COMPARATIVE STUDIES OF THE RELATIONSHIP BETWEEN SERUM THYROXINE AND THYROXINE-BINDING GLOBULIN

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ABSTRACT

COMPARATIVE STUDIES OF THE RELATIONSHIP
BETWEEN SERUM THYROXINE AND
THYROXINE-BINDING GLOBULIN

By

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A new non-electrophoretic technique is presented for determining binding capacities of the specific thyroxine-binding globulin (TBG) in blood serum. This method is based on complete saturation of the binding sites on TBG using a mixture of radiothyroxine and unlabeled endogenous as well as exogenously added thyroxine (T_4). Barbital buffer (pH = 8.6) is employed to inhibit T_4 binding by prealbumins. The high dilution factor of 30-35 is employed to inhibit T_4 binding by albumins. Previous reports have indicated that albumins, prealbumins and α-globulins are the principal carriers of T_4 in blood. Under the conditions of the present experiments, the binding capacity measured is almost entirely that of TBG.

Since binding capacities of carrier proteins have been reported to vary with temperature, all serum and thyroxine mixtures are incubated in a 37°C water bath in order to obtain comparable results.

Serum T_4 levels are measured by a competitive binding procedure. Using the new method for measuring thyroxine binding capacity, a binding curve is documented by plotting µg T_4/100 ml serum bound to TBG on the ordinate and µg total T_4/100 ml serum used on the abscissa. Binding capacities usually rise with increasing concentrations of total thyroxine until a plateau or saturation point is reached. A point on the plateau is selected as the total thyroxine concentration to be used in determining the maximum binding capacity of TBG in individual serum samples of the species in question. All determinations are made in duplicate and these show good agreement.

In preliminary experiments, 20 measurements were made of the binding capacity of TBG in a pooled sample of bovine serum. The mean value of 12.02± 0.13* µg T_4/100 ml serum obtained indicates good repeatability for the technique.

Serum thyroxine and binding capacities of TBG were determined in normal nonpregnant women, Holstein cows, Suffolk sheep, pigmy goats, horses and white-tailed deer. The ratio of $\frac{\text{Serum } T_4, \mu g\ \%}{\text{TBG binding capacity, } T_4\ \mu g\ \%}$ in each species is termed Saturation Index (SI). The ungulates studied, except deer, have a mean Saturation Index of 0.73 ± 0.03 and this was not significantly different from

*Standard error of the mean.

the index in women. Saturation Indexes are also unaltered in pregnant cows, sheep and in women taking oral contraceptive pills. All these species have in common the fact that their TBG is the major carrier of T_4.

In deer, where albumins have been reported to transport quantitatively more thyroxine than globulins, the Saturation Index is significantly higher than that of other higher mammals studied. Deer also have significantly higher serum thyroxine levels than any other higher mammal studied. Since thyroxine-binding capacities of deer TBG is comparable to that of the other mammals, the higher Saturation Index indicates T_4 binding to proteins other than TBG.

In bovine fetuses, there is a gradual increase of serum T_4 and TBG capacity during the second and third trimesters of pregnancy. Quantitatively, the two parameters are almost identical and yield Saturation Index values of 1.05 and 1.13 during the second and third trimesters, respectively. After birth, when TBG levels rapidly decline, the excess thyroxine is released from protein binding thus becoming available for metabolic uses. It is suggested that this reserve of quickly available extrathyroidal T_4 plays a vital role in the adjustment of the newborn calf to its environment.

Serum thyroxine levels of thyroprotein-fed cows are significantly elevated over those of untreated cows but with no corresponding change in the thyroxine-binding

capacity of TBG. This results in a significantly higher Saturation Index in the thyroprotein-treated than in the untreated cows. The mean Saturation Index of 0.97 ± 0.04 in treated animals also indicates that TBG is the major T_4 carrier protein in cows and any T_4 in excess of the binding capacity of TBG is rapidly cleared.

Another physiological state during which the Saturation Index of cows is substantially altered is lactation. Here serum thyroxine levels are significantly depressed, probably because of the high intensity of lactation and consequent intense competition for iodine between the thyroid and mammary glands. Binding capacities of TBG are, however, unaltered and a significant depression of Saturation Index results.

In nonlactating pregnant cows, sheep and rats, serum T_4 and Saturation Index values are statistically no different from the values in open animals.

Open rats have serum T_4 levels of 6.20 ± 0.17 µg/100 ml serum. Since the binding capacity of their TBG is only 1.73 ± 0.23 µg T_4/100 ml serum, Saturation Index is 4.06 ± 0.41. Guinea pigs and birds show negligible T_4-binding TBG capacities and relatively low serum thyroxine levels.

Male chickens have higher T_4 levels than those of nonlaying chickens whose T_4 levels are higher than those of the layers. Male turkeys are different from other birds studied in having TBG T_4-binding capacities of

1.34 ± 0.24 μg T_4/100 ml serum, a value which is statistically higher than zero.

COMPARATIVE STUDIES OF THE RELATIONSHIP BETWEEN SERUM THYROXINE AND THYROXINE-BINDING GLOBULIN

By

Kevin M. Ogon Etta

A THESIS

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DEDICATION

This thesis is dedicated to the memory of my late grandmother, Lucy Baarong Obi, in appreciation of the shining example her life was to all her children and grandchildren. It is also dedicated to my--

mother, Maria-celine Okaja Odu,

wife, Maria Ndik Etta and

guardians, Joseph Obi Etta and

Hon. Michael Etta Ogon

for contributing in countless vital ways to the successful completion of my entire educational program.

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INTRODUCTION

The thyroid gland, through its hormones, affects the integrity and function of every major system in the bodies of higher animals. It is not only involved in basal metabolism and temperature regulation, but also in reproduction and lactation, cerebration and development, and even in mineral metabolism. Like all other hormones, thyroid hormones complement the nervous system in maintaining an effective communication and coordination between the various organs and tissues. An accurate assessment of the status of the thyroid in different physiological conditions is, therefore, of the utmost importance.

Over the years, thyroidal uptake of iodine, thyroid secretion rates, total serum thyroxine, thyroxine degradation rates, protein bound iodine (PBI) and metabolic rate have all been studied in an attempt to establish reliable indices of thyroid function in man and various animals. In comparatively recent years, it has become apparent that a parameter of possibly equal importance is the thyroxine-binding capacity of thyroxine-binding proteins.

Since 1952, it has been known that human serum thyroxine has a maximum affinity for α-globulins, an intermediate affinity for prealbumins and only feeble affinity for albumins. Extensive studies have been undertaken to determine alterations of thyroid status during different physiological states in man. The data during one such state, pregnancy, indicated augmented thyroidal avidity for iodine, thyromegaly and an increase in the circulating levels of thyroid hormone. Although this triad of anatomical and functional alterations is ordinarily considered to be diagnostic of thyrotoxicosis, it was associated in early pregnancy at least, with an unequivocally euthyroid state. It was postulated that alterations in the interaction of thyroid hormone with serum proteins might provide a clue to these paradoxical findings. Since early reports indicated that thyroid hormone is largely bound to a specific α-globulin in human serum, attention has been directed to this protein in most of the recent investigations.

Most investigators up to now have used various electrophoretic techniques in studying the capacity of the specific α-globulin to bind thyroxine. A comparison of the reported electrophoretic patterns of various animal sera shows that these patterns vary widely both in regard to the number of distinct components and with respect to the relative proportion of each component. Such variations are attributable, at least in part, to

heavy trailing and other drawbacks of electrophoresis that have been pointed out in recent reviews. Even the technique of reverse-electrophoresis that was introduced to obviate trailing still has several other drawbacks, not the least being its unsuitability for routine application.

In the present study, a new non-electrophoretic technique for measuring the binding capacity of TBG is presented. This technique is suitable for routine use, avoids the drawbacks of electrophoresis and employs a resin-impregnated sponge for separating protein-bound from unbound thyroxine.

The binding of thyroid hormone in human serum has been extensively studied. There have, however, been fewer reports of thyroxine-protein interactions in other species, even though currently available information suggests important species differences in the thyroxine-binding proteins. Experiments to be reported in this study were planned to obtain data on the relationship between plasma level of T_4 and TBG in several different species and to explore the possibility of changes within species during varying physiological states.

A new thyroidal parameter, the Saturation Index, is introduced to express the relationship between serum T_4 levels and the binding capacity of TBG.

REVIEW OF LITERATURE

Most of the early findings in the field of physiology were the results of investigations undertaken to improve the practice of medicine, understand human diseases, and cure them. Findings concerning thyroxine-binding proteins were no exception.

Thyroxine-Binding Proteins

The existence of a specific thyroxine-binding protein (TBP) with a very high affinity for thyroxine (T_4) was first convincingly demonstrated in 1952 by Gordon and his co-workers. When human serum containing ^{131}I-T_4 was subjected to zone electrophoresis on paper, the binding protein exhibited the properties of an α-globulin at pH 8.6. This protein was designated thyroxine-binding α-globulin (TBG). Subsequent to the discovery of TBG, the existence of a second important thyroxine-binding protein in human serum, thyroxine-binding prealbumin (TBPA), was demonstrated (1958). A third binding protein in human serum, albumin, appears to be of less importance physiologically than the other two carriers of the hormone.

Thyroxine affinity for globulin is much stronger than that for prealbumin and even stronger than the affinity for albumin.

Thyroid hormones secreted into the blood from the thyroid gland are bound by thyroxine-binding proteins. In mammals, the affinity of thyroxine for the binding proteins is about 3-4 times that of tri-iodothyronine. Evidence has been adduced to show that tri-iodothyronine is bound either feebly or not at all to TBG in vivo (Zaninovich et al., 1966; Robbins and Rall, 1957). Most of the review will, therefore, center around the binding of thyroxine by the binding proteins.

For thyroxine to participate in tissue metabolism, it has to be freed from its binding proteins. Considerable interest was, therefore, generated in the physiological role and physicochemical properties of the specific thyroxine-binding proteins. Many researchers have linked the variations of thyroxine-binding proteins in various physiological conditions with an alteration in the level of thyroxine in serum.

TBP During Pregnancy

Dowling et al. (1956b) reported a marked increase in TBP in human serum during pregnancy. It has also been observed that the thyroid glands of pregnant women have an augmented avidity for iodine (Pochin, 1952). Circulating levels of thyroxine are believed to rise during pregnancy

(Heinemann et al., 1948; Russel, 1954). These three functional alterations are ordinarily considered to be diagnostic of thyrotoxicosis. Paradoxically, however, the above findings are neither accompanied by the symptomatic stigma of hyperthyroidism, nor are they associated, at least during the first half of pregnancy, with an increase in the basal metabolic rate. The increased thyroxine-binding protein of pregnancy may thus be directly related to both increased avidity for iodine by the thyroid and increased circulating levels of thyroxine. Precise cause and effect relationships between these parameters are still difficult to establish.

The thyroid gland would need to take up more iodine at a faster rate to keep up with the increased synthesis of thyroxine and increased circulating levels in blood serum. More iodothyronines are therefore synthesized and secreted from the thyroid gland. Whether this increased level of thyroid hormone synthesis and production is the result or the forerunner of increased levels of thyroid hormone-binding proteins is a subject of intense debate and research. Many researchers prefer to merely implicate increased thyroxine-binding protein with increased circulating thyroxine levels (Dowling et al., 1956a). Others suggest that the increased circulating levels of thyroxine are a consequence of increased serum binding proteins (Tepperman, 1962). According to the latter view, a small amount of free thyroxine in the blood

is necessary for the feedback mechanism between the thyroid gland and the production of thyrotropic hormone by the adenohypophysis. If this free thyroxine level is decreased by an increase in the binding proteins, more tropic hormone is put out from the adenohypophysis. This stimulates thyroid gland activity and more thyroxine is synthesized and secreted to bring the unbound thyroxine level in the blood to a normal value for the species. The concept of a relationship between the serum thyroxine level and binding capacity of TBG might also be the key to the levels of T_4 and TBG capacity for T_4 during lactation, in fetal blood, in newborns and in near-term and postpartum animals.

Effects of Lactation

Thyroid secretion rates in rats have been shown to fall from 2.58 to 0.99 µg T_4/100 gm body weight/day during lactation (Lorscheider, 1970). At the same time serum T_4 declined from 3.94 to 1.96 µg T_4/100 ml. So both methods of thyroid evaluation show marked and significant reductions of more than 50% in lactating rats. Serum thyroxine levels have also been shown to drop significantly during peak lactation in ewes and in cows (Lorscheider, 1970). Flamboe and Reineke (1959) have reported a lower thyroid secretion rates in lactating goats compared to the nonlactating ones. In 1961, Iino and Greer reported marked decrease in the thyroidal uptake of radioiodine during lactation. Piecing together these several reports, a consistent picture emerges. During lactation, the

thyroid takes up less iodine, and so synthesizes less thyroid hormone. Thyroid secretion rate is consequently lower. This in turn results in a low serum thyroxine level. The low radioiodine uptake by thyroid glands during lactation appears to be related to the successful competition of mammary glands for iodine during this time. Flamboe and Reineke (1959) reported that considerable iodide is secreted in milk. It would be of interest to find out the picture of T_4-binding capacities of thyroxine-binding protein as the thyroid activity changes during lactation. So far as this writer is aware, there are no reports in the literature on this subject.

Fell et al. (1968) reported a striking rise in γ-globulins of blood serum during lactation in the ewe. However, γ-globulins are more important as immune proteins than as carriers of thyroid hormones. If the binding protein-thyroxine relationship during pregnancy is any guide, there should be a corresponding relationship between serum T_4 levels and the binding proteins during lactation.

