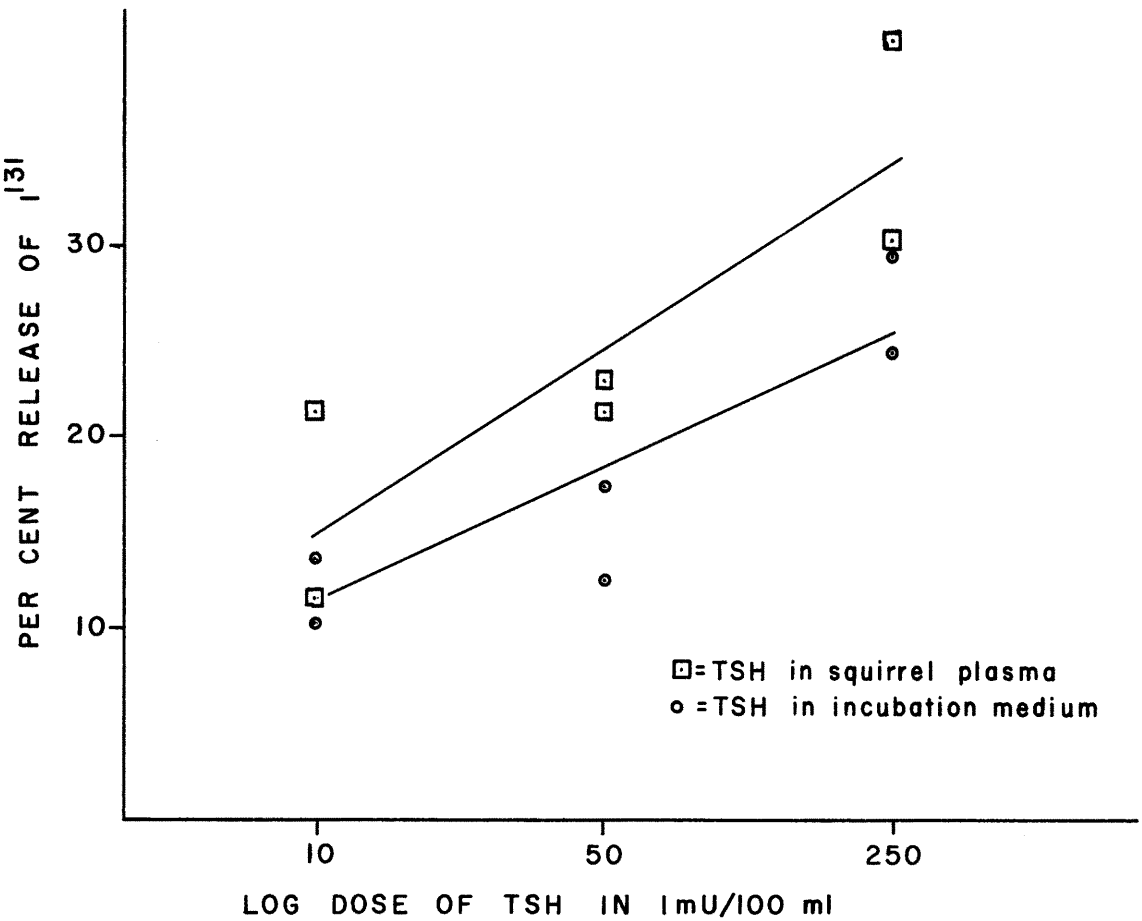

Figure 8: A comparison of dose-responses to U.S.P. reference standard TSH in incubation medium and ground squirrel plasma. The least squares equations are: incubation medium, Y=10.71 X -0.046; squirrel plasma, Y=13.81 X + 1.4. Deviation from parallelism is not significant, F_p=0.4.

activity to the assay system, and that no inhibition of the TSH-stimulated release of I^{131} occurs with rat plasma or squirrel plasma. The use of untreated plasma seems justified on the basis of the above findings. While Bottari's group has not published data supporting the absence of inhibitory effects, their assay results compare well with those obtained by other methods (15, 25, 26).

Specificity of the release response

Bottari, et al. (15) tested ACTH, vasopressin, serum gonadotrophin and heparin. None of these showed significant TSH activity.

A solution containing 25 units of ACTH per ml (Parke, Davis and Co.) was assayed and it did not produce responses significantly greater than were produced by the addition of Gey's solution.

The effect of storage on TSH activity

Bottari's group routinely freezes plasma until the time of bioassay, but no critical time of storage was given (15).

