A LONGITUDINAL STUDY OF SERUM CONCENTRATIONS OF THYROXINE AND TRIIODOTHYRONINE IN THE BUF RAT

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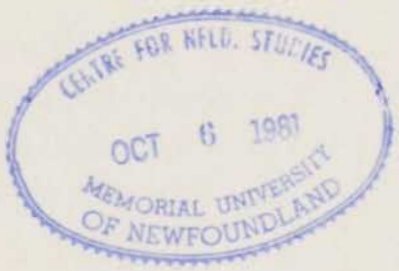
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A LONGITUDINAL STUDY OF SERUM CONCENTRATIONS OF THYROXINE AND TRIIODOTHYRONINE IN THE BUF RAT

by

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ABSTRACT

Thyroid function was investigated in a colony of BUF inbred rats in order to establish the normal baseline in this model for spontaneous autoimmune thyroiditis, and to thereby attempt to monitor the development of the disease (histologic examination of a small sampling of the BUF rats confirmed that thyroiditis did occur in animals of this colony). Sera were obtained by longitudinal sampling of males and virgin females, and concentrations of thyroxine (T4) and triiodothyronine (T3) were measured by radioimmunoassays. T4 and T3 levels were each found to be correlated with body weight in males, with $r = 0.264$ ($P < 0.002$) and $r = 0.261$ ($P < 0.005$) respectively, but neither hormone was significantly correlated with body weight in virgin females. T4 and T3 levels declined with age in both sexes; in males, $r = -0.309$ ($P < 0.001$) for T4 and $r = -0.369$ ($P < 0.001$) for T3, and in virgin females $r = -0.283$ ($P < 0.001$) for T4 and $r = -0.417$ ($P < 0.001$) for T3. Although these correlation coefficients do not indicate that there was a significant sexual difference in the variation with age of either T4 or T3 ($P > 0.05$ for each hormone), in general the females had slightly lower levels of T4 and slightly higher levels of T3 than did the males. For those samples that had been assayed for both hormones, the T4/T3 ratios were significantly ($P < 0.001$) lower in the females and were independent of age in both sexes. Serial samples revealed that T4 and T3 concentrations fluctuated greatly from month to month in animals of each sex. Values which fell outside of the normal ranges (95% confidence limits around the regression lines) could not necessarily be attributable to thyroiditis, with values from subsequent ages often returning to within the normal ranges.

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Lastly, but most importantly, I wish to thank the lady who has, during the period of this research, become my wife; she has spent many Saturday mornings bleeding BUF rats with me, in order to be able to spend more time with me. Thank you Bobbie, both for your practical assistance and for the spiritual assistance that you gave me.

TABLE OF CONTENTS

(continued)

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

1. INTRODUCTION

1.1 PHYSIOLOGY OF THE NORMAL THYROID

1.1.1 Anatomy of the thyroid

The thyroid (reviewed by Klinck, 1964; Schneeberg, 1970; Ermans et al., 1972) is composed of two lobes, one on either side of the trachea, connected by an isthmus; occasionally there is also a pyramidal lobe. Each lobe is composed of structural units called lobules (see Fig. 1); each lobule contains between 20 and 40 functional units called follicles (or acini), and in rats there is also a second system called the ultimobranchial follicles. Parafollicular cells (C cells) can also be found in the lobules. The follicles are bound by a thin sheath of connective tissue, in a framework called the stroma. Each follicle is a spherical sac, varying in size (200-300 um in diameter), composed of a single layer of epithelial cells which surround a lumen; a basement membrane encompasses each follicle. The follicular cells contain numerous mitochondria, an extensive rough endoplasmic reticulum, well-defined Golgi apparatus and, at their apical ends, numerous microvilli projecting into the lumen (greatly increasing the interface). The cells secrete a clear jelly-like fluid called the colloid (which is about 85% thyroglobulin, a glycoprotein) into the lumen. The amount of colloid and the mean follicular cell height tend to reflect the activity of the follicles.

The follicles have an extraordinarily rich and extensive blood supply. Thyroid arteries form a network over the surface of the thyroid, enter the thyroid and subdivide; a lobular artery supplies each lobule separately, branching to each follicle within the lobule, and each branch subdivides further to cover the follicle in a capillary plexus, with the endothelium of the plexus in close apposition to the basement membrane of the follicle.

The thyroid has an extensive autonomic nerve supply which exerts control over thyroid blood flow and, as well, releases autonomic amines which bind to receptors on the follicular cells. The sympathetic nerves release adrenergic amines (e.g., norepinephrine) which appear to promote secretion of thyroid hormones, whereas the cholinergic nerves release acetylcholine which is reported to be inhibitory (Melander & Sundler,

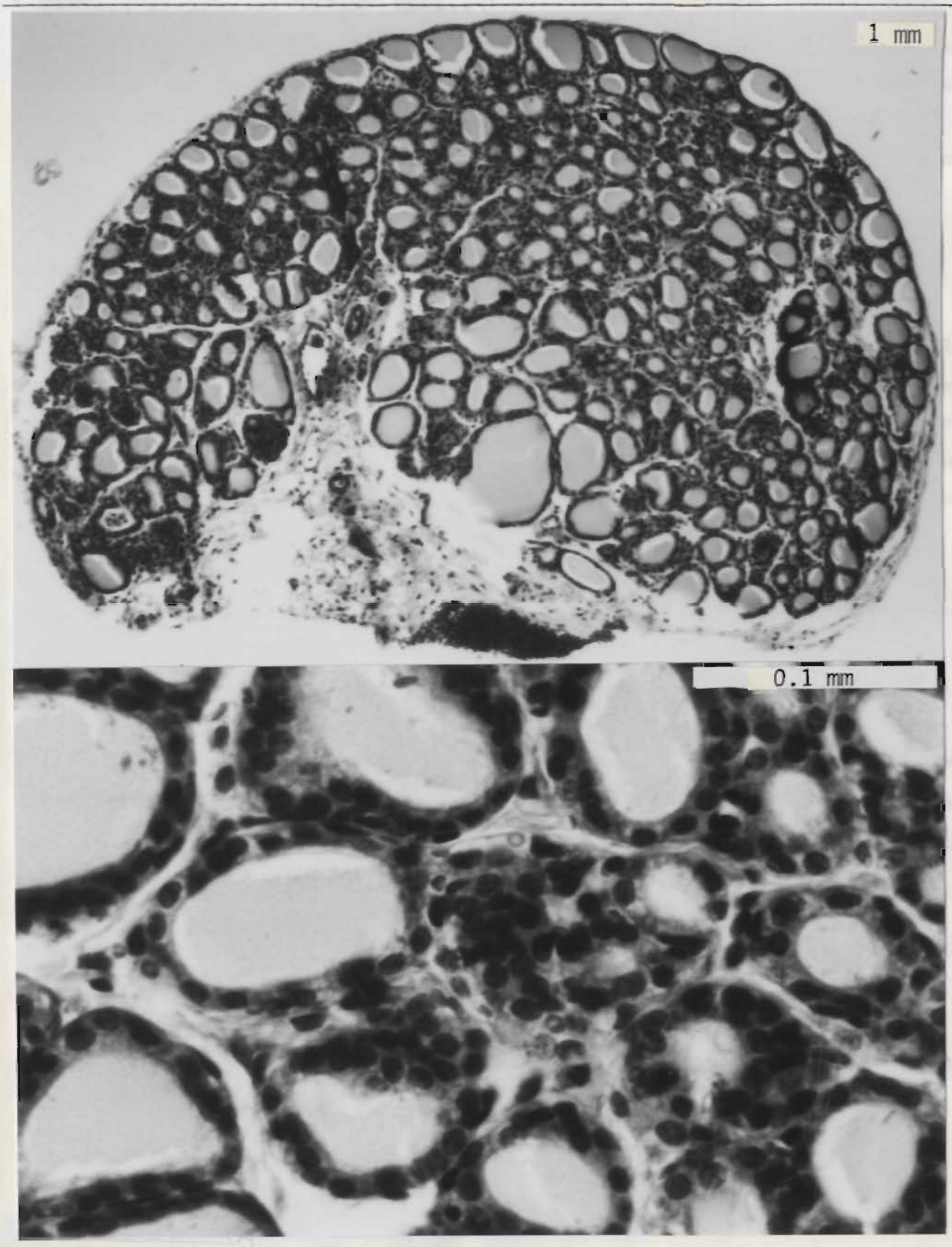


Fig. 1. Normal thyroid histology (BUF rat).

1979 and references cited therein).

The follicles are also served by a lymphatic network. Lymph vessels penetrate the thyroid, branch freely, and terminate in the interfollicular stroma; large cisternae in the interfollicular spaces are thus interconnected by smaller lymph channels, enabling a single lymph vessel to serve several follicles.

1.1.2 Chemistry of the Thyroid Hormones

Iodine. With an atomic weight of 127, iodine is the heaviest atom normally in living organisms. It has been cited as the only example of an authentic biologic requirement for a halogen atom (Frieden & Lipner, 1971). Iodine is concentrated to some extent in the stomach and in the salivary glands, but the principal storage form, thyroglobulin, is found in the thyroid gland. The iodinated residues of thyroglobulin give rise to the thyroid hormones.

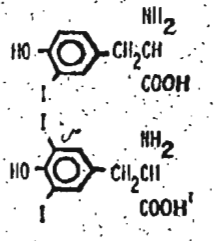
Thyroid hormones. (See Fig. 2) The tyrosine residue is defined by an alanine side chain on a phenyl ring; addition of an outer or beta ring defines the thyronine residue. Iodination can occur on the third and fifth carbon atoms of each ring (the 3, 5, 3', and 5' positions). The iodinated hormones are among a limited number of monoamino acids which have been shown to possess endocrine activity; they are also among the few naturally-occuring molecules which contain a biphenyl ether (i.e., two benzene rings are coupled by an oxygen bridge).

Experiments using analogues (see Frieden & Lipner, 1971 and references cited therein) suggest that the thyroid hormones may possess two active sites; the 3,5-diiodotyrosyl residue may be associated with binding, whereas the 3'-iodo- and the 4'-hydroxyphenyloxy parts of the thyronine molecules may be associated with such functional activities as calorogenesis.

TYROSINE DERIVATIVES

3-monoiodotyrosine
(MIT)

3,5-diiodotyrosine
(DIT)



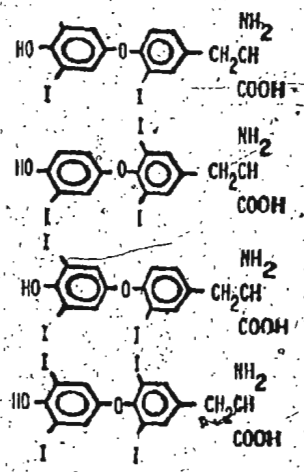
THYRONINE DERIVATIVES

3,3'-diiodothyronine
(T2)

3,5,3'-triiodothyronine
(T3)

3,3',5'-triiodothyronine
(reverse-T3)

3,5,3',5'-tetraiodothyronine
(thyroxine, T4)



SIDE CHAIN ANALOGUES

3,5,3',5'-tetraiodothyroacetic acid
(tetrac, T4A)

3,5,3',5'-tetraiodothyroformic acid
(tetraformic acid)

3,5,3'-triiodothyroacetic acid
(triac)

3,5,3'-triiodothyropropionic acid
(triprop)

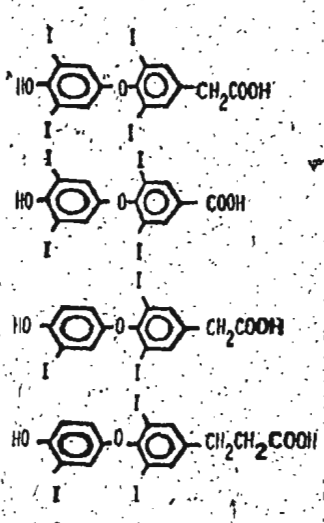


Fig. 2. Iodocompounds.

1.1.3 Biosynthetic Pathways in the Thyroid

Inorganic iodide is ingested in the diet, absorbed in the gut, and circulated in the plasma. At the thyroid there is a halogen-pumping mechanism which causes the iodide to accumulate in the thyroid. A peroxidase enzyme converts the iodide into "active molecular iodine" (active I_2). In this active form the iodine can bind to tyrosine residues on polypeptide chains which are destined to become subunits of thyroglobulin, producing 3-monoiodotyrosine (MIT) and 3,5-diiodotyrosine (DIT) residues (see Fig. 2). It is generally believed that the formation of a free radical then leads to the coupling of iodotyrosine residues, forming iodothyronine residues on the thyroglobulin molecules. The predominant iodothyronine was named "thyroxine" by Kendall (1914); it is 3,5,3',5'-tetraiodothyronine (T4). The other iodothyronine residues are 3,5,3'-triiodothyronine (T3), 3,3',5'-triiodothyronine (reverse-T3 or rT3) and 3,3'-diiodothyronine (T2) (see Fig. 2).

The thyroglobulin is released at the apical end of the follicular cells and fills the follicular lumen. In order to secrete the thyroid hormones, the follicular cells use pinocytosis to take up colloid (including the thyroglobulin) into cytoplasmic vesicles, the vesicles join with lysosomes, proteases digest the thyroglobulin into its constituent amino acid residues, and the iodothyronines T4, T3 and reverse-T3 are secreted. In one report (Chopra, 1974), 1 mg of protein preparation of thyroglobulin contained 3.0 ug T4, 0.16 ug T3 and 0.042 ug reverse-T3; the average yield of one molecule of thyroglobulin has also been calculated to be 12 molecules of MIT and DIT (which are not secreted from the thyroid), 2 molecules of T4, one-third of T3 and traces of others (which would include reverse-T3) (Frieden & Lipner, 1971 and references cited therein).

The iodothyronines are secreted and enter the capillary plexus which surrounds the follicle. In the bloodstream, thyroid hormones bind principally to three serum proteins: thyroxine-binding globulin (TBG), thyroxine-binding prealbumin (TBPA) and albumin. A proportion of each hormone binds reversibly to these carrier proteins according to the law of mass action. In humans, TBG binds over 70% of the plasma T4 and about 90% of the T3; TBPA binds about 15% of the T4 and 7-9% of the T3; and

albumin binds about 10% of the T4 and some T3 (Beeson & McDermott, 1971; Davis et al., 1972). Thus, about 0.03% of the T4 and about 0.3% of the T3 in the blood at any moment is "free" (dialyzable). Only free thyroid hormone molecules can react with hormone receptors in target tissues, and it appears that homeostasis seeks to maintain the absolute level of free molecules; when the amount of carrier protein changes (e.g., due to estrogen contraceptives), total hormone levels tend to follow, so that the number of free molecules is preserved.

1.1.4 Peripheral Metabolism of Iodothyronines

Thyroid hormones, principally T4, can be metabolized by several mechanisms (Owen & Flock, 1964). Firstly, the alanine side chain can be altered (e.g., conversion to acetic acid yields 3,5,3',5'-tetraiodothyroacetic acid, abbreviated tetrac or T4A, or the triiodo-analogue triac; see Fig. 2). Secondly, T4 can be conjugated (with glucuronic acid, or to a lesser extent with sulfates) in the liver, and then excreted in the bile; in an enterohepatic cycle, however, some of the conjugate can be hydrolyzed in the gut and the liberated T4 can be resorbed. Thirdly, and physiologically the most important, is monodeiodination.

It has been recently suggested that there may be monodeiodinases which are specific with respect to target iodothyronine ring (Chopra, 1974; Gavin et al., 1978). One enzyme, if it removed an iodine from the inner ring, would convert T4 into reverse-T3 and thereby "inactivate" the T4 (since reverse-T3 is not thyromimetic). A different enzyme could remove an iodine from the outer ring instead, which would convert T4 into T3 and reverse-T3 into T2; this would both "activate" the T4 (T3 is about four times more active than T4; Danowski, 1962) and "inactivate" the reverse-T3 (which is a potent competitive inhibitor of T4-to-T3 conversion; Kaplan & Utiger, 1978).

The extrathyroidal conversion of T4 to T3 in humans was first clearly demonstrated when circulating T3 was measured in patients who were receiving only T4 as replacement therapy after removal of their thyroid glands (Braverman et al., 1970). It has been clearly established that T4 is indeed an important source of the body pool of T3 in humans

and in experimental animals (Pitt-Rivers et al., 1955; Sterling et al., 1970; Pittman et al., 1971; Schwartz et al., 1971; Fisher et al., 1972, Surks et al., 1973). Of the total daily T4 turnover in humans (100 nmoles), 35% leads to T3. (Because 80% of the T4 turnover is by monodeiodination, therefore almost one-half of the T4 that is deiodinated (35/80 nmoles) is converted to T3. In the rat as well, about one-half of the deiodinated T4 is converted to T3; Oppenheimer et al., 1972.). Since the total daily T3 production in humans is about 40 nmoles, about 85% of the body pool of T3 appears to be produced by peripheral 5'-monodeiodination and only about 15% by synthesis within the thyroid (Ingbar & Braverman, 1975; Cavalieri & Rapoport, 1977 and references cited therein). Moreover, humans have been reported to be capable of providing virtually all of the T3 requirement by deiodination of T4 alone (Stock et al., 1974).

Investigations have also indicated that the liver is involved in peripheral outer ring monodeiodination (Plaskett, 1961); kidney homogenates were as active as those from liver, but homogenates from intestine, spleen, heart muscle, lung and brain were ineffective with regard to converting T4 into T3 in vitro (Kaplan & Utiger, 1978). The cellular location of the deiodinase appeared to be in the plasma membrane (Maciel et al., 1978). A source of sulfhydryl groups, such as reduced glutathione, appears to be a cofactor required for enzyme activity (Chopra, 1978).

Reverse-T3, like T3, has been reported to be derived, in humans, predominantly by peripheral monodeiodination (Gavin et al., 1976). Of the 80 nmoles of T4 deiodinated daily, about 45 nmoles was reported to produce reverse-T3 (Cavalieri & Rapoport, 1977). Additionally, reverse-T3 levels have been reported to be decreased in hypothyroid patients and to be increased in hyperthyroid patients, relative to control subjects (Chopra, 1974). Decreased serum T3 levels (believed to result from decreased extrathyroidal 5'-monodeiodination of T4) were usually associated with increased levels of reverse-T3 in patients with a variety of systemic illnesses (Kaplan & Utiger, 1978 and references cited therein). Similarly, anaesthesia has been reported to decrease T3 levels while increasing reverse-T3 (Georges et al., 1977).

1.1.5 Normal Variation of Thyroid Hormone Concentrations

There are a variety of factors which have been associated with normal variations in the circulating levels of T4 and/or T3. Thus, it may be necessary for an investigator to control for the effects of sex, stage of estrous cycle in females, age, time of sampling (with regard to diurnal and seasonal variations) and diet. Experimental factors, such as sampling procedure (especially if it includes anaesthesia) can also be suspected of affecting hormone levels. These factors are described below; variations in thyroid hormone concentrations which can be associated with autoimmune thyroiditis are reviewed in Subsections 1.2.2 and 1.3.4.

Variation with sex.

T4. There are conflicting reports regarding variations in T4 due to sex. In rats, T4 levels were reported to be higher in adult females than in males (Reuber, 1970c; Kieffer et al., 1976). However, if the mean for each stage of the estrous cycle (Kieffer et al., 1976) were averaged, the overall mean would be only slightly higher than the mean which was observed for the males. Furthermore, Reuber (1970c) measured Protein Bound Iodine levels in BUF strain rats; in the first place, Protein Bound Iodine assays are not as accurate or as specific as radioimmunoassays, and in the second place, thyroid function in BUF rats may be altered, especially in females (see Subsection 1.3.4). Two other papers have reported that T4 levels were slightly, but significantly, higher in male rats: Rapp & Pyun (1974) measured T4 in adult Wistar rats, using the Murphy-Pattee Competitive Protein Binding method (which is an improvement over measuring Protein Bound Iodine levels, but it too is not as accurate or as specific as a radioimmunoassay); whereas Fukuda et al. (1975), measuring T4 in Sprague-Dawley rats, did use a radioimmunoassay.

A further note is that Kieffer et al. (1976) reported that they could not detect a sexual variation in T4 levels in neonatal rats (2, 4, 16 and 17 days old) and Fisher et al. (1977) could not detect a significant sexual variation in children between 1 and 15 years of age.

T3. Fukuda et al. (1975) reported that T3 levels were lower in male rats than they were in females (in contrast with their observation that

T4 levels were higher in males). In humans, Fisher et al. (1977) failed to detect a significant sexual variation in T3 levels in children between 1 and 15 years of age (similar to their finding with T4).

Variation with estrous cycle.

T4. Small but statistically significant differences were found between T4 concentrations at different stages of the estrous cycle in rats (Kieffer et al., 1976). Values were lower at proestrus and diestrus-1, but higher at estrus and diestrus-2; thus, T4 levels appeared to cycle twice during the estrous cycle.

T3. No reports of T3 variations during the estrous cycle were found.

It is worth noting that thyrotropin (TSH) concentrations do not appear to vary signficantly during the estrous cycle (Kieffer et al., 1976; Fukuda et al., 1975). Thus, the pituitary would not appear to contribute to any thyroid hormone variation which may be associated with the estrous cycle.

Variation with age.

T4. Data obtained by Kieffer et al. (1976) indicated that T4 levels rose in neonatal rats until they reached adult levels. In humans, T4 levels were found to decline between the ages of 1 and 15 years (Fisher et al., 1977); this was attributed to a concommitant decline in TBG levels which was also observed. Bermudez et al. (1975) reported that a progressive decrease of T4 concentrations also occured in adults. The earlier findings of Fisher et al. (1974) did not agree with this, but it must be noted that the less sensitive and less specific Protein Bound Iodine and Butanol-Extractable Iodine assays were used.

T3. No investigations of T3 variations with age in rats were found. In humans, T3 values in the fetus and in newborns were reported to be initially low, increase greatly within the first several hours after birth, and decline over the first three days post partum to adult normal values (Abuid et al., 1973, 1974; Erenberg et al., 1974). Decline of T3

with age was observed between the ages of 1 and 15 years (Fisher et al., 1977) and after the age of 30 years (Rubenstein et al., 1973). Bermudez et al. (1975) also reported that the decline observed in children was continued in adults. In two other reports which failed to show a significant decline of T3 in children, the authors may have failed to ensure that all of the children were healthy "normal" subjects (Fisher et al., 1977 and references cited therein). The decline of T3 concentrations with age in children, like that of T4, may be due to the concommittant decline in TBG levels (Fisher et al., 1977).

Diurnal variation.

T4 and T3. Fukuda et al. (1975) reported that plasma T4 and T3 levels did not exhibit significant diurnal variations in either male or female rats (although they reported that TSH levels did vary). Their conclusions were based upon two analyses of their data; analysis of variance, and the Neuman-Keuls comparison test. In contrast, in humans, T4 levels have been reported to exhibit diurnal variations (DeGroot, 1971; O'Connor et al., 1974; Balsam et al., 1975; Pekary et al., 1976), although the latter report noted that only 38% and 50% of the subjects had diurnal variations of T4 or T3, respectively. In chicks, too, both T4 and T3 exhibited diurnal variations of both T4 and T3; Newcomer (1974) showed that the data fit sinusoidal regression curves, allowing for a more efficient analysis of variation than was referred to earlier (Fukuda et al., 1975).

The diurnal variation of T4 in humans has been attributed to changes in plasma volume (DeGroot, 1971), which depends upon whether the subject is upright or lying down. T4 and T3 levels, corrected for changes in total protein concentrations (and thereby for changes in plasma volume) have been reported to exhibit both a diurnal variation and rapid fluctuations; when the subjects were supine and motionless, however, the ratio of each hormone to total protein concentration was stable (Pekary et al., 1976). Thus, posture may indeed be the factor which most contributes to diurnal variations in T4 and T3 concentrations in humans. It remains to be determined whether posture is a significant factor in rats (since rats tend to remain prone throughout most of the

day and night), but this might account for the inability of Fukuda et al. (1975) to detect significant diurnal variations of T4 or T3 levels in rats.

Seasonal variation.

T4 and T3. No reports of seasonal variations of either T4 or T3 concentrations in rats were found. In humans, however, there were conflicting reports. Among people who work outside (11 postmen), significant differences were observed between the monthly mean Protein Bound Iodine levels for January and February (the highest values) versus those for September and October (the months with the lowest mean levels) (Thompson & Kight, 1963). Osiba (1957) and Du Ruisseau (1965) also found a monophasic, seasonal variation related to outside temperatures, whereas Watanabe et al. (1963) reported that there was a diphasic variation (in men and in women), with peaks in spring and fall and nadirs in summer and winter. In contrast, seasonal variations could not be detected in other investigations (Thompson & Kight, 1963; Nuttal, 1970; Postmes et al., 1974; Morris & McCall, 1977). The latter two reports assayed T3 levels also, and found that T3 did not exhibit seasonal variation either.

None of the above investigations compared hormone levels with photoperiod changes during the year, attempting only to relate hormone levels and outside temperatures. In reviewing these papers, moreover, inadequacies were recognized, with regard to analysis of data and to the experimental conditions.

Regarding analysis, the data were not analyzed using efficient or sensitive tests of significance for cyclic variations; no attempts were made to fit the data to regression lines (e.g., sinusoidal curves). Moreover, the data from both sexes were usually pooled. Only Thompson & Kight (1963) and Watanabe et al. (1963) performed separate analyses for males and for females. Nuttal (1970) showed that the mean Protein Bound Iodine levels for males and for females were "essentially the same", but did not test to see if either sex, taken separately, showed a significant seasonal variation. Thus, their report that there was no seasonal variation does not rule out the possibility that, as reported

by Thompson & Kight (1963), one sex, or even both sexes separately, might exhibit significant seasonal variation when analyzed statistically.

Regarding experimental conditions in the above papers, most of the subjects lived and worked in buildings which were not temperature-controlled during the summers, but the fact that most of the subjects did spend most of their winters inside temperature-controlled buildings strongly suggests that the subjects were not actually subjected to the temperature changes with which their hormone levels were compared. If the temperatures in the buildings varied only slightly during the year, it would not be surprising that hormone levels did not exhibit seasonal changes related to outside temperatures.

Two further questions were prompted by this review of the literature. Firstly, how variable are the environmental conditions to which laboratory rats are subjected when housed in Animal Care facilities, and secondly, would the rats exhibit seasonal variations of T4 or T3 concentrations as a result of any such variation in their environment?

Variation with diet.

T4 and T3. Reports have related serum concentrations of T4 and T3 to two components of diet in particular; iodine, and calories (although it is also possible to subdivide calories in terms of carbohydrate versus non-carbohydrate sources).