Binding Protein-Thyroid Hormone Relationships During Fetal Life in Newborn and in Maternal Serum

Thyroid follicles begin to appear in the human fetus between the 7th and the 12th week of pregnancy (French and Van Wyk, 1964; Chapman et al., 1948). In the bovine, measurable amounts of iodine were first detected in fetal thyroids at 60 days of age (Wolff et al., 1949). A direct

relationship appears to exist between calf fetal thyroid iodine and each of the three growth parameters--body weight, crown-rump length and calculated age (Nichols et al., 1949; Wolff et al., 1949). Wolff and his co-workers also reported a progressive increase in the iodine concentrating capacity of fetal thyroid tissue with age. Gorbman et al. (1952) sacrificed two pregnant cows 24 hours after radioiodine injection and found that fetal thyroids contained twice as much ^{131}I as the maternal thyroids. Fetal thyroglobulin was also found to be 27 times more radioactive than in the mothers, more than one-fifth of the radioactivity in fetal thyroids being ascribed to freshly synthesized thyroxine. In sheep, with an average gestation period of 150 days, the fetal thyroids start to accumulate iodine by the 50th day of gestation and formulation of the thyroid follicles is observed histologically on the 42nd day (Barnes et al., 1957). Rats (gestation period 21-22 days) acquire the functional ability to store iodine around the 18th-19th day of gestation. This ability may be correlated with the first differentiation of follicles complete with lumen. At 21 days of gestation the rat is able to synthesize mono-and di-iodotyrosine as well as tri- and tetra-iodothyronine. For most of the species discussed above, therefore, fetal thyroids start functioning quite early during gestation. In humans, sheep, cows and goats, fetal thyroids are already metabolizing iodine by the end of the first trimester of

pregnancy. The binding capacities of the thyroid hormone carrier proteins can be expected to play a role in regulating observed levels of thyroid activity in the fetus.

Russel et al. (1964) reported a mean fetal thyroxine-binding globulin T_4-binding capacity of 29.1 µg T_4/100 ml of serum as compared to a maternal capacity of 42.1 µg T_4/100 ml serum in humans. Dowling et al.(1956b) reported that the capacity of TBG for T_4 in fetal humans was 1.33 times greater than the level in nonpregnant adults but still about 0.5 times the level in their mothers. There seems to be agreement that pregnant women have much higher TBG capacities for T_4 than the binding capacities of the fetal TBG. This raises several questions. Is the placental barrier which separates the fetal from the maternal circulation permeable to the thyroxine-binding proteins? Is the lower binding capacity for T_4 in fetal blood serum a reflection of a higher saturation and so a higher level of serum thyroxine?

Several workers have reported that placentae in many animals are permeable in varying degrees to thyroxine (Osorio and Myant, 1962; Hoskins et al., 1958; Reineke and Turner, 1941; Zondek, 1940; Contopoulos et al., 1964; Monroe et al., 1951). An increase in the transplacental passage of thyroid hormones as human pregnancy advances has also been demonstrated by Osorio and Myant (1962). This increase has been attributed to either an increase in the permeability of the placenta, a decrease in the

thickness of the membranes which separate fetal from maternal circulations or an increase in placental blood flow. The most important factor controlling the transfer of T_4 across placentae seems to be the difference in composition, affinity and binding capacity of the serum T_4-binding proteins between mother and fetus. The higher the thyroid hormone gradient, the greater the transfer across the placenta within the limits of the placental permeability. From reports by Myant (1964) and Robin et al. (1969), the slightly higher concentration of free thyroxine in the fetal blood results in a positive net diffusion from fetus to mother, at least in man.

Higher levels of free thyroxine in human fetal serum reflect the lower binding capacity by the thyroxine-binding proteins that has been reported by Russel et al. (1964). So the same kinds of binding protein-thyroxine interrelationships which have been observed in pregnancy and during lactation may also obtain in fetal, neonatal and postnatal animals. For instance, there have been several reports of very marked increases in the serum thyroxine levels of human babies within the first 30 minutes after birth (Fisher et al., 1964a; Fisher and Odell, 1969; Robin et al., 1969). Fisher and his co-workers suggest that cold exposure of the newborn is responsible for the initial thyroid hyperactivity. It would be of interest to find out what changes, if any, occur in the thyroxine-binding proteins during gestation and soon after birth.

The view of a close relationship between the serum thyroxine-binding proteins and the circulating level of thyroxine brings into focus an important question. Is there really an absolute increase in the thyroxine-binding globulin? Or is there rather an increase in those molecular or chemical characteristics of the proteins which determine their affinity for thyroxine? To date, there is no conclusive answer, but some light has been shed on this question by studies of factors which influence the affinity of thyroxine-binding proteins for thyroxine.

The following factors have been known to influence this affinity:

pH and buffers

Temperature

Dilution

Sexual maturity

Estrogen

Effects of pH and Buffer on T_4-Binding Capacities of TBG

The effect of pH on the capacity of serum proteins to bind thyroxine has been studied by Robbins and Rall (1960), Keane et al. (1969), and Lutz and Gregerman (1969). At pH 8.6, using barbital buffer, the binding of thyroxine to TBG is unaffected, but binding to prealbumin is markedly reduced (Keane et al., 1969; Robbins and Rall, 1960). Lutz and Gregerman (1969) used sodium phosphate buffer solution prepared by mixing ratios of mono- and

disodium salts. They found that the binding of thyroxine to albumin was maximal at pH 8.6 using sodium phosphate buffers. At pH values below and above 8.6, the binding to albumin was markedly reduced (Antoniades, 1960). Robbins and Rall (1960) in a very extensive review suggested that barbital buffer competed with thyroxine for the binding sites on the prealbumin. On the basis of electrophoretic studies using agar gels of physiologic pH (7.4), Hollander and co-workers (1962), estimated that the normal distribution of hormone between the three carriers is approximately 60 per cent with TBG, 30 per cent with TBPA and 10 per cent with albumin. Barbital buffer seems to be unique in competitively preventing the binding of thyroxine to prealbumins.

Effect of Temperature

Temperature importantly affects the binding of thyroxine to its carrier proteins. Perhaps the strongest evidence of this effect was provided by the work of Murphy and Pattee (1964). Standard curves for the binding of thyroxine by its carrier proteins were determined after equilibrating serum samples at various temperatures. Results indicated that binding was increased at lower temperatures. Thyroxine-binding capacity at 4°C is higher than at 23°C. Binding is lower at 30°C and even less at 40°C. For binding capacities to be comparable, therefore, they must be determined at similar temperatures.

Effect of Dilution

Besides temperature effects, Murphy and Pattee (1964), as well as Keane et al. (1969) have shown that dilution of either plasma or serum decreases the normally feeble binding of thyroxine to albumin. The higher the dilution, the less thyroxine is bound.

A decrease of the weak binding to albumin meant that almost all binding could be attributed to TBG when using barbital buffer at pH 8.6. This finding offered a subtle method for determining the thyroxine-binding capacity of TBG. Murphy and Pattee (1964) found that the binding capacity at 1:12th dilution was much more than double that at 1:32nd dilution. If determinations of binding capacity were made at pH 8.6 using barbital buffer and at about a 1:32 dilution, this would be a measure of the binding capacity of almost entirely TBG. The limiting factor here has to be the lower limit of serum dilution at which the thyroxine-binding globulin capacity can still be measured.

Sexual Maturity and Estrogen Secretion

Another factor which determines the binding capacity of thyroxine carrier proteins is the maturity of the animal. In a study of thyroxine-binding to the serum protein of adolescents and children, Riecansky (1967) demonstrated the importance of puberty. Thyroxine-binding to TBG in prepuberal children was shown to be unaffected

by either sex or age. Binding to prealbumin was not different in boys or girls at the age of ten. In 15-year-old subjects, however, the girls showed a higher binding capacity in their serum TBPA than the boys. Riecansky suggested that these differences were probably due to a higher level of sex hormone secretions in girls of this age. The greater ability of TBPA to bind thyroxine in these circumstances has also been shown by Ingbar (1963).

Other workers have demonstrated more specifically, the link between sex hormones and thyroxine-binding proteins. Alterations in TBG capacity for thyroxine have been repeatedly demonstrated for serum of gravid women. In an attempt to explain the origin of such alterations, several researchers suggested that the profound changes in the metabolism of estrogen during pregnancy might at least be contributory. During both the pre-partum and post-partum periods, changes in the level of serum PBI and thyroxine-binding are qualitatively similar and temporarily coincident (Dowling et al., 1965b). It has also been demonstrated that large doses of estrogen when administered to males or to nonpregnant females induced increases in the serum PBI level comparable to those which occur during normal pregnancy (Engstrom et al., 1952). Finally, a profound and progressive increase in the elaboration of estrogen is a concommitant of normal pregnancy (Sunderman and Boerner, 1949). It therefore seemed logical to study the effects of estrogen on the

thyroxine-binding capacity of TBP in various subjects. Such a study was undertaken by Dowling and his co-workers (1956a). All the human subjects received diethylstilbesterol in daily oral doses of 30 mg for 5 weeks and 60 mg after that. One patient with treated myxedema received 30 mg daily for four weeks and subsequently, 60 mg daily. Also included in this study was a patient with panhypopituitarism. Measurement of thyroidal accumulation of ^{131}I, BMR, serum PBI level and thyroxine-binding capacity of TBP were made at intervals prior to, during and following the administration of estrogen.

In all eumetabolic patients, the administration of diethylstilbesterol was associated with an increase in the percentage of added thyroxine bound to TBG. This increase was accompanied in all such patients with the previously observed increase in PBI levels and these alterations were comparable to those occurring during normal pregnancy. These effects of diethylstilbesterol were not dependent on the normal functioning of the thyropituitary axis, since they were noted in patients with hypopituitarism and with treated primary myxedema. The parallel between pregnancy and the administration of synthetic estrogens is thus very close. This parallel has also been borne out by the work of Musa et al. (1969). Dowling and his group also observed an increase in the capacity of TBG for thyroxine during estrogen administration. These findings suggest that the marked augmentation of thyroxine-binding by TBG which

occurs during human pregnancy may, at least in part, result from the influence of endogenous estrogen.

Techniques for Measuring Thyroxine-Binding Capacities

From the foregoing, it is apparent that a measure of TBG capacity is an important tool for evaluating not only thyroid economy but also sexual maturity and thyroidal changes during pregnancy.

Almost as soon as Gordon et al. (1952) characterized the three thyroxine-binding proteins, modifications of existing electrophoretic methods were employed to determine the capacity of each of the binding proteins for thyroxine. One of the first findings of such early studies is the dependence of the results on the method of determination. Methods range from dialysis to electrophoresis and, lately, immunoadsorption. Perhaps the most widely used and most variously modified is electrophoresis. An outline of this technique is presented here as the basis of a review of other techniques.

As employed by Robbins (1956), electrophoresis involved passing a constant electric potential of 100 volts for 24 hours through strips of Whatman number 3MM filter paper. Barbital buffer, pH 8.6 and ionic strength 0.1 was used. Each strip of paper was 3.75 cm wide and was suspended horizontally in a closed system between glass plates by means of taut silk threads along each edge. The ends of the paper strips dipped into two vessels, each

containing 1325 ml of buffer. The strips were moistened with buffer and placed in the chamber approximately 30 minutes before the serum was added. Glass plates, which were 3.4 cm apart, were lined with moistened filter paper. In a conventional run, Robbins (1956) applied 30 µl of a serum ^{131}I-L-T_4 mixture in a band near the cathodal end of the strip, 9 cm from the fluid level. The equalizing tube between buffer vessels was then closed and a constant electric potential applied for 24 hours. After the strips were dried, radioactivity was measured with a Geiger Muller counter. The proteins in the strips were then stained with bromphenol blue and quantified.

Tiselius and Flodin (1953) reported that in conventional electrophoresis, adsorption of migrating substances on the supporting medium frequently occurred. This adsorption was probably why Robbins (1956) could not get a saturation point for TBG--when he flooded--human serum with exogenous thyroxine. Robbins then introduced reverse electrophoresis as one method of obviating the difficulty of adsorption on the supporting medium. Reverse electrophoresis could presumably eliminate artifacts due to the adsorption of albumin-bound thyroxine on the filter paper medium since the serum globulin would no more migrate in the path of albumin. The movement of albumin toward the anode was just balanced by a flow of buffer in the opposite direction. This resulted in a displacement of globulins toward the cathode. But reverse-flow

electrophoresis itself produced more diffusion and some distortion of the protein bands.

All electrophoretic media currently used for the study of serum thyroxine-binding proteins present inherent disadvantages (Launay, 1966). Gordon and his co-workers as well as the other investigators who first discovered the existence of a specific thyroxine-binding protein in human serum used paper, the adsorptive properties of which produced heavy trailing and poor resolution of the protein bands. These bands were very clearly separated in starch-gel (Rich and Bearn, 1958), and starch block (Larson et al., 1952). The preparation of these media was, however, delicate and time consuming. Localization and quantification of radioactive fractions were also difficult and imprecise. Electrophoresis on slides coated with agar gel (Digiulio et al., 1964) presents the same drawbacks. The great variation in the results of electrophoretic methods was perhaps best illustrated in a review of these methods by Woeber and Ingbar (1968). They pointed out that of an endogenous concentration of T_4 about 30 per cent migrated with TBPA in agar gel at pH 7.4 (Hollander et al., 1962), 30-45 per cent migrated with TBPA in filter paper at pH 8.6 (Ingbar and Freinkel, 1960), while in starch gel values which vary from 10-60 per cent have been reported (Blumberg and Robbins, 1960).

Electrophoretic methods which have commonly been employed to assess the apportionment of endogenous thyroxine

among the binding proteins inevitably raised the possibility that artifacts were produced by the pH, the supporting media or buffers used. Artifacts could also be produced during the separation of proteins from one another or by the electrical field itself. It is not surprising therefore that the properties of endogenous T_4 associated with TBPA as judged from electrophoretic analyses have varied greatly.