During this study it was observed that solutions of standard TSH stored at room temperature lost all TSH activity within 2 days. Storage in a refrigerator prolonged, but did not prevent the loss of activity. Since samples of plasma, particularly from hibernating squirrels had been frozen and stored at

-10° C, the possibility of diminished activity during storage at this temperature was investigated.

Reference standard TSH was added to fresh ground squirrel plasma so that the concentration was 4.0 International milliunits per ml (400 ImU/100 ml). Plasma samples of volumes appropriate for bioassay were immediately frozen. Plasma was assayed on the day of preparation (day 0) and at 1, 42, and 43 days after storage. Table 2 summarizes the assay results on this stored plasma.

Table 2: The effect of storage at -10° C on TSH activity

Storage time in days	Estimated TSH activity in ImU/100 ml	Mean for period
0	374 (210--670)*	
1	387 (270--554)	380 ImU/100 ml
42	132 (118--152)	
	132 (26--246)	
43	143 (41--495)	
	135 (48--390)	136 ImU/100 ml

*95% fiducial limits on the estimate of activity

The mean assayed value of TSH at the beginning of the storage period was 380 ImU/100 ml, while the mean after storage was 136 ImU/100 ml. The difference between these means was found to be statistically significant (P less than 0.001) using the "t" test. During the same period bioassay results for freshly prepared U.S.P. reference standard TSH showed no significant variations in tissue response.

It was concluded that storage of plasma samples for even 5 weeks would introduce significant error into assay results. This finding seemed to explain early failure to detect TSH in ground squirrel plasma samples which had been stored for 6 months. In further assays of ground squirrel plasma only fresh plasma was used.

Bioassay of TSH in rat plasma

Fresh plasma from Holtzman strain male rats was assayed. Laparotomy was quickly performed under light ether anesthesia. Blood was collected from the abdomenal aorta using a heparinized 10 cc syringe and 21-guage needle. The blood was centrifuged immediately, after which the plasma was removed and assayed. The standard 2 X 2 assay design was used; results of 8 assays are reported in table 3, and a sample plot is found in figure 9.

The results indicate a lower level of TSH than estimated by Bottari, et al. (15) in male Wistar rats housed at 70° F. For Wistar rats the range was from 43 to 55 International milliunits per 100 ml, and for a Lister rat, 43 International milliunits per 100 ml. Results reported in table 3, besides representing a different strain, are based on samples from animals housed at somewhat higher temperatures, ranging between 75° and 80° F.

Table 3: Bioassay of TSH in rat plasma, male Holtzman rats.

Date of sacrifice	TSH, ImU/100 ml	95% limits
5-27-64	19	4--102
9-30-64	105	62--238
12-28-64	14	6--29
12-30-64	29	13--65
1- 2-65	37	16--90
1- 2-65	22	9--54
1- 3-65	47	6--384
1-12-65	33	10--110

