STUDY OF THE METHODOLOGICAL VARIABLES OF TRIIODOTHYRONINE RADIOIMMUNOASSAY AND EVALUATION OF THEIR EFFECTS ON THE T_3 VALUE

by

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INTRODUCTION

Section I

The Hormone

Tri-iodothyronine, usually abbreviated T_3, was identified in human plasma by Gross and Pitt-Rivers (1) in 1952. Since then, this naturally occurring iodinated amino acid has been the subject of investigation and speculation by thyroid physiologists. The interest in this hormone has been heightened by the observation by Gross, Pitt-Rivers (2) and Lerman (3) that the potency of T_3 is 3-4 times as great as that of thyroxine (T_4) in the treatment of myxedema patients. Despite this interest, little data accumulated on this subject in the fifteen years following its discovery. This was primarily due to the low concentration of the hormone in human serum and the tedious methods required for its quantitation. In the last six years several chromatographic methods have been developed for the determination of this hormone and a new interest in the role of T_3 in the thyroid pathophysiology was raised. It has become apparent, based on data collected using these methods and more recently using radioimmunoassay techniques, that T_3, despite the small quantities in plasma, plays an important role in the maintenance of the euthyroid state. Initial estimates were that perhaps one-half of the body's thyroid hormone needs were supplied by T_3 (4). This is due to the shorter half-life of T_3 (about one day) as compared to the half-life of T_4 (about 7 days). More recently, extrathyroidal conversion of T_4 to T_3 has been demonstrated in vivo and the original speculation of Gross and Pitt-Rivers that T_4 is converted

to T_3 before it can exert its metabolic action is revived (2). On the clinical level, the finding of some hyperthyroid patients with normal or low T_4 but elevated T_3, has increased the demand for its determination as an essential part of the evaluation of the thyroid status in increasing numbers of thyroid patients.

A. Biosynthesis of the Iodothyronines (T_3 and T_4)

1. The Thyroid follicle

The structural and functional unit of the thyroid gland is called the follicle and is composed of spheroid space (lumen) surrounded by a single layer of epithelial cells. Both the space and the surrounding cells vary in size, shape, and content with the stage of activity of the thyroid. It is in these units that the thyroid hormones are manufactured, stored and from there they are passed to the surrounding capillaries and venules to reach other tissues.

2. Iodine trapping

The biosynthesis of T_3, as well as of T_4, begins with the inorganic iodide (I^-) ingested with food and absorbed into the circulation. I^- enters the epithelial cells of the follicle by an active transport mechanism at the basal membrane. This mechanism is generally referred to as the iodide trapping. Trapped iodide is passed in to the follicular lumen, probably by the same active mechanism, to iodinate the thyroglobulin which has migrated to the same site.

3. Synthesis of Thyroglobulin (Tg)

Thyroglobulin is a glycoprotein formed from noniodinated amino acids on the polyribosomes of the epithelial cells of the thyroid gland. The

newly synthesized protein leaves the polyribosomes and migrates to the Golgi zone, where the protein acquires its carbohydrate moiety, and then passes to the colloid region, where it becomes iodinated. It is now known that iodinated amino acids are not used for the synthesis of the primary peptide chain of the thyroglobulin, and iodination reaction takes place on the thyroblobulin molecule rather than with free amino acids.

4. Iodination

Iodination is believed to occur at the interface of the apical cell membrane and the follicular lumen, catalyzed by a thyroid peroxidase (autoradiography studies show that no iodination of the thyroglobulin occurs during its passage through the cell). The result is the formation of Monoiodotyrosine (MIT) and Diiodotyrosine (DIT) by the iodination of tyrosyl residues on the thyroglobulin. Iodothyronine (T_3 and T_4) are formed by a coupling reaction of the Iodotyrosines (MIT and DIT) with extrusion of alanine side chain (5, 6).

From a teleological point of view it is thought that the relative formation of T_3 and T_4 would depend on the relative quantities of MIT and DIT and this in turn is dependent on the degree of iodination of the thyroglobulin.

5. Storage and Secretion of the Iodothyronines

In the normally functioning thyroid only a portion of the synthesized thyroid hormone is released in the blood. The rest is stored in the colloid. The degree of stimulation by TSH (thyroid stimulating hormone), a thyrotropic hormone which originates in the anterior lobe of the pituitary gland, and the quantity of available iodine are the principle factors controlling the ratio of thyroid hormones to be stored

as colloid to that which is to be secreted. Both T_3 and T_4 are stored in the colloid as part of the thyroglobulin, and under stimulation by TSH, some proteolytic enzymes hydrolyze the thyroglobulin releasing T_3, T_4, DIT and MIT. The last two are generally deiodinated in the gland by a specific enzyme known as deiodinase, which has no effect on T_3 or T_4. Thus iodide produced by deiodination is recycled (6).

B. Iodothyronine Transport Proteins

Based on in vivo and in vitro observations, T_3 is transported in human blood by three serum proteins: TBG (thyroxin binding globulin), TBPA (thyroxin binding prealbumin), and human serum albumin.

1. Physiological Significance of Transport Proteins

The physiological significance of the binding between proteins and small molecules, as T_3, is not completely understood. In contrast to the peptide hormones, T_3, T_4, and many other small-molecule hormones are relatively hydrophobic and exist in aqueous solution in blood by virtue of their firm binding to one or more serum protein carriers. The degree of binding to serum proteins seem to have great effect on the disappearance, or utilization rate of the hormones in the organisms. In that line of thought, it is observed that the biological half-life of a hormone varies with the magnitude of the free or non protein-bound moiety. Thus cortisol, which is 95% bound and 5% free, has approximately 1 hour half-life, T_4 is 99.95% bound and less than 0.05% free, has a biological half-life of one week. The much shorter half-life of T_3, compared to T_4, can be attributed to its less firm binding to serum proteins 99.5% (7). Clinically good correlation between the level of free hormone (T_4 or T_3)

and the metabolic status of human is usually found. Since the hormonal activity is suppressed in the hormone-protein complex, the complex formation provides a mechanism to store relatively large quantities of the hormone in the circulation in an inactive form.

2. The Nature of Hormone-Protein Binding

Hormone-protein interaction is made up of low energy bonds between the two. They are noncovalent bonds and affected by several physical factors: temperature, pH and certain chemicals. Their reactions are highly stereospecific much as those between antigen and antibody. The term "binding" includes a large number of molecular interactions in which association and disassociation occurs spontaneously and rapidly. The relation of T_3 bound to individual protein sites and the free T_3 can be described in terms of multiple-equilibrium system obeying mass-action principles (10, 11). Thus free hormone concentration will be influenced by the concentration of the proteins, the association constant and the total concentration of T_3.

$$T_3 + TBG \rightleftharpoons T_3 - TBG$$

$$K = \frac{[T_3] \cdot [TBG]}{[T_3 - TBG]}$$

$$T_3 = \frac{[T_3 - TBG]}{K \cdot [TBG]}$$

T_3 represent the concentration of free or unbound T_3.

TBG the concentration of Thyroxin Binding Globuline.

T_3 - TBG the concentration of the bound T_3.

K is the equilibrium constant of the reaction.

3. Serum Proteins Which Bind T_3

In early in vitro studies (12) it was shown that T_3, like T_4, was significantly bound to serum protein in humans, with 99.5% of T_3 being in the "bound" state and 0.5% in the "free" state. Further in vitro studies using electrophoretic methods showed that T_3 binds primarily to TBG and secondarily to serum albumin (13, 14) and does not, in contrast to T_4, bind to thyroxin binding prealbumin (TBPA) (15). However, using an ultrafiltration technique, Larsen (4) was able to demonstrate that T_3 binds to TBPA. Also, Davis et al (16) showed T_3 binding to TBPA using polyacrylamide gel electrophoresis. Evidence(s) for in vivo binding of T_3 to TBG is based on the correlation between serum T_3 and TBG concentrations. Hoteling et al (17) provided further evidence for in vivo binding to TBG by showing a statistically significant rise in T_3 as pregnancy advances, paralleling the rise in T_4 and TBG. Several investigators have reported elevated serum T_3 in pregnancy and idiopathic elevation of TBG (18). Conversely low serum T_3 was reported in genetic TBG deficiency (19). This is consistent with TBG being important in vivo transport protein for T_3. T_3 is less firmly bound to serum proteins than T_4 (12, 13). The reported estimate of the relative binding affinities of the two hormones T_4 and T_3, vary widely. One estimate is T_3/T_4=1/3 using whole serum (20, 21) and another is 1/30 using highly purified TBG (22). The presence of other serum proteins affect the distribution of T_3 and T_4 on TBG (10). While the affinity of T_3 to Alb. and TBPA is the same (4), the fact the concentration of Alb is some 200 fold greater than the concentration.

of TBPA in human serum would suggest that more T_3 is bound to Alb. than to TBPA in vivo.

4. Characteristics of T_3 Transport Proteins

a. Thyroxine Binding Globulin (TBG)

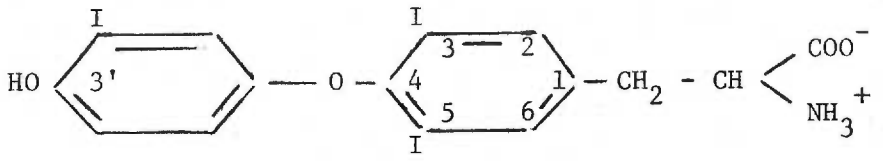
TBG is a glycoprotein with 32% of its weight as carbohydrate. It is present in a lower concentration than the other T_3 and T_4 binding proteins but has the highest affinity and specificity. It has a MW of 60,000 and is found between the two globulins (α_1 and α_2) on the electrophoretic pattern of serum proteins. It has been purified and a RIA (radioimmunoassay) for its determination in human serum has been developed (23). Based on this methodology, its concentration was found to be 2.0 - 4.8 mg/100 ml in normal subjects, up to 7.6 mg/dl in pregnancy and decreased in cirrhosis of the liver. Another approach for assessing the TBG level in serum is the determination of its maximum binding capacity for T_4(MBC) using T_4 - I^{125} and unlabeled T_4. This measurement has been shown to correlate very well with TBG concentration as determined by RIA (23). In normal subjects T_4MBC of TBG is 13-26 $\mu g T_4$/dl. TBG is transmitted as a dominant x-chromosome linked trait (24, 25). Therefore familial prevalence of TBG elevation and deficiency have been described.

b. Thyroxine Binding Prealbumin (TBPA)

TBPA is present in human serum in higher concentration than TBG, (23-35 mg/100 ml serum) and has a MW of 73,000. An immunologically pure TBPA has been prepared by Oppenheimer (10). It is found toward the anodal site from albumin (thus the name prealbumin). Its MBC is

216-342 μg T_4/100 ml serum. However, since it has a lower affinity for thyroid hormones than TBG, only 20% of the total T_4 in serum is bound to TBPA. This ratio probably applies for T_3 also. No abnormality in TBPA has been attributed to genetic etiology (10).

c. Specificity of Binding



3,3',5- Triiodothyronine (T_3)

At pH 8.6 and using highly purified TBG, it was shown that the binding of TBG to T_4 and T_3 is structurally very specific. Using analogues, it was found that the diphenyl ether, the four iodines, and the free phenolic group are essential for optimal binding. The alanine side chain is also involved in the binding. Both TBG and TBPA show preference for tetraiodinated over tri-iodinated analogues (20, 22). Albumin appears to have no specificity requirement for binding.

5. Variation in T_3 Binding to Serum Protein (in vivo)

As pointed out previously, the amount of T_3 bound to TBG, albumin, or TBPA will be influenced by the concentration of each protein, the association constant of each class of binding sites and total concentration of T_3. Thus changes in the concentration of one of those proteins or its association constant will lead to a variable level of free hormone and will change the overall distrubition of the hormone among

different proteins.

a. Causes of Variation in the Proteins Concentration

(1) Since TBG is transmitted as a dominant x-linked trait, familial prevalence of TBG elevation and TBG deficiency is observed. A number of asymptomatic euthyroid subjects with elevated PBI (protein bound iodine) and TBG binding capacity, and others with low PBI and TBG binding capacity have been described (13). With the availabilty of T_3 determination, correlation of the same type have been observed and patients with low serum T_3 and genetically determined low TBG have been described (25).

(2) In Pregnancy and Estrogen Useage

The effect of estrogens in increasing serum PBI by augmenting the binding capacity of TBG is now well established (26). Several authors have shown similar elevation in serum T_3 (17, 18, 27) due to pregnancy or the administration of estrogens. Elevation of the serum thyronine in these cases is due to increased concentration of TBG in the serum rather than to alteration in the binding capacity of the TBG molecule, as confirmed by Velayo et al (23).

(3) Androgen Useage

Testosterone decreases the PBI and T_4 binding capacity of TBG and it has the same effect on the T_3 level (26).

b. Causes of Variation in the Interaction of T_3 with the Binding Proteins

Several drugs are known to decrease serum T_3 by either displacement of T_3 from these proteins or by enhancing its cellular metabolism.

Patients receiving "Dilantin" (Diphenylhydantoin DPH) have lower levels of serum PBI, T_4, and T_3 (28). The in vitro ability of this drug to displace T_4 and T_3 from plasma proteins has been used to explain the lower levels of these hormones upon its administration. The basis of the displacement of T_3 and T_4 by DPH is thought to be the structural analogy between the diphenyl-ether linkage of the thyronines and the diphenyl grouping about carbon 5 in the diphenylhydantoin molecule (29).

Salicylate and dinitrophenol were found to displace T_4 from TBPA (30) and more recently, it was shown that salicylate displaces T_3 from TBG at a much lower concentration than needed to displace T_4 from the same protein (31, 32).

Tetetrachlorothyronine (TCT) given in vivo lowers serum PBI in intact and in thyroidectomized, thyroxine treated guinea pigs, which means that (TCT) does not lower the PBI through the pituitary TSH mechanism (30) but by affecting serum protein binding to the thyronines. In studies done on purified TBG, TCT was found to inhibit T_4 binding to this protein (22). This was not, however, observed in whole serum studies because TCT was found to inhibit T_4 binding to TBPA and thus T_4 binding to TBG appeared unaffected (22). Barbital ion is known to inhibit T_4 and probably T_3 binding to TBPA (20). Veronal markedly decreases the binding capacity of TBPA for acetic acid analogues of T_4 and T_3. Several other substances are known to interfere with this binding and are used for in vitro studies. These will be discussed in the pertaining areas of the study.

6. Factors Known to Affect the T_4 and T_3 - Serum Protein Binding In Vitro

a. pH

The binding capacity of TBG for T_4 is pH dependent. At pH 9.0, the MBC of TBG is less than at pH 7.4. Meanwhile, TBPA has higher MBC at pH 7.4 than at pH 9.0. Binding by human serum albumin is unaffected by pH (33).

b. Temperature

Increase in temperature above $4^{o}C$ decreases the binding capacity of both TBG and TBPA. TBG completely loses its binding capacity for T_4 by heating at $60^{o}C$ for 20 minutes (34).

C. Physiological Role and Clinical Significance of Triiodothyronine

1. Physiological Role of Triiodothyronine (T_3)

The hormones elaborated by the thyroid gland appear to stimulate the rate of cellular oxidation and maintain the normal level of metabolic activity in virtually all tissues (35). It has been long recognized that thyroxine (T_4) constitutes about 90% of the organic iodine in the blood (10). Twelve years after the initial demonstration of T_4 in plasma, Gross and Pitt-Rivers (1) identified triiodothyronine T_3 as the second circulating iodoamino acid. It was rapidly shown that T_3, like T_4, is secreted by the thyroid (36). T_3 has been found in the thyroid venous effluent in concentration substantially higher than those in concurrently sampled arterial blood (36).

The ease with which T_3 and T_4 are labeled with radioiodine has allowed for many studies of the detailed knetics of the metabolism of these compounds, in man as well as experimental animals (11, 37). However, there is a wide variation in the results of these studies. This has been attributed to variation in serum T_3 levels which in turn is due mainly to the methodological variation (4, 38). Considering the average serum T_3 level as determined by radioimmunoassay, it is estimated that T_3 secreted by the thyroid gland is about 10 µg/day compared with 80 µg T_4/day. The rest of circulating T_3, about 10-20 µg per day, come from the peripheral conversion of T_4 to T_3. It is estimated that one-half to two-thirds of the circulating T_3 is produced through this process (4). This estimate has been supported by studies on athyrotic patients who were given purified T_4 orally or intravenously, and a level of 2.4-6.8 ng/ml T_3 was found in their serum (39). No specific site has been identified for this conversion. The biological half-life of T_3 is so short that approximately half the extrathyroidal pool is turned over in a day (4, 8). In contrast, the daily fractional turnover of T_4 is about 10%. The fast rate of disappearance of T_3 has been ascribed to the weaker binding to serum proteins. Since the average normal serum T_3 level as reported by most RIA methods is 1.2 ng/ml, the kinetic analysis suggests that the turnover rate of T_3 is about 30-35 µg/day (40, 41). Since it also has been demonstrated that the metabolic activity of T_3 is about 3-4 times that of T_4 by weight (2), it is apparent that T_3 and T_4 make approximately equal contributions to the total biological activity.

The evidence of T_4 to T_3 conversion has again raised the possibility that T_4 must be converted to T_3 in order to exert its metabolic effect. This was suggested originally by Gross, Pitt-Rivers (2) and an accurate answer for it awaits some more knowledge of the precise mechanism of action of the thyroid hormones.

2. Effect of Iodine Intake on Serum T_3 Level

There is a well documented observation regarding the qualitative changes in thyroid gland thyronines during iodine deficiency. Elevation in T_3/T_4 ratio due to an increase in T_3 secretion and/or decrease in T_4 secretion was demonstrated by Greer (43) in rats which were put on iodine deficient diet, and in humans who were put on less than 50 µg iodine/day (44, 27). These changes revert when iodine deficiency is corrected by iodine administration.

In areas of Endemic Goiter due to iodine deficiency, the T_3/T_4 ratio is found to be elevated either due to increase in T_3 level and decrease in T_4 (45) or solely to decrease in T_4 level (46). Furthermore, Charib et al (38) have shown a variation in human serum T_3 level due to a regional difference in iodine intake. A group of Minnesota people were found to have higher serum T_3 values and lower iodine intake than a group of Californians when identical T_3 (RIA) were used in two laboratories in these areas.

These observations strongly suggest that the normal thyroid can alter its secretion of T_3 relative to T_4. This may be an adaptive mechanism to the availability of iodine and may be regulated intrathyroidally (43).

3. Clinical Significance of T_3 Determination

There are an increasing number of clinical situations in which available evidence is compatible with a major physiological role of T_3. The clinical application of its determination is increasing with the availability of the RIA technique. The following five clinical areas are known to be validly correlated with serum T_3 level.

a. In Conventional Hyperthyroidism

Whether hyperthyroidism is caused by toxic diffuse goiter, Graves' disease, toxic nodular goiter, or autonomously functioning adenoma, T_3 concentration in serum is invariably elevated (18, 27). The mean T_3 level in hyperthyroid patients is roughly four times the euthyroid level. The relative increase in T_3 over normal is greater than increase in T_4 in such patients. Therefore, there is a decrease in the ratio of T_4/T_3 in hyperthyroidism. This may be due to an increase in the rate of T_4 to T_3 conversion. There is no evidence for such increase yet. Consequently it is thought that T_3 determination is more sensitive than T_4 determination as a clinical laboratory test for the diagnosis of hyperthyroidism.

b. In T_3 Hyperthyroidism (T_3 Thyrotoxicosis)

T_3 hyperthyroidism is thyrotoxicosis due to elevation in T_3 secretion with normal secretion of T_4. This variant of hyperthyroidism is becoming more recognized now and the clinical picture for its presence has been established. The first case of this type was reported by Maclagan and associates in 1957 (47).

The first well-studied cases of T_3 thyrotoxicosis were reported

by Hollander (48) using a gas chromatographic method to measure circulating T_3 and T_4. This was followed by a group of patients reported by Sterling (49) using a paper chromatographic method. The incidence of T_3 hyperthyroidism was evaluated by Bellabara (50) who found seven cases in 62 consecutive cases of clinical hyperthyroidism. Stevenson in Chile (51) found that the incidence of T_3 hyperthyroidism is much higher in areas of iodine deficiency. Patients with this type of hyperthyroidism are heterogenous including those with toxic diffuse goiter, toxic nodular goiter, and some recurrent hyperthyroidism following treatment with surgery or radioiodine.

The clinical picture in patients with T_3 hyperthyroidism is not distinguishable from those with conventional thyrotoxicosis. Thus the diagnosis must be based on laboratory tests which should provide:

1. Evidence of thyroid abnormality with the presence of hypermetabolism or hyperthyroidism.
2. Normal level of T_4.
3. Abnormal T_3 suppression test to establish the autonomous function of the gland.
4. Elevation of serum T_3 level.
5. Absence of protein binding abnormality because conventional thyrotoxicosis with deficiency in TBG lead to the same laboratory results as in T_3 hyperthyroidism, i.e., normal T_4 and elevated T_3 and abnormal suppression test (52).

c. In the Early Detection of Thyrotoxicosis

The finding by Hollander (27) that hyperthyroid patients may pass through a stage of T_3 toxicosis before developing the usual form

of thyrotoxicosis has potential clinical importance in the early detection of hyperthyroidism. The existence of such groups would support the concept that relative increases in T_3 versus T_4 secretion is inherent in thyrotoxicosis itself rather than due to an acquired factor such as iodine deficiency (4).

d. In Iodine Deficiency

As has been pointed out, iodine deficiency leads to elevated T_3 level with normal T_4 (46). Thus T_3 determination is an indispensable measurement for evaluation of the sufficiency of iodine diet in certain areas.

e. In Patients with Hyperthyroidism During Treatment with Anti-Thyroid Agents

Changes in T_3 level provide a sensitive index of abrupt changes in the thyroidal secretion. If a patient is given propylthiouracil and intravenous Na Iodide, within 24 hours, there is a threefold decrease in T_3 level, with only slight fall in T_4 (18). Also, in patients treated with radioiodine and appearing clinically euthyroid, T_3 is normal but T_4 is low. Thus T_3 level is more correlated with the clinical appearance of these patients.

Section II

Methodology of T_3 Level Determination in Human Serum

A. The Chromatographic Methods

Most of the earlier exploratory approaches for T_3 determination have sought to demonstrate radioactive T_3 in the circulation of human subjects following the administration of I^{131}. Patient's serum, collected after I^{131} administration, is subjected to chromatographic separation, and radioactivity in the T_4 and T_3 areas is compared. While important information was gained from such studies, the figures thus obtained do not reflect the relative concentration of the two hormones in the circulation owing to differences in their specific activities which may be attributed to the difference in metabolic turnover of the two hormones.

Efforts were made to use direct physical and chemical methods without the need for injecting radioiodine. Because of their low solubility in water, the iodothyronines are strongly adsorbed to such support media as cellulose, dextran, gel or ion exchange resin and often behave in a nonideal manner. Therefore, several chromatographic methods have been developed for the determination of T_3. Most of these methods involve three major steps:

1. Extraction of the iodothyronine (T_3 and T_4) from plasma with a solvent or with an ion exchange resin.
2. Separation of T_3 from T_4 using paper, TLC (thin layer chromatography), column, or gas chromatography.

3. Quantitation of T_3: following separation, T_3 is quantitated by iodometry or competitive protein binding methodology (CPB).

The methods developed varied in their complexity. Out of these, Sterling's method (49) is the least complex and that is what made it more widely used until the development of the radioimmunoassay. This method depends on:

1. Removal of thyroid hormones from serum with small columns of cation exchange resin.
2. Complete separation of T_3 from T_4 by descending paper chromatography with a solvent consisting of a mixture of tertiary amyl alcohol, hexane and ammonia.
3. Elution of T_3 from the paper with methanol-ammonia and quantitation using competitive protein binding in which TBG is binding protein.

This method which had several methodological advantages over the other methods, clearly demonstrated that alteration in T_3 level in various clinical states paralleled the recognized change in T_4 level. The T_3 concentration in normal human serum was about 200 ng/100 ml, roughly about 1/40 of T_4 concentration by weight.