Disc electrophoresis has proved unsuitable for the study of binding proteins because a significant proportion of the radioactivity of ^{131}I-labeled thyroxine was lost in the sample gel. Besides, most of the radiothyroxine migrated far ahead of any protein fraction (Launay, 1966). Cellulose acetate membranes seemed more promising. Since they were introduced by Kohn (1957) their use for the study of thyroxine-carrying proteins has been suggested by Tata et al. (1961) and by many others. Cellulose acetate appears to be a medium ideally suited for this purpose, since it is ready to use, can be handled and seems like paper. It gives fast separation and high resolution like gel media and is reported as not adsorbing protein, thereby eliminating trailing. Initial experiments using cellulose acetate, however, revealed the presence of a thyroxine-binding component cathodal to all three thyroxine-binding proteins so far recognized. Further investigation showed this component to be due to protein

trailing. This would substantially affect the determination of the capacities of the thyroxine-binding proteins.

Recently Woeber and Ingbar (1968) presented an immunoadsorption technique. This employed a rabbit antiserum specific for human serum and designed to remove thyroxine-binding prealbumin from serum completely without affecting the T_4-binding activity of the TBG. Only about 15 per cent of the endogenous thyroxine was bound to TBPA judged from results by this method. But this method, like the electrophoretic methods, is unsuitable for routine work. Some method that is reliable and obviates the drawbacks of electrophoresis while being suitable for routine work would be an invaluable tool for research in this area. Such a method was suggested by the work of Murphy et al. (1963) on corticosteroid-binding globulin (CBG).

The relationship between thyroxine and thyroxine-binding globulin is in many respects similar to that between cortisol and corticosteroid-binding globulin. When methods based on the principle of protein-bound isotopic competition were developed by Murphy and her group for cortisol and other steroids in plasma, this similarity prompted the investigation of the application of the same principle for the determination of plasma thyroxine. In 1969, Keane et al. modified Murphy and Pattee's technique to allow the study of plasma protein-thyroxine interactions.

Very few comparative studies on T_4-binding proteins in diverse species have been made. An investigation by Tanabe et al. (1969) was perhaps the most extensive of these. They employed radioautography after cellulose acetate electrophoresis. This technique may have a few of the drawbacks of electrophoresis and may not be suitable for routine work but seems to be quite reliable. The thyroxine-binding capacity of α-globulin was found, by this technique, to be high in ungulates as compared with other mammalian orders and the lower vertebrates. In horses and dogs most radioactivity was found in albumin and α-globulin. No apparent thyroxine-binding α-globulin was detectable in Rodentia. Bound radioactivity was found only in plasma albumin in guinea pigs. In rats most radioactivity was bound to albumin and very little to post-albumin. In lower vertebrates such as Aves, Reptilia, Amphibia and Pisces, no thyroxine-binding α-globulin was found. Radio-thyroxine concentrated in plasma albumin in some species (chicken, duck, fish) but in other species (pigeon, snakes, lizards, frogs) the radio-thyroxine concentrated both in albumin and prealbumin. The study cited above showed that specific thyroxine-binding α-globulin occurred only in mammals. All other vertebrate classes including birds had proteins that were capable of binding thyroxine less tenaciously (Farer et al., 1962b; Tanabe et all, 1969).

STATEMENT OF PROBLEM

In the research to be reported endogenous T_4 in each serum sample is first determined by the Tetrasorb-125 method.* Then a measurement of the total T_4-binding capacity of TBG is made by the new method devised in our laboratory. In this method cold T_4 and ^{131}I-labeled T_4 are added in excess to completely saturate the T_4-binding sites on TBG. The excess or unbound T_4 is absorbed by a resin-impregnated sponge. From the difference in radioactivity counts before and after this separation and the specific activity of the total T_4 in the liquid mixture the binding capacity can be expressed in terms of T_4 bound per 100 ml of serum. Barbital buffer at pH 8.6 is employed to competitively inhibit binding of T_4 on prealbumin (Robbins and Rall, 1960), and high dilution of the serum is used to minimize binding to albumin (Tata and Shellabarger, 1959).

Comparative data on serum T_4 and T_4-binding capacity of several species of mammals and birds are presented together with data on physiological variations observed in serum of women, cattle, sheep, rats and guinea pigs.

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*Abbott Radiopharmaceuticals, North Chicago, Illinois.

MATERIALS AND METHODS

Chemicals

Resin-impregnated polyurethane sponges were donated by Abbott Radio-Pharmaceuticals, North Chicago, Illinois. When stored at 4°C these sponges remained reliable media for separating free thyroxine from protein-bound thyroxine for several months. A primary standard, crystalline, free thyroxine was purified by Professor E. P. Reineke from monosodium thyroxine pentahydrate. The free thyroxine showed only a single component when checked by thin-layer chromatography. A standard aqueous stock solution was prepared using this purified thyroxine to give a concentration of 5 μg T_5 per ml. ^{131}I-L-T_4 was purchased from Abbott Radio-Pharmaceuticals. Working solutions of various concentrations were prepared from both the stock T_4 and the labeled T_4 solutions as described in detail in Appendix I--4 and 5. All solutions were stored at 4°C. At this temperature the solutions remained stable and usable for upwards of 2-3 months. All containers used for storing stock and working standards were siliconized. This greatly reduces the adsorption of thyroxine to glass.

Serum Samples

Most of the samples used in this study were obtained from the Michigan State University Farms and Small Animals Laboratory. Blood samples were generally allowed to stand for 4-6 hours at room temperature before being centrifuged for at least 20 minutes at 1000g. Avian blood samples which yielded relatively less serum than others were spun for as long as 60 minutes. Serum obtained from the blood samples was frozen and stored for periods of several months. Before use, the serum samples were thawed and, along with thyroxine solutions, were brought to room temperature.

Blood samples of female University students and workers, taking oral contraceptive pills and those not taking them, were obtained from Olin Medical Center, Michigan State University, East Lansing.

In the study of the effects of thyroprotein treatment on thyroid parameters of cows, blood samples were taken from pregnant cows on the day before the initiation of thyroprotein treatment. Ten grams/day of thyroprotein were then orally administered to each cow for 6 days. Blood samples were again obtained from the cows the day after the last daily dosage of thyroprotein. Cows #840 and 841 were 3 to 4 years old and the rest were first-bred heifers.

Deer blood samples were obtained from white-tailed deer (Odocoileus virginianus) in captivity. These were

being fed a well balanced diet but otherwise retained their wild instincts.

All cows used were Holsteins and the sheep were Suffolks.

Apparatus

Gamma radiation of the ^{131}I-T_4 was counted in a scintillation counter (Nuclear Measurements Corporation). Thorough mixing was achieved in about 30 seconds using a Vortex Deluxe Mixer (Scientific Products Division of the American Hospital Supply Corporation, Evanston, Illinois). Since the reliability of the results of this technique depends very much on the precision of the small volumes measured, a Syringe Microburet (Micro-Metric Instrument Company, Cleveland, Ohio) was used for all volume measurements except the barbital buffer. Each syringe used in these measurements was calibrated to determine the equivalence of one division of the micrometer by weighing the water delivered using that syringe. In this way, all volumes were measured to the nearest 0.5 µl.

Serum thyroxine levels were determined by the Tetrasorb-125 resin-sponge technique as modified by Hernandez (1971).

A curve for the binding capacity of TBG at varying thyroxine concentrations was documented for each animal species. The concentration of thyroxine required to saturate the thyroxine-binding sites of TBG at 37°C was selected from the plateau of the binding curve and this

concentration was used to determine the binding capacities of the TBG of several individuals of the particular species. Determinations of T_4-binding capacities were, in each case, run in duplicate according to the sequence detailed in Appendix I-7.

RESULTS

Repeatability

In the initial experiments, a binding curve was established for a pool of steer serum. Twenty replications of the TBG T_4-binding capacity of this serum yielded a mean value of 12.02 ± 0.13* µg T_4/100 ml serum (Appendix F).

Thyroxine-Binding Globulin
T_4-Binding Curves

Thyroxine-binding curves for four species are shown in Figure 1. Binding curves were established in the same manner for all of the species studied. All curves except those for the birds and guinea pigs, show gradually rising levels of protein-bound thyroxine with increasing concentrations of total thyroxine used until a plateau is reached. The plateau indicates saturation of the capacity of TBG for T_4.

Sodium barbital buffer (pH 8.6) inhibits thyroxine binding to prealbumins. The very high dilutions of 30-35 employed in these experiments reduce even further the usually feeble binding of thyroxine to most mammalian

*Standard Error of the mean.

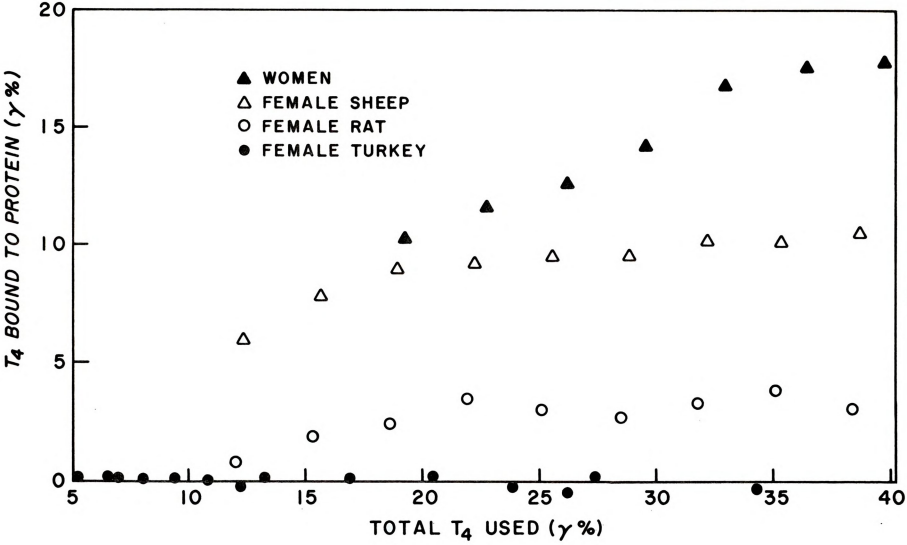

Figure 1.--T_4-binding curves of TBG in women and three species of animals.

albumins. The saturation capacities represented by the plateaus are, therefore, almost entirely those of the thyroxine-binding α-globulins since these are the only other thyroxine-binding proteins of consequence. The binding curves in all species studied plateaued at total thyroxine contents ranging from 5-35 μg/100 ml serum. So, for these species, total thyroxine concentrations of 35-42 μg/100 ml serum were chosen for use in the determination of the TBG T_4-binding capacities of the sera of individual animals.

Thyroid Parameters of Man and Other Species

The mean capacities of TBG for T_4 with the corresponding mean serum thyroxine levels for women, cattle, sheep, horse, deer, rats, guinea pigs, turkeys and chickens are presented in Table 1. The mean ratios of serum T_4: binding capacity or mean Saturation Index (SI) of women and various species are also given. The capacities of TBG for T_4 are generally higher than the serum thyroxine levels in man and all ungulates except the deer. The rodents and the birds, however, have higher serum thyroxine levels than the capacities of their TBG (if any is present) to bind thyroxine. The difference is succinctly dramatized by the much higher SI in rats (4.06 ± 0.41) compared to those of the ungulates and man ($p < 0.01$). It is not possible to calculate meaningful Saturation Indexes for guinea pigs and birds studied because these

TABLE 1.--Thyroid Parameters of Women and Various Species of Animals.

Species (Nonpregnant)	No. of Samples	Mean T_4-Binding TBG Capacity (μg T_4/100 ml Serum)	Mean Serum T_4 ± Std. Error (μg T_4/100 ml Serum)	Mean Saturation Index ± Std. Error
Women	11	17.45 ± 1.20*	12.29 ± 0.52	0.82 ± 0.52
Heifers	10	9.83 ± 0.42	6.79 ± 0.46	0.70 ± 0.04
Sheep	6	15.65 ± 0.65	12.61 ± 1.14	0.81 ± 0.07
Pigmy Goats	9	15.92 ± 0.77	12.08 ± 0.59	0.76 ± 0.02
Horses	5	3.60 ± 0.36	2.22 ± 0.30	0.66 ± 0.18
Deer	19	8.29 ± 0.46	16.17 ± 0.45	2.04 ± 0.11
Rats	10	1.73 ± 0.23	6.20 ± 0.17	4.06 ± 0.41
Guinea Pigs	10	Neg.**	2.37 ± 0.12	-----
Chickens (nonlayers)	10	Neg.	1.75 0.14	-----
Turkey (nonlayers)	8	Neg.	1.18 0.25	-----

SI of all ungulates except deer: 0.73 ± 0.03

Ungulate SI vs. deer SI ($p < 0.01$)

*Standard error of the mean.

**Value not significantly different from zero.

showed negligibly low levels of thyroxine-binding capacity.

Mean capacity of TBG for T_4 in horses is 3.60 ± 0.36 μg T_4/100 ml serum. TBG capacity for the other ungulates averaged 12.42 ± 1.97 μg T_4/100 ml serum. Horse TBG thus has a relatively lower thyroxine-binding capacity than that of the other ungulates ($p < 0.05$). Serum thyroxine in the horse has been previously reported to be primarily bound by albumins. However, from the present data, the TBG Saturation Index is 0.66 (Table 1) indicating that there is more than enough TBG present to serve as carrier for the existing thyroxine. In this respect the horse does not differ from the other ungulates.

The white-tailed deer (Odocoileus virginianus) is peculiar among ungulates studied in having a much higher mean Saturation Index of 2.02 ± 0.11 compared to the mean for other higher animals of 0.76 ± 0.03. The capacity of deer TBG to bind T_4 is generally comparable to that of other ungulates. But serum thyroxine levels are generally higher than those of other ungulates. This leaves a substantial level of T_4 in excess of the TBG T_4-binding capacity. The excess may be bound by one or more other T_4 carrier proteins. It has been reported, as cited earlier, that 48 per cent of an administered dose of ^{131}I-T_4 is bound to albumin and 36 per cent to α-TBG in the Muntjak deer. Our results thus reinforce this earlier finding.