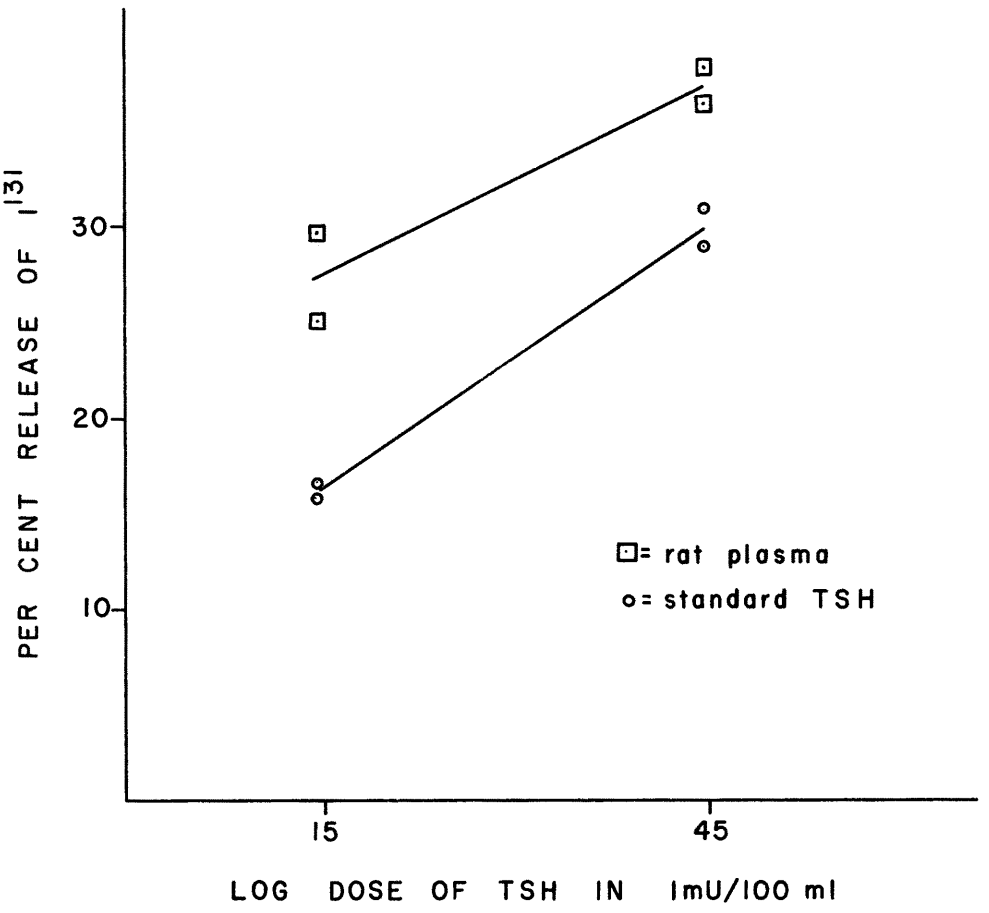

Figure 9: A 2 x 2 bioassay of rat plasma; male Holtzman rat, sacrificed 1-12-65. The estimated plasma concentration of TSH: 105 ImU/100 ml (62--238).

Bioassay of TSH in ground squirrel plasma

During the summer of 1964 the plasma of captive squirrels was assayed. Storage of samples was for less than two weeks in all cases. Fresh plasma from two winter squirrels, housed in the cold room but not hibernating, was assayed (table 4: M-5-64 and F-3-64). Descent of the testes and increased food and water consumption was observed in a single male after being brought from the cold room to 70° F for 1½ weeks (table 4: M-4-64).

In stored samples from hibernating ground squirrels no TSH activity was detected, and no fresh plasma was obtained during 1964-65 from hibernating animals. It was felt that the assay results from these samples were invalid, since storage time was approximately 6 months, and the samples were accidentally thawed and refrozen during the storage period. Assay results for ground squirrel plasma are summarized in table 4, and a sample assay plotted in figure 10.

More variation between duplicate estimates was found than reported by Bottari, et al. (15). This is noted in the case of squirrels F-68-64 and M-4-64. Such variability from assay to assay is most likely due to differences in the responsiveness of the guinea pig thyroid used.

Many assayists calculate the quantity lambda, which is a measure of precision, defined by $\lambda = S/b$ where S is the experimental error and b is the slope of the regression line. Lambda estimates the standard deviation in log-dose (X), and

Table 4: Bioassay of TSH in ground squirrel plasma

Identification number	Date of sacrifice	TSH in ImU/100 ml	95% limits
F-52-64	6-9-64	14	8--22
M-31-64	6-9-64	3	1--10
F-50-64	7-7-64	13	1--293
M-51-64	7-28-64	97	34--379
F-68-64	7-29-64	57	14--227
"	"	21	8--55
M-5-64	12-29-64	3	2--5
F-3-64	12-29-64	5	4--6
M-4-64	1-9-65	65	55--75
"	"	129	50--345
"	"	153	57--410

Note: Male animals are denoted by the prefix M and females by the prefix F in identification numbers.