With rats, TSH increased while T4 and T3 both declined when dietary iodine was decreased (Riesco et al., 1976); T4 declined at a greater rate than did T3, presumably due to the thyroid's shift towards increased T3 production in response to increased TSH stimulation. Excess iodine had comparable effects (Yamada et al., 1976). After two days on a diet containing excess iodide, thyroid and plasma levels of both T4 and T3 were significantly decreased, and plasma TSH levels were increased; therefore, acute excess appeared to block the synthesis of T4 and T3 in the thyroid. After 14 days on the diet, however, thyroid levels of both T4 and T3, and plasma levels of T4, were normal, but plasma T3 and TSH levels were significantly decreased and pituitary levels of TSH were slightly (but not significantly) increased; thus, chronic excess iodide

did not appear to maintain the block of thyroidal synthesis of T4 and T3, but the conversion of T4 to T3 did appear to be impeded.

Investigations of the effect of caloric intake have been performed with humans. Increases in calories from carbohydrate sources, and to a lesser degree from non-carbohydrate sources, were not associated with changes in T4 concentrations, but T3 concentrations did increase up to a plateau (Chopra & Davidson, 1976). An excess of calories appears to be dissipated by an increase in heat production (Miller et al., 1967) as well as by an increase in body fat (Sims et al., 1973). It is not known whether these responses are mediated by, or lead to, the suggested changes in T4-to-T3 conversion.

A decrease in nutritional status, using decreases in the serum levels of transferrin and retinol-binding protein as indices, was associated with a significant decrease in T3 but not in T4 (Olusi et al., 1976). Although acute weight loss, or gain, was correlated to a decrease or an increase in T3, respectively, the T4 levels exhibited variable responses (Bray et al., 1976). Moreover, various indices of body weight (e.g., percentage of ideal body weight, body-mass index, and ponderal index) were found to exhibit significant (positive) correlations with T3, but not with T4 (Bray et al., 1976). The relevance of these findings (from studies on humans), with regard to rats, remains to be seen.

Variation with anaesthesia.

T4 and T3. Of the papers which were reviewed, only one investigated whether anaesthesia affected T4 or T3 concentrations in rats. Dohler et al. (1977) reported that, relative to decapitation without anaesthesia, chloroform had no effect on T4 or T3 concentrations, but did slightly increase TSH levels. Although ether slightly increased T4 and T3 levels (the increases generally were not significant) and significantly increased TSH levels, Nembutal on the other hand was associated with significant decreases of both T4 and T3, but did not affect TSH levels. In addition, TSH levels in rats were not significantly affected by a variety of anaesthetics which required injection, but were decreased by those which were administered by inhalation (Mannisto et al., 1976).

Thus, different anaesthetics appeared to have different influences on T4, T3 and TSH concentrations in rats.

In humans, T4 increased and T3 decreased with general anaesthesia, even before actual surgery began (Brant et al., 1976a,b). In contrast, T4 declined with epidural anaesthesia (Brant et al., 1976a,b). The suggestions have been put forward that certain anaesthetics may cause T4 release from hepatic stores (Brant et al., 1976b), and that anaesthesia (in baboons) may be associated with a shift of the monodeiodination of T4, away from T3 and toward reverse-T3 (Georges et al., 1977).

Based upon the information presented in the review of diurnal variation, the following questions arose. To what extent are the changes in T4 and T3 related to changes in plasma volume which might accompany anaesthesia (wherein the subject is supine)? Is it possible that changes in the rats were in fact due to differences in the positions of the animals during different types of serum collection? If there are direct metabolic influences of anaesthesia, would they be counteracted by changes in blood volume (due to posture)?

1.2 AUTOIMMUNE THYROIDITIS

1.2.1 Introduction

Thyroiditis. Goitre (swelling of the thyroid) can be a sign of dietary iodine insufficiency (resulting in compensatory thyroglobulin synthesis) or of a disease. Thyroiditis ("inflammation" of the thyroid), in humans, has been classified into four principal forms (Beeson & McDermott, 1971; Woolner, 1964).

Acute suppurative thyroiditis is a very rare disorder, resulting from bacterial infection (usually in an abnormal thyroid) (see Hamburger, 1973 and references cited therein).

Riedel's struma (invasive fibrous thyroiditis) is rare and usually recognized only at operation. The most common clinical manifestations, when they occur, are difficulty breathing or swallowing (due to a slightly enlarged thyroid) or hypothyroidism.

De Quervain's thyroiditis (subacute or granulomatous thyroiditis) is more common; it is reputed to be a viral infection. The disease tends to spontaneously remit and soon recur, cycling in this manner for 4 to 12 months before finally subsiding.

The fourth and most common form of thyroiditis is Hashimoto's thyroiditis, described in detail below.

1.2.2 Hashimoto's Thyroiditis

Clinical definition. In Hashimoto's thyroiditis (struma lymphomatosa, lymphadenoid goitre, lymphocytic or autoimmune thyroiditis), a patient usually has a diffusely enlarged, firm-to-hard goitre. Some patients have a reduced thyroid hormone-synthetic activity; this hypothyroidism may become evident as sluggishness, weight-gain and increased sensitivity to cold (due to a greatly decreased basal metabolic rate).

Thyroid function. Hashimoto's thyroiditis can be associated with varying degrees of thyroid function; about two-thirds of the patients are euthyroid and about one-third are hypothyroid (Bastenie, 1972; Hamburger, 1973). In a small fraction of the cases, the patient is hyperthyroid (the basal metabolic rate is increased, usually related to increased T4 levels).

In euthyroid patients with Hashimoto's thyroiditis, neither T4 nor T3 levels were significantly different from those of control subjects. In hypothyroid patients, T4 was reduced but T3 tended to be increased; T4/T3 ratios were significantly lower than in other other groups of Hashimoto's thyroiditis patients. In hyperthyroid patients, both T4 and T3 levels were increased, but T4/T3 ratios remained equal to those of euthyroid patients (Gharib et al., 1972).

Incidence. The ratio of females to males is approximately 10:1 (Woolner, 1964; Nève et al., 1972). It is usually suggested that the incidence is especially increased in older women, but at least one report (Fursyfer et al., 1970) indicated that the incidence remained high more-or-less from the second decade (10-19 years) to the sixth (50-59 years).

Familial incidence of Hashimoto's thyroiditis has been reported, which strongly suggests that there is (are) genetic factor(s) involved in the incidence of the disease (Delespesse et al., 1972).

Histopathology. The prototypical patients for Hashimoto's thyroiditis were four middle-aged women whose thyroids had the following histologic characterisitics: diffuse infiltration by round cells; numerous lymphoid follicles; striking changes in the follicular epithelium and contents; and, extensive connective tissue formation (Hashimoto, 1912). This description, however, is more indicative of the patient whose disease has advanced than it is of the full spectrum of possible severity. Thus, diffuse infiltration, if untreated, appears to lead to the stage wherein the development of lymphoid follicles or germinal centres can be observed.

Hashimoto's thyroiditis can be diagnosed using histopathologic criteria (Beall, 1978): the epithelial cells are often unusual in size and shape, with large nuclei and eosinophilic cytoplasm; the follicles are small and depleted of colloid and thyroglobulin; and, lymphocytic infiltration is always present. Fibrosis is common and may be extensive.

Evidence of autoimmunity. In the mid-1950's, Witebsky and his coworkers (principally Rose) found that a form of thyroiditis which resembled Hashimoto's thyroiditis could be produced in rabbits, by injecting them with either thyroid extracts or with purified thyroglobulin (Witebsky et al., 1955; Rose & Witebsky, 1955, 1956; Witebsky & Rose, 1956). This disease, which could be termed "experimental autoimmune thyroiditis", was among the first indications that horror autotoxicus (Ehrlich, 1900) did indeed occur. Roitt and co-workers soon showed that the serum from patients with Hashimoto's thyroiditis contained antibodies to thyroglobulin (in the colloid) or to a distinct antigen present in the microsomal fraction of thyroid homogenates (Roitt et al., 1956; Roitt & Doniach, 1958). This led to the suggestion that lymphocytic thyroiditis in humans (Hashimoto's thyroiditis), like experimental autoimmune thyroiditis in rabbits, might be directly related to the presence of circulating antibodies (Doniach & Roitt, 1957). When transfer experiments were performed, however, serum from Hashimoto's thyroiditis patients (containing thyroid antibodies) failed to produce thyroiditis after injection into a monkey (Roitt & Doniach, 1958). Thus, although autoantibodies can usually if not always be found in the serum of patients, and the titres broadly parallel the severity of the disease (Senhauser, 1964), it appears that the antibodies themselves are not solely responsible for the thyroid pathology.

It is worth describing the thyroid antibodies which are found, as well as the techniques used to detect them. In order of ascending sensitivity, the assays are: precipitation in agar gel by the Oudin or Ouchterlony techniques (e.g., see Doniach & Roitt, 1957; Roitt & Doniach, 1957); hemagglutination techniques such as that of Boyden (e.g., see Roitt & Doniach, 1958); the complement-fixation technique of Donnely (again, see Roitt & Doniach, 1958); immunofluorescence techniques such as the Coons test or indirect immunofluorescence tests (e.g., see Noble et al., 1976); and radioimmunoassays (e.g., see Takeda et al., 1975).

Precipitation and hemagglutination techniques detect antibodies which react with thyroglobulin; high titres of these antibodies usually occur in only the more severe cases (Senhauser, 1964). Complement-fixing antibodies, which react with the microsomal antigen (Roitt & Doniach, 1958), can be found in a high percentage of patients with Hashimoto's thyroditis (Kunkel & Tan, 1964). Immunofluorescence techniques can detect thyroid antibodies in every case of Hashimoto's thyroiditis (Senhauser, 1964); immunofluorescence assays can distinguish antibodies which react with thyroglobulin in the colloid and to a lesser extent in the apical ends of the follicular cells, antibodies which react with a second colloid antigen that is distinct from thyroglobulin, and antibodies which react with the microsomal antigen (Kunkel & Tan, 1964). Radioimmunoassays for anti-thyroglobulin antibodies (Salabe et al., 1974) and for anti-T4 or anti-T3 antibodies (Staeheli et al., 1975) have recently been developed which allow semi-quantitative measurement of the circulating levels of these antibodies.

In view of the evidence that antibodies do not appear to be directly responsible for the lesions, investigators have addressed themselves to the question of whether antigen-specific T cells (thymus-dependent lymphocytes) participate in the pathogenesis of the disease. Several in vitro tests for cell-mediated immunity have been used: skin reaction to intradermal immunization; migration inhibition factor (MIF) assays; lymphoblastogenic (i.e., mitogenic) assays; and, cytotoxicity assays. The evidence is reviewed briefly below; for more detail, the reader is referred to the review of Calder & Irvine (1975) and several other references cited below).

Positive skin reactions have been produced by intradermal injections of thyroid extract into patients with Hashimoto's thyroiditis. However, these may have been due to Arthus reactions (due to circulating antigen-antibody complexes) rather than cell-mediated (delayed hypersensitivity) immune reactions.

Migration inhibition factor (or MIF) assays are designed to allow leukocytes (usually macrophages) to react with a soluble factor produced by antigen-specific T cells in the presence of the antigen; however, in some cases, macrophage inhibition may be an artifact of serum antibodies

or soluble immune complexes (Calder & Irvine, 1975 and references cited therein). Nonetheless, there were many reports that patients with Hashimoto's thyroiditis had leukocytes which were MIF-positive in the presence of crude thyroid extract, thyroglobulin, thyroid microsomes and/or thyroid mitochondria; there was no correlation between the amount of inhibition and either the presence or the titre of the thyroid antibodies measured in the patients' sera (again, see references cited in Calder & Irvine, 1975).

Antigen-induced mitogenesis (the transformation of small lymphocytes into lymphoblasts) is usually estimated by the incorporation of tritiated thymidine during DNA synthesis. The lymphocytes from some patients respond to either thyroglobulin and/or crude thyroid extract, whereas lymphocytes from other patients do not; these conflicting results suggest that either only some of the patients have lymphocytes which undergo blastogenesis (which may indicate that another pathologic mechanism exists in other patients), or else some patients may have lymphocytes which react with other thyroid antigens.

Cytotoxicity experiments have revealed that lymphocytes from Hashimoto's thyroiditis patients have a significantly increased capability for lysing target cells coated with either human thyroglobulin or thyroid microsomes (Calder & Irvine, 1975 and references cited therein). Pretreatment of patients' lymphocytes with anti-human-thymus globulin (to remove thymus-associated cells, which may include non-T cells) was found to reduce the cytotoxicity; Lagyea et al. (1973) suggested that the requirement for anti-thymus globulin-sensitive cells indicated that T cells were involved. However, peripheral lymphoid cells from Hashimoto's thyroiditis patients exhibited increased cytotoxicity in assays designed to detect K cells. The activity of these antibody-dependent "killer" lymphocytes, which do not appear to be either T or B cells (Calder & Irvine, 1975 and references cited therein), was increased in patients, whereas the number and proportion of T and B cells (assayed by rosette-forming techniques) were unchanged (Calder et al., 1976). There is obviously a need for more research into the nature of K cells, in general, and specifically with regard to Hashimoto's thyroiditis.

1.2.3 Spontaneous Autoimmune Thyroiditis in Animal Models

There are several reasons for searching for animal models for Hashimoto's thyroiditis. Many experimental procedures, such as thyroid biopsy and thymectomy (removal of the thymus; refer to Subsection 1.3.2), would not be readily available using only human subjects. As well, animals with shorter lifetimes would allow longitudinal (serial) and vertical (multigenerational) studies. Furthermore, animals facilitate controlled breeding, useful for genetic studies.

Spontaneous autoimmune thyroiditis, the animal model of Hashimoto's thyroiditis, has been reported in several species; the reader is referred to a review by Bigazzi & Rose (1975). Two models are discussed below and the BUF rat model is described in the following section.

Beagle dogs. Tucker (1962) found thyroiditis in several colonies of beagles. Although the histology and thyroid function in this model appear to closely resemble the human disease, relatively few workers have studied this model; perhaps this is due to the absence of inbred strains derived from animals with the disease (consider how many years it would require to acheive the 20 generations of brother x sister matings), or the lack of commercial suppliers of Beagle dogs from "high incidence pedigrees" (Bigazzi & Rose, 1975 and references cited therein).

OS chickens. Spontaneous autoimmune thyroiditis has also been reported in a closed flock of White Leghorn chickens (van Tienhoven & Cole, 1962) which have since been selectively bred to produce the OS (Obese Strain) chicken. Pathology of the thyroid was greatly accelerated and very severe in this model (Bigazzi & Rose, 1975 and references cited therein). There was a significant decrease in thyroid function within 2-3 weeks after hatching; serum T4 and T3 were almost absent and TSH was increased. Mononuclear cells, macrophages and lymphocytes were observed to infiltrate the thyroid 1-5 weeks after hatching. The incidence of thyroiditis in the OS chicken is over 90% in both sexes. Thus, despite the differences betweeen thyroiditis in humans and OS chickens, these birds could provide investigators with another valuable tool. Again, however, the availability of stock OS chickens or maintenance may have been problems.

1.3 BUF RATS

1.3.1 Introduction

Originally designated as the Buffalo rat, this strain was used in studies of experimentally-induced thyroiditis for several years before it was recognized as a model of spontaneous autoimmune thyroiditis. A brief review of the background of this rat strain (see also Festing & Staats, 1973; Bigazzi & Rose, 1975 and references cited therein) and a more detailed account of thyroiditis follow.

Inbreeding. The BUF strain was probably derived from Wistar stock; rats were obtained from Buffalo, NY, USA in about 1931. The Genetics Research Unit, Laboratory Aids Branch, of the National Institutes of Health, Bethesda, MD, USA obtained stock animals at the tenth generation of brother x sister matings, and rats have now been inbred at least to the 58th generation. There do appear to be differences, nonetheless, between BUF rats available from different sources (see Subsection 1.3.3).

Known genes. BUF rats have been typed as follows: alleles A, B6 (H-1b), and C1 of the rat major histocompatability (Ag) region; homozygous cc for coat colour (albino); and, allele b of the RI-1 allotype (an immunoglobulin marker). With regard to metabolism, they have a genetic defect in the liver's methyl transferase system; they are also good responders to LDH-A_4 isozyme.

Susceptibility to spontaneous tumours. Thymomas have been reported in about 50% of males and 40% of females; 40% of males develop adrenal medullary tumours. The incidence of anterior pituitary tumours increases with age; the incidence of this tumour (about 75% in females) and of adrenal cortex tumours (about 67% in females older than 18 months) are about twice those in males. BUF rats will also grow several transplantable tumours, including Morris hepatoma and pituitary tumour.

Susceptibility to other spontaneous diseases. Older animals are susceptible to high incidences of a variety of diseases: myocarditis (30%); otitis media (70%); and respiratory disease (bronchiectasis) (80%). Spontaneous thyroiditis is described in Subsection 1.3.3.

1.3.2 Experimentally-induced thyroiditis

Reuber and his co-workers used BUF rats from the National Institutes of Health, Bethesda, MD, USA. By feeding a high fat, low protein, choline deficient diet (Reuber, 1969b) or by injecting carbon tetrachloride (Glover & Reuber, 1967; Reuber & Glover, 1967a,b), they were able to induce cirrhosis of the liver in the BUF rats; thyroiditis was also observed in treated rats, although it could not be correlated with the incidence or the severity of the cirrhosis. Thyroiditis was also observed after treating BUF rats with either trypan blue (Reuber, 1969a, 1970a), 7,12-dimethylbenz(A)anthracene (Reuber & Glover, 1969c), N-4-(4'-fluoro-biphenyl)acetamide (Stromberg & Reuber, 1970), 3'-methyl-4-dimethyl-aminoazobenzene or N-2-fluorenyldiacetamide (Reuber & Glover, 1973, 1976). A series of papers (Reuber & Glover, 1968; Glover & Reuber, 1968; Glover et al., 1968; Reuber & Glover, 1969a,b; Glover et al., 1969; Reuber, 1970b,c) characterized the thyroiditis resulting from various regimens and routes of administration of 3-methylcholanthrene. Methylcholanthrene altered thyroid function and prolactin levels in non-BUF rats (Moon, 1964; Newman & Moon, 1966, 1967), but only BUF rats appeared to be susceptible to developing thyroiditis after methylcholanthrene treatment; Glover et al. (1969) reported that Fischer, Marshall, Osborne Mendal, and ACI inbred rats did not.

In general, Reuber and his co-workers observed that both the incidence and the severity of experimentally-induced thyroiditis were greater in the female BUF rats than in the males, and younger animals were usually more susceptible than older ones (e.g., thyroiditis was observed in each of eleven rats which were fed methylcholanthrene for 12 weeks beginning at the age of 4 weeks; Reuber & Glover, 1969a).

Silverman, working with Rose, examined experimentally-induced autoimmune thyroiditis in BUF rats obtained from a different source: Simonsen Laboratories, Gilroy, CA, USA. The susceptibilities of males and females were not generally compared, as they usually used females only. They reported that LEW strain inbred rats were not susceptible to methylcholanthrene-induced thyroiditis either (Silverman & Rose, 1975a), and suggested that, since (BUF x LEW)F_1 and (LEW x BUF)F_1 hybrids did not develop thyroiditis (except in one rat), therefore susceptibility

was neither transmitted maternally nor inherited as a Mendelian dominant (Silverman & Rose, 1975b). Most of the rats which developed thyroiditis also had antibodies to rat thyroid gland extract, detectable by indirect immunofluorescence techniques, and, in some of the more severe cases, by hemagglutination techniques as well (Silverman & Rose, 1971) and it was subsequently shown that injecting BUF rats with thyroid extract would protect them from developing methylcholanthrene-induced thyroiditis (Silverman & Rose, 1974a). Although thymectomy did not affect the incidence when performed on adults, neonatal thymectomy (with or without subsequent methylcholanthrene treatment) increased the incidence to virtually 100% (compared to 13% in the untreated BUF controls; Silverman & Rose, 1974b).

1.3.3 Spontaneous Autoimmune Thyroiditis in BUF Rats

Hajdu & Rona, performing comparative endocrine pathology studies, found a high incidence of spontaneous thyroiditis among BUF rats (which had been obtained from Microbiological Associates, Bethesda, MD, USA), but not in several other rat strains (Sprague-Dawley, Long Evans, Wistar, LEW, F344 and RAH; Hajdu & Rona, 1969). The incidence appeared to be decreased in older animals, but the small sample size and the few ages that were tested prevent one from making firm conclusions. Similarly, although the males appeared to have a higher incidence than the females (since thyroiditis was observed only in six of the eleven 36 wk old males), it must be recognized that Hajdu & Rona only examined ten females -- five at each of 4 and 52 wk of age.

Reuber and co-workers, using BUF rats from the National Institutes of Health, rarely observed spontaneous thyroiditis. Out of at least 256 male and 277 female control BUF rats examined in all of their papers cited above, only 2 of the males (0.8%) and 4 of the females (1.4%) had developed the disease.

Silverman & Rose (1971) examined 15 untreated 60-64 week old female BUF rats which had been obtained from Microbiological Associates also. They found that 6 of these rats (40%) had developed thyroiditis, but the small sample size again prevents one from making any firm comparisons with the animals used by Hajdu & Rona. In their series of papers,

however, out of a total of at least 226 untreated non-breeder (virgin) females which had been obtained from Simonsen Laboratories, 18 (8.0%) had developed spontaneous thyroiditis. This was more than five times higher than the incidence in females that Reuber and his co-workers observed in their BUF rats from NIH.

With regard to the incidence of spontaneous autoimmune thyroiditis, Silverman & Rose (1975a) investigated two other factors: sex and multiparity or breeding. Females had higher incidences than males, which was comparable to the situation in experimentally-induced thyroiditis but was in contrast to the suggestion made by Hajdu & Rona (1969). It must be remembered that not only did Silverman & Rose use animals from a different source, but they also examined many more females than did Hajdu & Rona. When Silverman & Rose examined multiparity or breeding, they observed that retired breeders of both sexes (but especially the females) had higher incidences than did the corresponding virgin animals.

Rose and his co-workers also examined immunity in BUF rats; Noble et al. (1976) observed that the incidence and titre of thyroid antibodies were correlated with the incidence of thyroiditis (only female retired breeders and neonatally-thymectomized females -- with and without subsequent "reconstitution" -- were investigated).

1.3.4 Thyroid Function in BUF Rats

Thyroid weight. A rough indication of the state of the thyroid can be obtained by measuring the weight of the thyroid gland. In general, in the papers from Reuber's and Rose's laboratories (cited above) and in a paper from Maloof's laboratory (Kieffer et al., 1978), thyroid weights were higher in BUF rats with experimentally-induced thyroiditis than they were in the treated rats which had not developed thyroiditis (which themselves were approximately equal to the thyroid weights of untreated controls). Treated and untreated females that had developed thyroiditis both tended to have heavier thyroids than the comparable males, although usually there was little difference between males and females that had not developed either spontaneous or experimentally-induced thyroiditis.

Protein Bound Iodine assays. Protein Bound Iodine levels were previously measured either at one age only or else by cross-sectional (rather than longitudinal) studies of BUF rats at a variety of ages (Reuber & Glover, 1969a,b; Glover et al., 1969; Reuber, 1970b). In the BUF rats that did not have thyroiditis, untreated females had higher levels than untreated males. Protein Bound Iodine levels were increased in BUF rats that had been treated with methylcholanthrene (although methylcholanthrene had reduced plasma Protein Bound ^{131}I levels in Sprague-Dawley rats to approximately one-half of the levels in controls; Newman & Moon, 1967), and the levels were increased further in the treated animals that had developed thyroiditis. It must be noted that, in humans with thyroiditis, the thyroid releases increased levels of iodoproteins (such as thyroglobulin and iodoalbumin) which are measured by Protein Bound Iodine assays.

Thyroid hormone assays. More recent investigations used more sensitive and specific assays than the Protein Bound Iodine assays, measuring T4 concentrations. Kieffer et al. (1978) reported that female BUF rats had slightly lower levels of plasma T4 than did the males. Moreover, those rats which had unusually high plasma TSH concentrations, or increased "relative thyroid weights", almost always had T4 values that were below the mean which was calculated for the rest of the BUF rats; some of the thyroids which gave unusually high "relative thyroid weights" were examined microscopically, and they were found to exhibit lymphocytic infiltration and disruption of the normal architecture of the follicles.

With regard to the reduced T4 levels in the females, however, it must be recalled that female Wistar and Sprague-Dawley females have also been reported to have lower levels of T4 than the corresponding males (Rapp & Pyun, 1974; Fukuda et al., 1975; see Subsection 1.1.5 regarding variation with sex).

None of the investigations measured T3 levels in BUF rats, although Fukuda et al. (1975) reported that T3 levels were higher in females Sprague-Dawley rats than in the males. As well, it may be recalled that, in Hashimoto's thyroiditis, T3 levels were increased only in a very small minority of the hypo- and hyperthyroid patients (see Subsection 1.2.2).

1.4 OBJECTIVES

Previous BUF rat studies almost always examined small numbers of animals (i.e., 6-12 animals per sex at each of 1-6 ages); only one study (Noble et al., 1976) sampled rats as they aged (although the antibody titres were measured only in neonatally-thymectomized females at 2, 3 and 4 months of age).

A longitudinal study, to examine the natural history of the development of spontaneous autoimmune thyroiditis in a large control population, would necessarily preclude examining histological specimens at each age. This investigation was therefore undertaken with the purpose of maintaining a colony of BUF rats, collecting serial samples from males and virgin females (thereby excluding the effects of multiparity), and measuring serum total T4 and T3 concentrations (using sensitive and specific radioimmunoassays). Not only would this yield information about the relationships between thyroid hormone levels and thyroiditis in the BUF rat model for spontaneous autoimmune thyroiditis (which could serve as a baseline for future studies), but there would also be more general findings with respect to thyroid function (e.g., variations due to sex, age and body weight).

2. MATERIALS AND METHODS

(continued)

2. MATERIALS AND METHODS

2.1. EXPERIMENTAL CONDITIONS

2.1.1 Rats

BUF rats. Mature BUF rats (6 males and 14 females) were obtained from Simonsen Laboratories, Gilroy, CA, USA in January of 1975; these formed the nucleus of the colony used in this investigation. In order to begin collecting serum samples from young animals, without having to wait for the mature rats to breed, young rats (9 males and 11 females) were purchased at the same time. During the summer of 1976, rats of various ages (including 20 female retired breeders) were obtained for a supplementary investigation; these rats were not incorporated into the colony, but were sacrificed within one week after their arrival.

Sprague-Dawley rats. Also used in this investigation were Sprague-Dawley rats, kindly provided by the Animal Care Unit, Faculty of Medicine, Memorial University, from a small colony which they maintain.