Effect of Pregnancy on Serum T_4 and TBG Capacities

Serum thyroxine levels of pregnant sheep, cows, rats, and guinea pigs were slightly higher than in nonpregnant animals (Tables 2 and 3). The capacities of TBG for T_4 were also slightly higher during pregnancy than in nonpregnant animals. But, unlike previously cited reports, in man, pregnancy in these animals did not cause any significant elevation in either the serum thyroxine levels or the capacity of TBG to bind T_4. Of perhaps greater significance is the fact that the mean TBG Saturation Indexes (where these could be calculated) of the nonpregnant animals were no different from those during pregnancy (Table 2). The mean TBG Saturation Index of 0.81 ± 0.07 in nonpregnant sheep is statistically no different from the SI of 0.70 ± 0.04 in pregnant sheep. This ratio is also essentially unchanged during pregnancy in cows when compared to the ratio in nonpregnant cows. The rats, which have the very high SI of 4.06 ± 0.41 show only a slight alteration to 2.58 ± 0.36 during pregnancy, no significant change even here.

Effect of Oral Contraceptives on Serum T_4 and TBG Capacities

Serum thyroxine in eight women taking oral contraceptive pills averaged 14.31 ± 0.55 μg T_4/100 ml serum (Table 4). The mean serum thyroxine for eleven control women was 12.29 ± 0.52 μg T_4/100 ml serum. Serum T_4 levels

TABLE 2.--Means ± Std. Error of Thyroidal Parameters in Open, Lactating and Pregnant Sheep and Cows.

Physiological State of Animal	No. of Samples	Serum T_4 (µg/100 ml Serum)	T_4-Binding TBG Capacity (µg T_4/100 ml Serum)	Saturation Index
Sheep:				
Open (control)	6	12.61 ± 1.14	15.65 ± 0.65	0.81 ± 0.07
Pregnant sheep	6	11.47 ± 0.83	16.26 ± 0.64	0.70 ± 0.04
Lactating sheep	6	9.32 ± 0.66	13.53 ± 0.43	0.68 ± 0.04
Cows:				
Open (control) heifers	10	6.79 ± 0.46	9.83 ± 0.42	0.70 ± 0.04
180 days pregnant heifers	10	5.91 ± 0.31	9.03 ± 0.39	0.68 ± 0.60
264 days pregnant heifers	6	6.67 ± 0.59	9.06 ± 0.49	0.84 ± 0.10
All heifers	26	6.42 ± 0.25	9.35 ± 0.25	0.70 ± 0.03
Lactating open*	5	3.75 ± 0.39	8.51 ± 0.66	0.44 ± 0.03
Lactating pregnant**	6	5.65 ± 0.29	9.20 ± 0.31	0.61 ± 0.02

*Producing an average of 30 kg milk/day.

**Producing an average of 13 kg milk/day.

TABLE 3.--Mean (± Std. Errors) Thyroidal Parameters of Open and Pregnant Rats and Guinea Pigs.

Animal and State	No. of Samples	Serum T_4 (µg T_4/ 100 ml Serum)	TBG T_4-Binding Capacity (µg T_4/100 ml Serum)	Saturation Index
Rat:				
Open (control)	10	6.20 ± 0.17	1.73 ± 0.23	4.06 ± 0.41
Pregnant	7	7.00 ± 0.27	2.07 ± 0.2	3.58 ± 0.36
Guinea Pig:				
Open (control)	10	2.37 ± 0.12	Neg.*	-----
Pregnant	10	2.44 ± 0.44	Neg.	-----

*Value not significantly different from zero.

TABLE 4.--Thyroxine-Binding Capacities of TBG and Serum Thyroxine Levels (μg T_4/100 ml Serum) in Control Women and Those Taking Oral Contraceptives.

	Control			On Contraceptive Pills		
	TBG T_4-Binding Capacity	Serum T_4	SI	TBG T_4-Binding Capacity	Serum T_4	SI
	23.88	14.31	0.60	28.23	14.83	0.53
	14.17	11.64	0.82	26.50	11.48	0.43
	12.81	13.50	1.05	26.57	15.76	0.59
	14.63	10.53	0.72	21.85	21.08	0.96
	19.25	10.74	0.56	21.55	17.82	0.83
	13.32	9.43	0.71	24.17	19.58	0.81
	18.39	18.02	0.98	22.86	16.88	0.74
	26.29	17.80	0.68	21.79	14.11	0.65
	17.48	17.35	0.99			
	14.61	13.77	0.94			
	17.70	17.14	0.97			
No. of Samples	11	11	11	8	8	8
Mean	17.45	12.29	0.82	24.19	14.31	0.69
± Std. Error	± 1.20	± 0.52	±0.52	±0.92	±0.55	±0.06

SI of control vs. women on pill ($p > 0.10$).

were therefore significantly higher ($p < 0.02$) in women taking contraceptive pills compared to T_4 levels in controls. Thyroxine-binding capacities of TBG in women taking the pill averaged 24.19 ± 0.92 µg T_4/100 ml serum. Binding capacities in control women averaged 17.45 ± 1.20 µg T_4/100 ml serum. The pill seems to be associated with an even greater increase in the capacity of the TBG for thyroxine ($p < 0.01$). In spite of these observed changes the relationship between serum thyroxine levels and the T_4-binding capacity of TBG did not change significantly. The mean Saturation Index of 0.82 ± 0.05 in women not taking the pill is statistically no different from that of women on the pill ($p > 0.10$), as shown in Table 4.

Saturation Index in Thyroprotein-Treated Cows

Thyroprotein-treated cows had a mean T_4-binding TBG capacity of 10.03 ± 0.65 µg T_4/100 ml serum (Table 5). This was statistically no different from the mean of 9.39 ± 0.41 µg T_4/100 ml serum of cows before thyroprotein treatment. Mean serum thyroxine levels were, however, significantly raised ($p < 0.01$) by thyroprotein treatment over pretreatment levels (Table 5). Saturation Indexes significantly higher than those in non-treated cows ($p < 0.01$) were therefore obtained. Since serum thyroxine levels were greatly increased with only slight corresponding increases in the capacity of T_4-binding TBG, the values of serum T_4 and binding capacity became almost identical. In

TABLE 5.--TBG T_4-Binding Capacities, Serum T_4 (μg T_4/100 ml Serum) of Pregnant Thyroprotein-Treated and Untreated Cows. II Saturation Indexes of Both Groups.

	Control (Untreated Cows)			Thyroprotein-treated		
	T_4-Binding TBG Capacity	Serum T_4	SI	T_4-Binding TBG Capacity	Serum T_4	SI
	9.10	5.81	0.64	8.27	8.62	1.04
	12.72	9.59	0.75	9.11	8.89	0.98
	9.94	7.96	0.80	12.64	10.35	0.82
	8.73	5.78	0.66	11.13	10.15	0.91
	9.65	4.94	0.51	9.31	9.05	0.97
	11.04	4.92	0.45	9.70	10.89	1.12
	9.18	6.07	0.66			
	8.63	6.58	0.76			
	7.79	6.40	0.82			
	8.46	7.17	0.85			
	9.99	6.22	0.62			
	10.11	4.14	0.41			
Mean ± Std. Error	9.39 ± 0.41	6.34 ± 0.39	0.66 ± 0.03	10.03 ± 0.65	9.66 ± 0.38	0.97 ± 0.04

Control vs. thyroprotein-treated binding capacities: ($p > 0.10$).

Control vs. thyroprotein-treated serum T_4: ($p < 0.01$).

Control vs. thyroprotein-treated SI: ($p < 0.01$).

other studies currently in progress in our laboratory serum thyroxine levels were determined daily starting from the day after termination of thyroprotein treatment. A very fast rate of thyroxine degradation was recorded until much of the high levels of thyroxine had been metabolized and cleared. This would re-establish the species--specific thyroxine-TBG interrelationship with a Saturation Index more nearly like that of untreated cows.

Thyroxine-Binding Capacities of TBG During Lactation

The mean thyroxine-binding capacity of TBG in control nonpregnant sheep was found to be 15.65 ± 0.65 μg T_4/100 ml serum (Figure 2). Thyroxine-binding capacity in lactating sheep averaged 13.53 ± 0.43 μg T_4/100 ml serum. A significant decrease in the capacity of TBG for T_4 is thus observed in the lactating sheep compared to normal nonpregnant controls ($p < 0.02$). Control nonpregnant cattle showed a mean thyroxine-binding capacity of 9.83 ± 0.42 μg T_4/100 ml serum. When compared to the mean binding capacity of 8.01 ± 0.88 μg T_4/100 ml serum in nonpregnant lactating cows a non-significant decrease in binding capacity is observed. Serum thyroxine levels in lactating sheep and cows are both significantly less than control values ($p < 0.01$) as shown in Table 2.

In cows, there is a significant decline in the mean Saturation Index from 0.70 ± 0.04 in nonpregnant controls to 0.47 ± 0.03 in lactating open cows. In

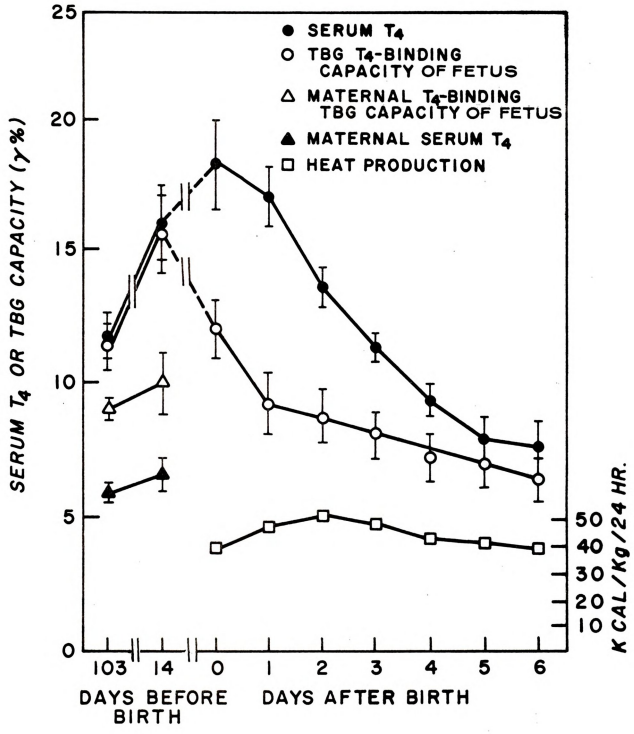

Figure 2.--Thyroidal Parameters of Bovine Fetus, Neonate and Mother. (Serum T_4 Values by Hernandez, 1971; Heat Production Data by Roy et al., 1957.)

pregnant cows during the period of declining lactation, SI returns to the open nonlactating level. In order to explore the meaning of these observed alterations it is necessary to look at other thyroid parameters.

Thyroxine-Binding Capacities of TBG in Bovine Fetus and Newborn Calves

Thyroxine-binding capacities of TBG in second and third trimester bovine fetuses averaged 11.39 ± 0.85 and 15.68 ± 1.40 μg T_4/100 ml serum, respectively. The corresponding fetal serum thyroxine levels were 11.82 ± 0.85 and 16.08 ± 1.38 μg T_4/100 ml serum (Figure 2). Both the serum thyroxine and the thyroxine-binding capacity of the TBG increase from the second to the third trimester in the fetuses ($p < 0.02$). The mean Saturation Index of fetal TBG during the third trimester (1.13 ± 0.21) is, however, not substantially changed from the level during the second trimester of 1.05 ± 0.05 (Table 6).

If the serum T_4 and T_4-binding capacities of the fetus are compared to those of the mother, interesting differences become apparent. Fetal serum thyroxine levels of 11.82 ± 0.85 μg T_4/100 ml serum during the second trimester and 16.08 ± 1.37 μg T_4/100 ml serum in the third trimester are significantly higher than the corresponding maternal values of 5.91 ± 0.31 and 6.67 ± 0.59 μg T_4/100 ml serum. Thyroxine-binding TBG capacities of the fetus

TABLE 6.--Mean TBG Saturation Indexes (± Std. Errors) of Fetus, Mother and Neonatal Calves.

A. Fetal and Maternal SI

	Fetus	Mother
180 days gestation (2nd Trimester)	1.05 ± 0.04 (10)*	0.68 ± 0.06 (10)
264 days gestation (3rd Trimester)	1.13 ± 0.21 (7)	0.84 ± 0.10 (7)

B. Neonatal SI

Days After Birth	0	1	2	3	4	5	6
Mean TBG Saturation	1.50	1.98	1.65	1.49	1.41	1.20	1.24
Index ± Std. Error	±0.20	±0.27	±0.18	±0.13	±0.17	±0.13	±0.12
	(10)	(10)	(9)	(10)	(10)	(10)	(10)

*No. of samples.

during the second and third trimesters are similarly higher ($p < 0.05$) than the maternal levels during the same periods (Appendix B).

In contrast, several workers have reported that human fetuses show lower serum thyroxine and TBG T_4-binding capacities than their mothers. The differences in fetal and maternal thyroid parameters between the human and the bovine probably set the stage for the peculiar thyroidal activities that are observed in bovine neonates.

There is a rise in TBG mean Saturation Index of 1.50 ± 0.20 on the day the calf is born to 1.98 ± 0.27 on the day after birth. Whereas the serum T_4 declined from 18.28 ± 1.67 µg T_4/100 ml serum on the day of birth to 17.11 ± 1.13 one day after birth, the T_4-binding TBG capacity dropped rapidly from 12.42 ± 0.94 µg T_4/100 ml serum to 9.65 ± 0.99 µg T_4/100 ml serum during the same period. The net result is liberation of T_4 from TBG for metabolism during the first critical days of neonatal life.

Avian Thyroid Parameters

Thyroxine-binding capacities are negligibly low in male chickens as well as in laying and nonlaying chickens and turkeys (Table 1 and 3; Appendix H; Table 7). Male turkeys, however, have measurable though low T_4-binding capacities of 1.34 ± 0.24 µg T_4/100 ml serum and serum T_4 values of 1.51 ± 0.21 µg T_4/100 ml serum.