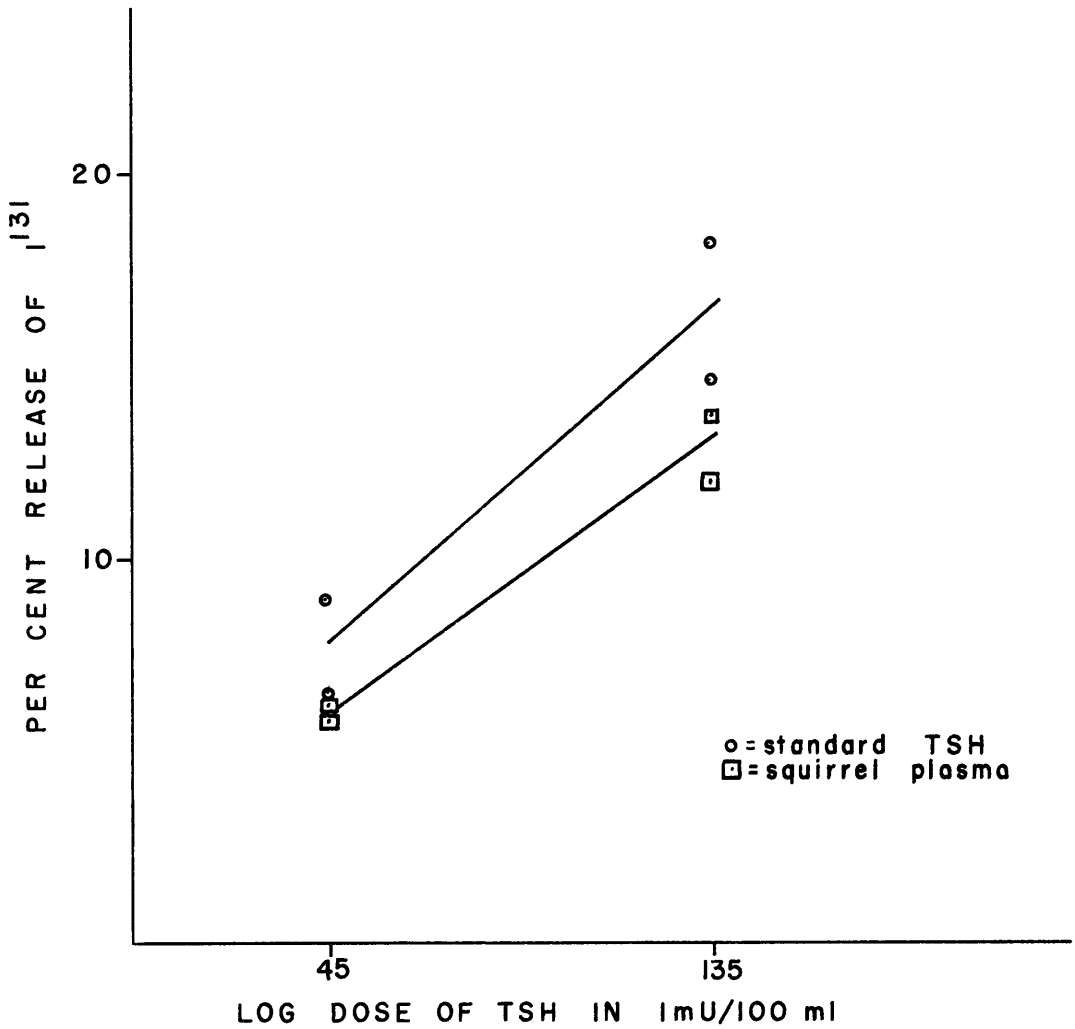

Figure 10: A 2 x 2 bioassay of C. richardsoni plasma, M-51-64, sacrificed 7-28-64. The estimated plasma concentration of TSH: 97 ImU/100 ml (34--279).

expresses the efficiency of the response as an assay method. A small value of lambda indicates greater inherent precision of estimates. Bottari, et al. (15) reported a range of lambda between 0.095--0.267 for a series of 10 standard assays, and the values for rat plasma assays were of the same order. In this study lambda ranged between 0.160 and 0.431. The higher values of lambda were due in most cases to slopes of lower magnitude than obtained by Bottari.

Considering the squirrel data, with these limitations in mind, one observes definite trends in spite of the limited data. The group of squirrels sacrificed during the early summer had higher levels of TSH than the two squirrels removed from the cold room in December (table 4: M-5-64, F-3-64). This is consistent with histological observations that thyroid activity is greatest during the summer (62) and does not increase upon exposure to cold (23, 47).

While the 2 animals taken from the cold room were not hibernating at the time of sacrifice, the low TSH titer observed (M-5-64, F-3-64) represents a striking contrast when compared with TSH levels in the male squirrel sacrificed on 1-9-65. These assays were carried out on fresh plasma and within a short period of time. Squirrels sacrificed on 12-29-64, exposed to cold, showed low activity and food consumption, while the animal sacrificed on 1-9-65 was more active and showed increased food and water consumption as well as descent of the testes.

The association of increased circulating TSH with increased activity and food consumption and with descent of the testes correlates well with indirect evidence. Endocrine function in hibernators appears greatest during the early spring (52, 62). The data also indicate a tendency toward lower levels of TSH in the summer as opposed to the early spring.

CHAPTER V

SUMMARY

The method studied was essentially that described by Bottari, et al. (15) with the exception that incubations were carried out in stoppered Erlenmeyer flasks using a Dubnoff metabolic shaking apparatus instead of the standard roller tube technique. Furthermore, guinea pigs of the same strain as used by Bottari's group were not available, nor were the animals used of a single strain.