Housing. The Animal Care Unit maintained all of the rats in their facilities in the Health Sciences Centre; a "Small Animal Room" was set aside for the colony of BUF rats. They were housed in transparent plastic cages, usually 1-3 adults per cage (on occasion, up to 5 adults could share a cage); individual rats were therefore identified by ear-punching. Three racks were used to hold the cages: one for males only, one for females only, and a third was used for breeding.

Throughout the course of this investigation, the Health Sciences Centre was under construction (although partly occupied by the Faculty of Medicine); the temporary arrangements often caused problems with regard to heat and humidity. During the summers there were periods of excess heat and/or humidity, whereas during the winters there periods of excess heat and/or insufficient humidity. Low birth rates (reduced drastically during periods of excess heat) and high death rates were encountered. On a few occasions, large numbers of BUF rats died within only a few weeks (although other rats in the Animal Care Area were little affected). The most extreme example was when about 40 BUF rats died in November 1975 during an epidemic of respiratory disease. (During

this epidemic, the apparently healthy BUF rats were temporarily moved into another Small Animal Room in order to segregate them from those rats which were showing obvious signs of illness.)

Lighting. The BUF rat colony received a 12 hour light/dark cycle; fluorescent lights were automatically turned on at 0800 h. On a number of occasions, the "night-light" switch (controlling one of the four double-bulb fixtures) was found to have been inadvertantly placed in the "on" position.

Feeding. All of the rats were fed Purina Rat Chow (Ralston Purina of Canada, Woodstock, Ontario, Canada) and tap water ad libitum. Purina Rat Chow contained 1.17 ppm iodine (information provided by the manufacturer) and daily consumption (which can be expected to have averaged about 15 g per rat) would have provided about 18 ug iodine. The municipal water supply in St. John's, Newfoundland contained 0.2 ppb iodine (as determined by the Water Analysis Laboratory, Chemistry Department, Memorial University; provided through the courtesy of Mrs. M. Hooper) and daily intake (which can be expected to have averaged about 35 ml per rat) would then have been a negligible source of iodine.

The supplementary BUF rats, while at Simonsen Laboratories, were fed Simonsen Custom Lab Diet G 4.5 which contained a minimum of 1.5 ppm iodine (information supplied by Mr. J. Russell, Simonsen Laboratories); they were shipped with a "travelling diet" (e.g., raw potatoes and carrots), and between their arrival and their sacrifice (i.e., for less than one week) they were fed Purina Rat Chow.

2.2 COLLECTION OF SAMPLES

2.2.1 Serum Collection

Design. The initial plan was to collect serum samples as follows: from male and female BUF rats, each week up to six months of age, with about 5-10 samples in each sex/age category. Later however, based upon new publications (e.g., Silverman & Rose, 1975a), it was decided to change the experimental design to the following: serum samples would be collected from male and virgin female BUF rats only, once per four-week interval up to one year of age, and an attempt would be made to include more samples (i.e., up to 20 in each sex/age category). Virgin females, which after weaning were housed separately from males, were used in order to exclude the effects of breeding upon the incidence of thyroiditis in females; breeding had less effect on males (Silverman & Rose, 1975a). A bleeding schedule was charted to show, for the rats which were born on different days, the calendar dates which corresponded to the four-week intervals beginning at the birth of each litter. Thus, this chart grew as each new litter was born; it finally contained 18 groups of dates (for 64 males, 57 virgin females, and 19 females which were used for breeding). It was decided, for convenience, to collect samples only on one day per week, bleeding those animals which had been due for their four-weekly bleeding during the preceding week. Several of the BUF rats were bled at 4 wk, but not enough serum was obtained to be able to assay both T4 and T3, and it was therefore decided to bleed rats only from the age of 8 wk.

Fig. 3 illustrates, for each rat, the ages at which serum was collected and assayed; some of the samples were assayed for T4, some for T3 (with a considerable number assayed for both). An attempt was made to exclude from sampling animals that appeared to have respiratory disease, and, as stated previously, many of the rats died before the age of 52 wk. Additionally, since each sample was obtained by excising the end of the rat's tail (as described below), it was anticipated that, with some rats, it might not be possible to collect so many samples (i.e., every four weeks from 8 to 52 wk); however, by omitting the taking of samples from some of the rats at the younger ages, it was possible to take samples from those rats at later ages.

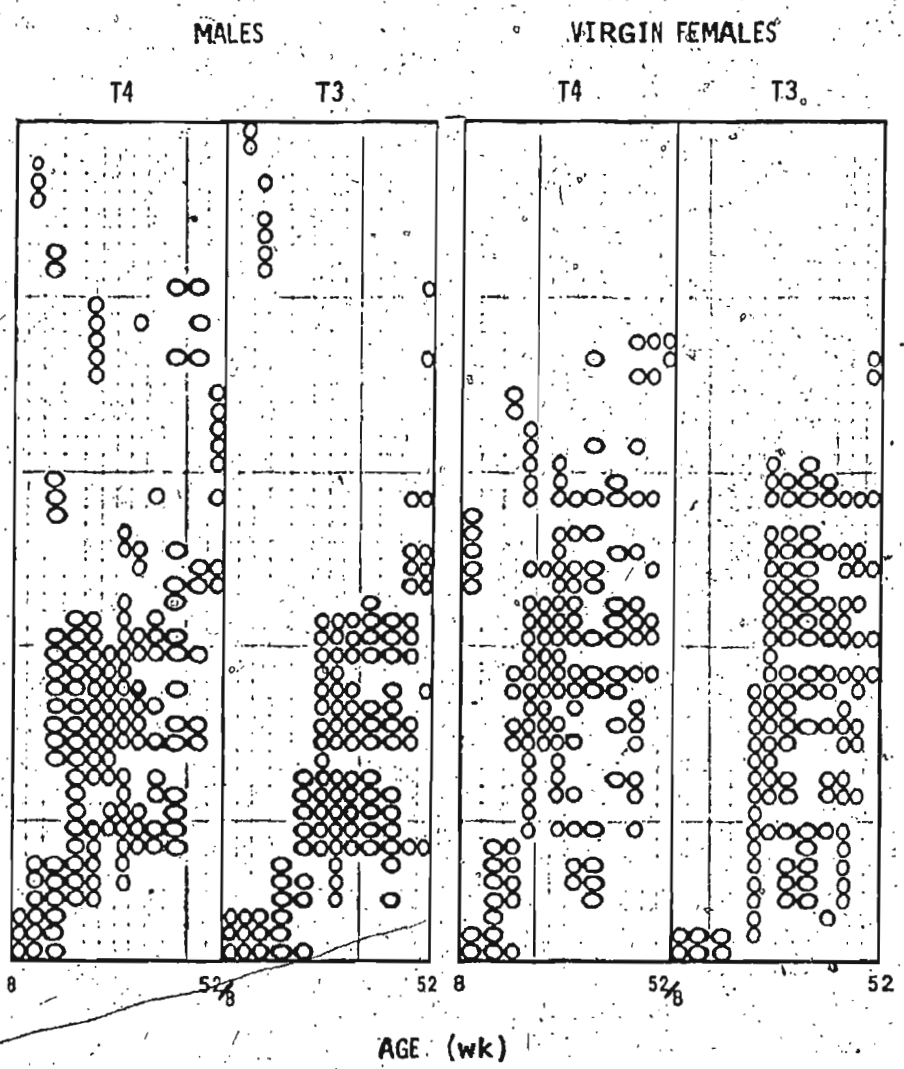


Fig. 3. Distribution of the BUF rat serum samples. One line in each panel represents four-week intervals in the life of an individual rat. Not all of the samples that were obtained were assayed for T4 and/or T3; the circles show those ages at which a hormone was assayed (T4 on the left side of the panel, T3 on the right).

Procedure. Each BUF rat was weighed immediately prior to being bled. For speed and convenience, their weights were determined to a one decade range (i.e., a 272 g rat would have been recorded as 270–280 g, and this would have been regarded as 275 g in all subsequent calculations). After being weighed, the rat was placed into an anaesthesia jar (a large glass dessicator jar containing some ether-soaked cotton in the lower level). When the rat was anaesthetized, which generally took about 5 min, it was removed from the jar. A small container which could fit over the rat's muzzle, containing some ether-wetted cotton, was used when necessary in order to maintain anaesthesia. Using a scalpel, the tip of the rat's tail was cut off; the excised portion ranged from about 5 to 20 mm in length, depending upon the length and diameter of the tail. The cut end was allowed to bleed into a clean test tube (glass and plastic tubes, e.g., 10 x 75 mm, were both used); in most cases there then ensued a wait of several minutes, but in some instances the blood flowed extremely rapidly (e.g., 5 ml in under 30 sec). The bleeding usually stopped naturally after about 3 ml had been collected, but in some cases self-closing hemostatic forceps were placed on the cut end of the tail in order to prevent further bleeding once enough blood had been collected. On the other hand, however, there were a few occasions when not even 1 ml could be collected. Rarely, the tail was then squeezed ("milked") to try to obtain more blood (although it was preferable to avoid this procedure, since extracellular fluids as well as vascular blood could be collected), and sometimes a second piece of the tail was excised in order to cause more blood to flow. Preheating of the rat, or its tail, or applying xylol to the tail in order to dilate the vessels did not appreciably improve the collection rate. As the rat was being bled, it was allowed to begin to regain consciousness, so that when the collection of the sample was completed the rat could be returned to its cage. Blood samples were allowed to clot while the rest of the rats were being bled (up to about 2 h), rimmed with glass capillary tubes and placed into a refrigerator (to enhance clot retraction and thereby to improve serum yields). Either that evening or else on the next day, the samples were spun at about 2000 x g at 2–8 C for about 5 min. As much serum as was possible was transferred to clean tubes, using Pasteur

pipets; a large number of cells were therefore carried over, and so the second set of tubes were also spun as above. The resulting sera were transferred by Pasteur pipets into larger-mouthed containers (20 ml scintillation vials or 12 x 100 mm plastic test tubes) in order to allow the use of a 250 ul Eppendorf pipet (which could not fit into the smaller blood collection tubes); 250 ul aliquots were then dispensed into 6.35 x 38.1 mm polystyrene tubes (LP/2 precipitin tubes, Luckham Ltd., Sussex, England). Microtitration plates (e.g., flat- or U-bottomed 96 well Microtitre plates, Cooke Engineering, Alexandria, VA, USA) were used as racks for the LP/2 tubes. Each tube was labelled with a consecutive aliquot number, the rat's identification (sex and number) and age. The tubes were covered with several thicknesses of Parafilm, frozen, and stored in an ultra-low temperature freezer (below -60 C) until used.

2.2.2 Diurnal Variations

BUF samples. The first few BUF rat serum samples were obtained without regard to the time of day. A review of the literature (performed in early 1975) failed to reveal any rat studies which had shown whether T4 and/or T3 levels varied significantly during the day, but, for instance, diurnal rhythms were found in thyroid hormone levels in chicks (Newcomer, 1974); thereafter, therefore, each day's bleeding began between 1000 and 1100 h and was completed by 1200 or 1300 h, in order to reduce variations which might have been caused by diurnal rhythms in serum T4 and/or T3 levels.

Sprague-Dawley rats. In addition, it was decided to collect sera from rats throughout the course of a day, in order to determine to what extent thyroid hormones did exhibit diurnal variation in rats (especially between 1000 and 1300 h). Sprague-Dawley rats were used so that the animals could be sacrificed, in order to obtain larger sample volumes, without depleting the BUF rat colony.

Serum was collected from six groups of four 24-28 wk old Sprague-Dawley males, one group every four hours. Each group was transferred from the Small Animal Room which housed the Sprague-Dawley

colony into a Procedure Room in the Animal Care Area one-half hour before sampling; lighting in the two rooms was synchronized. At the appropriate times, the rats were weighed, anaesthetized (in pairs, using the method described for the collection of serum samples from BUF rats) and then decapitated. Trunk blood was collected from each rat in the group, the serum fractions were obtained, and 250 ul aliquots were stored in an ultra-low temperature freezer (below -60 C) until assayed.

2.2.3 Other Possible Causes of Variation

No attempt was made to evaluate whether the stage of the estrous cycle, the season, or the sampling procedures (other than time of day) affected thyroid hormone levels. It was assumed that, over the course of the collection of the sera, random selection with regard to the stage of estrus would tend to average out the effects of the estrous cycle. Similarly, the variations associated with changes in season would have been averaged out since different animals in the colony reached each age at different times; moreover, environmental conditions (such as temperature and photoperiod) in the Animal Care facilities remained relatively constant in comparison with the outside conditions, and so should have little, if any, effect on thyroid hormone levels. It can also be assumed that the effects, on each animal, of bleeding (e.g., changes in blood volume, etc.) would have been reduced to negligable levels by the time of the next bleeding (at least four weeks later). Anaesthesia, using ether, might have had a small effect on thyroid hormone levels (see Subsection 1.1.5), but since nothing is known about whether the effects would be sex-, age-, or weight-dependent, one cannot speculate on whether all of the animals could have been affected equally.

2.2.4 Thyroid Collection

Histology specimens. Over the course of this investigation, there were 14 BUF rats which were found shortly after they had died, and 6 rats 52 wk old or more which were sacrificed; the sex/age distribution is shown in Fig. 4. The thyroid glands were immediately removed from these rats and placed into phosphate-buffered formalin to fix the tissues. The twenty thyroids were given over to the Histology Laboratory of the Faculty of Medicine and slides were prepared as follows: the specimens were imbedded in paraffin, sectioned through three levels, mounted and stained with hematoxylin and eosin. The finished slides were returned so that microscopic analysis for histopathology could be performed.

Supplementary specimens (including spleens). Supplementary BUF rats were obtained as described in Subsection 2.1.1. Within one week after their arrival, some had been bled for serum samples and all had been sacrificed (using ether inhalation); the thyroid glands had been immediately removed and placed into phosphate-buffered formalin and the spleens were removed and weighed. The fixed thyroids were weighed, and then given to the Histology Laboratory for slide preparation as described above. Serum samples and thyroid specimens from the supplementary rats were analyzed separately from the BUF rats maintained within the colony.

2.3 HISTOPATHOLOGY

The thyroid sections described above were screened in order to establish that spontaneous autoimmune thyroiditis did indeed occur in these BUF rats (especially those in the colony), and therefore thyroiditis was diagnosed simply on the basis of lymphocytic infiltration (although alterations in follicular architecture were also apparent); no attempt was made to grade the severity of the disease.

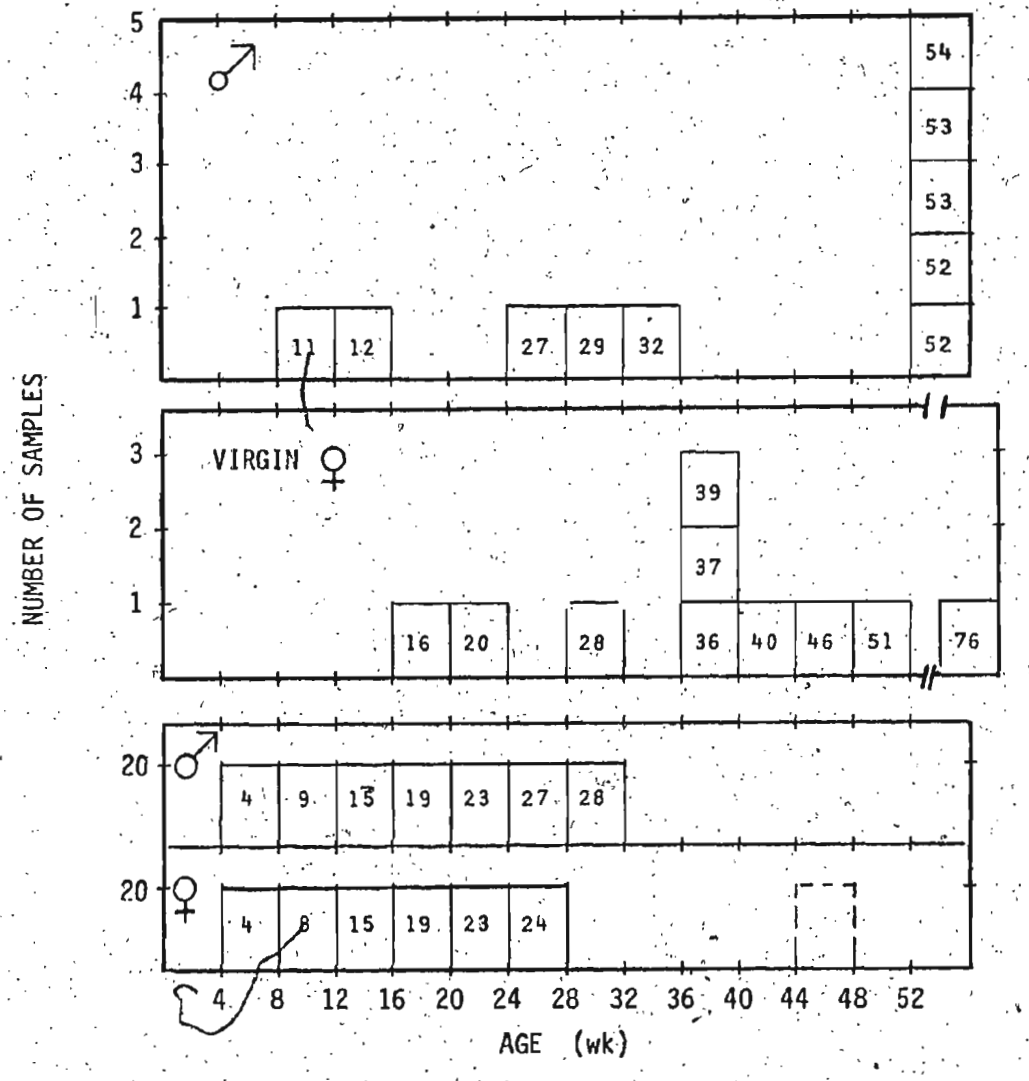


Fig. 4. Distribution of the BUF rat thyroid specimens. *upper*: thyroid histology specimens; *lower*: supplementary specimens (from rats that were not incorporated into the colony). Although the ages are divided into intervals of four weeks, the actual age of the specimen is shown inside each box. With regard to the supplementary specimens, note that there are 20 animals in each sex/age group, including female retired breeders about 11 months of age (indicated by the broken box).

2.4 RIA FOR T4

2.4.1 Materials

Serum levels of T4 were determined by a radioimmunoassay, using T4RIA (PEG) Diagnostic Kits (Abbott Laboratories, Diagnostics Division, North Chicago, IL, USA). These kits contained the following reagents.

Thyroxine standards. The standards contained 0, 3, 6, 12 and 24 ug/dl T4 and 2 g/l sodium azide in serum (horse serum; information supplied on request). Henceforth, these standards are referred to as 0, 39, 77, 154 and 309 nmol/l (or nM).

Buffer. The assay buffer consisted of 0.05 M barbital buffer (pH not specified) with 7.5 g/l bovine gamma globulin, 0.3 g/l ANS (8-anilino-1-naphthalenesulfonic acid) and 0.1 g/l thimerosal.

(^{125}I)Thyroxine tracer. The tracer solution contained 0.46 uCi or less per ml (specific activity not specified) in a 0.05 M barbital buffer (pH not specified) which contained 1 g/l bovine serum albumin and 0.1 g/l thimerosal.

Thyroxine antiserum. The antiserum consisted of sheep antiserum (information supplied on request) in 0.05 M barbital buffer (pH not specified) with 7.5 g/l bovine gamma globulin, 0.3 g/l ANS and 0.1 g/l thimerosal.

Polyethylene glycol (PEG). A solution of 180 g/l polyethylene glycol 6000 in 0.09 M barbital buffer (pH not specified) was provided to precipitate the antibodies in the assay.

2.4.2 Assay Procedure

Standards (at room temperature) and freshly-thawed serum samples were transferred, using a 25-ul Eppendorf pipet, into appropriately labelled 10 x 75 mm glass tubes (Pyrex disposable culture tubes, #14-962-15A, Fisher Scientific, Montreal, Quebec, Canada). The antiserum was added to each of the tubes, using a 400 ul Centaur pipet, and then the tubes were vortex-mixed for 3-5 sec. The tracer was added to each of the tubes, using a 100 ul Eppendorf pipet, and then the tubes were vortex-mixed for another 3-5 sec. The tubes were capped and allowed to incubate at room temperature for 120-140 min. During that time, 3-5 of the tubes were counted in a Beckman Gamma 310 Radiation Counter; the "total cpm" were about 25000 and 33000 with the two kits used. After the 2 h incubation, 2 ml PEG solution were added to each of the tubes, using a Cornwall Pipettor. The tubes were then vortex-mixed for 5 sec. Within 15 min after the addition of the PEG, the tubes were in a centrifuge and were spinning at room temperature at about 2000 x g for a 10 min spin. Within 15 min after the centrifuge stopped, the supernatant fractions were decanted, the tubes were allowed to drain, and their lips were blotted on paper towels. The pellets, containing antibody-bound tracer, were counted for 1 min (greater than 4000 cpm). Subsequent calculations were based upon these "bound cpm" values.

2.4.3 Validation Experiments

Dose-response graphs. Linear graph paper was provided with the T4RIA (PEG) Diagnostic Kits; however, when either the % bound (i.e., bound cpm divided by total cpm) or B/B_0 (i.e., bound cpm at a dose, divided by the mean of the bound cpm of the replicates of the zero dose standard) values were plotted against the dose, the data produced a curve. Manual curve-fitting was cumbersome and reduced the accuracy of dose-interpolation. The dose-response data more closely approximated a straight line when plotted on semi-logarithmic graph paper (i.e., B/B_0 versus $\log_{10}$ dose); this facilitated line-fitting. Moreover, it was thus possible to analyze the dose-response data using electronic calculators and the method of least squares fitting. At first, the analysis was performed using a Wang 600 programmable calculator (with an accessory papertape

reader which allowed the papertape output from the gamma counter to be fed directly into the calculator). Later, however, a Hewlett-Packard 9815A programmable calculator became available; HP pre-recorded tape cartridge, Volume 2 (Analysis of Variance and Regression Analysis, part no. 09815-15014, rev. B), was used to perform Bartlett's test for homogeneity of variances (to confirm the validity of using B/B_0-transformed data in the dose-response graphs and another tape cartridge, Volume 1 (General Statistics, part no. 09815-15000, rev. C, containing programs for a family of regression analyses), was used to re-analyze the dose-response data and to confirm the validity of logarithmic regression analysis.

One feature of the dose-response graphs, the slope, was thus obtained in the regression analyses; other features were calculated using a Hewlett-Packard 97 programmable calculator and HP-97 Standard Pac card SD-03A (which could perform logarithmic curve-fitting, as well as interpolation of doses and responses). These other features were the midrange (the dose which corresponded to $B/B_0 = 0.5$), and, the least detectable dose (the lowest dose which would be significantly different from zero; the level of significance was chosen to be two standard deviations of the zero dose replicates assayed for the given dose-response graph). The HP-97 also performed dose-interpolation of the response data from the rat serum samples.

Within-run variability. Several aliquots of one BUF rat serum sample were assayed, one with each of 8 batches of samples assayed one afternoon. The eight T4 values were then obtained by dose-interpolation from that day's dose-response graph, and the mean, standard deviation and coefficient of variation were calculated.

Between-run variability. The same BUF rat serum sample which was used above (to determine within-run variability) was also assayed (in duplicate) on three other days. Thus, the T4 concentration of that sample was determined on four independent occasions, and the mean, standard deviation and coefficient of variation were calculated.

Recovery experiments. Recovery data, using human serum, were supplied with the kits; 39 and 77 nmol/l T4 were added to sera from a variety of patients. The overall mean recoveries were 104.8% and 104.5%, respectively. Recovery experiments, using rat serum, were not performed for this investigation.

Specificity experiments. In response to a request for specificity data, Abbott Laboratories kindly provided a copy of the graph and table which had resulted from their own specificity experiments; the information is provided in Fig. 5. The cross-reactivity at 97 nmol/l was not significant for any of the compounds tested except for tetrac (T4A), which exhibited 16% cross-reactivity. However, the normal adult serum level of tetrac in humans, for instance, is only about 1.5 nmol/l (Burger, 1976).

Comparison with an independent assay. Dr. N. R. Farid has kindly provided two graphs illustrating experiments which compared the T4RIA (PEG) Diagnostic Kit with two other Abbott Laboratories kits for human serum T4 (Fig. 6). Values obtained using the T4RIA (charcoal) Diagnostic Kit correlated well ($r = 0.95$); values obtained using the Competitive Protein Binding (Murphy-Pattee) Kit were generally lower and did not correlate as well ($r = 0.88$) (see Farid & Kennedy, 1977).

As well, aliquots of 8 rat serum samples were assayed by the Radioisotope Laboratory of the St. John's General Hospital (using the Abbott kit) and by Dr. P. Walfish's Laboratory (using a double antibody T4 assay developed for rat serum samples). The resulting T4 values again showed an acceptable correlation, with $r = 0.87$ ($P < 0.01$) (unpublished data).