However, further examination of this method (53) suggested that there were artifactual elevations when T_3 was measured by this technique. This elevation was found due to incomplete separation of T_3 from T_4 on the paper chromatograph, deiodination of T_4 to T_3 during extraction and separation, and technical problems associated

with the use of paper extracts (54). With correction for deiodination (estimated as 0.4% of T_4 converted to T_3), T_3 normal value became about 150 ng/100 ml.

Hollander et al (27) have demonstrated that there is contamination of the T_3 area on the paper chromatogram with .25 to .5% of the endogenous T_4 in serum, as well as conversion (deiodination) of 0.2 - 0.75% of the endogenous T_4 to T_3 during the extraction and separation. They, therefore, developed another method for T_3 determination using gas-liquid chromatography. As with the previous method, T_4 to T_3 conversion was evident and high T_3 values were obtained. After correction for deiodination there was a good correlation between the gas-liquid chromatographic method and the radioimmunoassay.

The Major Disadvantages of the Chromatographic Methods :

1. It was evident in all chromatographic methods used, that iodoaminoacids, especially T_4, are subject to rapid deiodination during drying on filter paper or similar support (54), while in solution, or during gas-liquid chromatography. The mechanism of this process is not well understood. Several factors were found to have an effect on the rate of deiodination. It can be decreased by the presence of propylene glycol, and thus most commercial preparations use this substance in the iodothyronine solutions. Deiodination decreased also in the presence of serum proteins. It is less on cellulose-starch thin layer than on silica gel medium. Prewashing of the silica gel to remove iron decreases the deiodination process (55).

Even the deiodination inherent in using the chromatographic

methodology does not significantly affect the T_4 concentration. However, the T_3 level is significantly affected because it is present in human serum at a much lower level than T_4. Thus, if 0.4% of T_4 is converted to T_3, serum T_3 level is increased by 75%.

2. All the described chromatographic methods are tedious and do not fit routine clinical use. They require special technical skills, large samples of blood (5 ml or more in Sterling method), and the number of samples that can be done in one run is limited.

B. The Radioimmunoassay

It was apparent that for physicological studies, and clinical usefulness, a more accurate, practical, and sensitive methodology was needed for T_3 determination. Basic to the desired method is the ability to discriminate T_3 from much larger amounts of the very similar hormone T_4. This ability was sought in antibodies to T_3. Brown et al (56) were the first to produce these antibodies by injecting rabbits with a carbodimide (CDI) condensation product of T_3 and succinylated polylysine. Surprisingly high specificity antibodies were obtained through this approach. Good antibodies were also produced using T_3 - BSA (T_3-Bovine Serum Albumin), T_3 - RSA (T_3-Rabbit Serum Albumin), and T_3 polyglutamic acid as immunogens. With the production of specific antibodies to T_3, several radioimmunoassays have been developed. For proper evaluation of these methods, the general principles and methodology of RIA will be discussed.

1. General principle of RIA

One of the most exciting advances in the field of hormones methodology and drug analysis has been the development of the radio-

immunoassay technique. Originally described in 1960 by Yalow and Berson (57), this technique in its basic principle exploits the ability of unlabeled antigen in plasma or other solutions to compete with labeled antigen for a limited number of antibody binding sites made against that antigen and thereby to inhibit quantitatively the binding of labeled antigen to the antibody.

Labeled Antigen (F)	Ag*		Ag–Ab	Unlabeled antigen bound to the antibody
		+ Ab ⇌		
Unlabeled Antigen in standard solution or unknown samples	Ag		Ag*–Ab	Labeled antigen bound to the antibody (B)

Diagram 1. The dynamic competition between the labeled and unlabeled antigen for antibody binding sites. F = Free fraction of the labeled antigen. B = Bound fraction of the labeled antigen.

As the result of this competitive inhibition between the labeled and unlabeled antigen, the amount of labeled antigen bound to the antibody is decreased as the concentration of the unlabeled antigen is increased. Thus the quantitative indicator of the unlabeled antigen concentration is the distribution of the radioactive antigen between the two factions, bound and free. It is evident here that to measure the final distribution of the labeled antigen, a means of separating these two fractions is needed. Several methods are commonly used to achieve this step; they rely on either precipitating the bound fraction or adsorbing the free fraction. Of the first type is the use of saturated

ammonium sulfate, polyethylene glycol, or second antibody. Of the second type is the use of dextran coated charcoal, ion exchange resins or Florsil. It should be noted here that the separated fractions, bound and free, each contain both labeled and unlabeled antigen, yet we only measure the radioactive antigen in each of the fractions or in one of them to know the distribution of the labeled antigen between the two.

2. Methodology

The performance of a radioimmunoassay involves, generally, four major steps:

a. Simultaneous preparation of a series of standards and unknowns, all of which are set up in buffered solution and contain identical concentrations of labeled hormone and antiserum. Increasing known amounts of the unlabeled antigen are added to the standards, and plasma (or other biological fluid) is added to the unknown tubes.

b. Incubation: The length and temperature of incubation is established for optimal sensitivity and reproducibility.

c. Separation of the two fractions: free antigen fraction from antibody-bound antigen fraction. This step is achieved by one of several known methods and usually requires centrifugation of the assay tubes and decanting or aspirating one of the fractions.

d. Counting and calculation. Radioactivity in one of the fractions or in both of them is determined and the results obtained in the standards are plotted as: Added antigen concentration vs. %B, B/F, B/B^{O}, or B^{O}/B. Or logit B/B^{O} is plotted against log dose. Where %B = the percent of the total counts added to the assay mixture that are in the

bound fraction. $\%B^{O}$ = the % bound in the standard which contains no unlabeled antigen.

There are many other plots to represent the dose-response curve, the choice of which depended on the linearity of competitive inhibition and the ease with which these variables are calculated for a particular assay and laboratory. The antigen concentration in an unknown sample is obtained by comparing the inhibition observed in the unknown, i.e., %B, B/F, B/B^{O}, etc. to the graph plotted from the standards.

3. Parameters of Radioimmunoassay (RIA)

a. Validity

(1) Objective

The validity studies of the RIA aim at providing evidence(s) that the assay system is qualitatively and quantitatively measuring the antigen it is set up to measure, and not misinterpreting nonspecific factors in the biological sample.

(2) Requirement

The validity of a RIA requires that:

(a) Changes in antigen concentration as measured by this assay are consistent with what is known about the physiology of that antigen.

(b) Demonstration of correlation between the level of the measured antigen in serum and the physiological and pathological status of the gland producing this antigen.

(c) Demonstration that the concentration of the antigen in the unknown fall linearly with dilution, so that the

concentration in undiluted plasma is independent of the dilution at which the plasma is assayed.

(d) Demonstration of specificity of the assay, i.e., freedom from interference by substances other than the one intended to be measured.

(e) Obtaining good recovery for an added amount of the standard antigen to samples containing various amounts of the endogenous antigen.

(3) Effect of Nonspecific Factors on the Validity

Several nonspecific factors in the environment of the assay can affect the accuracy of the RIA. Of these, pH, ionic strength, temperature and serum proteins. These factors affect the assay by modifying the rate of the primary Ag-Ab reaction and/or by exerting some effect on the antigen making it not free to bind to the antibody (serum protein binding). Since RIA is dependent entirely on a comparison between the standards and unknowns, identity between the two must be achieved by methodological modification of the assay. For that reason, and since serum protein are found to affect Ag-Ab interaction (58) and bind some of the antigen in the serum, it is necessary when assaying samples directly (without extraction) that the standard antigen be prepared in antigen-free plasma, or Ag-free plasma added to the standards assay tubes. Binding of the Ag to serum protein in the standard and unknown can be estimated by running simultaneously a nonspecific binding control which determines the % labeled antigen bound to plasma proteins in absence of the Ab. Optimal condition for pH and incubation temperature

should also be selected as to avoid or minimize the effect of these factors on the antigen-antibody interaction.

b. Sensitivity

The sensitivity of the RIA is defined as the smallest amount of unlabeled antigen that can be distinguished from no antigen (59). Also, it can be defined in terms of the slope of the dose-response curve. Maximum sensitivity of an assay is ultimately dependent on the affinity constant, K, which characterize the energy of the Ag-Ab reaction (60).

For a given antisera, sensitivity of the assay is greatest at higher dilution of that antisera. For a given antibody and labeled antigen, sensitivity is greatest when labeled antigen with highest specific activity is used. This is because less labeled antigen can be used, thus relatively greater change in the labeled Ag distribution is brought about by minute amounts of unlabeled antigen. One of the greatest advantages of the RIA over other clinical procedures is the high sensitivity resulting from the nature of Ag-Ab interaction and the high specific activity of the labeled antigen produced.

c. Precision and/or Reproducibility

Precision is defined as "the extent to which a given set of measurements of the same sample agrees with the mean of that set" (59). It, thus, refers to the magnitude of the random error and it demonstrates what is called the reproducibility of the measurement. For a validated assay, precision and reproducibility are the same. The main factor influencing reproducibility, the duplication of the

values within or between assays, is the difference in individual technique in carrying out the same operation. To determine the reproducibility, one should perform replicate determination for the same sample(s) in the same assay or in separate assays.

In RIA precision is not uniform at different concentrations of the measured antigen, and it depends on the extent of change in the labeled antigen distribution between the bound and free fractions brought about by a given amount of antigen. However, unlike sensitivity, it is not dependent on the K value of the antibody (61).

STATEMENT OF THE PROBLEM

The chromatographic methods for the determination of serum T_3 level in humans have been found to be too tedious for large scale clinical laboratory usefulness. Also, these methods were found to give artificially elevated values due to the deiodination of T_4 to T_3 during the separation of the two hormones. With the rising interest in serum T_3 level, antibodies against T_3 were produced in rabbits and several radioimmunoassay procedures were reported. Wide variation of methodological approaches were used in these reports, especially to eliminate TBG effect on the assay. It was also evident in these reports that the normal T_3 level varies within wide range. It is the goal of this study to study the methodological variables of T_3 RIA and to evaluate their effect on the T_3 value, after developing an accurate and practical procedure which utilizes the commercially available antibodies.

In that quest the following variables were studied, compared, and optimal conditions leading to an accurate determination were selected.

1. T_3 antibodies (from commercial sources)
2. Methods of eliminating TBG effect on the assay
3. Methods for separating the bound and free fractions
4. Effect of pH, incubation time and temperature

The final procedure thus selected was evaluated for its accuracy, sensitivity and reproducibility. In the second phase of this study the effects of the methodological variables on the T_3 value were evaluated.

Section III

Review and Evaluation of T_3 Radioimmunoassays

Several radioimmunoassays for the determination of serum T_3 have been developed in the past four years. However, wide variations in the reported serum T_3 value have been observed in these reports and there seems to be no general agreement on the T_3 level in human serum. This variation has been attributed mainly to methodological variation and partially to regional variation in iodine intake (38). In order to evaluate the result of the reported methods, it is necessary to recognize the technical problems associated with this assay and the complication raised by the different approaches used to resolve these problems.

I. Major Problems Encountered in the Direct Radioimmunoassay of T_3 Are:

A. The presence of T_4, which is very similar in structure and exists in human serum at much higher concentrations than T_3. This fact requires antibodies used in this assay to be highly specific for T_3.

B. The in vitro binding of T_3 to some serum proteins with rather high affinity; TBG and to a lesser extent to TBPA and human serum albumin. This phenomenon is of critical importance for the T_3 assay, and it might affect the T_3 value in several ways depending on the assay conditions:

1. When serum proteins bind the labeled antigen, T_3 - ^{125}I, it becomes not free to compete with the unlabeled antigen for binding sites on the Ab. This would result in either an overestimation or underestimation of the T_3 value depending on the method used to separate the bound and the free fraction as it will be discussed later in Paragraph 4.

2. By binding of the unlabeled antigen, T_3 to serum proteins it becomes unavailable to compete with the free tracer T_3 - ^{125}I, thus leading to underestimation of T_3 value.

3. The extent of binding of both labeled and unlabeled antigen will vary depending on the affinity of the antibodies used in the assay, and other assay conditions. If the antibodies used have very high affinity to T_3, the binding to serum protein becomes less significant than when low affinity antibodies are used.

4. The end result effect of binding to serum protein on serum T_3 value will depend on the method used to separate the free fraction from the bound fraction upon completion of the assay reaction. If the second antibody technique is used to precipitate the bound fraction, this precipitate will not include the serum protein-bound tracer, thus leading to less T_3 - ^{125}I in the bound fraction of the unknown than in the standard containing the same amount of T_3 but contains no serum proteins, this is interpreted as overestimation of the displacement produced by endogenous T_3 in the unknown. Whereas, if DCC (dextran coated charcoal) is used to adsorb the free fraction, the bound fraction will contain the serum protein-bound T_3 - ^{125}I leading to falsely

higher %B in the unknown over the standard which contains similar level of T_3 but no serum proteins which in turn is interpreted as lower than the actual T_3 value. This effect will be significant only if no serum proteins are used in the standard curve which should be set up to bring about the same protein environment as in the unknowns.

C. Approaches Used to Eliminate or Minimize the Effect of Serum Proteins

1. Use of Inhibitors which block T_3 binding to TBG and sometimes TBPA. Inspection of the reported direct RIAs for T_3 show that this methodology has varied not only in the substance used to block T_3 binding to serum proteins, but also in the concentration of this substance.

Substances used to block T_3 - binding to serum TBG:

a. Merthiolate (ethylmercurithiosalicylic acid)

b. ANS:8 anilino-1-napthalene sulphonic acid

c. TCT (Tetrachlorothyronine)

d. T_4 (Thyroxine)

e. Sodium salicylate (Na-Sal)

f. DPH (Diphenylhydantoin) or Dilantin

These substances are added in vitro to inhibit binding of T_3 - ^{125}I to TBG and to displace T_3 already bound to that protein, thus making both labeled and unlabeled T_3 free to compete for antibody binding sites. Table (1) gives authors who have used each one and the concentration used.

The mechanism of action of these substances is not well under-

stood. It is thought that they act through some similarity in structure occupying the binding sites on TBG and sometimes TBPA. Thyroxine (T_4) and teterachlorothyronine (TCT) have almost identical structures, only one halogen more than T_3. ANS is a substituted napthalene and was found to inhibit T_4 binding to serum proteins (62). Merthiolate, which is commonly used as a bacteriostatic agent, belongs to the salicylate group. Salicylates are known to increase free T_4 by blocking its binding to TBG and TBPA (20, 30, 31). DPH has diphenyl structure which is one of the chemical structures that bind TBG. The ideal performance of these inhibitors is effective inhibition of T_3 binding to TBG, noninterference with T_3 - Ab interaction, and having good solubility and stability at the assay conditions. Criterion for effective inhibition have not, unfortunately, been established by many authors who used these substances. A good criterion set up by Chopra et al in their second report (63) requires that the binding of T_3 - ^{125}I to the Ab in buffer environment be equal to or close to, its binding to the Ab when serum proteins and the inhibitor are added to the system. This would indicate that the presence of serum proteins (T_3 - free serum) has no significant effect on the reaction and that the inhibitor does not affect the antibody (no cross reactivity). Therefore, the type and concentration of the inhibitor should be selected on this basis to achieve effective inhibition and valid T_3 value.

Chopra and coworkers (64) have attempted to saturate the assay system with T_4. Theoretically, T_4 will displace T_3 from TBG binding sites because of its much greater affinity for TBG. But the known

contamination of T_4 preparations with varying amounts of T_3, carry the risk of introducing an unknown quantity of exogenous T_3, because the amount of T_4 added to each sample depends on the endogenous T_4 of the sample which has to be determined beforehand. Furthermore, the added T_4 might augment difficulties due to cross reaction with T_3 antibody. In their second report, Chopra et al (63) found ANS to be an effective inhibitor which showed no cross reactivity at the level used for the assay (Table 1). In a comparison study done by Hufner (65), ANS was found to be a very potent inhibitor; however, those authors found it to affect the antigen antibody reaction. This effect decreased if T_3 - free serum is present.

Merthiolate was reported not to interfere with T_3 - Ab binding (65). However, Sterling (66) has shown there is cross-activity when the compound is used in high concentrations. Gharib et al (67) obtained higher values for serum T_3 than any other reports on T_3 RIA (Table 3). In their complete report they used merthiolate, however, they show no evidence for effective inhibition of T_3 binding to serum proteins. Furthermore, T_3 - free serum was not used in the setting of their standard curves. This may have led to the overestimation of T_3 value due to the nonidentical protein environment in the standards and the unknowns. Merthiolate was found to be a weaker inhibitor by Hufner (65) who used it however, with higher pH (9.0-9.2), because at higher pH the binding affinity of T_3 and T_4 is decreased (33, 68).

Diphenylhydantoin, used by Utiger and Lieblich (18), does not

seem to interfere with the antigen antibody reaction. In their method, however, Utiger et al used 4% BSA solution in the standard curve to substitute for T_3 - free serum. This is close to human serum albumin concentration but does not represent all serum proteins. The main disadvantage of DPH is its limited solubility at the pH usually used for RIA. Teterachlorthyronine was used by Mitsuma et al (27) and found to be an effective inhibitor, but in later evaluation by Hufner, it was found to be an unstable compound and that it interfered in the Ag-Ab reaction by cross reacting with T_3 antibody, probably due to its content of trichlorothyronine. Addition of these compounds to the reaction mixture introduces an important methodological variable, and unless their effectiveness and concentration has been established for the particular assay system, they might lead to artifacts. Sodium salicylate was found by Larsen (31) to be an effective inhibitor of T_3 binding to TBG. Comparing it to ANS, Hufner found salicylate to be a potent blocker to TBG binding and it has less effect on T_3 antibody than ANS. Another practical advantage of salicylate is its stability in solution, thus not requiring the preparation of fresh solution for each run. This is an advantage over ANS which should be made up for each run because of its photosensitivity.

2. Heat Inactivation of TBG

Another approach was sought to eliminate or minimize TBG effect on this assay is the use of heat inactivation of serum proteins at $60^{o}C$. Sterling and his coworkers (34) found that TBG loses its ability to bind T_4 if serum is heated for 20 minutes at $60^{o}C$. Recently

those authors have applied this observation to T_3 radioimmunoassay (66). They used heat inactivation for 3 hours at 60°C; however, they found that the addition of merthiolate was essential to block, binding of T_3 to TBPA and without the use of merthiolate higher serum T_3 values were obtained. Despite the use of the heat and merthiolate at 1 µg/µl of serum, the values obtained by those authors were higher than most reported values and close to the values of Mayberry et al who used only merthiolate at 1 µg/µl but did not include T_3 - free serum in the standards assay. Therefore, this approach does not seem to offer any advantage over the use of a good inhibitor. They also did not study the effect of heating at 60°C on the antigen-antibody reaction.

II. Sensitivity of T_3 Radioimmunoassays

Sensitivity is an essential requirement of T_3 RIA because of the very low level of T_3 in human serum. From Tables 1 and 3 it is apparent that most of the methods were able to detect the low hypothyroid level, except in the first report of Chopra et al (64). The lack of sensitivity in that assay can be attributed in part to the low affinity of the antibody which was produced using human thyroglobulin as an immunogen (Table 1), to the use of T_4 as the blocking agent of T_3 - TBG binding, and partly to the presence of some T_3 in the hypothyroid sheep serum used in setting up the standard curve.

There is no specific correlation between the type of immunogen used and the affinity of the Ab produced by that immunogen. It should be noted that, besides the affinity of the antibody, other factors such

as antibody dilution, effective inhibition of T_3 binding to serum proteins, delayed addition of the tracer, and method of separation, have significant effect on the sensitivity. Therefore, sensitivity obtained in the various methods cannot be attributed only to the affinity of the Ab. The lower detectable limit of T_3 RIA is between 10-60 pg/assay tube (Table 2). 10 pg/tube corresponds to 40 ng/100 ml, if 25 µl of serum is used and to 10 ng/100 ml if 100 µl of serum is used as a sample. In most reported assays hypothyroid patients were within the detectable limits.

III. Sepcificity of the Reported T_3 - Antibodies

The production of antibodies specific for T_3 was the key for the valid application of direct RIA to the determination of this hormone. It is very essential that the antibody used has no or insignificant cross reactivity with T_4 which is similar in structure and present in relatively high concentration. The percent cross reactivity of T_4 with the reported T_3 - antibodies varied between .02% to 2.5% (Table 2). These values can't be taken to represent true T_4 cross reactivity with T_3 - antibodies, because commercial T_4 preparation used in most of these studies contains variable amounts of T_3 (67, 38). Mitsuma et al (70) used purified T_4 in their study; therefore, they obtained a value of .02% which can be considered the true cross reactivity of their antibodies. Chopra et al (64) and Stanley et al (69) obtained the highest values (Table 2). This has been attributed to the use of human thyroglobulin (which has T_3 and T_4) as an immunogen

by Chopra who later used T_3 - thyroglobulin conjugate (produced by coupling more T_3 to thyroglobulin) to obtain better specificity. Stanley observed an improvement in specificity if the same antibodies are used in solid phase methodology (69). All the antibodies listed in Table 2 appear to possess satisfactory specificity to be used for serum T_3 measurement. There is no correlation between the relatively higher T_3 values obtained by Gharib et al (67) and Sterling (66) and the cross reactivity of their antibodies with T_4. This is probably due to the other methodological variable in each assay.