The method of incubation did not alter to a great degree the time characteristics of uptake and release. Time to maximum uptake was found to be the same as reported by Bottari (15), but time to maximum release was 1 hour and 45 minutes whereas Bottari suggested 2 hours.

Animal strain variability is suggested as the major factor in producing dose-response variation. Both magnitude of slope and variance at each dose level contributed to lower precision than reported by Bottari (15). Error arising from the method of pipetting aliquots was not a significant factor contributing to low precision.

The method was successfully applied to the recovery of TSH from various diluting fluids. In addition to demonstrating quantitative recovery of TSH from horse serum, rat plasma, and ground squirrel plasma, these data indicated the absence of a TSH-inhibitor in all of these materials.

When the method was applied to the assay of TSH in rat plasma, the estimated levels in Holtzman strain rats were less than those reported by Bottari's group (15) in Wistar and Lister strain rats. The observed difference was possibly the result of strain variation or of temperature-dependent changes in thyroid activity.

From the assay of ground squirrel plasma, certain trends in seasonal fluctuations of TSH were observed. TSH was found lowest in squirrels exposed to cold during the winter months and highest in a single animal at the time of endocrine reactivation in the spring.

A more comprehensive study would now seem possible, with some reservations and recommendations drawn from experience. Bottari's method appears to be capable of detecting TSH in non-hibernating ground squirrels during all seasons, and would be applicable to monitoring TSH throughout the year. The schedule should allow for the assay of fresh plasma, or for a storage procedure more suitable than freezing. The capability of the method to detect hibernating levels of TSH is not known. It is possible that the sensitivity of the assay would be insufficient, since in hibernators the thyroid shows little sign of activity during hibernation (47, 65).

APPENDIX

I. Figure 1: Uptake of I^{131} by guinea pig thyroid fragments *in vitro* at 25° C.

Time in hours	Per cent uptake of I^{131}
0.5	9.0
1.5	17.3
3.5	35.8
6.0	38.5
7.7	38.6
8.5	32.3
9.7	37.7

II. Figure 2: Per cent uptake of I^{131} as a function of tissue weight per flask.

Tissue weight in mg	Per cent uptake of I^{131}
10	12
15	28
"	28
"	21
"	20
16	24
25	31
"	32
"	39
"	38
30	40
35	32
"	32
"	34

II. (continued)

Analysis of variance

Source	D.F.	S.S.	M.S.	F_c	
Total	14	12840.70			
Mean	1	12042.31			
Total'	13	789.39			
Group mean	5	691.785	138.36	10.39	S
Regression	1	450.32	450.32	33.81	S
Error	8	106.605	13.32		

Least squares equation of the line: $Y = 0.681\ X + 13.741$

III. Figure 3: Per cent release of I^{131} as a function of tissue weight per flask. TSH dose: 80 ImU/100 ml.

Tissue weight in mg	Per cent release of I^{131}
15	25.9
"	29.4
"	24.8
"	37.7
16	34.6
25	30.1
"	35.6
"	24.2
"	29.2
30	23.5
35	33.4
"	33.9
"	33.8

III. (continued)

Analysis of variance

Source	D.F.	S.S.	M.S.	F_c	
Total	13	12339.77			
Mean	1	12068.86			
Total'	12	270.91			
Group mean	4	103.03	25.8	1.23	NS
Regression	1	5.45	5.45	2.6	NS
Error	8	167.87	20.89		

IV. Figure 4: Standard response curve, U.S.P reference standard TSH.

Dose of TSH ImU/100 ml	Per cent release of I^{131}	
5	4.8, 4.2	log interval, I=0.477
15	7.2, 7.5	Least squares equation
45	11.2, 12.6	of the line: Y= 7.757 X - 1.205
Saline	0.2, 0.5	T_c= 13.7 (S)

Analysis of variance

Source	D.F.	S.S.	M.S.	F_c	
Total	6	432.97			
Mean	1	370.04			
Total'	5	62.93			
Group mean	2	61.72	30.86	76.7	S
Regression	1	54.57	54.57	136.2	S
Error	3	1.20	0.402		

V. Figure 5: Standard response curve, U.S.P. reference standard TSH.

Log interval, I=0.477. Least squares equation of the line: Y= 19.2267 X - 5.952.