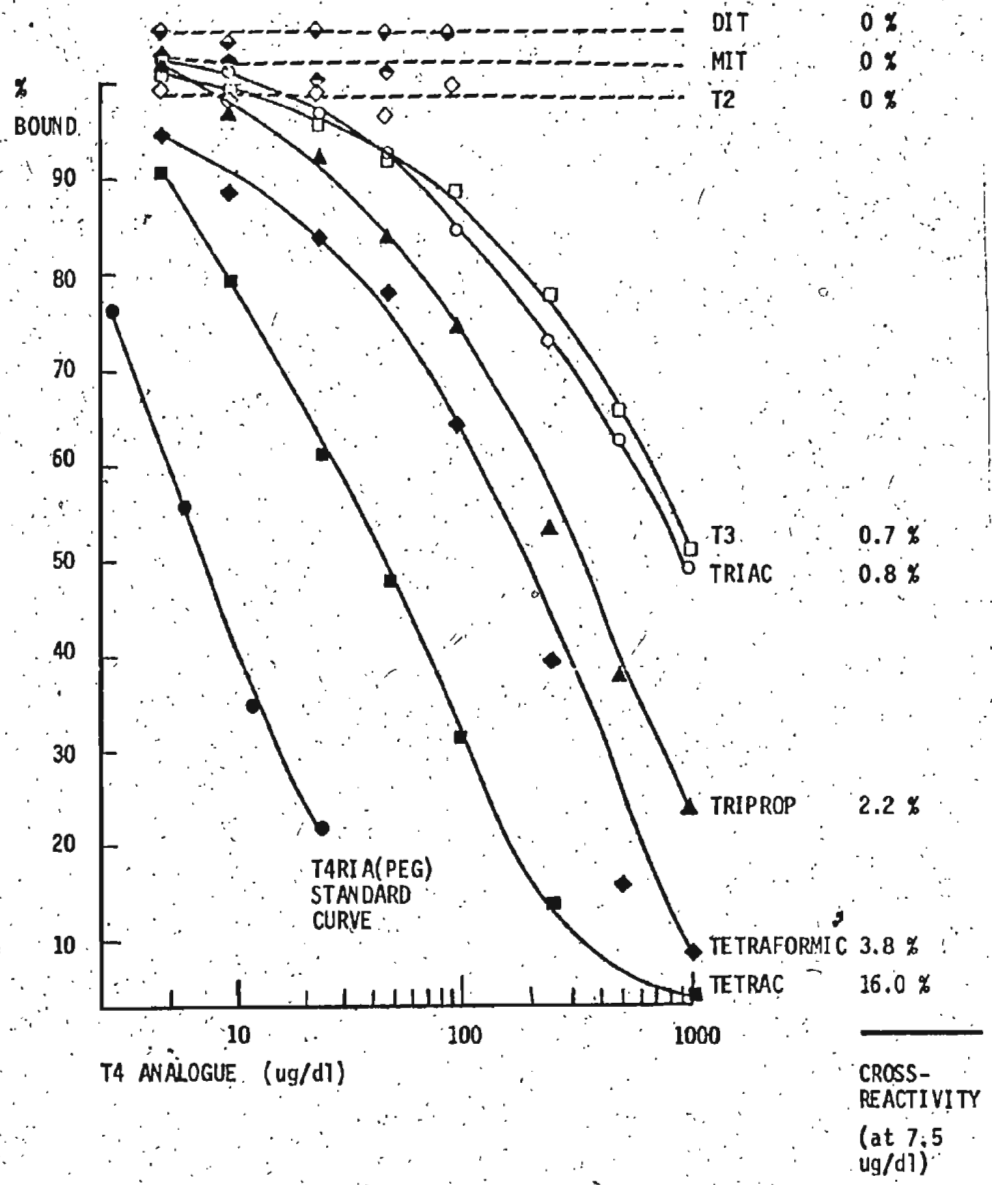


Fig. 5. Specificity of the assay for serum total T4. In response to a request for specificity data on the T4RIA (PEG) Diagnostic Kit, Abbott Laboratories kindly provided the table and rough graph from which this figure has been produced.

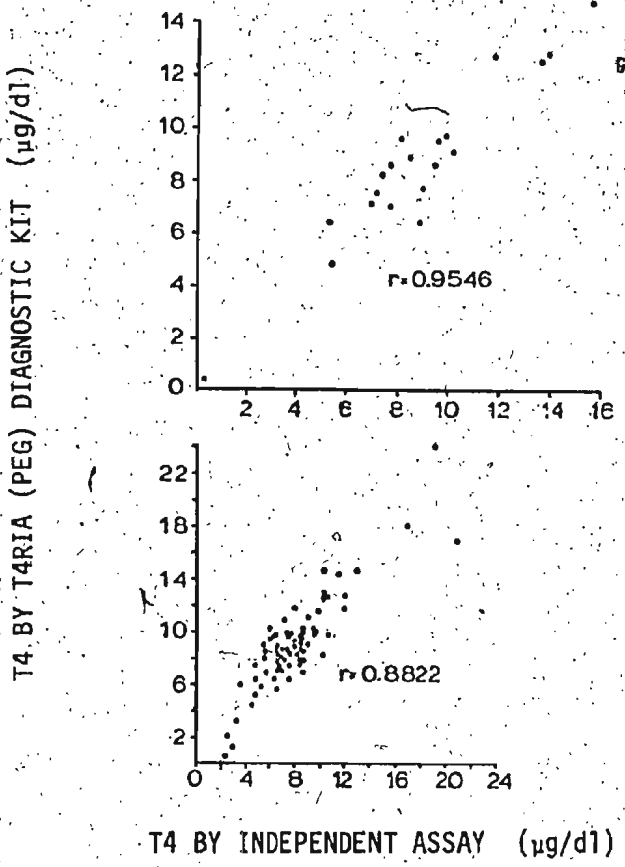


Fig. 6. Comparison with independent assays for serum T4. In a previous study, human sera were assayed using two other assay kits from Abbott Laboratories and the results were compared with those from the T4RIA (PEG) Diagnostic Kit. The upper panel shows the comparison with the T4RIA (charcoal) Diagnostic Kit (courtesy of Dr. Farid); the lower panel shows the comparison with the Tetrasorb kit, a Competitive Protein Binding assay which uses the Murphy-Pattee technique (from Farid & Kennedy, 1977). The correlation coefficients were both significant ($P < 0.001$). Comparisons, using rat serum, were not performed in this investigation.

2.5 RIA FOR T3

Serum T3 was determined by a radioimmunoassay based on the method of Mitsuma et al. (1972). Development of the assay, as reported herein, can be divided into four stages: preparation of the T3 antiserum, preparation of the T3 tracer solution, titration (to determine the optimum working dilutions for both the antiserum and the tracer), and validation of the assay.

2.5.1 Preparation of T3 Antiserum

Preparation of the immunogen. The antigen was conjugated to a protein carrier by following the method of Gharib et al. (1971): 50 mg bovine serum albumin (Sigma, St. Louis, MO, USA) were dissolved into 25 ml distilled water and passed through a Millipore filter; 30 mg carbodiimide (1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluene sulfonate, Aldrich, Milwaukee, WI, USA) were dissolved into the carrier solution; 20 mg L-T3 (free acid, also from Sigma) were dissolved into 5 ml N,N-dimethylformamide (Fisher, Fair Lawn, NJ, USA); the antigen solution was added dropwise to the carrier solution, while stirring constantly and maintaining a pH of 5.5; after 10 min, an additional 10 mg carbodiimide were added; the beaker and magnetic stirrer were covered with a cardboard box and the solution was stirred in the dark, at room temperature, for 18 h; the solution was then dialyzed against distilled water (changed three times daily) for 72 h; and the conjugate was lyophilized.

The immunogen was prepared for injection as follows: 10 mg lyophilized antigen conjugate were dissolved in 5.0 ml phophate buffered saline; the conjugate solution was transferred into a large syringe; 10 ml Freund's Comlete Adjuvant (Difco, Detroit, MI, USA) were aspirated into a second large syringe; the two syringes were coupled by a female-to-female adaptor (No. 3110LL/LL, Becton-Dickinson, Rutherford, NJ, USA); and the conjugate was emulsified in the adjuvant by repeatedly transferring the contents of the two syringes back and forth. In order to assess the water-in-oil emulsification process, two drops of the mixture were dropped onto the surface of water in a beaker; the conjugate was deemed to have been adequately emulsified in the adjuvant

when the second drop would not spread across the surface of the water. Later, in order to boost the antibody response, a second emulsion was produced, this time using Freund's Incomplete Adjuvant instead.

Immunization. The antisera were raised in New Zealand White rabbits. Two animals (6-7 kg) were each injected subcutaneously with 1 ml of the initial immunogen (T3-BSA conjugate in Complete Adjuvant) divided equally between four dorsal sites. Subsequent immunizations, with either Complete or Incomplete Adjuvant (as shown in Table 1), were injected in the same manner.

Collection of antisera. Both of the rabbits were bled from the marginal ear vein, after swabbing with xylol, by cutting across the vein and allowing 30-50 ml to drop into tubes. Each serum sample was separated, divided into aliquots and stored at -20 C. Sera were obtained prior to the booster injections during weeks 2, 6 and 9, and these were screened together as described below. During week 19, sera were again obtained from both rabbits (note that, during week 12, rabbit 1 had been injected with the last prepared dose of the immunogen); when these were screened, it was found that the T3 antibody level in rabbit 1 had been sufficiently increased by the last booster. Therefore, during week 20, rabbit 1 was exsanguinated by cardiac puncture in order to obtain a large quantity (about 50 ml) of useable T3 antiserum to be used in the assay.

2.5.2 Screening of the Antisera

Screening for T3 antibodies was performed as described by Mitsuma et al. (1971, 1972). The materials and methods which were used are described below.

Barbital buffer. The diluent used, both in the screening and later in the assay itself, consisted of 0.08 M sodium barbital (Fisher, Fair Lawn, NJ, USA), pH 8.4, with 1 g/l bovine serum albumin (Difco, Detroit, MI, USA).

Table 1. Immunization.

TIME (wk)	ADJUVANT USED	
	Rabbit #1	Rabbit #2
0	Complete	Complete
2	Complete	Complete
6	Incomplete	Incomplete
9	Incomplete	Incomplete
12	Incomplete	----

Tracer solution. For the screening experiments, (^{125}I)T3 (Triomet-125, Abbott Laboratories, North Chicago, IL, USA, 70-90 uCi/ug) was diluted in the barbital buffer to give 0.3 ug/l label (also see Subsection 2.5.3).

Dextran-charcoal slurry. Rather than using a slurry of activated charcoal and methyl cellulose (as described by Mitsuma et al., 1971) to separate free T3 from antibody-bound T3, a dextran-charcoal slurry (as described by Mitsuma et al., 1972) was used: 0.5 g Dextran T110 (Pharmacia, Uppsala, Sweden) were dissolved in 300 ml barbital buffer; 5.0 g Norit A charcoal (activated, Matheson, Coleman & Bell, East Rutherford, NJ, USA) were mixed in 300 ml barbital buffer; and, while the slurry of charcoal continued to stir, the dextran solution was poured into it. The dextran-charcoal slurry was stored at 2-8 C and mixed thoroughly before (and during) each use.

Procedure. To screen the rabbit serum samples to determine which, if any, could be used as a T3 antiserum, the samples were thawed and used neat and diluted 1:10, 1:50, 1:100, 1:500, $1:10^3$, $1:10^4$, in barbital buffer. 9.5 x 63.5 mm polystyrene tubes (LP/3 tubes, Luckham Ltd., Sussex, England) were labelled in duplicate; using a 100 ul Eppendorf pipet, each dilution of serum was added to the approporiate tubes, followed by the same quantity of (^{125}I)T3 tracer (30 pg/100 ul). The tubes were vortex-mixed, capped, and incubated at 0-4 C for 24 h. 1.0 ml dextran-charcoal slurry was then added to each tube; preliminary experiments had shown that maximum binding of the tracer could be acheived with this volume of the slurry. The tubes were mixed gently, incubated at 0-4 C for 5 min, and then spun for 15 min at 675 x g. The supernatant fractions, containing the antibody-bound tracer, were decanted into a second set of appropriately labelled LP/3 tubes; the "free" fraction of the tracer was thus left adsorbed to the dextran-charcoal sediment. Bound and free cpm were determined using either a Beckman Gamma 300 or 310 Radiation Counter. A bound/free ratio (B/F) in excess of 2 (i.e., % bound greater than 66.7%) at the lower serum dilutions (1:10 and 1:50) was taken as an indication that the serum contained potentially useable antibody.

Antisera. None of the sera collected during weeks 2, 6 or 9 gave a B/F ratio greater than 2 when diluted 1:50. The serum collected during week 19 from rabbit 1 (which had been injected again during week 12) did contain a sufficient level of T3 antibodies; therefore, as described in Subsection 2.5.1, rabbit 1 was exsanguinated during week 20 and the resulting serum was stored to be used as the T3 antiserum in the assay.

2.5.3 Preparation of T3 Tracer Solution

(^{125}I)Triiodothyronine. Labelled triiodo-L-thyronine (Triomet-125, Abbott Laboratories) was purchased to screen the antisera and later to titrate the antiserum and tracer (described in Subsection 2.5.6). Triomet-125 has a nominal activity of 70-90 uCi/ug, but the two containers received had only 59 and 76 uCi/ug respectively (according to the information on their labels). In order to increase the counting rates, and eventually, therefore, the sensitivity of the assay, thereafter 125-I Triiodothyronine Research Preparation (Abbott Laboratories, 300-500 uCi/ug) was purchased. The desired concentration of (^{125}I)T3 was obtained by dilution in the barbital buffer.

8-Anilino-1-naphthalenesulfonic acid (ANS). Mitsuma et al. (1972) incubated 30 pg (^{125}I)T3 with 0.1 ml diluted serum (1/2 or 1/4 in barbital buffer) in a total volume of 0.5 ml; the reaction mixture also contained 0.2 ml barbital buffer containing 350 ug ANS (for serum diluted 1/2) or 175 ug ANS (for serum diluted 1/4). Thus, a separate step was used to add 175 ug ANS per 25 ul serum in order to block binding to TBG. However, as in several other RIAs for T3 (e.g., Lieblich & Utiger, 1972; Sekadde et al., 1973), the ANS (magnesium salt, Eastman Kodak, Rochester, NY, USA) was incorporated into another reagent. In the titration experiments, the ANS was incorporated into the barbital buffer which was used instead of standards or sample sera; in the subsequent assays, the ANS was incorporated into the tracer solution. Thus, 50 ul of tracer solution added to 25 ul standard or sample serum provided 200 ug ANS.

2.5.4 Preparation of Standards

Stock T3 standards. Stock solutions of unlabelled T3 were prepared twice during this investigation, both times following the method of Alexander & Jennings (1974). To produce a 1.5 mmol/l solution, L-T3 (free acid, Sigma) was dissolved in 80 mM sodium hydroxide and immediately diluted ten-fold in distilled water; the concentration was tested spectrophotometrically. Alexander & Jennings, citing Gemmill (1955), stated that absorbance of a 1 mg/ml solution (i.e., about 1.54 mmol/l), diluted ten-fold in NaOH, should be 0.715 ± 0.005 (SD). The extinction coefficient, equal to the absorbance divided by the concentration (in mM), would equal about 0.465; therefore, the concentration of the stock T3 solutions (diluted ten-fold in NaOH) would equal the absorbance divided by this extinction coefficient. The two preparations of stock T3 had absorbances of 0.676 and 0.675; therefore, both contained 1.45 mM T3. Aliquots of 1.0 ml were dispensed into tubes, covered, and stored in an ultra-low temperature freezer (below -60 C) until used.

T3-free serum. Working T3 standards were prepared by diluting stock T3 standard in T3-free sheep serum. T3 was absorbed from sheep serum using the methods of Larsen (1972) and Mitsuma et al. (1972). On several occasions, (^{125}I)T3 was added to an aliquot of sheep serum, pre-counted in a gamma counter, treated (i.e., incubated with charcoal, centrifuged three times, and passed through a Millipore filter to remove the fines) and then re-counted. The final supernatant was found to contain less than 1% of the initial radioactivity.

Working T3 standards. A series of working solutions of T3 standards were prepared weekly. An aliquot of stock solution was thawed and diluted in barbital buffer to 10 umol/l, and then serially diluted in T3-free sheep serum to produce working T3 standards of 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 nmol/l. These working T3 standards were stored at 2-8 C and used within one week.

Quality control standards. Sera from Sprague-Dawley rats were pooled to use as a within-run and between-run quality control standard (as opposed to assaying several aliquots of one BUF rat serum sample, as was done in the RIA for T4). Sera from eleven 27 wk old females were collected in October 1976 (using the procedure described for the collection of BUF rat serum samples, except that these rats were bled between 1730 and 2100 h), the sera were pooled prior to the second centrifugation, and the 250 ul aliquots were stored at -20 C.

2.5.5 Assay Procedure

Each assay run consisted of a number of batches of samples and/or one batch of standards, which were set up over the course of one day and were completed over the course of the next day. The following protocol was used for each batch, and was used in both the titration experiments and in the assay of the rat serum samples. LP/3 tubes were approporiately labelled in quadruplicate and placed into a cold-rack (a test tube rack which had been modified so as to enclose a cold water bath in an outer jacket; a commercial cold-rack, the Kryorack Kold-Holder, is now available from Isolab, Akron, OH, USA) to keep the tubes cold while the reagents were being added (Sekadde et al., 1973) without freezing the small volumes involved. Working T3 standards (at 2-8 C) and quality control serum (freshly thawed), or up to 10 serum samples (freshly thawed aliquots), were added to the LP/3 tubes, using a 25 ul Eppendorf pipet. In the titration experiments, as mentioned previously, ANS in barbital buffer was added instead of standard or sample serum. The following three steps were performed on the replicates of half of the standards or samples, and then on the replicates of the other half, in order to minimize any differences due to time of addition: T3 tracer solution was added using a 50 ul Hamilton repeating dispenser; T3 antiserum (diluted in barbital buffer) was added using a second 50 ul Hamilton dispenser; and the tubes were vortex-mixed for 3-5 sec. When the last half of the tubes had been set up, all of the tubes were capped and transferred to another test tube rack, and the batch was incubated at 2-8 C for 24 h. Once a batch had been put into the refrigerator, another batch could be set up. After the 24 h had

passed, the batch was removed from the refrigerator, returned to a cold-rack, and the caps were removed. Well-mixed dextran-charcoal slurry was added to the replicates of half of the samples, using a Cornwall Pipettor set to deliver 1.0 ml (the refilling accessory was removed because the charcoal caused it to clog and the buffer tended to foam), and without delay the tubes were vortex-mixed for 3-5 sec each; these two steps were then performed on the second half of the batch. All of the tubes were then incubated for a further 10 min at 2-8 C. Next, all of the tubes in the batch were spun for 15 min at 2-8 C at maximum speed (1000-2000 x g, depending upon the number of tubes) in order to produce, as rapidly as possible, a dextran-charcoal pellet containing the non-antibody-bound ("free") tracer and a supernatant containing antibody-bound ("bound") tracer. The next step was performed on half of the tubes in the batch and then on the other half, again to reduce any possible differences due to reaction time: without delay, the supernatants were decanted into a second set of LP/3 tubes (which had been appropriately labelled); each pair of tubes were tapped together to try to get as much as possible of the supernatant to drop into the second tube. A Beckman Gamma 310 Radiation Counter was used to determine the bound and free cpm, counting each tube for 1 min (greater than 6000 cpm). Data from the titration experiments were transformed into % bound values (i.e., bound cpm divided by the total of the bound cpm and the free cpm, multiplied by 100), whereas the data in the assays were transformed into B/B_0 values (i.e., bound cpm at a dose, corrected for non-specific cpm, divided by the mean of the bound cpm of the zero dose replicates, also corrected for non-specific cpm).

2.5.6 Titration of Antiserum and Tracer

Titration experiments were performed in order to determine the optimum working dilutions of the antiserum and the tracer. In the first experiment, assays were run using 25 ul barbital buffer (containing 50 ug ANS), 50 ul antiserum diluted to 1/100, 1/200, 1/400, 1/800, 1/1600 and 1/3200 in barbital buffer (without ANS), and 50 ul tracer solution containing 10, 20, 30, 40, 50 or 100 pg T3 (200, 400, 600, 800, 1000 or 2000 ng/l, respectively) in barbital buffer (without ANS). In a

second experiment, the antiserum was diluted to 1/1000, 1/2000, 1/4000, 1/8000 and 1/16000 and the tracer solutions contained 25, 30, 50 and 100 pg T3 per tube. Optimum working dilutions were found to be the tracer solution containing 25 pg/tube and the antiserum diluted to 1/1000 (See Fig. 7); Mitsuma et al. (1972), for comparison, used 30 pg Triomet-125 per tube and their antiserum was diluted 1 to 2000. Subsequently, the titration experiment was performed using the tracer with the higher specific activity (recall Subsection 2.5.3), and the working dilutions determined before continued to produce the steepest dose-response graphs (and therefore the greatest sensitivity to differences in serum T3 concentrations). Thus, the RIA for serum total T3 used herein employed (^{125}I)T3 diluted to 500 ng/l in barbital buffer (containing 1 g/l ANS) and a rabbit T3 antiserum diluted to 1/1000 in barbital buffer (described in Subsection 2.5.2).

2.5.7 Validation Experiments

Dose-response graphs. The dose-response data were analyzed in the same manner as was described for the RIA for T4: a Hewlett-Packard 9815A programmable calculator was used to perform Bartlett's test for homogeneity of the variances (of the replicates of the standards) and to perform a family of regression analyses. The coefficients of the dose-response equation for each assay run were thereby determined, and a Hewlett-Packard 97 programmable calculator and Standard Pac card SD-03A (for logarithmic curve-fitting) were used to calculate the midrange and least detectable dose values for each dose-response graph, as well as for dose-interpolation (see Subsection 2.4.3).

Recovery experiments. An aliquot of a stock T3 solution was diluted in T3-free sheep serum to produce three recovery standards: 0.5, 1.0 and 1.5 nmol/l. An assay was performed, using working T3 standards for calibration and the recovery standards as serum samples, in order to compare the values which were interpolated with those which were expected.

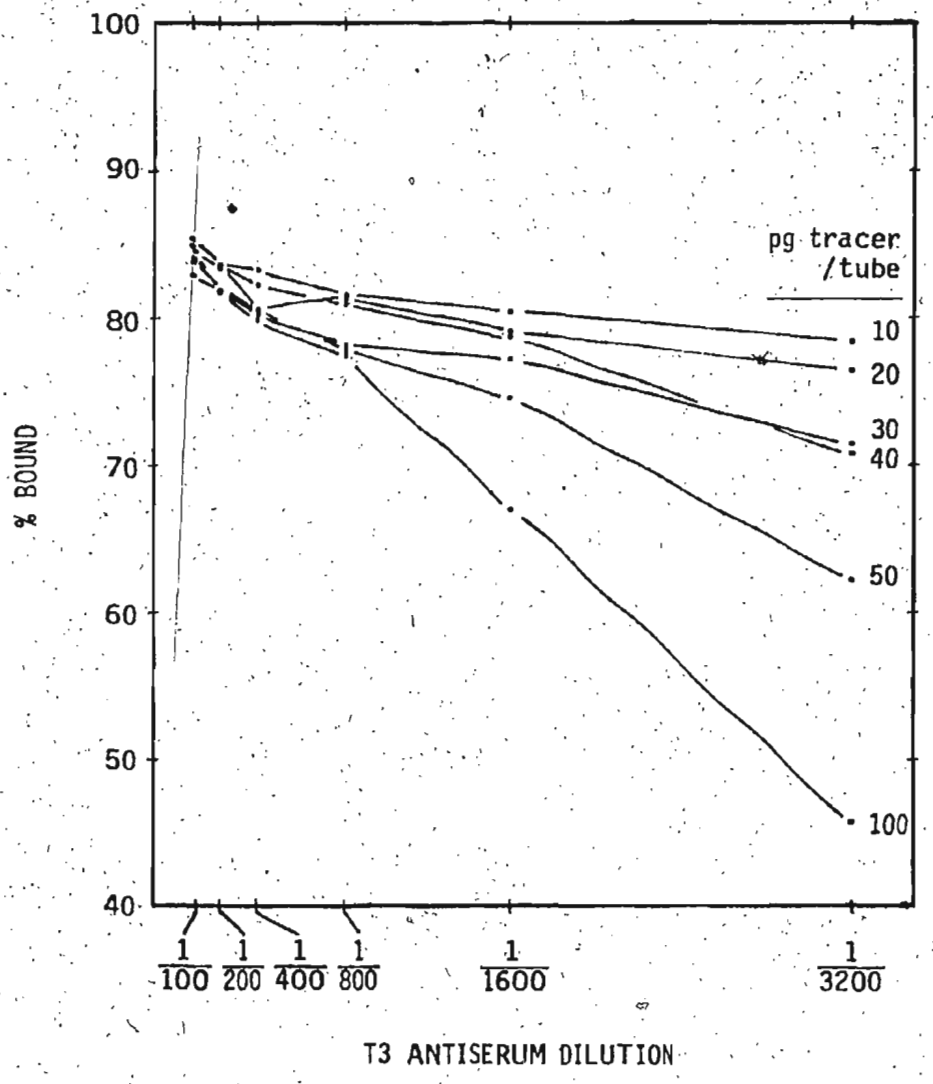


Fig. 7-A. Titration experiments for the T3 assay. It was apparent that 10 and 20 pg tracer/tube were insufficient, and that the antiserum could be diluted more than 800-fold. A second experiment was performed to establish working dilutions (see Fig. 7-B).

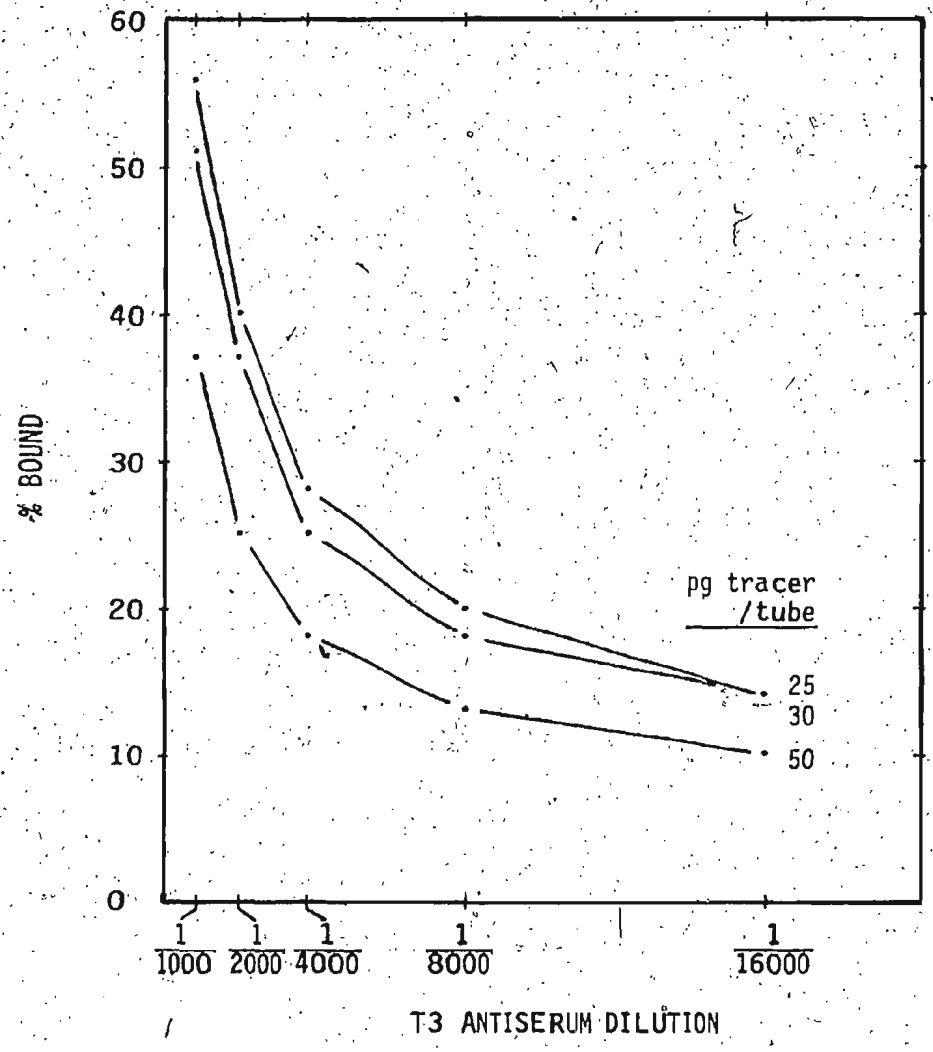


Fig. 7-B. Titration experiments for the T3 assay. There was little difference between 25 and 30 pg tracer/tube, and the steepest decline occured between 1000- and 2000-fold dilutiond of the antiserum. Dose-response graphs produced in subsequent experiments confirmed that working dilutions of 25 pg tracer/tube and antiserum diluted 1/1000 produced satisfactory calibration curves.