TABLE 7.--Mean (± Std. Error) Thyroidal Parameters of Chickens and Turkeys.

Species and State	No. of Samples	TBG T_4-Binding Capacity (μg T_4/100 ml Serum)	Serum T_5 (μg T_4/ 100 ml Serum)	Saturation Index
Chicken:				
Nonlayer	10	Neg.*	1.75 ± 0.14	-----
Layer	10	Neg.	1.00 ± 0.16	-----
Turkey:				
Nonlayer	10	Neg.	1.18 ± 0.25	-----
Layer	8	Neg.	1.39 ± 0.30	-----
Male Chickens	9	Neg.	2.10 ± 0.42	-----
Male Turkey	7	1.34 ± 0.24	1.51 ± 0.21	1.75 ± 0.46

*Value not significantly different from zero.

Saturation Indexes could thus be calculated only for male turkeys and these were 1.75 ± 0.46 μg T_4/100 ml serum.

Nonlaying turkeys have 1.18 ± 0.25 μg T_4/100 ml serum and this value is not significantly different from either the male level above or the laying T_4 level of 1.39 ± 0.30 μg T_4/100 ml serum.

Serum thyroxine levels are 2.10 ± 0.42 μg T_4/100 ml serum for male chickens and 1.75 ± 0.14 μg T_4/100 ml serum for nonlayers. There is no significant difference between these two. Layers have 1.00 ± 0.16 μg T_4/100 ml serum, a value which is significantly lower than that of either the nonlayers ($p < 0.05$) or the male chickens ($p < 0.01$).

GENERAL DISCUSSION

Differences in the thyroid parameters of various species may be related to the adaptations that occurred in each species as it adjusted to particular environments during its evolutionary development. More specifically the differences between the primate and ungulate groups on the one hand and the rodents and birds on the other are related to the binding protein which has the greatest capacity for thyroxine in each group.

In previously cited reports it was shown that α-globulins are the major thyroxine-binding proteins in primates and most ungulates. These findings are borne out by the fact that binding capacities of the TBG in all the higher mammals studied except the deer exeeed the serum thyroxine levels (Table 1). Mean SI values below 1.0 were therefore obtained. In rodents and birds, the meagre, if any, thyroxine-binding globulins that occur have correspondingly lower capacities to bind T_4 (Table 1: Appendix G and H) because thyroxine is almost entirely bound by the albumins.

The data clearly indicate that when the value of the Saturation Index is less than 1.0, α-globulins are

the major carriers of thyroxine as is the case in humans and most ungulates. If however, the SI is significantly greater than 1.0 as is the case in deer, rodents and birds, then other proteins, presumably the albumins and prealbumins become carriers for the excess of T_4. In this regard Saturation Indexes constitute quite sensitive indicators of the role played by TBG in binding the serum thyroxine of various animals.

During pregnancy, the thyroid parameters of sheep, cows and rodents are not substantially altered from those in open animals. This is in contrast to reports in humans that show significant increases in both serum thyroxine levels and T_4-binding capacities of TBG during pregnancy. The lack of any significant change in the TBG T_4-binding capacities of pregnant sheep is in accord with reports by Annison and Lewis (1958). The values reported by these authors range from 12-16 µg T_4/100 ml serum for the T_4-binding TBG capacities of nonpregnant sheep. Our experiments yielded a mean value of 15.65 ± 0.65 µg T_4/100 ml serum. These results suggest an important species difference between primates on one hand and lower mammals on the other. The mean Saturation Indexes of the pregnant animals are not significantly different from the indexes of nonpregnant ones. Even in rats, which have a much higher SI, this was only slightly altered during pregnancy. These findings emphasize the constancy

of the serum T_4: TBG relationship in different physiological conditions.

Data on the effects of contraceptive pills on thyroid parameters of women further bear out this constancy, despite individual variations in the items that determine Saturation Indexes (Table 4). The observed alterations confirm previously reported findings of the effects of contraceptive pills.

Standeven (1969) has reported a significant increase of the PBI values in women taking the pill for longer than 29 months. Mishell et al. (1969) report slight elevations of PBI values in women within the first week of therapy and a definite further progression upward after one month. Many of the PBI values of such women after one month of therapy were in the hyperthyroid range. Williams et al. (1966) reported significant changes in PBI as early as 14-20 days after the beginning of therapy. All these reports bear out the effect of contraceptive pills on thyroidal activity. What could be responsible for such observed alterations in thyroid indexes in women on contraceptive pills?

As has already been pointed out in the literature review, investigators have found significant elevations in the thyroxine-binding capacities of the TBG of humans given diethylstilbesterol that were comparable to those which occur during pregnancy. More recently, Gimlette and Piffanelli (1968) reported significant increases in total

serum thyroxine of both pregnant subjects and those taking oral contraceptive pills but no significant difference between the values of the two groups. Williams et al. (1966) suggested that all the changes occurring in women who took the pill reflected the effects of estrogen on the thyroid hormone binding capacity of serum proteins. By increasing the amount of bound thyroid hormones, estrogen would decrease the relative amount of free thyroid hormone and so cause raised PBI values. This would be the case if serum thyroxine levels remained unchanged. Our results, however, showed a substantial increase in serum thyroxine levels as a result of pill therapy to match the increase in T_4-binding TBG capacities. This truly fits the classical picture of thyroidal activities.

In the classical way of looking at thyroid hormone-binding protein relationships TBG capacities are raised during contraceptive pill therapy as they are during human pregnancy probably as a result of elevated levels of plasma estrogen occurring during these periods. The resulting increase in unsaturated TBG causes a lowering of free thyroxine (reflected as reduced T_3-I^{131} uptake in the Hamolsky* test). The thyroid-pituitary feedback mechanism then stimulates the production of pituitary thyrotropin (TSH) which causes increased activity of the thyroid gland and the release of more thyroxine into the

*Hamolsky and Freedberg, 1960.

bloodstream giving rise to increased serum thyroxine and PBI levels. Thus, the two factors tending to regulate free thyroxine are again brought into proper relationship, but at individually elevated levels. This classical scheme is fully borne out by the mean Saturation Indexes of the control and in women taking contraceptives. The control SI value of 0.82 ± 0.05 is statistically no different from the value of 0.69 ± 0.06 obtained in women taking the pill.

Most of the contraceptive pills currently in use like all those employed in the works cited above contain relatively small quantities of estrogen. One example, Ortho-Novum*, contains 1 mg of the progestational substance norethindrone (17-α-ethinyl-17-hydroxy-4 estren-3-one) together with 0.05 μg of the estrogenic compound, mestranol (ethinyl estradiol 3-methyl ether). Is it possible that such relatively low levels of estrogen would be responsible for the significant increases in serum T_4 and TBG capacities that have been observed in our laboratory and reported by several other workers?

Alexander and Marmorston (1961) have demonstrated that as little as 0.01 μg of ethinyl estradiol or mestranol will raise PBI values in post-menopausal women treated for one to eleven months. They report that such changes occasionally occurred as early as 7 days after therapy. If

*Ortho Pharmaceutical Corporation, Raritan, New Jersey.

there still were any doubts as to the role of estrogen in the observed changes, Hollander et al. (1963) demonstrated that medroxy progesterone acetate, a compound with 40-100 times the progestational activity of norethynodrel and without significant estrogenic activity had no effect upon PBI values in male subjects. Only in combination with some estrogen did medroxy progesterone acetate alter PBI values. It therefore becomes apparent that it is the estrogen component, ethinyl estradiol, used by Hollander and coworkers, by Standeven and involved in our experiments which are responsible for the alterations observed.

Thyroid parameters of cows treated with thyroprotein and during lactation constitute examples of conditions under which the T_4: TBG relationship is substantially altered.

As can be seen from Table 5, thyroprotein treatment substantially increases serum thyroxine levels with no corresponding elevation of T_4-binding TBG capacity. The TBG is therefore more saturated as evidenced by the significantly higher SI value in these than in untreated cows. The present data also indicate that most of the serum thyroxine in cows is bound by thyroxine-binding globulin for two reasons:

1. When the level of serum T_4 rises above the binding capacity of TBG, the excess is rapidly metabolized and cleared as previously

mentioned. In animals with other binding proteins, T_4 in excess of TBG binding capacity has been shown to be bound by albumins and prealbumins.

2. Even with elevated thyroxine levels during thyroprotein treatment, the Saturation Index is not significantly different from 1.0. In deer, rodents and birds where other proteins play a major role in binding serum thyroxine, the Saturation Index is substantially higher than 1.0 (Table 1; Appendix G and H).

The effects of thyroprotein treatment contrast very sharply with the effects of lactation in cows. During heavy lactation, there is a steep decline in serum thyroxine associated with a nonsignificant drop in the T_4-binding capacity of TBG. The mean SI is consequently lower here than in nonlactating open cows. A new factor may be responsible for this discrepancy.

A marked decrease in the uptake of radioiodine by thyroids of lactating rats has been reported by several workers. This decrease seems to be related to the successful competition of the mammary glands for iodine during lactation. Thyroid secretion rates have been shown to fall from 2.58 in control rats to 0.99 μg T_4/100 gm body weight/day in lactating rats (Lorscheider, 1970). Other reports have shown lower thyroid secretion rates in lactating compared to nonlactating goats. These reports

suggest that during lactation, at least in the rat and the sheep, thyroid glands are relatively poor competitors for iodine when pitted against the mammary glands. A low iodine uptake results in the low thyroid secretion rate and low serum thyroxine levels that have been observed in the reports cited above and in our data.

In cows, the intensity of competition for iodine between the mammary and thyroid glands is probably responsible for the steep decline in serum T_4. These cows were producing an average of 30 kg of milk/day at the time of sampling. At such a high intensity of lactation, the drop in serum T_4 was steep enough to significantly change the Saturation Index.

With lactating pregnant cows, when the cows were producing about 13 kg of milk/day, the competition for iodine drops to the point where serum T_4 levels were virtually back to nonlactating open levels. Since the TBG T_4-binding capacity was unchanged in the process SI values return to the level characteristic of open nonlactating cows. In the third trimester, when lactation stops, the competition is removed. This boosts serum T_4 levels even closer to those of open cows. Since the binding capacity of TBG remains virtually unaffected, the Saturation Index at this time is again within the range characteristic of open nonlactating controls.

The factor that makes all the difference during lactation appears to be the competition for iodine. Sheep

are not usually selected for their high milk production. It is possible, therefore, that the intensity of lactation of the sheep studied was lower than that of cows. This would explain the relatively smaller decline in the serum T_4 of lactating compared to the non-lactating sheep (Table 2). A corresponding decrease in the binding capacity of TBG leaves the mean Saturation Index of lactating sheep virtually the same as that of the nonlactating animals.

In iodine deficient rats, there is a compensatory shift to the secretion of a higher proportion of T_3 (Heninger and Albright, 1966). If this is also the case during the lactation-induced iodine deficiency in cows and sheep, our methods would not detect the T_3 since, as previously mentioned, the affinity of T_3 for carrier proteins is much lower than that of T_4.

Saturation Indexes are also useful in the interpretation of data on bovine fetuses, newborn calves and deer which are unique among ungulates. As already pointed out in the "Review of Literature," the functionality of bovine fetal thyroids starts as early as 60 days of age (gestation period: 284-286 days). Functionality seems to increase with the progress of gestation. This is reflected in the fact that both serum T_4 and the binding capacity of TBG in third trimester fetuses are elevated over second trimester levels (Figure 2).

The fetuses live in an optimal amniotic fluid environment. Their basic metabolic demands would, therefore, be minimal and so very little T_4 would be expended. This also helps in the observed gradual build up of fetal T_4 levels.

As earlier pointed out, the serum T_4 in cows appears to be almost entirely bound to TBG. If the elevated levels of T_4 were to exceed the binding capacity of the existing TBG, the excess would either be cleared or would shut off any further T_4 production by the thyroids. Two possible factors militate against this:

1. The physiological characteristics of the existing level of TBG may change in such a way as to allow the same quantity of TBG to bind more T_4. This possibility has been raised by many workers.
2. There may be some diffusion of small quantities of fetal T_4 in excess of the binding capacity of fetal TBG to the maternal circulation and a simultaneous diffusion of some TBG from the mother across the placental membranes to fetal blood. Fell, et al. (1968) have raised the possibility that some varieties of globulin could cross placental barriers.

By some such mechanism, the capacity of TBG for T_4 increases in almost perfect unison with the increasing

levels of fetal T_4 through the last two trimesters of pregnancy. This is well borne out by the fact that the fetal SI is 1.05 and 1.13 in the second and third trimesters, respectively. In this regard, the TBG may act as a storage vehicle for the attendant high levels of thyroxine during this period in preparation for life immediately after birth.

Thyroxine carrier proteins in the white-tailed deer may also have a storage role. Deer are peculiar among the ungulates studied in having twice as much serum thyroxine as there is the capacity of TBG to bind T_4. This is well reflected in the deer Saturation Index of 2.04 ± 0.11. The deer used in this study were fed a well-balanced diet and kept in captivity. They were not, however, domesticated and still retained their wild instincts. The blood samples for this study were obtained on December 1, 1970 just as the winter started in earnest. With the cold exposure that deer in the wild have to face, they probably need an extra thyroidal store of thyroxine that can be rapidly drawn upon in times of heavy demand. It is therefore postulated that the thyroxine in excess of the capacity of TBG for T_4 would be loosely bound to other carrier proteins, presumably albumins, and would be the first line of defense against the cold. Previous studies on the Muntjak deer have revealed that more T_4 is bound to albumin than to TBG. Should the albumin-bound thyroxine become depleted, there

probably would be a release of the T_4 bound to TBG as a second line of defense against cold exposure.