TABLE 1

Inhibitors, Extraction Methods, and Separation Methods Used in Different Reports on T_3RIA

Author & Ref.	Method of Eliminating Serum Proteins Effect	Concentration of Inhibitor Used Per µl Serum	Method Used for Separation
Brown et al (56)	Extraction	--	--
Mitsuma et al (70)	TCT	2 µg/µl	MCCC
Gharib et al (67)	Merthiolate	1 µg/µl	DA
Chopra et al (64)	T_4	6 µg/µl	DA
Chopra et al (63)	ANS	1 µg/µl	DA
Sekadde et al (75)	ANS	1.1 µg/µl	PEG
Utiger et al (18)	DPH	0.5 µg/µl	DA
Surks et al (78)	Extraction	--	DCC
Patel & Burger (77)	Extraction	--	DA
Evered & Hesch (74)	Na-Salicylate	50 µg/µl	DCC
Larsen, P. L. (73)	Na-Salicylate	30 µg/µl	DCC
Hufner & Hesch (68)	Merthiolate	1 µg/µl	DCC
Sakurada et al (72)	DPH or DNP or Extra	--	DCC
Shimizu et al (71)	Extraction	--	Sephadex
Stanley et al (69)	Solid Phase	--	--
Sterling, K. (66)	Heat at 60°C and Merthiolate	1 µg/µl	PEG
Rubenstein et al (79)	Extraction	--	DA

Abbreviations :

Merthiolate

T_4 Thyroxine

ANS 8-Anilino-1-napthalene-sulfonic acid

DPH Diphenyl hydantoin

DNP Dinitrophenol

DA Double Antibody

DCC Dextran Coated Charcoal

MCCC Methyl Cellulose Coated Charcoal

PEG Polyethylene Glycol

TCT Teterachlorothyronine

TABLE 2

Characteristics of T_3 - Antibodies as Obtained by Different Authors

Author & Ref.	Immunogen used for Ab Production	T_4 Cross Reactivity %	Sensitivity* Pg/assay tube
Brown et al (56)	T_3ME-SPLL	2.0	20
Mitsuma et al (70)	T_3ME-PLL	0.02 (P)	12.5
Gharib et al (67)	T_3-HSA	0.1-0.3	50
Chopra et al (64)	Tg (human)	1.3	400
Chopra et al (63)	T_3 - Tg	0.05	--
Sekadde et al (75)	T_3 - HSA	0.2	--
Utiger et al (18)	T_3 - HSA	0.14	60
Surks et al (78)	T_3 - BSA	0.1-0.2	30
Patel and Burger (77)	T_3 - BSA	0.2-1.2	--
Evered and Hesch (74)	T_3ME - BSA	0.1	--
Larsen, P. L. (73)	T_3 - BSA	0.11	25
Hufner and Hesch (68)	T_3ME - BSA	0.3-0.5	10
Sakurada et al (72)	T_3ME - BSA	0.21	--
Shimizu et al (71)	T_3 - BSA	0.071	--
Stanley et al (69)	T_3 - BSA	2.5	50
Sterling, K. (66)	T_3 - BSA	0.5-1.4	10
Rubestein et al (79)	T_3 - BSA	0.3	25

Abbreviations

T_3ME = T_3 Methyl ester
SPLL = Succinylated Poly-L-Lysine
PLL = Poly-L-Lysine
HSA = Human Serum Albumin
BSA = Bovine Serum Albumin
(P) = Purified T_4 used for cross reactivity study
Tg = Thyroblobulin
* Minimum detectable amount

TABLE 3

Concentration of T_3 (ng/100 ml) in Human Serum in Normal and Abnormal Thyroid States as Obtained by Different RIAs

Source & Ref.	Euthyroid Mean ± SD or Range	Hypothyroid Mean ± SD or Range	Hyperthyroid Mean ± SD or Range
Brown et al (56)	75 - 160	<75	290 - 860
Mitsuma et al (70)	138 ± 23	62 ± 9	494 ± 265
Gharib et al (67)	215 ± 43	103 ± 43	760 ± 289
Chopra et al (64)	100 - 170	undetectable	160 - 1300
Chopra et al (63)	112.8 ± 3.3	40.1 ± 7.6	490.7 ± 42.3
Sekadde et al (75)	146 ± 25	50	367 ± 99
Utiger et al (18)	145 ± 25	99 ± 24	429 ± 146
Surks et al (78)	146 ± 24	44 ± 26	665 ± 289
Hesch & Burger (77)	130 (mean)	38 - 127	450 (Mean)
Evered & Hesch (74)	126 ± 23	- - 107	177 -1188
Larsen, P. R. (73)	110 ± 25	39 ± 21	188 - 2380
Hufner & Hesch (68)	120 ± 30	--	--
Sakurada et al (72)	--	--	--
Shimizu et al (71)	148 ± 24	52 ± 20	425 ± 110
Stanley et al (69)	--	--	--
Sterling et al (66)	189 ± 30	47 ± 39	838 ± 398
Rubenstein et al (79)	119 ± 6.3(*)	20 - 92	76 - 556

(*) Difference due to age was observed by this group
(5-9) years old have 140.8ng/100 ml
(80-93) years old have 92.2 ng/100 ml

MATERIALS AND METHODS

I. Materials and Equipment

A. Materials

1. Tri-iodothyronine antibodies

a. From Wien Laboratories, Inc., Succasunna, N. J.

b. From Endocrine Sciences, Tarzana, California

c. From Pantex, Malibu, California

2. The following materials were purchased from Sigma Chemical Company, St. Louis, Mo.

a. Tri-iodothyronine (T_3)

3, 3', 5-Triiodo-L-thyronine, free acid

b. Thyroxine (T_4)

3, 3', 5, 5'-teteraiodo-L-thyronine, free acid.

c. Glutamic acid

L - aminoglutaric acid, free acid.

3. Triiodothyronine (T_3): 3,3', 5-Triiodo-L-thyronine sodium from Schwartz/Mann, Orangeburg, New York

4. T_3 - ^{125}I

a. L-Triiodothyronine, ^{125}I (liothyronine, T_3) supplied as the Na-salt in 50% proplylene glycol, Specific activity (80-100 mci/mg) and concentration of 0.1 mci/ml. From Industrial Nuclear Company, St. Louis, Mo.

b. ^{125}I Triiodothyronine solution with specific activity (300-400 mci/mg) from Abbott Laboratories. Supplied in small quantities through Wien Lab., Inc., Succasunna, N. J.

5. ANS, 8-Anilino-1-Napthalene-sulfonic acid. Sodium salt from K and K Laboratories, Inc., Plainview, N. Y.
6. Merthiolate (Thimerosal Powder) from Eli LIlly & Co. Indianapolis, Ind.
7. Barbital Buffer, B-2 Buffer, pH 8.6, ionic strength 0.075 contains 2.76 gm Diethyl Barbituric Acid, 15.40 gm Sodium Diethyl Barbiturate. From Spinco Division of Beckman Instruments, Palo Alto, California.
8. The following materials were obtained from Mallinckrodt Checmical Works, St. Louis, Mo.:
   Boric Acid, Na-salicylate, ammonium sulfate, Amberlite IRA-400 (AR), concentrated HCl, concentrated ammonium hydroxide, and sodium hydroxide.
9. The following materials were obtained from Matheson Colman and Bell, Los Angeles, California:
   Sodium Azide, Activated Charcoal Norit A, Tetra-Aml Alcohol (2 methyl-2-butanol).
10. Ion exchange resins (all were Analytical Grade)
    AG 50 W - X 2 100-200 mesh (H) form
    AG 50 W - X 8 20-50 mesh (H) form
    AG 50 W - X 4 50-100 mesh (H) form
    Ion exchange columns "Tri-count" and Ion exchange columns "Tetra count"
    All were purchased from BioRad Laboratories, Richmond, California.

11. Bovine Serum Albumin powder, Metrix, Chicaog, Illinois (Reagent Grade).
12. Polyethylene Glycol 6000 and Hexane, from J. T. Baker Checmical Co., Phillipsburg, N. J.
13. Propylene glycol from Fisher-Scientific Company, Fairlawn, N. J.
14. Dextran T70 from Pharmacia Fine Chemicals, Inc., Piscataway, N. J.
15. Versatol Pediatric Control serum from Warner-Lambert Company, Morris Plains, N. J.
16. Anti IgG (second antibody) solution from Pantex, Malibu, California
17. Gamma Globulin, Fraction II from Sigma Chemical Company, St. Louis, Mo.

All reagents were Analytical or Reagent Grade

B. Equipment

1. Test Tubes

   12 x 75 mm and 17 x 150 mm from Falcon Plastics, Oxnard, California.

2. Micropipets

   a. Lang-Levy type: Ultrapette pipets size from 10 µl - 200 µl from Scientific Products, Evanston, Illinois or from Interex Corp., Waltham, Mass.

   b. Eppendorff microliter pipet 100, 200, 500 µl from Brinkman Instrument, Inc., Cartiague Road, Westbury, N. Y.

3. Packard Automatic Counter with Tri-Carb Scintillation Spectrometer Model 2002. Packard Instrument Company, Inc., Downers Grove, Ill.

4. Automatic Gamma Well Counting Systems. Nuclear-Chicago Corporation, Des Plains, Ill.

5. Automatic Refrigerated Centrifuge
   Sorval General Purpose RC-3 from Ivan Sorval, Inc., Newton, Connecticut.

6. Water Bath A
   Temp. Range room temperature to 70^{o}C from Chicago Surgical and Electrical Company, Chicago, Illinois.

7. Water Bath B
   Temp Range room temperature to 70^{o}C from Chicago Surgical and Electrical Company, Chicago, Illinois.

8. pH Meter
   Cenco, Centeral Scientific Company

9. Millipore Filter Swinnex-25, Millipore Corporation, Bedford, Mass.

10. Calculator
    Litton Monroe 1320, Monroe, Orange, N. J.

11. Computer

Oregon State University Computer Center through Terminal at the University of Oregon Medical School, Department of Clinical Pathology.

II METHODS

A. Reagents Preparation

1. T_3 - Free Serum Preparation (T_3 1 FS)

To establish a similar protein environment in the standards and in the unknowns, human serum from which T_3 was removed was added to the standards. T_3 - free serum was prepared as follows: T_3 - ^{125}I, to give approximately 10,000 counts/ml, was added to 100 ml of pooled human serum and incubated at $4^{o}C$ for two hours. Then about 7 g of Norit A declorizing charcoal were added per 100 ml of serum and mixed at room temperature for a period of three hours with a magnetic bar. The mixture was then centrifuged initially for 15 minutes and then for four successive 45 minute periods at 3800 g in a refrigerated centrifuge. The clear supernate was removed after each centrifugation. Following that, it was found essential to remove the very fine remaining charcoal particles by filtration through a 0.45 μ millipore filter. Efficiency of the treatment in removing T_3 was calculated as follows:

$$0/0\ T_3 - I^{125}\ \text{Remaining} = \frac{\text{CPM/ml of serum after treatment - CPM background} \times 100}{\text{CPM/ml of serum before treatment - CPM background}}$$

In earlier studies to estimate the required time of incubation with charcoal, longer time fo incubation was used but found to be not necessary (Table 7). Ion exchange resin IRA 400 was also used but found to require

longer incubation time and is more expensive; however, it is "cleaner" to work with.

Quality control testing on T_3 - free serum preparation

a. To evaluate the T_3 binding capacity of this preparation, the T_3 resin uptake (Abbott Trisorb) was performed (Table 9).

b. To determine the amount of T_4 remaining, thyroxine level by competitive protein binding was done before and after treatment of serum (Table 9).

c. To further investigate T_3 content of this preparation, T_3 determination by RIA was done on 50 µl of a sample with and without 50 µl of T_3 - FS (Table 8).

2. Dilutent is a buffer (Barbital or Borate) containing 0.5 Gamma Globulin Fraction II or 0.5% BSA (Bovine Serum Albumin). This solution was kept refrigerated when not in use.

3. Preparation of standard solutions of T_3

25 mg of T_3 (Free acid, Sigma) was dissolved in a 250 ml volumetric flask with 9N NaOH which was added until a clear solution was obtained. The volume then was completed to 250 ml with buffer (Barbital or Borate) containing 0.5% Gamma Globulin Fraction II. That gave a 100 µg/ml solution. Working standards containing 10, 5, 1, and 0.5 ng/ml were made by further dilution of the stock standard with buffer containing 0.5% BSA and sodium azide 0.2%. These working standards were aliquoted in 1 ml amounts into plastic tubes and kept frozen at -20°C. For useage, one tube or more of each working standard was

thawed and 100 µl was used per assay tube to give 50, 100, 500, 1000 pg/tube. When 250 pg/tube was included, 50 µl of 5 ng/ml working solution was used and diluted to 100 µl with 50 µl diluent.

4. Preparation of T_3 - ^{125}I Solution

Radioactive T_3 - ^{125}I was supplied from two sources: the Wien Laboratories Inc., material which was supplied in small amounts was diluted with buffer to give 80-100 pg/100 µl of solution. The tracer supplied by Industrial Nuclear was also diluted with buffer (60 - 100 pg/100 µl). All solutions were kept at $4^{o}C$ when not in use and brought up to room temperature before use.

5. Preparation of T_3 Antibody Solution

The antibody was supplied in lypholyzed form and reconstituted with diluent as follows:

a. Antibody from Wien: each vial was reconstituted with 5 ml diluent to give a 1:500 dilution of the original antiserum. Further dilution was made according to the desired final dilution to be used in the experiment 1:5000 to 1:10,000.

b. T_3 - Antibody from Endocrine Sciences: Each small vial containing freeze-dried antibody was reconstituted with deionized water according to the instructions on the vial to give a 1/20 dilution. This was further diluted to 1:70 in diluent solution to give 1:1400 dilution. One hundred µl of the this solution was used per assay tube which had a total volume of 0.5 ml. Thus the final dilution of the antibody is 1:7000.

All antibodies were kept refrigerated when not in use.

6. Buffers

a. Borate Buffer 0.2 M pH 8.5 or 9.0, 0.05% sodium azide. In a one liter volumetric flask 12.35g reagent grade boric acid crystals, 6 ml of 9N NaOH, and 0.5g of sodium azide were mixed and the volume was completed gradually to the 1000 ml mark with deionized water. The solution was mixed until all the crystals were dissolved. The pH was checked with a pH meter and adjusted to 8.5 or 9.0 with 1N NaOH or 1N HCl.

b. Barbital Buffer pH = 8.6 ionic strength 0.075 supplied in bags. Contains 2.75 g diethyl barbituric acid, 15.40 gm sodium diethyl barbiturate. The contents of bag was dissolved in 1000 ml deionized water and the pH adjusted when needed to 8.6 with 1N NaOH or 1N HCl.

c. Glutamate Buffer, pH = 3.3

Glutamic acid, 1.2g was dissolved in 200 ml of water. One ml of KCN solution (4g/liter) was added. The pH was checked and adjusted to 3.3 with molar HCl or NaOH if necessary. Stable for six months at room temperature.

7. Ammonium Sulfate

To 1000 ml of deionized water increasing amounts of $(NH_4)_2SO_4$ was added and mixed till excess crystals were seen despite mixing for 2 hours using automatic shaker. Various solutions, 80%, 60% and 40% were prepared by diluting the saturated solution with deionized water or buffer.

8. Polyethylene glycol solution

To prepare a solution of PEG the weighed amounts were put in volumetric flasks and deionized water added and mixed slowly. Then the mixture was left at room temperature for 20 minutes (to hydrate PEG), and the volume completed with deionized water. Forty, 30, 20 and 17% W/V solutions were prepared in that manner.

9. Dextran Coated Charcoal Suspension:

a. Solution of dextran: One-half gram of Dextran 70 was mixed till dissolved in 100 ml barbital buffer pH = 8.6.

b. Charcoal suspension: 5 g of charcoal, Norit A was suspended in 100 ml of buffer using a magnetic bar. Equal volumes of (a) and (b) were mixed for 1 minute and then refrigerated for at least 2 hours before use. It was always used cold. This suspension was found to be stable for at least three months.

B. Equipments Check

1. Automatic Gamma Counter (Packard)

a. Spectrometer setting for ^{125}I counting - The optimal gain level was found at 900 (coarse) and 10 (fine gain) and kept constant after calibration. Base of 26 and window of 200 KEV was found best for optimal efficiency and elimination of instrument noise. Background counts at these settings was 40-60 CPM and the use of wide window (200) did not increase the background radiation and led to better stability than the use of narrow window of 100 or less.

b. Estimation of counting error at different levels.

Counting error was estimated by counting the same tube 15-24 times each for 1 minute. The standard deviation (SD) and coefficient of variation (CV) was calculated to represent % counting error. This procedure was followed at different levels of counts ranging from background level (50 CPM) to 50,000 CPM. Table (4) gives the results of this study.

TABLE 4

Estimation of Counting Error of Gamma Counter (Packard)

Exp. No.	CPM Range	Mean	SD	No. of Determination	CV or % Error
1	43-62	54	6.5	17	11.8
2	1465-1603	1451	43.83	21	2.84
3	9599-9978	9795	110.41	24	1.12
4	20310-21060	20617	177.72	20	0.80
5	54900-55400	55100	250.00	15	0.41

It is clear that the counting error decreases significantly as the accumulated counts increased.

c. Effect of change in volume of solution counted on the efficiency of the gamma counter (Packard). The effect of increasing the volume of the solution counted was assessed by increasing the volume of the same amount of T_3 ^{125}I with deionized water and counting

the tube 10 times after each increase. Table (5) gives the observed change.

TABLE 5

Effect of Volume on the Counts in Packard Counter

Volume	Counts (Mean)	# of Determination
0.3 ml	20617	(10)
0.6 ml	20390	(10)
1.4 ml	19454	(10)

Only increase in volume above 1 ml has decreased the observed counts significantly. This experiment was done under the same conditions (geometry and type of tubes) used for routine counting throughout the study.

2. Precision of microliter pipets

   a. Lang-Levy pipet (100 µl)

   b. Eppendorf pipet (100 µl)

Those pipets were usually used to add T_3 ^{125}I to the assay. To determine the CV of the amount of tracer delivered by these pipets, T_3 ^{125}I solution was pipetted into a series of 15 plastic tubes and a one minute count was taken for each tube.

Pipetting error was estimated by subtracting the counting error from the observed error in this experiment (Table 6).

TABLE 6

Comparison of the Microliters Pipets

	Lang-Levy (100µl)	Eppendorf (100 µl)
Counts Range	54121-55300	19432-20540
Mean	54608 .	19874
SD	422	329.8
CV	0.77%	1.60
No. of Determination	15	15
Counting error	0.50%	0.86
Pipetting error	0.27%	0.74

Lang-Levy gave better precision; however, using this pipet for many tubes is tedious and for the purpose of adding the tracer Eppendorf pipet was used routinely.

C. Experimental T_3 Radioimmunoassay Procedure

This basic procedure was followed while studying the effect of different variables on the assay. Any departure from this procedure is specified in the appropriate experiment.

1. Dose Response Curve (standard curve)

A standard curve was set up for each run of T_3 RIA following this basic protocol.

In 15 x 75 plastic tubes, add:

100 µl	T_3 - free serum
100 µl	T_3 standard solution containing 0, 50, 100, 250, 500, or 800 pg T_3/100 µl
1-400 µl	Diluent. The amount added depends on the antibody dilution and the method used to separate free and bound fractions
100 µl	Inhibitor solution (when no inhibitor was used, 100 µl of diluent replaced it.)
100 µl	$T_3{}^{125}I$ solution (contains 60-100 pg $T_3{}^{125}I$)
100 µl	T_3 - antibody solution (dilution of which is determined for the particular experiment.)

Each standard was run in duplicate or tiplicate. Standard with no T_3 antibody was called Blank or B^o. Nonspecific binding controls contain all constituents listed above except T_3 antibody and T_3 standard solution. To replace these, 200 µl of diluent is added.

2. Samples

Samples were set up the same way as standard except for the use of 100 µl of the serum sample to replace T_3 - free serum and 100 µl of diluent to replace the standard solution. When less than 100 µl of serum was used to determine the T_3 level of a sample, the volume was completed to 100 µl with T_3 - free serum.

3. Incubation Time and Temperature

Initially two incubation periods were used, first at 37°C for 30-45 minutes, and second at 4°C for 90-120 minutes.

4. Separation of Bound from Free Fraction

Separation of the bound from free fraction was achieved by one of the following:

a. Dextran coated charcoal (DCC)

b. 45% saturated ammonium sulfate (SAS)

c. Polyethylene glycol (PEG)

d. Ion exchange resins

The procedure followed when these methods were used will be described under Separation Method Study.

5. Calculation and Standard Curve Plot (graphic representation)

Two types of plots were used:

a. T_3 standards concentration in picogram/tube (horizontal axis) against B/B^O on the vertical axis. To obtain B/B^O values the following formula was used.

$$\% \; B/B^O = \frac{B - NSB}{B^O - NSB} \times 100$$

Where B = CPM in the bound fraction of the standard or unknown. NSB = Average counts found in the nonspecific binding control (Triplicate). B^O = Average counts found in the zero standard (Blank B^O) (Triplicate). Linear graph paper was used.

b. T_3 standards concentration in picogram/tube against B^O/B. To obtain B^O/B values the following formula was used:

$$\frac{B^{O}}{B} = \frac{B^{O} - NSB}{B - NSB}$$

Average of the ratios of each standard level was plotted.

Linearization: To obtain the equation for the line of best fit, these ratios (B^{O}/B) were fed into the department terminal of a computer with the corresponding T_3 concentration as ng per tube. Pearson r (correlation coefficient) and the equation of the line was obtained. Linearization of the standard curve was considered valid only if the correlation coefficient (r) was 0.99 and above for the five points usually used (.05, 0.1, 0.25, 0.5, and 1ng/tube).

D. Titration of Antisera

Antibody titer was assessed by adding increased amounts of the antisera to a series of assay tubes containing constant amounts of T_3 - ^{125}I (100 pg) in diluent. Volume was completed to 0.9 ml in all tubes with diluent. After incubation at $37^{O}C$ for 30 minutes and at $4^{O}C$ for 2 hours, free and bound fraction were separated by DCC. The %B radioactivity was determined (NSB was not subtracted) and plotted against the amounts of antisera added. Figure (1) shows Wien Ab titration curve and Figure (2) shows Endocrine Sciences Ab titer curve. The amount of these antisera which bind 60-65% of the T_3 - ^{125}I added was used for radioimmunoassay of T_3. However, at certain occasions higher dilution of these antibodies were used (30-40%B).

Comparison of two lots of antisera from Endocrine Sciences was done. Table (10) shows the results of simultaneous titer of both lots.

E. Evaluation and Selection of a Mean to Eliminate the Effect of T_3-Binding Proteins on T_3 Radioimmunoassay

1. Evaluation of substances used to inhibit T_3-binding to TBG

The objective of this study was to compare the effectiveness of the three most commonly used inhibitors, ANS, sodium salicylate, and merthiolate, and to determine the optimal concentration for effective inhibition under actual assay conditions.

Preliminary experiments by several authors (63, 64, 70), showed that T_3-binding proteins in serum, such as TBG, interfere in T_3 RIA by competing with T_3-antibody for the tracer T_3-^{125}I and T_3. Thus the amount of T_3-^{125}I bound to the antibody is decreased when T_3-free serum is added to the assay. The criterion of effectiveness of an inhibitor is its ability to make the binding of T_3-^{125}I to the Ab in the presence of T_3-free serum and the inhibitor equal to its binding to the Ab in absence of T_3-free serum.

To compare these substances, a series of blanks (contain 100 µl T_3-free serum, 100 1 T_3 ^{125}I and 100 1 diluent) was set up and put into three groups, A, B, and C. To group A, 0 - 0.15 g/dl of ANS was added. To group B increased amounts of 0 - 1.5g/dl sodium salicylate was added. To group C, 0 - 0.15 g/dl merthiolate was added. In the same run a quadruplet of tubes containing the same amounts of Ab and T_3-^{125}I but no T_3-free serum was run. Volume of final assay mixture was kept at 0.5 ml in all tubes by adding buffer when needed.

The experiment above was also set up using normal serum sample instead of T_3-Free serum); but only with sodium salicylate. This was done

to compare the inhibition pattern in the presence of unlabeled T_3, (Figure 4).

All tubes were incubated for 30 minutes at 37°C and then at 4°C for 2 hours. Separation was achieved by 45% saturated ammonium sulfate (SAS) and radioactivity in the bound fraction was determined for each tube. %B (B/T x 100) was determined and averaged for each duplicate and plotted against the inhibitor concentration on linear graph paper, (Figure 3 and Figure 4).

2. Evaluation of heat inactivation of TBG.

a. Heat inactivation of TBG at 60°C.

To study this method, the following experimental run was set up.

(1) Standards and four serum samples were set up with the use of ANS (0.06 g/dl) as inhibitor following the basic T_3 RIA procedure.

(2) Standards and the four samples were set up with the use of heat inactivation at 60°C for 45 minutes. T_3-free serum in the standard tubes, and serum samples were put in plastic tubes and heated at 60°C in a water bath for 45 minutes then cooled on ice for 10 minutes. Following that, the T_3 RIA procedure described was followed.

(3) A third group of standards and the four samples were set up without any inhibitor or heat inactivation.

General T_3 RIA procedure was followed and separation by DCC. Ab from Wien was used at dilution of 1:5,000. Figure (6) compares the standard curves obtained under these conditions and Table (12) compares the samples T_3 values obtained from the corresponding standard curve.

b. Heat inactivation of TBG at 100°C

In order to decrease the time of heat inactivation, we sought the use of higher temperature. In preliminary trials, we found that heating the sera above 60°C led to precipitation of the denatured proteins, and the use of higher temperatures requires low pH to avoid that precipitation.

Our preliminary work to determine the assay condition using heat inactivation at 100°C led to the following findings.

1. Low pH is very essential in the range of 3.3 (glutamic buffer) (80) because when acetate buffer at pH 4.6 was used, serum protein precipitated upon heating.

2. The amount of glutamic buffer with which the serum is heated is dependent on the amount of serum used, for 100 µl of serum, we found that 0.6 ml of buffer is essential to insure solubility of the denatured proteins.

3. Restoring the pH usually used for the assay (8.6-9.0) is possible by adding high pH solution of 12.2 (Barbital buffer and 1 N NaOH), and does not cause reprecipitation of serum proteins.

4. Plastic tubes generally used cannot tolerate heat at 100°C for 15 minutes. Therefore, precoated glass tubes were used (coating was achieved by filling 15 x 75 glass tubes with barbital buffer containing 0.5% BSA and transferring its contents to the next tube, etc.)