Specificity experiments. Several solutions of T3 analogues (up to 10^4 nmol/l) were assayed in order to measure percent cross-reactivity (Weeke & Orskov, 1975) of the T3 antiserum. The percent cross-reactivity of each analogue was calculated by comparing the doses (of T3 with each analogue) required to inhibit half of the binding of tracer (i.e., from the zero dose, which was approximately 50% bound, to the dose which resulted in a % bound value of 25%). T3, T4, MIT and DIT were obtained from Sigma; reverse-T3 was obtained from Henning Berlin GMBH, Berlin, FRG.

Within-run variability. A quality control standard (aliquots of pooled serum from Sprague-Dawley rats) was assayed, in ten replicates, over the course of a day's run. The serum total T3 concentration for each replicate was determined by dose-interpolation, and the mean, standard deviation and coefficient of variation were calculated.

Between-run variability. Aliquots of the quality control standard were assayed, in quadruplicate, each day on which samples were run. The serum total T3 concentrations were interpolated from each day's dose-response graph, and the overall mean, standard deviation and coefficient of variation were calculated.

Comparison with an independent assay. The T3 assay which was developed in this investigation was compared with a commercial assay kit that came on the market in 1977 (the T3RIA (PEG) Diagnostic Kit from Abbott Laboratories). Nineteen BUF rat serum samples (which had been assayed earlier) were assayed using this kit, and the two values for each sample were compared using linear regression analysis.

3. RESULTS

3. RESULTS

3.1 BODY WEIGHTS

3.1.1 Variation

Body weight is an indicator of the general well-being of an animal; the data from the BUF rat colony is illustrated in Fig. 8.

Regarding the males first, there are several points to note. Generally, they gained weight progressively between the ages of 8 and 52 wk, although the body weights varied greatly within each age. At several of the ages, there were a few values which were distinctly lower than the others of that age; usually these were either serial measurements from the same rats (e.g., male #54 weighed less than 300 g at each of the six ages from 20 to 40 wk), or else they were the last values obtained from those particular animals (which suggests that those rats may have died within the next four weeks). However, in regard to three very low values (less than 200 g at 12 wk of age): these rats had been weighed together on the same day, and when re-weighed two weeks later they all weighed between 300 and 350 g (i.e., in the expected range); this suggests that the values at 12 wk may be incorrect.

Regarding the virgin female BUF rats (Fig. 8), the mean weights declined slightly at the older ages, and when the serial data were examined, moreover, a progressive weight loss was evident in a few of the animals. It has been previously reported that virgin female rats, as they age, frequently go into a state of constant estrus (Clemens & Meites, 1971) and they are therefore much smaller than males or breeder females.

In addition, the variability of the body weights within each age was generally much smaller for the virgin females than for the males, and fewer of the values were noticeably higher or lower than those of the rest of the animals within each particular age.

Thus, a few "deviant" males appeared to contribute a large amount of variation in the weights of the males, whereas the virgin females tended to have body weights which were less variable as well as lower. Some of the virgin females lost weight at the older ages, but it is not possible to determine whether such weight loss was due to a state of constant estrus, maintenance conditions (see Subsection 2.1.2 regarding

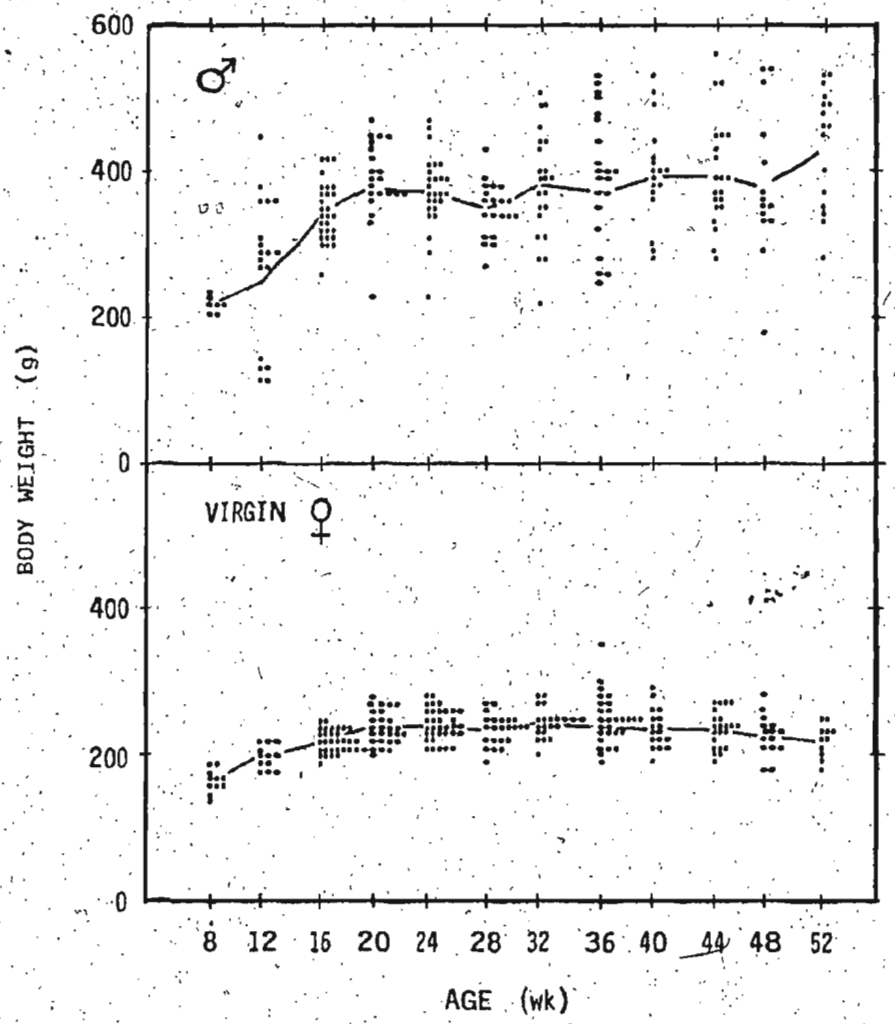


Fig. 8. BUF rat body weights. The rats in the colony were weighed (to a decade range) prior to being bled. The values within each age are shown as a histogram.

temperature and humidity), non-thyroidal disease (see Subsection 1.3.1 regarding susceptibility to myocarditis, otitis media and bronchiectasis), or thyroiditis.

3.1.2 Variation with Age

Linear regression analyses were performed on the data for the BUF rat body weights; the body weights for each sex were found to be positively correlated with age: $r = 0.495$ ($P < 0.001$) for 217 values obtained when bleeding males, and $r = 0.337$ ($P < 0.001$) for 241 values from virgin females.

These two correlation coefficients were compared, using the z-transformation and the appropriate two-tailed t test, and body weight versus age was found to exhibit a significant sexual variation ($P < 0.05$).

3.2 HISTOPATHOLOGY

Thyroid specimens from twenty of the rats in the BUF colony were examined (see Subsection 2.2.3 and Section 2.3); the slides were coded to hide the identity of the rats during this examination. The majority of the thyroids exhibited lymphocytic infiltration. In some cases the infiltration did not occur in both lobes, or in each of the three levels examined, and the extent of loss of follicular architecture (i.e., the severity) varied, but nonetheless thyroiditis could be diagnosed in animals in the BUF rat colony; Fig. 9 illustrates the thyroiditis observed.

It must be recalled that all of the specimens, except those from animals 52 wk of age or older, were obtained from animals which were found shortly after they had died; this would bias the sampling in favour of diseased rats, but this does not necessarily mean that the disease was thyroiditis. The minimum value for the incidence of thyroiditis in the BUF colony can be calculated by assuming that the sampling provided only, and every, case of thyroiditis in the colony: just over 14% (20 out of the 140 rats in the colony).

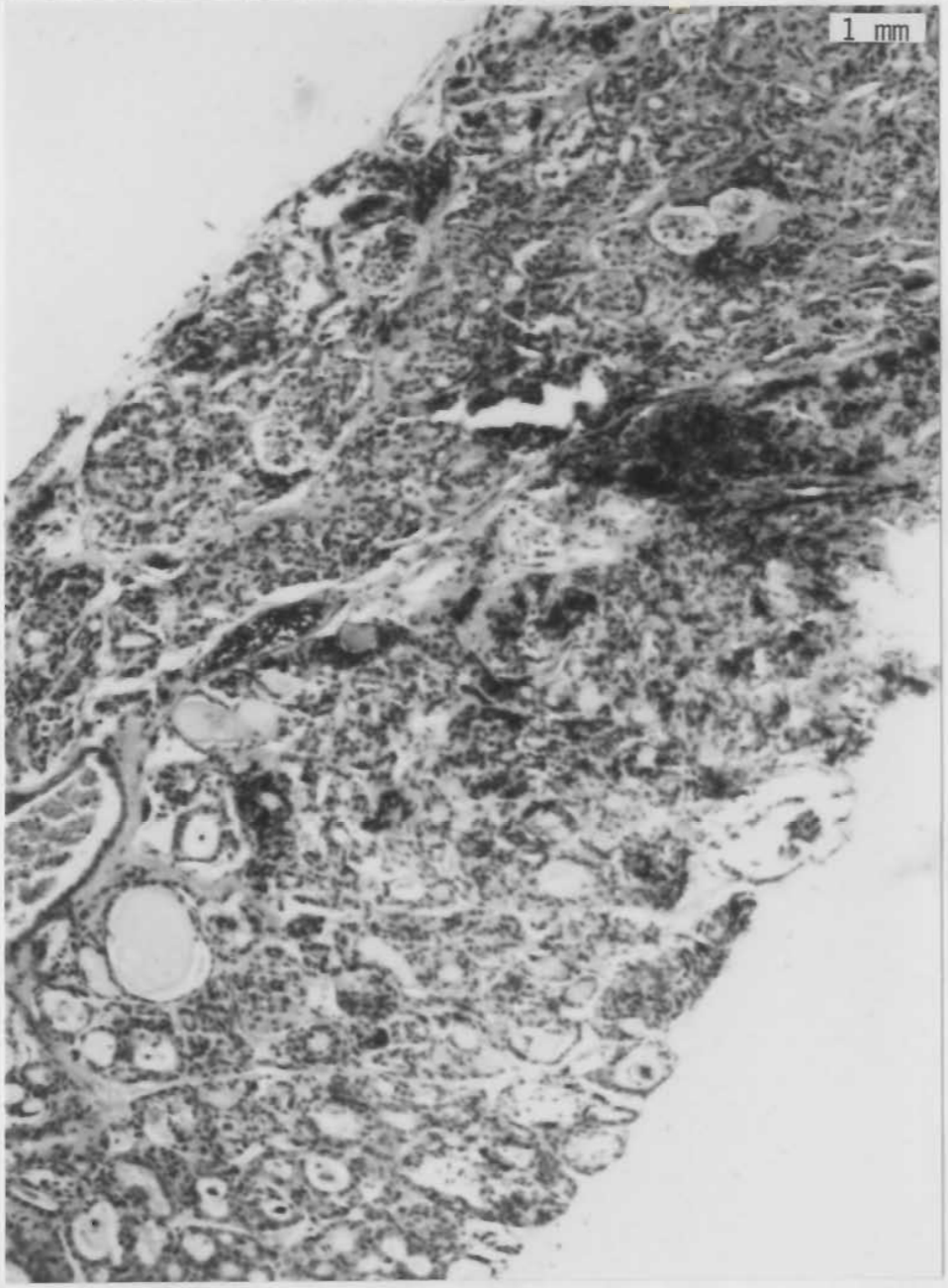


Fig. 9-A. Histopathology of the BUF rat thyroid. Note that this lobe is much larger than the normal one shown in Fig. 1, and that most of the follicular architecture has been obliterated. There is a diffuse infiltration of round cells (see Fig. 9-B).

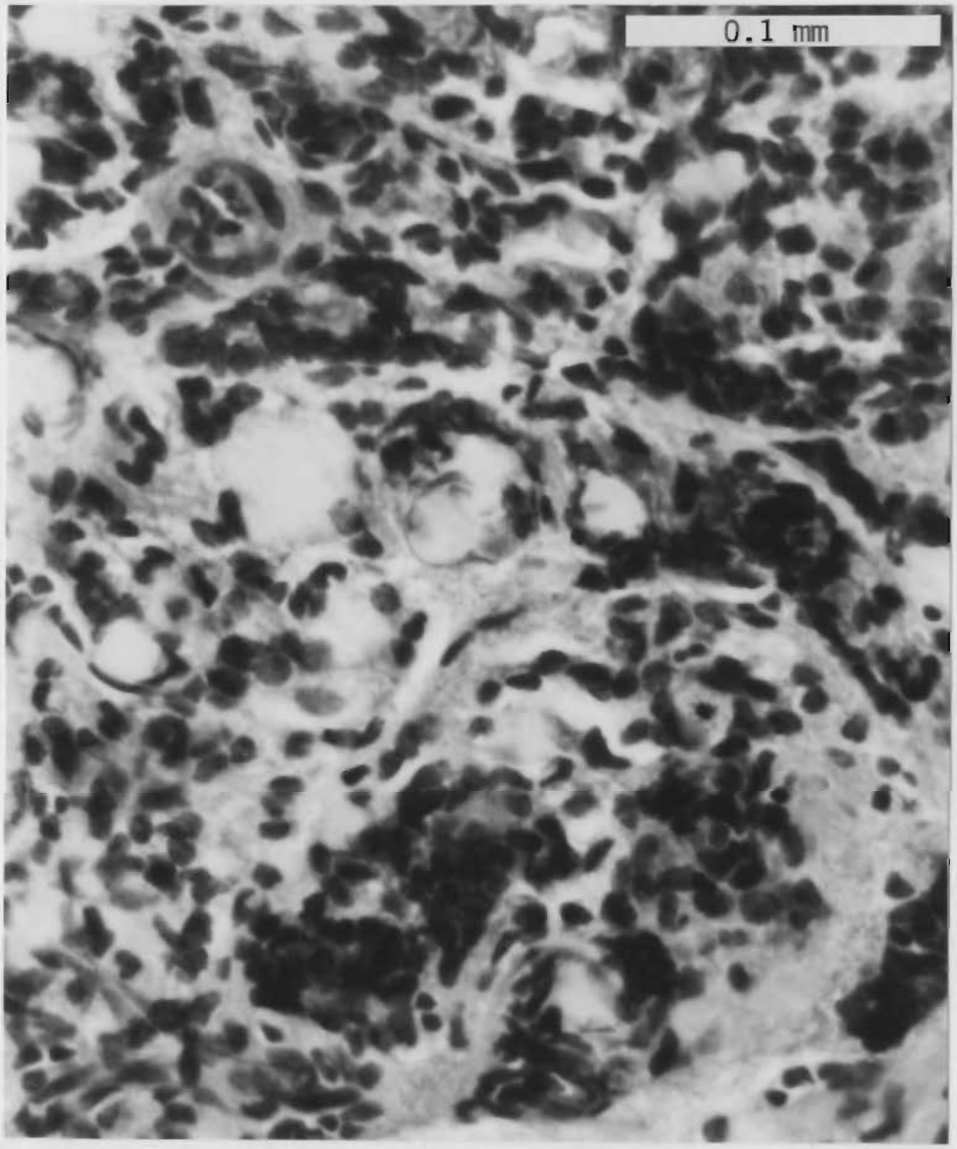


Fig. 9-B. Histopathology of the BUF rat thyroid (continued). Infiltrating cells were recognizable as lymphocytes.

3.3 VALIDATION OF THE RADIOIMMUNOASSAYS

3.3.1 RIA for T4

Dose-response graphs. Dose-response data for the serum total T4 RIA were analyzed as described in Subsection 2.4.3; a typical graph is shown in Fig. 10. BUF rat serum samples were assayed in six runs; the features of the dose-response graphs (mean $\pm$ SD) were as follows:

$$B/B_0 = A + B \log_n \text{DOSE (in nmol/l)}$$

A = 1.516 $\pm$ 0.043

B = -0.218 $\pm$ 0.007

MIDRANGE = 105.1 $\pm$ 7.5 nmol/l

LEAST DETECTABLE DOSE = 11.6 $\pm$ 1.1 nmol/l.

Within-run variability. Each of the eight batches of samples assayed one afternoon included an aliquot of one BUF rat serum sample, and the eight T4 concentrations were interpolated from that day's dose-response graph. The mean and SD of these values were 66.1 $\pm$ 7.3 nmol/l; therefore, the within-run variability was 11.1%.

Between-run variability. Each of the three subsequent runs also included an aliquot of the above sample; the mean and SD of the four T4 values were 69.9 $\pm$ 10.1 nmol/l, giving a between-run variability of 14.5% for the RIA for T4.

3.3.2 RIA for T3

Dose-response graphs. Dose-response data from the serum total T3 RIA were analyzed as described in Subsection 2.5.7; a typical dose-response graph is illustrated in Fig. 11. The BUF rat serum samples were assayed in six runs over a two week period (using a different set of working T3 standards each week); the six dose-response graphs had the following features (mean $\pm$ SD):

$$B/B_0 = A + B \log_n \text{DOSE (in nmol/l)}$$

A = 0.637 $\pm$ 0.029

B = -0.243 $\pm$ 0.013

MIDRANGE = 1.77 $\pm$ 0.19 nmol/l

LEAST DETECTABLE DOSE = 0.26 $\pm$ 0.05 nmol/l.

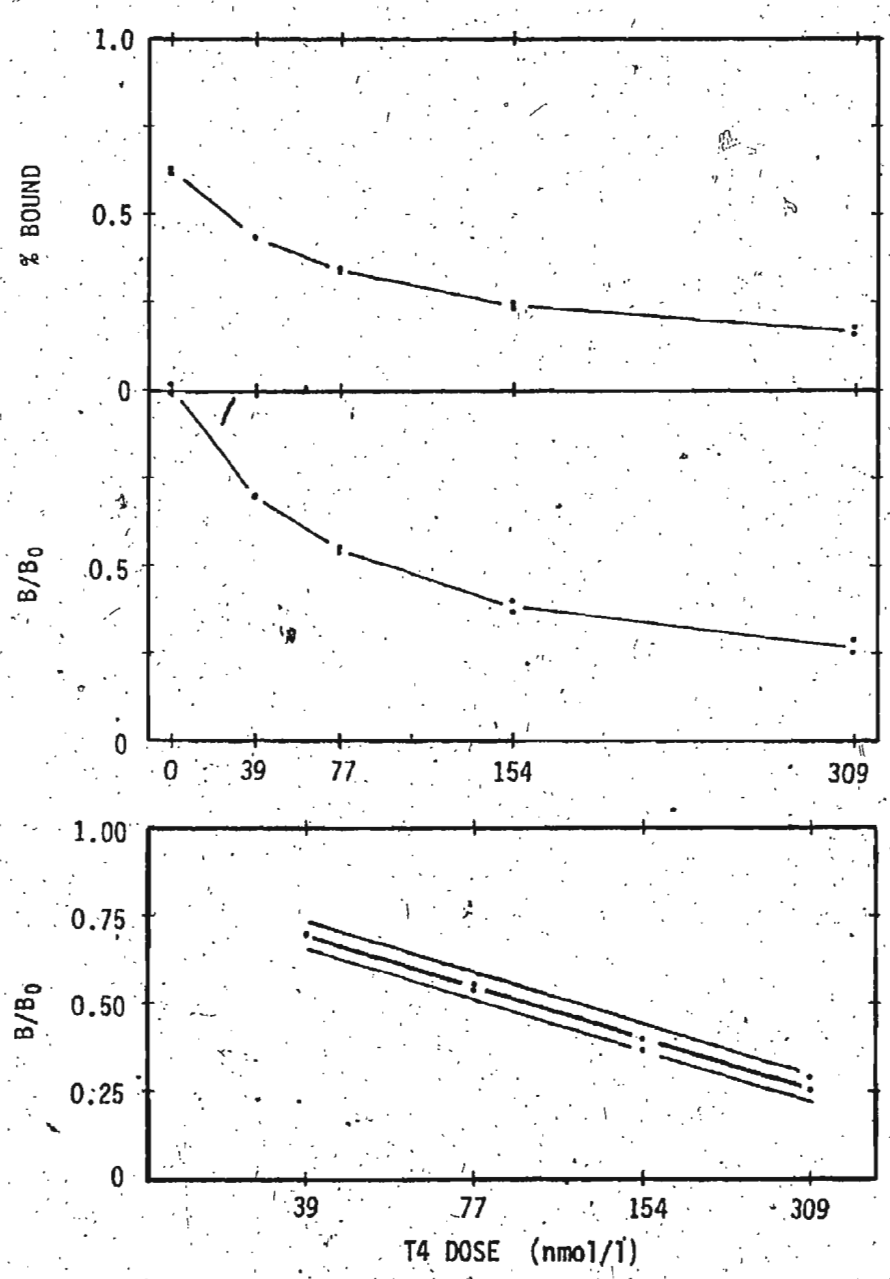


Fig. 10. T4 dose-response graphs. The lower panel shows the linear regression of B/B_0 with the $\log_n$ of the dose (± 2 SE of the estimate).

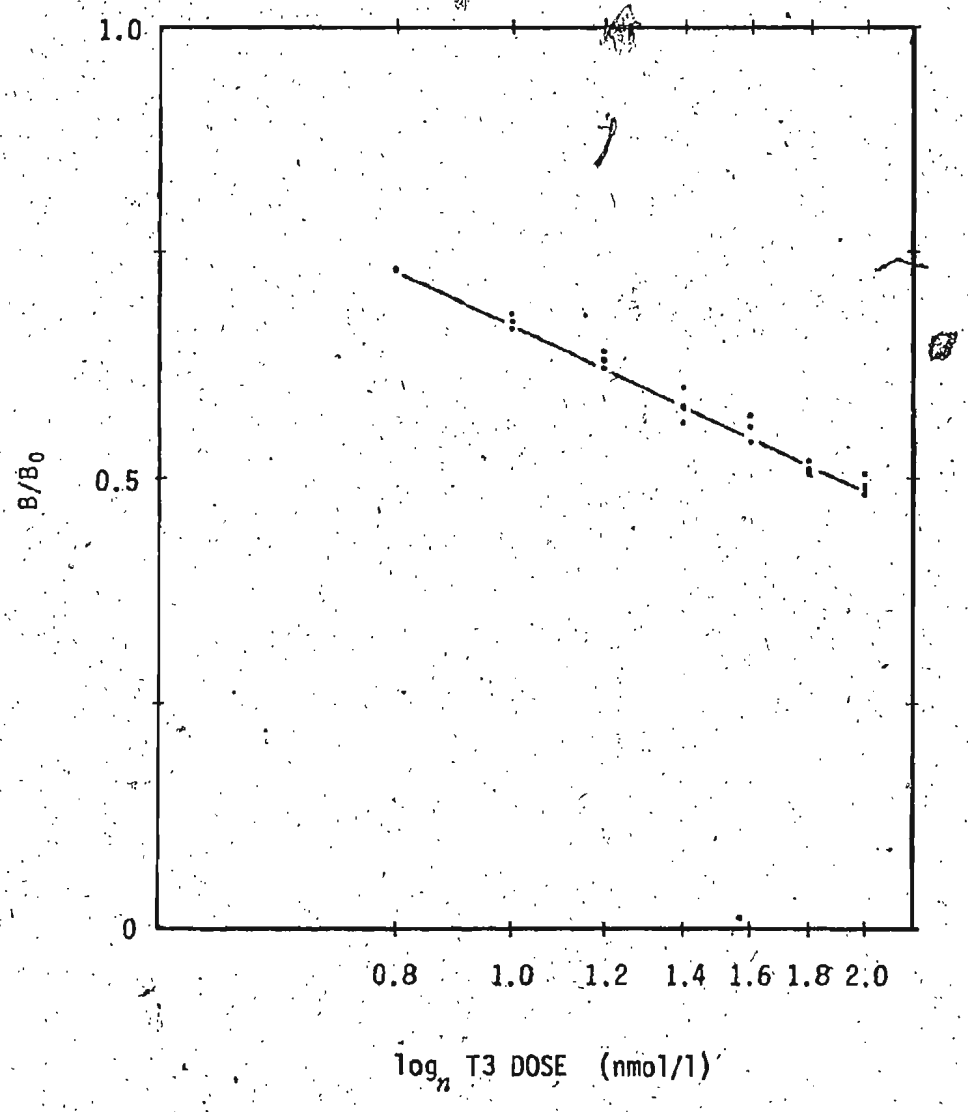


Fig. 11. A typical T3 dose-response graph. The linear regression equation is $B/B_0 = 0.646 - 0.237 (\log_n \text{DOSE})$. The midrange (where $B/B_0 = 0.5$) is 1.79 nmol/l. The least detectable dose (with a 2 SD confidence limit) is 0.17 nmol/l.

Recovery experiment. For the 0.5, 1.0 and 1.5 nmol/l recovery standards (assayed in quadruplicate; see Subsection 2.5.7), the recoveries were 91.0%, 101.3% and 98.4%, respectively.

Specificity experiment. (See Fig. 12) The highest % cross-reactivity (the dose of analogue, compared to the dose of T3, required to inhibit one-half of the binding of of the tracer) which was found (0.28%, for T4) was not considered to significantly affect the RIA for T3.

Within-run variability. Ten replicates of a quality control standard (pooled serum from Sprague-Dawley rats) were assayed during a preliminary experiment. The mean ($\pm$SD) of nine T3 values (one tube broke) was 1.11 ± 0.100 nmol/l; thus, the coefficient of variation was 9.0%.

Between-run variability. A quality control standard was run, in quadruplicate, each of the six days on which BUF rat serum samples were assayed; the serum total T3 concentration averaged 1.01 ± 0.100 nmol/l; hence, variation was 9.8%.