V. (continued)

Dose of TSH ImU/100 ml	Per cent release of I^{131}
15	17.6, 18.0
45	22.4, 29.1
135	31.5, 32.1
405	47.7, 45.0

Analysis of variance

Source	D.F.	S.S.	M.S.	F_c	
Total	8	8305.28			
Mean	1	7405.44			
Total'	7	899.84			
Group mean	3	873.49	291.16	44.2	S
Regression	1	841.0	841.0	27.6	S
Error	4	26.35	6.59		

VI. Figure 6: Comparison of U.S.P. reference standard TSH in incubation medium and horse serum.

TSH added ImU/100 ml	Per cent release incubation medium	Per cent release horse serum
5	16.6, 22.0	16.2, 10.4
15	31.7, 24.4	30.4, 28.0
45	40.3, 59.4	35.0, 28.0
135	53.3	46.8
	$Y= 27.345 X - 0.496$	$Y= 20.867 X + 0.45$

VII. Figure 7: Comparison of U.S.P. reference standard TSH in incubation medium and rat plasma.

TSH added ImU/100 ml	Per cent release incubation medium	Per cent release rat plasma
10	11.4, 9.9	18.6, 20.7
50	12.9, 18.0	26.1, 30.1
250	26.8, 22.3	23.7, 31.0
	Y= 9.944 X - 0.0148	Y= 7.073 X + 12.71

VIII. Figure 8: Comparison of U.S.P. reference standard TSH in incubation medium and ground squirrel plasma.

TSH added ImU/100 ml	Per cent release incubation medium	Per cent release rat plasma
10	10.4, 13.9	11.9, 21.2
50	12.6, 17.8	23.0, 21.6
250	24.6, 29.6	30.1, 41.6
	Y= 10.71 X - 0.046	Y= 13.81 X + 1.4

IX. Figure 9: Bioassay of rat plasma.

Per cent release at standard and unknown dose levels:

S_1	S_2	U_1	U_2
16.5	30.9	29.5	38.0
16.0	28.9	25.0	36.1

Log-dose interval, I= 0.477

S_1 = 15 ImU/100 ml; S_2 = 45 ImU/100 ml

IX. (continued)

Analysis of variance and factorial analysis

Source	D.F.	S.S.	M.S.
Total	8	6560.73	
Mean	1	6099.61	
Total'	7	461.12	
Error	4	14.05	3.51

	S_1	S_2	U_1	U_2	$f\Sigma x^2$	$\Sigma x_i T_i$	$(\Sigma x_i T_i)^2 / f\Sigma x^2$
C_1	-	+	-	+	8	46.9	274.95
C_2	-	-	+	+	8	36.3	164.71
C_3	+	-	-	+	8	-7.7	7.41
T_i	32.5	59.8	54.5	75.1			

F_{c1} = 78.3 S ; F_{c2} = 46.8 S ; F_{c3} = 2.1 NS.

Potency ratio (log units), $M' = \dfrac{I \Sigma x_2 T_2}{\Sigma x_1 T_1} = 0.369$

Potency ratio, M= 2.34

Potency of unknown sample: $P = M \times S_2 = (2.34)(45 \text{ ImU/100 ml})$
$P = 105$ ImU/100 ml

Estimated error in M, $S_M = (MSE)(I)(MSC_1 + MSC_2)^{\frac{1}{2}} / MSC_1$

$S_M = 0.128$ (in log units)

95% fiducial limits: $M' - S_M T < M' < M' + S_M T$

$62 < 105 < 238$ ImU/100 ml

X. Figure 10: Bioassay of ground squirrel plasma, M-51-64.

Per cent release at standard and unknown dose levels:

S_1	S_2	U_1	U_2
8.0	18.3	6.4	13.7
6.5	14.7	6.0	12.0

S_2 = 135 ImU/100 ml, I = 0.477

Analysis of variance and factorial analysis

Source	D.F.	S.S.	M.S.
Total	8	1065.88	
Mean	1	915.92	
Total'	7	149.96	
Error	4	14.64	3.66

	S_1	S_2	U_1	U_2	$f\Sigma x^2$	$\Sigma x_i T_i$	$(\Sigma x_i T_i)^2 / f\Sigma x^2$
C_1	-	+	-	+	8	31.1	120.9
C_2	-	-	+	+	8	-9.4	11.04
C_3	+	-	-	+	8	-5.2	3.38
T_i	14.5	33.0	10.4	25.7			

F_{c1} = 33.03 ; F_{c2} = 3.01 ; F_{c3} = 0.9, NS.

M = 0.717

P = 97 ImU/100 ml

S_M = 0.166

34 < 97 < 279 ImU/100 ml

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