For bovine neonates, the period of heavy T_4 demand is just after birth. Neonatal thyroxine levels are at least double the maternal levels and exponentially decline during the first 6 days after birth. This contrasts with the reported increase in both TSH and thyroid secretion rate of human neonates. Bovine neonates are morphologically and even physiologically more developed than human neonates. In consequence, calves are probably better equipped to handle the stresses of extrauterine life than are neonates of many other species. How, specifically, does the neonatal calf withstand its new environment?

Perhaps the most revealing thyroid parameter in this respect is the Saturation Index both in utero and soon after birth. As indicated in Figure 2 and Table 6, the mean Saturation Index of 1.13 ± 0.21 of the third trimester fetus increases to 1.50 ± 0.20 on the day of birth and peaks at 1.98 ± 0.27 one day after birth. The SI value then exponentially falls to 1.24 ± 0.24 on the 6th day after birth. Apparently, there is a rapid metabolism of binding protein soon after birth. This accounts for the rise in SI on the day of birth compared to the level in the third trimester in utero. Metabolism of TBG sets free the T_4 that was bound. Even more TBG is metabolized one day after birth with the release of yet more T_4.

The bovine newborn, like that of other mammals, experiences cold exposure. Unlike any other mammals described to date, however, the bovine is able to draw from its extrathyroidal store of thyroxine to meet the demands of this critical period. The large quantities of T_4 in excess of the exponentially decreasing capacity of TBG are used for metabolism and thermogenesis soon after birth. In this way, the new born calf is able to withstand near-freezing temperatures.

The concept of an increase in heat production by the bovine neonate has been well documented by Roy et al. (1957). These workers showed a curvilinear increase in the heat production of newborn calves from the first through the 5th day, and then an exponential decline from the 5th through the 8th day after birth (Figure 2).

Bovine neonates effectively use their enhanced thermogenesis to raise and maintain their body temperature. So, like the deer in winter, neonatal calves withstand the extrauterine cold environment by drawing from their extrathyroidal store of T_4 as a quick and very early defense.

In birds, any changes in thyroidal parameters during laying would be expected to differ from those during pregnancy in mammals because of two possible reasons:

1. The hormonal and oviductal changes that accompany pregnancy are not exactly duplicated during laying.

2. In most mammals studied TBG is the major T_4 carrier protein. In birds, however, all previous reports and the present data for female birds indicate a negligible presence or total absence of TBG. Most of the thyroxine appears to be transported by albumin.

On this account, if the capacity of carrier proteins for T_4 were influenced by hormonal changes during laying, this would be more profoundly expressed in carrier proteins other than TBG. The finding in male turkeys is a bit startling. A pooled sample of serum from 7 male turkeys was therefore used to repeat the documentation of a TBG T_4-binding curve and this too yielded binding capacity values that were statistically higher than zero. To the knowledge of this writer, there has been no previous report on the binding capacities of TBG in male turkeys.

In the one instance of male turkeys where Saturation Indexes could be calculated, these averaged 1.75 ± 0.46 and again reflect the same picture as in rodents where albumins are considered the major carriers of T_4.

The serum thyroxine data of chickens point up the possible effects of estrogen that have been reported by Common et al. (1948). They report that estrogen tends to suppress thyroidal activity in chickens. From the data, in Table 7, male chickens with presumably minimal quantities of estrogen, show significantly higher serum

T_4 levels than either the nonlayers or layers. In the same way, nonlayers have higher serum T_4 levels than layers, in which estrogen levels are expected to be highest.

The generally low serum thyroxine levels in birds are apparently related to the absence or negligible presence of TBG. Albumins bind T_4 very loosely and readily release it for metabolism. These protein: T_4 relationships probably account for the fact that if T_4 is administered to chickens, only a transient effect on metabolic rate is observed (Mellen, 1958; Singh et al., 1968).

APPRAISAL OF NEW TBG-SATURATION TECHNIQUE

The underlying principle of the new method is that the sum total of endogenous T_4, exogeneously added cold T_4 and the ^{131}I-labeled T_4 completely saturate the binding sites on the TBG. Excess T_4 is bound to resin-impregnated sponges and binding capacities are expressed as µg T_4/100 ml serum bound to TBG. In most of the work previously reported, a dose of labeled thyroxine was added to serum, the proteins were separated by electrophoresis and the results were expressed as a percentage of the label that migrated with each of the thyroxine-binding proteins. So far as this writer is aware, none of the previous studies except that of Keane et al. (1969) have included the endogenous T_4 in their computations. In the other methods, the total thyroxine available for binding was not determined. The thyroxine-binding capacity of TBG could not, therefore, be calculated.

In the new technique, three conditions insure that the protein-bound thyroxine is associated almost exclusively with TBG:

1. At pH 8.6 using barbital buffer, thyroxine binding by prealbumins is competitively inhibited by the buffer.
2. At the high dilution factor of 30-35 the usually feeble thyroxine binding by albumin is reduced to a negligible minimum.
3. Equilibration of the reaction mixture at 37°C minimizes thyroxine-binding to all proteins other than TBG.

The above principle and conditions have been incorporated with a system of small volume measurements and equilibration which enhances the accuracy and reliability of the new technique. Evidence of this reliability is the results of 20 determination of the T_4-binding capacity of TBG in the serum samples of one animal. A mean value of 12.02 ± 0.13 µg T_4/100 ml serum was obtained. This indicates quite a high degree of repeatability.

Our method of expressing T_4-binding TBG capacity as "µg T_4/100 ml serum" has been used by only a few others. Besides, some of those who have expressed results in this way have usually measured PBI. One group of workers who measured the binding capacity of the TBG in nonpregnant sheep reported values of between 12-16 µg T_4/100 ml serum. Our study yielded substantially similar mean TBG T_4-binding capacity for nonpregnant sheep (15.65 ± 0.67 µg T_4/100 ml serum). Even in studies where proportions of T_4 bound to each carrier protein were expressed as per cent of the

total radioactivity, our results were again in general agreement.

Tanabe and his associates (1969) found that among the ungulates the Muntjak deer TBG has a relatively low T_4-binding capacity because most of the serum thyroxine was bound to the albumins. Results in our studies reinforce the concept of a proportionately minor T_4-binding role in white-tailed deer TBG compared to other ungulates. The mean Saturation Index of 0.73 ± 0.03 for ungulates was significantly lower than the deer mean of 2.02 ± 0.11.

In chickens, where very low, if any, TBG has been reported, our findings confirm the earlier reports. Apparently most of the thyroxine in birds is bound by albumin not globulin. In guinea pigs and rats where reports have indicated that the albumins or prealbumins are the major carriers of thyroxine in serum, our results again show negligible or very low thyroxine-binding capacities in TBG. So a comparison of the results of our studies with reports on the same animal groups by other workers show a high degree of qualitative and even quantitative agreement. However, the new TBG saturation technique is very suitable for routine use and eliminates the drawbacks that have been observed in electrophoretic techniques. It is, therefore, considered an improvement on existing techniques and could become an invaluable investigative tool.

Perhaps the most significant single application of data from the new technique is their utility in determining the TBG Saturation Indexes of different animal groups in various physiological conditions. Saturation Indexes reflect the relationship between serum thyroxine levels and the T_4-binding capacities of TBG. These indexes seem to be tenaciously maintained at some species-specific constant through various physiological states. In the sheep, for instance, the SI is 0.84 ± 0.08 in nonpregnant controls, 0.70 ± 0.04 during pregnancy and 0.69 ± 0.04 during lactation. Even though the serum thyroxine and T_4-binding TBG capacities are individually altered during pregnancy and lactation, their relationship (SI) is maintained virtually constant. In somewhat abnormal physiological conditions such as when thyroprotein is administered to the bovine the mean Saturation Index does change from the level characteristic of the species. Mean SI values much closer to 1.00 are obtained apparently because with the observed increase in serum T_4 the TBG is substantially saturated.

Saturation Indexes of TBG have also sensitively indicated the role played by the globulin as a thyroxine carrier when compared with other thyroxine-binding proteins. In the deer, rodents and birds where mean Saturation Indexes are substantially higher than 1.0, the bulk of the thyroxine is apparently bound by albumin as confirmed by other reports. In humans and the ungulates

other than deer, where the Saturation Index is less than 1.0, the capacity of TBG for thyroxine is not normally saturated by endogenous thyroxine and most of it is bound by the globulins. This is again in agreement with several previous reports.

The new technique for measuring the T_4-binding capacities yields not only the proportion of thyroxine bound by TBG but goes further to quantify it. Results from the new technique are more quantitatively consistent than those from electrophoretic techniques. On the other hand, the great variation in results from electrophoretic methods has been well illustrated in a review by Woeber and Ingbar (1968).

The reliability of the new technique is in some measure due to the clean and quantitative separation of bound from unbound thyroxine by the resin-impregnated sponges. Another factor that aids in the accuracy of this technique is the virtually complete blocking of thyroxine-binding by albumin and prealbumin. Clearly then, the new technique for measuring T_4-binding TBG capacities not only eliminates the drawbacks of most current techniques but also has some new advantages.

SUMMARY

1. A new technique for measuring the Saturation capacity of TBG to bind endogenous, radioactive and exogenously added cold thyroxine (T_4) has been presented. The conditions of this method block T_4-binding to prealbumins and albumins and resin-impregnated sponges are employed to separate protein-bound from unbound T_4. The new technique is suitable for routine use and avoids the major drawbacks of electrophoresis. Results obtained indicate good repeatability and are in good qualitative and quantitative agreement with data from previous reports by workers who used other methods.

2. Thyroxine-binding TBG capacities are generally higher than serum T_4 levels in man and most ungulates. In rodents and birds, the capacities are lower than serum T_4 levels because, as has previously been reported, in these groups most of the thyroxine is bound by albumins or prealbumins. This is well reflected in the higher ratio of serum T_4: TBG capacity (Saturation Index) in rodents and birds compared to those of humans and the ungulates.

3. Thyroxine-binding globulins appear to be the only T_4-carrier proteins in the bovine. Thus when there is an increase in serum thyroxine in excess of the binding capacity of TBG, the excess is rapidly metabolized and cleared as observed in thyroprotein-treated cows.

4. Unlike the case in man, pregnancy in cows, sheep, and rats is not accompanied by a significant increase in serum thyroxine-binding capacity of TBG. The slight alterations in serum T_4 which occur during pregnancy are accompanied by equally slight alterations in the capacity of TBG to bind thyroxine. Mean Saturation Indexes during pregnancy therefore remain at the levels characteristic to each species.

5. During lactation in cows and sheep, the previously reported decrease in serum thyroxine is accompanied by a nonsignificant decline in the capacity of TBG for T_4. In sheep, both parameters are equally depressed. The mean Saturation Index of lactating cows is consequently lower than that of the nonlactating controls, whereas this index is unchanged in sheep.

6. Women on oral contraceptive pills show significant increases in both their serum thyroxine and capacities of TBG to bind T_4 when compared to those not taking the pills. Both groups of women thus yield substantially similar mean Saturation Indexes.

7. Serum thyroxine and TBG T_4-binding capacities of bovine fetuses gradually rise at least from the second trimester up to term. Since the T_4 levels and binding capacities of TBG are nearly identical during this period there is little or no unbound thyroxine and the Saturation Index of second and third trimester fetuses are 1.05 ± 0.05 and 1.13 ± 0.21, respectively. At term, the neonatal thyroxine level is 3-4 times the adult level. With the rapid decline of binding capacities of TBG after birth, the thyroxine freshly released from binding is available for producing heat which is necessary at this time to counteract the extrauterine cold exposure of the first few days of life.

8. Deer are unique among the ungulates studied in having very high serum T_4 levels and Saturation Indexes. The high indexes indicate that substantial amounts of T_4 are carried by a protein or proteins other than TBG.

9. Laying chickens have lower serum thyroxine levels than nonlayers whose T_4 levels are lower than those of male chickens.

10. Male turkeys are unique among the birds studied in having TBG T_4-binding capacities that are significantly higher than zero.