Comparison with an independent assay. Nineteen BUF rat samples (evenly distributed between 0.50 and 1.50 nmol/l) were re-assayed using Abbott Laboratories' T3RIA (PEG) Diagnostic Kit; values from the two assays were significantly correlated ($r = 0.61$, $P < 0.01$).

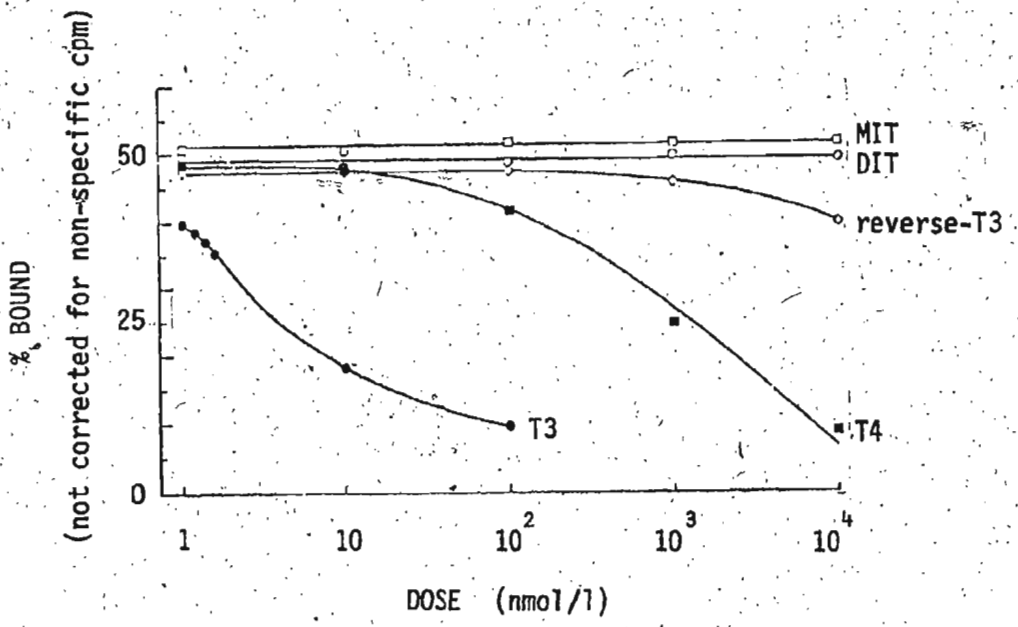


Fig. 12. Specificity of the assay for serum T3. The percent cross-reactivity with T4 was 0.26%; *i.e.*, the inhibiting dose (which reduced binding by one-half) of T3 was 0.26% of the inhibiting dose of T4.

3.4 DIURNAL VARIATION OF T4 (IN SPRAGUE-DAWLEY RATS)

Fig. 13 illustrates the serum total T4 concentrations which were obtained when six groups of four 24-28 wk old Sprague-Dawley male rats were sacrificed, at four hour intervals, and aliquots of the resulting sera were subsequently assayed (in a randomized order). In general, the T4 levels appeared to cycle -- when the lights were on, the levels rose, and when the lights were off, the levels declined -- but the significance of this variation (e.g., sinusoidal regression) was not analyzed. Between 1000 and 1300 h, when BUF rats were routinely bled, the T4 concentrations in the Sprague-Dawley rats rose about 15% (from about 68 nmol/l to about 78 nmol/l).

With these results, plus a paper which was published in the meantime (Fukuda et al., 1975), it was deemed to not be necessary to assay the T3 levels in these samples; diurnal variations between 1000 and 1300 h would not be expected to significantly affect the BUF rat samples.

3.5 SERUM TOTAL T4 CONCENTRATIONS

Fig. 14 shows the T4 concentrations in samples from 44 of the males and 36 of the virgin females in the colony of BUF rats; on average, T4 was assessed at three ages per animal, although some rats were evaluated at up to nine of the twelve ages. T4 has been reported to decline with increasing age in rats (see Subsection 1.1.5); thus, the general trend in the BUF rats was as expected. Within most of the ages, however, the variation was large. In the males, the large variation appeared to be particularly due to only a few of the rats; for instance, one of the males was evaluated at five ages, and each of its T4 concentrations was the lowest value within the age (see also Sections 3.1,3.9 and Fig. 23).

T4 levels in the virgin females tended to rise in the younger animals, with a peak at 24 wk, and thereafter declined gradually (at approximately the same rate as in the males). The T4 concentrations within each age were generally lower in the virgin females than in the males; however, a two-tailed t test for each age (preceded by the F_{max} test for equality of variances) showed that the differences were significant ($P < 0.05$) at only five of the twelve ages (see Fig. 15): 8,

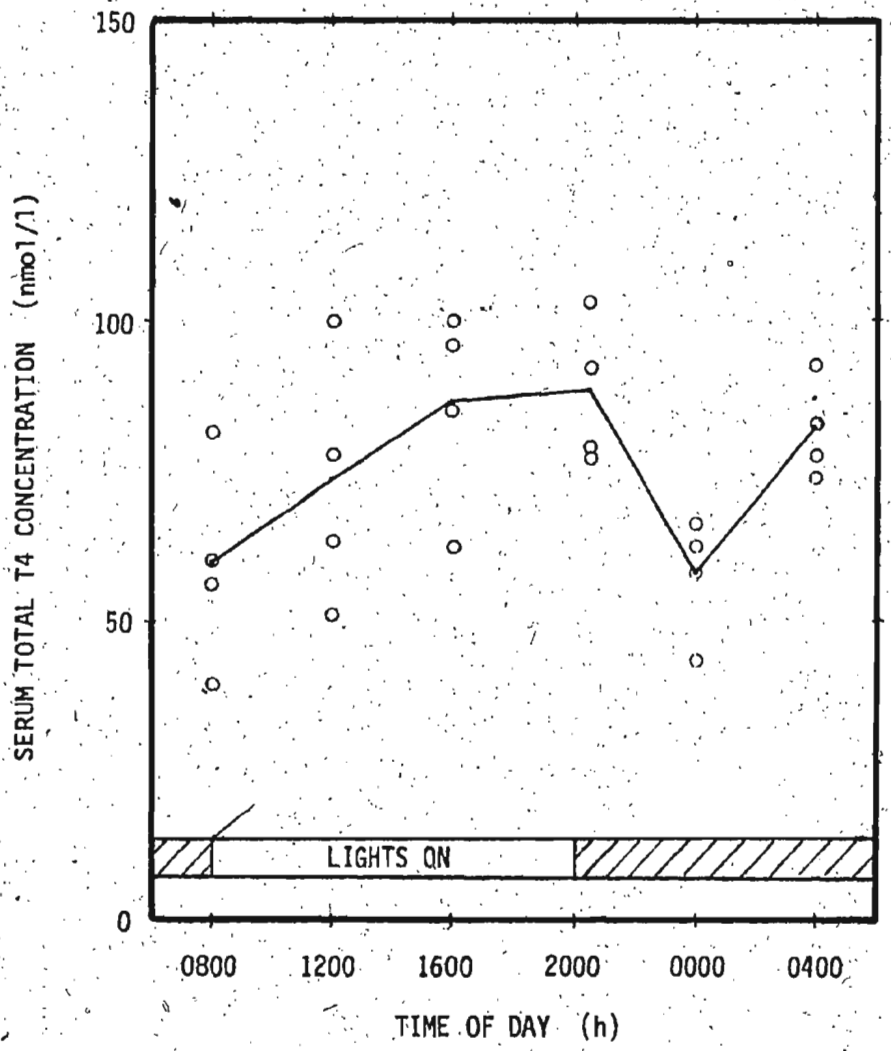


Fig. 13. Diurnal variation of serum T4 in Sprague-Dawley rats. Each 24-28 wk old male was anaesthetized with ether and decapitated to provide a sample of trunk blood for assay by the T4RIA (PEG) Diagnostic Kit. The average T4 level rose from about 68 nmol/l to about 78 nmol/l (15%) between 1000 and 1300 h. A diurnal rhythm was apparent.

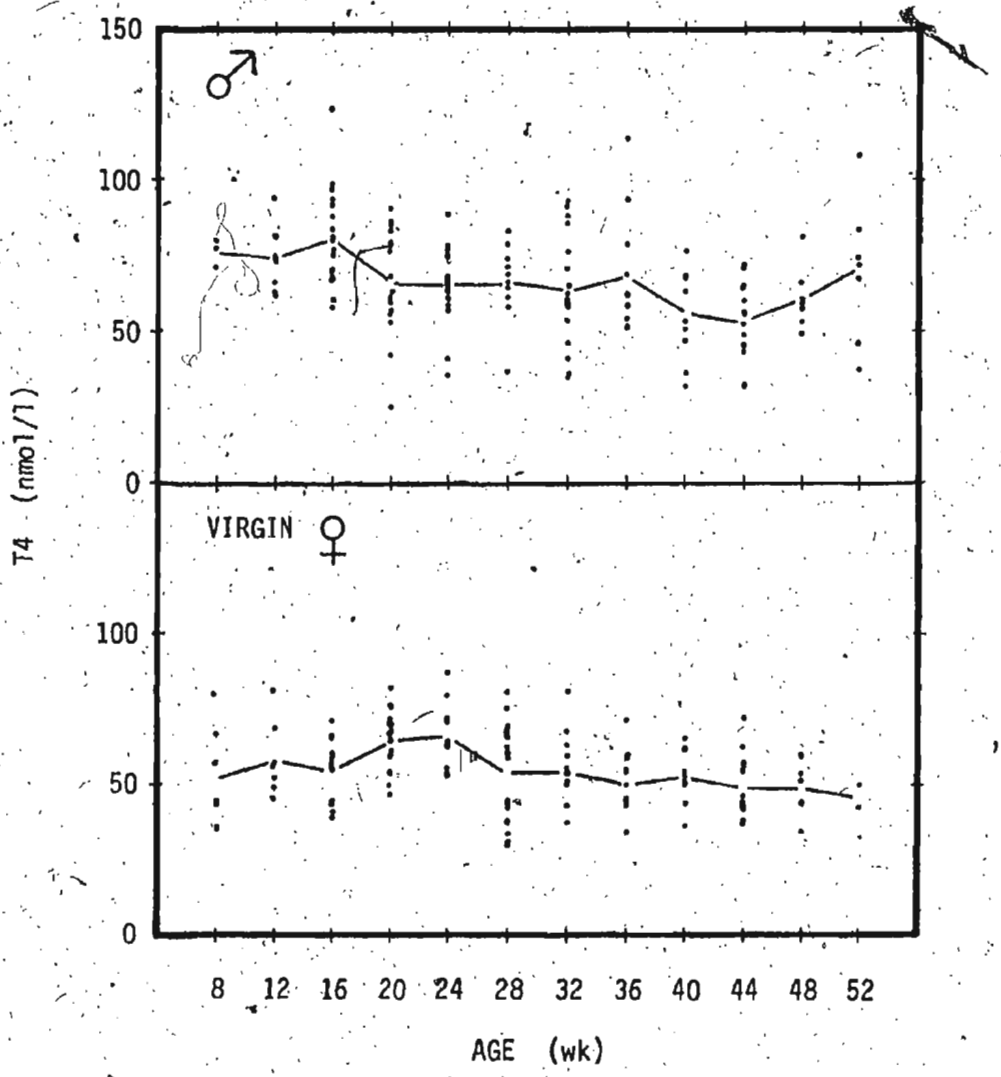


Fig. 14. Serum total T4 concentrations in BUF rats.

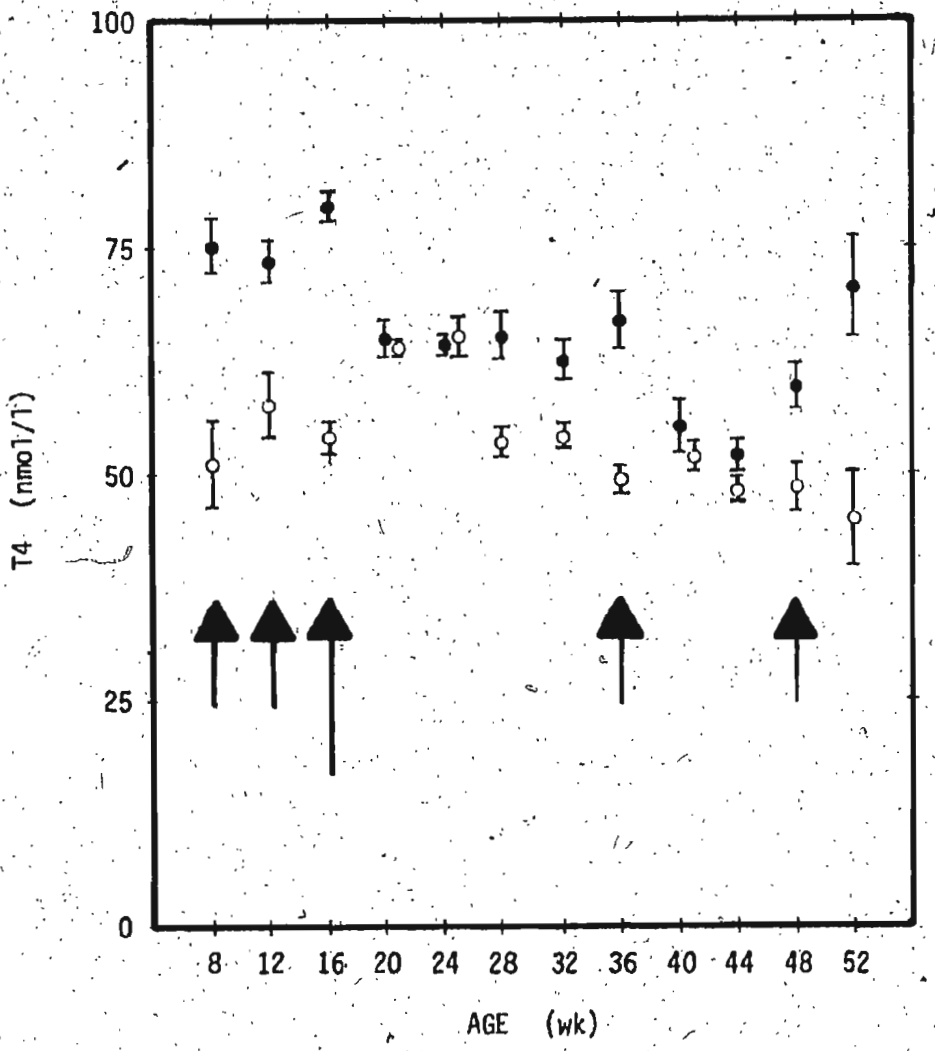


Fig. 15. T4 in males *versus* virgin females. For each age, the closed circle (•) is the mean of the males, the open circle (o) is the mean of the virgin females, and the vertical bars are ± 2 SE. The arrows indicate the ages at which the differences between BUF males and virgin females were significant: short arrows for $P < 0.05$; a long arrow for $P < 0.001$.

12, 16, 36 and 48 wk. The first three ages were those when the values of the females appeared to be rising towards those of the males. The T4 data is analyzed further, with respect to T3, age and weight, in Sections 3.7 through 3.9.

Comparing T4 values from BUF and Sprague-Dawley rats, the mean $\pm$ SD of the T4 concentrations obtained from twenty 24 wk old male BUF rats (64.4 $\pm$ 12.0 nmol/l) was slightly lower, but not significantly different from the values obtained from the four 24-28 wk old Sprague-Dawley males which had been bled at 1200 h (68.8 $\pm$ 20.6 nmol/l); recall that the BUF rats were routinely bled between 1000 and 1300 h.

3.6 SERUM TOTAL T3 CONCENTRATIONS

Fig. 16 shows the T3 concentrations in samples from 34 male and 30 virgin female BUF rats, with an average of 4 ages per rat. It has been previously reported (in humans at least) that T3 levels tend to decline with increasing age, and in general the data from the BUF rats showed a similar trend (although the mean T3 values for each sex were relatively constant from 8 wk until about 28 wk). The variability within each age was very large; for 48 wk old males, the most extreme example, the coefficient of variation was more than 50%.

F_{max} and t tests were used to compare the males with the virgin females within each age (excluding 20 wk, for which there were no values from virgin females). Although the means of the T3 concentrations were consistently higher in the virgin females than in the corresponding males (see Fig. 17), the differences were significant ($P < 0.05$) at only two of the eleven ages (40 and 44 wk). Further analyses involving the T3 data are presented in the following three sections.

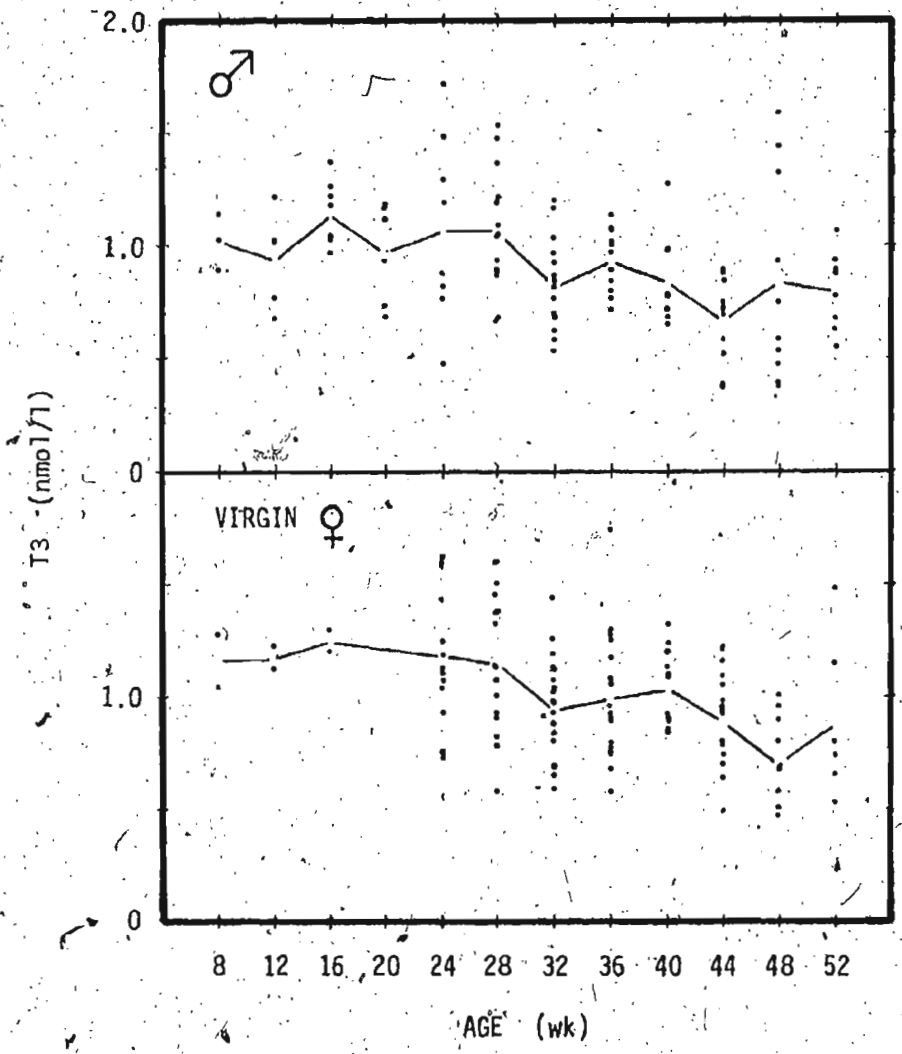


Fig. 16. Serum total T3 concentrations in BUF rats.

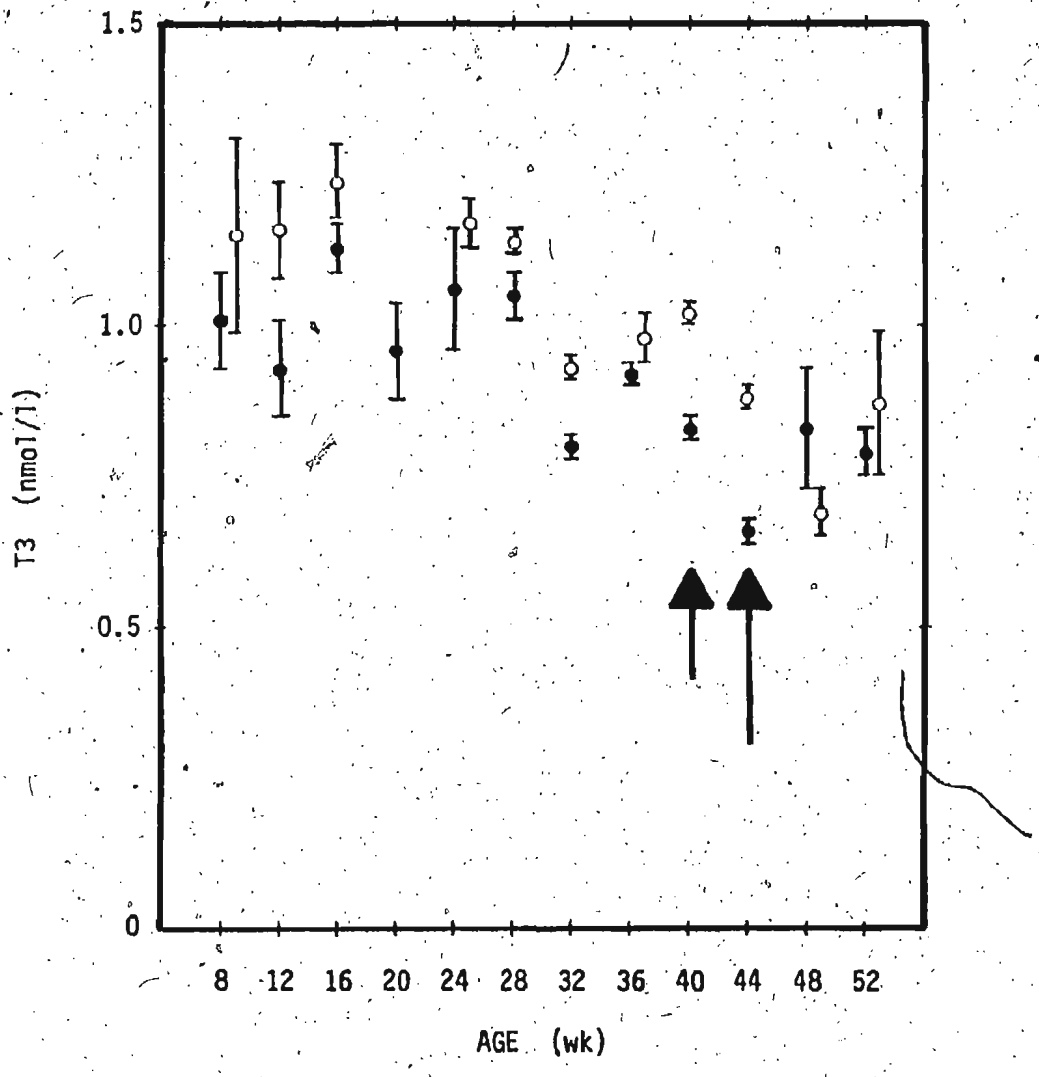


Fig. 17. T3 in males *versus* virgin females. For each age, the closed circle (●) is the mean of the males, the open circle (o) is the mean of the virgin females, and the vertical bars are ± 2 SE. The arrows indicate the ages at which the differences between BUF males and virgin females were significant: a short arrow for $P < 0.05$; a long arrow for $P < 0.01$.

3.7 RELATIONSHIPS BETWEEN T4 AND T3

3.7.1 T4 versus T3

T4 and T3 concentrations were both obtained in 78 samples from 25 of the male BUF rats and 82 samples from 26 virgin females. Linear regression analysis was performed (see Fig. 18), and positive correlations between T4 and T3 were exhibited by each sex: $r = 0.503$ ($P < 0.001$) for the males and $r = 0.602$ ($P < 0.001$) for the virgin females.

3.7.2 T4/T3 Ratios

T4/T3 ratios were also calculated for the above samples (see Fig. 19). Neither sex exhibited significant variation of T4/T3 ratios with age ($P > 0.05$). The values from the males were compared with those from the virgin females, using two tests of significance: the Mann-Whitney U test, performed with a Hewlett-Packard 9815A programable calculator and pre-recorded tape cartridge Statistics, Volume 3 (Non-Parametric Analysis, part no. 09815-15020, rev. A), and the t test; the differences between the sexes were significant ($P < 0.001$) using both tests. The T4/T3 ratios averaged 72.5 ± 20.0 ($\pm$SD) for the males and 52.9 ± 12.4 for the virgin females.

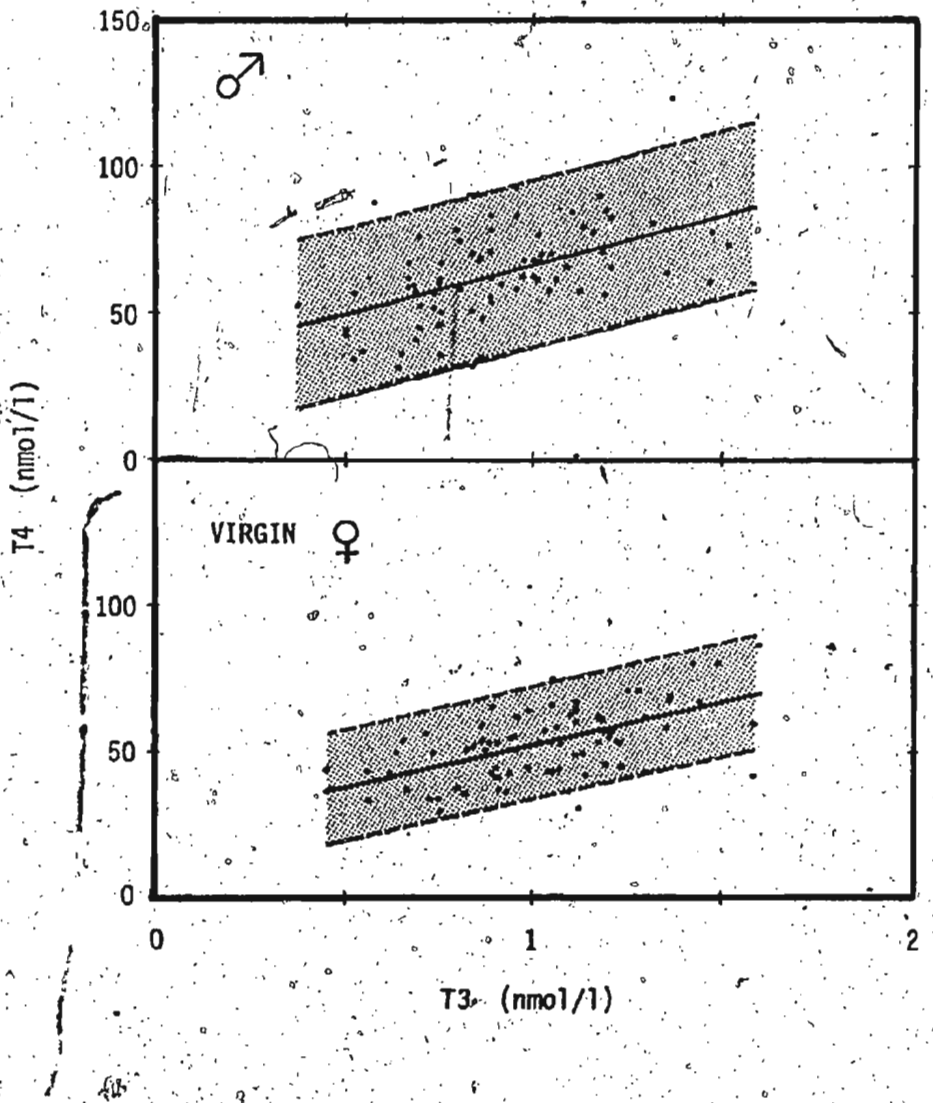


Fig. 18. T4 *versus* T3. The shaded areas are the normal ranges, defined by ± 2 SE of the estimate.