5. T_3 standard solutions have to be prepared in glutamic buffer to keep the pH low when heated with T_3-free serum.

Based on these findings, the following protocol for T_3 RIA with the use of heat inactivation at 100°C was adopted.

Standards

In precoated glass tubes (15 x 75) add

glutamic buffer pH 3.3	600 µl
T_3-free serum	100 µl
T_3 standard solution	100 µl

Heat in boiling water bath for 15 minutes, then cool on ice to room temperature and add

high pH solution (pH 12.2)	200 µl
T_3-^{125}I	100 µl
T_3-antibody	100 µl

Samples were set up in analogous way except for the use of 100 µl of serum sample to replace T_3FS in the standard and 100 µl buffer to replace T_3 standard solution.

Following that incubation, separation, and calculation was completed as in regular T_3RIA procedure. To compare this method with the use of an inhibitor, two standard curves and some samples were run, one following the T_3RIA with ANS and one using heat inactivation as described above. Figure (6) gives the standard curve obtained and Table (12) compares the T_3 values of the samples. The heat inactivation method gave consistently higher serum T_3 values. Since no significant difference is seen between the dose-response curve obtained with the use of heat inactivation and the one obtained with ANS, conversion of T_4 to T_3 or to a similar substance which cross react with T_3 antibody was hypothesized and this hypothesis was tested in the next experiments.

3. Effect of heat inactivation of human serum at 100°C on the stability of T_4

To test the hypothesis that T_4 might be converted to T_3 upon heat inactivation of human serum at 100°C for 15 minutes, T_3 radioimmunoassay using heat inactivation procedure was carried out on two groups of assay tubes (1) and (2). Group (1) assay tubes containing 300 pg T_3/tube and to which 10 or 20 µg T_4/tube was added before the heat inactivation step (A) or after heat inactivation (B). Group (2) assay tubes contain only T_4, to which 20 µg T_4/tube was added before heat inactivation (A), or after heat inactivation (B). The heat inactivation procedure was followed as described earlier. Separation was achieved by dextran coated charcoal. T_4 working solution was prepared in glutamic buffer and the T_3 antibody employed in this experiment was from Wien laboratory.

The T_3 values obtained were compared in Table (13). Difference between A and B values can be attributed only to the effect of heat on T_4 preparation. Net increase of T_3 value between A and B was calculated for each level of T_4 and the rate of increase in the T_3 value was estimated by dividing the net increase in T_3 value in these two groups over the amounts of T_4 added, Table (14).

F. Selection of the optimal pH for T_3 RIA

The binding of T_3-^{125}I to T_3-Ab in series of blanks at pH range from 7.1 to 9.5 was determined. Using barbital buffer to establish pH from 7.1 to 8.4 and borate buffer to establish pH from 8.5 to 9.5 (pH adjusted with 1N HCl or 1N HaOH). Radioimmunoassay procedure was

carried out on these blanks. Separation of the bound fraction was achieved by 45% saturated ammonium sulfate. Percent T_3-^{125}I bound was determined and plotted against the pH used. Figure (7). The nonspecific binding was checked at pH 7.1, NSB = 10.4% and pH 9.0, NSB = 5.3%.

Upon the finding that optimal binding is at pH = 9.0, the effect of this maximal binding to T_3-Ab on the sensitivity of the assay and on serum T_3 value was studied. Two standard curves and eight samples were run with each one at pH 8.4 and 9.0. Figure (8) and Table (15) compare the results obtained. Borate buffer was used in both cases and regular RIA and calculation was done. Linearization was valid because correlation coefficient is 0.999 in both conditions.

G. Study of Methods of Separation

In the search for a specific, reproducible and practical method for separating the free antigen from antibody-bound antigen, the following methods were evaluated:

1. Polyethylene glycol (PEG)
2. Saturated amonium sulfate (SAS)
3. Ion exchange resins (IER)
4. Dextran coated charcoal (DCC)

1. Polyethylene glycol (PEG)

Polyethylene glycol has been used originally to precipitate the antibody-bound antigen in the radioimmunoassay of growth hormone and of T_3 (75, 81). The concentration of the PEG used to achieve optimal separation (specific precipitation of the antibody-bound antigen), depends mainly on the serum protein concentration and the pH of the

assay. For a particular assay, the concentration of PEG has to be selected to give optimal separation and minimal nonspecific precipitation.

a. Determination of PEG optimal concentration for T_3 radioimmunoassay.

To determine the optimal concentration, two experiments were set up and carried out at pH 9.0 (Barbital buffer).

(1) A series of blanks and a series of nonspecific binding controls were run (duplicates of each for each concentration of PEG to be studied). ANS was used as an inhibitor. Regular T_3 RIA protocol was followed. At the end of incubation PEG solutions concentration of which are 10, 15, 20, 25, 30 and 40 g/dl were added in equal volume to the assay total volume (0.5 ml). Thus the final PEG concentration was 5, 7.5, 10, 12.5, 15 and 20 g/dl. To enhance the precipitation, 100 µl of T_3-free serum was added to each tube immediately after the PEG. All tubes were mixed well (on a Vortex mixer) and kept in an ice bath for 10 minutes after which they were centrifuged at 3200 g for 12 minutes. The supernate was decanted and discarded. Absorbent paper used to remove the few drops at the top of each tube. Radioactivity in the precipitate in each tube was determined. A plot of percent T_3-^{125}I in the precipitate (in both the blanks and the nonspecific binding controls) against PEG final concentration is given in Figure (10).

(2) To compare the precipitation pattern obtained in the

actual assay conditions (as done in the previous experiment) with the precipitation pattern of the antigen-antibody complex in diluent only (no serum proteins), a series of tubes containing T_3- ^{125}I 100 µl, 200 µl T_3-antibody and 700 µl barbital diluent were set up and treated as described in the previous experiment. The percent T_3-^{125}I precipitated was determined and plotted against PEG final concentration (Figure 11). Specific precipitate is percent T_3-^{125}I precipitated in the blanks which contain both the antibody and T_3FS minus the NSP (%B - %NSP).

b. The effect of some methodological factors on the separation by PEG.

(1) The addition of T_3-FS to enhance precipitation.

(2) The preparation of PEG in DW (deionized water) instead of buffer.

(3) The use of BSA vs. the use of Gamma Globulin II as a protein in the diluent.

To study the effect of these three factors, five blanks were set up under each condition and compared with 5 blanks set up under the opposite condition; 5 blanks with T_3-FS as carrier protein vs. 5 blanks without T_3-free serum, etc. Tables (17) and (18) give the results of this comparison.

(4) Upon finding the optimal concentration of PEG between 10-14%, another approach was used to minimize the nonspecific precipitate. This depends on the use of larger volume of a less concentrated PEG to reach the same final concentration; therefore, 1.5 ml of 17% PEG was added to 0.5 ml assay final

volume to reach final concentration of PEG 12.75.

2. Use of saturated ammonium sulfate (SAS) for separation.

Two concentrations of ammonium sulfate were studied for their ability to precipitate the antibody-bound fraction, 50% and 45%.

a. Use of 50% saturated ammonium sulfate.

Saturated solution of ammonium sulfate was added in equal volume to the assay mixture (0.5 ml), mixed well (Vortex mixer) and incubated at $4^{o}C$ for 10 minutes, then centrifuged at 3200 g for 30 minutes. The supernatant was decanted and discarded and the precipiate was counted. Despite the long centrifugation time, the precipitate was not firm. Therfore, decanting the supernate has to be fast.

b. Method used for 45% SAS

1.5 ml of 60% SAS was added to each tube (contining 0.5 ml assay mixture); thus the final concentration of ammonium sulfate is 45%. Tubes were then mixed (Vortex mixer), incubated at $4^{o}C$ for 10 minutes and centrifuged at 3200 g for 12 minutes. Supernatant was discarded and radioactivity in the precipitate was determined if assay was run without washing step.

To wash the precipitate 2 ml of 45% SAS in borate diluent was added to each tube and the precipitate was resuspended (on Vortex mixer) and centrifuged again as described. The final precipitate was counted. This has been found to reduce the nonspecific binding precipitate to 2-5%.

3. The use of ion exchange resins for separation

In the search for an ion exchange resin which has the ability to

separate the free T_3 from the bound T_3, the following resins were tested.

AG 50W - X2 Mesh (100-200) H form

AG 50W - X4 Mesh (50-100) H form

AG 50W - X8 Mesh (20-50) H form

Amberlite IRA 400

Also, a prefilled resin column used for T_3 resin uptake (Biorad T_3 resin uptake kit) was evaluated for the same purpose.

Preparation: The resins were washed several times with deionized water and then let to dry. Biorad columns were shaken before washing with purified water present in the columns. The bottom tip of each column was broken and the top lid opened to let the resin settle and the water drain out. This was done immediately before the run.

To evaluate these resins a blank, 500 pg/tube standard and a nonspecific binding control, each in triplicate was set up for each resin. Regular procedure for T_3 RIA was used to set up these assay tubes in barbital buffer pH = 8.6, T_3 antibody from Wien at 1:5000 (final dilution), and ANS (0.06 g/dl) as an inhibitor. Total assay mixture volume was 1 ml.

Addition: Resins were added in batch type procedure 1 ml of resin to each tube (using special small scoop), mixed well and left on ice for 10 minutes, then centrifuged at 1500 g for 10 minutes. One-half ml of the supernate (bound fraction) was transferred to a clean counting tube and counted for 2 minutes. Counts obtained were multiplied by 2 to determine the radioactivity in the entire fluid phase, then the percent T_3-^{125}I was calculated in regular manner.

For the use of resin columns, each tube's contents was transferred to the corresponding column and followed by 1.5 ml of buffer used to wash each assay tube. The eluate was collected in clean tube and counted. The percent T_3-^{125}I in the eluate was calculated as percent of the activity of the "total" tube which contains the same amount of T_3-^{125}I in 2ml buffer. Table (20) gives the results of these studies.

4. Dextran coated charcoal

In optimizing the conditions for the use of dextran coated charcoal, timing between the first tube to which DCC was added and the last tube was found to have no effect if kept below 3 minutes. This was established by running three blanks as the first three tubes and as the last three tubes and no significant difference in the percent T_3 ^{125}I bound was observed. This was true if the tubes were incubated at $4^{o}C$ for 10 minutes after adding the DCC to the last tube. Cold temperature was used because it gave better reproducibility from run to run. To achieve good separation centrifugation for 15 minutes at 2700 g was found sufficient. After centrifugation, the bound fraction (the supernate) was transferred to new clean tubes and radioactivity in this fraction was counted.

H. Cross reactivity of T_3 antisera with T_4

Cross reactivity with T_4 was estimated by adding T_4 (Sigma) to blanks, and serum samples and determining the effect this addition has on the T_3 value. T_4 was added at two levels, 10 and 20 ng of T_4/tube which corresponds to 10 and 20 µg T_4/dl. The percent cross reactivity was calculated as percent of the added T_4 which reacted as T_3. T_4 solution used was made in barbital buffer and the T_4 used was not purified.

This study was done on antibodies from both Wien and Endocrine sciences. Table (26) lists the values obtained and compares these values with the ones reported by the supplier.

J. Incubation time and temperature

Two phase incubation time was evaluated for sensitivity (slope) and linearity of the standard curve (correlation coeficient). The two phase incubation time at 37°C followed by 4°C was reported to decrease the total required time of T_3 RIA (72).

The following incubation times were studied:

	at 37°C		at 4°C
(1)	30 min.	and	30 min.
(2)	30 min.	and	1 hour
(3)	30 min.	and	2 hours
(4)	2 hours	and	1 hour

Four dose-response curves were set up and incubated one at each condition. Table (16) gives the slope, correlation coefficient, and y intercept of the B^{o}/B plot. Fig. 9 compares the standard curves. In this experiment Wien Ab at 1:5000 dilution, barbital buffer pH 8.6 was used, and separation was achieved by Dextran coated charcoal (DCC).

K. Methods of Evaluating the Final Assay Procedure

Upon selecting the optimal methodological conditions the final RIA procedure was evaluated for accuracy, sensitivity, and reproducibility.

1. Accuracy (validity) of the assay

Accuracy of the T_3 RIA procedure developed has been evaluated

by the following studies

a. Recovery studies

b. Extraction studies

c. Linearity of results obtained using increased volumes of samples and the effect of serum proteins on the assay,

d. Correlation of serum T_3 value with serum T_4 value (clinical correlation) and correlation between this method and another locally used T_3 RIA.

a. Recovery studies

Standard T_3 solution was added to different samples. To each sample two levels of T_3 were added (50 and 200 pg/tube). T_3 RIA was performed simultaneously on these samples with and without the added T_3. The percent T_3 recovered was determined from the formula

$$\frac{\text{Observed } T_3 \text{ value} - \text{Endogenous } T_3}{\text{Added } T_3} \quad X\ 100$$

Table (21) gives the results of this study. Each determination was run in triplicate.

b. Extraction studies

To insure that the serum T_3 value is free of protein effect, 6 samples were run with extraction and without extraction. Solvent extraction was performed as follows: 1 ml of serum mixed with 100 µl of T_3-^{125}I of high purity (100 Pg T_3-^{125}I) and total CPM was taken, then 1.9 ml of cold absolute ethanol were added. Tubes were centrifuged at 3100G for 10 minutes in

a refrigerated centrifuge. One ml of the supernate was transferred from each extract into clean plastic tubes and dried in a water bath at 45°C. To the dried extract of each sample 500 µl of T_3-FS was added and mixed on a Vortex mixer and radioactivity in each tube was determined to calculate extraction efficiency. Tubes were incubated at 4°C for two hours to insure solubility of the extract in serum proteins. Direct T_3 RIA was run on these extracts and corrected for extraction efficiency. Comparison of T_3 value with and without extraction is given in Table (22).

c. Linearity and serum protein effect on the assay

To assess the linearity of the assay, a high serum T_3 control (Versatol Pediatric) was assayed without and with dilution with T_3-free serum. The increase in T_3 value as the assayed amounts of the sample increase is compared to the standard curve run simultaneously (Figure 13). In another experiment different portions of the same sample were assayed (each time the assayed serum was completed to 100 µl with T_3-free serum to bring to the same volume used in the standard curve (100 µl). Thus if 50 µl of a sample was used, 50 µl of T_3-free serum was added. Table (23) gives the T_3 value on full size sample (100 µl) and on portion of the sample (50 and 25 µl).

Also, T_3 value when 50 µl of a sample + 50 µl of T_3-FS was assayed compared to T_3 value obtained if only 50 µl of the sample was assayed with no T_3-free serum added. Table (24) gives the

result of this comparison.

d. Correlation of serum T_3 value with T_4 and with another locally used T_3 RIA.

(1) The T_4 values of 35 samples from hyper, euo, and hypothyroid patients were compared with T_3 values and correlation coefficient was calculated.

(2) To compare the serum T_3 value obtained by this method with other RIA locally used, 40 samples were run by both laboratories. Figure (15) compares the results obtained.

2. Sensitivity

To determine the minimum detectable limits of T_3, decreasing amounts of standard T_3 solution were assayed. The least amount of T_3 giving a %B that is significantly different (lower) from %B observed in the blanks run simultaneously was considered the minimum detectable level of T_3. The student t-test was used to determine if the difference was significant, N = 7 for each level of T_3, and $P < .005$.

3. Reproducibility

a. Within run reproducibility

The reproducibility within a run was estimated by studying the variation in T_3 value of the duplicates of 25 samples. The following formula was used.

$$\text{Coefficient of Variation (CV)} = \frac{\Sigma d^2}{2n}$$

Where: $$d = \frac{\text{highest value of the duplicate}}{\text{lowest value of the duplicate}} - 1 \quad X\ 100$$

n = Number of samples (duplicates) being studied

b. Between runs reproducibility

Twenty samples with wide range of T_3 were assayed in five runs. The average, the standard deviation, and coefficient of variation of each sample was calculated. The average of the coefficient of variation of all samples was considered the between runs coefficient of variation, Table (29).

L. Evaluation of the Effect of Some Methodological Variables on the T_3 Value

1. Effect of the antibodies cross reactivity with T_4

Seven samples were assayed under identical conditions except for the source of the antisera. Dilution of the antisera was selected to obtain similar binding at zero dose (Net $\%B^o$ = 33-35%). Table (27) compares the values obtained and shows its relation to the T_4 concentration of these samples.

2. Effect of the separation method on the T_3 value

Four dose-response curves were set up and treated identically following the final procedure except for the method of separation. Eight samples were run with each of the four curves. Table (28) summarizes the results of this study.

3. Effect of the type and concentration of TBG binding inhibitors on the T_3 value

In one run, four standard curves with six samples were treated identically except for the inhibitor used. Two of them were treated with sodium salicylate at different concentrations (0.4% and 0.8%). The third with ANS and the fourth with merthiolate. All inhibitors were used at the concentration which was found optimal from previous studies. Table (30) compares the T_3 values and the dose-response curve parameter.

4. Effect of pH on the T_3 value

Two dose-response curves with eight samples were run at two different pHs (8.4 and 9.0). Table (15) compares the values of the eight samples.

5. Effect of the antibody dilution on the T_3 value

Using the same antisera (Wien Lab.) at two different dilutions (1/5000 and 1/10000) two standard curves and seven samples were analyzed, following otherwise identical conditions (Table 31).

Serum T_3 normal level

The normal level of serum T_3 by this method was determined on 45 samples from individuals with no complaint of thyroid problems and who have T_4 level within the normal range (medical technology students and laboratory personnel).

RESULTS

1. T_3-Free Serum Preparation

Table (7) shows that removal of T_3 from pool serum by treatment with charcoal does not require long incubation time, neither is it temperature dependent. 96.7 to 98.0% of the T_3-^{125}I added to pool serum before treatment is removed by charcoal. Two-hour incubation was used for routine preparation of T_3-free serum. Since the tracer used to follow up the removal of T_3 is not completely pure it is likely that 2-3% of the radioactivity remaining is due to ^{125}I iodine. The fact that no detectable T_3 remained in this preparation is shown in Table (8). Adding 50 µl of T_3-free serum to 50 µl of pool serum did not change the T_3 value as determined by the RIA procedure.

This preparation still has the binding capability to T_3 as determined by T_3-resin uptake. The observed decrease in the T_3-resin uptake is due to the removal of T_3 and T_4 from that preparation (Table 9). The use of charcoal to prepare T_3-FS is an efficient way, but requires a long time for centrifugation and a final filtration through millipore filter (.45 µ). This was found to be essential to remove the very fine particles remaining that could not be removed by centrifugation. Comparable results were obtained if anion exchange resin IRA 400 was used. The resin requires longer incubation with the serum (6-12 hours) and is more expensive. However, it does not require long centrifugation, and is cleaner to work with.

TABLE 7

Effect of Length of Incubation of Pool Serum With Charcoal on the Efficiency of Removing T_3-^{125}I Added to the Serum

Time and Temperature	% Radioactivity Removed
24 hours at 4°C	97.2
12 hours at 4°C	96.2
12 hours at room temp. (R.T.)	98.0
2 hours at R. T.	96.7

TABLE 8

Effect of T_3-Free Serum on the T_3 Value of a Sample

Sample Assayed	T_3 Value ng/100 ml	N
50 µl pool + 50 µl diluent	62 ± 2.35	4
50 µl pool + 50 µl T_3-FS	62 ± 1.70	4
100 µl pool only	125 ± 3.6	4

TABLE 9

Effect of Charcoal Treatment on T_3-Resin Uptake and T_4 Value of Pool Serum

Test	(N)	Before Treatment With Charcoal	After Treatment With Charcoal
T_3 Resin Uptake	(4)	28.5%	22.6
T_4 (CPB)	(4)	10 µg/100 ml	0.2 µg/100 ml

2. Antisera Titration

Figures (1) and (2) demonstrate the titration curves for two commercial T_3 antisera: Wien Laboratories and Endocrine Sciences. The dilution needed to obtain certain %B is usually indicated by the supplier, but since the amount of tracer used, assay conditions and method of separation vary, it is necessary to confirm the antibody titer. Dextran coated charcoal is used in this study and the %B in both figures (1) and (2) includes the nonspecifically bound (NSB) T_3-^{125}I. This has been taken into consideration in selecting the antisera dilution to obtain 30-50% specific binding. Comparison of two lots number in Table (10) show consistency of the titer.

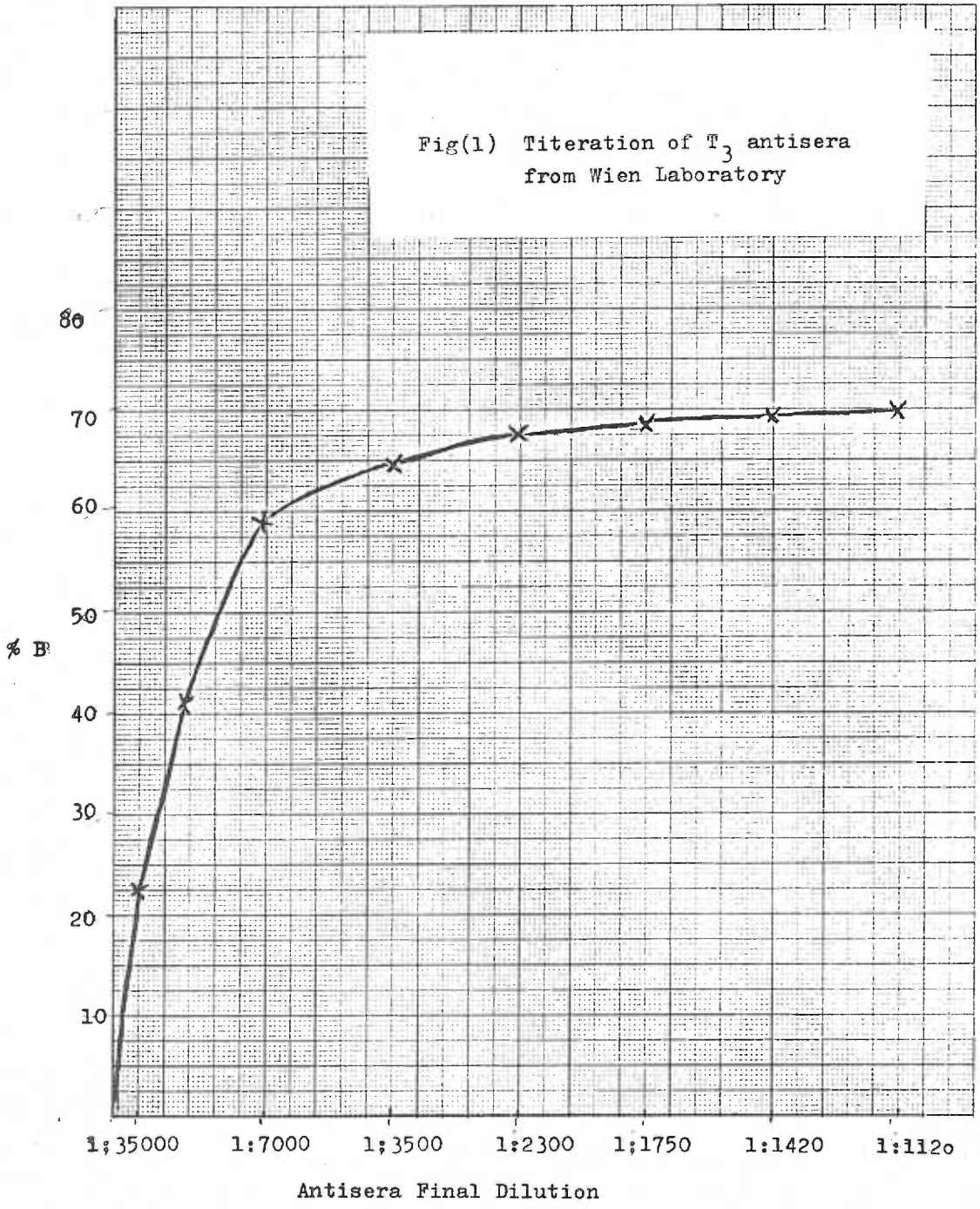
TABLE 10

Comparison of the Titer of Two Different Lots of Commercial Antisera (Endocrine Sciences)