APPENDIX A

THYROID PARAMETERS OF DEER, PIGMY GOATS AND HORSES

Deer

Sample Code No.	Serum T_4 (µg T_4/100 ml Serum)	T_4-binding TBG Capacity (µg T_4/100 ml Serum)	Saturation Index
589	16.72	10.68	1.57
620	14.28	10.93	1.31
604	16.37	10.39	1.58
602	14.22	6.01	2.37
600	17.97	11.88	1.51
617	14.78	9.05	1.63
603	18.01	11.63	1.55
623	13.54	5.63	2.40
619	15.65	8.40	1.80
614	11.37	6.63	1.71
11	15.52	6.65	2.33
12	17.17	7.13	2.41
616	16.13	6.19	2.61
572	15.83	9.22	1.72
580	16.03	7.29	2.20
574	17.81	7.20	2.47
50611	17.72	8.57	2.07
582	19.84	6.15	3.23
589	18.22	7.80	2.34
Means ± Std. Error	16.17 ± 0.45	8.29 ± 0.46	2.04 ± 0.11

Pigmy Goats

Sample Code No.	Serum T_4 (µg T_4/100 ml Serum)	T_4-binding TBG Capacity (µg T_4/100 ml Serum)	Saturation Index
GC-A_1	12.91	14.81	0.87
GC-A_2	9.03	11.22	0.80
GC-A_3	10.47	14.71	0.71
GC-30	13.51	18.27	0.74
GC-32	11.38	16.40	0.69
GC-34	13.48	18.50	0.73
GC-36	13.23	16.95	0.78
GC-38	13.87	17.62	0.79
GC-43	10.85	14.77	0.73
Means ± Std. Error	12.08 ± 0.57	15.92 ± 0.77	0.76 ± 0.02

Horses

Sample Code No.	Serum T_4 (µg T_4/100 ml Serum)	T_4-binding TBG Capacity (µg T_4/100 ml Serum)	Saturation Index
205595-6	1.45	4.51	0.33
205581	3.19	2.47	1.29
205501	1.00	4.05	0.40
205588	2.15	3.88	0.48
205550	2.50	3.10	0.78
Means ± Std. Error	2.22 ± 0.30	3.60 ± 0.36	0.66 ± 0.18

APPENDIX B

THYROID PARAMETERS OF COWS IN

VARIOUS PHYSIOLOGICAL CONDITIONS

Control Heifers (Average: 17 months old)

Sample Code No.	Serum T_4 (µg T_4/100 ml Serum)	T_4-Binding TBG Capacity (µg T_4/100 ml Serum)	Saturation Index
431	6.38	7.32	0.87
434	5.61	9.21	0.60
1035	9.72	10.10	0.96
1036	5.71	9.38	0.61
1038	7.05	11.80	0.60
429	7.30	9.84	0.74
1039	6.41	12.03	0.53
1041	4.95	9.33	0.53
1045	6.16	9.91	0.62
1046	8.58	9.41	0.91
Means ± Std. Error	6.79 ± 0.46	9.83 ± 0.42	0.70 ± 0.04

Lactating Open Cows (Average age 6 years)

Sample Code No.	Serum T_4 (µg T_4/100 ml Serum)	T_4-Binding TBG Capacity (µg T_4/100 ml Serum)	Saturation Index
800-6	3.14	6.87	0.45
319-C	3.08	7.07	0.43
796-C	3.52	9.95	0.35
904-C	5.22	8.84	0.59
716-C	3.81	9.84	0.38
Means ± Std. Error	3.75 ± 0.39	8.51 ± 0.66	0.44 ± 0.03

Lactating Pregnant Cows
(79 days pregnant, average age 5 years)

Sample Code No.	Serum T_4 (µg T_4/100 ml Serum)	T_4-Binding TBG-Capacity (µg T_4/100 ml Serum)	Saturation Index
952-A	5.71	8.92	0.64
902-A	5.70	9.09	0.63
869-A	6.14	9.73	0.63
838-A	4.39	7.95	0.55
811-A	5.52	9.47	0.58
702-A	6.46	10.05	0.64
Means ± Std. Error	5.65 ± 0.29	9.20 ± 0.31	0.61 ± 0.02

Dry Pregnant Heifers
(Average 180 days pregnant, age 18-24 months)

Sample Code No.	Serum T_4 (µg T_4/100 ml Serum)	T_4-Binding TBG Capacity (µg T_4/100 ml Serum)	Saturation Index
561	5.78	8.73	0.66
554	4.94	9.65	0.51
556	6.92	6.75	1.03
555	4.92	11.04	0.45
559	6.07	9.18	0.66
558	6.58	8.63	0.76
546	6.40	7.79	0.82
567	7.17	8.46	0.85
551	6.22	9.99	0.62
557	4.14	10.11	0.41
Means ± Std. Error	5.91 ± 0.31	9.03 ± 0.39	0.68 ± 0.06

Dry Pregnant Heifers
(264 days pregnant on average, age:18-22 months)

Sample Code No.	Serum T_4 (µg T_4/100 ml Serum)	T_4-Binding TBG Capacity (µg T_4/100 ml Serum)	Saturation Index
549	7.24	11.38	0.64
562	5.14	8.78	0.58
550	7.89	8.18	0.96
563	8.10	8.04	1.01
560	7.00	8.99	0.78
564	4.66	8.96	0.52
Means ± Std. Error	6.67 ± 0.59	9.06 ± 0.49	0.84 ± 0.10

APPENDIX C

BOVINE FETAL THYROID PARAMETERS DURING THE SECOND AND THIRD TRIMESTERS OF PREGNANCY

180-Day-Old-Fetus

Sample Code No.	Serum T_4 (μg T_4/100 ml Serum)	T_4-Binding TBG Capacity (μg T_4/100 ml Serum)	Saturation Index
561	6.62	5.65	1.17
554	13.32	11.04	1.21
556	8.89	8.42	1.06
555	13.26	12.11	1.09
559	12.81	11.48	1.12
558	10.26	11.99	0.86
546	14.66	15.08	0.97
567	14.58	11.40	1.28
551	10.23	12.67	0.81
557	13.56	14.06	0.96
Means ± Std. Error	11.82 ± 0.85	11.39 ± 0.86	1.05 ± 0.05

264-Day-Old Fetus

Sample Code No.	Serum T_4 (μg T_4/100 ml Serum)	T_4-Binding TBG Capacity (μg T_4/100 ml Serum)	Saturation Index
549	18.68	13.70	1.36
566	8.72	17.89	0.49
562	20.18	9.05	2.23
550	17.39	14.44	1.20
563	15.76	15.87	0.99
560	16.23	19.85	0.82
564	15.57	18.97	0.82
Means ± Std. Error	16.08 ± 1.37	15.68 ± 1.40	1.13 ± 0.21

APPENDIX D

THYROID PARAMETERS OF SHEEP DURING VARIOUS PHYSIOLOGICAL CONDITIONS

Open Nonlactating Sheep

Sample Code No.	Serum T_4 (μg T_4/100 ml Serum)	T_4-Binding TBG Capacity (μg T_4/100 ml Serum)	Saturation Index
8-38	16.34	15.56	1.05
8-42	13.15	14.71	0.89
8-04	13.97	14.65	0.95
8-21	11.16	16.69	0.67
8-26	12.99	18.32	0.71
8-25	8.05	14.00	0.57
Means ± Std. Error	12.61 ± 1.14	15.65 ± 0.65	0.81 ± 0.07

Pregnant Sheep

Sample Code No.	Serum T_4 (μg T_4/100 ml Serum)	T_4-Binding TBG Capacity (μg T_4/100 ml Serum)	Saturation Index
4-35	10.53	14.67	0.72
7-29	10.91	15.24	0.72
4-13	8.19	15.32	0.53
4-33	12.47	16.15	0.77
7-23	13.94	17.37	0.80
4-29	12.79	18.81	0.68
Means ± Std. Error	11.47 ± 0.83	16.26 ± 0.64	0.70 ± 0.04

Lactating Sheep

Sample Code No.	Serum T_4 (µg T_4/100 ml Serum)	T_4-Binding TBG Capacity (µg T_4/100 ml Serum)	Saturation Index
4-35	7.71	12.37	0.62
7-29	8.75	13.11	0.67
4-13	10.49	14.79	0.71
7-23	10.83	13.03	0.83
3-06	10.83	14.90	0.73
6-30	7.30	12.95	0.56
Means ± Std. Error	9.32 ± 0.66	13.53 ± 0.43	0.68 ± 0.04

APPENDIX E

THYROID PARAMETERS OF NEWBORN CALVES FROM THE 1ST TO THE 6TH DAY OF BIRTH

Thyroid Parameters on Day of Birth

Sample Code No.	Serum T_4 (µg T_4/100 ml Serum)	T_4-Binding TBG Capacity (µg T_4/100 ml Serum)	Saturation Index
405	10.36	10.80	0.96
431	14.00	9.10	1.10
1031	24.71	11.60	2.13
408	22.77	12.43	1.83
994	27.87	9.43	2.95
420	15.71	10.82	1.45
427	17.61	18.34	0.96
417	16.73	13.76	1.22
406	17.18	16.48	1.04
413	15.82	11.44	1.38
Means ± Std. Error	18.28 ± 1.67	12.42 ± 0.94	1.50 ± 0.20

Day-old Calves

Sample Code No.	Serum T_4 (µg T_4/100 ml Serum)	T_4-Binding TBG Capacity (µg T_4/100 ml Serum)	Saturation Index
405	11.56	10.71	1.08
431	21.04	7.96	2.64
1031	19.12	9.62	1.99
408	18.14	5.44	3.33
994	22.51	6.91	3.25
420	16.16	10.31	1.57
427	19.38	13.09	1.48
417	16.09	14.85	1.08
406	14.54	11.83	1.23
413	12.51	5.81	2.15
Means ± Std. Error	17.11 ± 1.13	9.65 ± 0.99	1.98 ± 0.27

2-day-old Calves

Sample Code No.	Serum T_4 (µg T_4/100 ml Serum)	T_4-Binding TBG Capacity (µg T_4/100 ml Serum)	Saturation Index
405	12.80	10.22	1.25
431	14.66	5.94	2.47
1031	17.55	9.03	1.94
994	11.33	4.37	2.59
420	14.15	10.79	1.31
427	12.11	9.61	1.26
417	15.86	12.41	1.28
406	14.55	11.61	1.25
Means ± Std. Error	13.68 ± 0.77	8.96 ± 0.91	1.65 ± 0.18

3-day-old Calves

Sample Code No.	Serum T_4 (µg T_4/100 ml Serum)	T_4-Binding TBG Capacity (µg T_4/100 ml Serum)	Saturation Index
405	9.92	9.21	1.08
431	11.35	6.80	1.67
1031	14.54	9.52	1.53
408	12.55	5.83	2.15
994	10.58	4.92	2.15
420	9.55	10.02	0.95
427	10.26	8.31	1.23
417	13.57	12.39	1.10
406	11.85	9.41	1.26
413	10.07	5.71	1.76
Means ± Std. Error	11.42 ± 0.53	8.21 ± 0.74	1.49 ± 0.13

4-day-old Calves

Sample Code No.	Serum T_4 (µg T_4/100 ml Serum)	T_4-Binding TBG Capacity (µg T_4/100 ml Serum)	Saturation Index
405	10.16	8.36	1.22
431	9.74	4.74	2.05
1031	11.29	8.64	1.31
994	9.18	3.94	2.33
420	7.70	8.55	0.98
427	8.42	9.25	0.91
417	10.91	10.51	1.04
406	10.58	10.95	0.97
413	5.22	4.39	1.19
Means ± Std. Error	9.48 ± 0.61	7.48 ± 0.83	1.41 ± 0.17

5-day-old Calves

Sample Code No.	Serum T_4 (µg T_4/100 ml Serum)	T_4-Binding TBG Capacity (µg T_4/100 ml Serum)	Saturation Index
405	10.40	9.16	1.14
431	7.90	4.96	1.59
1031	12.35	8.50	1.45
408	8.15	5.47	1.49
994	6.73	3.56	1.89
420	6.37	8.61	0.74
427	6.66	8.09	0.82
417	11.08	12.95	0.86
406	6.82	5.55	1.23
413	3.52	4.69	0.75
Means ± Std. Error	7.80 ± 0.83	7.15 ± 0.89	1.20 ± 0.13

6-day-old Calves

Sample Code No.	Serum T_4 (µg T_4/100 ml Serum)	T_4-Binding TBG Capacity (µg T_4/100 ml Serum)	Saturation Index
405	7.40	6.15	1.20
431	7.47	4.30	1.74
1031	13.60	8.61	1.58
408	7.12	4.37	1.63
994	6.99	4.22	1.66
420	8.60	7.87	1.09
427	8.11	9.65	0.84
417	10.76	10.60	1.02
406	4.40	5.67	0.78
413	2.90	3.55	0.82
Means ± Std. Error	7.74 ± 0.94	6.50 ± 0.80	1.24 ± 0.12

APPENDIX F

REPEATABILITY STUDIES: TBG T_4-BINDING CAPACITIES AND SATURATION INDEXES OF SERUM SAMPLES FROM ONE HEIFER

Serum Thyroxine (μg T_4/100 ml Serum)	T_4-Binding TBG Capacity (μg T_4/100 ml Serum)	Saturation Index
6.41	11.92	0.54
6.41	11.92	0.54
6.41	11.90	0.54
6.41	11.95	0.54
6.41	11.39	0.56
6.41	12.66	0.50
6.41	12.67	0.51
6.41	12.60	0.51
6.41	11.62	0.55
6.41	11.95	0.54
6.41	11.19	0.57
6.41	11.49	0.56
6.41	12.47	0.51
6.41	12.54	0.51
6.41	11.11	0.58
6.41	11.48	0.56
6.41	12.96	0.49
6.41	12.83	0.50
6.41	11.53	0.56
6.41	12.14	0.52
Mean ± Std. Error	12.02 ± 0.13	0.53 ± 0.01

APPENDIX G

THYROID PARAMETERS OF RODENTS

Nonpregnant Control Rats

Sample Code No.	Serum T_4 (μg T_4/100 ml Serum)	T_4-Binding TBG Capacity (μg T_4/100 ml Serum)	Saturation Index
Rp1	6.66	1.51	4.41
Rp2	6.31	1.23	5.13
Rp3	6.62	1.65	4.01
Rp4	6.40	1.37	4.67
Rp5	5.62	1.09	5.16
Rp6	6.15	2.48	2.48
Rp7	5.71	1.05	5.44
Rp8	5.99	1.23	4.87
Rp9	5.46	2.47	2.21
R-1	7.13	3.26	2.18
Means ± Std. Error	6.20 ± 0.17	1.73 ± 0.23	4.06 ± 0.41

Pregnant Rats

Sample Code No.	Serum T_4 (μg T_4/100 ml Serum)	T_4-Binding TBG Capacity (μg T_4/100 ml Serum)	Saturation Index
Rc1	5.81	1.74	3.39
Rc2	7.80	2.45	3.18
Rc5	7.80	2.48	3.15
Rc6	7.29	2.36	3.09
Rc7	6.69	1.22	5.48
Rc8	6.57	1.59	4.13
Rp12	7.04	2.66	2.65
Means ± Std. Error	7.00 ± 0.27	2.07 ± 0.21	3.58 ± 0.36

Guinea Pigs

Nonpregnant Controls		Pregnant Guinea-pigs	
Sample Code No.	Serum Thyroxine (μg T_4/100 ml Serum)	Sample Code No.	Serum Thyroxine (μg T_4/100 ml Serum)
A-169	1.83	A-29	0.49
A-168	2.22		
A-167	2.51	A-77	2.89
A-166	2.68	A-105	2.39
A-165	2.94	A-102	2.63
A-163	1.78	A-78	2.40
A-150	2.68	A-97	0.89
A-145	2.55	A-99	4.21
A-144	2.12	A-108	3.63
A-143	2.34		
Mean ± Std. Error	2.37 ± 0.12		2.44 ± 0.44