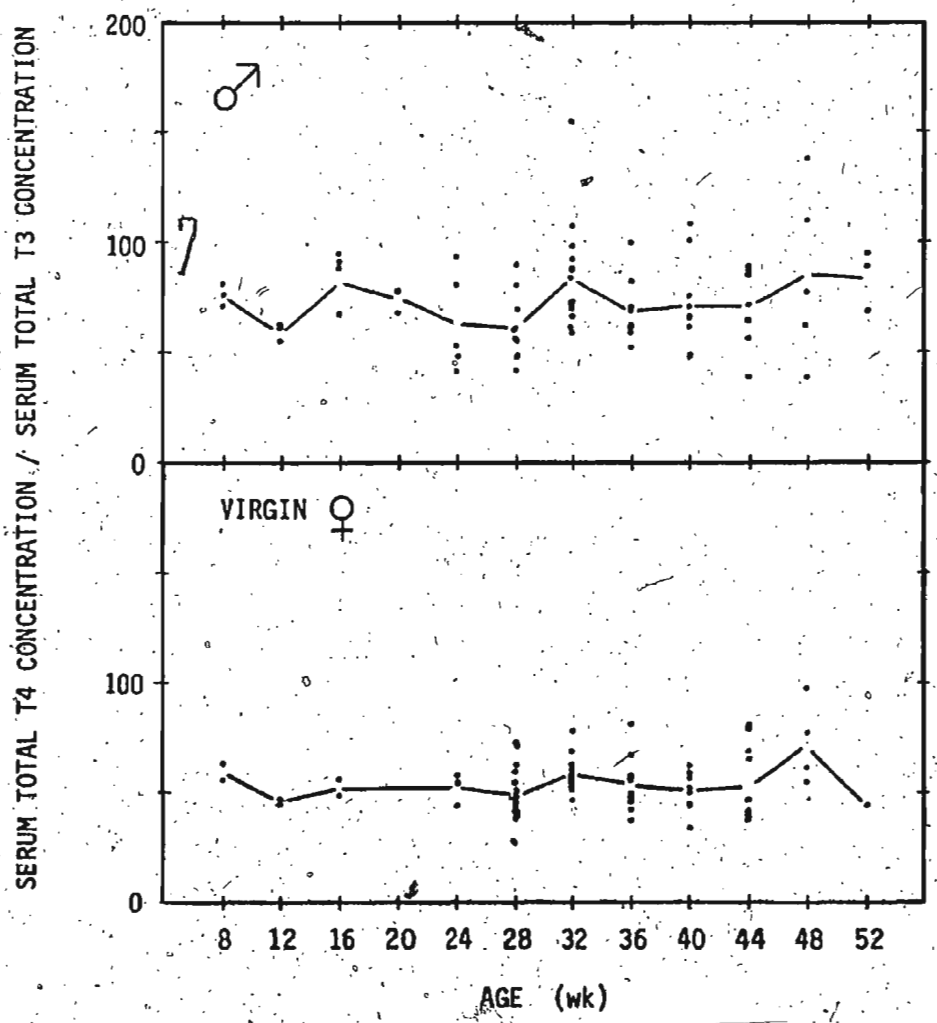


Fig. 19. T4/T3 ratios. Serum T4 and T3 concentrations were both obtained for 78 samples from 25 male BUF rats and for 82 samples from 26 virgin females.

3.8 RELATIONSHIPS BETWEEN HORMONE CONCENTRATIONS, AGE AND WEIGHT

Table 2 shows the results of linear regression and partial correlation analyses involving T4 or T3 and age and/or body weight; these analyses were performed using subroutines in the Statistical Package for the Social Sciences, SPSS (Nie et al., 1975).

3.8.1 Variation with Age

In each sex, T4 and T3 levels declined as age increased (Fig. 20 and 21). For both hormones, the differences between males and virgin females were not significant (comparing the regression coefficients, using the z-transformation and a two-tailed t test). Moreover, when the data for both sexes were combined, both hormones again showed negative correlations with age (data not shown).

For the purpose of comparing this data with Kieffer et al. (1978), the T4 values from ages greater than 24 wk were excluded (80% of the BUF rats used in Kieffer et al. were between 2 and 6 months of age). Recalling that the T4 values from males and from virgin females were significantly different at 8, 12 and 16 wk (see Fig. 15), it is not surprising that the combined data did not exhibit a significant variation with age ($P > 0.05$), whereas the data for each sex, separately, did ($P < 0.001$ for each sex). T3 data for the ages of 8 to 24 wk did not show a significant correlation with age, whether the sexes were separated or combined.

3.8.2 Variation with Age, Controlling for Weight

Serum T4 and T3 levels rose as body weight increased, although the correlation coefficient was significant only in the males (see Table 2). Partial correlation analysis was employed to control for this effect of weight, in order to further investigate the relationships between age and hormone concentrations.

As shown in Table 2, in males, the correlation coefficient for each hormone was greatly increased by controlling for the effect of weight, whereas in virgin females there was little change in the r value (as would be expected, since weight did not have a significant correlation with T4 and T3 levels in the females).

Table 2. Linear regression and partial correlation analyses of the BUF rat data

	MALES			VIRGIN FEMALES		
	r	P <	df	r	P <	df
T4 *versus* AGE	-0.309	0.001	145	-0.283	0.001	135
T4 versus AGE, CONTROLLING FOR WEIGHT	*-0.522*	*0.001*	*136*	*-0.308*	*0.001*	*128*
T4 *versus* WEIGHT	0.264	0.002	139	0.021	NS*	131
T4 versus WEIGHT, CONTROLLING FOR AGE	*0.502*	*0.001*	*136*	*0.129*	*NS**	*128*
T3 *versus* AGE	-0.369	0.001	113	-0.417	0.001	123
T3 versus AGE, CONTROLLING FOR WEIGHT	*-0.592*	*0.001*	*111*	*-0.465*	*0.001*	*121*
T3 *versus* WEIGHT	0.261	0.005	112	0.059	NS	122
T3 versus WEIGHT, CONTROLLING FOR AGE	*0.547*	*0.001*	*111*	*0.234*	*0.01*	*121*

* NS = not significant (P > 0.05)

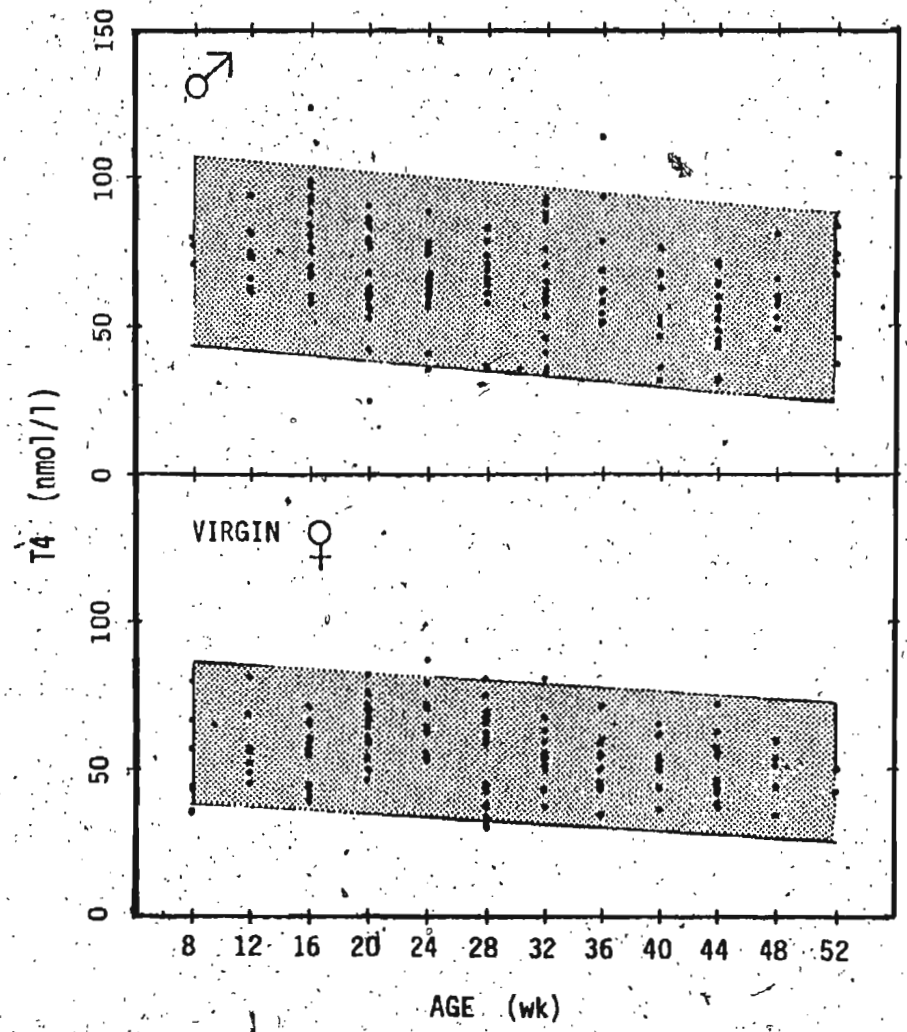


Fig. 20. T4 *versus* age in BUF rats. The shaded area for each sex is the normal range (*i.e.*, ± 2 SE of the estimate) for the linear regression. T4 = 78.249 - 0.429 x AGE for males, and 63.511 - 0.305 x AGE for virgin females.

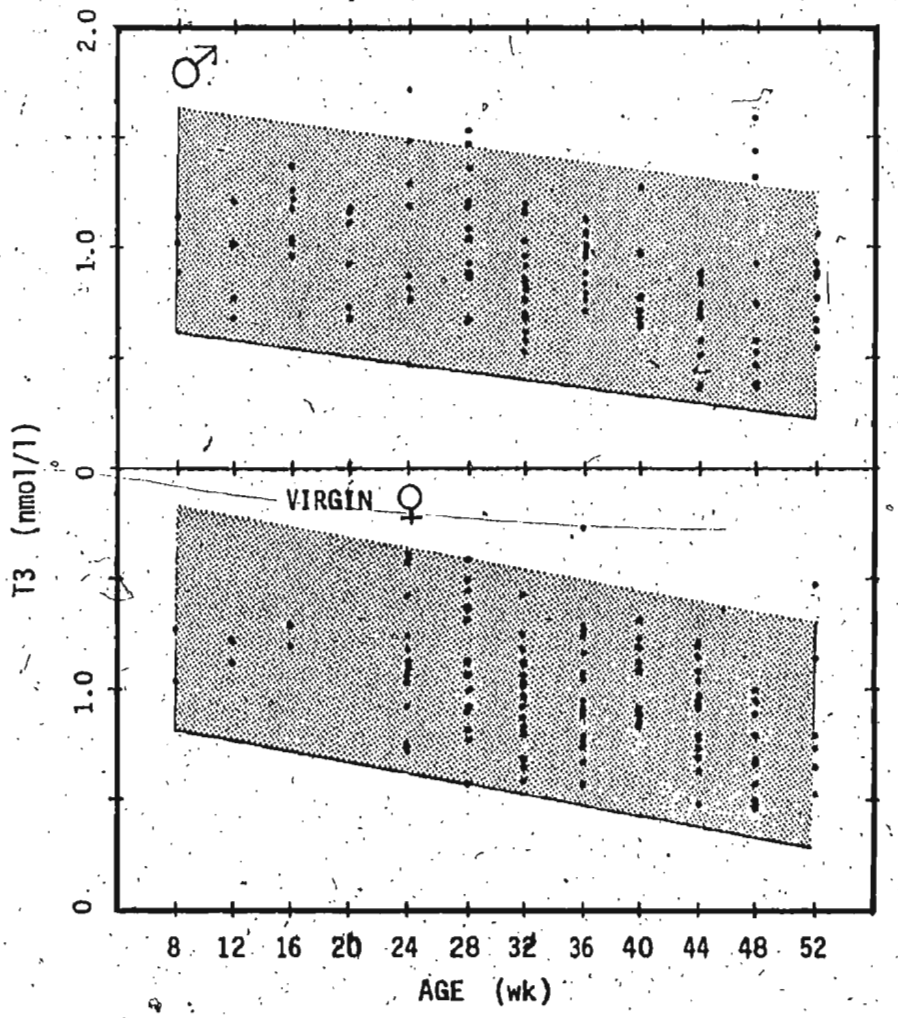


Fig. 21. T3 *versus* age in BUF rats. The shaded area is the normal range (*i.e.*, ± 2 SE of the estimate) for the regression line. T3 = 1.1745 - 0.0086 x AGE for males, and 1.4148 - 0.0122 x AGE for virgin females.

3.8.3 Variation with Weight, Controlling for Age

Partial correlation analysis was again employed -- this time controlling for the effect of age -- in order to examine the relationships between body weight and T4 or T3 concentrations (see Table 2). In males, the correlation coefficients for T4 and for T3 with weight were approximately doubled just by controlling for the counteracting effect of age. In virgin females, too, the correlation coefficients were greatly increased by controlling for the effects of age; so much so, in fact, that the variation of T3 with body weight achieved a level of significance ($P < 0.01$). However, T4 concentrations in virgin females were still not significantly related to body weights ($P > 0.05$) after the effect of age was "partialled out".

3.9 SERIAL SAMPLES

Fig. 22 shows, for T4, the variations with age for each rat that was evaluated at more than one age; due to the complexity of this data, and the data for T3, the natural histories of a few animals that are representative are shown in Fig. 23. It appeared to be normal for the hormone concentrations to vary greatly from month to month, showing both large and sudden changes without any apparent trend. Although such changes usually reversed themselves the following month, this cannot be confirmed for those rats which were either not bled the next month or whose sample for the next month has not been assayed.

For each sex and hormone, less than 5% of the values lay outside of the "normal range" (defined as the calculated regression line $\pm$ 2 SE of the estimate; this is what one would expect statistically ($\pm$ 2 SE of the estimate approximates the 95% confidence limits). Only one rat (male #54; see Section 3.1) contributed more than one "deviant" value; it had T4 concentrations below the normal range at 20 and 24 wk, and values just above the borderline at 28, 32 and 36 wk (these were all of the ages for which that particular rat had been evaluated). Although there were a few rats that showed steep drops, dying before the next month, there was no rat which could be recognized as having hypothyroidism suggestive of thyroiditis.

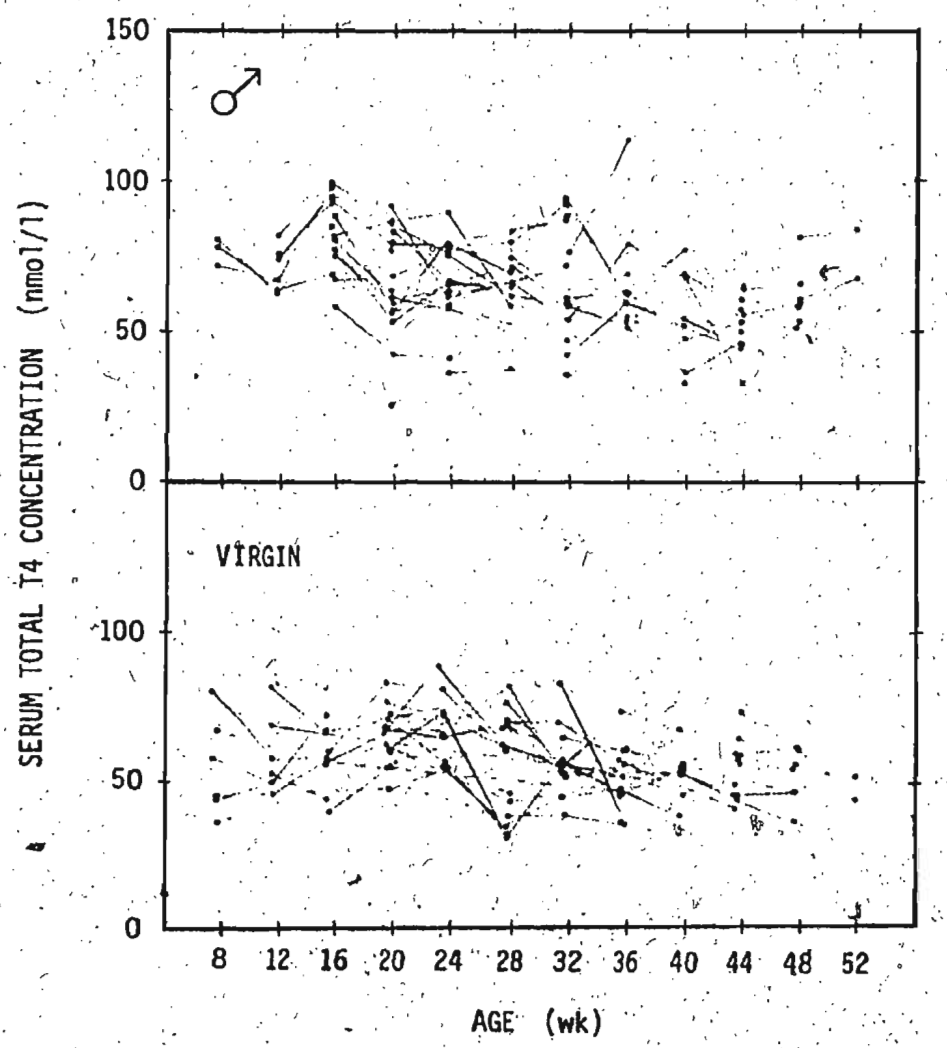


Fig. 22. T4 in serial samples. Solid lines connect sequential samples (obtained at four-week intervals); broken lines connect samples spanning more than one age interval (*i.e.*, when there is a missing value). Because of the complexity of the serial data, both for T4 and for T3, Fig. 23 shows a few individuals that represent the different patterns seen.

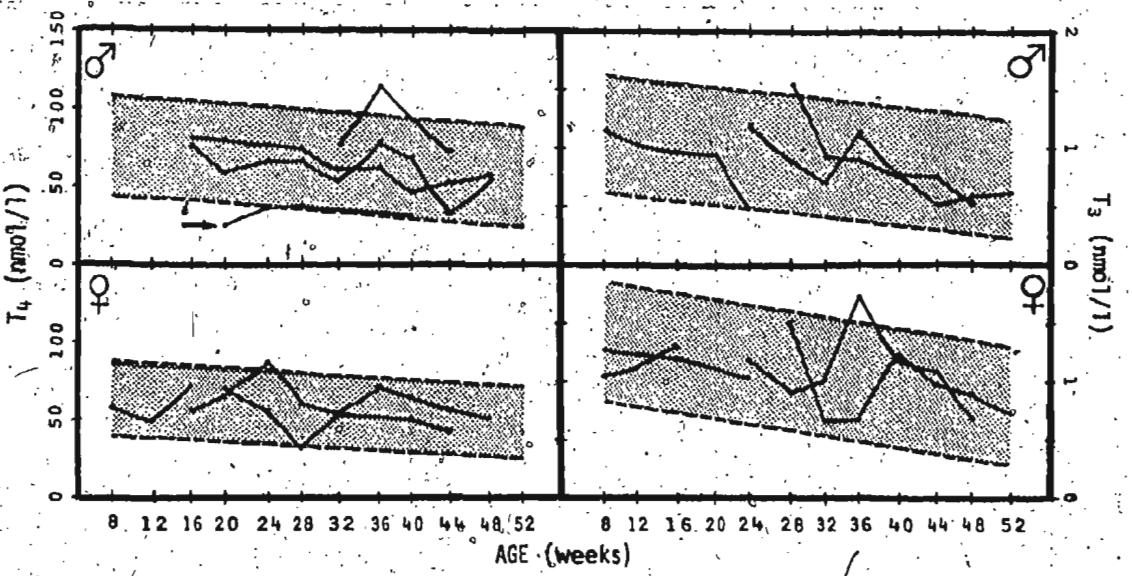


Fig. 23. Representative individuals. Four patterns were observed: some animals showed a steady, progressive decline with age; some animals exhibited large fluctuations (staying within the normal range); some animals exhibited fluctuations in which a single value was observed outside of the normal range; and, one animal (indicated by the arrow) had two such "deviant" values and three more values which were just within the normal limits.

3.10 SUPPLEMENTARY FINDINGS

This section deals with the data obtained from BUF rats which were sacrificed within one week after arriving from Simonsen Laboratories, Gilroy, CA, USA.

3.10.1 Thyroid/Body Weight Ratios

Fig. 24 shows the thyroid/body weight ratios of 280 rats (20 males and 20 females in each age group); note that there are odd ages (e.g., 15 wk) and 20 female retired breeders (about 11 months old). 4-5% of the rats had very markedly high values, indicative of goitre. Although "deviant" T4 and T3 values could perhaps be attributed to errors of technique (e.g., radioimmunoassays are extremely sensitive to inaccuracies in pipetting microlitre volumes), these unusually high thyroid/body weight ratios could not so easily be discounted.

Examples of a normal thyroid and an unusually heavy one are depicted in Fig. 25.

3.10.2 Spleen/Body Weight Ratios

Fig. 26 shows the spleen/body weight ratios of the same rats. "Deviant" values, indicative of splenic enlargement as might accompany a large increase in immune activity, were scarce; moreover, the incidence was not related with "deviant" thyroid/body weight ratios. Thus, the few animals with enlarged spleens may have had some non-thyroidal disease, such as bronchiectasis (which is believed to be due to an infection).

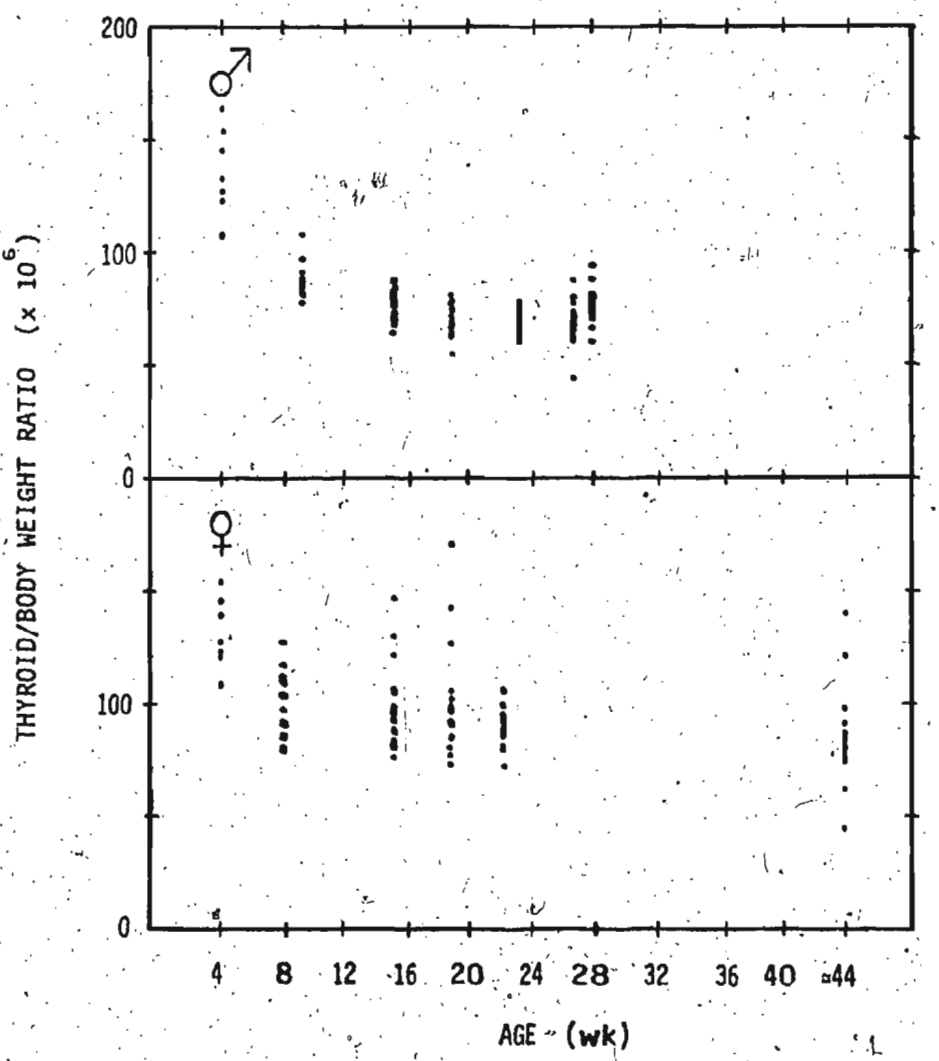


Fig. 24. Thyroid/body weight ratios of the supplementary BUF rats. The thyroids were fixed (in phosphate buffered formalin) before they were weighed. As was shown in Fig. 4, the ages of the rats did not follow the four-week intervals. Note that the ratios in the females did not decline with age as noticeably as in the males; moreover, the females exhibited markedly "deviant" values (especially the retired breeders).

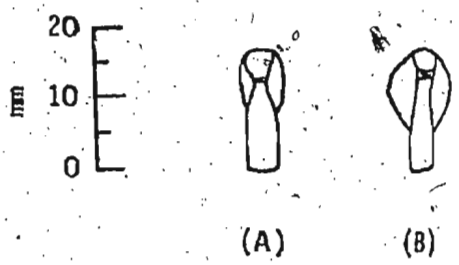


Fig. 25. Thyroid glands of supplementary BUF rats. These line drawings of the gross appearance at the time of dissection, showing portions of trachea with thyroid lobes attached, were obtained by outlining the specimens on a piece of paper and then pencilling in details of the edges of the thyroid lobes. The twenty 19 wk old females had a mean ($\pm$ SD) thyroid weight of 27 $\pm$ 20 mg. (A) is a "normal" gland weighing 16 mg; (B) is an enlarged gland weighing 111 mg.