Antisera Dilution N=3	%B Lot #1	%B Lot #2
1:35000	22.42 ± 2.0	23.00 ± 1.9
1:14000	41.40 ± 2.4	44.81 ± 1.3
1:7000	59.73 ± 1.6	60.95 ± 2.8
1:3500	64.99 ± 3.0	65.03 ± 1.5
1:1750	68.52 ± 1.9	67.17 ± 2.0

3. Effectiveness of the Inhibitors Used to Block Binding of T_3 to Serum Proteins

Table (11) shows that when T_3-free serum was added to the assay mixture of the Ab and T_3-^{125}I (Group 1) the precent T_3-^{125}I was decreased


Fig(1) Titeration of T_3 antisera from Wien Laboratory
80
70
60
50
% B
40
30
20
10
1;35000
1:7000
1;3500
1:2300
1;1750
1:1420
1:1120
Antisera Final Dilution

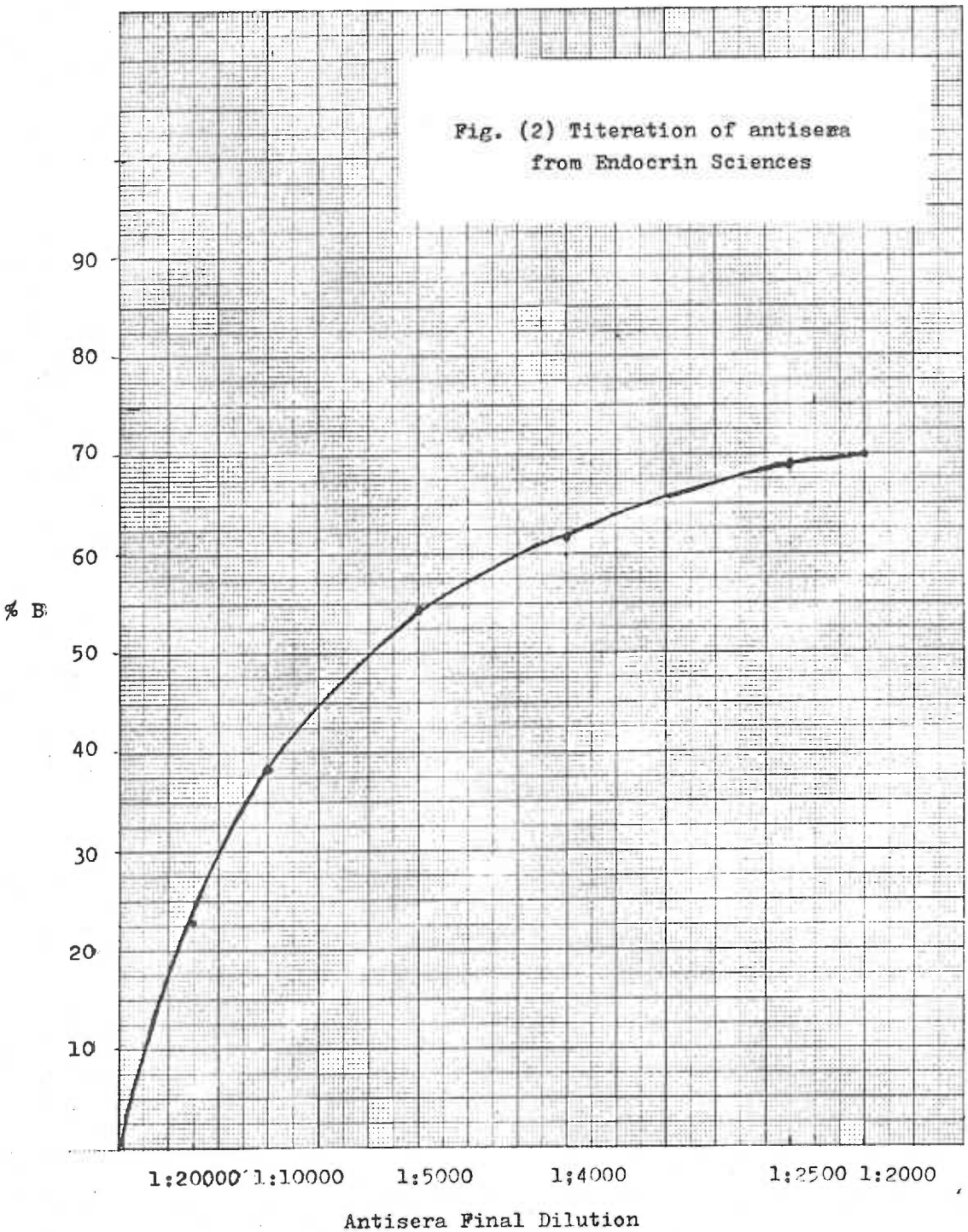


Fig. (2) Titeration of antisera
from Endocrin Sciences
90
80
70
60
% B
50
40
30
20
10
1:20000
1:10000
1:5000
1;4000
1:2500
1:2000
Antisera Final Dilution

from 62% to 45%. Adding the inhibitor to the mixture led to elimination or minimization of this decrease to 60.5% (Group 3).

TABLE 11

The Effect of T_3-free Serum and an Inhibitor on the Binding of T_3-^{125}I to T_3-antibody

Tubes Group Number (N=5)	1	2	3
Tube Content			
T_3-antibody	100 µl	100 µl	100 µl
T_3-^{125}I	100 µl	100 µl	100 µl
T_3-free serum	--	100 µl	100 µl
Inhibitor	--	--	100 µl
Buffer	800 µl	700 µl	600 µl
%B	62.0±2.0	45.7±1.53	60.5 ±1.90

Based on that observation, the effectiveness of the substances used to block the binding of T_3 to serum proteins is assessed by its ability to restore the binding of T_3-^{125}I to the antibody in the presence of serum proteins to the level observed when there is no serum proteins present (i.e., T_3-^{125}I and T_3-antibody only).

Figure (3) represents comparison of the effectiveness of the three most effective inhibitors at wide ranges of concentration. The T_3-^{125}I binding to the antibody with no T_3-free serum is 36%. When T_3-FS was added, it decreased to 17.5%, then the %B is sharply increased as the

concentration of the inhibitors is increased. It is observed here that both ANS and Sodium Salicylate restored the percent T_3-^{125}I bound close to the original value of 36.5%, meanwhile merthiolate brings it up to 28.5%. The use of higher concentration of any of these inhibitors is known to affect the T_3-^{125}I binding to the Ab leading to a decrease in the %B (65, 66). It is evident from Figure (3) that merthiolate is inferior and an inadequate inhibitor. ANS is an effective inhibitor, but its solution is photosensitive, and therefore has to be prepared daily. Sodium Salicylate is stable in solution, and at the optimal concentration 0.8% has only a minor effect on the antibody binding to T_3-^{125}I as seen in Figure (5), which represents two dose-response curves, both set up with no T_3-free serum but one with Sodium Salicylate and the other without Sodium Salicylate. The fact that this salicylate is used by rheumatoid arthritis patients should not affect the assay because the therapeutic level in the blood does not exceed 0.03g/dl (66, 73). Figure (4) indicates that the same pattern of inhibition is obtained in the presence of T_3.

4. Use of Heat Inactivation to Eliminate TBG Effect on the T_3 RIA

   a. Heat inactivation at $60^{o}C$

In Figure (6) the dose-response curve set up with neither an inhibitor nor heat inactivation show very shallow displacement (low slope) indicating the effect of the serum proteins on the assay. The similarity of the two standard curves obtained by using ANS at 0.06% and using heat inactivation at $60^{o}C$ indicate that the two approaches are equivalent. However, heat inactivation at $60^{o}C$ is time consuming (45 min.).

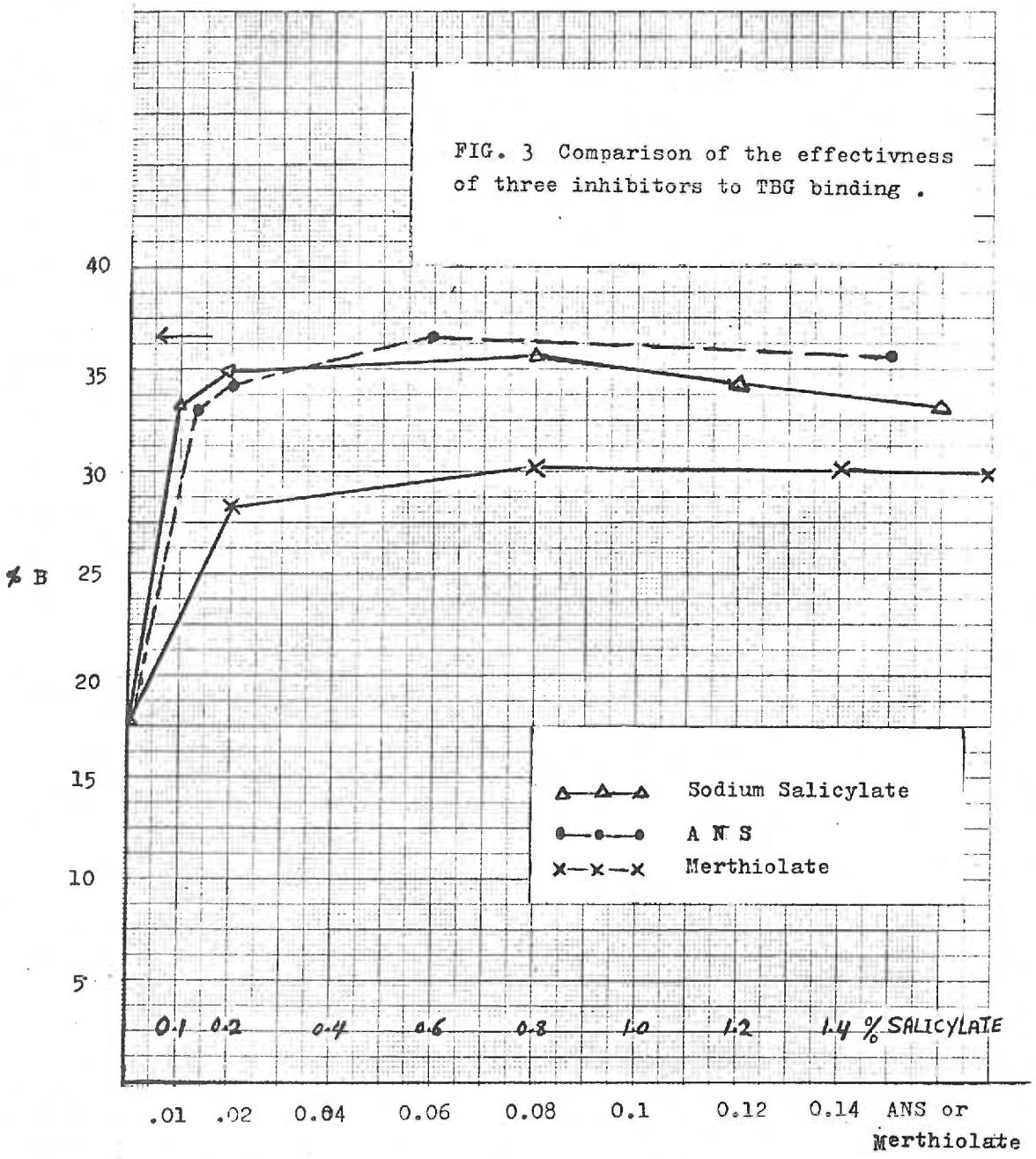


FIG. 3 Comparison of the effectivness of three inhibitors to TBG binding .
40
35
30
% B 25
20
15
10
5
Sodium Salicylate
A N S
Merthiolate
0.1 0.2 0.4 0.6 0.8 1.0 1.2 1.4 % SALICYLATE
.01 .02 0.04 0.06 0.08 0.1 0.12 0.14 ANS or Merthiolate
INHIBITOR CONCENTRATION

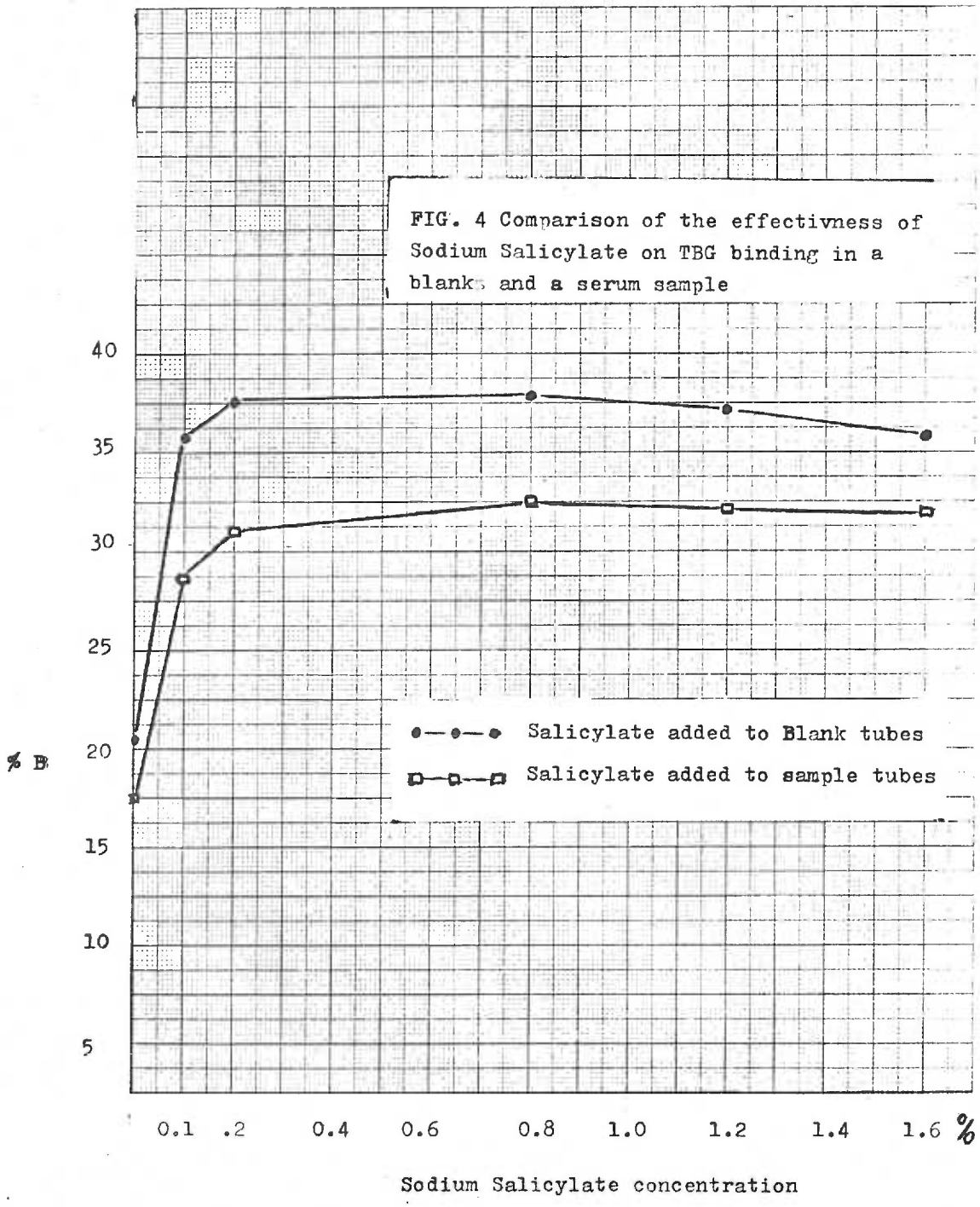


FIG. 4 Comparison of the effectivness of Sodium Salicylate on TBG binding in a blank and a serum sample
40
35
30
25
20
15
10
5
% B
Salicylate added to Blank tubes
Salicylate added to sample tubes
0.1
.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
%
Sodium Salicylate concentration

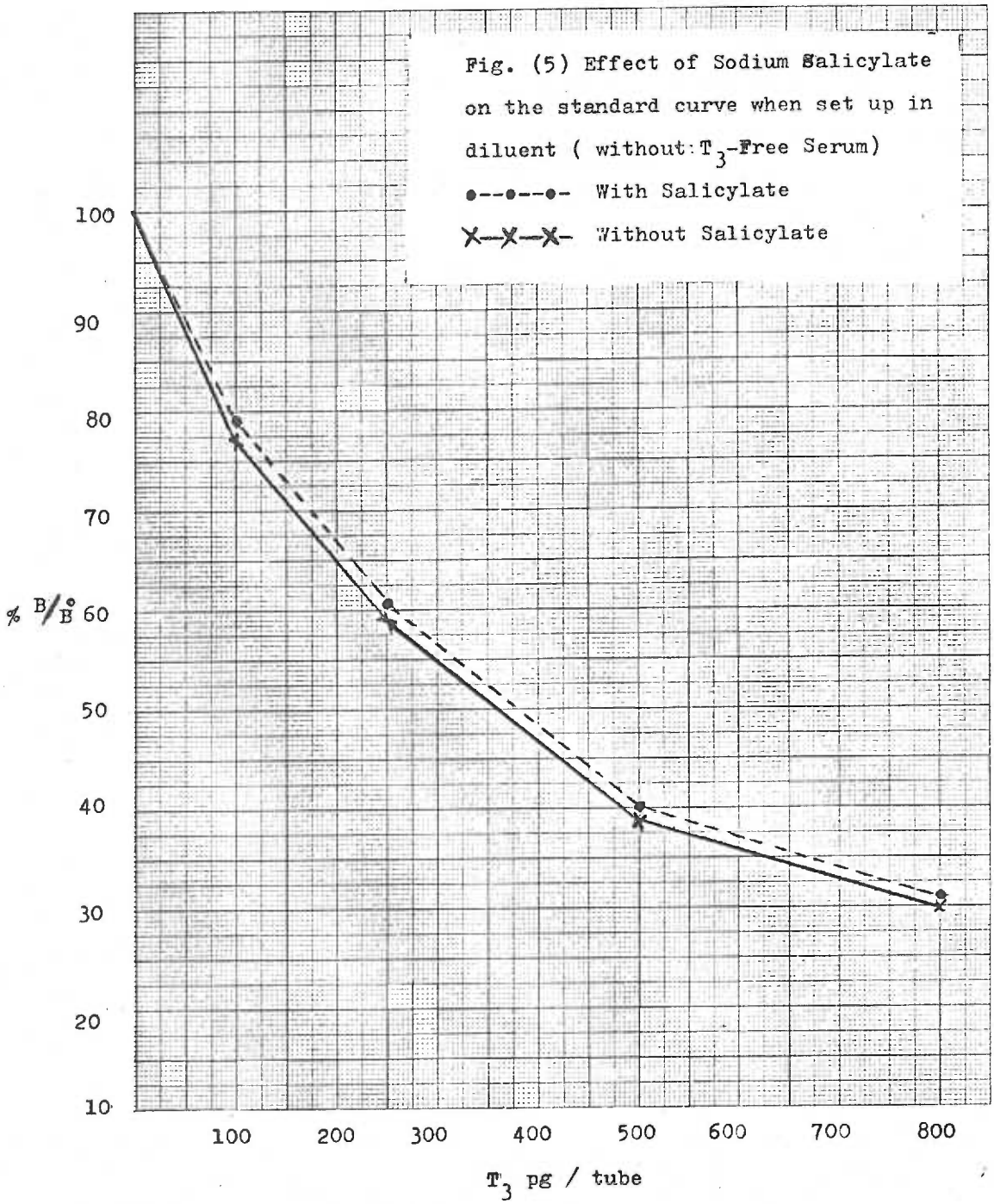


Fig. (5) Effect of Sodium Salicylate on the standard curve when set up in diluent (without T_3-Free Serum)
●--●--●- With Salicylate
X—X—X- Without Salicylate
100
90
80
70
% B/B° 60
50
40
30
20
10
100
200
300
400
500
600
700
800
T_3 pg / tube

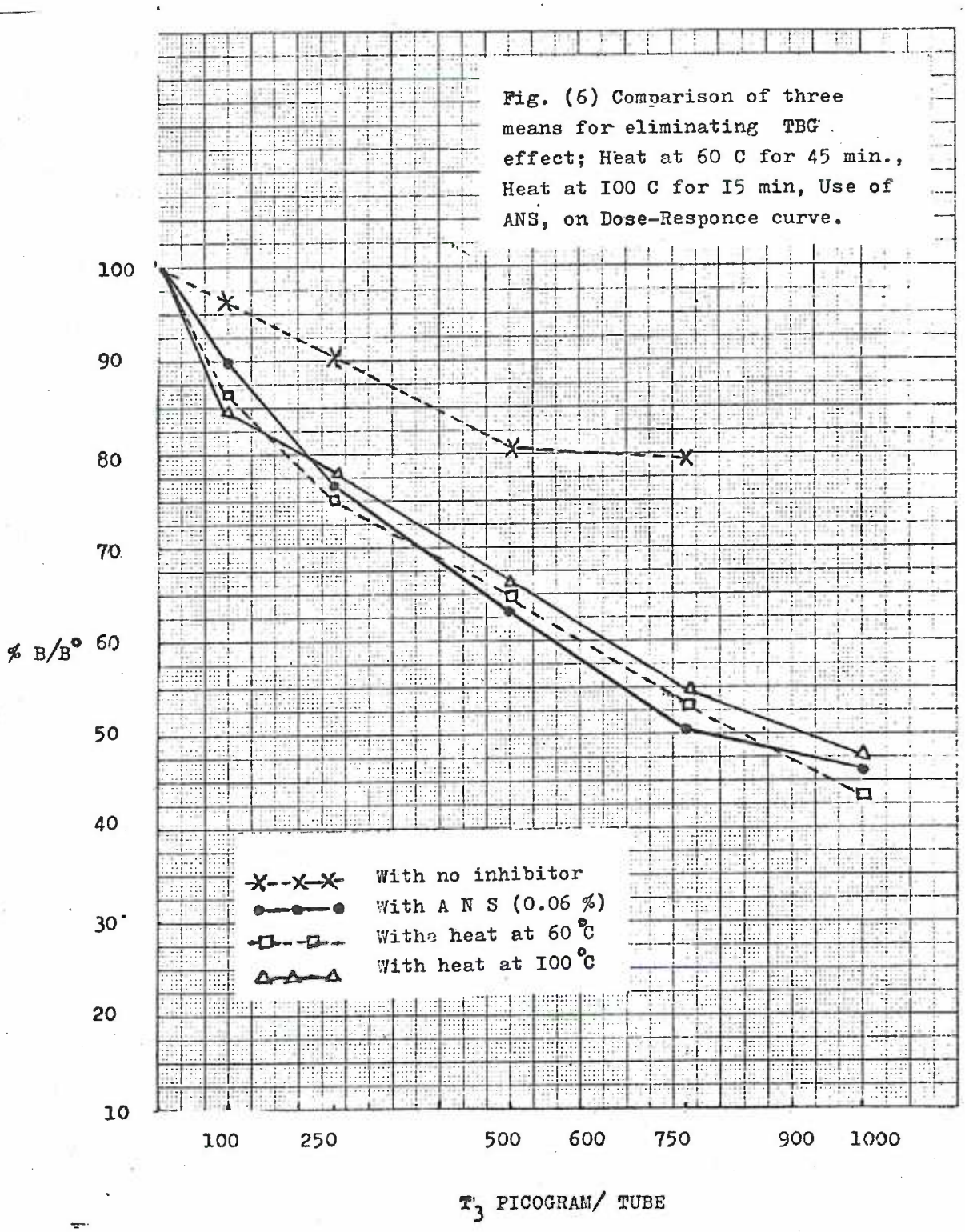


Fig. (6) Comparison of three means for eliminating TBG effect; Heat at 60 C for 45 min., Heat at I00 C for I5 min, Use of ANS, on Dose-Responce curve.
100
90
80
70
% B/B° 60
50
40
30
20
10
100
250
500
600
750
900
1000
With no inhibitor
With A N S (0.06 %)
Withe heat at 60 °C
With heat at I00 °C
T3 PICOGRAM/ TUBE

TABLE 12

Comparison of the T_3 Value of Serum Samples
Using Different Approaches to Eliminate TBG Effect

Approach Used to Eliminate TBG Effect	T_3 Value (ng/dl) of Five Samples					
	1	2	3	4	5	N
None	250	325	340	290	210	3
ANS (0.06%)	100	186	210	160	103	3
Heat inactivation at 60°C	136	228	240	204	152	3
Heat Inactivation at 100°C	195	298	326	305	176	3

It is evident from Table (12) that without heat or inhibitor the T_3 values are very high and this is due to the effect of TBG on the binding of T_3-^{125}I and T_3 to the Ab. Values obtained using heat inactivation at 60°C are consistently higher than those obtained using ANS despite the similarity of the standard curves obtained under either condition, Figure (6).