APPENDIX H

AVIAN THYROID PARAMETERS

Male Turkey

Sample Code No.	Serum T_4 (μg T_4/100 ml Serum)	T_4-Binding TBG Capacity (μg T_4/100 ml Serum)	Saturation Index
TM_1	1.52	1.65	0.92
TM_2	1.06	1.92	0.55
TM_3	1.09	2.17	0.50
TM_4	0.81	0.58	1.39
TM_5	1.62	1.55	2.79
#2	2.31	0.64	3.61
#3	2.14	0.86	2.49
Mean ± Std. Error	1.51 ± 0.21	1.34 ± 0.24	1.75 ± 0.46

Female Turkey
(Negligible T_4-Binding TBG Capacities)

Nonlayers		Layers	
Sample Code No.	Serum Thyroxine (μg T_4/100 ml Serum)	Sample Code No.	Serum Thyroxine (μg T_4/100 ml Serum)
TNL_1	0.74	TL_1	0.52
TNL_2	1.16	TL_2	1.16
TNL_3	0.16	TL_4	0.59
TNL_4	1.13	TL_5	0.94
TNL_5	1.55	TL_6	0.74
TNL_6	1.12	#6	2.68
TNL_7	0.99	#7	2.17
TNL_8	2.60	#8	2.34
Mean Std. Error	1.18 ± 0.25		1.39 ± 0.30

Chickens
(Negligible T_4-Binding TBG Capacities)

Males		Nonlayers		Layers	
Sample Code No.	Serum T_4 (µg T_4/100 ml Serum)	Sample Code No.	Serum T_4 (µg T_4/100 ml Serum)	Sample Code No.	Serum T_4 (µg T_4/100 ml Serum)
CM_1	2.73	CNL_1	2.76	CL_1	1.35
CM_2	4.44	CNL_2	1.47	CL_2	0.88
CM_3	3.46	CNL_3	1.81	CL_3	1.04
CM_6	0.44	CNL_4	1.29	CL_4	1.48
CM_7	1.80	CNL_5	1.94	CL_5	0.23
CM_9	0.78	CNL_6	1.51	CL_6	0.78
CM_{10}	1.65	CNL_7	1.68	CL_7	0.49
CM_{11}	1.57	CNL_8	1.52	CL_8	1.71
CM_{12}	2.03	CNL_{10}	1.76	CL_9	0.45
				CL_{10}	1.55
Mean ± Std. Error	2.10 ± 0.42		1.75 ± 0.14		1.00 ± 0.16

Males vs. Nonlayers ($p < 0.05$)
Layers vs. Nonlayers ($p < 0.01$)

APPENDIX I

SATURATION TECHNIQUE FOR MEASURING
BINDING CAPACITY OF TBG

1. N/10 HCl Solution

   Weigh out 9.856 gm of concentrated HCl solution (37% HCl).

   Dilute to 1 L using distilled water.

   Standardize with Na_2CO_3 solution using phenolphthalein indicator.

   Adjust to 0.1N if not at the correct pH.

   Label this solution B.

2. M/10 Sodium Barbital Solution

   Weigh out 20.618 gm of powdered sodium barbital.

   Dissolve in distilled water and dilute to 1 L.

   Label this solution A.

3. Barbital Buffer pH 8.6

   Add 12.1 ml of solution B from a burette into a 1000 ml volumetric flask.

   Dilute to 1000 ml with solution A.

   Mix thoroughly by inverting the stoppered flask several times.

   Check the pH of the buffer solution using a pH meter.

   The pH should be 8.6.

4. Stock Cold T_4 Solution

Weigh out 10 mg of pure L-T_4 and transfer to a 100 ml siliconized volumetric flask.

Dissolve in glass distilled water with the aid of a minimum quantity of NaOH solution and dilute to 100 ml to give a 100 µg T_4/ml solution.

Pipette out 1 ml of this solution to dilute to 20 ml with glass distilled water to give a stock 5 µg T_4/ml standard.

Various working standards can be prepared by aqueous dilution of the stock solution. Working standard concentrations varied from 0.1-0.5 µg T_4/ml depending on the endogenous T_4 level of serum being investigated.

5. ^{131}I-L-T_4 Solution

Portions of the stock radiothyroxine solution are diluted such that a volume of 0.05 ml of the working standard gives 2,000-40,000 cpm.

6. Polypropylene Tubes and Resin-impregnated Sponges

Each polypropylene tube has the following dimensions: 1.3 cm I.D. X 8.6 cm (Abbott Radiopharmaceuticals, North Chicago, Illinois).

Polyurethane resin-impregnated sponges* capable of adsorbing free T_4 have the following dimensions:

*In the absence of resin-impregnated sponges the technique can be modified to allow the use of IRA-400 anion-exchange resin.

1.1 cm O.D. X 1.95 cm (Abbott Radiopharmaceuticals, North Chicago, Illinois).

7. Procedural Sequence

a. Measure into each of 3 polypropylene tubes the volume of 0.087M sodium barbital buffer (pH = 8.6) which sums up with the combined volumes of ^{131}I-labeled and unlabeled T_4 solutions as well as serum to give a total volume of 2.0 - 2.5 ml and a serum dilution of 30-35. Two of these tubes are duplicates, the third will be a serum-free blank. The amount of endogenous T_4 in the serum volume used in duplicates is exactly replaced by cold T_4 in the blank.

b. In sequence, measure the calculated volumes of labeled T_4, cold T_4 and lastly serum (see Appendix I-9, equations 1-4) into the buffer using a syringe microburet.* After the addition of each of the three sources of T_4, the external tip of the syringe needle used is rinsed with 2-4 drops of barbital buffer in order to effect complete quantitative transfer of whatever is measured.

c. To ensure complete equilibration of all ions in each polypropylene tube, the liquids are vortex-mixed for 15 seconds after the addition of either cold or radioactive T_4 solution, and 30 seconds after the addition of serum.

*Micro-metric Instrument Corporation, Cleveland, Ohio.

d. All tubes are then incubated for 60 minutes in a 37°C water bath.

e. The tubes are taken out of the bath and a resin-impregnated sponge is immediately added to each tube.

f. Each sponge is gently depressed three times with a plastic plunger.

g. Initial radioactivity (CPM) from each tube is determined using a scintillation well counter and gamma ray spectrometer set for counting at the ^{131}I peak.

h. The tubes are again returned to the 37°C water bath for 30 minutes and then taken out.

i. Each tube is immediately filled with distilled water. The sponges are each washed 3 times with distilled water using a suction apparatus and special plastic aspirators for sucking out the liquids.

j. A final count of radioactivity from each tube is then taken.

8. Special Critical Steps in Procedure

1. All syringes and needles used in this technique have to be specially cleaned using in sequence the following:

Aqueous Alconox Solution.

Radiac wash (for radioactive syringes and needles).

Tap water.

Glass distilled water.

Ethyl alcohol (for dissolving and washing off any thyroid hormone).

The needle and syringe are washed six times using each of the above liquids.

All instruments so washed are dried in an oven and cooled to room temperature before use.

2. When adding thyroxine solutions or serum to special polypropylene tubes (Tetrasorb-125 Kit) care must be taken to let the drops of solution fall to the bottom of the tube. The drops should not be allowed to run down the sides of the tubes. If some drops do run down the tube wall, their path must be rinsed down with 2-3 drops of barbital buffer. This ensures that there is no significant adsorption of thyroxine to the sides of the polypropylene tubes.

3. All thyroxine solutions and serum must be added into a buffer medium. A reversal of the order detailed above results in binding of thyroxine to other proteins in addition to TBG.

9. Computation of the Concentrations of Thyroxine to be Employed in Each Experiment

The total quantity of thyroxine needed to saturate the thyroxine-binding capacity of TBG in serum of a particular animal species is read from the flat part of the binding curve as set forth in the results section. This concentration includes the endogenous

thyroxine of the serum in question which is determined by the Tetrasorb-125 resin-sponge technique. Some ^{131}I-labeled thyroxine is employed as a tracer. Since the combined endogenous and labeled thyroxine in the amounts used are usually insufficient to saturate the serum binding proteins, some cold thyroxine is also added.

In the subsequent reactions, the optimal quantity of serum used has been found to be in the order of 0.07 ml. The contribution of endogenous T_4 is obtained by the equation

$$\frac{a \times b}{100} = X \qquad (1)$$

where,

x = µg T_4.

a = serum volume in ml, and

b = serum T_4 concentration in µg%.

Amount of ^{131}I-T_4 contributed by the labeled T_4 solution is given by the equation

$$y = a_1 \times b_1 \qquad (2)$$

where,

y = µg ^{131}I-T_4.

a_1 = volume of T_4 solution used, ml.

b_1 = concentration of ^{131}I-T_4 solution, µg/ml.

Sum of T_4 from serum and ^{131}I-T_4 solution

$$= (x + y)\ \mu g$$

Amount of cold T_4 needed is given by

$$m = Z - (x + y) \qquad (3)$$

where,

m = amount of cold T_4, μg.

Z = amount of total T_4 (μg) needed to saturate binding sites on TBG.

Then, volume of cold T_4 solution needed is given by the equation

$$a_2 = m/b_2 \qquad (4)$$

where,

a_2 = volume of cold T_4 solution, ml.

b_2 = concentration of T_4 solution, μg/ml.

10. Correction for Thyroxine Not Bound by Resin Sponges

Since the resin-impregnated sponges do not have an unlimited capacity to bind free thyroxine, blanks are employed for each level of thyroxine. The blanks have the same amount of thyroxine as corresponding duplicates but no serum. The difference between the first and second counts of radioactivity of the blanks gives the amount of thyroxine not taken up by the sponges. This difference is calculated as a percentage of the

initial count and the percentage is then used to correct final counts of serum-containing tubes as follows:

The figures used in this illustration are part of the results of an actual experiment.

	Initial counts/min	Final cpm.	Difference	% Difference
Blank	13,544	12,213	1,331 cpm	$\frac{1331}{13544} \times 100 = 9.83$
Serum-containing tube	13,669	6,737		

From the blank, the final cpm of the serum-containing tube was underestimated by $\frac{13668 \times 9.83}{100} = 1344$ cpm.

The corrected final count should, therefore, be 6737 + 1344 = 8081. It is the corrected final count that is used in the calculation of the µg T_4 bound to protein.

11. Computation of the Capacity of TBG for Thyroxine

The basic principle of this technique is that thyroxine-binding globulin will bind both labeled and unlabeled thyroxine to saturate all its binding sites at 37°C. The thyroxine in excess of the maximal binding capacity of carrier proteins at the temperature employed is bound to the resin-impregnated sponges. After the sponges are washed, only the non-protein-bound thyroxine is left bound to them. The difference in radioactivity between the initial and corrected

final counts is therefore attributable to protein bound thyroxine.

The initial count represents the total thyroxine used in the experiment. From this, the number of counts that represent one µg of thyroxine can be calculated. A knowledge of the counts per µg T_4 enables a calculation of the µg of T_4 bound to protein from the difference between the initial and final counts. Final results as µg% T_4 bound to TBG are calculated by the equation,

$$a = \frac{b}{c} \times \frac{100}{d} \qquad (5)$$

where,

a = µg % T_4 bound to TBG.

b = radioactivity bound to TBG, cpm.

c = initial cpm/µg total T_4.

d = serum used, ml.

12. Saturation Index of Thyroxine-Binding Globulin

The term "saturation index" (SI) was adopted as a concise description of the relationship between serum thyroxine levels and TBG T_4-binding capacities. "Saturation" was considered appropriate since the T_4-binding capacities were obtained when serum samples were flooded with such concentrations of thyroxine as would more than saturate the binding capacities of the thyroxine-binding globulins. The Saturation Index is calculated according to the equation,

$$\text{Saturation Index} = \frac{S}{C} \qquad (6)$$

where

S = serum T_4 µg/100 ml.

C = T_4-binding TBG capacity µg/100 ml.

Significance of differences within and between various animal species was obtained by the student "t" test (Li, 1964). The Chauvenet criterion* was employed to determine whether or not extreme values of a series should be discarded.

13. Rationale of Procedure

The pH of sodium barbital buffer as well as the 30-35 times dilution of serum create conditions which virtually block the binding of thyroxine by carrier proteins other than TBG.

Incubation at 37°C simulates the average body temperature of most mammals. In order for data from other animal classes to be comparable, their sera are also incubated at 37°C even though the normal body temperature of these animals might differ from 37°C. A maximum of 60 minutes is required for all the available binding sites on the α-globulin to become filled by thyroxine. In several preliminary experiments it was found that 30 minutes is sufficient for free thyroxine binding by the sponges.

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*Documenta Geigy, Scientific Tables, 5th Edition, Basle (Switzerland): S. Karger, New York, p. 47, 1959.

Since the sponges have a maximum affinity for thyroxine of about 0.17-0.20 g, blanks or controls were run for each concentration of total T_4. Whatever excess of free T_4 is left unbound by the sponges in the blanks, is calculated as a percentage of the total T_4 used. The tubes containing sera are each corrected by this percentage excess as explained in the section on calculations. The initial count represents the radioactivity of both the protein bound and unbound thyroxine. After the sponges are washed three times all the protein-bound thyroxine (labeled and unlabeled) is removed in the washings. The final count represents the radioactivity of the thyroxine that was in excess of the saturation capacity of the binding proteins. Neither the binding proteins nor the resin sponges can distinguish between the labeled and unlabeled thyroxine. The higher the concentration of the total unlabeled thyroxine relative to the labeled species, the less labeled thyroxine is bound to either thyroxine binder. However, if the two species of thyroxine are fully equilibrated (the reason for vortex mixing) the radioactivity counts can be assumed to represent total thyroxine. The difference between the first and the second counts represents the radioactivity of the thyroxine bound to proteins.

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