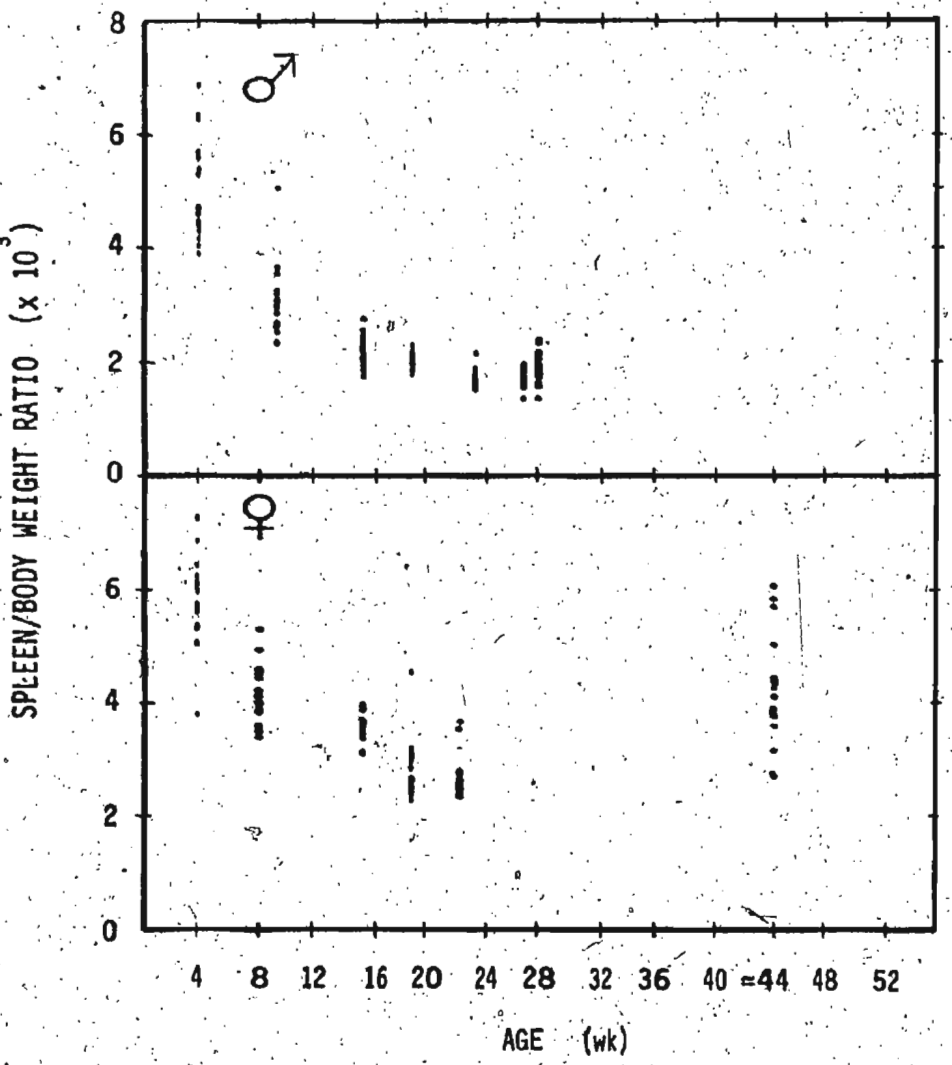


Fig. 26. Spleen/body weight ratios of the supplementary BUF rats. In both the males and the females, the ratios tended to decline with age. Although there were a small number of values which were unusually high for the age, note that most of the female retired breeders had much higher values than did the 19 or 22 wk old females.

4. DISCUSSION

4. DISCUSSION

4.1 THYROID FUNCTION

4.1.1 Variation due to Sex

T4. The present study confirmed a recent finding (Kieffer et al., 1978) that untreated female BUF rats had slightly lower levels of T4 than did the males; the virgin females generally had lower levels than the males, and indeed, at 5 of the 12 ages tested herein, the virgin females had an average serum total T4 concentration that was significantly lower than that of the males.

T3. Serum total T3 concentrations were also measured in this investigation; there were no previous reports of T3 levels in the BUF rat. Virgin females tended to have higher levels of T3 than did the males.

T4/T3 ratio. In animals of each sex, T4 and T3 concentrations were correlated, and T4/T3 ratios were significantly lower in the virgin females than in the males.

The observed differences between males and virgin females, with regard to thyroid function, appear to be a reflection of sexual variation in thyroid function itself rather than a result of sexual variation in the incidence or severity of spontaneous autoimmune thyroiditis in BUF rats. Two previous studies (Rapp & Pyun, 1974; Fukuda et al., 1975), on rats of other strains, have indicated that the decrease of T4 and the increase of T3 in females (relative to males) appear to be normal aspects of thyroid function (the differences in T4 and T3 levels are apparently regulated by differences in TSH levels (Fukuda et al., 1975) although it is not known why the thyrotrophs in the pituitary would be "set" at different levels in males and females).

4.1.2 Variation due to Age

T4. In contrast to the earlier finding that BUF rats did not exhibit a significant variation of T4 with age (Kieffer et al., 1978) this investigation has found that the decline of T4 with age was significant in each sex (with no significant difference between the two sexes). This discrepancy appears to be due to the fact that the previous study pooled

the data for both sexes; the sexual variations which exist at some ages could cause the pooled data to give the appearance of not varying with age, as was shown when the comparable data in this study was pooled and re-analyzed (see Subsection 3.8.1). Aside from providing new information about the variation with age, this serves to stress the importance, when looking at such experimental subjects as BUF rats, of allowing for variation due to sex by analyzing the sexes separately.

T3. The decline of T3 was significant in each sex; as with T4, there was no significant difference between the decline in males and in virgin females.

T4/T3 ratio. In view of the evidence that T4 and T3 levels vary with age, it must be emphasized that the T4/T3 ratios for each sex did not vary significantly between 8 and 52 wk of age.

4.1.3 Variation due to Body Weight

It was evident from the present study that body weight was also an important factor in thyroid hormone levels (especially with T4). It is not known why the hormone levels would rise with increasing body weight, although it may be suggested that the increased bulk might require a proportionately higher concentration of the thyroid hormones in order to maintain the same level of metabolic activity; recall, also, the relationships that have been found in association with acute weight gain (or loss), which may, however, depend upon some other mechanism(s) (see Subsection 1.1.5 regarding variation with diet, and Bray et al., 1976 and references cited therein).

4.1.4 Diurnal Variations

A diurnal variation was observed in the T4 levels of Sprague-Dawley rats tested in this investigation; there were no previous reports in the literature regarding diurnal variations in either T4 or T3 levels in rats. Although BUF rats were not tested, the results suggest that diurnal variation may be a normal contributor to the variation observed in T4 levels (both in these BUF rats, and, in rats in general).

4.1.5 Hypothyroidism

It was previously suggested (Kieffer et al., 1978) that a mild form of hypothyroidism was widespread in BUF rats, based on the reduced levels of T4 when compared with rats of another strain (Kieffer et al., 1976). The finding must be viewed with caution; the values from BUF rats were only compared with those from outbred CD rats, animals in which the females were found to have higher levels of T4 than did the males (which contrasts with the relationship reported in other strains; see Subsection 1.1.5). Moreover, the present investigation found generally higher levels in BUF rats than did Kieffer et al. (1978), and the T4 levels in 24 wk old BUF males were shown to not be significantly different from those of a similar group of Sprague-Dawley males.

4.2 SPONTANEOUS AUTOIMMUNE THYROIDITIS

4.2.1 A Model for Hashimoto's Thyroiditis

The need for a model. Hashimoto's thyroiditis is a significant human disease for at least two reasons. Firstly, the incidence can be relatively high -- about 0.1% in females older than 40 years. Advances in diagnosis, treatment, or especially, prevention would alleviate much physical, psychological and financial discomfort; such advances depend upon research aimed at understanding the underlying bases of the disease. This brings in the second reason, namely that Hashimoto's thyroiditis is believed to be an example of an autoimmune disease, in which the body's immune system has somehow been re-directed to act against a normal constituent. As such, understanding Hashimoto's thyroiditis could perhaps unlock the secrets of many other autoimmune diseases.

One possible avenue of investigation has involved the characterization of immune recognition and responsiveness in patients with autoimmune thyroiditis. Alternatively, investigators have sought to elucidate the genetics of susceptibility (in terms of incidence, age of onset, severity, etc.). Genetic markers have been used in attempts to associate disease susceptibility with a variety of geno- or haplotypes.

Clearly there are limitations on this kind of research in the clinical setting, both moral and physical; e.g., human generation time is prohibitively long, and can only be overcome by examining large families (as can be found in geographically-isolated Newfoundland communities. A genetically-defined, well-characterized and preferably inbred animal model with a relatively short generation time would be invaluable in enabling investigators to study the immunologic and immonogenetic factors involved in autoimmune diseases such as Hashimoto's thyroiditis. Moreover, an animal model might overcome the moral objections to experimental manipulations designed to determine the roles of thyroid antigens, of components of the immune system (including B cells, plasma cells, antibodies, the thymus, T cells and their factors), and of environmental and other host factors (e.g., bacterial or viral infection) which might be involved in the regulation of autoimmunity.

BUF strain inbred rats, therefore, appear to be an excellent tool for the researcher. They are relatively inexpensive to obtain and/or maintain, they have a short generation time (facilitating back-crossing, hybridizing with other strains, etc.), and, spontaneous autoimmune thyroiditis in the BUF rat, in contrast to in the OS chicken model, is comparable to the disease in humans (at least with regard to thyroid antibody specificities and age- and sex-related incidences, and perhaps also with regard to thyroid function). BUF rats exhibit a relatively high incidence of spontaneous autoimmune thyroiditis, and experimental manipulations (e.g., neonatal thymectomy, ingestion of methylcholanthrene, etc.) easily, yet dramatically, increase the incidence further.

Incidence(s) of spontaneous autoimmune thyroiditis. From a review of the literature, BUF rats from two different sources have appeared to exhibit different incidences of the disease (see Subsection 1.3.3). The cumulative incidence which can be calculated for Reuber and his co-workers' female BUF rats (from NIH, Bethesda, MD, USA) was 1.4%, well below the 8.0% calculated for Silverman & Rose's non-breeder females (from Simonsen Laboratories, Gilroy, CA, USA. Although the present investigation was not intended to determine the incidence of histologically-defined thyroiditis, a small sampling of the rats in the colony (which was derived from Simonsen stock) indicated that they too had a higher rate of spontaneous thyroiditis (see Section 3.2). The incidences observed by the different investigators would depend upon the age distributions of the rats used, but the possibility of environmental factors (e.g., diet, infectious agents, etc.) or of genetic differences (even though BUF rats are highly inbred) cannot yet be ruled out as potential causes for the differences in incidences.

4.2.2 Markers for Autoimmune Thyroiditis

Thyroid function. Although it was deemed to be desirable to monitor the natural history of the BUF rat, in order to define a baseline for future investigations, it was impossible to assess lymphocytic infiltration in the thyroid glands as the rats aged. For this reason, it was decided to see whether thyroid function (in terms of serum concentrations of T4 and T3) could serve as a marker for thyroiditis.

Among patients with Hashimoto's thyroiditis, only about onethird are hypothyroid. In this longitudinal investigation of the BUF rat model, a small proportion of the sample values lay outside of the normal range (defined as ± 2 SE of the estimate around each regression line: T4 versus age, T3 versus age, and T4 versus T3). However, such "deviant" values did not usually appear at more than one age in a given rat; they are not likely to be good indications of animals with thyroiditis. Thus, one conclusion of this investigation must be that thyroid function studies, with regard to T4 and/or T3, are not suitable markers for the study of spontaneous autoimmune thyroiditis in the BUF rat model.

Thyrotropin. It remains to be seen whether a longitudinal study of serum TSH, rather than T4 and T3, levels would be any more valuable. In that respect, a recent study (Kieffer et al., 1978) was able to correlate "deviant" TSH concentrations (more than 3 SD above the mean calculated for the rest of the BUF rats) with "deviant" relative thyroid weights (all of which were more than 3 SD above the mean value for the rest of the animals and were associated with lymphocytic infiltration of the thyroid gland). Thus, TSH may indeed be a better marker for thyroiditis than T4 and T3.

Immune activities. Another recent report (Noble et al., 1976) has indicated that in the BUF rat, as in the human, assays for circulating thyroid antibodies are more likely to be diagnostic of spontaneous autoimmune thyroiditis than are thyroid function studies. Therefore, it would seem that a longitudinal study of anti-thyroid immunoglobulin levels would form a valuable baseline for future studies in the BUF rat model.

To speculate on the pathogenesis of spontaneous autoimmune thyroiditis, and thus to suggest other future investigations, it is worth recalling what was reported in previous clinical and animal studies (see Subsection 1.2.2). The presence of thyroid antibodies alone (in transfer experiments) was not sufficient to produce thyroiditis (Roitt & Doniach, 1958), although the presence of antibodies to certain thyroid antigens is considered to be sufficient to make a diagnosis. It appears certain that thyroid-directed cell-mediated immunity, involving cells and/or their soluble factors, is a prerequisite for thyroiditis, but it is not possible to say at this time whether the root of the problem is a particular gene product, an acquired "fault" (perhaps precipitated by hormonal levels associated with pregnancy, stress, or other aspects of normal life), an external factor (perhaps infectious or chemical), or some combination of these.

Although there have been extensive clinical studies of immunocompetance in thyroiditis, few studies have been carried out to evaluate immune function in the BUF rat. BUF rats produce antibodies to thyroglobulin, both spontaneously and following treatments such as immunization with a rat thyroid extract and either a complete or an incomplete adjuvant, feeding with a diet containing 3-methylcholanthrene, or neonatal thymectomy (Noble et al., 1976; Silverman & Rose, 1971, 1974b, 1975a). Plaque-forming cells have yet to be enumerated in BUF rats, but the incidence and titre of thyroid antibodies in neonatally-thymectomized females have been observed to increase with age (Noble et al., 1976). It is possible that these observations are the sequelae of changes in a sub-population of suppressor T cells, but again there has yet to be an examination of the numbers or types of T and B lymphocytes present in BUF rats (with or without thyroiditis). Similarly, although "killer" cells have been implicated in thyroiditis, little enough is known about this type of cell at all, let alone specifically in the BUF rat.

In conclusion, therefore, one is left with the hope that measurements of a variety of immune activities may turn up the most suitable markers for spontaneous autoimmune thyroiditis, and in the process allow a better understanding of Hashimoto's thyroiditis (in particular) and of immunity, and autoimmunity (in general).

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Appendix I. Body weights of male BUF rats.

RAT #	AGE (wk) 4	8	12	16	20	24	28	32	36	40	44	48	52
1			110										
2			130										
3			130										
4			110	365									
5			140										
6				375									
7				375									
8				395									
9				345									
10											445	445	445
13				295		345							
14				295		375			255		325	345	335
15				315		365							
16				335		385			245		315	325	325
17				315		385							
18													455
19													365
20													495
21													455
22													525
23													485
24				365		365							
25				355		385				415	415	405	395
26				375		405							
27			285		325								
28			285										
29			275										
30			265					215					
31								435	465	485	515	515	515
32								485	515	525	555	535	485
33								455	475	505	515	535	525
34					355								
35					385								
36					395		300	275	275	275	275		
37					365								
45				365	365	395		385	385	395	385	175	
46		235	265	305		355	335	305	315	295	285	285	
47		215		295		335	335	335	345	385	345	335	
48		205		255		285	295	305					
49		205		305		345	355	365	365	365	365	355	275
50				325		365	365	365	385	375	345		
51				315		355	355	345	365	355	355	345	
52				335		385	385	395	395	395	385	365	
53				305		335	295						
54					225	225	265	275	255	285			
55					365	405	375	385	395	375	445		
56					415	405	425	425	435	435	365		
57					335	365	375	395	405	405	355		
58					385	355	375	385	395	395	385	325	345
59			355	415	435	445		485			425		475
60			355	415	445	455		435					
61			355	415	445	465		505			445		
62	45	215	295	335	365								
63	55	225	305	345	375								
64		215	285	325	365	305							

Appendix II. Body weights of virgin female BUF rats.

RAT #	AGE (wk) 4	8	12	16	20	24	28	32	36	40	44	48	52
6				225									
7				215									
8				205									
9				205									
10				205									
12											265	275	245
13	75			215		255							
14	75			215		245			185	205	215	235	225
15				205		245			255				
16				225		255							
17						225							
21										215			
22										225	225	235	225
37				225		235							
38				245									
39				245									
40				235		275							
41			215		225								
42			185		205								
43			215		265								
44			215		255			245	245	255	255		
45				195									
46				235	245	265	265	275	265				
47				205	205	215	215	225	205	205	195	205	205
48				195	195	205	205	215	205	205	205	205	195
49		155			205								
50		185			235	235	245	235	205				
51		185			225	225	235	245	245	235	195	175	
52		165			225	235	235	245	245		215	225	215
53		175			215	215	215	225	215				
54				195		205	185	195	195	185	185	175	
55		155		205		225	215	235	225	225	235	205	185
56		165		205									
57		155		205		205	215	215	225	215	205	215	175
58		165		215		225	205						
59				205		205	205	215	225	215	225	225	215
60				235	245	255	255	255	245	245		225	
61				235	235	245	235	245			245		
62				235	255	255	245	265	265	255	265	255	
63				235	245	245	245	245				245	
64					265	265	245						
65					265	255	235	245	245	235	245		
66					265	265	265	265		275	265	215	
67					235	235							
68					275	275	265	275	275	285	255		
70			195	215		235			235		235		
71			195	215		225		235	225		235		
72			195	215		235		235	245		235		255
73			205	225		245		245	245		235		245
74			175			225				245			
75	45	145	185	195		205							
76		135	175	185									

Appendix III. Serum T4 concentrations of male BUF rats.

RAT #	AGE (wk) 8	12	16	20	24	28	32	36	40	44	48	52
3		73.4										
4		80.8										
5		93.3										
8			122.5									
9			69.7									
10										63.2	65.0	
13					67.0							
14					64.1			51.0			48.3	
15					63.1							
16					60.3					55.0	58.2	
17					65.7							
18												71.0
19												45.3
20												106.9
21												73.2
22												82.5
24			59.8									
25			69.0						67.2			36.5
26			66.7									
30							35.6					
31							75.6	112.8		71.1		
32								92.6			59.9	66.7
33										63.9	80.6	82.7
36							63.8			30.8		
45				82.0	66.0		61.3		62.3			
46			57.3	41.7	40.4		57.3	50.4	35.5	44.8		
47			74.1	58.3	64.9	65.4	52.7	77.9	66.7	31.3	52.2	
48			87.1	56.2	57.8	82.4	87.4					
49			75.7	52.2	64.5	70.3	58.4	67.6		69.8		
50			79.3	60.2	62.1	63.6		61.2	75.2			
51			65.8	67.2	74.0	60.6	57.6	53.6		58.8	56.7	
52			79.2	78.2	75.7	73.0	59.6	61.4	45.8	51.8	56.5	
53			80.4	85.6	87.5	57.0						
54				23.9	34.5	36.0	34.0		30.8			
55				62.0			45.4		67.2	55.3		
56				75.1		77.8	90.6	61.2		47.6		
57				54.6	77.1	67.5	91.9	57.6	52.3	44.1		
58				59.4	55.8		40.1	57.4	49.6	42.2		
59		74.2	90.9	77.1	73.3		84.7					
60		72.0	93.0	89.7	59.9		69.9					
61		80.1	97.5	84.1	73.9							
62	76.3	62.4	67.3									
63	70.5	65.1	95.9									
64	79.3	61.0	82.9									

Appendix IV. Serum T4 concentrations of virgin female BUF rats.

RAT #	AGE (wk) 8	12	16	20	24	28	32	36	40	44	48	52
12										71.1	58.5	41.4
14								59.0				48.8
22										56.5	58.9	
38			43.8									
39			59.7									
42				63.4								
44				71.1				58.3		53.4		
46				58.6		44.3						
47				59.2		41.7			52.2			
48				53.3		79.7	49.3	53.3	53.2	61.7	53.1	
49	34.8											
50	66.2					36.6	36.3	33.7				
51	35.6					66.6			43.1	54.7		
52	44.2			45.9	51.9	68.6	62.1	57.9			43.2	
53	43.0					74.1	53.5	48.8				
54				68.9	54.9	30.5	52.8		50.2	42.3		
55				65.8	62.8	67.1	50.6		49.2	41.2	33.2	
57				74.9	69.9	59.1	54.4	42.7	51.6	42.1	42.5	
58				60.5	70.7	29.3						
59			58.3	80.9	78.3	57.6	53.1	70.2	64.3	56.2	50.5	
60			38.6	52.6	53.1	33.1	42.3	44.7	35.0			
61				66.7	63.3		50.5			45.6		
62			40.3	68.7	61.7	64.4			60.3	43.5		
63			54.3	63.9	86.0	59.5	53.0			41.3		
64				49.0		37.4						
65				75.1		61.8			60.7	35.9		
66				68.9		42.6	58.2			41.0		
67				66.1								
68				65.9		65.3	66.4	53.8		37.3		
70		51.4	42.6									
71		67.7	64.7				53.1	42.2				
72		80.5	65.4				79.6	33.5				
73		44.7	55.6					43.8				
74		55.1										
75	78.9	56.3	56.8									
76	56.8	48.3	70.5									

Appendix V. Serum T3 concentrations of male BUF rats.

RAT #	AGE (wk) 8	12	16	20	24	28	32	36	40	44	48	52
1		0.67										
2		0.76										
4			1.21									
6			1.17									
7			1.25									
8			1.36									
9			1.04									
10												1.05
16												0.92
25											0.92	0.54
31											1.43	0.86
32											1.58	0.76
33											1.31	0.88
36									1.26			
45						1.03	0.67	0.70	0.97	0.57	0.46	
46						0.67	0.69	0.83	0.76	0.70	0.36	
47						1.08	0.61	0.79	0.67	0.83	0.38	
48						1.20	0.57					
49						1.18	0.67	1.00		0.83		0.66
50						1.35	0.83	0.75	0.70	0.71		
51						1.46	0.80	0.88	0.77	0.68	0.74	
52						1.52	0.91	0.88	0.76	0.74	0.52	
53						1.04						
54					0.86	0.65	0.52	1.05	0.64			
55					1.28	0.92	0.75	0.99	0.97	0.88		
56					1.70	0.88	0.85	1.06	0.96	0.86		
57					1.47	0.85	0.95	0.95	0.70	0.50		
58					1.18	0.87	0.69	1.12	0.76	0.50	0.57	0.61
59				1.15			1.19			0.37		
60				1.17	0.75		1.02					
61				1.10	0.80		1.15			0.35		
62	1.01	1.00	1.01	0.72								
63	0.88	1.20	1.02	0.67								
64	1.13	1.01	0.95	0.92	0.46							

Appendix VI. Serum T3 concentrations of virgin female BUF rats.

RAT #	AGE (wk) 8	12	16	20	24	28	32	36	40	44	48	52
14												1.13
22												1.46
46						0.99		0.56				
47						1.58	0.58	0.78	1.07			
48						1.49	0.64	0.66	1.22	0.96	0.88	0.72
50						0.91	0.67	0.73				
51						1.44	0.79	1.04	1.30	1.20	0.99	
52						1.36	1.10	1.06		0.68	0.45	0.51
53						1.05	0.86	0.74				
54						1.12	0.91	1.28	0.87	0.94	0.49	
55						1.36	0.82	1.15	0.89	0.90		
57						1.58	0.95	1.04	0.84	0.62	0.56	0.64
58						0.76						
59						1.35	1.02	1.25	1.11	0.72	0.94	0.78
60					1.23	0.56	0.68	1.23	0.82		0.49	
61					1.11	1.05	0.96			1.19		
62					1.17	0.89	1.00	1.72	1.18	1.07	0.67	
63					1.60	1.11	1.05			1.14	0.78	
64					1.09	0.80						
65					1.41	1.30	0.82		1.18	0.93		
66					1.60	1.05	0.86		0.90	0.91	0.65	
67					1.58							
68					1.56	1.05	1.11	0.94	0.82	0.47		
70					0.74			0.87		0.78		
71					0.74		1.17	0.89		0.68		
72					0.71		1.42	0.75		0.76		
73					1.05		1.24	0.90		1.03		
74					0.91				1.08			
75	1.26	1.21	1.19		1.02							
76	1.03	1.11	1.28									

Appendix VII. T4/T3 ratios in BUF rats, males & Virgin females.

RAT #	AGE (wk) 8	12	16	20	24	28	32	36	40	44	48	52
8			90.1									67.6
9			67.0								37.9	87.8
25											61.5	94.0
32												
33							91.5		64.2			
45							83.0	60.7	46.7	64.0		
46						60.6	86.4	93.6	99.6	37.7	137.4	
47						68.7	153.3					
48						59.6	87.2	67.6		84.1		
49						47.1		81.6	107.4			
50						41.5	72.0	60.9		86.5	76.6	
51						48.0	65.5	69.8	60.3	70.0	108.7	
52						54.8						
53					40.1	55.4	65.4		48.1			
54							60.5		69.3	62.8		
55						88.4	106.6	57.7		55.3		
56					52.4	79.4	96.7	60.6	74.7	89.2		
57					47.3		58.1	51.3	65.3	84.4		
58				67.0			71.2					
59				76.7	79.9		68.5					
60				76.5	92.4							
61												
62	75.5	62.4	66.6									
63	80.1	54.3	94.0									
64	70.2	60.4	87.3									
14												43.2
46						44.7						
47						26.4			48.8			
48						53.5	77.0	80.8	43.6	64.3	60.3	
50						40.2	54.2	46.2				
51						46.3			33.2	45.6		
52						50.4	56.5	54.6			96.0	
53						70.6	62.2	65.9				
54						27.2	58.0		57.7	45.0		
55						49.3	61.7		55.3	45.8		
57						37.4	57.3	41.1	61.4	67.9	75.9	
58						38.6						
59						42.7	52.1	56.2	57.9	78.1	53.7	
60					43.2	59.1	62.2	36.3	42.7			
61					57.0		52.6			38.3		
62					52.7	72.4			51.1	40.7		
63					53.8	53.6	50.5			36.2		
64						46.8						
65						47.5			51.4	38.6		
66						40.6	67.7			45.1		
68						62.2	59.8	57.2		79.4		
71							45.4	47.4				
72							56.1	44.7				
73								48.7				
75	62.6	46.5	47.7									
76	55.1	43.5	55.1									