Even higher values are obtained when a higher temperature was used (100°C).

It is observed here that the standard curves obtained using heat inactivation method are comparable to those obtained using an inhibitor, but much higher values for the samples obtained when heat inactivation is used. We attributed this to the effect of heat on the stability of T_4 which is present in the samples but not in the standards.

C. Effect of Heat Inactivation on the Stability of T_4

In Table (13) is shown results of experiments in which 10 or 20 ng T_4 was added to standard assay tubes which contained 300 pg T_3/tube before or after the heat inactivation step. The same additions were also made to tubes containing no T_3 at all. It is clear that added T_4 causes elevation in the T_3 value to much higher extent when added before heat inactivation step than when added after that step (compare A to B values). The difference between B and A (B-A) can be attributed only to the effect of heat on the added T_4. The increase in T_3 value over the originally present T_3 (300 pg) when T_4 was added after the heat inactivation step is due to the T_3 content of T_4 preparation (T_4 was not purified) and to cross reactivity effect.

However, for the purpose of this experiment there is no need to use pure T_4 because we are interested in the difference between A and B. T_4 to T_3 conversion (monodeiodination) is known to take place in vitro on chromatographic media (paper, thin layer, or gas) and during extraction of T_4 from serum. The increase in T_3 value due to heating T_4 can be attributed to the conversion of T_4 to T_3 or to another heat degradation product which is highly cross reacting with T_3-antibody.

TABLE 13

The Effect of Heat Inactivation for 15 Minutes at 100°C on T_3 Values in the Presence of T_4

T_3 Present	T_4 Added and Phase of Addition	T_3 Value by RIA in pg Mean SD (N)	Increase in T_3 Value Due to Heating T_4
300 pg/tube	(A) 10 ng after heat	453 ± 4.15 (6)	149 pg
	(B) 10 ng before heat	602 ± 13.2 (6)	
300 pg/tube	(A) 20 ng after heat	511 ± 52.8 (6)	269 pg
	(B) 20 ng before heat	781 ± 15.7 (6)	
0 pg/tube	(A) 20 ng after heat	200 ± 28.43(5)	265 pg
	(B) 20 ng before heat	543 ± 24.66(5)	

TABLE 14

The Rate of Conversion of T_4 at Different Levels

T_4 Added	Increase in T_4 Value Due to Heat (pg)	Rate of Conversion*
10 ng	149	1.49%
20 ng with T_3	269	1.34%
20 ng without T_3	265	1.32%

*Rate was calculated by dividing the increase in T_3 value (pg) over the added T_4 (in pg) and multiplying the result by 100.

TBG heat inactivation at 100°C has been used for T_4 RIA (80). The effect of heat on T_4 was not considered in that report. However,

as seen in Table (14), the rate of conversion is very low and would not affect the T_4 value significantly (1.32-1.49%). Since serum T_3 level is much lower than T_4, such a rate of conversion increased T_3 level significantly. Therefore, this approach was considered inapplicable to T_3 RIA.

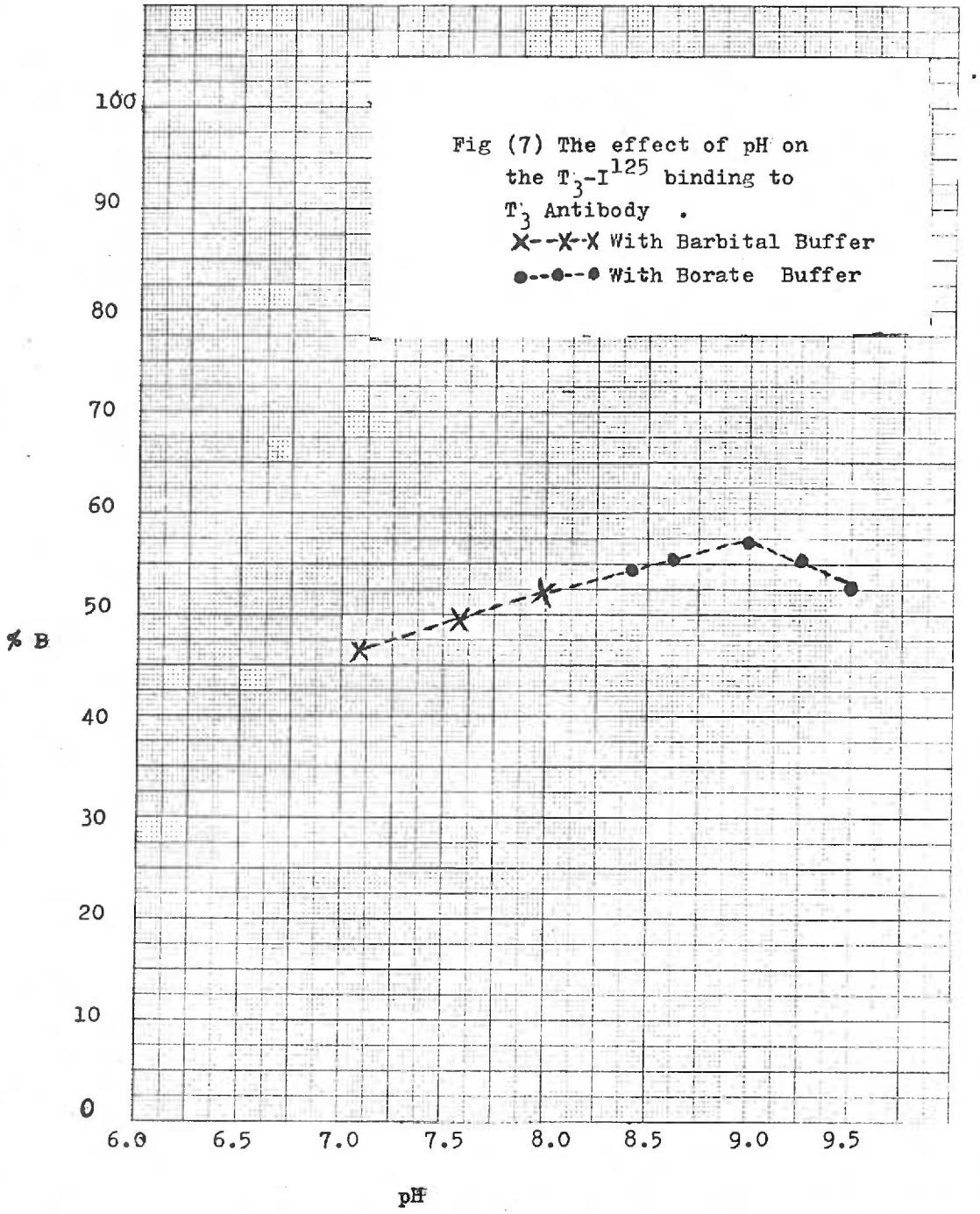
5. Selecting Optimal pH for the assay

Figure (7) represents the pattern of T_3-^{125}I binding to T_3 antibody as function of pH. Increase in binding observed between pH 7 and 9. This can be attributed to decrease in binding of T_3-^{125}I to serum proteins at higher pH. It is known that at alkaline pH, the thyronines dissociate from serum protein probably due to increase in ionization of these substances. Figure (8) demonstrates that pH 9.0 produces a slight but consistent shift to the left of pH 8.4, with increase in the y intercept, indicating slight improvement in the sensitivity (least detectable dose).

Table (15) lists the T_3 values of eight samples run with two standard curves. No significant difference in the T_3 value is observed. pH 9.0 using borate buffer which has better buffering capacity in that range was selected. Sodium Salicylate was found to increase the pH; therefore Borate buffer was adjusted only to 8.8. The pH increases to 9.0 when the salicylate is added at the effective concentration.

6. Incubation Time and Temperature

Figure (9) represents four standard curves. Each was set up the same way except for incubation time. Table (16) gives the parameters;


100
90
80
70
60
50
40
30
20
10
0
% B
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
pH
Fig (7) The effect of pH on the T_3-I^{125} binding to T_3 Antibody .
With Barbital Buffer
With Borate Buffer

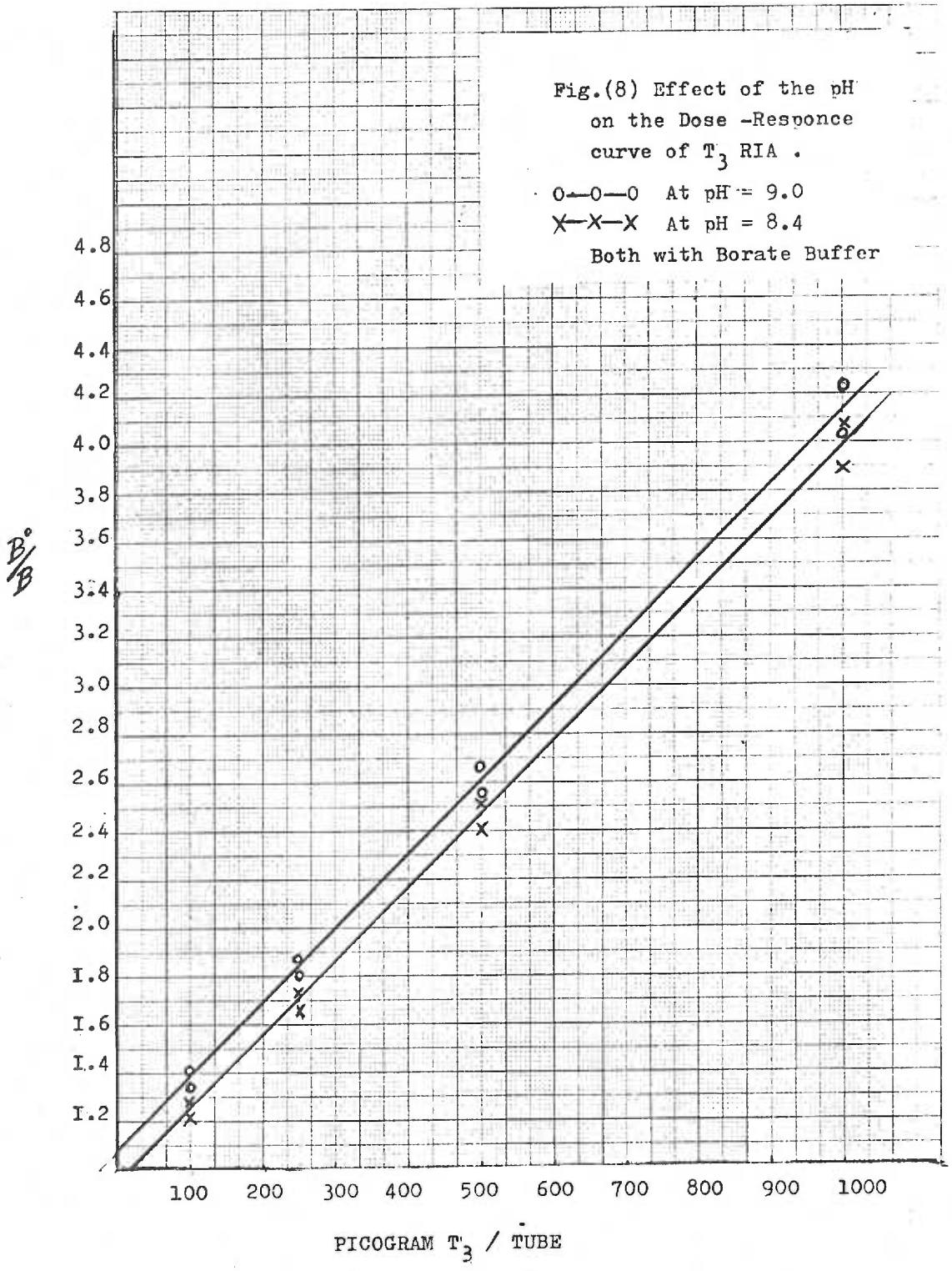


Fig.(8) Effect of the pH on the Dose -Responce curve of T_3 RIA .
O—O—O At pH = 9.0
X—X—X At pH = 8.4
Both with Borate Buffer
B°/B
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
I.8
I.6
I.4
I.2
100
200
300
400
500
600
700
800
900
1000
PICOGRAM T_3 / TUBE

TABLE 15

Serum T_3 Concentration (ng/100 ml) of Eight Samples at Two Different pHs

Sample Number	T_3 Value at pH 8.4	T_3 Value at pH 9
(1)	420 ng/100 ml	405 ng/100 ml
(2)	270	243
(3)	65	82
(4)	127	135
(5)	151	165
(6)	132	137
(7)	128	140
(8)	135	148

of slope, y intercept and linearity correlation coefficient of the B^o/B vs. T_3 concentration plot (linearization plot). It is observed in Figure (9) and Table (16) that as the incubation time at 4^oC increased, both the slope (therefore the sensitivity) and the linearity of the assay (indicated by r) is increased.

TABLE 16

Effect of Incubation Time and Temperature on Standard Curve Parameter (Sensitivity, Linearity)

	Incubation Time		Correlation Coefficient (r)	Slope	y Intercept
	at 37^oC	at 4^oC			
A	30 min	30 min	0.964	0.88	1.20
B	30 min	60 min	0.992	1.14	1.03
C	30 min	120 min	0.998	1.244	1.07
D	120 min	60 min	0.999	1.169	1.11

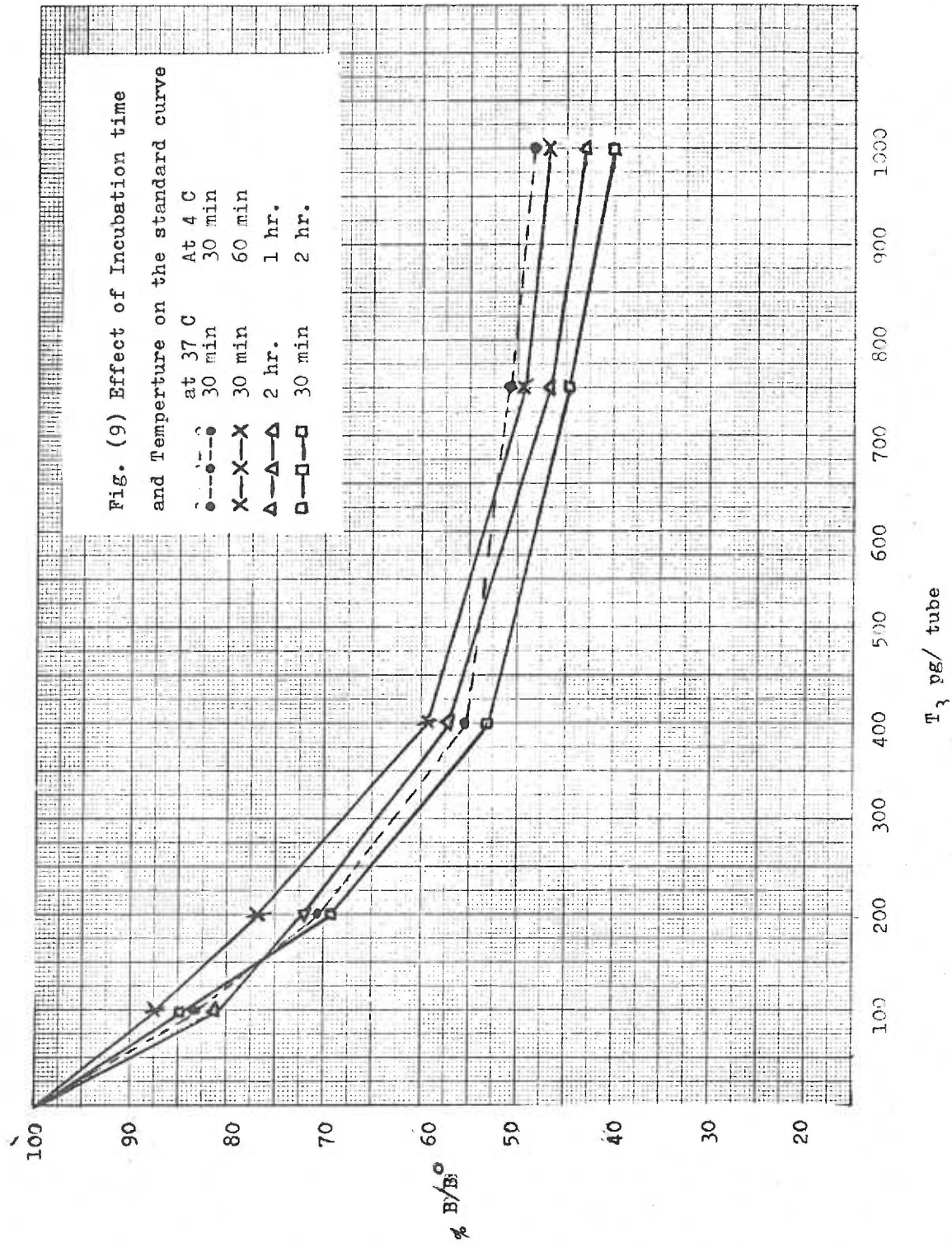


Fig. (9) Effect of Incubation time
and Temperture on the standard curve
at 37 C
30 min
30 min
2 hr.
30 min
At 4 C
30 min
60 min
1 hr.
2 hr.
B/Bo %
T3 pg/ tube
100
90
80
70
60
50
40
30
20
100
200
300
400
500
600
700
800
900
1000

There is no advantage of increasing incubation time at 37^{o} for 2 hours. Thus, 30 minutes at $37^{o}C$ and 2 hours at $4^{o}C$ was found practical and gave satisfactory sensitivity and linearity.

7. Selecting a Method for Separating the Bound and Free Fractions

A. Separation by Polyethylene Glycol

(1) Selection of optimal concentration of PEG

Abbreviations:

NSP = Nonspecific precipitate, represents T_3-^{125}I precipitated in assay tubes containing no T_3-antibody.

TP = Total precipitate, is T_3-^{125}I precipitated under actual assay conditions, i.e., T_3-^{125}I bound to the antibody and the NSP.

SP = Specific precipitate, is calculated by substracting the NSP from TP.

Figure (10) demonstrates that the precipitation of T_3-^{125}I (as TP or NSP) is a function of PEG final concentration. The same pattern of precipitation is obtained in Figure (11) where there is no serum proteins included. This shows that the precipitation under assay conditions (Figure 11) is due mainly to the precipitation of the antibody bound T_3-^{125}I. The total TP reaches a plateau in both Figures (10) and (11), however the NSP continue to rise as PEG concentration increases. This explains why the calculated SP (TP - NSP) declines above 10% PEG. The optimal PEG final concentration is when all the antibody bound antigen is precipitated but with least NSP. Figure (12) shows that opti-

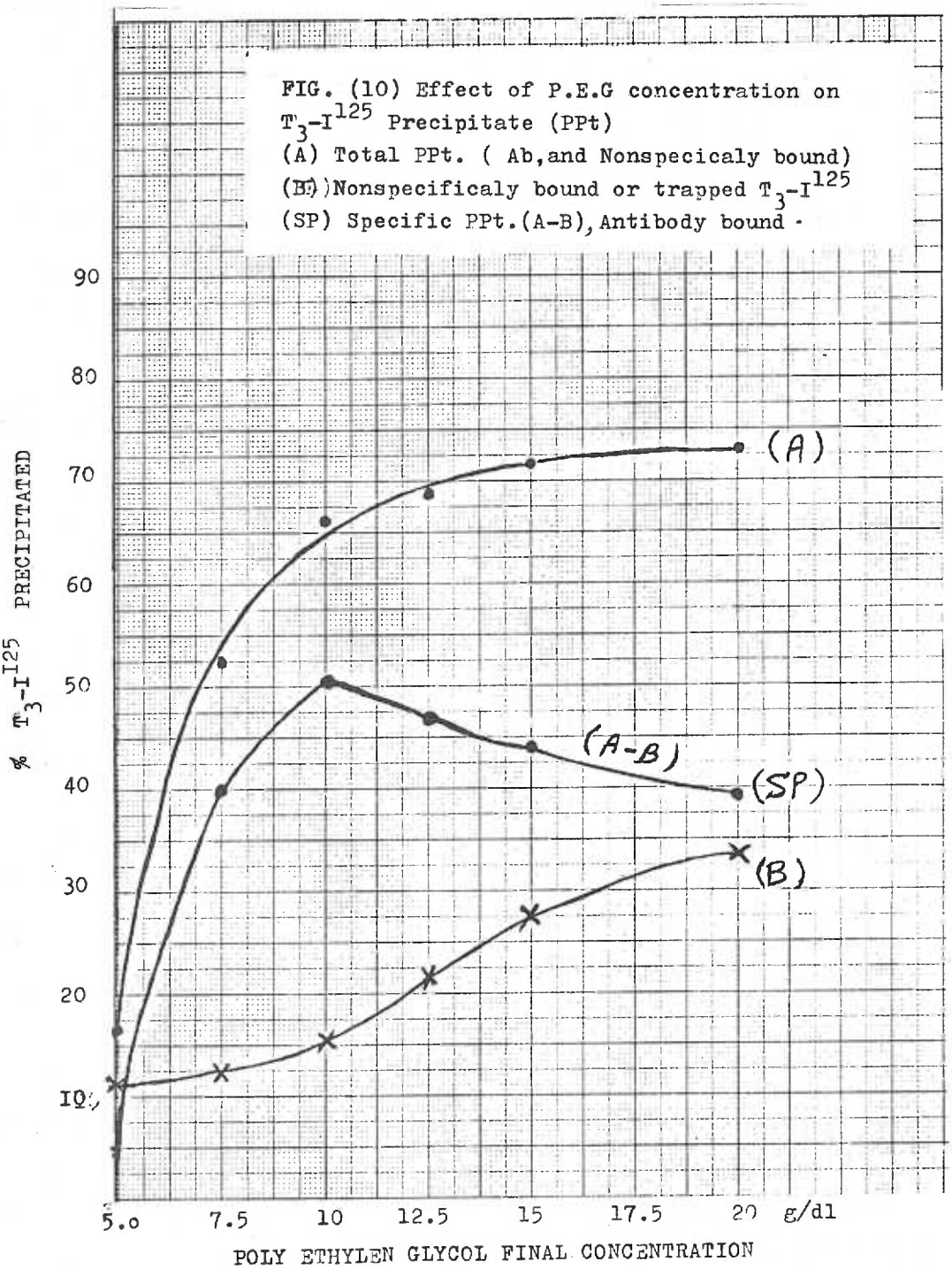


FIG. (10) Effect of P.E.G concentration on T_3-I^{125} Precipitate (PPt)
(A) Total PPt. (Ab,and Nonspecicaly bound)
(B) Nonspecificaly bound or trapped T_3-I^{125}
(SP) Specific PPt.(A-B), Antibody bound -
% T_3-I^{125} PRECIPITATED
90
80
70
60
50
40
30
20
10
(A)
(A-B)
(SP)
(B)
5.0
7.5
10
12.5
15
17.5
20
g/dl
POLY ETHYLEN GLYCOL FINAL CONCENTRATION

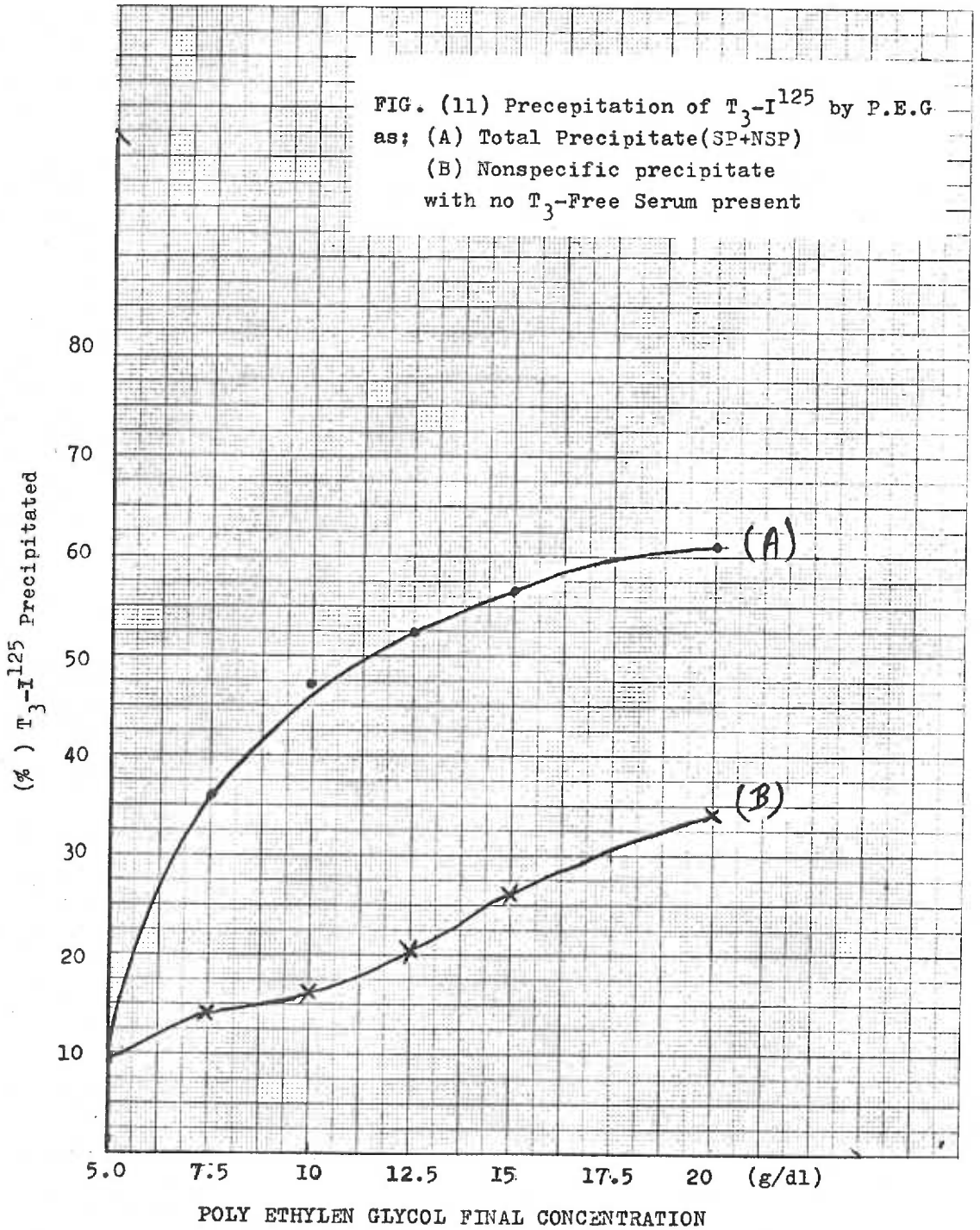


FIG. (11) Precepitation of T_3-I^{125} by P.E.G as; (A) Total Precipitate(SP+NSP)
(B) Nonspecific precipitate with no T_3-Free Serum present
(%) T_3-I^{125} Precipitated
80
70
60
50
40
30
20
10
(A)
(B)
5.0
7.5
10
12.5
15
17.5
20
(g/dl)
POLY ETHYLEN GLYCOL FINAL CONCENTRATION

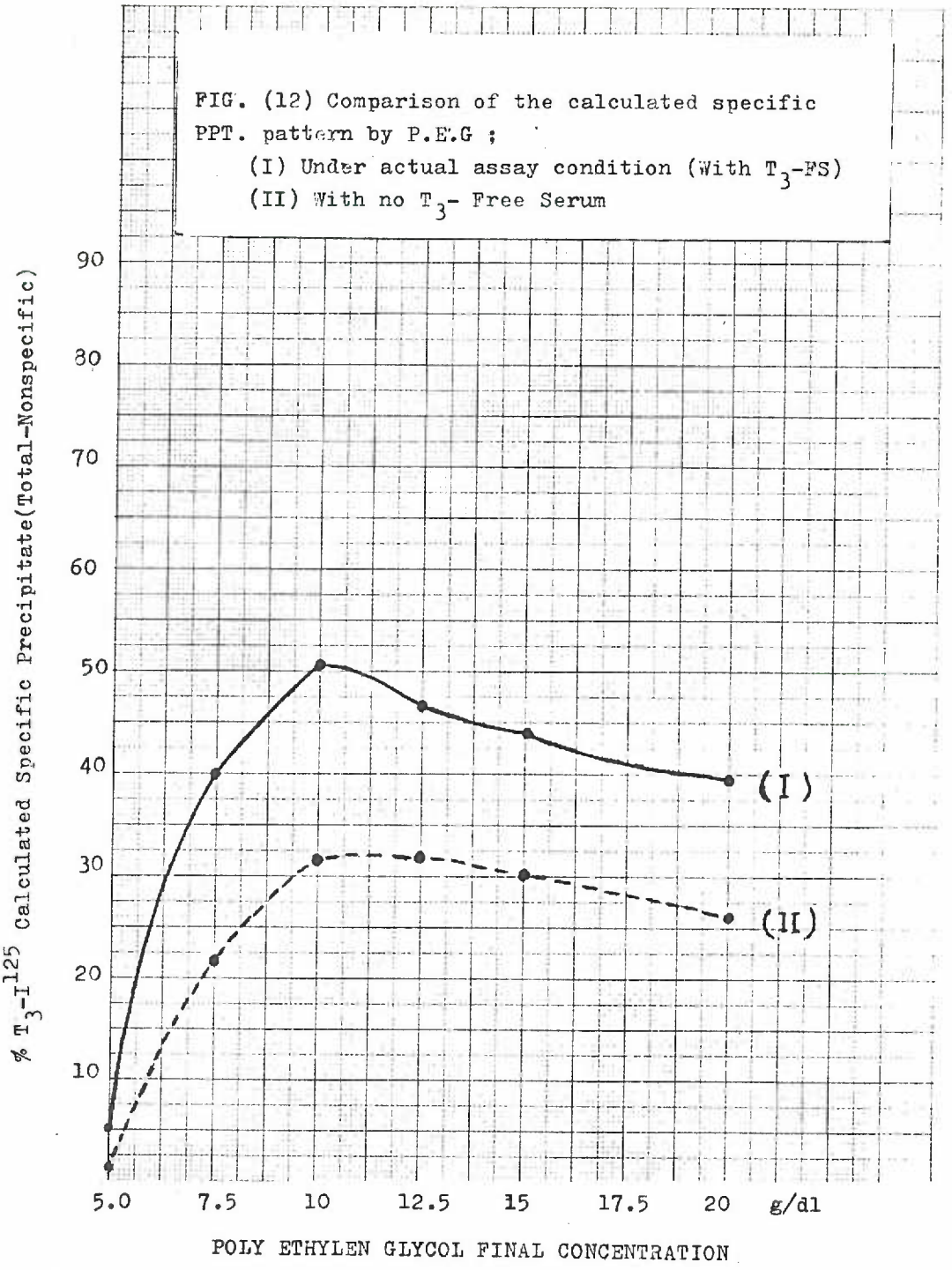


FIG. (12) Comparison of the calculated specific PPT. pattern by P.E.G ;
(I) Under actual assay condition (With T_3-FS)
(II) With no T_3- Free Serum
% T_3-I^{125} Calculated Specific Precipitate(Total-Nonspecific)
90
80
70
60
50
40
30
20
10
(I)
(II)
5.0
7.5
10
12.5
15
17.5
20
g/dl
POLY ETHYLEN GLYCOL FINAL CONCENTRATION

mal concentration for this assay is 10%, with, or without serum proteins. To ensure precipitation of all the bound fraction, 12.75% PEG was selected. To reach this concentration, 0.5 ml of 25.5% PEG solution was added to each assay tube containing a total of 0.5 ml assay mixture. Using this procedure the NSP was 12-15%. To further reduce the NSP which is partially due to some of the free T_3-^{125}I being trapped in the precipitate or by the viscous PEG solution, a 1.5 ml of 17% PEG solution was added to each tube containing 0.5 ml total assay mixture, thus the final PEG concentration is 12.75 and the total volume is 2 ml. Using this modification led to decrease in the NSP to about 8%. Therefore this method was preferred.

(2) Effect of some factors on the precipitation by PEG

(a) Effect of adding serum proteins as carrier proteins to enhance the precipitation

Routinely, when PEG solution was added to each tube, it was followed by 100 µl of T_3-free serum to enhance the precipitation. Since serum proteins are present as part of the assay mixture in a relatively high concentration (20%), it was questionable whether the addition of T_3-free serum is essential. Table (17) demonstrates that adding T_3-free serum as carrier protein has no observable effect on the precipitation of the bound fraction or on the NSP.

(b) Effect of proteins added to the buffer

In Table (18) it is evident that the presence of 1% BSA in

the assay mixture has caused a significant decrease in the percent T_3-^{125}I precipitated by PEG. This is because PEG does not precipitate albumin (81). This supports strongly that T_3 binds to BSA. However, the effect of BSA or any other binding protein will depend on the affinity of the antibody used. Gamma Globulin II (Bovine) 0.5% was selected as an alternative protein to inhibit adsorption of the reaction constituents to the glassware and plastic.

(c) Effect of the use of buffer in PEG solution

Usually PEG solution was prepared in the buffer used in the assay. It is shown in Table (17) that the use of deionized water to prepare this solution is equivalent to buffer.

TABLE 17

The Effect of Some Factors
on precipitation by PEG

Factor Being Studied	%B (TP) M±SD	%NSP M±SD	(N)
PEG solution made in buffer	47.14±1.0	16.5±1.2	(5)
PEG solution made in dist. water	48.62±1.5	16.2±1.1	(5)
With T_3-FS as carrier protein	47.14±1.5	16.1±1.0	(5)
Without T_3-FS as carrier protein	47.34±1.6	15.8± .9	(5)

TABLE 18

The Effect of BSA and Gamma Globulin on the Total and Nonspecific Precipitation by PEG

Protein Added to the Buffer	%B (TP)	%NSP	N
None	48.66 ± 1.4	15.87 ± .9	5
BSA 1%	38.34 ± 1.1	12.6 ± 1.1	5
Gamma Globulin II (Bovine) 1%	47.14 ± 1.0	16.15 ± 1.0	5

B. Separation by Ammonium Sulfate

The use of 50% saturated ammonium sulfate (SAS), achieved by adding equal volume of SAS to 0.5 ml assay mixture, gave a large and soft precipitate, which requires 30 to 40 minutes of centrifugation. Furthermore, the nonsepcific precipitate (NSP) was high (25-40%) (Table 19). Meanwhile 45% SAS which was reached by adding 1.5 ml of 60% SAS to the assay mixture of .5 ml, gave a smaller, compact precipitate which required about ten minutes centrifugation, and reduced the NSP (Table 19). We also found that by washing the precipitate with 45% SAS the NSP was reduced to 2.5-5%. It is known that gamma globulin precipitate between 42-45% SAS therefore the bound fraction is completely separated by this method with minimal NSP.

TABLE 19

The Effect of Ammonium Sulfate Concentration on
The Nonspecific Precipitation and Centrifugation Time

Ammonium Sulfate Concentration	N	NSP Range	Centrifugation Time At 3200 G
50%	5	25-40%	35-40 minutes
45%	5	8-16%	10-12 minutes
45% with washing	5	2.5-5%	10-12 minutes

C. Separation by Ion Exchange Resins

The ability of several ion exchange resins to separate the free T_3 fraction from the bound is shown in Table (20), which gives the %B obtained when each of these resins was used. Since the amount of antibody used binds 50-55% of the tracer, it is expected that the resin which only exchanges with the free T_3 should give about 50-55% B. AG 50 x 2 and commercial T_3 resin columns achieve this. The column gave the lowest NSB value. Other resins do not seem to achieve this step as effectively. Despite the good separation achieved by the commercial T_3 columns, they were not favored for use on our method because they require more technical work, especially when used on large scale. Furthermore, they are too expensive.

Evaluation of the final procedure

Upon studying the different methodological aspects of T_3 RIA, a new procedure was established based on the optimal conditions found. The final procedure depends on the use of ES antisera, sodium salicylate at 0.8 g/dl as blocking agent, pH = 9 with borate buffer, and the incubation of assay mixture at 37°C for 35 minutes then at 4°C for 2 hours. The free and bound fraction are separated by 12.75 PEG (final concentration) which we selected because it did not require washing of the precipitate. The final procedure was evaluated for its accuracy, reproducibility and sensitivity.

TABLE 20

Separation of the T_3-^{125}I Bound Fraction
By Different Ion Exchange Resins

Ion Exchange Resin	% B (T_3-I^{125} in the Bound Fraction) Range			
	Blank	500 pg/tube std.	NSB Control	N
AG50 x 2	48.0--52.5	30.1--32.1	12.82--13.4	3
AG50 x 4	41.4--44.23	26.33--30.00	14.61--15.46	3
AG50 x 8	37.0--39.0	23.94--24.74	13.8--14.8	3
Amberlite IRA 400	71.7--79.4	53.8--53.79	21.1--25.8	3
Commercial* Resin Columns for T_3 Separation	54.4--56.8	34.3--34.6	2.1--8.8	3

*From Biorad Laboratories

a. Recovery Study

Table (21) demonstrates that practically all T_3 added to various samples was recovered. This indicates that under this procedure all added T_3 is made available and free to compete with T_3-^{125}I.

Otherwise, lower recovery values would have been obtained.

TABLE (21)

Recovery of T_3 Added to 3 Samples
Determined Using T_3-RIA Final Procedure

Sample No.	T_3 Value pg/tube	T_3 Added pg/tube	Expected Value	Observed Value	T_3 Recovered*
1	55	50	105	100	90%
		200	255	265	105%
2	115	50	165	162	94%
		200	315	306	95.5%
3	280	50	330	334	108%
		200	480	462	91%

*Calculated as described in Methods section

b. Extraction Study

Table (22) demonstrates the comparison of the T_3 value obtained by direct measurement with values obtained after extracting the six samples using absolute alcohol. This indicates that the level of TBG of the sample has no observable effect on the T_3 value as determined by this procedure. Furthermore, it indicates that the immunoreactive material present in serum could be removed by procedures known to extract much or all of the endogenous T_3.

TABLE 22

T_3 Value of Six Samples Determined Using the Final Procedure (Direct) and After Extraction With Alcohol

Sample No.	N	T_3 ng/100 ml (Direct)	T_3 ng/100 ml (Extracted)*
1	4	80 ± 4.3	91 ± 5.0
2	4	200 ± 6.9	186 ± 4.9
3	4	260 ± 7.1	270 ± 7.9
4	4	363 ± 8.1	351 ± 10.3
5	4	149 ± 5.6	158 ± 4.7
6	4	445 ± 9.4	470 ± 10.9

*Values are after correction for extraction efficiency (75-86%)

c. Linearity and Effect of Serum Proteins on T_3 Concentration in the Final Procedure

This has been evaluated in several experiments. In Table (23) it is evident that T_3 concentration is not affected by the size of the sample used.

Also Figure (13) shows excellent linearity when increased amounts of the same sample was assayed and compared to the standard curve set up with increased amounts of standard T_3. The linearity and the parallelism seen are indicative of accuracy of the T_3 value.

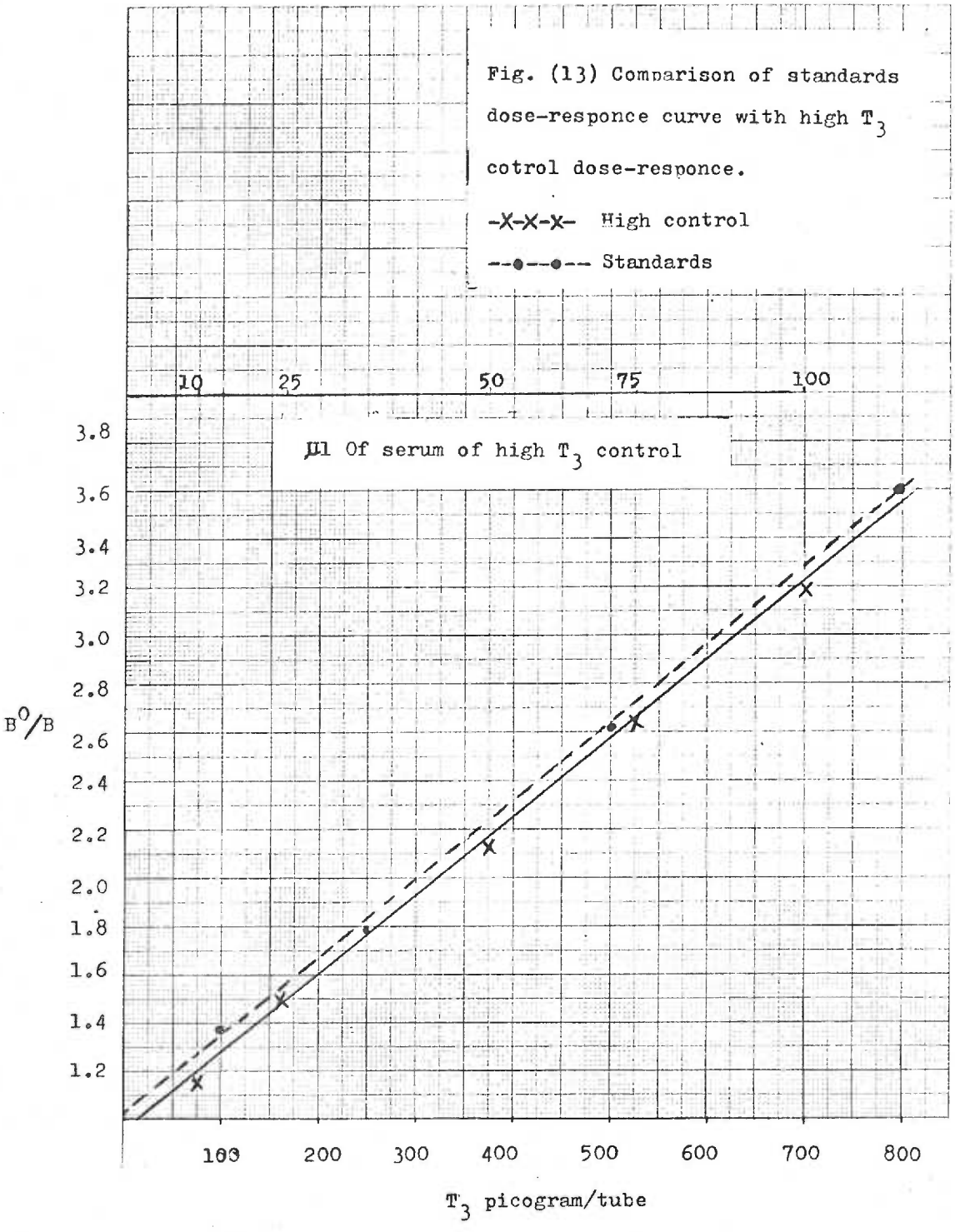


Fig. (13) Comparison of standards dose-responce curve with high T_3 cotrol dose-responce.
-X-X-X- High control
--●--●-- Standards
10
25
50
75
100
µl Of serum of high T_3 control
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
B^0/B
100
200
300
400
500
600
700
800
T_3 picogram/tube

TABLE (23)

The Effect of Dilution of Three Samples With T_3-FS on T_3 Value

Sample No.	Amount Assayed (µls)	T_3 Concentration (M ± SD) ng/100 ml N = 5
1	50	117 ± 3.1
1	100	130 ± 2.9
2	25	213 ± 4.5
2	100	230 ± 4.9
3	25	400 ± 6.1
3	50	385 ± 5.6

Further support to the absence of serum protein effect is indicated in Table (24) where the volume of the sample was completed to 100 µl with diluent to replace T_3-free serum.

TABLE 24

Effect of Dilution of the Samples With Diluent Instead of T_3-free Serum

Sample assayed	T_3 Value µg/100 ml M ± SD
50 µl Pool + 50 µl diluent	154 ± 4.0 (N=5)
50 µl Pool + 50 µl T_3-FS	155 ± 3.6 (N=5)
100 µl Pool	154 ± 2.3 (N=5)

Table (25) shows that the assay is well buffered against TBG level variation because the addition of up to 50 µl ot T_3-FS has no significant effect on the T_3 value. This indicates that variation in TBG level encountered in pregnancy should have no significant effect on the T_3 value. However, adding 100 µl of T_3 FS caused significant increase above the original value.

TABLE 25

Effect of Variation in Serum Protein Level on the T_3 Value. T_3-free Serum was Added in Increasing Amounts to 100 µl of a Sample

T_3-free Serum Added	T_3 Value ng/100 ml	N
0 µl	158 ± 3.4	4
10 µl	158 ± 3.9	4
20 µl	157 ± 3.5	4
30 µl	159 ± 2.9	4
50 µl	161 ± 4.9	4
100 µl	179 ± 3.9	4

d. Antisera Cross Reactivity With T_4

In Table (26) the cross reactivity of two commercial antisera is compared. It is clear that Endocrine Sciences (ES) antibody have much lower cross reactivity with T_4 than Wien Laboratories (WL). Also, with WL antisera the value we obtained is higher than what is given by the supplier of the antisera.

TABLE 26

Estimation of the Cross Reactivity of Two Commercial T_3 Antibody

Antibody Source	T_3 Value pg/tube	T_4 Added ng/tube	T_3 Value (After T_4 was Added) pg/tube	Cross Reactivity Value*	Supplier Value of Cross Reactivity
WL	0 (blank)	10	100	1.0%	0.75%
WL	0 (blank)	20	220	1.1%	
WL	150 (sample)	10	255	1.05%	
ES	0 (blank)	10	25	0.25%	0.25%
ES	250 (standard)	20	295	0.225%	
ES	200 (sample)	20	255	0.27%	

*Cross reactivity is determined by this formula

$$\frac{\text{Increase in the } T_3 \text{ value (pg/tube)}}{\text{Added } T_4 \text{ in pg/tube}} \times 100$$

It has been shown that cross reactivity estimates vary depending on the purity of the T_4 preparation (67). This also indicates that these values are not due to true cross reaction of the Anti-T_3 with T_4 but also to its content of T_3.

The difference in cross reactivity values between the two antisera in Table (26) can be attributed to difference in stereospecificity of the two antibodies because the same T_4 preparation is used.

TABLE 27

Effect of T_3 Antibody Cross Reactivity
With T_4 on T_3 Value

Ab Source and Dilution	Net %B	T_3 ng/100 ml Mean and Range (N=3) 1	2	3	4	5	6	7
Wien 1/5000	35%	127 (120-134)	160 (150-170)	132.5 (123-141)	433 (420-446)	315 (299-331)	94.5 (90-99)	263 (255.5-270.5)
Endocrine Sciences 1/6000	33.5%	85 (82-88)	148 (140-156)	118 (108-128)	350 (345-355)	246 (240-252)	75 (69.8-80.2)	217 (214-222)
T_4 µg/100 ml		4.8	5.2	6.1	17.2	15.2	3.2	7.1

TABLE 28

The Effect of the Separation Method on the T_3 Value

Separation Method	Dose-Response-Curve Variables			T_3 ng/100 ml Mean and Range (N=3)							
	Net %B°	Slope	NSB	1	2	3	4	5	6	7	8
PEG	36%	3.9	6.8	75 70-80	65 60-69	190 185-194	95 91-106	210 203-217	340 326-354	474 465-483	606 580-632
DA	37.2%	3.6	4.5	75 65-79	50 46-56	186 175-196	100 92-108	183 171-195	331 324-340	493 483-504	626 615-636
SAS	29.6%	6.4	8.0	150 143-157	100 86-114	260 241-281	140 129-150	290 268-301	345 335-336	610 581-639	760 698-819
DCC	39%	5.4	16.0	112 110-118	75 66-85	251 238-269	110 103-117	245 231-257	400 390-410	592 571-621	700 676-725

e. Reproducibility

Table (29) shows the coefficient of variation observed in duplicates of 25 samples within a run. Samples studied were of wide range 45-365 ng/100 ml. A higher coefficient of variation is obtained when 20 samples are assayed in five different runs. Generally coefficient of variation was lower at the normal range of T_3 (60-195 ng/ml).

TABLE 29

Within Run and Between Run Coefficient of Variation

Reproducibility	No. of Samples	No. of Runs	Coefficient of Variation
Within run	25	4	6.4 ± 1.5
Between runs (day to day)	20	5	7.9 ± 2.6

f. Methodological Variables Effect on the T_3 Value

(1) Effect of the T_3 antibody cross reactivity with T_4 on T_3 value.

It is evident in Table (27) that the use of Wien Laboratory antisera, which is the only variable in this experiment, gave higher T_3 values than Endocrine Sciences antisera. This elevation seems to correlate with T_4 level and is attributed to the higher cross reactivity of the WL antisera with T_4. These results indicate clearly that the

specificity of the antisera used in T_3 RIA has significant effect on the T_3 value.

(2) Effect of the separation method on the T_3 value.

In Table (28) it is shown that the method used to separate the free from bound fraction of T_3-^{125}I has consistent effect on the T_3 value. The overall effect of this variable correlates with the parameter of the dose-response curve, thus the higher the slope and NSB, the higher the T_3 value is. A good correlation is found between the double antibody method and polyethylene glycol. However, a much higher value is obtained with SAS and DCC.

(3) The effect of the type and concentration of the inhibitor on the T_3 value.

Table (30) compares the T_3 value of six samples with wide range when different inhibitors are used at optimal concentration. T_3 values obtained are not significantly different when ANS or sodium salicylate is used, however slightly lower values are observed with merthiolate. This seems to correlate with lower slope obtained with this substance. Decreasing sodium salicylate concentration to 0.4% did not affect the T_3 value. This is because 0.4% is within the effective range of this substance (Figure 3).

(4) Effect of antibody dilution on the T_3 value

In Table (31) a slight but consistent increase in the T_3 value is observed when higher dilution of the same antisera is used. This can be attributed to increase in the sensitivity which is known to be increased with dilution.

TABLE 30

Effect of the Type of Inhibitor or its Concentration on the T_3 Value

Inhibitor and Concentration	Dose-Response-Curve Parameter			T_3 ng/100 ml Mean and Range (N=3)					
	Slope	%B°	NSB	1	2	3	4	5	6
Merthiolate .15%	2.58	30.4	6.0	42 (41-45)	80 (76-86)	152 (148-156)	410 (392-438)	126 (118-134)	180 (163-195)
ANS 0.06%	3.38	31.5	4.6	65 (56-74)	73 (66-80)	190 (181-199)	485 (463-507)	148 (140-156)	222 (210-233)
Sodium Salicylate 0.4%	3.4	32.8	7.0	36 (30-42)	95 (91-101)	193 (186-199)	532 (509-551)	161 (151-171)	226 (218-235)
Sodium Salicylate 0.8%	3.1	36.7	4.5	40 (32-48)	92 (90-94)	200 (193-207)	561 (531-589)	169 (159-179)	220 (201-229)

TABLE 31

Comparison of Serum T_3 Values at Two Dilutions of the Same Antibody

Ab Source and Dilution	%B	T_3 ng/100 ml Mean and Range (1)	(2)	(3)	(4)	(5)	(6)	(7)
Wien 1:5000	48%	101 98-104	105 100-110	110 104-114	425 410-441	227 217-227	82 71-94	290 280-301
Wien 1:10000	35%	127 120-134	160 147-173	132 126-140	483 459-507	263 248-276	96 91-101	336 305-366

Normal T_3 value by this RIA

The normal level (Euthyroid) of serum T_3 by this method is 60-195 ng/100 ml or 128 ± 35 ng/100 ml. Which is in close agreement with many of reported T_3 RIA.

Correlation of the T_3 value by RIA with T_4 value by CPB

A good correlation was found in the samples analyzed. The correlation coefficient is 0.932. All samples which were in the hyperthyoid range by T_4 were in the same range by T_3. This also was found to be true in the normal range. Three samples out of ten were in the hypothyroid range by T_4 but gave borderline results (Hypo-Euo) by T_3 RIA.

Correlation of this T_3 RIA with another T_3 RIA

Figure (15) shows that generally lower values were obtained by this method. However, since the normal range of PCL method is higher 80-220 ng/100 ml, the diagnostic accuracy of the two methods is equivalent. It is observed in Figure (15) that most of the values are

on the PCL side of the 100% correlation line. The actual correlation value (r) between the two methods is 94.5%. PCL method uses double antibody techniques for separation which makes the procedure much longer (overnight incubation).

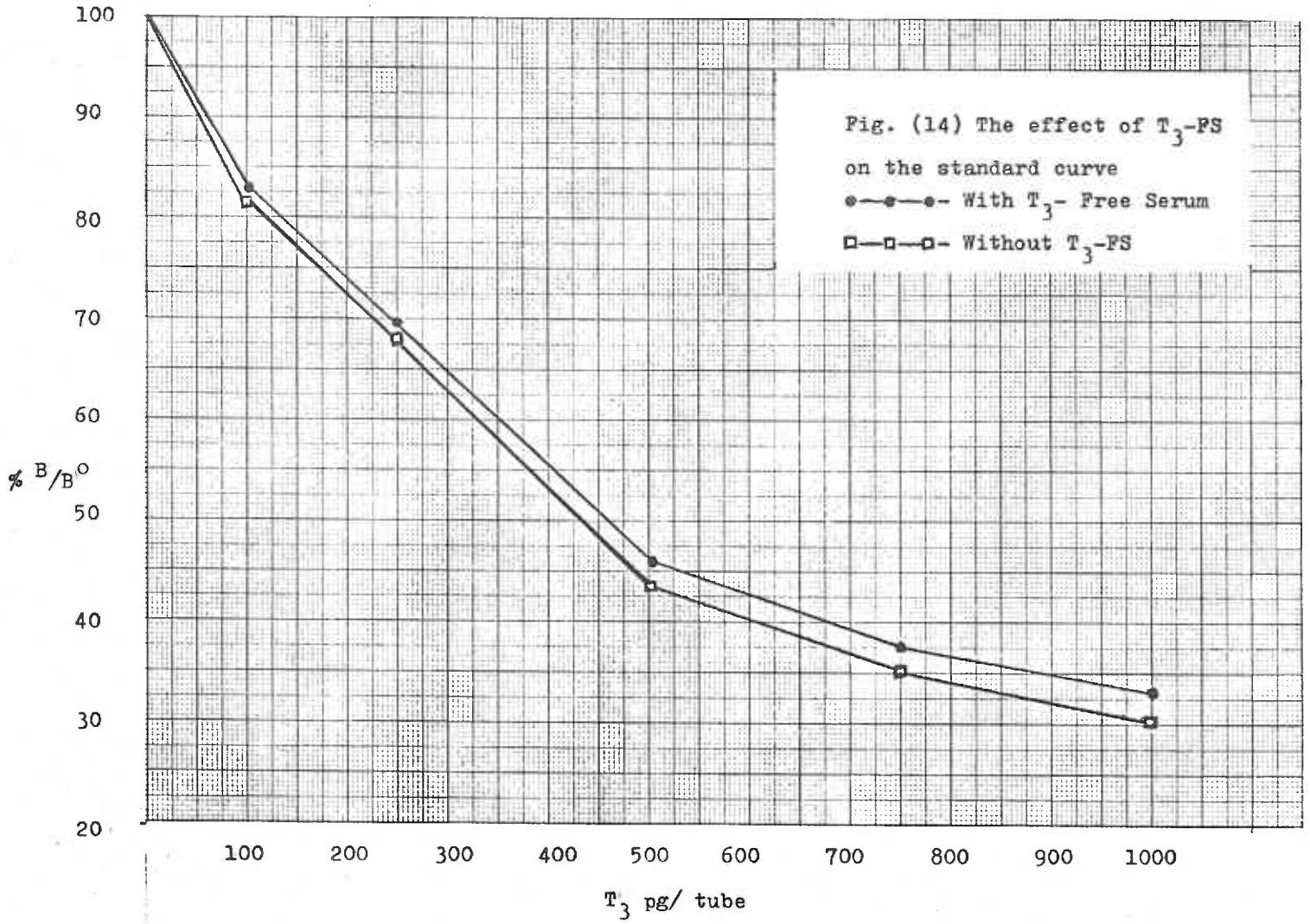


Fig. (14) The effect of T_3-PS on the standard curve

●—●—●- With T_3- Free Serum

□—□—□- Without T_3-PS

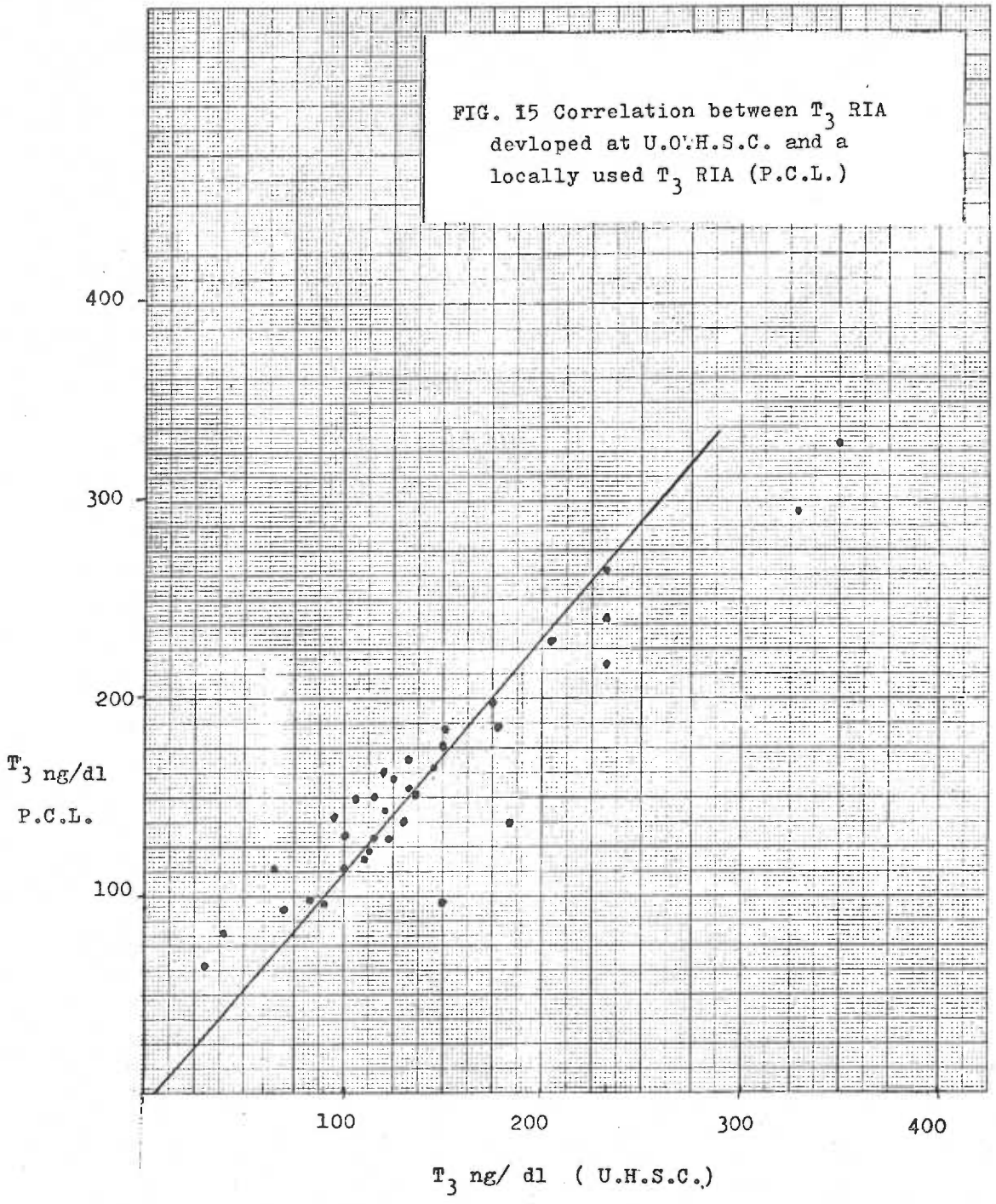


FIG. 15 Correlation between T_3 RIA devloped at U.O.H.S.C. and a locally used T_3 RIA (P.C.L.)

DISCUSSION

A. Development of a T_3 Radioimmunoassay

The radioimmunoassay of T_3 in human serum is a relatively new technique. Several reports have been published on this subject with wide variation in methodology and normal values (Table 3). The reported procedures vary mainly in the following aspects:

1. The method used to inhibit or eliminate the TBG effect on the assay
2. The quality of the antisera (especially the cross reactivity with T_4)
3. The method used to separate the free from bound fraction

The invitro competition for T_3 by TBG was emphasized as the most important factor in T_3 RIA (63). As demonstrated in Table (11), adding T_3-Free serum led to substantial decrease in the percent T_3-^{125}I bound to the T_3 antibody. To eliminate the effect of TBG on the assay two methods were evaluated. The use of binding inhibitors and the use of heat inactivation of the TBG.

Several compounds have been used to block T_3 binding to TBG, (Table 1). Some of these were found to have serious drawbacks such as instability (tetra chlorothyronine, T_4) or insolubility at the assay pH 8-9 (diphenylhydantoin). The effectiveness of the best three inhibitors; ANS, sodium salicylate and merthiolate were compared, (Figure 3). In agreement with Hufner et al (68), we found ANS and sodium salicylate to be superior to merthiolate. It is useful that these substances show a plateau type response within which the inhibitor can be used

in excess to buffer the assay against TBG level variation. However, if they are used above a certain level they will inhibit binding of T_3 to the antibody. Merthiolate does not show that in our study but it was shown by Sterling (66) to exhibit the cross reactivity effect if used at higher concentration. Sodium salicylate was selected for use in our assay because it showed no effect on the T_3-anti T_3 interaction, (Figure 5), and because it is more practical to use than ANS which is photosensitive, thus a fresh solution should be prepared for each run (63). Furthermore, it is reported that sodium salicylate inhibits the binding to TBPA (73) which eliminates the need for using barbital buffer. Heat inactivation of TBG at $60^{o}C$ for 45 minutes was compared with ANS. Despite similarity in the standard curves obtained under both conditions, Figure (6), higher T_3 values for the same samples were obtained with heat inactivation method, Table (12). The same observation was found (with greater elevation in T_3 values) when serum was heated at acid pH to $100^{o}C$ for 15 minutes. This increase can be attributed to the effect of heat on the T_4, as observed in Table (13). We assumed that the in vitro conversion of T_4 to T_3 due to heat is the reason for this elevation. Whatever the end product of the effect of heat on T_4, clearly it is leading to significant false elevation in the T_3 value. Therefore, we considered this approach unapplicable for T_3 RIA. Sterling et al (66) has used heat inactivation at $60^{o}C$ for 3 hours. The normal values they reported are higher than the great majority of other reports (Table 3). They are only in close agreement with Gharib et al (67) who did not use T_3 FS in the standard curves at all. Heat inactivation may be the reason for elevation of the T_3 value in Sterling report.

Our study documents for the first time the effect of heat on the T_4 stability.

The antisera cross reactivity with T_4 is of major significance here. Observable difference in the sample T_3 value was found when two commercial antisera were compared Table (27). This was because Endocrine Sciences antisera had much less cross reactivity with T_4 than Wien Laboratories antisera Table (26). It is worth noting here that the cross reactivity values in Table 26 are determined using unpurified T_4 which contains some T_3. Therefore, they are not true cross reactivity values. The fact we found higher values than the ones provided by Wien Laboratories may be due to the use of T_4 with different purity. All T_4 cross reactivity values reported in Table (2) are determined on unpurified T_4 except for Mitsuma et al (70) which represents a true value for their antibody.

The reason we don't see correlation between the cross reactivity value of T_4, Table (2), and the normal T_3 value, is that other methodological variables have different effects on the T_3 value as will be discussed later. Endocrine Sciences antisera, due to its lower cross reactivity with T_4 (0.25%) was selected for our assay.

In evaluating methods used to separate the free from the bound fraction, Polyethylene Glycol, used at optimal concentration, was found most effective and practical method. It gave us results which agreed with the double antibody method Table (28). PEG final concentration was found dependent on the serum protein concentration (81). Therefore, the optimal concentration should be determined for each assay. 12.75%

PEG was found optimal in this study to insure complete precipitation of the bound fraction with least nonspecific precipitation (Figures 11, 12, and 13). Sekadde et al (75) have added T_3-FS immediately after adding PEG presumably as a carrier protein to enhance the precipitation. We found no need for this in this assay probably because serum protein concentration is sufficient to obtain a good precipitate. Unlike PEG, 50% saturated ammonium sulfate gave a large but soft precipitate with very high NSP or (NSB). The use of 45% SAS proved to be better if the precipitate was washed. This additional step and the lower reproducibility observed with this method made us prefer PEG.

In agreement with Hufner et al (68) and Twomey et al (82) the optimal pH for T_3 RIA is 9. It is known that T_3 and T_4 have lower binding affinity to TBG at alkaline pH. Borate buffer was selected to achieve pH 9 because of its higher pK over Barbitol buffer.

The use of T_3-Free serum in the standard curve is essential for the accuracy of the assay. Any RIA must be viewed as depending entirely on a comparison between the standard and unknowns. Therefore, essential identity should be achieved between standards and specimen by methodological modification. Standard curves set up with or without T_3-FS (both having inhibitor) are not usually the same Figure (14). This observation has been found in most reports on T_3 RIAS (64, 73). Four percent BSA solution has been used to replace T_3-FS by Utiger et al (18). This only brings about similar albumin environment but does not as accurately duplicate the specimen matrix.

To account for the nonspecific binding (NSB) which represents

the T_3-^{125}I which is not bound to the antibody, however it is trapped or remains in the bound fraction, pool serum was preferred over T_3-FS, because the presence of unlabeled T_3 and T_4 makes the NSB thus determined closer to the actual NSB in the unknowns.

The final RIA method thus developed is accurate as indicated by the good recovery of T_3 added to serum specimen with different amounts of endogenous T_3 Table (21), and by the similarity of the T_3 values obtained by direct determination versus those obtained by extraction Table (22). Both the recovery and extraction studies show that all the endogenous T_3 in serum is made available to the antibody in the course of the reaction and that serum proteins, especially TBG, had no significant effect on the T_3 value by this method. The absence of serum protein effect on the assay was confirmed in several ways. The dose response curve set up by increased amounts of hyperthyroid control was found very similar to the dose response set up using standard T_3 and the linearity is excellent Figure (14). Furthermore, adding T_3 Free serum to a sample did not change the T_3 value significantly Table (25), indicating that the assay is satisfactorily buffered against normal variation in the concentration of TBG and other thyroid binding proteins in the blood. The minimum detectable level in this method is 12 ng/dl, a concentration rarely found in the hypothyroid level. Most reports on RIA of T_3 show the ability to detect hypothyroid patients except for Chopra's first (64). The lack of sensitivity by Chopra's method has been attributed to the use of hypothyroid sheep serum to construct the standard curve and T_4 as an inhibitor. Since there is some T_3 present

in the sheep serum and in T_4 preparation both factors caused the elevation in the detectable limit Table (2). The clinical validity of this assay is indicated by the good correlation (r = .932) between the T_3 values determined by this method and the T_4 values of the same samples, as determined by competitive protein binding (Abbott).

B. Study of the T_3 Value Variation

The normal level of serum T_3 by this method is 60-195 ng/dl. Our normal value is in relatively close agreement with most reports on T_3 RIA Table (3). Significantly higher values have been reported by Garib et al (67) 215 ± 55/dl and by Sterling (66) 189 ± 30.

In analyzing the reason for this variation in normal T_3 level, two etiologies must be considered. First, geographic differences in iodine intake lead to variation in T_3 level in normal individuals. This observation has been supported by a study by Garib et al (38) in which the same T_3 RIA method was used in two different areas (Minnesota and California). It was found that slightly but significantly higher values were obtained in areas of lower iodine intake. The second reason for the variation is the methodological variables of T_3 RIA. A recent inter-laboratory comparison of serum T_3 by Amirav et al (83) found a mean variation of 36% between 16 laboratories who analyzed the same samples. This study, however, was not able to determine which factor(s) of the various procedures are responsible. The question remained, how do these variables affect the T_3 value? Our study considered first the variation in T_3-Ab cross reactivity with T_4. This, as shown in Table (27), caused significant variation in the T_3 value; thus the

higher the cross reactivity, the higher the T_3 level. This observation might not have been possible if more than one methodological variable was involved at the same time. The other variables which are found to have an effect on T_3 value are: the type of inhibitor Table (30), the method of separation Table (28), and the antisera dilution Table (31). It is observed that there is a consistent and positive relationship between the slope of the standard curve and the T_3 value of the unknowns. This relation indicates a non-identical response of the standards and the unknowns to methodological variations. This can be attributed to some intrinsic differences between the standards and the unknowns. These differences are not completely resolved by the methodological modification used so far. Using a special quality control study on T_3 RIA, Moser et al (76) support the relationship found here between the slope, NSB, and the T_3 value. The exact T_3 value, thus remains unknown, and if the direct T_3 RIA is to remain the procedure of choice, the use of international standards would be a good way to reduce the heterogeniety in T_3 value among laboratories.

SUMMARY AND CONCLUSIONS

REFRENCES

SUMMARY AND CONCLUSIONS

The methodological variables of T_3 radioimmunoassay were studied, a new procedure was developed and the effects of the methodological variables on the serum T_3 value were evaluated.

Among the different methods used to eliminate the effect of TBG on the T_3 value, sodium salicylate was found the most effective and practical inhibitor. Also it showed no significant effect on the T_3-anti T_3 interaction. Heat inactivation of TBG causes false elevation in the T_3 value by affecting T_4 present in the unknowns. Therefore, this approach was considered unapplicable to T_3 RIA. The binding of T_3 to anti-T_3 was found optimal at pH 9.0 and this was established with borate buffer. To separate the bound from the free fraction, PEG at final concentration 12.75 was preferred over 50% saturated ammonium sulfate, dextran coated charcoal, ion exchange resins and double antibody. Effective separation and lower nonspecific binding was obtained by PEG.

The final procedure depends on the use of commercial antisera at dilution which bind 40-50% of the added $T_3{}^{125}I$ (60-100 pg). Reaction mixture, 0.1 ml serum or standard, 0.1 ml antibody, 0.1 ml sodium salicylate, 0.1 ml $T_3{}^{125}I$ and 0.1 ml diluent, was incubated at 37°C for 30 minutes followed by 2 hours at 4°C. Separation was then achieved by 1.5 ml of 17% PEG solution. This procedure was found to be accurate by recovery and extraction studies, and sensitive to detect 12 ng/dl. The within run variation is 6.4 ± 1.5% (CV ± SD) and between runs 7.9 ± 2.6% (CV ± SD). Normal T_3 value by this method is 60-195 ng/dl.

Several methodological variables affect the T_3 value significantly. The cross reactivity of antisera with T_4 has been shown here as a factor leading to variation in the T_3 value. Other variables as the separation method, the antibody dilution, the type of inhibitor used, affect the T_3 value by varying the parameters of the standard curve, i.e. slope, nonspecific binding. It is found that the higher the slope the higher the T_3 value. This may be due to differences in the matrix of the standards and the unknowns. These differences have not been resolved by the methodological modification used so far. It is concluded here that the methodological variables are responsible for the heterogeniety in the T_3 value.

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